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Determinants of smoke induced lung damage and relationship with metabolic syndrome

by

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UNIVERSITY OF SOUTHAMPTON - ABSTRACT

FACULTY OF MEDICINE, HEALTH, AND BIOLOGICAL SCIENCES, SCHOOL OF MEDICINE

Doctor of Medicine

DETERMINANTS OF SMOKE INDUCED LUNG DAMAGE AND RELATIONSHIP WITH METABOLIC SYNDROME

By DINESH BAGMANE

Abstract

Smoking is the major risk factor for COPD. Smoking also has systemic effects and is considered as one of the risk factors for metabolic syndrome (MeS). It is unclear whether it is smoking per se or the systemic effects of COPD that cause metabolic syndrome in smokers.

Smokers with and without COPD and non-smoking controls were studied by pulmonary function testing, skin prick tests, body composition, fasting glucose, CRP, and lipids analysis to diagnose MeS. This showed a gradual increase in prevalence of MeS, but with only difference between non-smokers on one hand and smokers with or without COPD on the other being significant. This suggested that smoking, rather than the systemic effect of COPD, was the cause of MeS.

All smokers were then grouped and smokers and non-smokers compared in respect of lung function, inflammatory markers (CRP, a series of inflammatory cytokines), insulin resistance, and body composition. Smokers had increased central obesity and total body fat, which is in contrast to the common belief that smoking reduces weight. Male smokers demonstrated increased abdominal fat, while females showed an increase in total body fat. FEV_1 was reduced when comparing all smokers with MeS and those without MeS, and there was a greater reduction in males who had a greater prevalence of MeS, but had better quality of life even though they smoked more. However, whilst smokers with MeS had higher levels of insulin resistance, as measured by Homeostasis Model Assessment (HOMA-R), none of the plasma inflammatory markers, except for IL-12, was raised, suggesting that these indices of inflammation were not the reason for MeS.

Smoking is associated with a gradual decline in lung function in smokers with and without COPD. A previously recruited cohort of smokers with and without COPD and healthy non-smoking subjects were followed up over a period of 5 years. Amongst a whole series of measurements, including HRCT, measures of lung density/emphysema, only sputum neutrophilia (both absolute and percentage counts) predicted the annual decline in FEV_1.

In summary, this study suggests that there is an increased prevalence of MeS in smokers associated with insulin resistance caused by smoking but it fails to show an association between MeS and COPD. Smoking is also associated with central obesity and increased body fat, contributing to a reduction in FEV_1. Sputum neutrophilia, but not smoking pack years or lung HRCT measurements, predicts the annual FEV_1 decline in smokers.

Tables and figures

Declaration of authorship

I, Dinesh Bagmane declare that the thesis entitled '**Determinants of smoke induced lung damage and relationship with metabolic syndrome**' and the work presented is my own.

I confirm that

this work was done wholly or mainly while in candidature for a research degree at the University of Southampton.

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Signed……

Date ……

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Abbreviations

AAT	Alpha-1 antitrypsin
ATP	Adult treatment programme
ATS	American Thoracic Society
BIA	Bioelectrical impedance analysis
BMI	Body mass index
BTS	British Thoracic Society
CF	Cystic fibrosis
CO	Carbon monoxide
COPD	Chronic Obstructive Pulmonary Disease
CRP	C- reactive protein
DTE	Dithioerythritol
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immuno sorbent assay
ERS	European Respiratory Society
ESR	Erythrocyte sedimentation rate
FEV_1	Forced expiratory volume in 1 second
FFM	Fat free mass
FFMI	Fat free mass index
FM	Fat mass
FRC	Functional residual capacity
FVC	Forced vital capacity
GM-CSF	Granulocyte macrophage colony stimulating factor
GOLD	Global initiative for obstructive lung disease
HBS	Hepes buffered saline
HC	Healthy non-smoker control
HDL	High-density lipoprotein
He	Helium
HOMA-R	Homeostasis Model Assessment-Resistance
HRCT	High resolution computed tomography
HS	Healthy smoker
HU	Hounsfield units

ICAM	Intercellular adhesion molecule
IDF	International Diabetic Federation
IFNg	Interferon gamma
IL	Interleukin
IR	Insulin resistance
LDL	Low-density lipoprotein
MeS	Metabolic Syndrome
MRC	Medical Research Council
m-RNA	Messenger ribonucleic acid
NCEP	National cholesterol education programme
NICE	National Institute for Clinical Excellence
NS-N	Non-smokers with no metabolic syndrome
NS-Y	Non-smokers with metabolic syndrome
PEF	Peak expiratory flow
PFT	Pulmonary function test
PAI-1	Plasminogen Activator Inhibitor-1
QOL	Quality of life
SGRQ	St George's Respiratory Questionnaire
Sm-N	Smokers with no metabolic syndrome
Sm-Y	Smokers with metabolic syndrome
SOP	Standard operating procedure
TGF	Transdermal growth factor
TLC	Total lung capacity
TLco	Carbon monoxide transfer factor
TNF-α	Tumour necrosis factor alpha
VC	Vital capacity
VLDL	Very low-density lipoprotein
W/h	Waist-hip ratio
WHO	World health organisation
WTCRF	Wellcome Trust Clinical Research Facilities

1.0 Chapter-General introduction

1.1. Introduction

Chronic obstructive pulmonary disease (COPD) is a major health problem. The World Health Organisation (WHO) has predicted that it will become the fifth largest disease burden and the third greatest cause of death by 2020[1]. The reason for this is that COPD is not usually detected until patients seek medical attention for shortness of breath or an exacerbation of the disease[2]. It is an under diagnosed disease and thus the available data regarding the mortality and morbidity are likely to underestimate the total burden to patients and the healthcare providers.

1.1.1. Different COPD definitions

COPD has been defined by many organisations and respiratory societies in different ways in an attempt to define the disease severity and thus help physicians in the classification and management of the disease. According to the Global Initiative on Obstructive Lung Disease (GOLD), COPD is a preventable and treatable disease with some significant extrapulmonary 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 lung to noxious particles or gases[3]. It is now termed 'Syndrome' by many, as it is composed of chronic bronchitis, small airway disease (Bronchiolitis) and emphysema that vary in proportions between affected individuals.

The British Thoracic Society defines COPD as a chronic, slowly progressive disorder characterized by airflow obstruction (reduced FEV_1 and FEV_1/FVC ratio) that does not change markedly over several months. Most of the lung function impairment is fixed, although some reversibility can be produced by bronchodilator (or other) therapy[4].

According to the American Thoracic Society, COPD is a disease state characterized by the presence of airflow obstruction due to chronic bronchitis or emphysema: the airflow obstruction is generally progressive, may be accompanied by airway hyperreactivity and may be partially reversible[5].

European Respiratory Society defines COPD as a disorder characterized by reduced maximum expiratory flow and slow forced emptying of the lungs:

features, which do not change markedly over several months. Most of the airflow limitation is slowly progressive and irreversible. This airflow limitation is due to varying combinations of airways disease and emphysema: the relative contribution of the two processes is difficult to define in vivo[6]. These various definitions are meant to help in understanding the disease burden, the treatment costs, as well as the mortality and morbidity of COPD.

1.1.2. History and burden of the disease

COPD is one of the leading causes of death in many countries and it has been on the rise because of smoking and an increasingly ageing population. In Europe there is a decreasing trend in many countries[7]. In the United Kingdom over the last 30 years, mortality has decreased in men but increased in women[8]. Surprisingly women who are exposed to solid biomass fuels (animal dung, crop residue, and wood) and develop COPD have a similar picture of loss of quality of life and mortality as cigarette smokers[9, 10].

COPD is thought to be the fourth most common cause of mortality in the world and WHO predicts that it will be third by 2020. This could be due to changing smoking habits in the developing world[11]. In 2004, more than 27,000 men and women died of COPD in the UK and most of the deaths were in the age group over 65years[12]. There is a socio-economic trend in COPD mortality with men aged 20-64 employed in unskilled manual occupation being 14 times more likely to die of COPD than those in a professional occupation[12]. It is difficult to assess the severity of the disease by symptoms alone or by the various definitions being used in the community. A systematic review of all population based studies of COPD across the world and published between 1999-2004 estimated that the prevalence of COPD in adults over 40 years was 9-10%[13]. However, there was a variation in age specific rates and variation in current or past smoking history. In their study, de Marco *et al* measured the prevalence of COPD in adults under the age of 45 years living in Europe. They found a rate of 2.5% for GOLD stage 1 and approximately 15% for stages 2 and 3[14]. Women had more respiratory symptoms and less of airflow obstruction, however COPD was diagnosed in older subjects. There was no measure of alpha-1 antitrypsin deficiency in that study.

COPD has increased the burden on the health services in the UK. It is estimated that there are 1.4 million consultations in primary care for COPD which is four times that of angina[15]. The overall cost to the health services is around £800 million[16], however, the National Institute of Clinical Excellence (NICE) estimates slightly lower costs[17]. The disease remains underdiagnosed or misdiagnosed as something else, because of few or no symptoms. Early intervention in terms of mass screening for COPD diagnosis may give a better understanding of prevalence. This early diagnosis could also help the clinician in early treatment, prevention of morbidity and mortality as well as improvement in quality of life.

COPD is a syndrome, which includes chronic bronchitis (previously classified as stage0), small airways disease, which is bronchiolitis and emphysema. There are clinical and pathological differences between chronic bronchitis and emphysema.

1.1.3. Chronic bronchitis and emphysema

The description of chronic bronchitis and emphysema was made in the early nineteenth century, but interest developed in the late 1950's when many people died during the London smog[18]. During this period, spirometry was becoming available, and this led to the observation that airflow obstruction was the key factor in determining disability and mortality.

Chronic bronchitis is defined clinically, as the presence of cough with sputum production for most days for a minimum of three months per year for at least two successive years which cannot be attributed to other pulmonary or cardiac causes[19]. The prevalence of chronic bronchitis in the United States is high, with approximately 12–15 million Americans suffering from this syndrome.

The word emphysema is derived from Greek and means, "to blow into," hence "air containing" or "air inflated". The term emphysema was initially applied to air within the tissues (i.e. subcutaneous emphysema). In 1721, Ruysch described large air spaces in lung specimens of humans as emphysema. In 1799, Bailey subsequently provided the first clear illustration and description of emphysema as enlarged air spaces[20]. Laennec contributed considerably to the understanding of emphysema and recognized that emphysema is associated with chronic bronchitis.[21]. In 1939, Cournand

introduced the concept that emphysema causes airflow obstruction. In 1952 Gough described the differences in the centrilobular and panacinar emphysema which opened up the understanding of the pathology of the airways [22]

The US Department of Health looked at the prevalence of COPD[23]. Accordingly, the prevalence of chronic bronchitis was lowest in the 25-44 age groups. Across the age groups, females had a higher rate than males for both white and black races. Among the 25-44 year olds and the over 65s, prevalence was higher in whites than blacks for each group. The prevalence of emphysema was higher for the 65 and above age group and higher in males and in whites. Over the past two decades prevalence of emphysema has been consistently higher for the 65 and above age group[23].

1.1.4. The pathology of emphysema and bronchitis

The lung appears first as an epithelial bud at the caudal end of the laryngotracheal groove on the 26th day after ovulation[24, 25]. The bud derived from the endoderm will form the epithelium of the airways and the acini. The mesenchyme develops into the connective tissue, cartilage, smooth muscle, and vessels of the lung. By 33 days, the trachea is separated from the foregut and the pouches represent the five lobes. By 41 days the dichotomous division leads to the development of full adult segments and by 16 weeks terminal bronchioles and the bronchial tree develop[26].

There are at least two genetic forms of chronic bronchitis, primary ciliary dyskinesia and cystic fibrosis (CF). In addition, the most common form of chronic bronchitis in North America is the acquired form, which typically reflects cigarette smoking and, to a lesser degree, air pollution[27]. In the United Kingdom the prevalence of chronic bronchitis in smokers aged over 45 is 45 for every 10000 people and around £120,000 would be saved for every 100 hospital admissions avoided[28]. Mucous gland enlargement is the histological hallmark of chronic bronchitis. The structural changes described in the airways include atrophy, focal squamous metaplasia, ciliary abnormalities, and variable amounts of airway smooth muscle hyperplasia, inflammation, and bronchial wall thickening.

Emphysema on the other hand develops at later stages of the disease. Over 3.1 million Americans have been diagnosed with emphysema, of which

91% were 45 years of age or older. Emphysema rarely occurs in those under 45 in smokers[29]. Emphysema is most likely to develop in cigarette smokers, but cigar and pipe smokers also are susceptible, and the risk for all types of smokers increases with the number of years and amount of tobacco smoked. Men are affected more often than women are, but this statistic is changing, as there is raising trends in smoking in women. Other risk factors include age, second hand smoking, occupational exposure to chemical fumes, and exposure to indoor and outdoor pollution. Severe alpha 1-antitrypsin (AAT) deficiency is a proven genetic risk factor for chronic obstructive pulmonary disease (COPD), especially in individuals who smoke[30, 31]. Unlike asthma, which occurs when the muscles in the airways tighten, emphysema causes a loss of elasticity in the walls of the small air sacs in the lungs. Eventually, the walls stretch and break, creating larger, less efficient air sacs that are not able to handle the normal exchange of oxygen and carbon dioxide.

The three described morphological types of emphysema are centriacinar, panacinar, and paraseptal. The first type, centriacinar emphysema, begins in the respiratory bronchioles and spreads to the periphery and it is also called centrilobular emphysema, this form is associated with long-standing cigarette smoking and predominantly involves the upper lobes of the lungs. It occurs with loss of respiratory bronchioles in the proximal portion of the acinus sparing the distal alveoli. Second type is panacinar emphysema, which destroys the entire alveolus uniformly and is predominant in the base of the lungs. This type of emphysema is generally observed in patients with homozygous alpha1-antitrypsin (AAT) deficiency, and is well established in pulmonary emphysema and infantile cirrhosis[32]. Alpha 1-Antitrypsin deficiency is a hereditary condition where there is a high risk of developing emphysema at a young age[33]. The third type, distal acinar emphysema (also known as paraseptal emphysema), preferentially involves the distal airway structures, alveolar ducts, and alveolar sacs. The process is localized around the septae of the lungs or pleura. Although airflow frequently is preserved, the apical bullae may lead to spontaneous pneumothorax. Giant bullae occasionally cause severe compression of adjacent lung tissue.

The main symptoms of emphysema are shortness of breath and a reduced capacity for physical activity, both of which are likely to become worse as the disease progresses. In time, sufferers may have trouble breathing even when lying down, and it may be especially hard to breathe during and after

respiratory infections, such as colds or the flu. Other symptoms include fatigue, loss of body fat and muscle mass and cough. Tissue remodelling associated with small airways inflammation is observed in patients with severe COPD[34]. Many studies carried out using transgenic mice have reported that overproduction of inflammatory cytokines like TNF-α, IFNg, IL-1β and IL-13[35-38] causes emphysema in these animal models. This evidence suggests that these cytokines may be involved in the pathogenesis of COPD. There are few studies, which investigated the relationship between peripheral airways and inflammation. O'Donnell et al reported that peripheral airway dysfunction which was measured by HRCT was a determinant of COPD severity. Airway neutrophils are also associated with severity but are not related to overall emphysema[39].

1.1.5. Classification of COPD

COPD is classified according the Global Initiative for Chronic Obstructive Lung Disease (GOLD). To understand the classification it is important to understand the pulmonary function testing used, as the classification is based on the forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) and their ratio (FEV_1/FVC).

1.1.6. Pulmonary function testing (PFT)

Pulmonary function testing has three components, a) Spirometry, b) measurement of lung volumes and 3) diffusion capacity. Spirometry is the most common pulmonary function test which measures lung function, specifically the measurement of the amount (volume) and/or speed (flow) of air that can be exhaled (FEV_1 and FVC). Body plethysmography is used to measure resistance to flow and lung volumes.

Pulmonary function testing will measure the different types of defect in health and disease. They are as, a) normal airways b) obstructive defect c) restrictive defect d) combined defect.

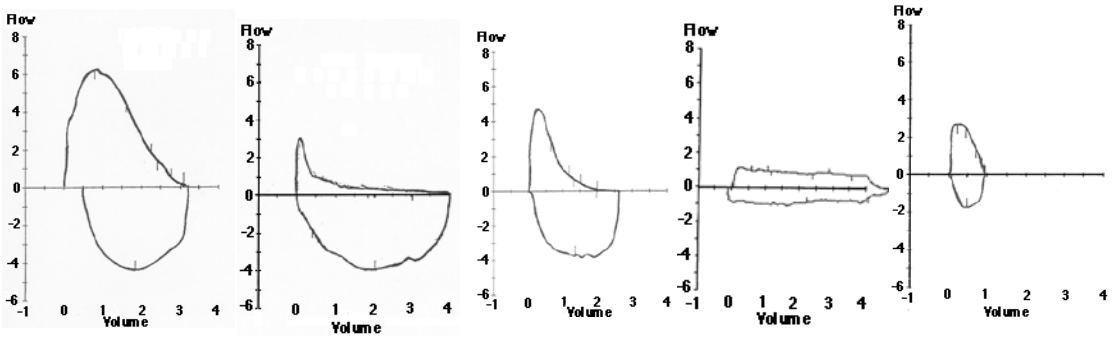

Figure 1.1 Flow volume loops in normal, obstructive, restrictive, upper airways obstruction and combined defects.

1.1.7. Obstructive defect

This is seen in airways disease such as bronchial asthma and COPD. In asthma, there is no defect in the lung volumes but there is a decrease in FEV_1 and FEV_1/FVC ratio. In COPD patients FEV_1 may be normal or reduced and FEV_1/FVC ratio is <70%, along with obstruction in the small airways. There is also air trapping.

1.1.8. Restrictive defect

The current gold standard tool to measure the restrictive pulmonary defect is by spirometry and by measuring the total lung capacity by helium dilution or by plethesmography[40]. A restrictive ventilatory defect has a typical shape with a downward and rightward shift of the static expiratory pressure–volume curve. Lung recoil is increased over the range of the inspiratory capacity with a reduction of total lung capacity (TLC) and vital capacity (VC)[41-43]. A low FVC could be seen in true restrictive lung disease or in the patients with obstructive defect with severe air trapping. In the absence of airflow obstruction, it has been recommended that restrictive defect can be measured by spirometry alone. So a low FVC by spirometry and normal or high FEV_1/FVC ratio is traditionally classified as restrictive defect[44]. Diseases of the parenchyma such as idiopathic lung fibrosis or sarcoidosis are examples of restrictive ventilatory defects.

1.1.9. Combined or mixed defect

Reduction in FEV_1/FVC ratio and a decrease in FVC are termed as combined ventilatory defect. In the majority of the cases this defect represents airway obstruction with lung hyperinflation[45]. This needs to be verified by plethysmography to find the reason for these changes.

1.1.10. GOLD classification of COPD

Stage 0	Chronic bronchitis	Normal lung function
Stage I	Mild	FEV_1/FVC < 0.70 $FEV_1 \geq$ 80% predicted
Stage II	Moderate	FEV_1/FVC < 0.70 50% $\leq FEV_1$ < 80% predicted
Stage III	Severe	FEV_1/FVC < 0.70 30% $\leq FEV_1$ < 50% predicted
Stage IV	Very severe	FEV_1/FVC < 0.70, FEV_1 < 30% predicted *or* FEV1 < 50% predicted *plus* chronic respiratory failure

Table 1.1 GOLD classification for chronic obstructive pulmonary disease illustrating different stages of COPD.[46]

Stage 0 has been omitted from the revised GOLD classification, which was used to describe chronic bronchitis without airway obstruction. As this study was started in 2004, I had recruited patients classified with stage 0 COPD.

1.1.11. Mild COPD

Patients with the milder form of disease have preserved FEV_1 i.e. they are able to expel all the air in the first second but the ratio between FEV_1/FVC is below 70% predicted for their height, age and sex.

1.1.12. Moderate COPD

Patients with moderate disease have FEV_1 <80% predicted and FEV_1/FVC <70%.

1.1.13. Severe and very severe COPD

Severe disease is defined in patients who have more symptoms and their FEV_1 is <50% predicted. These patients have restricted mobility and are often on oxygen supplement as well as both short and long acting inhaled bronchodilators, steroids, and anti cholinergic inhalers. In the most severe disease there is a severe defect in the pulmonary function test with an FEV_1 <30% predicted and in chronic respiratory failure.

1.1.14. COPD severity and symptoms

COPD symptoms differ with the different stages of the disease. Patients with a mild form of disease or with mild airflow limitation may be asymptomatic. This limits the clinician for the early intervention. The main symptoms of the disease are variant degree of cough with or without sputum production, predictable shortness of breath mainly due to exertion and wheeze[47]. These symptoms worsen as patients continue to smoke. Age in combination with smoking is a risk factor for COPD and any diagnosis for patients under 40 years old should bring suspicion of genetic predisposition for COPD i.e. α-1antitrypsin deficiency.

Cough is one of the early symptoms in COPD. It can be productive and more frequent in the morning. Generally, the sputum would be colourless or clear i.e. not infected. However, during an exacerbation of COPD, the sputum produced could be green, yellow, brown, or blood tinged. This change in colour may be due to bacterial infection or due to the neutrophilia observed upon exacerbation.

Breathlessness worsens as the disease progresses. It is related to the degree of airflow limitation and the onset of emphysema. Breathlessness is measured by either asking the patients about their activity levels or by Medical Research Council (MRC) dyspnoea scale[48]. This can also be determined by asking the patient to perform a six minute walk test[49]. The percentage of emphysema is measured by High Resolution Computed Tomography scan (HRCT). Unlike asthma, there is no diurnal variation in breathlessness in COPD. The degree of breathlessness in COPD increases if exposed to infections, variation in climate, or smoky environments.

Wheeze is also a common symptom in COPD patients. This varies with airflow limitations, but this is not a cardinal symptom as in asthma. Other features of COPD can include chest pain due to ischemia of intercostal muscles where other causes like ischemic heart disease or infection have been ruled out.

The major complication of COPD is development of Cor Pulmonale. In this condition, there is right heart failure due to chronic hypoxia and tissue damage and pulmonary vasoconstriction. This is one of the complications of COPD and around 5-10% of patients develop severe pulmonary hypertension and right heart failure and deteriorate clinically[50]. Patients may develop ankle swelling, shortness of breath and cyanosis and progress to right heart failure and death. There are different tests to classify and measure COPD severity for e.g. pulmonary function testing and HRCT.

1.1.15. Measurement of COPD severity

Lung function decline is a common feature in patients with COPD. It is important to choose correct definition to diagnose a defect especially in obstructive ventilatory defect. This is because according to the American Thoracic Society and the European Respiratory Society, obstructive defect is defined as occurring when the ratio of FEV_1 to the slow expiratory vital capacity (VC) is below the lower limit of normal[51]. However, according to GOLD and the British Thoracic Society, it is $FEV_1/FVC<0.70$ and $FEV_1< 80$ percentage predicted. Therefore, definitions are crucial when diagnosing the defect.

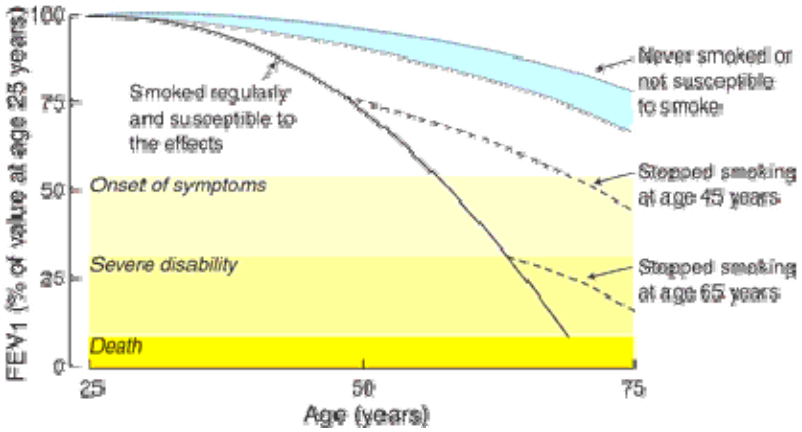

Table 1.2 CM Fletcher, R Peto, CM Tinker graph demonstrating FEV1 decline in health and disease [52]

Historically, the rate of decline in respiratory disease was first developed by Fletcher and Peto three decades ago[53]. More recently Anthonisen *et al* and Burrows *et al* observed the prognostic factors in COPD including age, FEV_1, dyspnoea scale, poor bronchodilator response, functional reserve capacity, exercise tolerance, exercise heart rate, smoking and decline in FEV_1 [54, 55]. Similarly there are other studies who have identified additional factors such as asthma, diet and other comorbid conditions which are associated with decline in lung function[56-58]. David *et al* studied the lung function decline as a predictor of morbidity and mortality in a huge population with and without COPD. They found that rapid lung function decline was independently associated with a modestly increased risk of COPD hospitalization and death[59]. Another interesting observation in this study was that, having a more rapid decline in lung function was a stronger predictor of death or COPD related hospitalizations among subjects who had GOLD stage 0 disease than with more severe disease.

There are many studies in the literature showing a relationship between lung function decline and mortality, but it is not clear whether this is independent of smoking. There are other studies which have examined the association between the rate of decline and total mortality during a long term follow up[60]. The Honolulu Heart Program looked at 17-year mortality in a follow up study and found that the change in rate of decline in lung function is associated with mortality in subjects with debilitating illnesses and smokers. They suggested that smoking is a confounder; however, they found that FEV_1

decline by itself might contribute to the causation of the illness, which ultimately causes death.

1.1.16. High resolution computed tomography (HRCT) in COPD

The development of HRCT has brought new dimensions in understanding lung disease in recent times. The scanner acts as a densitometer and the reconstructed image enables the viewer to identify low attenuation areas consistent with the loss of lung tissue that accompanies emphysema. The contrast between the solid bronchial walls and the air within the lumen enables the bronchi to be visualised with a diameter as low as 1 mm. The pattern of low density areas enables a diagnosis of centrilobular and panacinar emphysema to be made with reasonable accuracy [61].

Peripheral airway dysfunction, assessed by expiratory HRCT measurements, is a determinant of COPD severity[39]. There are many advantages of HRCT including diagnosis of bronchiectasis and interstitial lung disease. A further advantage of HRCT scanning is that densitometric analysis allows a quantitative measure of emphysema to be made, and this matches well with the macroscopic and microscopic assessment of pathological specimens[62].

1.1.17. Lung function decline in other major diseases

Lung function decline is a very gradual process, which can be accelerated by various factors that affect the lungs including smoking. Some of the diseases where this could be seen are discussed below.

1.1.18. Diabetes Mellitus

Pulmonary involvement in diabetes has been reported sporadically for many years. Chronic hyperglycaemia leads to wide spread non-enzymatic protein glycosylation and micro and macrovascular end organ dysfuction[63-65]. Diabetic microangiopathy targets the lung as it does to other organs.[66] With the introduction of inhaled insulin, there is recognition that the lung can be involved in diabetes. Even though the respiratory dysfunction with diabetes is subclinical and rarely do the patients have any respiratory symptoms, there are

several reasons to recommend a pulmonary assessment in diabetics: (1) it is non-invasive, (2) there may be subclinical loss of pulmonary reserve in stress, aging, hypoxia, volume overload, cardiac and renal failure (3) it is largely independent of physical fitness, (4) it is simple to measure and could provide useful information about systemic microangiopathy, (5) it can provide information regarding the long term effects of inhaled insulin on the lungs[66]. Up to one third of subjects with diabetes can be shown to have abnormal ventilator response to hypoxia, hypercapnia, or exercise. This is consistent with autonomic neuropathy which is depressed cholinergic bronchomotor tone and neuroadrenergic response[67]. Patients with diabetes without a smoking history consistently demonstrate a modest restrictive defect with proportional 8-20% reductions in lung volume, FVC, FEV_1, and vital capacity when compared with patients without diabetes[68-74]. Elevated fasting blood glucose on its own is associated with reduced lung function[75].

1.1.19. Cardiovascular disease

FEV_1 is associated with mortality in respiratory and cardiovascular diseases[76]. In a study by Ebi-Kryston *et al* found that, FEV_1 <65% predicts higher mortality. The 15 year mortality for men in the Renfrew and Paisley study increases from 15% among lifelong non-smokers to 28% among those smoking 20 cigarettes a day with a good FEV_1 of 48% for those smoking 20 cigarettes a day with a poor FEV_1 [77].

There are many confounders to COPD severity. The important ones are age, smoking and gender. The affinity to cigarette smoke is different in men and women, which increases the morbidity and mortality in smokers.

1.1.20. Pathogenesis of COPD

Cigarette smoke is the main cause for developing COPD. Inhalation will cause both local[34, 78, 79] and systemic inflammation[80-82]. There are cellular and structural changes in both peripheral and central airways in COPD and the inflammatory process extends to the lung parenchyma and pulmonary arteries. There is evidence to show that the cigarette smoking can elicit an inflammatory reaction involving the entire tracheo-bronchial tree even in the absence of established airflow limitation[83 84].

The central airways of smokers have shown that T lymphocytes and macrophages are the predominant cells infiltrating the airway wall, where as neutrophils, which are scarce in the airways wall are increased in the airway lumen[83, 85]. Analysis of the cell profile in alveoli and small airways show an increase in all of the cell types implicated in COPD, including macrophages, T-lymphocytes, B-lymphocytes and neutrophils[86]. Early lesions in the peripheral airways are inflammatory cell infiltration into the wall, predominantly mononuclear cells and clusters of macrophages in the respiratory bronchioles[84]. In established COPD patients the development of airflow limitation is associated with further increase of macrophages and T lymphocytes in the airway wall and neutrophils in the lumen[87-89].

1.1.21. Neutrophils in COPD

Neutrophils are increased in the airways in patients with COPD, including the submucosal glands[90], indicating an important role in the development of mucus hypersecretion in COPD. Increased interleukin-4 gene expression by large numbers of inflammatory cells infiltrating the bronchial sub epithelium and sub mucosal mucous secreting glands of smokers with chronic bronchitis has also been observed [91]. In smokers there is a strong association between the accelerated decline in lung function and increased number of neutrophils in the airway lumen[92]. In subjects with a more rapid decline in FEV_1, neutrophils exhibited increased expression of CD11B/CD18, an important activation marker of neutrophils which acts as an adhesion molecule whose ligand is intercellular adhesion molecule (ICAM)-1 [93].

Neutrophils produce amongst other chemokines, the ligands for CXCR3, CXCL10, CXCL9, and the IFN-□-inducible T cell chemoattractant CXCL11[94, 95]. There is involvement of the CXC and other chemokines in the inflammatory processes associated with COPD. There is evidence to show that the CXC family plays a role in pathogenesis and remodelling in COPD. Cytokines of the CXC family not only have a role in the control of inflammation but also in angiogenesis as a result of shared expression of their specific receptors by leukocytes and endothelial cells [96].

1.1.22. Role of Cytokine and Chemokines in COPD

Recent advances have shown insights into cytokine-regulated control of inflammatory processes. Most cytokines exert their function in a complex network where one cytokine can influence the production of and respond to another. Their important role is in inflammatory processes, in response to injury or infection. The disruption of their homeostasis can lead to local or systemic pathology as demonstrated in various chronic inflammatory conditions[97, 98] .

TNF-α plays an important role in chronic diseases and wasting disorders such as COPD emphysema, cachexia, cystic fibrosis, chronic heart failure [82]. Increased levels of TNF-α are seen in patients with severe COPD. Increased levels of IL-6 are found in exhaled breath condensate of patients with COPD[99], and stimulated primary cultures of human bronchial epithelial cells produced more IL-6 than unstimulated cells from patients with COPD[100].

Inflammation is characterized not only by upregulation of proinflammatory cytokines but also of the antinflammatory cytokine inhibitor including their soluble receptors. These include IL-10, transforming growth factor (TGF)-β[101], IL-11 and IL-1 receptor antagonist, which are released to limit the duration and extent of inflammatory responses. Limited data is available on these anti-inflammatory proteins in COPD. One study reported significantly lower levels of IL-10 and IL-10-positive cells in sputum of patients with COPD [102].

The role of chemokines is important in the pathogenesis of COPD. Smokers with COPD have increased numbers of CXCR3+ cells in the peripheral airways and these cells are CD8+ and produce interferon (IFN), which suggests a predominant role for CD8+ cells in the pathogenesis of COPD. In addition, such findings indicate that the CXCR3/CXCL10 axis may be involved in the recruitment of Th1 T cells to the peripheral airways. Recent data have shown the involvement of airway smooth muscle in the inflammatory response of patients with COPD. Thus, the airway smooth muscle may also serve as a source of CXCL10 in inflamed airways. Human airway smooth muscle cells release a variety of pro-and anti-inflammatory mediators such as IL-8 and granulocyte colony-stimulating factor (GM-CSF), an important survival and activation factor for neutrophils [103-105].

1.1.23. Role of inhibitory cytokines

Inhibitory cytokines are released to limit the duration and extent of inflammatory responses. They include IL-10, transforming growth factor TGF-β, IL-11, and IL-1 receptor antagonist. TGF-β is a multifunctional growth factor that modulates cellular proliferation and induces differentiation and synthesis of extracellular matrix proteins including collagens and fibronectin in many types of cells. It is also a potent chemotactic factor for mononuclear phagocytes, mast cells and T lymphocytes[106-109]. In the small airway epithelium from smokers and patients with COPD, it is reported that the levels of TGF-β1 mRNA are significantly higher compared with non-smokers and showed positive correlation with the burden of cigarette smoking.

The level of TFG-β, gene expression correlates with the degree of peripheral airway obstruction and cultured airway epithelial cells from smokers with or without COPD. There is an increased release of total TGF-β1 protein in COPD compared with non-smokers[110]. It has been reported that in subjects with COPD there is higher expression of TGF-β1 mRNA and protein in airway and alveolar epithelial cells as compared with subjects without COPD. Higher expression of TGF-β receptors has also been observed in macrophages from COPD subjects[107-109, 111]. The higher expression of TGF-β1 in bronchiolar epithelial cells correlated positively with both increased numbers of macrophages and mast cells present in the broncho alveolar epithelium in COPD current or ex-smokers. This suggests that TGF-β1 is implicated in the recruitment of macrophages and mast cells into the airway epithelium in COPD[111].

1.1.24. Inflammation in COPD

Increasing evidence suggests that the clinical features of COPD and airflow limitations are poorly correlated and there is a need for a comprehensive approach which may include imaging[112], assessment of exercise tolerance and body mass index[113]. There is evidence to show that COPD is not just a disease of the lung but also a systemic disease. Cigarette smoke causes not only inflammation in the lung but it also plays a major role in systemic cellular and humoral inflammation, oxidative stress, changes in vasomotor and endothelial function and increased circulating concentrations of procoagulant factors[114, 115].

COPD patients show changes in local and systemic inflammatory markers, which affect the disease progression.

1.1.25. Local inflammation

Inflammation caused by smoking is thought to create an imbalance between oxidative and antioxidative factors, locally in the lungs. This imbalance leads to oxidative stress, which has been hypothesised as the most important pro-inflammatory event in the pathogenesis of COPD[116].

Markers of oxidative stress and inflammation include interleukin (IL)-8, tumour necrosis factor-alpha (TNF-α), nitrous oxide, hydrogen peroxide, and isoprostanes, which may be measured in induced sputum, blood, expired air or bronchoalveolar lavage fluids[117, 118]. To date no epidemiological studies have so far included these specific measures. It is possible these could indicate early changes or even act as markers of susceptibility in subjects with normal lung function.

The association of an abnormal inflammatory response of the lungs to noxious gases with airflow limitation in COPD indicates the important role of inflammatory process in the pathogenesis of COPD. Airway inflammation is the key feature in COPD and it is thought to have a role in the pathogenesis of the disease[119]. There is a marked increase in neutrophils and inflammatory mediators such as IL-8 and soluble tumour necrosis factor receptor P55 (s TNF R55) in bronchoalveolar lavage fluid and sputum from patients with COPD and these correlate with increasing disease severity[120]. Immunohistochemical staining of bronchial biopsies from patients with mild disease shows infiltration of macrophages and CD8+ T cells and this infiltration was associated with airflow limitation. In contrast, influx of neutrophils and macrophages into the lungs was reported to be associated with severity of obstruction in patients with severe disease [121, 122].

Enhanced levels of the extracellular matrix compound hyaluronan are found in the sputum of patients with mild to severe COPD[123]. The presence of hyaluronan seems to be related to disease severity and to markers of local inflammation. Hyaluronan has also been shown to be a sensitive marker for COPD with increased levels seen in a group of patients with mild COPD in

which IL-8, s TNFR55 and s TNFR75 are not enhanced. There was also an increase in sputum neutrophils in smokers suggesting local inflammation.

1.1.26. Systemic inflammation

Many studies have shown convincingly that COPD is associated with increased levels of several proinflammatory cytokines including tumor necrosis factor alpha and its soluble receptors sTNF-R55 and sTNF-R75, interleukin-6 and interleukin-8, acute phase reactants such as C-reactive protein, oxidative stress and activation of several inflammatory cells specifically neutrophils, monocytes and lymphocytes[115, 124].

The origin of the systemic inflammation present in patients with COPD is still poorly understood, and several independent pathways may be involved. As smoking causes many extra pulmonary effects, cigarette smoke alone may contribute to systemic inflammation. Both systemic oxidative stress and peripheral vascular endothelial dysfunction have been reported in passive smokers and smokers with few pack years [125, 126].

Vernooy *et al* studied a group of subjects with mild to moderate[127] COPD and found that patients with COPD showed significant increase in neutrophil count and levels of soluble (s) TNF-R55 and IL-8 in sputum as compared with control subjects, whereas sputum sTNF-R75 levels tend to be higher in COPD. Sputum TNF-α level was similar in both groups. When comparing sTNF receptors in sputum and plasma, no direct correlation was found despite elevation of circulating sTNF-R75 levels in patients with COPD. In addition, sputum sTNF receptors was inversely related to the FEV_1 in patients with COPD, whereas circulating sTNF receptors were not, suggesting different regulation of inflammation in the pulmonary and systemic compartment. When subjects were divided according to their current smoking status, levels of sTNF-R55, sTNF-R75, and IL-8 in sputum were significantly elevated in ex-smokers versus currently smoking patients with COPD, suggesting ongoing inflammation in airways and circulation of patients with COPD after smoking cessation.

Recent study by Michel *et al*[128], where they subjected healthy people to inhalation of the proinflammatory stimulus, bacterial lipopolysaccharide, showed differential changes in body temperature, airway reactivity and FEV_1. Subjects with elevated body temperature showed increases in the inflammatory

response whereas those with an airway hyperreactivity showed an increase in airway inflammation, but no systemic inflammation, suggesting that the responses are independent. The evidence so far suggests that these processes in the local and systemic compartment are regulated differently.

Systemic inflammation is increasingly recognised as a risk factor for a number of different diseases including atherosclerosis[129], cachexia[130], anorexia[131] and osteoporosis[132]. All of the above changes are commonly observed in patients with COPD[133-138]. However, there is debate as to whether systemic inflammation is present in stable COPD and whether it is wholly or partially responsible for these associations. Studies, which addressed this issue, were all of very weak statistical power.

A systematic review carried out by W Q Gan *et al* on the association between COPD and systemic inflammation found that when compared with healthy controls, patients with chronic airflow limitation had significantly raised levels of serum CRP, fibrinogen, leucocytes and TNF-α indicating that persistent systemic inflammation is present in COPD[139]. Among ex smokers with chronic airflow limitation, there was still evidence of a low-grade systemic inflammation. This observation suggests that once patients develop COPD, smoking cessation may not fully halt the inflammatory process.

The presence of systemic inflammation in COPD is linked with various types of complications including weight loss[140-142], cachexia[130, 131], osteoporosis[131, 132] and cardiovascular diseases[133, 135, 143]. An interesting observation by Dahl *et al* suggests that individuals with increased inflammatory markers such as fibrinogen have an accelerated decline in lung function and have an increased risk of COPD exacerbations and hospitalization in the future [144].

1.1.27. Markers of systemic inflammation

The use of exhaled markers and markers measured systemically in blood could provide a means of measuring the extent of systemic involvement at different stages of the disease. In established COPD, a number of non-specific markers of ongoing inflammation are available. Neutrophils are increased in the airways of smokers[145] and patients with COPD[146]. If compared with local inflammation there is data from a small study in smokers and ex smokers that indicates an increase in the number of neutrophils in the sputum is associated with an accelerated decline in lung function[147], and a similar observation has also been reported among smokers in an (aluminium smelter workers) occupational

cohort[148]. In most studies, neutrophils provide a crude marker of generalised inflammation, not least because of the large-individual variation in leukocyte count.

1.1.28. C-reactive protein (CRP)

CRP is an acute phase reactant protein measured in plasma, which is synthesised by the liver in response to inflammatory stimuli. CRP is elevated in patients with stable COPD, regardless of severity[149-151]. This is also a biomarker for low grade inflammation in stable COPD[152]. In patients with acute exacerbation after treatment there is a decrease in CRP[149] levels. Schols *et al* demonstrated increased levels of CRP and lipopolysaccharide binding protein in patients with stable COPD, and this was more pronounced in a subset patients with COPD with an increasing resting energy expenditure and decreased fat-free mass[149, 150].

1.1.29. Fibrinogen

Fibrinogen is synthesised by hepatocytes and is an acute phase reactant and a blood-clotting factor, released into the circulation in response to the cytokine IL-6, which is produced by macrophages and the airway epithelium. Elevated levels have been reported in patients with stable COPD and in exacerbation[149, 153, 154].

1.1.30. Interleukin-6

IL-6 is the primary regulating cytokine of the acute phase reactant α1-antitrypsin, which is also considered the major inhibitor of neutrophil elastase in the lower respiratory system. IL-6 levels are reported to be high in acute exacerbation of COPD[155]. It has a well-recognised role as a hereditary risk factor for emphysema; measures of α1-antitrypsin can also indicate ongoing inflammation in COPD.

1.1.31. Tumour necrosis factor (TNF) – α

TNF-α is a potent pro inflammatory cytokine which exerts its activities by interaction with two structurally related but functionally distinct transmembrane receptors TNF-R55 and TNF-R75[149, 156]. TNF-α co ordinates the inflammatory process by stimulating increased expression of adhesion molecules on leukocytes and endothelial cells, with up regulation of other pro inflammatory cytokines such as IL-1 and IL-6, and inducing angiogenesis.

Increased levels of TNF-α in induced sputum in patients with severe COPD have been reported [157]. As well as increased levels of TNF, patients with COPD also have significantly higher levels of soluble TNF-R55 in sputum compared with control subjects and soluble TNF-R levels are related inversely to FEV_1 in patients with COPD[158]. These soluble receptors are also considered pro-inflammatory markers because they are shed from the cell membrane in response to endogenous TNF-α and other inflammatory mediators. Higher levels of TNF-□ in spontaneously expectorated sputum has also been reported in patients colonized with Haemophilus influenza in comparison with non colonized patients[159].

1.1.32. Determinants of COPD severity

1.1.33. Gender differences in COPD

The prevalence and mortality from COPD in females are on the rise compared to men[160]. It is also seen that females are more likely to report with symptoms compared to men[161-163]. Females also show greater baseline hyperresponsiveness to methacholine[164]. A study from Copenhagen showed greater impact of smoking on lung function and higher risk of hospital admission in women than in men[165, 166]. One of the reasons for this rise could be due to heavy smoking by women for e.g.Danish women are among the heaviest smokers amongst females in Europe[167].

Most of the studies are based on the lung function but there are concerns how lung function decline should be compared between genders. Some of the suggestions are that FEV_1 decline should not be taken as gold standard but studies are needed which are focused on other measures of morbidity. Prescott *et al* believed that hospital admission is an appropriate measure of the impact

of the disease and it is a more sensitive measure of morbidity with less delay[166].

Excess weight gain has been linked to reduction in lung function particularly FVC and this effect is greater in men[168] . This may be due to the central fat distribution in men compared to women[169]. Raida *et al* showed that body fat distribution has independent effects on lung function that are more prominent in men than in women[169].

There are studies which have investigated into the differences in small airways function in COPD emphysema. Martinez *et al* reported recently that in patients with severe COPD, women have an anatomically smaller airway lumen with disproportionately thicker airway walls relative to men[170]. They also showed that emphysema is less extensive with smaller hole size and less peripheral involvement.

1.1.34. Gene expression in COPD

The development of gene array technology in measuring hundreds of genes has changed the understanding of the many disease and the gene interactions. These methods can be used to screen for differentially expressed genes in disease state[171]. Epithelial cells play an important role in airway inflammation[172]. Airway epithelium is the first source of contact for the cigarette smoke and smoke is a rich source of oxidants [173], some of which are very reactive. Many studies have shown that there is an increase in oxidative stress in smokers including increased concentrations of H2O2 [174].

Pierrou *et al* studied the mechanism of oxidant/antioxidant stimuli and gene expression in smokers with and without COPD[175]. They hypothesised that the epithelial expression of genes in oxidant/antioxidant responses in smokers are different from non-smokers and also COPD patients may express different genes in addition to those expressed by smokers without COPD. Their observation was that cigarette smoke induces different oxidant responses but not in a linear pattern in patients with COPD.

Schulz *et al* showed that melanoma cell adhesion molecule (MCAM), a member of the immunoglobulin super family, is differentially expressed and induced in primary bronchial epithelial cells derived from patients with COPD[176]. MCAM is the single gene identified which is unregulated in primary bronchial epithelial cells. This was a comparison between ex smokers with

COPD and current smokers without COPD. It was thought that smoking might not be the reason for the expression; rather the results may indicate that MCAM expression may be related to pathophysiology of the disease. The important role of MCAM in different cell systems and its engagement in cell-cell interactions show the possibility that it contributes to airway remodelling in subjects with COPD. Recent studies have shown increased epithelial gene expression levels of transforming growth factor-β1[177], monocytes chemoattractant protein-1and interleukin-8 in COPD patients[178].

Cell adhesion molecule expression under inflammatory conditions are well known such as ICAM-1 in psoriasis[179]. In primary explant cultures, human bronchial epithelial cells from COPD patients seem to release high level of ICAM-1 compared to smokers without COPD when exposed to cigarette smoke[180]. ICAM-1 also serves as a receptor for activated T cells[180, 181]. This up regulation may suggest the recruitment and migration of these cells into the airway lumen[101].

There are many uncertainties about gene expression in patients with COPD, like differences in expression between men and women, the different pathways involved the role of gene polymorphisms and expression in patients with COPD who have never smoked.

1.2. Metabolic syndrome (MeS)

1.2.1. Definition

The MeS has also been called by various names in the past like syndrome-X and sometimes insulin resistance syndrome. Central obesity, insulin resistance, dyslipidemia, and hypertension characterize this syndrome. These features increase the risk of cardio vascular morbidity and mortality. Many expert groups have attempted to develop a unifying definition for MeS. However, most widely accepted and used definitions are International Diabetic Federation (IDF), World Health Organisation (WHO), The European Group for the study of Insulin Resistance (EGIR) and the National Cholesterol Education Programme-Third Adult Treatment Panel (NCEP-ATPIII). In my study, I have used the IDF and NCEP-ATP III definitions for diagnosis and for the analysis of the data.

According to the IDF for a person to have MeS, individuals should primarily have increased central obesity, which is ethnic specific (for Europeans

and American (≥ 94cms for men and ≥ 80cms for women) plus any of the two of four additional factors. The four factors are (1) raised triglycerides ≥ 1.7 mmol/l (150mg/dl) (2) reduced HDL-cholesterol ≤ 1.03 mmol/l (40mg/dl) in males and ≤ 1.29 mmol/l (50mg/dl) in females or specific treatment for these abnormalities,(3) raised blood pressure ≥130/85mmHg or on treatment for diagnosed hypertension and (4) raised fasting plasma glucose ≥ 5.6 mmol/l (100mg/dl) or previously diagnosed type-2 diabetes[182].

NCEP-ATP III definition was a slightly differed in two major ways. Firstly it didn't include a measure of insulin resistance as a component (Included in WHO and EGIR) and second treated glucose abnormalities as of equal important with other components in diagnosing MeS. It also includes waist measurement (≥ 102cms for men and ≥ 88cms for women)[182] slightly higher than that in IDF .

1.2.2. Epidemiology and prevalence

MeS is highly prevalent in adults globally but particularly in affluent westernised countries. A quarter of the US population aged 25yrs and above fit into ATP-111 criteria for MeS. There is also an increasing trend in children and adolescents [183]. There is 3 fold higher rate of mortality from coronary artery disease in subjects aged 41-60yrs with MeS compared with those without MeS when studied for more than 10years[184]. A very consistent finding is that MeS is age dependent, until recently with the increase rates of obesity in young people making clear that the disease can begin at any age group. According to the American Heart Association, three groups of people often have MeS, (1) people with diabetes who cannot maintain a proper level of glucose (glucose intolerance) (2) people without diabetes who have high blood pressure and secrete large amounts of insulin (hyperinsulinemia) to maintain blood glucose levels (3) heart attack survivors who have hyperinsulinemia without glucose intolerance.

MeS has become increasingly common in the United States. The prevalence in U.S. adults ≥20years of age was recently estimated to be 23.7% [185], and it is estimated that around 47 million U.S. adults suffer from this problem. The prevalence of MeS increases with age, reaching 43.5% and 42.1% for those 60-69 and ≥70 years of age respectively. MeS is closely associated with insulin resistance, where the body is unable to utilize insulin efficiently, hence MeS is also called the insulin resistance syndrome. Acquired factors such

as excess body fat and physical inactivity can elicit insulin resistance and MeS in these people. The biologic mechanisms at the molecular level between insulin resistance and metabolic risk factors are not fully understood, and appear to be complex.

1.2.3. Classification of MeS

There are many definitions and clinical classification for this vastly researched syndrome. The important and accepted worldwide are the International Diabetic Federation (IDF) and the National Cholesterol Education Programme Adult Treatment Panel 111(NCEP ATP111) classification and are discussed in detail below

1.2.4. International Diabetic Federation definition (IDF)

The IDF is an international body comprising 150 countries and its main objective is to guide physicians and other health care workers to prevent diagnose and treat diabetes in a way, which is uniform, and evidence based. The federation is committed to raise awareness globally for prevention and treatment of diabetes and working towards a cure for diabetes. According to IDF MeS is defined as having an abnormal waist (central obesity) circumference and two out of the other four variables, which are in the table 1.2.

Features of MeS	I D F	NCEP-ATP III
Raised triglycerides	≥150 mg/dl(1.7 mmol/L) or specific treatment for this abnormality	≥ 1.7 mmol/l (150mg/dl)
Raised blood pressure	≥130/85mmHg) or specific treatment for this abnormality	≥ 130/85 mmHg
Reduced HDL	≤ 40 mg/dL (1.03 mmol/L) in males ≤ 50 mg/dl (1.29 mmol/L) in females or specific treatment for this lipid abnormality	≤ 1.03 mmol/l (40mg/dl) Men ≤ 1.29 mmol (50mg/dl)
Raised fasting blood glucose	≥ 100 mg/dl (5.6 mmol/L), or previously diagnosed type 2 diabetes	≥ 5.6 mmol/l (100mg/dl)
Waist circumference	≥ 94 cms –Men ≥ 80 cms – Women	≥102cms(>40inches)Men ≥88cms(>35inches)Women

Table 1.3 Classification of features of MeS according to IDF and NCEP-ATPIII criteria.

1.2.5. National Cholesterol Education Programme Adult Treatment Panel 111 (NCEP ATP111)

There are many studies that show that there is an increase in coronary artery disease, which are not controlled, and a large proportion of patients whose lipids are not measured. The patients who are on lipid lowering medications

also did not attain the target. Therefore, NCEP produced recommendations for the global assessment for lowering cholesterol. The recommendations were that the high-density lipoprotein (HDL) should be secondary target in patients with increased triglyceride levels. Although low density lipoprotein (LDL) is generally considered as a primary target for treatment for the first time NCEP ATPIII classifies as a secondary target[186]. The recommendations are described in the table 1.3 below, and any three of the five features will be considered indicative of MeS.

Features	Male	Female
Waist circumference	>102cms (>40inches)	>88cms (>35inches)
Blood Glucose	≥110 mg/dl(6.1mmol/l)	≥110 mg/dl(6.1mmol/l)
Triglyceride level	≥150 mg/dl(≥1.69mmol/l)	≥150mg/dl(≥1.69mmol/l)
HDL level	<40mg/dl(<1.03mmol/l)	<50mg/dl(<1.29mmol/l)
Blood pressure	≥130/85mmHg	≥130/85mmHg

Table 1.4 NCEP ATP-III classifications of features of MeS in men and women

1.2.6. Clinical features

It is unusual to diagnose MeS early in life. The features of MeS are central obesity (excessive fat tissue in and around the abdomen), atherogenic dyslipidemia with high triglycerides and low HDL cholesterol, raised blood pressure (130/85 mmHg or higher), insulin resistance or glucose intolerance, prothrombotic state (e.g., high fibrinogen or plasminogen activator inhibitor in the blood), and proinflammatory state (e.g., elevated high-sensitivity C-reactive protein in the blood).

1.2.7. Pathogenesis of MeS

The exact cause of MeS is not yet known, however, most researchers believe it is caused by a combination of genetic and lifestyle choices, including the types of diet and level of physical activity.

MeS elicits a series of biochemical changes. Over time, these changes lead to the development of one or more associated medical conditions. The sequence begins when insulin, a hormone excreted from the pancreas, loses its ability to make the body's cells absorb glucose from the blood. When this happens, glucose levels remain elevated after eating. The pancreas, sensing a high glucose level in blood, continues to secrete insulin. Loss of insulin production may be genetic or secondary to high fat levels with fatty deposits in the pancreas. Dyslipidemia may result from raised plasma triglycerides, decreased HDL cholesterol, raised remnant lipoprotein, increased apolipoprotein B, small particles of low-density lipoprotein and HDL.

Hypercoagulability is another important aspect of MeS and is measured by raised plasma plasminogen activator inhibitor-1 and fibrinogen levels. MeS is also known to be a proinflammatory state (sub clinical Inflammation), however, clinically seen are elevated C reactive protein, elicited by inflammatory cytokines such as IL-6,TNF released from excess adipose tissues which are present in most subjects with MeS[187, 188]. Clinically there are no immediate physical symptoms and the syndrome's associated medical problems develop over time.

1.2.8. Complications of MeS

Consistently high levels of insulin and glucose are linked to many harmful changes including: (1) damage to coronary and other arteries, a key step towards the development of heart disease or stroke, (2) changes in the kidney's ability to remove salt, leading to high blood pressure, heart disease and stroke, (3) increase in triglyceride levels, resulting in an increased risk of developing cardiovascular disease, (4) increased risk of blood clot formation, which can block arteries and cause heart attacks and strokes, (5) slowing of insulin production, which can signal the start of type 2 diabetes, a disease that

may increase the risk for a heart attack or stroke, and may damage the eyes, nerves or kidneys.

1.2.9. Obesity, insulin resistance and dyslipidemia

Increased levels of free fatty acids, which are derived from adipose tissue in MeS, accelerate hepatic synthesis of very low-density lipo proteins (VLDL). Reduced triglyceride clearance may also contribute to dyslipidemia in diabetes via a relative decrease in lipoprotein activity[189]. The reciprocal relationship between elevated VLDL triglycerides and low HDL levels is driven primarily by the action of cholesterol ester transfer protein, which mediates the transfer of excess triglycerides from VLDL to HDL compounds. Increased HDL turnover has been documented in type 2 diabetic patients[189]. Increased triglycerides are also implicated as an underlying cause for the production of small particles of LDL cholesterol that are more easily oxidized, hence more atherogenic, and less readily cleared[189]. In addition, excess triglyceride levels give rise to highly atherogenic, cholesterol-rich remnants of triglycerides-rich particles[190].

1.2.10. Proinflammatory and prothrombotic state

It is now accepted that inflammation plays an important role in the pathogenesis of atherosclerosis[191]. Excess adiposity is associated with the release of inflammatory adipokines or adipocytokines. These factors may contribute to an increase in the level of C-reactive protein, a marker of inflammation and a recently recognised cardiovascular risk factor[192]. Elevated C-reactive protein has been found in subjects who meet criteria for the MeS[193]. Increased release of inflammatory adipokines may also mediate increases in fibrinogen and plasminogen activator inhibitor (PAI-1) levels. These findings are also reported in subjects with MeS. Elevated levels of PAI-1 may reflect impaired fibrinolysis and thus may be associated with the increased risk for arterial thrombosis which is a clinical association between increased PAI-1 levels and insulin resistance is also well established[194].

People with MeS are at increased risk of coronary heart disease, type 2 diabetes and other diseases related to plaque build-ups in artery walls for e.g. stroke and peripheral vascular disease[195]. Similar lipid changes in selective

hypothalamic neurons regulate insulin action and glucose homeostasis, in addition to food intake and body weight. This approach is integrated with the analysis of inflammatory reactions associated with end organ damage.

Free fatty acids provoke insulin resistance. In striated muscle, the accumulation of intramyocellular lipid inhibits the uptake of glucose, the synthesis of glycogen through the activation of protein kinase C and oxidation of glucose. Free fatty acids also inhibit the insulin-mediated suppression of hepatic glycogenolysis[196]. Leptin plays a protective role against obesity, at least in the early stages of weight accumulation by setting the plateau of body weight and by inducing compensatory oxidation of free fatty acids[197].

1.3. Relationship of MeS with other diseases

1.3.1. Cardiovascular disease and mortality

The clinical features of MeS are associated with an increased risk of cardiovascular disease[198], including the greater risk of coronary disease for a given level of low-density lipoprotein and cholesterol[199] and premature death[200]. MeS consisting of a disorder of lipid storage, insulin resistance and hypertension is extremely significant in the pathogenesis of cardiovascular diseases[201-205].

Although obesity is not a required element for the diagnosis of the MeS, there is considerable evidence that visceral intra abdominal fat, which increases with age [206], is closely linked to insulin resistance. This pathophysiologic relationship is consistent with the emphasis on abdominal girth in the NCEP-ATPIII criteria and waist/hip ratio in the criteria of the WHO, rather than body mass index as an indicator of obesity-related cardiovascular risk[207]. In fact, even in normal-weight subjects, increased waist circumference is associated with increased risk [208].

Several studies have attempted to quantify the positive association between the MeS and cardiac morbidity. For example Alexander *et al* reported that 19.2% of patients with MeS and type-2 diabetes had prevalent coronary artery disease based on the National Nutrition Examination Survey (NHANES III), compared to 13.9% with MeS but no diabetes and 7.5% with diabetes but no MeS[198]. Similarly, Girman *et al* found an approximately 50% greater risk of

major coronary events in patients with the MeS across two large clinical trials[209]. In both, low levels of HDL cholesterol were found to be associated with an increased risk of developing MeS a finding also supported by analysis of data from NHANES.

Several proinflammatory mechanise MeS contribute to atherosclerosis. These include monocyte recruitment and accumulation in vascular lesions as well as differentiation and activation of macrophages[191]. It is clear that obesity drives a proinflammatory state, contributing to the development of insulin resistance, glucose intolerance, and atherogenesis.

The pioneering work of Hotamisligil *et al* first established that TNF-□ was expressed in adipocytes, that its expression was induced in obese states, and it could contribute to systemic insulin resistance[210]. In addition the mechanism involving inhibition of proximal insulin signalling steps, and the attenuation of peroxisome proliferators activated receptor gamma activity have been implicated as the cause of TNF-□ induction of insulin resistance[211]. Several other proinflammatory molecules, including interlukin-6, serum amyloid A3, α1 acid glycoprotein, and monocyte chemo attractant protein-1, are expressed in adipose tissue and are potentially induced in response to obesity and diabetes[192].

Recent studies using gene microarray techniques found a broad proinflammatory gene expression program in adipose tissue from obese rodents[212]. The fact that these effects appear to precede the development of insulin resistance during high-fat feeding further supports the notion that adipose-derived inflammatory factors have a causative role in the development of insulin-resistant type-2 diabetes, and MeS.

1.3.2. Hypertension

Elevated blood pressure is a frequent component of MeS, and is more prevalent in subjects with either insulin resistance or obesity. Several studies have demonstrated that even modest weight gain can precipitate the onset of hypertension[213]. Hypertension plays a vital role in cardiovascular mortality and morbidity. It is also one of the important risk factors for stroke. Good control of blood pressure would reduce risk and morbidity and this could be achieved

by primary care physicians via early diagnosis and life style modifications and appropriate pharmacological intervention[214].

New epidemiological data has proved that there is a strong relationship between cardiovascular risk and blood pressure and also the importance of systolic blood pressure as a risk factor[215]. Recent WHO report concluded the importance of blood pressure as a preventable cause of morbidity and mortality in the developing and developed countries[216]. There is also growing evidence that people with borderline or high normal blood pressure will develop into patients with hypertension with age[217]. This observation has prompted the US Joint National Committee to coin a new classification of blood pressure called 'Pre hypertension' referring to those with high normal blood pressure[218]. According to the British Hypertension Society, there is underdiagnosis of hypertension in the UK, and treatment is suboptimal in the majority of the cases. The reason may be the use of monotherapy in treatment[219], which is in contrast to the recommended action.

Proposed mechanisms which link insulin resistance and hyperinsulinemia to increased blood pressure include, (a). Insulin normally mediates vasodilatation; however resistance to this effect has been reported in obese and diabetic patients [220]. Cellular cation transport may also be altered in association with insulin resistance. This effect on either Na/K-ATPase or on Na/Li counter transport has been linked to insulin action, and could have a role in promoting vasoconstriction, (b)Insulin enhances renal sodium retention, and therefore hyperinsulinemia is envisaged as a cause of increased sodium retention[221], (c)Increased hyperinsulinemia driven by the sympathetic nervous system has been documented in association with obesity, although sympathetic activity itself could cause insulin resistance[222]. It could also play a causative role in hyperinsulemia, (d) There is also a molecular link between obesity and the pathogenesis of hypertension, involving components of the renin-angiotensin system that are clearly present in adipose tissues [222].

1.3.3. Non-alcoholic fatty liver disease

Clinical, epidemiological and biochemical data strongly support the concept that non-alcoholic fatty liver disease is the hepatic manifestation of MeS. Insulin resistance is the common factor connecting obesity, diabetes,

hypertension, and dyslipidemia with fatty liver and the progression of hepatic disease to steatohepatitis, fibrosis, and cirrhosis and hepatocellular carcinoma. There may be, as in many other complex metabolic diseases, interactions between the genetics and the environment that determine phenotypic expression of the disease. The prevalence of MeS(22%) and the disease(20%) is similar in the US[223]. This could be the hepatic complication of MeS.

There is a close link between the components of MeS and non alcoholic liver disease[224]. In a study in China they found fatty liver in 48% of patients who had MeS with 39% of them having a body mass index of 25kg/m^2 or greater. They also found that 41% were diabetic and 32% had dyslipedemia[225]. Treatment aimed at reducing insulin resistance may help in preventing this complication of MeS.

1.3.4. Ischemic stroke and transient ischemic attack

The combination of risk factors known to cause MeS has generated a great deal of interest, however prospective data on the syndrome's association with ischemic cerebrovascular events is attracting less attention.

In a study by N.Koren-Morag who looked at more than 14,000 patients, of which 26% fulfilled the criteria of MeS, patients without diabetes exhibited a 1.49-fold increased risk for stroke or TIA, whereas there was a 2.29-fold increase in those who had diabetes[226]. They concluded that the presence of the MeS without diabetes in patients with pre-existing atherosclerotic vascular disease identifies patients at increased risk for ischemic stroke or TIA. The suggestion of more pronounced risk MeS in women needs future investigation[226].

1.3.5. Metabolic consequences (Musculo skeletal effects and adipokines) of COPD

COPD is a multicomponent disease and these components can act both locally (the lungs) and systemically. Furthermore, these effects can manifest in either a structural (including airway remodelling, emphysema, skeletal muscle wasting) or functional nature (Inflammation, apoptosis, senescence).

1.3.6. Muscle wasting in COPD

For many decades, weight loss was thought to be an irreversible component of terminal progression of the disease process. Wasting of body cell mass, the actively metabolising and contracting tissue is an important systemic manifestation. Loss of body mass can be clinically recognised by weight loss in general and loss of fat free mass in particular. In moderate to severe COPD patients who are clinically stable there is 20% depletion of fat free mass and 35% patients who are eligible for pulmonary rehabilitation[227, 228]. In patients with acute respiratory failure, nutritional depletion is reported in up to 70% of the cases[229].

Weight loss particularly loss of fat mass, occurs if energy expenditure exceeds dietary intake. Muscle wasting is a consequence of an imbalance between synthesis and breakdown of protein. Several studies have shown the involvement of systemic inflammation in the pathogenesis of tissue depletion in patients with COPD [230]. It is clear that the body composition is an important indicator and hence may be used as routine screening test for COPD [231].

Wasting is a generally occurring manifestation in many chronic conditions and could be an important systemic manifestation of the disease. Tissue depletion is commonly seen in patients with COPD, its prevalence increases from 20% in clinically stable outpatients to 35% of those patients eligible for pulmonary rehabilitation[232, 233]. Several reports have provided evidence that weight loss negatively affects the prognosis of COPD. Schols *et al* demonstrated that low body mass index (BMI), age and low PaO2 are independent predictors of increased mortality rates [234]. A BMI below 25kg/m2 has an increased mortality rate. Another from Copenhagen City Heart Study prospectively examined whether BMI was an independent predictor of mortality in subjects with COPD[235]. Celli *et al* postulated that assessment of body mass index (B), obstruction (O), dyspnoea (D) and exercise capacity (E) (BODE index) is better predictors of survival in COPD patients[113]

The presence of MeS was frequently observed in patients with COPD who participated in a cardiopulmonary program. Hence, this population should be considered for screening for MeS [236]. Progressive weight loss is characterized by disease-specific elevated energy requirements, unbalanced by dietary intake in many diseases including COPD. Weight gain can be achieved by caloric

supplementation while future studies should prove efficacy of amino acid modulation to stimulate protein synthesis and enhance muscle anabolism.

Disproportionate muscle wasting resembles the cachexia syndrome as described in other chronic wasting diseases (cancer, chronic heart failure, and acquired immunodeficiency syndrome). At present, there is no adequate nutritional strategy available to treat cachexia in COPD. Muscle substrate metabolism has hardly been investigated, but the data available points toward a decreased fat oxidative capacity which may show similarities with the MeS as described in type II diabetes and obesity, and could theoretically benefit from polyunsaturated fatty acid modulation.

To adequately target the different therapeutic options, more clinical (intervention) studies are needed in chronic obstructive pulmonary disease patients that are adequately characterized by local and systemic impairment and in which molecular and metabolic markers are linked to functional outcome [237].

1.3.7. Role of adipokines in inflammation

Leptin is a pleiotropic molecule which regulates food intake, and metabolic, endocrine function[238]. Adipose tissue, gastric mucosa, intestinal epithelial cells, placenta, mammary epithelium, and importantly skeletal muscle secrete it. It is considered a hormone because it regulates the balance between food intake and energy expenditure signalling to the brain the changes in the stored energy. Leptin has an influence on hypothalamic-pituitary-adrenal axis, the reproductive system, hematopoiesis and angiogenesis[239].

Leptin receptors are expressed in the kidneys, lung, adrenal gland, neutrophils, monocytes, and peripheral T-cells. It plays an important role in immunity, inflammation and haematopoiesis[238]. Leptin is an adipokine produced in proportion to the total body fat mass. The other important role of leptin is to assess the total amount of body fat which indirectly reflects food availability, to stop food intake and to increase metabolism[240]. The relationship between leptin levels, body fat in COPD, and between systemic inflammation particularly in patients with emphysema is been reported[238, 241, 242]. It acts independently and not with TNF-α which correlates with BMI suggesting its role in cachexia[243]. Recent data demonstrates that colon epithelial cells are a source

of luminal leptin, which activates NF-kB implicating further that leptin is a proinflammatory cytokine[244]. It has been shown recently by Poulain *et al* and colleagues that leptin levels are raised in obese patients with MeS and COPD[245].

Leptin has a similar structure to the type-1 family of cytokines which includes growth hormone, prolactin, erythropoietin, IL-3, IL-11, IL-2, oncostatin M and G-CSF. A long chain four helical bundle characterizes this family of cytokines structurally. Leptin levels are increased in acute infection and sepsis where it exerts direct effects on CD4+ T lymphocyte proliferation, macrophage phagocytosis and secretion of inflammatory mediators such as IL-1 and TNF-α.[239, 246, 247].

Increased levels of leptin have been observed in the peripheral circulation of COPD patients experiencing an acute exacerbation [248]. Leptin expression correlates well with different GOLD stages of the disease. It is expressed more on the epithelium in healthy people but its expression is more in the submucosa and is co localized mainly with activated T cells and CD8 positive cells and T lymphocytes[249]. Leptin correlates negatively with FEV_1 and FEV_1/FVC ratio signifying the proinflammatory role associated with functional impairment[249].

Adiponectin is an anti-inflammatory adipokine, which is secreted from the adrenal glands and adipose tissues. It is also said to have anti atherosclerotic effect[250, 250]. Elevated circulating adiponectin is observed in underweight patients with COPD. It also correlates with the body weight loss, hyperinflation, and systemic inflammation. Increased adiponectin levels may reduce cardiovascular events in underweight patients with COPD[250].

TNF-α is associated with cachexia[82] and TNF-α along with leptin are elevated in stable and acute exacerbation of COPD. Administration of endotoxin or cytokines like TNF-α or IL-6 produce a prompt dose dependent increase in serum leptin levels.[243].

In the recent past, there has been a lot of interest in understanding the systemic effects of smoking and COPD. There has been increasing evidence to prove COPD is an inflammatory disease. Increased number of neutrophils is found in the sputum and bronchial biopsies of COPD patients, which prove the point. Systemic inflammatory marker like CRP and fibrinogen also shown to play a key role in this process. The main driver for the inflammatory process is smoking. The systemic effect of smoking causes insulin resistance and in turn central obesity.

There are no single studies that address the issue of systemic effects of smoking or COPD causing the development of features of MeS. It is not clear about the mechanism involved in development of MeS in COPD. Inflammation may be driving this process. However, there are other confounders like physical activity, insulin resistance.

Gene environment interactions may be one important aspect, which will help to explore reason for development of both COPD and features of MeS. The answers will help the clinicians in the future to intervene early in the disease process to reduce mortality and morbidity and economic burden on the country.

1.4. Aims of the thesis

The overall aim of my thesis was to elucidate the role of MeS and systemic inflammation in smokers with or without COPD.

The specific aims were:
To establish the prevalence of MeS in healthy non-smoking subjects and in smokers with or without COPD.

To analyse mediators of inflammation in subjects with or without MeS in healthy non-smoking subjects, in smokers with or without COPD.

To identify determinants of lung function decline in patients with COPD through follow up and analysis of their annual FEV_1 decline and correlate the latter with a series of clinical and radiological parameters.

To explore the gender differences in smokers with features of MeS.

My hypotheses were:
Individuals who smoke have a higher prevalence of MeS than non-smokers.

Smokers with COPD have an even higher prevalence of MeS.

Smokers with COPD have more evidence of systemic inflammation as measured by a series of pro-inflammatory markers.

Any association between MeS and COPD would suggest either that MeS contributes to the development of COPD or that COPD causes systemic inflammation and that, in turn, results in MeS.

Neutrophilic airways inflammation is a determinant of lung function decline as measured by FEV_1 and TLco.

2.0 Chapter -Methods

2.1. Subjects characterisation

2.1.1. Subjects in the MeS study

The subjects for this study were recruited mainly from in and around Southampton city. Southampton is a major city in the South east of England with a population of around 230,000. According to the local council, it is the 91st most deprived city out 394 local authorities in UK (1 being most deprived). According to the Health and Lifestyles survey of Southampton population, cigarette smoke is the leading cause of preventable death in Southampton. This is a cross sectional study wherein subjects were recruited from the community. The socioeconomic status of the subjects was not recorded. However, they represented a cross section of the general population in and around Southampton. The publicity for the study was by word of mouth.

150 subjects were approached by providing an information sheet and 141 subjects were ultimately screened, out of which 131 were successfully recruited for the study. The rest of the subjects were excluded, as they did not fulfil the inclusion criteria: (criteria were non-atopic, non-asthmatic and smokers with reversibility of less than 12-15% to 400mcg of inhaled salbutamol). Subjects were also excluded from the study if they had a cold or on inhaled corticosteroids /oral steroids or treated with antibiotics in the previous six weeks for any infection. The subjects were grouped as (a) healthy non-smoker control subjects (n=44), (b) healthy smokers without COPD (n=46) and (c) smokers with COPD (n=41). Subject characteristics are described in Table 3.1. Subjects with COPD as well as the healthy smokers group were current smokers at the time of recruitment with history of >10 pack years. All subjects were non-atopic with minimal reversibility (<10%) in FEV_1 following inhalation of 400 ug of the β_2-agonist salbutamol. All the subjects in the groups were matched for age, sex and BMI

2.1.2. Subjects participating in a follow-up study with smokers and non-smoking control subjects

I inherited a second cohort of subjects from a past research fellow in my supervisor's group. These had been recruited into a study titled 'Differential Gene Expression in the epithelium of smokers with and without COPD and healthy non-smoking controls'[39]. For this present study, these subjects followed up yearly from 2004 until 2007, but as some of them had been first studied in 1999, many more years of follow up data were available. In total 102 were followed up once, of which 70 were also followed up once more after a further year. The analysis was done on 64 subjects comprised of 15 healthy controls, 21 healthy smokers and 24 COPD patients and 4 ex smokers.

All participants underwent medical examination, skin prick testing for airborne allergens, pulmonary function testing and induced sputum. Blood samples were collected and stored for future analysis.

2.2. Study design

2.2.1. Study 1: Prevalence of metabolic syndrome and body composition in smoking and non-smoking subjects.

Subjects attended the department on 2-3 occasions, depending on convenience of the volunteer. The first visit included history taking and full medical examination and fasting blood sample collected for blood glucose, full blood count, lipids, electrolytes, urea, creatinine, liver function test, C-reactive protein, insulin levels, urine albumin. Blood was also stored for cytokine analysis. Subjects underwent full lung function testing, which included FEV_1, FVC, and transfer factor (TLco). On a separate visit, subjects underwent metabolic analyses. For this assessment, subjects' height and weight was recorded and subjects completed quality of life (St George's respiratory questionnaire), the Baecke's physical activity questionnaire. Subjects had their body composition measured by air displacement plethysmography (BODPOD), bioelectrical impedance analysis (BIA), and skin-fold thickness measurements. Skin fold measurements were taken at four sites (sub scapular, biceps, triceps, and supra iliac). Furthermore, blood pressure in supine, sitting, and lying positions were measured. Oxygen saturation on room air was also measured in

all subjects. This study was slightly underpowered (According to the power calculation each group should have 65 subjects to get a significant difference)

2.2.2. Study 2: Inflammatory markers in smoking and non-smoking subjects – the role in metabolic syndrome

All subjects (131) were consented and bloods were taken. Blood was taken to separate serum and plasma. 10mls of clotted and 10 mls of EDTA blood were spun at 3000rpm for 10 minutes. Then plasma and serum was separately stored in 250µl of aliquots and stored at -80^{o}C. These bloods were later used to measure cytokines by multiplex ELISA by Luminex method.

2.2.3. Study 3 Insulin resistance, quality of life, and physical activity in smokers with MeS

This study was designed to understand and explore the relationship between insulin resistance by HOMA-R[251] (The Homeostasis Model Assessment which estimates beta cell function and insulin sensitivity , as percentages of a normal reference population) and smoking in subjects with and without MeS. The study population was divided into three groups as mention in subject characterisation in study 1. All subjects were consented and fasting bloods taken for glucose, lipids, and insulin. Blood was also collected to measure cytokines and adipokines at a later stage. Their lung function and body composition was measured as stated in study 1.

Subjects were asked to complete a quality of life questionnaire (SGRQ) in an environment with no prompting and with adequate time. This questionnaire ranged from 0-100% of possible distress and divided into symptoms, activity, impact scores (total of 76)[252]. They also completed a validated physical activity questionnaire (Baecke's) which measure work activity, sports activity and leisure activity. A total score was then generated, which was used for analysis.

2.2.4. Study 4: Gender influence on features of metabolic syndrome in smoking and non-smoking subjects

Gender differences are common in any chronic disease. This study was designed to understand the various differences in male and female smokers

and their metabolic and inflammatory profile. A Total of 131 subjects were recruited for this study. They were subdivided into three groups, healthy non-smoker controls, healthy smokers or smokers without COPD and smokers with COPD. In healthy controls there were 44 subjects 24 women, 20 men, second group consisted of 46 healthy smokers with 22 women and 24 men, and last group consisted of 41 COPD group with 19 women and 22 men (table 3.1 for details). Subjects were age matched. All subjects underwent similar visits as in study 1. In the second analysis all smokers were grouped (HS + COPD) and divided according to their gender (men=45 and women= 42). The groups were then subdivided into (a) with MeS (men=20, women=8) and (b) without MeS (men=25, women=34).

2.2.5. Study 5: Predictors of rate of decline in FEV_1 in follow up subjects with and without COPD

I inherited a cohort who had participated from a previously done study in my supervisors group. The study was about differential gene expression in COPD and healthy smokers. These subjects had undergone HRCT and densitometric measurements and bronchoscopy to obtain bronchial biopsies. My predecessor did all the analysis of the brushings and biopsies. There was an optional yearly follow-up visit in the study wherein volunteers were invited to participate. Those subjects who agreed to return on an annual basis underwent pulmonary function testing, induced sputum, and an interview about symptoms and quality of life. All of them consented for the study, completed medical history with full physical examination, and skin prick testing. All subjects completed St. George's quality of life questionnaire.

2.3. Ethics approval

The Southampton and South West Hampshire Research Ethics Committee approved all of the above study protocols and all subjects gave written informed consent. All research work was carried out at the Wellcome Trust Clinical Research Facility (WTCRF) at Southampton General Hospital.

2.4. Methodology

2.4.1. Medical examinations and questionnaires

All subjects had a full history taken and underwent complete physical examination. Subjects were requested to fill in St George's Quality of Life Questionnaire (SGRQ)[252, 253], a health-related quality of life questionnaire validated for COPD studies and subjects were also asked to complete the Baecke's physical activity questionnaire. This is a validated questionnaire to assess the level of physical activity in many disease states. It is also designed to assess the different domain of the physical activity[254], which were sports activity, activity at work and leisure activity.

2.4.2. Skin prick tests

Skin prick tests was performed to test allergies for different aeroallergens. This test was done on all subjects and smokers who were positive test was excluded from the study. Merck skin testing solutions by Allergo pharma Joachim Ganzer KG was used for the skin prick testing. The panel of aero-allergen tested were: aspergillus fumigatus, candida albicans, grass mix, tree mix (mid blossoming), dermatophagoides pteronyssinus, dermatophagoides farinae, dog dander, cat dander and feather mix (chicken, duck and goose). The criterion for a positive skin test was a wheal reaction (papule) 3 mm greater than positive (histamine) control.

2.4.3. Blood tests

All the subjects visited the department in the morning fasting from midnight. Blood was taken to measure glucose levels, insulin, full blood count, ESR, lipid profile which included total cholesterol, triglycerides, HDL, LDL, cholesterol/HDL ratio, C reactive protein (CRP). Urine was collected to measure albumin/Creatinine ratio. Blood was collected and centrifuged at 3000rpm for 10 minutes in Labofuge 400R (Heraeus instruments) and aliquoted into 5 plasma and serum samples of 250µl each and stored at -80^{0}C for future cytokine/adipokine measurements.

2.4.4. Pulmonary function testing: Spirometry and transfer factor (TLco)

Pulmonary function testing is a robust test used in research and clinical practice to diagnose airways disease like asthma and COPD. This test was carried out during visit 1 and diagnose of COPD was made accordingly. The test included spirometry with □-2 agonist reversibility (salbutamol), diffusion capacity of the lung and lung volumes. Spirometry was performed according to the American Thoracic Society guidelines(ATS)[255]. Machines available for spirometry are the vitalograph and 'COLLINS CPL' system, which is a volume displacement pulmonary function system was used to perform spirometry, functional residual capacity (FRC) and single breath diffusion test (TLco).

Procedure: The CPL machine was calibrated every morning prior to testing. Subjects were advised to avoid smoking for 24 hours and alcohol for 4 hours as per the protocol. Vigorous exercise was also avoided 30 minutes prior to the testing and importantly they were asked not to take short acting bronchodilators before the test for at least 4 hours. They were advised to relax as much as possible and seated for 5-10 minutes prior to the test.

The subject was instructed to connect to the mouthpiece of the machine. Subjects were asked to in a breath to fully inflate their lungs and then blow out all the air into the mouth piece of the machine forcibly and quickly in one second (FEV_1) and continued blowing out until all the air in the lung had completely emptied (FVC) into the machine. Three such tests were done and the best was recorded. From the results, FEV_1/FVC ratio was then calculated.

Carbon Monoxide Transfer Factor (T_{LCO}) was measured as described in ATS recommendations[256], via single-breath carbon monoxide test as follows

Each volunteer was seated upright wearing a nose clip and exhaled the air from the lung completely via a disposable mouthpiece. The volunteer was then immediately asked to breathe from a reservoir bag containing gases, including carbon monoxide (CO) and Helium (He) at a known concentration (0.3%CO,14%He,18%O_2) from which was rapidly inhaled to total lung capacity. The volunteer held the breath for approximately 10 seconds at maximal inspiration before making a rapid and complete expiration. The first 750mls assumed to be contaminated with anatomic dead space gas was discarded and the next 500mls was collected for the analysis of CO and He. The TLco value was then determined by (a) calculating the rate of disappearance of inhaled CO

from the alveolar gas Kco (units: min^{-1}). This was done by dividing the difference between the alveolar concentration of CO at the beginning and at the end of breath holding time by the duration of breath holding time. (b) secondly to calculate the lung volume through which the CO diffused during breath holding, the alveolar volume Va (mlSTPD). Va was calculated by the helium dilution method. As helium does not cross the blood gas barrier, and the inspired concentration was known, the alveolar volume was calculated from the new dilution of helium in the expired air. (c) finally from these results the TLco was calculated and expressed as,

TLco=kCOxVa per unit pressure= (kCOxVa)/Pb ($mmpl^{-1}kPa^{-1}$in SI units) where Pb is barometric pressure minus water vapour at 37 degrees. All tests were carried out either by me or by trained pulmonary technicians.

2.4.5. Bronchodilator reversibility test

Repeat measurement of FEV_1 following treatment with 400mcg of salbutamol can help to differentiate COPD from other reversible airway disease for e.g. asthma. All subjects in this study underwent reversibility testing before their diagnosis. All subjects were given 400mcg of inhaled salbutamol through a spacer and FEV_1 was recorded at base line and after 15 minutes later. Subjects who demonstrated a reversibility of ≥ 12% FEV_1 were excluded from the study.

2.4.6. Histamine provocation challenge

Histamine challenge is a standard technique used to determine the degree of non-specific bronchial reactivity of an individual. It is thought that the histamine may act in a direct way by causing contraction of the airway smooth muscle. The method used in the follow up study (section 2.2.5) is described by Chai *et al*[257]. Histamine solution (0.03-16mg/ml in normal saline) was prepared as per the departmental protocol. A Spira Electro Dosimeter or a hand held nebuliser was used to nebulise for the challenge. The volunteer was given adequate time to rest before the procedure and all the questions were answered. Volunteer's height was measured and their age noted as this was required to calculate the predicted value for FEV_1 and FVC.

Procedure: Prior to commencing the challenge, spirometry was measured and the base line reading was recorded. The highest FEV_1 was used to determine that it was more than 70% of the predicted value. The volunteer was then nebulised via dosimeter driven by compressed air at 8L/minute. If the predicted value was more than 70% predicted then the test was continued. Subjects inhaled five breaths of nebulised saline. This involved forced inhalation from the functional residual capacity (FRC) to total lung capacity (TLC). Measurement of FEV_1 was made at 1 minute and 3 minutes after the inhalation. The lowest of the best FEV_1 readings at 1 minute and 3 minutes were used to calculate the percentage fall from the baseline.

Calculation: $\frac{\text{Baseline } FEV_1}{\text{Predicted } FEV_1} \times 100 = \%\ \text{Predicted}$

$$\text{Percentage change} = \frac{\text{Post saline baseline } FEV_1 - \text{Post inhalation } FEV_1}{\text{Post saline baseline } FEV_1} \times 100$$

This reading was then used as the baseline for the future measurement to record the percentage (%) fall for the rest of the challenge. If there was a fall of greater than 10% after saline inhalation from the baseline value the challenge was aborted. If there was no fall in FEV_1 or if the fall was less than 10%, then the first dose of histamine was delivered starting with 0.03mg/ml solution and FEV_1 was recorded at 1 minute and 3 minutes and the lower of the best FEV_1 readings taken and the % of fall in FEV_1 was calculated and compared to that of the baseline. If there was no fall or a fall of less than 10% then the histamine concentration was doubled and nebulised. This procedure was continued to a total of 16mg/ml of histamine solution. If there was fall of more than 20% FEV_1 (PC_{20} positive), which was calculated as:

$$20\%\ \text{fall} = \frac{\text{Post saline } FEV_1 \times 100}{100}$$

At this stage, 200mcg of inhaled salbutamol was given and FEV_1 was measured after 15 minutes and when the FEV_1 was restored to 90% of the first baseline measurement and if there was no symptoms like cough, wheeze or chest tightness the volunteer was then discharged.

2.4.7. Sputum induction and processing

The ability to study and understand various inflammatory markers has changed with the development of techinics in induced sputum in research. The first description of a standardised method was in 1921 by Pin et al[258]. Since then researchers have published many papers about the advantages of induced sputum in the study various inflammatory cells in asthma and COPD. The first attempt to standarise and validate took place in a workshop in 1936 at Stockholm, Sweden. Subsequently ERS set up a task force in 1998 to validate the processing, read outs and its clinical implications [259].

Induced sputum is a technique used for research and diagnostic purposes. Trained doctors, nurses, or respiratory technicians who have been assessed and shown to be competent carried out this procedure. All the induction for my study took place at the WTCRF. The volunteer was given information about the procedure to read before hand with adequate time to ask questions.

Procedure: The sputum induction was performed as previously reported [260]. To begin, baseline peak flow was recording and 200mcg of inhaled ß-2 agonist salbutamol was give through the volumatic spacer device according to the protocol. After 10 minutes, post bronchodilator peak flow reading was recorded. Ensuring the volunteer was comfortable wearing the nose clip, 4.5% of nebulised saline was nebulised. The subject was advised to maintain normal tidal breathing during each 5 minutes of procedure. After 5 minutes, the volunteer was asked to rinse the mouth with water and encourage expectorating into the petri dish, which was kept on the ice to preserve the cells until the processing. After every 5minutes period, peak flow measurement was performed and if there was no decrease in PEF or the decrease was less than 20%, nebulisation was continued for further 5 minutes. However, if the PEF dropped by more than 20% then the procedure was discontinued and bronchodilator (salbutamol) was given. Where possible, the induction lasted for a total of 20 minutes.

In subjects with COPD, the procedure was modified according to the standard operating procedure (SOP) and according to ERS guidelines[259]. The procedure was started with 0.9% normal saline and as a safety precaution, PEF was measured at 30 seconds, 1min, and 5 minutes. If that failed to produce sputum, and provided there was no significant drop in peak flow values, the

concentration of saline was increased to 3 % and the procedure repeated at the same interval as above. If that fails to produce sputum then the concentration of saline was increased to 4.5%. Induction was stopped if the peak flow reading dropped by more than 20% or if the volunteer had excessive coughing/wheeze or was short of breath. Bronchodilator salbutamol 200mcg was given and peak flow reading was repeated after 15 minutes to ensure that it had returned to 90% of the baseline measurement.

Sputum processing was performed according to a standardised protocol. Sputum was placed on the petri dish on ice and processed within an hour. A portion of the sputum was sent to microbiology for analysis. Using forceps the more mucoid portion of the sample was selected and transferred into another petri dish. The sample was then thoroughly mixed with forceps and divided into two (A+B). Sample A was solubilised with DTE (Dithioerythritol), while sample B was solubilised with HEPES buffered saline (HBS). Using a cell scraper the sputum was removed from the petri dish into the falcon tube leaving the obvious saliva behind. The tube was weighed with the sample to get an accurate reading of the expectorate to be processed. The first portion (A) was treated with 4x the volume of DTE. The second portion (B) was treated with 4x the volume of HEPES. 22.5µl of protease inhibitor cocktail per gram of sputum was added to both A and B. The tubes were briefly vortexed, inverted several times to ensure thorough mixing, and placed on a bench roller for 30 minutes by inverting the samples once every 5 minutes. More viscous samples if needed was further mixed by aspirating with a Pasteur pipette (C), the contents were filtered through a 100µm filter into a pre weighed 50ml Falcon tubes to remove unsolubilsed mucus. Then it was reweighed to determine the weight of the filtrate recovered. g) Then the filtrate was centrifuged at 1500rpm (400g) for 10 minutes to pellet cells. (D). The supernatant was then carefully removed from the tube and put into labelled 1.5ml eppendorf tubes. (E) The supernatant was then aliquoted (250µl) into labelled eppendorfs (10 tubes depending on the sample) and any extra into a single tube and stored for future analysis at -80^{0}C. (F) The cell pellet from the DTE treated sample (A) was resuspended in 1ml PBS, k) 10µl of the cell suspension (A) was removed and mixed with 90µl of Trypan Blue in an eppendorf. The Total Cell Count was calculated per ml using the Trypan Blue exclusion method following the workings on the sputum processing work sheet. Cell viability was also calculated. It was diluted with PBS to obtain 1 x 10^{6} cells/ml, (G) 8 cytospins were prepared using 75µl per slide at

450rpm for 6 minutes the quality of the slides was assessed and repeated where necessary. Slides were air dried overnight, (H) One slide was stained with Rapi-Diff, and the remainder were wrapped in foil and stored at -20^{0} C for future analysis.

2.4.8. High resolution computed tomography (HRCT) and densitometry measurements

High resolution computed tomography (HRCT) is being used more frequently to assess the degree COPD and emphysema. It allows the early detection lung damage, and gives a quantitative measure of lung density, as well as small and large airways. Several studies has shown that HRCT is a safe and sensitive way of assessing pulmonary function, and that it often is a more sensitive test than PFT. However, most of these studies are concerned only with one sub-phenotype (e.g. emphysema).

HRCT is a radiographic test that supplies the details about the lung parenchyma. The lungs were divided into series of cross sectional radiographic images of 1mm thickness. This was measured in Hounsfield units (HU). The scan was performed on a General Electric Hi-speed CTi scanner, at 10mm intervals with a collimation (slice thickness) of 1mm. All scans were carried out in the supine, feet first position, on full inspiration and expiration, from the lung apices to the costrophrenic angles. Scanning voltage was 140kV and tube current was 250mA. Hard copy images were photographed at a window level of -650HU and the window width of 1500HU. The scans were evaluated both qualitatively by a blinded consultant radiologist and quantitatively by computerised density mask analysis.

High resolution CT images were acquired at both inspiration and expiration. A programme was written in IDL (Interactive Data Language, Research Systems Inc.,Boulder, CO, USA) to enable reasonably automated image processing of the CT images to be carried out. The images were converted into a suitable file format (interfile) and read by the program. The operator was then prompted to set an appropriate grey scale window width and level to enable structures in the lungs to be clearly visualised. The next stage of the process involved drawing one or two regions of interest on each slice, around each cross-section of the trachea and bronchi leading into the lung. These were set to a high pixel value of 5000HU. This was to effectively

block off the trachea and bronchi so that their volume would not be included in the automatically calculated lung volume.

The lung volume was determined using a seed-growing algorithm. A seed growing start point was placed in a low-density area of each lung. An IDL sub-routine was then initiated. This is detected in three dimensions, each connected voxel in the image volume within the range of HU values which had been established being lung tissue (-400 HU to -1000HU).

Once the lungs had been identified, several parameters could be calculated from their corresponding histogram of pixel values. The program automatically calculates the total volume of the lungs by multiplying the number of voxels identified as being lung tissue by the individual voxel volume. It also calculates the number of lung voxels below-950 HU (below which the tissue is thought to be emphysematous), the percentage that this was of the total lung volume, the CT number which 10% of voxels in the lung fell below, the mean and median CT number of the voxels in the lung and the ratio between the mean inspiration and expiration CT values.

2.4.9. Multiplex ELISA/Luminex

Cytokines are small proteins that mediate immune response in health and disease. Many of these cytokines were first identified and characterised by biological assays[261]. These assays are cumbersome and labor intensive and some of these tests are more expensive than others. Several multiples analysis technologies have been developed and used to measure cytokines in the recent years including flow Cytometry and the Luminex system. The advantages of multiplex cytokine assays over the standard ELISA assay include smaller sample volume, higher through put and low cost. Luminex is a well validated technique and a suitable alternative to the ELISA for most cytokines, provides a valid characterisation of cytokine expression profile and T helper bias and even may ve superior to ELISA which is commonly used in immunology[261]

Luminex assay (Bio-Rad, Hemel Hempstead, UK) was used to determine the concentrations of multiple cytokines (IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-α, IFNg) in patient plasma samples diluted 1:5 according to the manufacturer's instructions. Briefly, recombinant cytokines for standard curve generation were purchased from RandD systems (Abingdon, UK). Capture

and detection antibodies were from RandD (TNFα), Pharmingen, UK (IL-2, IL-4, IL-13 capture anti body; IL-5, IL-10, IL-12p70, IL-13 detection antibody), Endogen, UK (IFNg, IL-6, IL-8, IL-10, IL-12p70 capture antibody; IFNg, IL-4, IL-6, IL-8, IL-12p70 detection antibody), and GlaxoSmithKline, Stevenage, UK (IL-5 capture antibody).

Briefly, 50 µl of standards or diluted samples were placed on a 96-well filter plate (Millipore, Watford, UK) pre-wet with 50 µl of assay buffer (PBS, 1% BSA, 0.025% Triton-X100). Fifty micro litres of appropriate antibody-conjugated xMAP carboxylated microspheres (Applied Cytometry Systems, Sheffield, UK) were added to each well ($1x10^5$ microspheres/ml for each conjugated microsphere set) and the plate covered and incubated at 4ºC overnight. After two washes with wash buffer (PBS, 0.05% Tween-20), 50 µl of a cocktail of appropriate detection antibodies (500 ng/ml for each antibody) was added to each well and incubated on a shaker at 700 rpm for 1 h at room temperature. After two further washes, 50 µl of streptavidin-PE (1 µg/ml in assay buffer - BD Biosciences, Oxford, UK) and incubated on a shaker for 30 min at room temperature at 700 rpm. The plate was then washed twice and 120 µl of sheath fluid was added to each well before reading on a Luminex xMAP100 machine (Bio-Rad). Results were analysed using STarStation 2.0software (Applied Cytometry Systems).

2.5. Body composition measurements

Height was measured with the subject standing barefoot on the base of a vertical scale (SECA Model 220) to the nearest 0.1 cm. Weight was measured with the volunteers in their undergarments without any jewellery or eyeglasses, to the nearest 0.1 kg using the electronic weighing scale connected to the BODPOD. The weighing scale was calibrated to the nearest 0.001 kg prior to the test.

Other body composition measurements were measured by three methods which included (a) measurement of lean mass and fat mass by air displacement plethysmography (BODPOD) (b) anthropometric measurement by bioelectric impedance analysis (BODYSTAT) (c) measurement of lean and fat mass by skin folds (callipers).

2.5.1. BODPOD

Whole body plethysmography is a new body composition technique to measure total body fat and fat free mass. This systemic is been used for research purposes and been validated against some of the other available techniques like hydrodensitometry[262]. The advantages of this system are,

- It is very accurate
- It uses a dedicated system with data management
- It has an option to either measure a subject's Thoracic Gas Volume or use predicted equation
- Measures fat and fat free mass, resting metabolic rate and total energy expenditure[263]
- Quick, the test may take less than 5 minutes
- Good reproducibility
- Importantly non invasive and suited for repeated test
- Simple and easy for both subjects and operator and no special training needed

Procedure:Airdisplacement plethysmography was performed using the BODPOD® system according to the manufacturers' instructions and recommendations. The proportion of body density contributed by muscle and bone (lean body density) was standardised according to the subjects' age, height and sex[264]. BODPOD® system was calibrated to a strict standard deviation value of below 75ml each day before use. The calibration outcome was unpredictable as many factors such as room temperature and room pressure could affect the process[265].

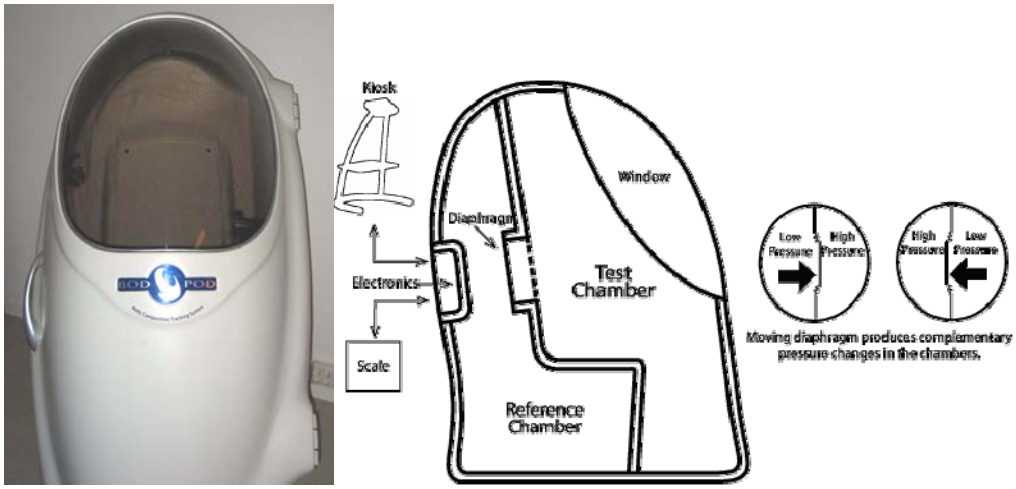

Figure 2.1 BODPOD and the chambers inside with a brief description.

Subjects were in their undergarments and wearing a swim cap before entering the system. The volunteers sat in a test chamber and were asked to limit movement and breathe normally. Subject age, gender and height were entered into the system using the kiosk connected to the BODPOD®. Measurement of volume was taken using the BODPOD® S/T Body Composition Tracking System, which consisted of taking a mean value from three repeated tests, each lasting approximately 40 seconds. The results that were shown included fat and lean mass in percentage and kilograms to the nearest 0.1% and 0.1 kg respectively and total body weight in kilograms to the nearest 0.1 kg.

The BODPOD® system utilises the principle of whole body densitometry to estimate amount of fat and lean tissue in the body. Body density was calculated using the formula: density = mass/volume. With the body density, percentage of fat was derived using the Siri Equation:

% Fat = (495 / Body Density – 450)

The percent lean mass was calculated as follows:
% Fat Free Mass = 100 - %Fat.
The amount of fat and lean mass was calculated from these results:

Fat Mass (kg) = [(%Fat) (Body Mass)] / 100%
Lean Mass (kg) = Body Mass – Fat Mass.

2.5.2. Bioelectrical Impedance Analysis (BIA)

The Bodystat® 1500 uses a BIA technique to measure body composition. The body's lean (muscle, bone, and water) compartment conducts electricity better than the body's fat compartment. These two compartments have very different resistance values to a high frequency electrical signal. The single impedance measurement reflects the degree of resistance to the flow of current in the body, water being a good conductor and fat a bad conductor, it calculates the percentage of these body components for an individual.

A variety of body composition methods is available and majority of them are limited to the research or clinical laboratories that include determination of body water, tomography, and electrical conductivity. Some of these methods are less reliable and not friendly. BIA on the other hand is a non-invasive, cheap and easy method of measuring body composition. BIA is a safe procedure and requires no special skills on the part of the operator or the subjects, cooperation is minimal and importantly measurement are reliable and rapid[266], The instrument is portable and inexpensive. BIA has been validated in adults[267-269] Further more this method has also has high rate of reproducibility in adults[266].

The principle behind this is based upon the conduction of an applied electrical current in the organism. In living organisms the application of a constant low level alternating current results in an impedance to the spread of the current that is frequency dependent[266]. This work was conducted by Nyboer of USA who studied arterial pulse wave forms and pulsatile blood flow to organs using electrical impedance plthysmography[270] . Lukaski et al demonstrated a strong relationship between BIA measurements and fat free mass, total body water and total potassium[266] and suggested that this method may be very useful as a tool routine measurement.

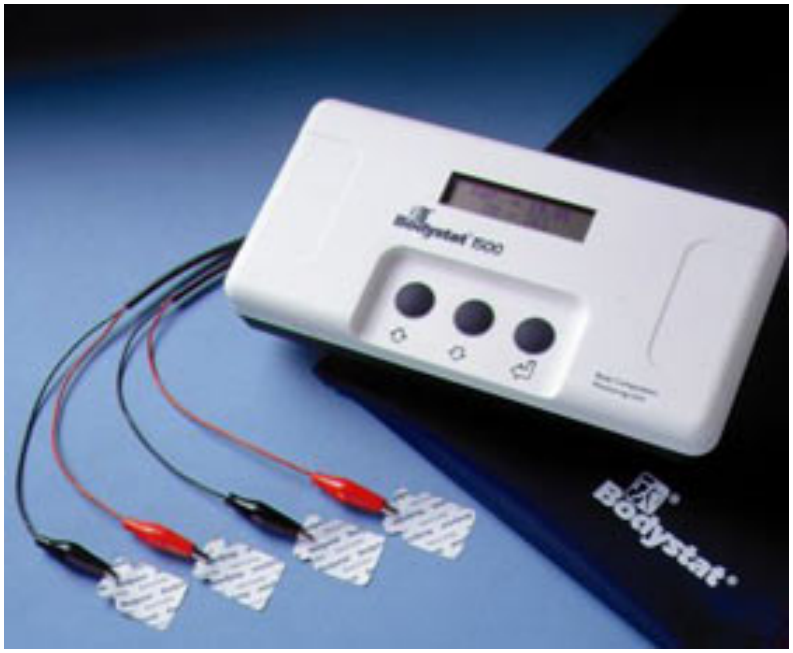

Figure 2.2 Bodystat® displaying the four electrodes that is used to measure fat and lean mass.

Procedure: Subject removed their shoes and socks from the right foot and lay down in supine position with no parts of the body touching one another. Two electrodes were used, one placed behind the second toe next to the big toe, and one on the ankle between the medial and lateral malleolus. A further two electrodes were used, one placed behind the knuckle of the middle finger, another one on the wrist next to the ulnar head. These electrodes were then connected to the Bodystat® 1500 by alligator clips. The volunteers' age, gender, height, weight and activity level were entered into the system before starting the test. Activity levels were categorised in 5 levels; very low, low/medium, medium, medium/high and high. The whole test took approximately 5 minutes and the readings were displayed on the screen of the Bodystat 1500®. The readings included fat and lean mass in percentage and kilograms to the nearest 0.1% and 0.1 kg respectively, total body weight to the nearest 0.1 kg, water percentage to the nearest 0.1%, total body water to the nearest 0.1 litre, estimated metabolic rate and estimated average energy required to the nearest 1 kcal, body mass index (BMI) to the nearest 0.1 and impedance to the nearest 1.

Although BIA does not require any complicated training to perform the test, minor difference in the distance between each electrode when placing the electrodes will produce significantly different results[271]. One investigator for

one subject throughout the recording day policy was practiced; however, individual variations in placing the electrodes could not be avoided since each electrode is of single use only.

2.5.3. Skinfold measurement

The skin fold measurements are the cheapest means to measure total body fat in adults. The measurements and validated and show a good correlation with Dexa scan [272], however there is always interpersonal variation. To minimize this technical error of measurements, differences between repeat skinfold measures, measurement sites and measurement techniques have been defined [273]. It has been suggested that anthropometry measurements should possible show an error in land marking if done by different people. Hence it is advised that the measurement are done by one [273] Few studies have investigated the magnitude of any error likely to derive from not measuring at exactly specified points. Ruiz et al reported that skinfold thickness varied by an average of 2.5mm when the calliper was placed 2.5 cm from the correct site[274]

Skinfold thickness is measured to the nearest mm, except for low values when it is taken to the nearest 0.5mm.The readings are made at four sites biceps, triceps, subscapular and supra iliac areas. These were done usually on the right side of the body with subject standing in a relaxed condition. , The circumference at the upper arm, upper thigh was obtained using a tape. Waist circumference, total leg length are also measured. Skinfolds and circumference are metered by the standard technique described by Weiner & Lourie (1969) except that the subscapular skinfold is taken at an angle of about 45^0 to the vertical and the position of the Suprailiac skinfold just above the iliac crest in the mid axillary line[275]

Figure 2.3 Callipers used for skin-fold measurements.

Procedure: Measurements were all performed on the volunteer's non-dominant side by measuring tape and recorded to the nearest 0.1cm. Skin fold measurements were repeated three times to obtain average readings. Volunteers were standing without their top garments and with their back to the measurer, arms hanging by their sides.

Firstly, the tips of the acromion at the point of the shoulders were palpated and marked with a cross. With the arm flexed at 90 degrees, the olecranon at the tip of the elbow were palpated and marked with a cross. The tape measure was placed on the mark on the acromion and dropped to the cross at the olecranon. Halfway between the acromion and the olecranon was marked. This indicated the half-upper arm length.

Volunteers relaxed their arms by their side when the tape measure was placed around the upper arm with the upper border of the tape at the level of the mark to measure the mid upper arm circumference. With the tape in position, a line was drawn on the skin posteriorly and anteriorly at the level with the first mark. The posterior line was used for the triceps fold and the anterior line was used for the biceps fold. With the palm facing forward, the mid point of the part, which sticks out the furthest, was marked vertically to form a cross. The skin was picked out in a vertical tube with two hands, at least 1cm above and below the cross. The skin fold callipers were applied at the level of the cross, with the cross on the apex of the fold. The readings were

recorded after the callipers were applied for 5 seconds. Three readings were repeated and the same was done for triceps skin fold posteriorly.

For the subscapular skin fold, volunteers stood topless with their back to the measurer with shoulders and arms relaxed. The lower most tip of the scapula were palpated and marked with a cross. The inferior angle of the scapula was palpated by following the medial border of the scapula downwards. The skin fold was then picked up obliquely and the callipers were applied at the level of the cross with the cross at the apex of the fold.

The iliac crest was then palpated on the non-dominant side and a cross was marked. The lower rib margin was marked as well on the same side. The midway point between these two marks was measured and marked indicating the half rib/ iliac distance. Suprailiac skin fold was measured at this point. The midpoint on the half rib and iliac distance, where the skin sticks out furthest, were marked with a cross. Suprailiac skin fold measurements were then performed using the technique described above. The measuring tape was then placed around the volunteer on the same location.

Body density was then calculated using the Durnin and Wormersley's linear regression equations[276], which is shown below,

BODY DENSITY = C-[M (LOG10 SUM OF ALL FOUR SKINFOLDS)]
Where C and M are constant depending on sex and age.

Male	17-19 yrs	20-29 yrs	30-39 yrs	40-49 yrs	50+ yrs
C	1.1620	1.1631	1.1422	1.1620	1.1715
M	0.0630	0.0632	0.0544	0.0700	0.0779

Female	16-19 yrs	20-29 yrs	30-39 yrs	40-49 yrs	50+ yrs
C	1.1549	1.1599	1.1423	1.1333	1.1339
M	0.0678	0.0717	0.0632	0.0612	0.0645

2.5.4. Waist circumference measurement

Volunteers relaxed and the reading of the waist circumference was measured to the nearest 0.1cm[277]. Circumference was measured with the volunteers removing objects bulging out in the trousers. The measuring tape was placed around the hip and few measurements were performed to get the largest measurement. Total leg length was measured with the volunteer standing straight and barefoot. The distance from the head of femur to the sole of the foot was measured. With the head of the measurement tape still in place on the head of femur, the kneecap was palpated. The distance from the head of femur to the kneecap was measured and the midpoint was recorded and marked with a tailor pen on the volunteer's pants. The mid thigh circumference was then measured by placing the tape around the mark on the volunteer's pants.

2.5.5. Blood pressure recording

Blood pressure was usually measured in the brachial artery using a manual or electronic sphygmomanometer. Blood pressure is the force of blood against the walls of the arteries. It is recorded as systolic over diastolic pressure. In my study the blood pressure was measured by DOSCH monitor. The blood pressure was measured in standing, sitting and lying positions at an interval of 2-3 minutes between each measurement. The mean of the three readings was then calculated and recorded for analysis.

2.5.6. Measurement of insulin resistance by HOMA

Homeostasis Model Assessment measures insulin resistance and beta cell function which was designed by Matthews *et al* [278] to understand and calculate insulin resistance from fasting glucose and fasting insulin levels. This is a computer-solved model used to predict the homeostatic concentrations which arise from varying degrees of beta-cell deficiency and insulin resistance. This model compares subjects fasting values with the models predictions, which allows a quantitative assessment of the contributions of insulin resistance and deficient beta-cell function to the hyperglycemia. The accuracy of this model has been compared with independent measures of insulin resistance and beta-

cell function using hyperglycaemic and euglycaemic clamps and an intravenous glucose tolerance test.

The formula for calculating insulin resistance (IR):

IR= (fasting insulin in μU/ml x fasting glucose in mmol/l)/ 22.

This assessment model is well validated and been used in many diabetic studies, however this less accurate than the clamp method.

2.6. Statistical methods

Results are presented as mean ± standard deviation (SD) for normally distributed variables and median and interquartile range otherwise. Initial statistical comparison was performed by ANOVA for normally distributed and Kruskal-Wallis for non normally distributed data. P values for differences between groups and linear trends across groups were analysed, taking $p<0.05$ as significant. Student's t-test and the Mann-Whitney U test were used to compare normally and non-normally distributed data between two groups respectively. The Chi-square test was used to compare proportions of features of MeS between the three groups. Pearson and Spearman correlation analyses were undertaken to examine associations between normally and non-normally distributed variables, respectively. Linear regression models were developed with FEV_1 to investigate the effect of variables on the outcome measure. All the analyses were undertaken using the SPSS 14.0 package, SPSS Inc., Chicago, IL, USA.

The statistical hypothesis were based on Austin Bradford Hill's criteria from 'The environment and disease association or causation' published in the, 'Proceedings of the the Royal Society of Medicine' in 1965[279]. Hills criteria of Causation outlines the minimal conditions needed to establish a causal relationship between two variables. The criteria have been applied in my study and in much epidemiological research to illustrate how this would be applied to research in the social and behavioural sciences. The criteria included temporal relationship, strength, Dose-response relationship, consistency, plausibility, consideration of alternative explanations, experiment, specificity, and coherence.

3.0 Chapter- Features of metabolic syndrome and body composition in smokers

3.1. Introduction

The clinical importance of diagnosing features of metabolic syndrome (MeS) in smokers with and without Chronic Obstructive Pulmonary Disease (COPD) has not been studied extensively. The systemic effects of smoking not only has an impact on exercise intolerance which is associated with COPD[115, 280, 281] but also lead to other co morbidities[115, 282-284], including cachexia [284], skeletal muscle abnormalities[281, 285, 286] hypertension[287, 288] and diabetes [289].

COPD is characterised by poorly reversible airflow limitation that is usually progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases, particularly cigarette smoke[290]. There is an increasing evidence that the airflow limitation including imaging and clinical features are poorly correlated[291]. Smoking is the most important risk factor for people to develop COPD and also for many chronic diseases and some cancers[292]. According to WHO it is estimated to kill 250 every hour[293]. COPD is also the leading cause of mortality and morbidity globally and it estimated to be the third leading cause of death by 2020[294, 295, 295, 296].

MeS is a global phenomenon and it is estimated to be on the rise, with one in four individuals in the US having features of metabolic syndrome. MeS is a cluster of cardio-metabolic risk factors consisting of hyperlipidemia, dysglycaemia, central obesity and hypertension that predisposes individuals to increased risk of developing cardiovascular and type-2 diabetes [297]. Various definitions have been proposed and commonly used ones are the International Diabetic Federation (IDF) and National Cholesterol Education Programme-Adult Treatment Panel III (ATP III). The definitions and classification have been discussed in the chapter I (section 1.7.3). Early recognition of metabolic syndrome would not only help patients with early diagnosis and treatment but also lessens the long-term health care costs and resources.

Body composition measurement in COPD plays a pivotal role in the disease progression. Low body weight and low body mass index (BMI) is found to be a major risk factor for mortality in COPD. Several studies have investigated factors affecting mortality[235, 298-301]. Marquis *et al* [301] in their study found that body composition measured as midthigh muscle cross sectional area obtained by computed tomography (CT) scan is a better predictor of mortality than BMI. It is also been reported that there is a relationship between total body water and electrical impedance based on the principal that fat mass

(FM) and fat free mass (FFM) which have different conductive and dielectrical properties[302]. This is due to the fact that FFM contains water and therefore has a lower resistance than FM[303].

What is less understood is the relationship between effects of COPD on the metabolic features and the relationship between body composition and COPD. My hypothesis was that COPD causes systemic inflammation and is driven by smoking. These systemic effects of COPD contribute to the development of features of metabolic syndrome and hence the prevalence of metabolic syndrome is high in COPD subjects when compared to smokers without COPD.

The aim of my study was to understand the prevalence of metabolic syndrome and the role of body composition in my cohort of smokers with and without COPD.

3.2. Methods

A total of 131 subjects including 44 healthy controls (HC), 46 smokers without COPD (HS) and 41 with COPD were recruited for this study and were matched for age and sex. Subjects were than categorised according to the GOLD criteria for diagnosing COPD.

All subjects had clinical history taken, underwent medical examination as outlined in Chapter 2 (section 2.2.1 study 1). Blood was taken for fasting glucose, fasting insulin, lipids, and for inflammatory marker CRP. Height and weight were measured as explained in chapter 2. Subjects underwent pulmonary function testing, measurement for body composition was measured by BODPOD, bioelectrical impedance analysis (BIA), and skin-fold measurements. All subjects had their blood pressure checked in sitting, standing and lying positions.

Metabolic syndrome was defined according to the IDF criteria which are discussed in detail in chapter 1 (section 1.16.4).

3.2.1. Statistical analysis

Descriptive data are expressed as means and standard deviation for the normally distributed data and median and quartiles for others. Independent t-test or Mann-Whitney-U test was used for the comparative analysis. Qualitative variables were compared by chi-square test. Linear regression analysis was

done to compare the characteristics of smokers and non-smokers. For the correlation analysis, Spearman's rank correlation was used, as the data was not normally distributed. Statistical Package for the Social Sciences 14 (SPSS Inc, Chicago IL, USA) software was used for the analysis. GraphPad prism 5 was used for the scientific graphing in this study.

3.3. Results

Subjects were characterised as described in Chapter 2 (study I). A total of 131 subjects were recruited for the study. The subjects were subdivided into healthy non-smoker controls (HC=44), smokers without COPD (HS=46) and smokers with COPD (COPD=41). Subject characteristics are in table 3.1.

Subject characterisation

	Non-smoker control	Healthy smoker	COPD
No of subjects	44	46	41
Sex M/F	20/24	24/22	22/19
Age (yrs)	*53 ± 8.5	*54.7 ± 8.1	*55.6 ± 8.4
Pack years	N/A	**31(21 to 40)	**45(39 to 57.5)
FEV_1 (%predicted)	**112 (102 to 121.7)	**106(98 to 114)	**73(60 to 90.5)
BMI	*25.8 ± 4.2	*26.5 ± 4.7	*26.8 ± 5.7
β_2 agonists use (long and short acting)	N/A	None	None
Inhaled/oral corticosteroids use	N/A	None	None

Table 3.1 Subject characteristics.

*** mean and standard deviation, ** median and quartiles.**

FEV1:Forced expiratory volume in 1 second, BMI:Body mass index, N/A:not applicable.

3.3.1. Prevalence of metabolic syndrome in smokers with and without COPD

When using the criteria of IDF to define the presence of MeS, the prevalence of MeS was 11.4% in healthy non-smoking individuals, 28.3% in healthy smokers and 36.6% in smokers with COPD (Figure 3.1)

B). Using the ATPIII criteria, the prevalence was 4.5%, 19.6%, 29.3%, in the three groups of subjects, respectively (Fig 3.1 A).

A

B

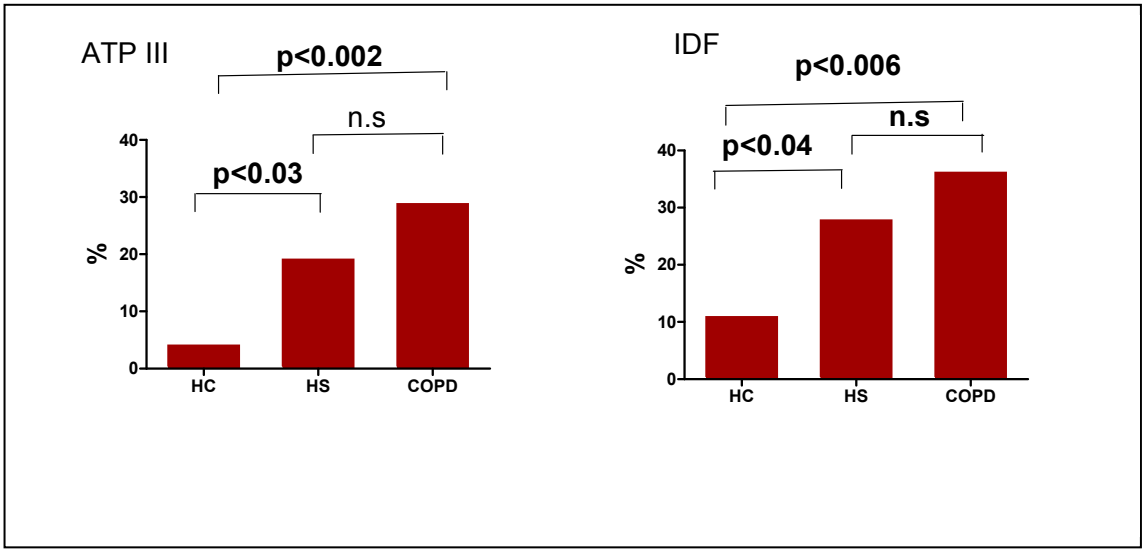

Figure 3.1 Prevalence of MeS in healthy non-smokers (HC), healthy smokers (HS) and smokers with COPD.

Data are represented as percentage of prevalence of metabolic syndrome. Chi square test was used to test the difference in prevalence between the groups. ATP: Adult treatment programme 111, IDF: International Diabetic Federation, n.s.: Non-significant, %: percentage.

3.3.2. Measurement of features of MeS

3.3.3. Fasting triglycerides

Hypertriglyceridemia is more common in coronary disease than high total cholesterol. The normal range of triglycerides is 0-2.0 mmol/L. In my study it was high in smokers with and without COPD ($p<0.004$ and $p<0.002$, respectively) when compared to non-smoker controls. However, I found no difference between smokers with and without COPD. It was noted from

correlation analysis that there was a weak inverse relationship between triglycerides and FEV_1 (r_s= -0.202, p<0.02).

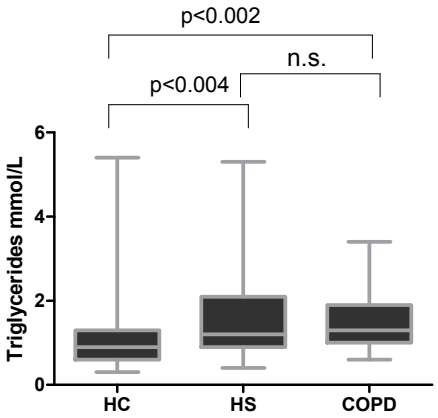

Figure 3.2 Triglyceride levels in healthy non-smokers (HC), healthy smokers (HS) and smokers with COPD

The data are expressed as median and quartiles. Mann-Whitney U test was used for analysis. n.s.: not significant, mmol: millimoles per litre.

3.3.4. Fasting high-density lipoprotein (HDL)

Reduced HDL cholesterol is accepted as a major risk factor for coronary disease. The normal range in our laboratory for HDL was 0.91-2.21mmol/L. The HDL levels in COPD subjects was lower than that in healthy non-smoker controls (p<0.01). There was a positive correlation (r_s= 0.201, p<0.02) between HDL and FEV_1 which was suggesting that HDL could be contributing directly or through a different mechanism in preserving the lung function in smokers.

HDL levels

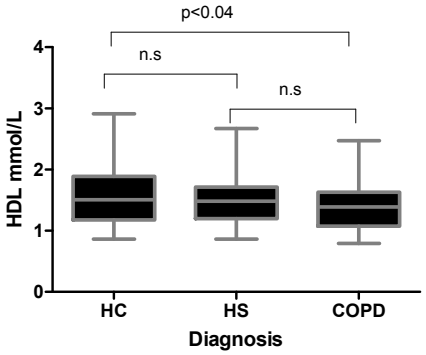

Figure 3.3 High-density lipoprotein levels in (HDL) healthy non-smokers (HC), healthy smokers (HS), and smokers with COPD.
The data are expressed as median and quartiles. Mann-Whitney U test was used for analysis. n.s.: not significant, mmol/L: millimoles per liter.

3.3.5. Fasting glucose

Increase fasting glucose or glucose intolerance is one of the main features of MeS (Normal range 4-6 mmol/L). It is well know that there is interrelationship between visceral abdominal fat and insulin resistance[185] which is a major risk factor for atherosclerotic cardiovascular disease. In my study I found that there was a difference between non- smoker controls and COPD ($p<0.01$), however, there was no difference between smokers with and without COPD. There was a strong negative correlation ($r_s = -0.251$ and $p<0.004$) between fasting glucose and FEV_1.

3.3.6. Waist circumference

There is a pathophysiological relationship between abdominal girth or central obesity and waist/hip ratio rather than the body mass index as obesity related cardiovascular risk[304, 305]. This is even true in normal weight subjects where central obesity is associated with increased cardiovascular risks[305, 306]. In my study COPD smokers had more central obesity than non-smoker controls

(p<0.03). There was a strong negative correlation between waist circumference and FEV_1 (r_s= -0.225, p<0.01).

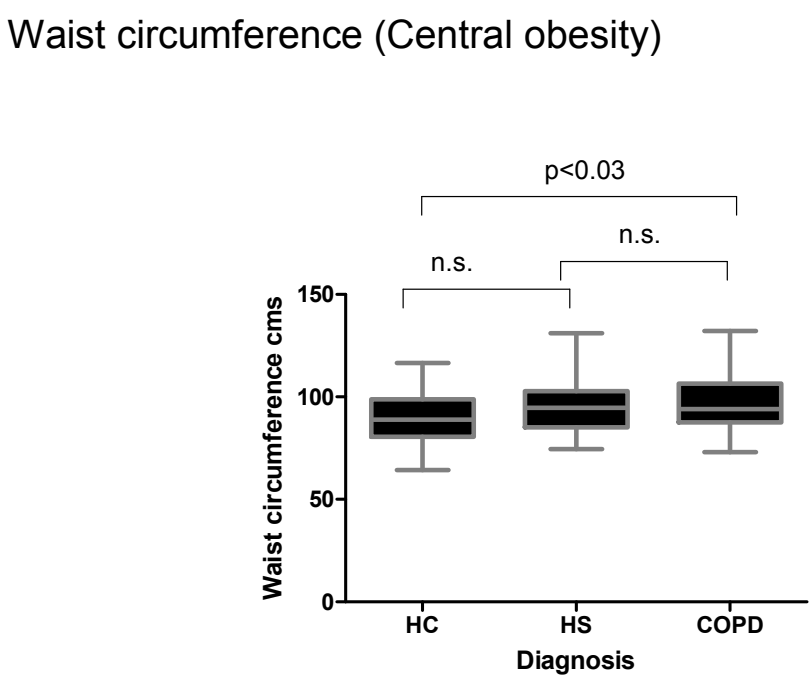

Figure 3.4 Waist circumference in healthy non-smokers (HC), healthy smokers (HS) and smokers with COPD.

The data are expressed as median and quartiles. Mann-Whitney U test was used for analysis. n.s.: significant, cms: centimetres.

3.3.7. Waist-hip ratio

The wait/hip (w/h) ratio is a well-standardized and simple measure of central obesity. A trend has been shown for smokers to have a lower BMI but higher W/H ratio[307]. In my study, COPD subjects had a higher w/h ratio than non-smokers (p<0.001). However, there was no difference between smokers with

and without COPD.

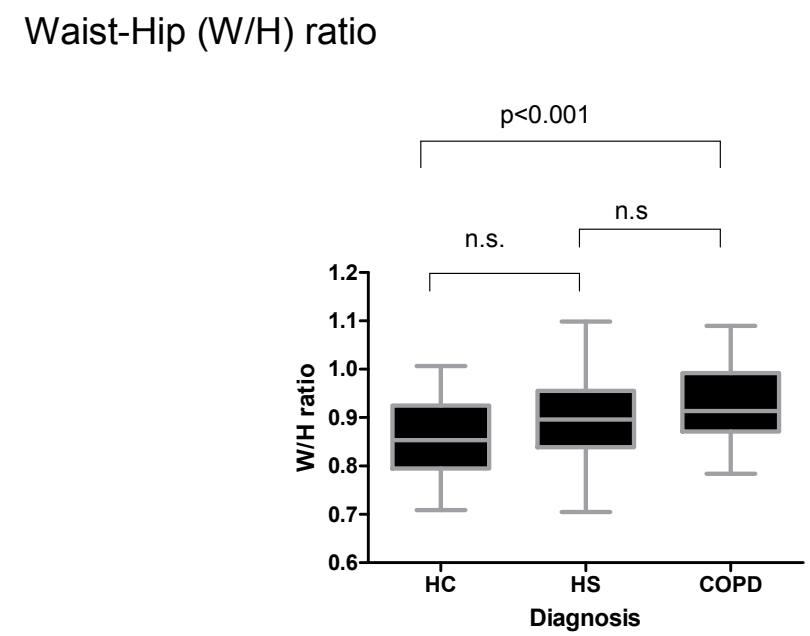

Figure 3.5 Waist/hip ratio in healthy non-smokers (HC), healthy smokers (HS), and smokers with COPD.

The data are expressed as median and quartiles. Mann-Whitney U test was used for analysis. n.s.:non significant, W/H:waist hip ratio.

3.3.8. Systolic blood pressure

Elevated blood pressure is a frequent component of MeS and hypertension is more frequent in subjects with either insulin resistance or obesity[185, 220, 308]. In my study, there was no difference in mean systolic blood pressure (mean of lying, sitting, and standing blood pressure) between non-smoker controls and smokers with or without COPD.

Systolic B P

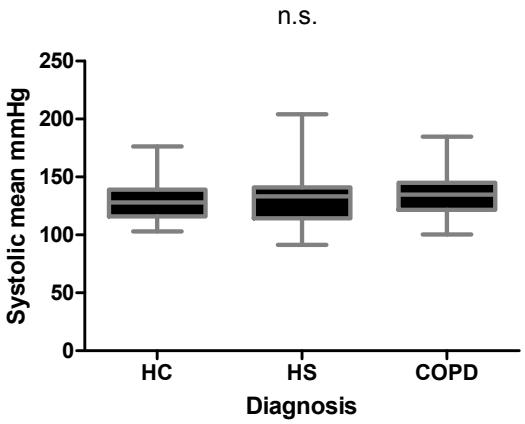

Figure 3.6 Systolic blood pressure in healthy non-smokers (HC), healthy smokers (HS), and smokers with COPD

The data are expressed in median and quartiles. Mann-Whitney U test was used for analysis n.s.:not significant, mmHg:millimetres of mercury.

3.3.9. Diastolic blood pressure

Diastolic blood pressure was measured in three positions: lying, sitting and standing. The means of those three measurements were recorded for the analysis. Blood pressure ≤85mmHg was accepted as normal. Any reading above 85mmHg or systolic blood pressure >130mmHg was considered as indicating presence of one of the features of MeS. There was no difference in diastolic blood pressure between the groups.

Diastolic BP

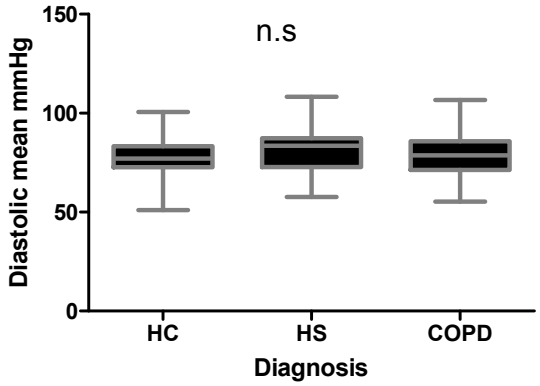

Figure 3.7 Diastolic blood pressure in healthy non-smokers (HC), healthy smokers (HS), and smokers with COPD.

The data are expressed as median and quartiles. Mann-Whitney U test was used for analysis. n.s.:not significant, mmHg:millimeters of mercury.

3.3.10. Body composition by bioelectrical impedance (BIA)

Body composition measuring fat free mass, lean mass, total body water and impedance (resistance) in the body were analysed. The measurements were done by three methods: BIA, BODPOD and by skin fold measurements in all subjects.

3.3.11. Percentage and total body fat (kg)

The Percentage of fat was no different in all three groups. However, there was a trend towards an increase in percentage fat in smokers with COPD. Correlation analysis with FEV_1 showed no significant correlation between

percentage body fat with FEV_1. There was no significant difference between non-smokers and smokers in fat weight and there was no correlation with FEV_1.

Fat %

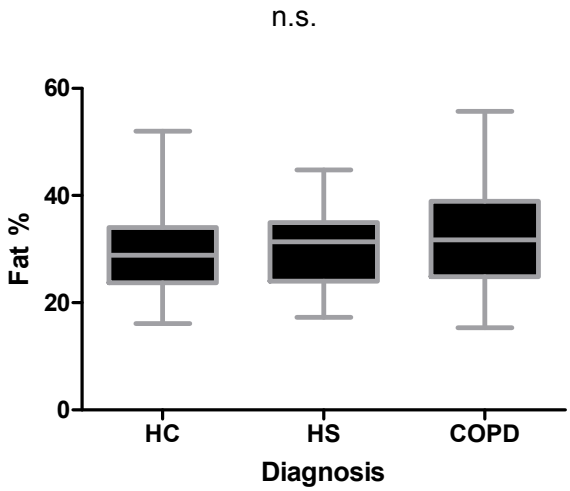

Figure 3.8 Percentage fat in healthy non-smokers (HC), healthy smokers (HS), and smokers with COPD

The data are expressed as median and quartiles. Mann-Whitney U test was used for analysis. n.s.:non significant.

Fat (Kg)

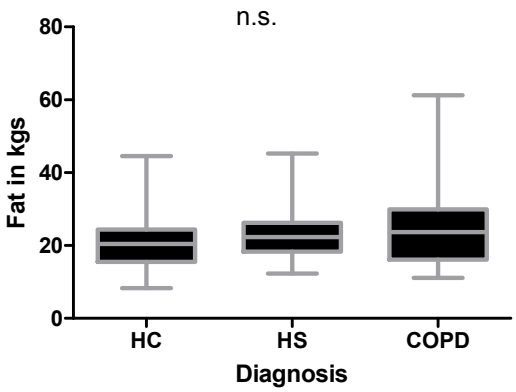

Figure 3.9 Total body fat (kg) in healthy non-smokers (HC), healthy smokers (HS), and smokers with COPD

The data are expressed as median and quartiles. Mann-Whitney U test was used for analysis.n.s. not significant, kgs: kilograms.

3.3.12. Measurement of percentage of lean mass

Muscle wasting is an important phenomenon in severe COPD and contributes significantly to the mortality and morbidity of the disease. It is, therefore, important to measure lean mass or fat free mass of the body. This was measured as both percentage of total lean mass and absolute weight expressed in kilograms. There was no difference in lean mass (absolute or percentage) between the groups.

Lean %

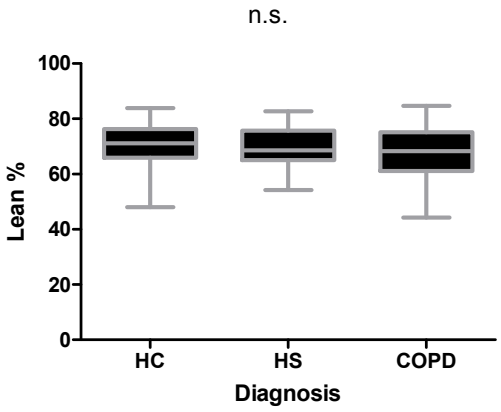

Figure 3.10 Percentage of lean mass in healthy non-smokers (HC), healthy smokers (HS), and smokers with COPD

The data are expressed as median and quartiles. Mann-Whitney U test was used for analysis.

Lean mass (Kg)

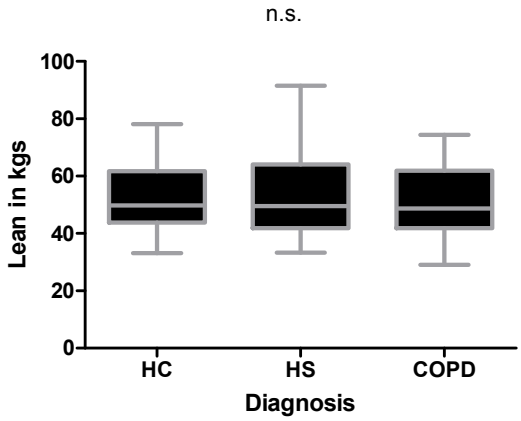

Figure 3.11 Lean (kg) mass in healthy non-smokers (HC), healthy smokers (HS), and smokers with COPD

n.s. not significant.

3.3.13. Comparison of non-smoker controls and smokers with and without metabolic syndrome

There was no significant difference in prevalence of MeS between healthy smokers and COPD as I hypothesized. The increase in prevalence of MeS in these two groups may be due to the effect of smoking rather than the disease, in COPD group. To understand the effect of smoking better, I therefore grouped all smokers and compared with non-smoker controls. There was a significant difference ($p<0.01$) between non-smokers and smokers (Fig 3.13).

3.3.14. Prevalence of metabolic syndrome

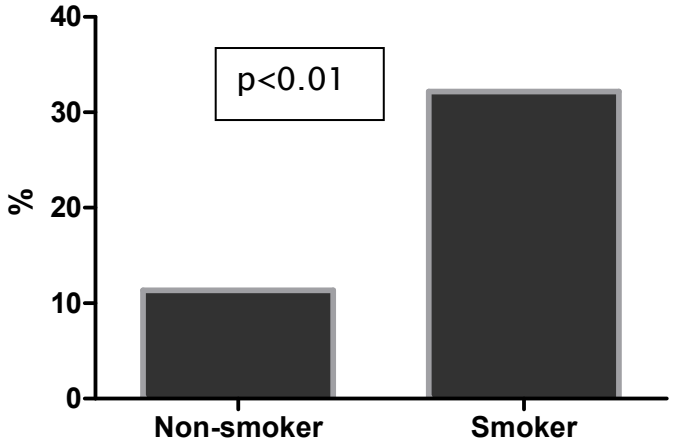

Figure 3.12 Prevalence of metabolic syndrome (using IDF criteria) in healthy non-smokers (n= 44) and smokers (n=87).

The data are expressed as percentage and chi-square test was used to analyse the data and to derive the p value (0.01). %: percentage.

3.3.15. Waist circumference

After finding that there was an increase in prevalence of MeS in smokers, I went on to analyse the individual features of MeS in smokers. The subjects were divided into four groups, non-smokers without MeS (NS-N, n=39), non-smokers with MeS (NS-Y, n=5) and smokers without MeS (Sm-N, n=59), smokers with MeS (Sm-Y, n=28) Measurement of waist predicted to be as good as abdominal fat. It is suggested to a simple clinical alternative to BMI[309]. In my study there was a significant difference in both groups ($p<0.01$, $p<0.0003$), however, the significance was more pronounced in smokers group.

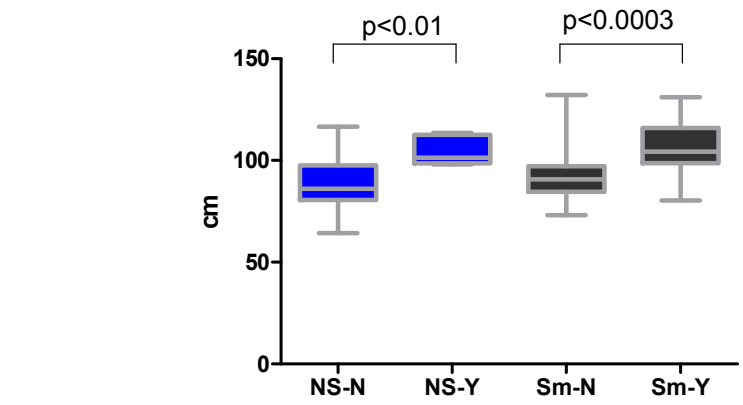

Figure 3.13 Waist circumference measurement in non-smoker controls and smokers with and without MeS.

The data are represented as median and quartiles. Non parametric Mann-Whitney U test was used for the analysis.

NS-N: non-smoker controls with no metabolic syndrome, NS-Y: non-smoker control with metabolic syndrome, Sm-N: Smokers with metabolic syndrome, Sm-Y: smokers with metabolic syndrome, cm: centimetre.

3.3.16. Waist Hip ratio

Smoking associated difference in w/h ratio contributes to the development of early diabetes particularly in smokers who has a smoking history of >10 pack years[310]. Hence, I analysed the w/h ratio in my group of subjects, and ratio of more than one was clinically significant and contributes to the morbidity and mortality of COPD. I found that there was difference in non-smokers ($p<0.002$) and in smokers ($p<0.0004$). In the correlation analysis w/h ratio was negatively

correlated with FEV_1 (r_s=-0.301, p<0.0004). W/h ratio was also positively correlated with insulin resistance (r_s=0.536, p<0.0006).

3.3.17. Measurement of fat

There is evidence that stopping smoking increases body weight, however, it is less understood the relationship of smoking and increase body fat. In my study, the total body fat in kilograms in smokers with and without MeS was measured. There was an increase in total body fat in smokers with MeS (p<0.003), however, there was no difference in non-smokers (Fig 3.15).

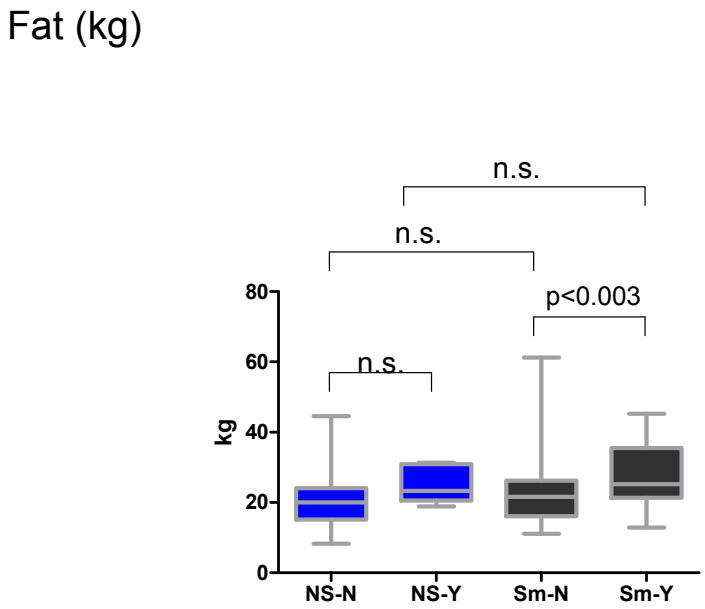

Figure 3.14 Total body fat (kg) measured by the bioelectrical impedance method in non-smoker controls and smokers with and without MeS.

NS-N: non-smoker controls with no metabolic syndrome, NS-Y: non-smoker control with metabolic syndrome, Sm-N: Smokers with metabolic syndrome, Sm-Y: smokers with metabolic syndrome, n.s: not significant, kg: kilogram.

3.3.18. Measurement of lean mass

The lean mass in my subjects was increased in smokers and non-smokers with MeS as compared to subjects without MeS ($p<0.04$ and $p<0.0004$, respectively).

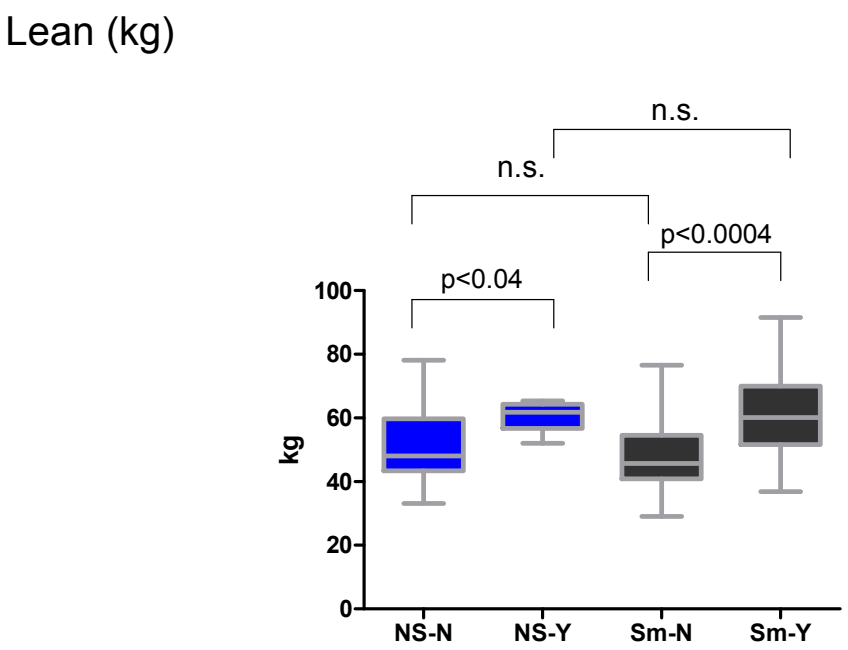

Figure 3.15 Total lean mass (kg) by bioelectrical impedance method in non-smoker controls and smokers with and without MeS.

The data are represented as median and quartiles.

NS-N: non-smoker controls with no metabolic syndrome, NS-Y: non-smoker control with metabolic syndrome, Sm-N: Smokers with metabolic syndrome, Sm-Y: smokers with metabolic syndrome, kg: kilogram, n.s.: not significant.

3.3.19. Measurement of systolic blood pressure

Increased systolic blood pressure in smokers contributes to cardiovascular disease and also to haemorrhagic stroke[311]. I found that systolic blood pressure was high in both groups ($p<0.005$ and $p<0.002$). There was a strong negative

correlation between systolic blood pressure and FEV_1 percentage (r_s= -0.273, p<0.002) which explains the effect of the blood pressure on the lung function.

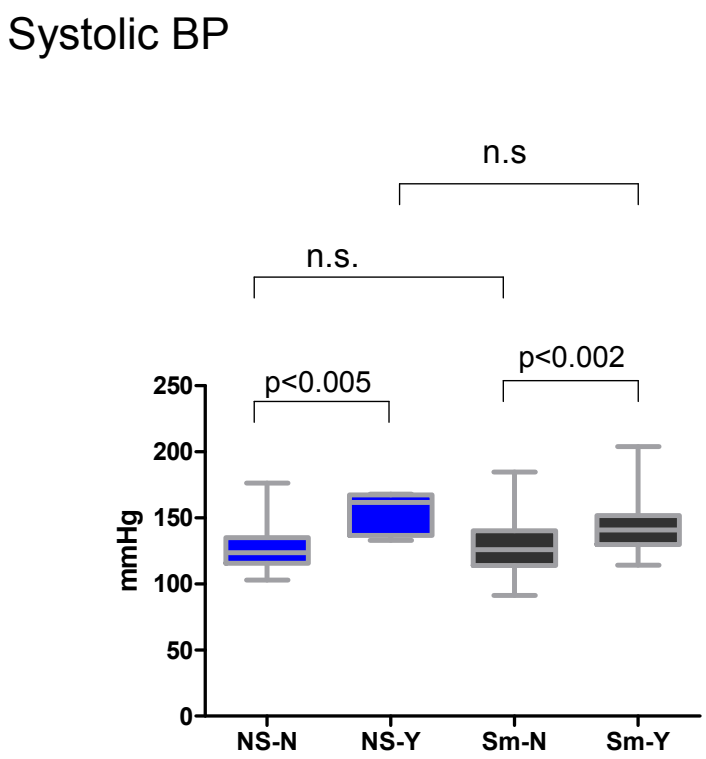

Figure 3.16 Systolic blood pressure (mmHg) in non-smoker controls and smokers with and without MeS.

The data are represented as median and quartiles.

NS-N: non-smoker controls with no metabolic syndrome, NS-Y: non-smoker control with metabolic syndrome, Sm-N: Smokers with metabolic syndrome, Sm-Y: smokers with metabolic syndrome, n.s: not significant, mmHg: millimetre of mercury.

3.3.20. Measurement of diastolic blood pressure

Diastolic blood pressure is as important as the systolic blood pressure. This is strongly associated with MeS. There is evidence to say that this could be due to a insulin receptor gene polymorphism[312]. In my study the diastolic blood

pressure was high in both groups (p<0.005 and p<0.05 respectively) but more so in non-smokers. With Spearman's rank correlation, there was no correlation between diastolic blood pressure and FEV_1 percentage.

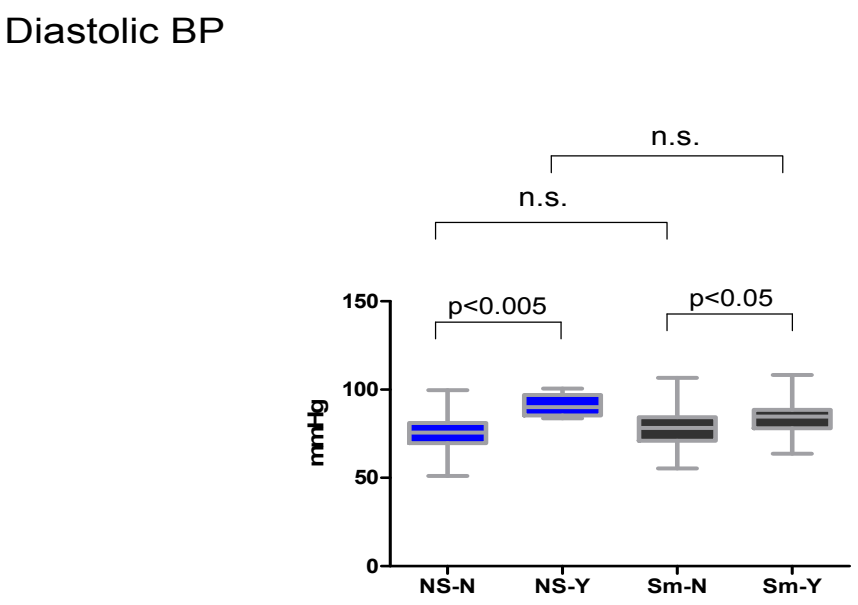

Figure 3.17 Diastolic blood pressure (mmHg) in non-smoker controls and smokers with and without MeS.

The data are represented as median and quartiles.

NS-N: non-smoker controls with no metabolic syndrome, NS-Y: non-smoker control with metabolic syndrome, Sm-N: Smokers with metabolic syndrome, Sm-Y: smokers with metabolic syndrome, n.s.: not significant, mmHg: millimetre of mercury.

3.3.21. FEV_1 and TLco in smokers with MeS

FEV_1 percentage and TLco percentage was measured in all smokers and non-smokers with and without MeS. Smokers with MeS had reduced FEV_1 percentage (p<0.04), however, there was no difference in non-smokers group. There was no difference in TLco percentage in smokers or non-smokers, however there was a difference in TLco percentage between non-smokers and smokers with and without MeS (Fig 3.19, p<0.05 and p<0.0001 respectively).

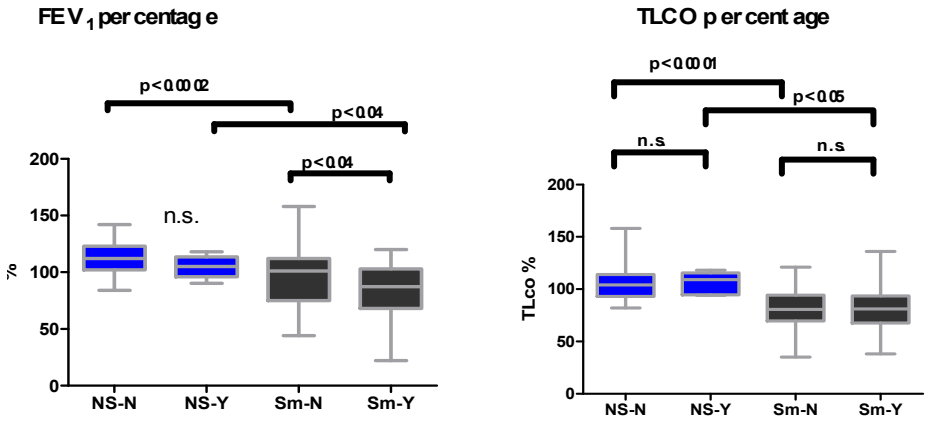

Figure 3.18 FEV1 percentage and TLco percentage in non-smoker controls and smokers with and without MeS.

control with metabolic syndrome, Sm-N: Smokers with metabolic syndrome, Sm-Y: smokers with metabolic syndrome. FEV_1: Forced expiratory volume in 1 second, TLco: transfer factor for carbon monoxide, n.s.: not significant.

3.4. Discussion

To date there have been no studies of the prevalence of features of MeS in smokers with COPD although limited research has shown an increased prevalence of MeS in smokers but has not taken into account the presence or absence of airways disease. The main aim of my study was to understand the prevalence of features of metabolic syndrome in smokers and COPD and to confirm whether it is smoking or systemic effects of COPD, which is, associated with the prevalence of features of MeS. In this well characterised cohort, there was an increase in prevalence of MeS in smokers regardless of the diagnosis of COPD when compared with non-smoker control subjects, suggesting that it is current smoking that is associated with MeS regardless of airways disease. The strong association between smoking and features of metabolic syndrome confirms the findings of Oh *et al* that smoking is associated with features of metabolic syndrome. Furthermore, that study went on to show that there was a dose-dependent association between pack years and the presence of MeS[245, 313].

There is good evidence to suggest that smoking has systemic effects[314]. Life-long smokers have an increased prevalence of cardiovascular disease [314]. Large population-based studies in smokers have confirmed an increase in serum CRP that reflects low-grade inflammation but the driver for this increase remains unknown. Metabolic Syndrome and the association with cardiovascular and type-2 diabetes [297] is well studied. It is also known that smokers with abdominal obesity are at high risk of developing coronary artery disease[315]. Smoking has been associated with increased abdominal fat, which has been shown to be associated with glucose intolerance and dyslipidemia. It has been established that central obesity measured by waist circumference predicts obesity-related health risk[316-319]. Whether smoking is at the origin of the abdominal obesity[320] has been debated. There was an increase in central obesity in smokers when compared to non-smokers in my cohort. This was also shown to the case when they were assessed for waist circumference and waist hip ratio. This confirms that smokers gain weight around the waist compared to healthy.

There is an important public message about the relationship between smoking and weight gain. This may be used as a tool in smoking cessation clinics, which may increase the success rate in future. However, it is unclear

from the results in my study whether smokers with central obesity would loose this feature of MeS if they stopped smoking. The other important feature of MeS is increased triglycerides and high-density lipoprotein (HDL). My study contrasts the data in the literature, Stefania Basili *et al* found that there was no difference in triglyceride and HDL levels in smokers and COPD compared with healthy subjects[321] but in my study I found that triglycerides were raised in both smokers with and without COPD when compared to healthy non-smokers. It was also noted that there was no difference between smokers with and without COPD suggesting the effect of smoking, rather than disease, on triglyceride levels.

There was a decrease in HDL level in COPD compared to healthy subjects. However, there was no difference in smokers without COPD and healthy or between smokers with and without COPD. This is again contrasts the study done by Tisi *et al* [322] where in they described an increase in HDL levels in COPD subjects. Others have reported an association between smoking and hypertension[323] however in my study there was a difference in systolic and diastolic blood pressure in healthy smokers with MeS but not in COPD group. This is again an indication of the effect of smoking and its role on endothelial damage.

There is a coexistence of type-2 diabetes and COPD and an increase trend of diabetes in elderly patients[324]. Abnormal glucose concentration and fasting insulin are important contributors for the development of feature in MeS. This opens a new treatment approach, which could be developed in such patients where both diseases co exist. The question why smokers have an increase in blood glucose remains open. One possible explanation is that smoking and indulgence in food go hand in hand. The other is that smoking induces insulin resistance. Indeed, in my study I found that COPD subjects had elevated fasting glucose compared to healthy and a borderline difference with smokers without COPD. There was an association between smoking and insulin resistance, which is discussed in chapter 5.

I found that fat mass was increased in both smokers with and without COPD (fig 4.2.5 and fig 4.2.8). This important finding suggests that smoking can increase fat-mass and also contradicts the widely held view amongst the public and doctors that smoking keeps people slim. It was also confirmed by increase in waist/hip ratio which showed increase with smoking (Fig 4.2.7 and 4.3.1).

After these analysis I realised that more of the changes seen are due to the effect of smoking rather than COPD. I therefore grouped both HS and COPD. The combined smoking group, as expected had high prevalence of MeS. The percentage of smokers having MeS was 32.18% and 11.36% in non-smoker controls. There was no difference in blood glucose, smokers had high triglycerides, and HDL was same when comparing smokers with and without MeS. This suggests that these features are not key in differentiating smokers with and without MeS. However, smokers with MeS had more fat mass and had increased waist, raised fasting insulin, and insulin resistance. It was also seen that both systolic and diastolic blood pressure were high in smokers with MeS. Other important finding was that the subject with MeS had decreased FEV_1 percentage and TLco was decreased compared to non-smokers with MeS. The reason could be that the features of MeS are probably driven by decrease in FEV_1. The possible hypothesis is that smoking causes decline in lung function, which in turn drives oxidative stress, and insulin resistance, which ultimately effects in early developlement of MeS in smokers. Alternatively, MeS could contribute to the progression of airways damage and development of COPD, i.e. decline in FEV_1.

Much remains to be understood about the association between smoking and metabolic features. It is unclear why some smokers develop features of MeS and what might be the protective mechanism in others. We also need evidence of reversal of these features if patients stop smoking and it remains unclear what is the role of early intervention such as diet and physical activity. There are no studies on gene environment relationship in COPD and metabolic syndrome which could shed some light on the contributing factors.

The limitation of this study was that the subjects all had mild-moderate disease and normal to overweight subjects with relatively small number. Future study should focus on severe COPD subjects and focus separately on obese and cachextic patients where the interaction between lung disease and the metabolism may be different.

4.0 Chapter- Systemic inflammatory markers in COPD and metabolic syndrome

4.1. Introduction

Inflammation is important in the pathogenesis of COPD[79]. Cigarette smoke known to cause local inflammatory changes in the airways and also systemic cellular and humoral inflammation, systemic oxidative stress and endothelial dysfunction and increased procoagulant factors[314, 325]. Inflammatory markers play an important role in the pathogenesis of COPD and there is a suggestion that inflammation is an important pathway between lung disease and vascular disease[326, 327]. Studies have explored the role of specific inflammatory cells in COPD and suggested the importance of neutrophils[328-330], CD8+ T lymphocytes[331-333], macrophages[334-336]. In chronic smokers neutrophils are increased in blood and bronchoalveolar lavage fluid[337]. IL-6 is considered to play a role in innate and adaptive immunity and studies have showed that IL-6 activity was decreased after smoke exposure. IL-8 measurement is used to help to discriminate between smokers with and without COPD and COPD patients reported to have a raised IL-8 levels[338].

There is an increasing evidence suggesting that clinical features of COPD and airflow limitation are poorly correlated and there is consensus about a comprehensive approach, including radiological evidence[112] , inflammatory markers, assessment of body composition, exercise tolerance[113] for the diagnosis and to classify the severity of the disease.

MeS is a cluster of specific risk factors for cardiovascular disease with common underlying abnormality in insulin production. The aetiology of metabolic syndrome is uncertain but it is accepted that MeS is associated with inflammation and it has been suggested that subclinical inflammation is a contributory factor in the development of MeS[339]. The inflammatory markers which are raised in MeS are CRP[340], TNF-α, fibrinogen and IL-6 and leptin among others[341]. The association between inflammation and MeS is complex. One of the accepted reasons is that the proinflammatory cytokines are released by adipocytes into the systemic circulation which in turn triggers hepatic CRP production, thereby increasing inflammation. A second view is that there is an increase in the prothrombotic molecule plasminogen activator inhibitor-1 (PAI-1) in individuals with MeS[342], which correlates with visceral adiposity[343].

Loss of body weight and fat free mass is associated with increased mortality, poor functional status and quality of life of patients with COPD[298, 301, 344]. However, there is an inverse relationship between mortality and BMI in

these patients and putting on weight in cachextic patients improves prognosis[113, 231, 298]. The association of features MeS like dyslipidemia, inflammation, insulin resistance, type 2 diabetes is associated with increased visceral fat seen in obese subjects[308, 345, 346]. There is a debate whether the presence of metabolic syndrome alone can predict global cardiovascular disease risk or is it abdominal obesity, the most prevalent manifestation of metabolic syndrome and a marker of dysfunctional adipose tissue, that is of central importance[346].

There is a view that COPD is a chronic systemic inflammatory syndrome which, apart from age and smoking, contributes to develop chronic heart failure, features of MeS and increased CRP. It is well known that inflammation plays an important role in COPD and there is evidence that inflammation may be associated with MeS but what is less understood is that which inflammatory cells, markers and inflammatory mediators play a crucial role in smokers and COPD who develop features of MeS. My hypothesis is that there is a strongh relationship between inflammatory mediators and smoking and that systemic effect of smoking contributes to the development of features of MeS in smokers and COPD.

The aim of this study was to understand the role of common inflammatory cells and markers, mediators released in smokers who develop COPD and to compare the same with non-smoker controls who also develop features of metabolic syndrome.

4.2. Methods

A total of 131 subjects were recruited for the study. They were sub divided into non-smoker controls (HC), smokers without COPD (HS) and smokers with COPD. Subjects underwent medical examination, body composition measurements. Bloods were taken for full blood count, CRP. Cytokine measurement was done on 98 subjects by multiplex ELISA (Luminex). The study design is explained in detail in chapter 2.0 (section 2.4.9).

4.2.1. Systemic inflammatory marker and inflammatory cells in smokers with and without COPD

4.2.2. C-Reactive protein (CRP)

Smoking increases the levels of CRP by causing tissue damage, however, there are inconsistencies with the results from many studies. In clinical practice CRP is routinely used as a marker of systemic inflammation. In my study CRP was measured from the peripheral blood and, the normal range was between 0-7.5mg/L. When CRP was analysed in all subjects there was a significant difference between HC and HS ($p<0.04$), HC and COPD ($p<0.0001$) and HS and COPD ($p<0.0003$). Smokers with COPD had high levels of CRP compared to smokers without COPD. This suggests that there is an increased inflammation in COPD subjects and confirms that CRP is raised in smokers in general.

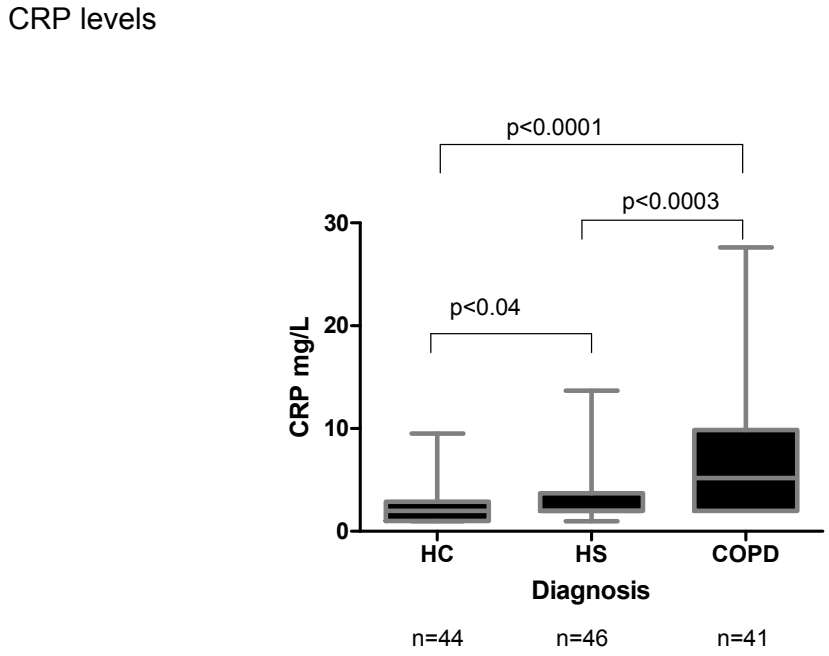

Figure 4.1 C-Reative Protein (CRP) levels in peripheral blood in healthy non-smokers (HC), smokers (HS) with and without COPD.

Data are represented as median and quartiles.

4.2.3. Blood neutrophils

Neutrophils are important mediator of inflammation in smokers and COPD. Neutrophils are short lived and transient cells in the body and they are recruited into the lung during inflammation and infection. In COPD patients, they play pivotal role in host defence. Neutrophil count was measured in peripheral blood in all subjects in the study. Bloods was taken after making sure that subjects had not exercised nor smoked for 12 hours. The normal peripheral blood neutrophil level is between 2.0-7.5*10^9/L in our laboratory and there was a difference between non-smoker controls (HC) and smokers with and without COPD ($p<0.0002$ and $p<0.0004$ respectively), however, there was no statistical difference between smokers with and without COPD.

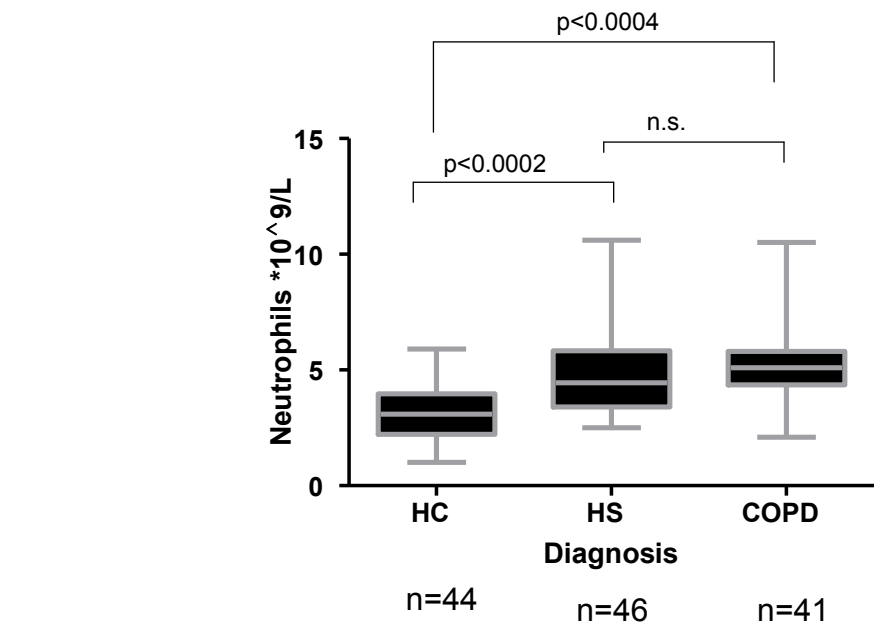

Figure 4.2 Peripheral blood neutrophils in non-smoker controls (HC), smokers (HS) and COPD subjects (COPD).

Data are shown as median and quartile and Mann.n.s: not significant.

4.2.4. Blood monocytes

The role of monocytes is to participate and help in host defence in COPD patients. Peripheral blood monocytes are the circulating precursors of alveolar macrophages[347]. Monocytes was measured in the peripheral blood in all subjects in the study. The data suggests that there was difference between HC and HS ($p<0.0001$), HC and COPD ($p<0.0001$), however, there was no difference between HS and COPD.

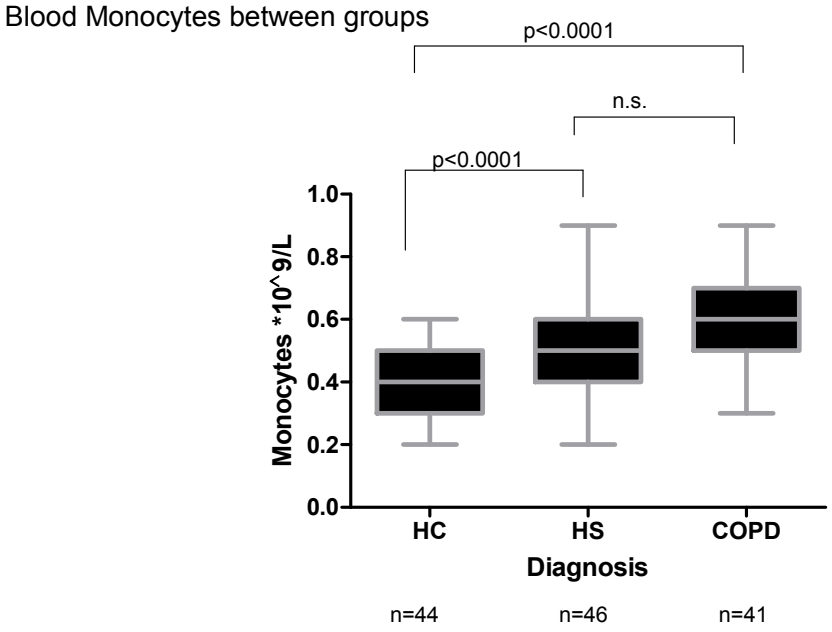

Figure 4.3 Peripheral blood monocytes in non-smoker controls (HC), smokers (HS) and COPD subjects.

Data are shown as median and quartiles and Mann Whitney U test used to analyse the difference between the groups. n.s: not significant.

4.2.5. Blood lymphocytes

Total lymphocyte was measured in the peripheral blood in all subjects in the study. There was difference between HC and HS ($p<0.0002$) and HC and COPD ($p<0.0001$), however, there was no difference found between HS and COPD.

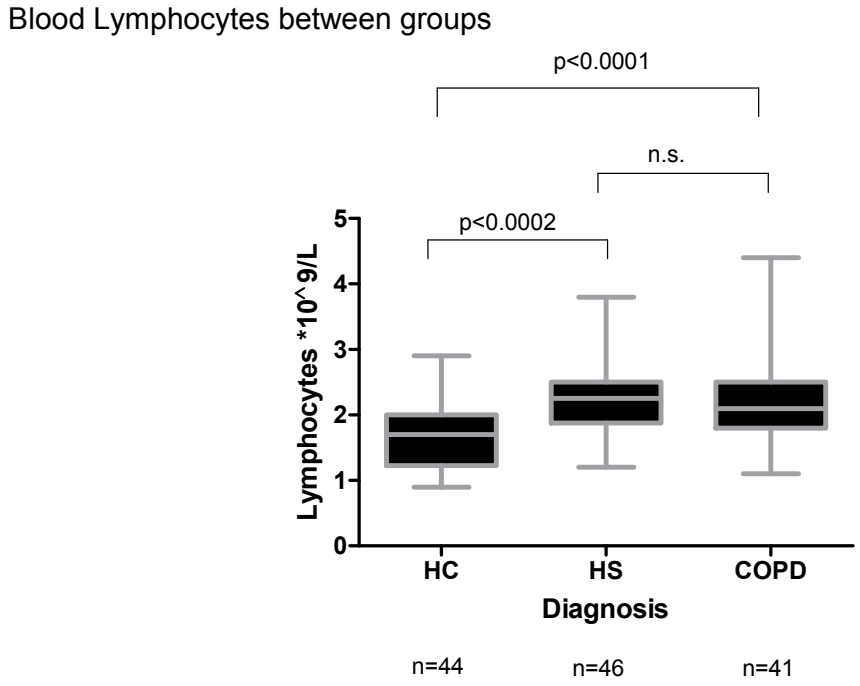

Figure 4.4 Peripheral blood lymphocytes in non-smoker controls (HC), smokers (HS) and COPD subjects (COPD).

Data are presented as median and quartiles.

4.2.6. Systemic inflammatory marker (CRP) and inflammatory cells in smokers combined

The inflammatory marker CRP levels and inflammatory cells neutrophils, monocytes and lymphocytes were higher in smokers and subjects with COPD. However, there was no difference between smokers with and without COPD except for CRP. This suggests that the inflammatory changes are due to smoking rather than COPD as a disease. All the smokers were then grouped to analyse the differences in inflammatory marker and inflammatory cells in smokers and non-smokers with and without MeS.

CRP was strongly associated with inflammation and there are numerous studies, which demonstrate this in smokers. In my study there was a steep increase in levels when compared with non-smokers (Fig 4.5 $p<0.0004$). In the

bivariate analysis CRP showed a strong negative correlation to FEV_1 percentage (r_s change=-0.514, p<0.0006), TLco (r_s change=-0.507, p<0.0006).

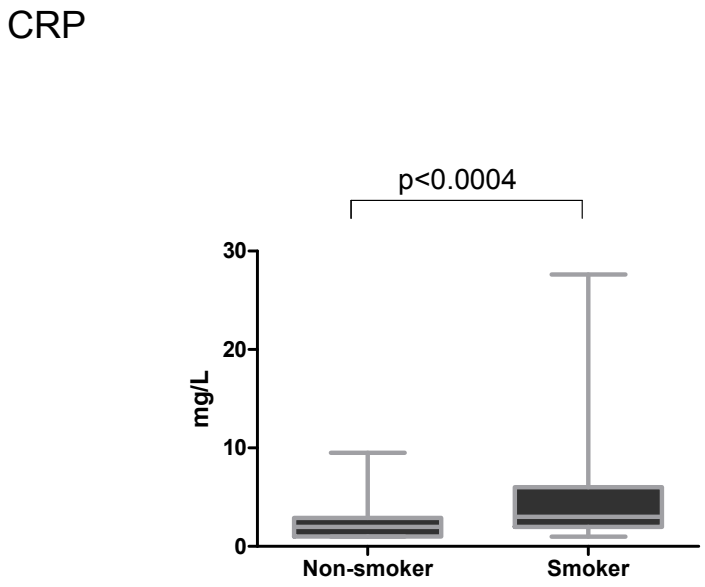

Figure 4.5 C-Reactive Protein (CRP) levels in healthy non-smokers and smokers. The data are represented as median and quartiles. mg/l: milligram per litre.

4.2.7. Blood inflammatory cells, neutrophils, monocytes, and lymphocytes

The results were analysed and found that there was difference (Fig 4.5A, p<0.0001) between smokers and non-smokers. Subsequently multivariate analysis was done with other inflammatory markers and FEV_1 as dependent variable. I found that neutrophil counts were not associated with FEV_1. However, there was a strong association between transfer factor (TLco) and neutrophils (r^2 change=0.311, p<0.002).

Blood monocytes were also measured in the study. The effect of smoking on monocytes is important to understand as blood monocytes are the precursors of tissue macrophages and the defective microbicidal function may represent an additional pathogenic factor in the diminished host defence observed in smokers. In my study I found that the monocyte counts were

raised in smokers when compared with non-smoker controls (Fig 4.5 B, p<0.0005). In the multivariate analysis there was no association of monocytes with FEV_1 percentage or transfer factor.

Many studies have shown that lymphocytes play an important role in the pathogenesis of asthma but there are few studies which have looked at their role in COPD. Lymphocyte counts in blood were raised in smokers when compared with non-smokers (Fig 4.5 C p<0.0006). In the multiple variant analysis there was a positive correlation between lymphocytes and transfer factor (r^2 change 0.311, p<0.02).

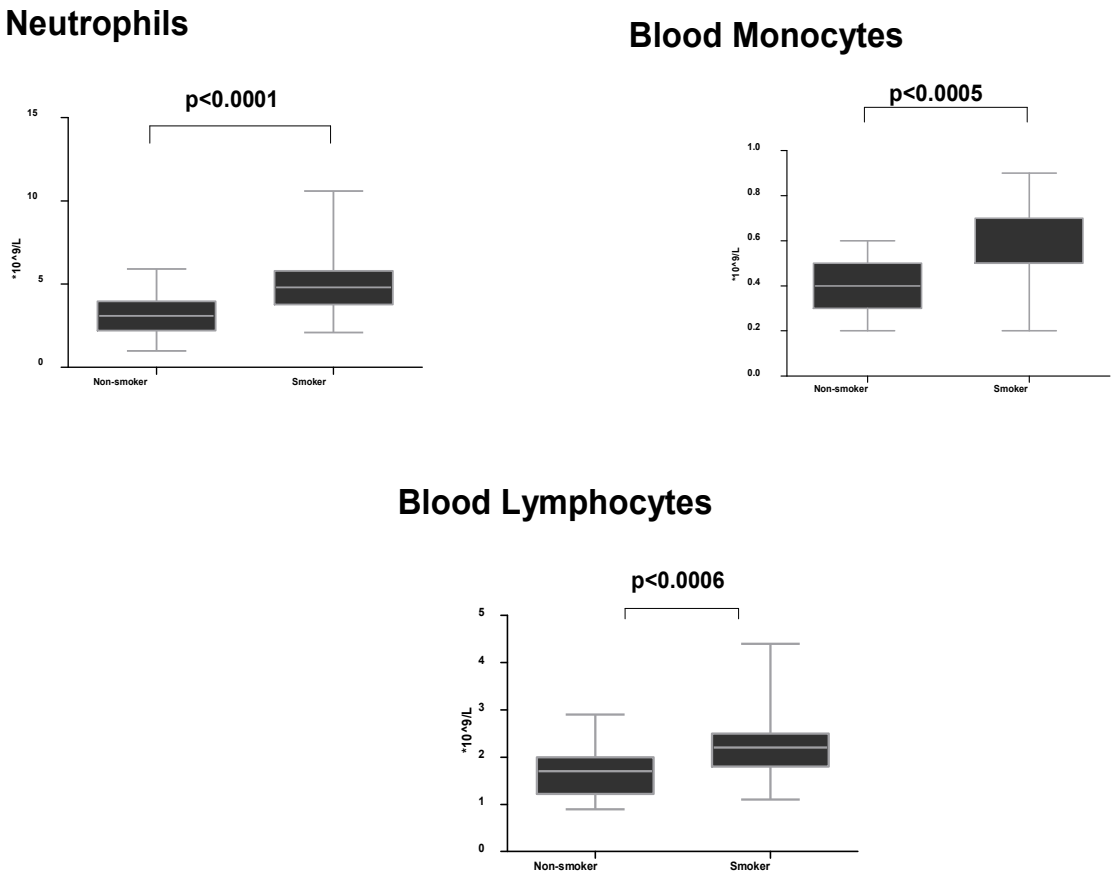

Figure 4.6 Blood neutrophils, monocytes, and lymphocytes between healthy non-smokers and smokers.

The data are represented as median and quartiles. Mann Whitney U test was used for the analysis.

4.2.8. Systemic inflammatory markers in smokers with and without MeS

4.2.9. C-Reactive protein

CRP is a strong marker of systemic inflammation. It has been shown to be raised in smokers and in COPD and is known to be raised in MeS. In this present study when analysed in smokers with and without features of MeS there was no difference between the groups and even in non-smoker controls (Fig 4.7). However there was a correlation with individual features of MeS: triglycerides (r^2= 0.255, p<0.003), HDL (r^2= -0.302, p<0.0004), waist circumference (r^2= 0.306, p<0.0003), glucose (r^2=0.202, p<0.02) and with systolic blood pressure (r^2= 0.271, p<0.002).

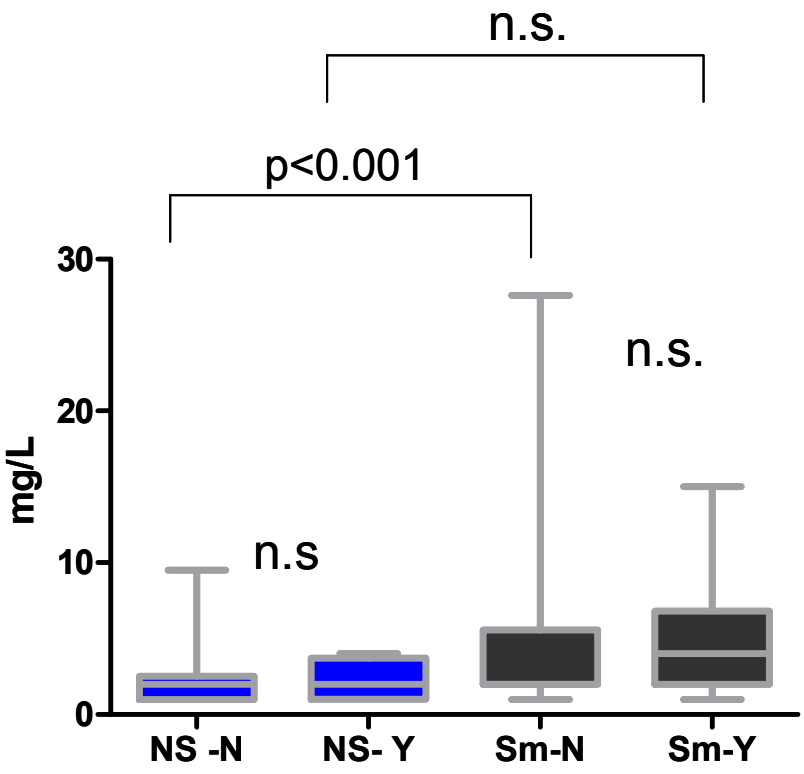

Figure 4.7 CRP levels in non-smoker controls and smokers with and without MeS

The data are represented as median and quartiles and p<0.05 was considered significant. NS-N: non-smoker controls with no metabolic syndrome, NS-Y: non-

smoker control with metabolic syndrome, Sm-N: Smokers with metabolic syndrome, Sm-Y: smokers with metabolic syndrome, n.s.: not significant, mg/L: milligram per litre.

4.2.10. Blood neutrophils, monocytes and lymphocytes

Neutrophils play an important role in smokers with COPD but its role in features of MeS is less studied. It has been found to be elevated in men with MeS[348]. Neutrophils, monocytes and lymphocytes were measured from the peripheral blood in my study. They were analysed using Mann Whitney-U non-parametric test. There was no difference in neutrophils (Fig 4.8), monocytes (Fig 4.8) and lymphocytes (Fig 4.9) between subjects with and without features of MeS.

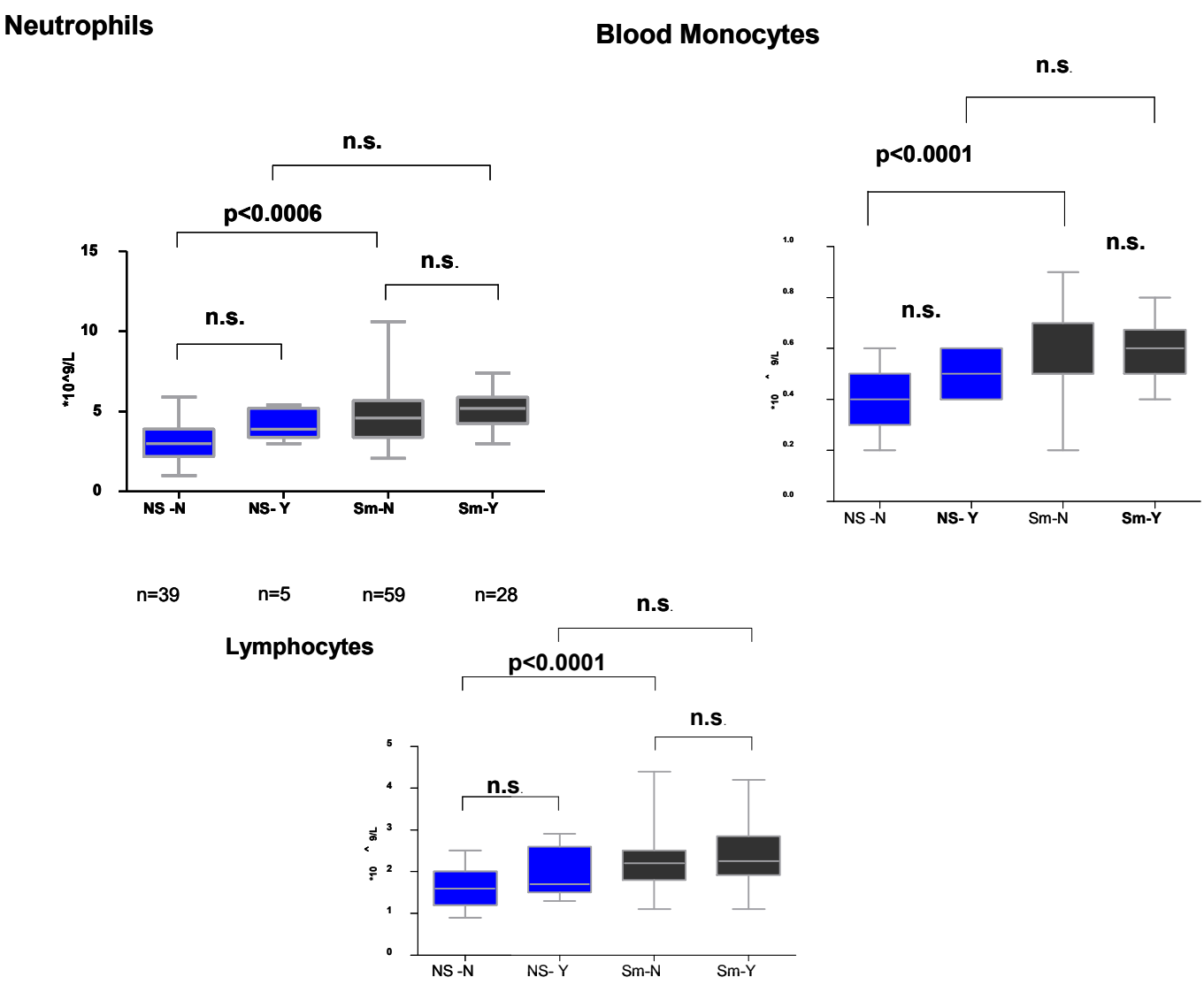

Figure 4.8 Inflammatory cells neutrophils, monocytes, lymphocytes in non-smoker controls and smokers with and without MeS

The data are expressed as median and quartiles and $p<0.05$ was considered significant.

NS-N: non-smoker controls with no metabolic syndrome, NS-Y: non-smoker control with metabolic syndrome, Sm-N: Smokers with metabolic syndrome, Sm-Y: smokers with metabolic syndrome, n.s.: not significant.

4.2.11. Inflammatory cytokines smokers with and without MeS

Plasma cytokines were measured by multiples ELISA (Luminex method). The total number of subjects sampled were 97: of these, 27 were healthy non-smokers without MeS, 3 were healthy non-smokers with MeS, 46 were smokers without MeS and 21 were smokers with MeS. The cytokines measured were IL-13, IL-8, IL-6, IL-10, IL-4, IL-12p70, IL-2, IL-5, TNF-α, IFNg and Apolipoprotein 1

(apo 1) which is a protein component of HDL cholesterol, which plays a role in autoimmunity.

4.2.12. Plasma IL-13

Pro inflammatory cytokine IL-13 is associated with MeS and type 1, 2 diabetes. In my study, I measured IL-13 in plasma of 97 subjects with and without MeS. The measurement was done by Luminex method. There was a difference in non-smokers group with and without MeS ($p<0.002$), however, there was no significant difference in smokers group (n.s.).

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IL-13

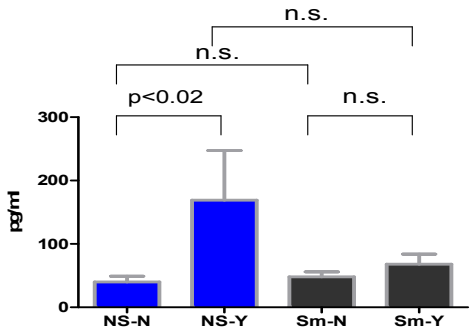


Figure 4.9 Plasma IL-13 levels in non-smoker controls and smokers with and without MeS.

The data are expressed as mean and standard error of mean.

NS-N:non-smoker controls with no metabolic syndrome, NS-Y:non-smoker control with metabolic syndrome, Sm-N:Smokers with metabolic syndrome, Sm-Y:smokers with metabolic syndrome, n.s.: not significant, pg/L:picogram per liter.

4.2.13. Plasma IL-12

IL-12 is a heterodimeric 70kDa glycoprotein (IL-12p70) consisting of 40kDa subunit. It is secreted by peripheral lymphocytes and is produced mainly by B-cells and to a lesser extent by T-cells. Plasma IL-12 with polypeptide subunit p70 was measured by multiplex Luminex method. There was significant

difference in smokers with and without metabolic syndrome (p<0.04). This was also raised in non-smokers with MeS (p<0.009).

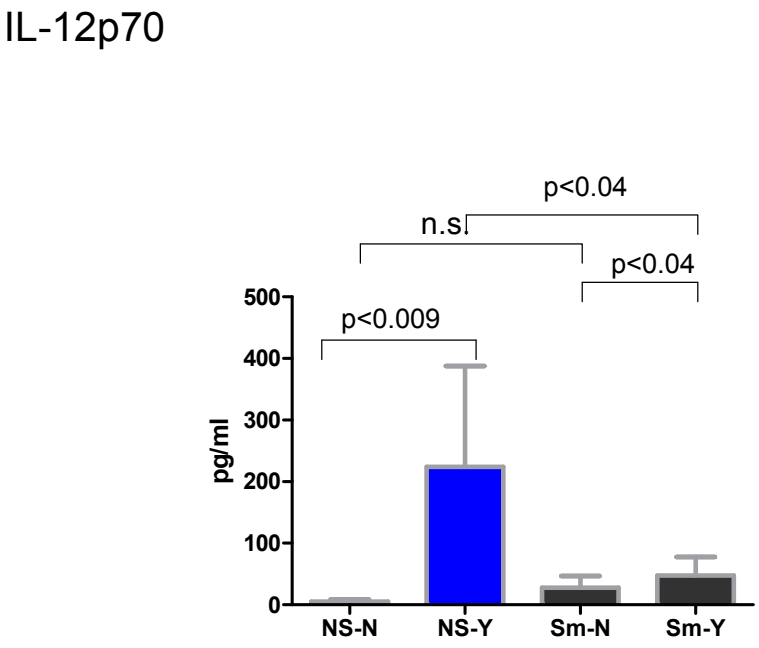


Figure 4.10 Plasma IL-12p70 levels in non-smoker controls and smokers with and without MeS.

The data are expressed as mean and standard error of mean.

NS-N:non-smoker controls with no metabolic syndrome, NS-Y:non-smoker control with metabolic syndrome, Sm-N:Smokers with metabolic syndrome, Sm-Y:smokers with metabolic syndrome, n.s.:not significant, pg/L:picogram per liter.

4.2.14. Plasma IL-8

IL-8 is chemotactic factor for neutrophils. They are secreted by macrophages and epithelial cells to attract neutrophils so that they could adhere to the vascular endothelium to migrate into the tissue. It is also produced by endothelial cells which store them in their vesicles[349, 350]. In the study IL-8 was measured by Luminex and there was no difference in smokers group, however it was found to be raised in non-smokers with MeS (p<0.02).

IL-8

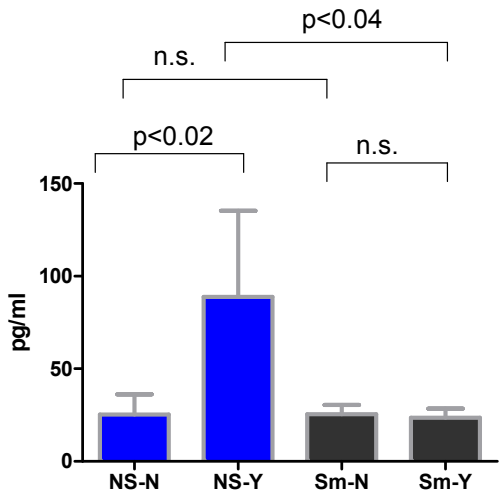


Figure 4.11 Plasma IL-8 levels in non-smoker controls and smokers with and without MeS.

The data are shown as means and standard error of mean.

NS-N: non-smoker controls with no metabolic syndrome, NS-Y: non-smoker control with metabolic syndrome, Sm-N:smokers with metabolic syndrome, Sm-Y:smokers with metabolic syndrome,IL:Interlukin, n.s.: not significant, pg/l: pictogram/litre.

4.2.15. Other inflammatory cytokines measured in plasma

Measurement of IL-2 and IL-4 showed differences in non-smokers (Fig 4.12 A and B, $p<0.02$ and $p<0.04$) and IL-5 (Fig 4.13 E, $p<0.03$), however, there was no difference in smokers with and without MeS.

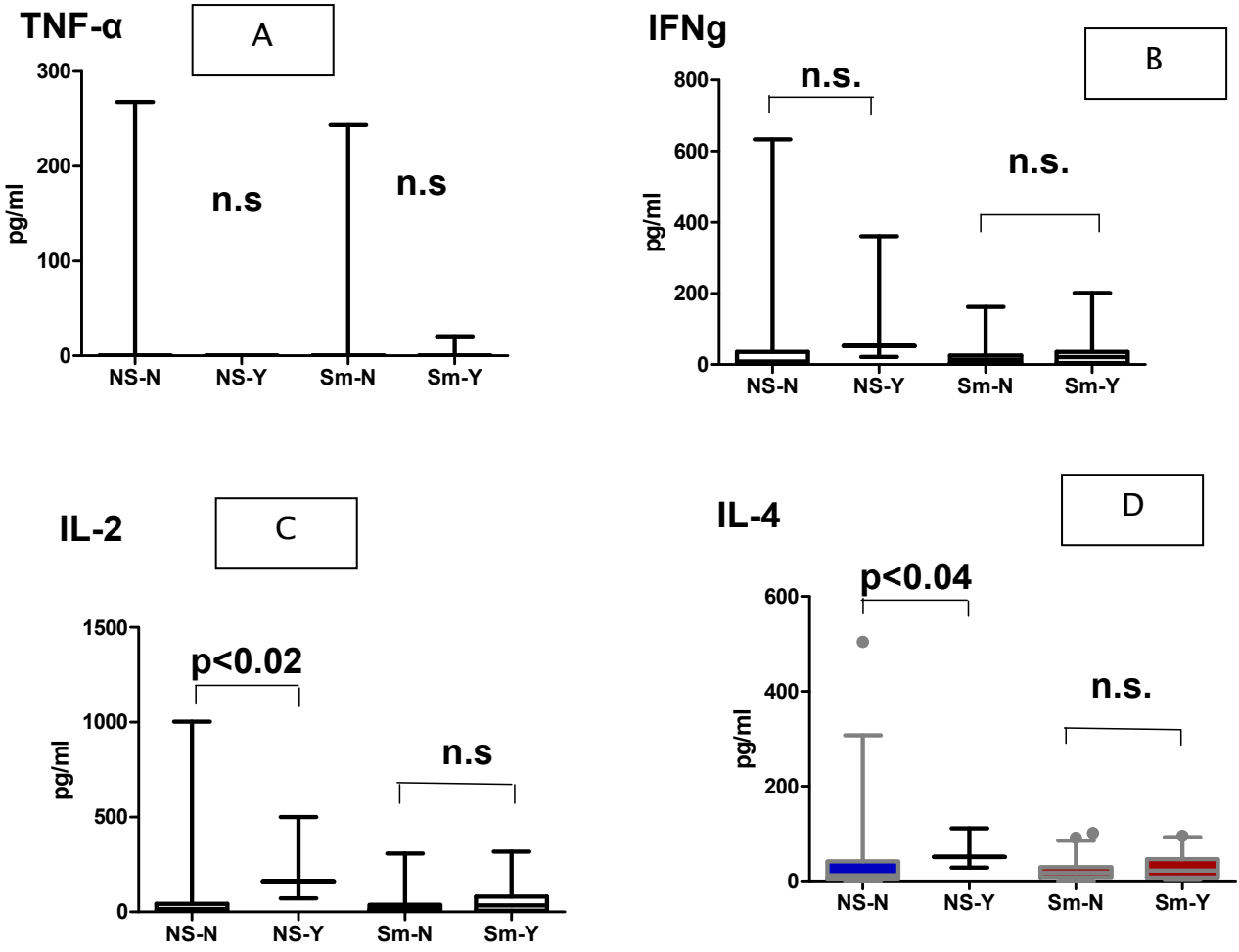


Figure 4.12 Inflammatory cytokines measured in plasma in non-smoker controls and smokers with and without MeS, (A) TNF-α, (B) IFNg, (C) IL-2, (D) IL-4).

The data are represented as median and quartiles.

NS-N:non-smoker controls with no metabolic syndrome, NS-Y:non-smoker control with metabolic syndrome, Sm-N:Smokers with metabolic syndrome, Sm-Y:smokers with metabolic syndrome.TNF-α:Tumor necrosis factor alpha, IFNg:Interferone gamma, IL:interlukin, n.s.: not significant, pg/L:picogram per liter.

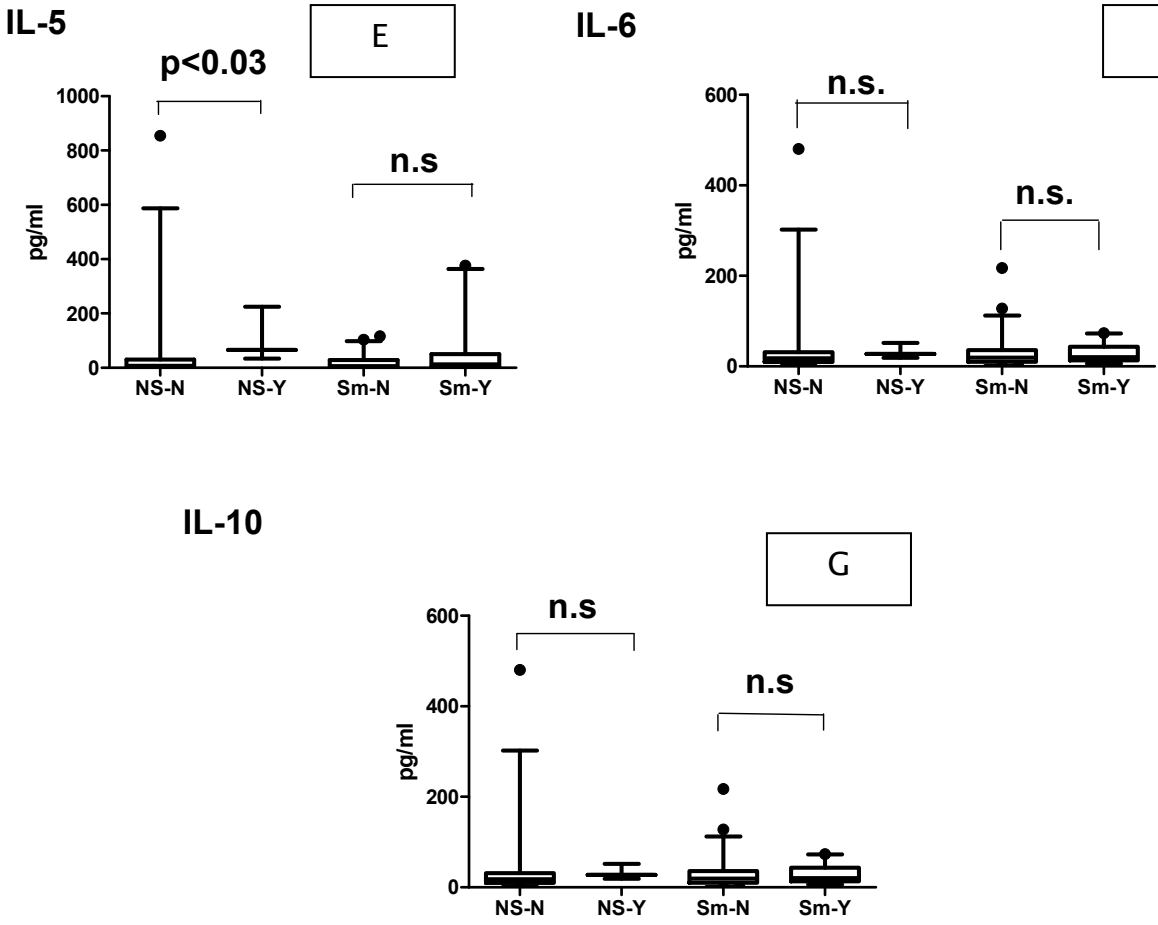


Figure 4.13 Inflammatory cytokines measured in non-smoker controls and smokers with and without MeS,

(E) IL-5, (F) IL-6, (G) IL-10. The data are represented as median and quartiles. NS-N:non-smoker controls with no metabolic syndrome, NS-Y:non-smoker control with metabolic syndrome, Sm-N:Smokers with metabolic syndrome, Sm-Y:smokers with metabolic syndrome.

4.3. Discussion

The aim of this study was to improve the understanding of the role of inflammation in COPD but more importantly, to better understand the effects of metabolic syndrome. The important findings in this study are, a) CRP levels have been shown to rise with increasing severity of COPD[327, 351]. I have shown in this study that CRP levels increased in a linear fashion in the three groups and that COPD subjects had the highest CRP levels, b) Result alos demonstrates an inverse relationship of CRP with FEV_1 which was consistent with the previous study that showed in general population there is an inverse relationship between CRP and FEV_1 predicted[352] c) Inflammatory cells are not raised in stable COPD compared to healthy smokers.

IL-6 and CRP have related roles in the inflammatory response: IL-6 induces CRP production in the liver by activating Janus kinases, signal transducers and activators of transcription subsequently switch on the CRP gene expression, leading to the production of CRP[353]. There is also a relationship between IL-6 and CRP in driving inflammation[354]. However, when CRP was measured in the my study, suggested no association between blood CRP and MeS and there was no correlation between IL-6 and CRP in MeS. This is in contrast to the available evidence about CRP levels[193]. One of the limitation here was CRP was not high sensitive and the number of subjects with MeS were small. There are inconsistent reports on the relationship between CRP level and lung function. CRP was also correlated with smoking. It is difficult to understand whether it is secondary to smoking or due to the systemic inflammation in COPD[355]. It is also seen that CRP reduces after treatment with inhaled corticosteroids[355]. In the regression analysis in my study, the CRP was independently associated with smoking and COPD. It is certain that CRP is playing a significant role in driving inflammation in COPD.

Inflammation plays an important role in the pathogenesis of features of metabolic syndrome. It is suggested that there is an ongoing subclinical inflammation in these people and there are very few studies that have looked at these markers in MeS in smokers. It has been suggested that the production of cytokines such as TNF-α[356], IL-6[357] and leptin[358] by adipose tissue may be involved with acute phase response in obesity related co morbidities. In this study, I found that inflammatory cells play a major role in smokers regardless of presence of COPD. Neutrophils. monocytes and lymphocytes were all raised

in smokers. There was not enough evidence in this study to confirm that inflammation in smokers play a role in the development of features of MeS. Of note, IL-4, IL-5, and IFNg were elevated in non-smokers without MeS.

Airway remodelling and destruction of the parenchyma is associated with inflammation in COPD[359]. Different cells and mediators of inflammation are involved in the pathogenesis of COPD[338]. Activation of monocytes/macrophages and oxidative changes in low density lipoprotein are important in atherogenesis[360]. In my study neutrophils, monocytes and lymphocytes were all elevated in healthy smokers. What was less understood was that these cells did not show any increase in COPD group in my study. One of the possible reasons may be that, the COPD subjects were mild-moderate and clinically stable at the time of recruitment.

The other interesting observation in my study is the raised eosinophils in healthy smokers and COPD. Even though the levels were within the laboratory accepted normal range, eosinophil counts were raised in COPD. Eosinophils are the inflammatory cells, which are commonly seen in asthmatic airways. However there is little evidence of eosinophilia in stable COPD subjects[361]. One of the other reasons is that raised eosinophils are seen in subjects with chronic bronchitis[362] and there were smokers with chronic bronchitis in my study group of healthy smokers.

In addition, other inflammatory cells have been shown to be raised in smokers compared to non-smokers (neutrophils, monocytes, and lymphocytes). However, inflammatory cells were not raised in COPD when compared to healthy smokers in my study. To examine the relationship between cellular immune status and features of MeS I measured total lymphocytes count in my subjects. There was no difference in the total count in subjects with and without MeS. In the Spearman' rank correlation I found that lymphocytes were stongly correlated with triglycerides (r_s= 0.424, $p<0.0007$) and weakly with waist circumference (r_s= 0.173, $p<0.048$) suggesting the role of inflammation in driving these two specific features of MeS.

In summary my study has shown that there was no association between inflammatory markers and features of MeS in either smokers or non-smokers. However, this study confirms the role of inflammation in smokers with and without COPD. I believe that there is more than one pathway involved and contributing to the development of MeS in smokers. Therefore, it is important

to understand other possible causes, which are contributing to the features for e.g. central obesity, insulin resistance, or physical activity, which are discussed in chapter 5.

5.0 Chapter- Role of insulin resistance, physical activity, and quality of life in smokers and COPD with metabolic syndrome

5.1. Introduction

Insulin resistance is a condition that increases the chances of developing diabetes and heart disease. This salient features is also thought to be the key in the development of metabolic syndrome (MeS), however, the relationship between smoking habits, insulin resistance, and related risk factors is less understood. It is also less known how insulin resistance has any role in the development of COPD.

MeS is a cluster of cardiovascular risk factors consisting of dyslipidemia, glucose intolerance, central obesity, and hypertension[363]. There are several additional possible risk factors that have been included recently, for example, increased total body fat including non-alcoholic fatty liver. The mechanisms behind this complicated syndrome are debated even today. Some believe that this syndrome is caused by insulin resistance and others think it is a combination of insulin resistance, subclinical inflammation and physical activity. One of the arguments about insulin resistance is that obesity mainly visceral adiposity contributes to insulin resistance[364]. As the prevalence of obesity is increasing globally, this new clinical disorder, which is associated with central obesity and insulin resistance, emerged and identified as the major risk factor for cardiovascular disease[364]. It is also been suggested that hyperinsulemia per se as a driver for muscle insulin resistance and anabolic effects, which could lead to hepatic steatosis and insulin resistance[365]. Reaven coined this cluster of features as' Syndrome X' and was named 'insulin resistance syndrome' later by others[185].

Smoking on the other hand is the main cause for development of COPD in the over 50-age group and it is an airway disease which is partially reversible in the early stages of the disease. There is a strong association between smoking and features of MeS (chapter 3). However, there is a large disparity in smokers who develop this condition. Smoking also contributes to elevated blood pressure, which is more frequent in insulin resistance and obese patients[185, 220]. One of the mechanisms of developing insulin resistance in smokers is that the acute affects of smoking increases the sympathetic activity and circulating levels of catacholamines[366], and these catacholamines are antagonist to insulin action[367] and hence smoking may have a link to insulin resistance. Smoking has local and systemic effects and the local effect of smoking is the cause for the development of COPD, which is accepted as an inflammatory disease.

Inflammation is considered to be one of the main reasons for development of MeS, however there was no evidence of that in my study (chapter 4). The other possible causes like insulin resistance, physical activity and diet is less explored.

Physical activity plays a major role in development of obesity and type-2 diabetes. Ekelund *et al*. examined a large population with no MeS at the baseline and found that physical activity energy expenditure independently contributes towards development of MeS[368]. This finding is important in context of my study because smokers tend to do less exercise.

The physical activity is reduced in COPD. This is seen more in GOLD stage III and stage IV diasease[369] . Hence there are reports of early referral to pulmonary rehabilitation to strengthen the respiratory muscle tone and to raise the quality of life in patients with COPD.

Quality of life (QOL) is an important part in any chronic disease management. It is more so in COPD patients who suffer from symptoms mainly shortness of breath. It has been shown previously that COPD patients have decreased quality of life[370]. The reasons for this reduced QOL apart from the symptoms are physiological, psychosocial, and sometimes functional. QOL is less understood in various stages of the disease process in COPD. In this study I hypothesise that QOL may be worse in smokers with features of metabolic syndrome.

I tested the hypothesis by studying the relationship between insulin resistance, physical activity, and QOL in smokers with and without features of MeS.

5.2. Methods

A total of 131 subjects were recruited for this study. The subjects were stratified into two groups: non-smoking control subjects, and smokers. The subjects underwent pulmonary testing, body composition mesaurements and bloods for fasting glucose and insulin (n=128). By HOMA-R insulin resistance was calculated. Subjects completed quality of life (SGRQ) and physical activity (n=120) questionnaires (Baecke). The details of the questionnaire are discussed in chapter 2 (section 2.2.3).

5.3. Results

5.3.1. Fasting insulin and HOMA-R measurement

5.3.2. Fasting insulin levels

Blood was taken from subjects (n=128) who were fasting overnight for the measurement of insulin. Mann-Whitney-U non-parametric tests were undertaken as the data was normally distributed. The results showed that there was no difference in insulin levels between non-smoker controls and smokers.

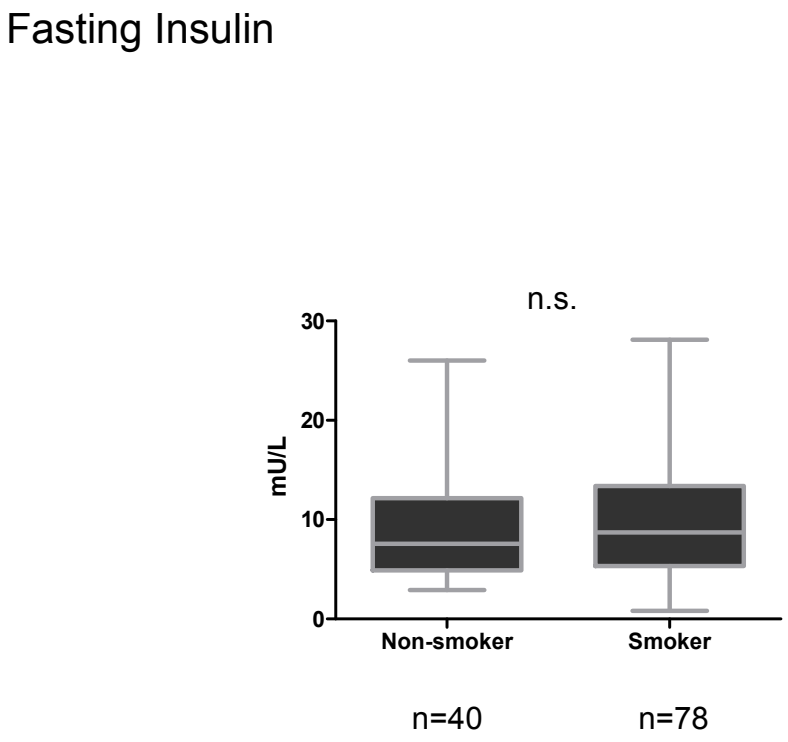


Figure 5.1 Fasting insulin levels (mU/L) in non-smokers and smokers.
The data are represented as median and quartiles. n.s.:not significant.

5.3.3. Insulin resistance measurement in non-smoker controls and smokers

Insulin resistance was calculated by Homeostasis Model Assessment (HOMA-R). This was calculated taking into account fasting insulin and fasting glucose

levels. In the analysis it showed no difference in insulin resistance between non- smokers and smokers (Fig 5.1.2).

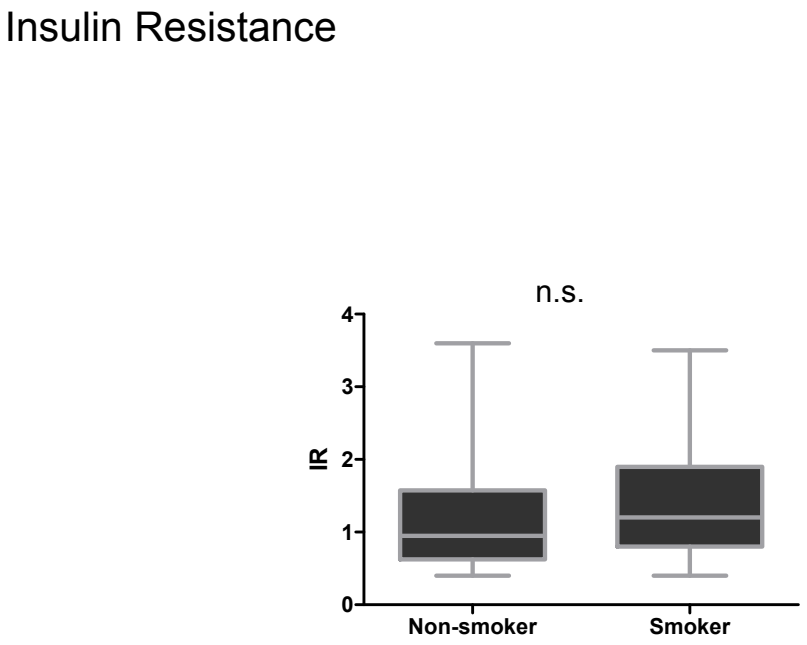


Figure 5.2 Insulin resistance measured by HOMA-R.
The data are represented as median and quartiles.
IR:Insulin resistance, n.s.:not significant.

5.3.4. Relationship between fasting insulin and insulin resistance in non-smokers and smokers with and without MeS

The subjects were divided into non-smokers with and without MeS (NS-N and NS-Y) and similarly in smokers group (Sm-N and Sm-Y). The analyses were done by non parametric Mann-Whitney U test. Firstly there was a significant difference in fasting insulin levels both in non-smoker controls and smokers with MeS (Fig 5.3 A, $p<0.009$ and $p<0.0003$ respectively). Secondly there was a difference in insulin resistance in non-smokers and smokers with MeS (Fig 5.3 B, $p<0.007$ and $p<0.0006$).

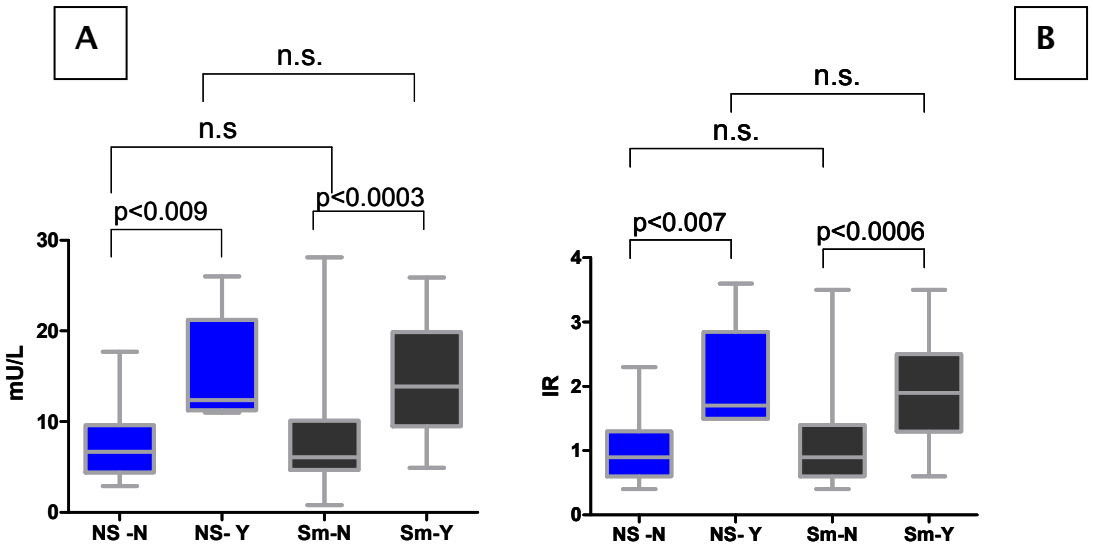


Figure 5.3 Fasting insulin (A) and insulin resistance (HOMA-IR) (B) measured in non-smoker controls and smokers with and without MeS.

The data are represented as median and quartiles and p<0.05 was considered significant. NS-N:non-smoker controls with no metabolic syndrome, NS-Y:non-smoker control with metabolic syndrome, Sm-N:Smokers with metabolic syndrome, Sm-Y:smokers with metabolic syndrome, n.s.:not significant, IR:Insulin resistance

5.3.5. Quality of life measured in non-smokers and smokers with MeS

Quality of life was measured by St George's Respiratory Questionnaire (SGRQ), which is a validated and reproducible questionnaire. This questionnaire has a range from 0-100 and with total of 76 points to be scored from their symptoms, impact, and activity scores. The analysis showed that in the smokers group the quality of life was significantly reduced compared to non-smokers (p<0.0001). When this was compared in smokers with and without MeS, there was no difference; however, there was trend towards reduced quality of life in smokers. When analysed between non-smokers with MeS and smokers with MeS there was reduced score in smokers (p<0.009). In the linear regression analysis model quality of life and physical activity were independently associated with FEV_1 (Adjusted r^2 = 0.48). However, insulin resistance was not independently associated with FEV_1 in a regression model when controlled for smoking, age, and gender.

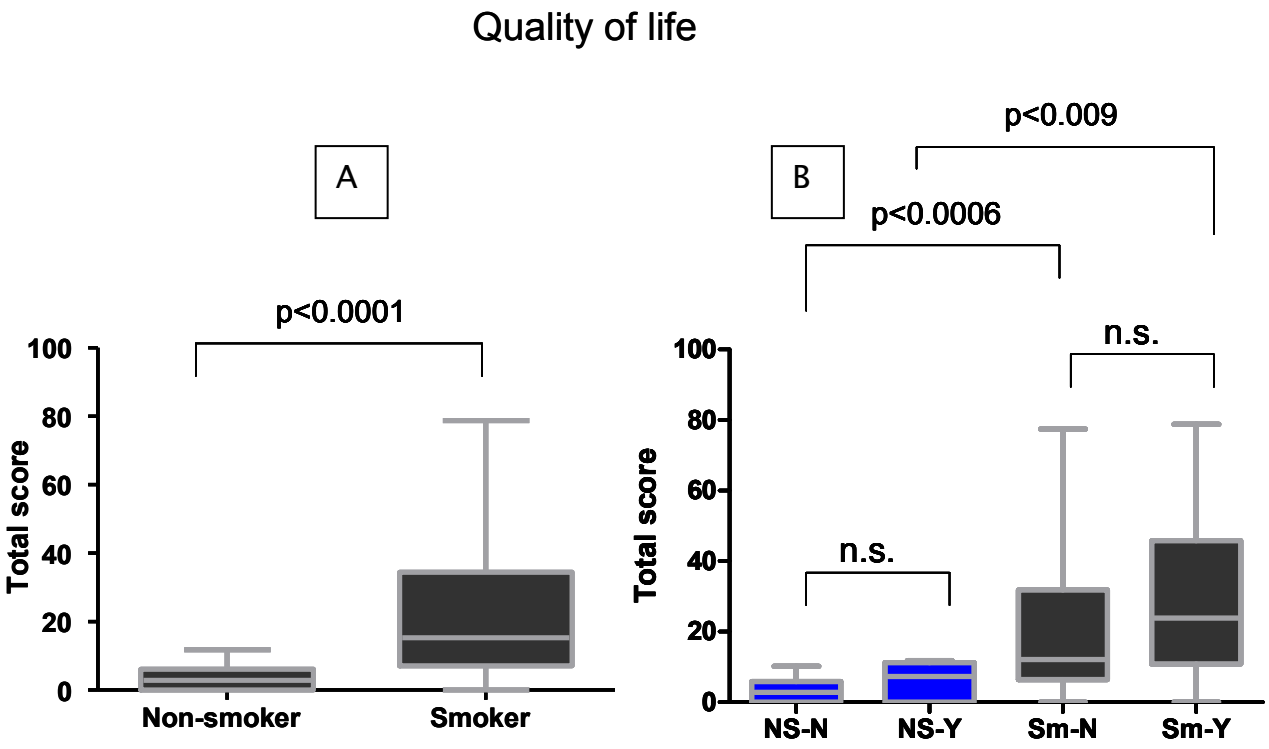


Figure 5.4 (A) Quality of life measured by SGRQ in non-smoker controls and smokers and (B) Quality of life in non-smoker controls and smokers with and without MeS.

The data are represented as medians and quartiles.

NS-N:non-smoker controls with no metabolic syndrome, NS-Y:non-smoker control with metabolic syndrome, Sm-N:Smokers with metabolic syndrome, Sm-Y:smokers with metabolic syndrome, n.s.:not significant.

5.3.6. Physical activity scores in smokers with and without MeS

A total of 120 subjects (44 non-smokers and 76 smokers) completed the Baecke habitual physical activity questionnaire. The questionnaire included scores from sports activity, activity at work and leisure activity. Then a total score was generated according to the calculation given in the guidance notes[371]. When the analysis was done in this study, there was a difference in the total score in non-smokers and smokers. Smokers were less physically active (Fig 5.5 A, $p<0.01$) compared to their counterpart, however, when the analysis was done in smokers with MeS there was no difference between the non-smokers and smokers (Fig 5.5 B). In the linear regression analysis the physical activity was independently associated with FEV_1 (Adjusted r^2 = 0.49 and

p<0.0001) when controlled for smoking, age, and gender and inflammatory markers.

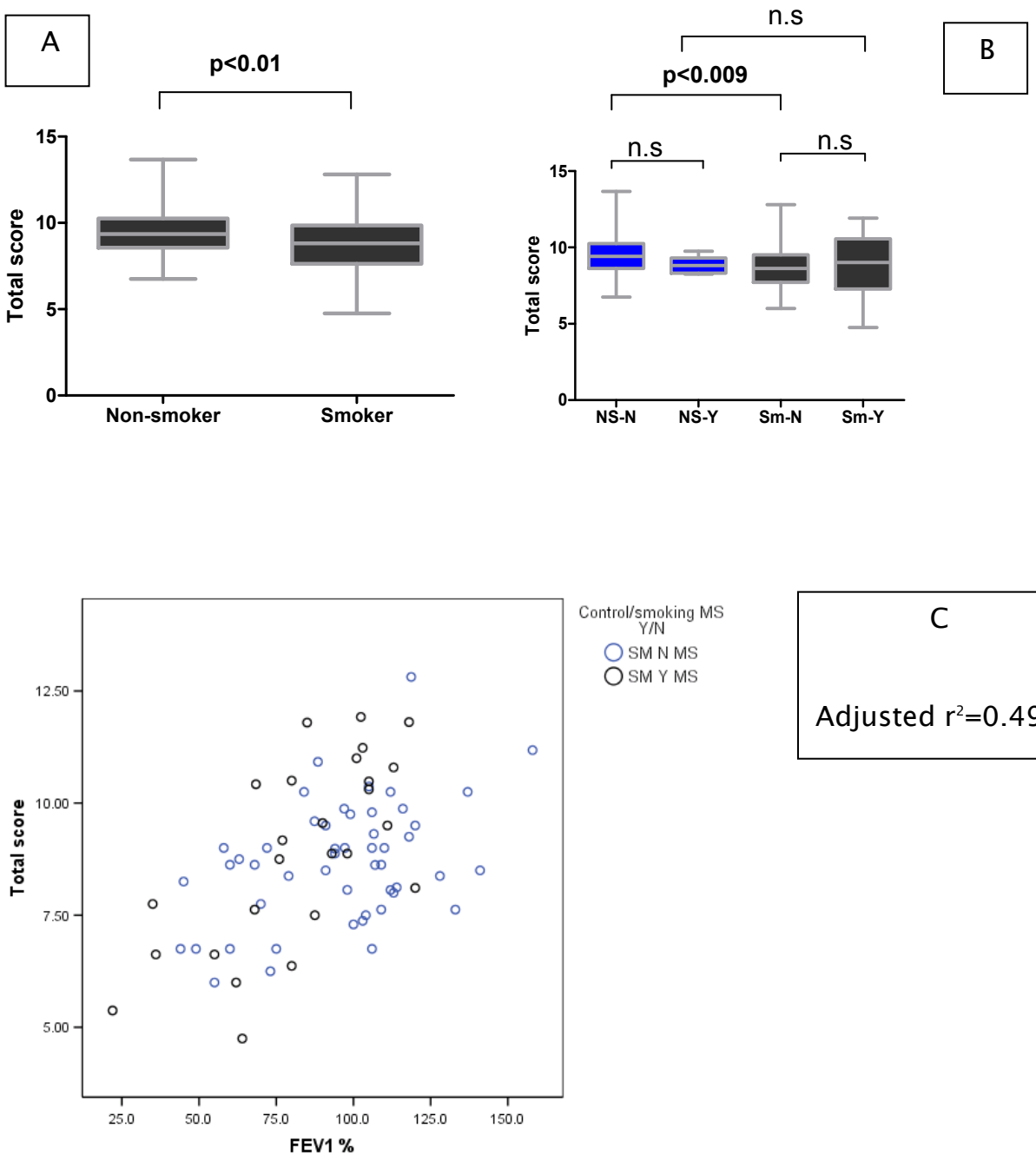


Figure 5.5 Total score measured from Baecke's physical activity questionnaire and scatter plot of correlation of physical activity with FEV1 percentage

(Adjusted r^2=0.49, p<0.0001). NS-N:non-smoker controls with no metabolic syndrome, NS-Y:non-smoker control with metabolic syndrome, Sm-N:Smokers with metabolic syndrome, Sm-Y:smokers with metabolic syndrome, FEV_1:Forced expiratory volume in 1 second, n.s.:not significant.

5.4. Discussion

This study was aimed to investigate the relationship between the measure of insulin resistance, features of MeS and respiratory related outcomes such as FEV_1 in smokers with MeS. The important findings in this study are, a) there was non increase in fasting insulin in smokers, b) there was no increase in insulin resistance in smokers, c) Physical activity measured by Baeck's questionnaire was and quality of life measured by SGRQ was independently associated with impaired FEV_1 in smokers,

Insulin resistance is a state in which supra-physiological concentrations of insulin are required for glucose homeostasis. Key insulin sensitive tissues that are normally affected by this state are adipose tissue, liver and skeletal muscle resulting in impaired insulin-mediated suppression of hepatic glucose production and impaired insulin-mediated peripheral glucose utilization in skeletal muscle and adipose tissue. In affected individuals, there is continual demand for insulin hypersecretion to compensate for insulin resistance and to maintain glucose homeostasis leading to eventual islet cell exhaustion and the development of glucose intolerance and type 2 diabetes mellitus[372].

There are many reasons for developing insulin resistance in normal individual and in smokers, e.g. excess visceral or hepatic fat insulin resistance tends to run in families and has a strong genetic predisposition[372, 372]. There are other possible mechanisms whereby smoking can elicit insulin resistance mediated via cardiovascular, neurohormonal and endocrine effects [373, 374]. Smoking could also have an effect on central obesity that is driven by insulin resistance and contributes to the development one of the early clinical signs of MeS.

A common clinical feature in the development of MeS is central obesity or abdominal fat and this is highly indicative of increased insulin resistance [375]. Central obesity is common in smokers and this has been discussed in chapter The results were surprising, central obesity measured by waist circumference or waist/hip ratio was raised in smokers with or without COPD. It is known that there is a strong correlation between systemic effects, COPD and insulin resistance[376]. One of the common reasons for individuals developing central obesity and insulin resistance is physical inactivity. Smoking can acutely impair insulin action[377] and causes insulin resistance. The amount of nicotine smoked per day is shown to be directly related to degree of insulin resistance[378]. In

contrast to the finding, in my study there was no increase in fasting insulin or insulin resistance amongst smokers or non-smoking control subjects. However, in my study there was a difference in fasting insulin levels and insulin resistance between smokers with and without MeS and the results were similar in non-smoker controls. Regardless of smoking effect, insulin resistance is a common mechanism by which individuals develop features of MeS. In smokers, this could be one of the many pathways by which smoking contributes to the development of MeS.

Insulin resistance and physical activity obviously play a major role in quality of life (QOL) in chronic disease subjects. Improved QOL is one of the indicators for the improvement in management of the disease. The questionnaire which was used (SGRQ) was subjective and not only depends on the physiological parameters but also on the psychological frame of mind. SGRQ questionnaire is a reproducible tool to measure quality of life in subjects with airway disease mainly COPD. The main observation in this study was that the quality of life was reduced in smokers and in COPD subjects. There was similar results seen when compared smokers with or without MeS. However, the difference in QOL was only found to be significant when smokers with or without MeS was compared with non-smoker controls. This explains that all smokers have reduced quality of life, which may be due to increased body fat and central obesity.

Pulmonary function abnormalities are seen in both insulin dependent and non-insulin dependent diabetes mellitus[74, 379]. In non diabetic individuals there is an inverse relationship between forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV_1)[380, 381] in men. In my study the linear regression analysis showed that insulin resistance was not independently associated with FEV_1 when controlled for age, gender, smoking. This could only be due to excessive fat accumulation in the abdomen which is known to influence both insulin resistance and ventilatory function[382].

Physical inactivity is a global public health problem and there is compelling evidence to suggest its contribution to many chronic diseases[383, 384]. The change in lifestyle contributes to an epidemic of obesity globally not only in health but also in chronic disease such as COPD. There are many reasons for this epidemic for example increased portions of unhealthy food intake[383], lack of physical activity and also increasing levels of stress in everyday activities. However, there is not enough evidence to support this argument. There are

many recommendations as to the amount and duration of physical activity to be undertaken to stay healthy and one of the recommendations is to undertake physical activity for 60minutes/day to prevent unhealthy weight gain or 30minutes moderate-intense activity, which provides substantial health benefit[385]. The main benefit of undertaking physical activity is to keep the balance between energy expenditure to energy stored. This principle is essential in chronic diseases like COPD. Henceforth there is new recommendation for early referral to pulmonary rehabilitation for smokers with mild to moderate COPD who develop exercise-induced symptoms. This programme has shown to be beneficial in COPD with MRC dyspnoea score 2 and above.

In this current study, Baecke's physical activity questionnaire was used to assess the daily physical activity in all the subjects. When the smokers in this study were analysed for physical activity scores it was found that they were less active when compared to non-smoker controls. However, when physical activity scores were assessed in smokers with and without features of MeS there was no measurable difference between the two. The inference from this data is that in this particular cohort the physical activity was not influencing the development of insulin resistance and features of MeS. When physical activity scores were analysed by linear regression analysis, there was a strongh independent relationship between physical activity and FEV_1 percentage when controlled for smoking, age, and gender. However, one of the limitations with this questionnaire was, it was a subjective assessment, and there are less comparable studies with physical activity devices that quantitatively assess physical activities in smokers and COPD. Historically features of MeS are related to physical activity, however, this was not found in my data in smokers.

In conclusion, the data in my study show evidence of insulin resistance in people with MeS. There was no evidence of increased insulin resistance in smokers versus non-smokers. There was no evidence of an association between a measure of insulin resistance (HOMA-R) and respiratory outcomes such as FEV_1 percentage. In univariate, analyses there were associations between Baecke physical activity score and FEV_1. I have also shown that physical activity and quality of life were independently associated with reduced lung function.

Future work should be guided towards more precise assessment of insulin sensitivity and physical activity energy expenditure in people who

smoke to understand the relationship between smoking and insulin resistance in subjects with MeS.

6.0 Chapter-Gender differences in healthy smokers and COPD in relation to MeS

6.1. Introduction

Interest has grown over the last few years regarding the long-term effect of smoking in men and women. Data suggests that women are more susceptible to develop airway obstruction compared to men for the same number of cigarettes smoked[386]. Smoking is the single largest preventable cause of death in the world. There are many adverse health effects for which smoking is the main cause which includes cardio-respiratory, vascular, reproductive system, and cancers[387]. These diseases affect differently both in men and women smokers across the globe. There has been an impact on clinicians' interest to understand the gender differences in smokers and COPD. Smoking was rare in women in the early 20th century and became more prevalent in recent times. In 2006 it was reported that 17.6% of women smoked in the US[388]. European women are historically heavy smokers and Danish women in particular are heaviest amongst all[389].

The effects of gender difference depends on the exposure and susceptibility to tobacco smoke and also to other environmental pollutants which causes harmful effects to the airways[390-395]. The adverse effects of smoking are not uniformly distributed between both sexes. In one of the studies, they found that smoking affects pulmonary vasculation in women and airways in men[396].

COPD is one of the local complications of smoking and there are recent evidence to show more women with COPD than men[160]. It is also observed that mortality from COPD is increasing in women in Europe and North America[397]. One of the views is that female smokers more than male smokers tend to inhale deeper and breath hold for a long time which may predispose them to more airway damage[398].

Metabolic syndrome on the other hand is more prevalent in men than in women[399], however, it is noted that the first isolated component of MeS occurs earlier in women than in men[399], however there is no data on which of these features appear first in women. MeS has been observed in many ethnic groups and the influence of gender varies between population and on the definition used[400]. The development of MeS is slower in subjects who have normal BMI and follow good exercise habits[399]. Age, genetic inheritance, race, and gender specific hormones influence fat distribution in the body. It is observed that in women due to estrogens, fat is deposited on the hips, thighs and abdomen

and in men fat is deposited around abdomen and upper body because of the hormone testosterone.

In the Lung Health study the gender differences were produced a result of differential abdominal deposition of fat in men compared with women and this had a greater impact on FEV_1 and FVC than peripheral obesity, which is seen in women[401]. However, there is also an association between abdominal fat and visceral fat. The increase in abdominal fat deposition is associated with an increase in visceral fat in the abdomen which could reduce vital capacity[402]. There are other important features between gender, which could affect, for e.g. quality of life, and physical activity. How quality of life and physical activity influence the development of MeS in men and women smokers is not yet known.

The relationship between gender and MeS in smokers is less well understood. There is also less evidence to confirm the pathways, which drive MeS differently in men and women smokers. For the first time in this study I aim to find out how different smoking habits in men and women could affect lung function, inflammation, metabolic features and body composition, quality of life and physical activity and to understand the role played by the combination of smoking and gender in worsening the features of MeS and COPD.

6.2. Methods

A total of 131 subjects were recruited for the study. They were sub-divided into three groups according to their smoking habits, non-smoker controls (HC, M=20, F=24), healthy smokers (HS, M=24, F=22) and smokers with COPD (M=22, F=19). The subject characterisation is discussed in chapter 2 (section 2.1.1). Non-smokers and healthy smokers had normal predicted values for FEV_1 and TLco.

6.3. Results

6.3.1. Smoking habits (pack years), FEV_1, and TLco% in smokers and COPD

There was no difference between men and women. In the COPD group, there was a trend in reduction in FEV_1 and TLco in men compared to women. Men smoked more in COPD ($p<0.0002$) group than women.

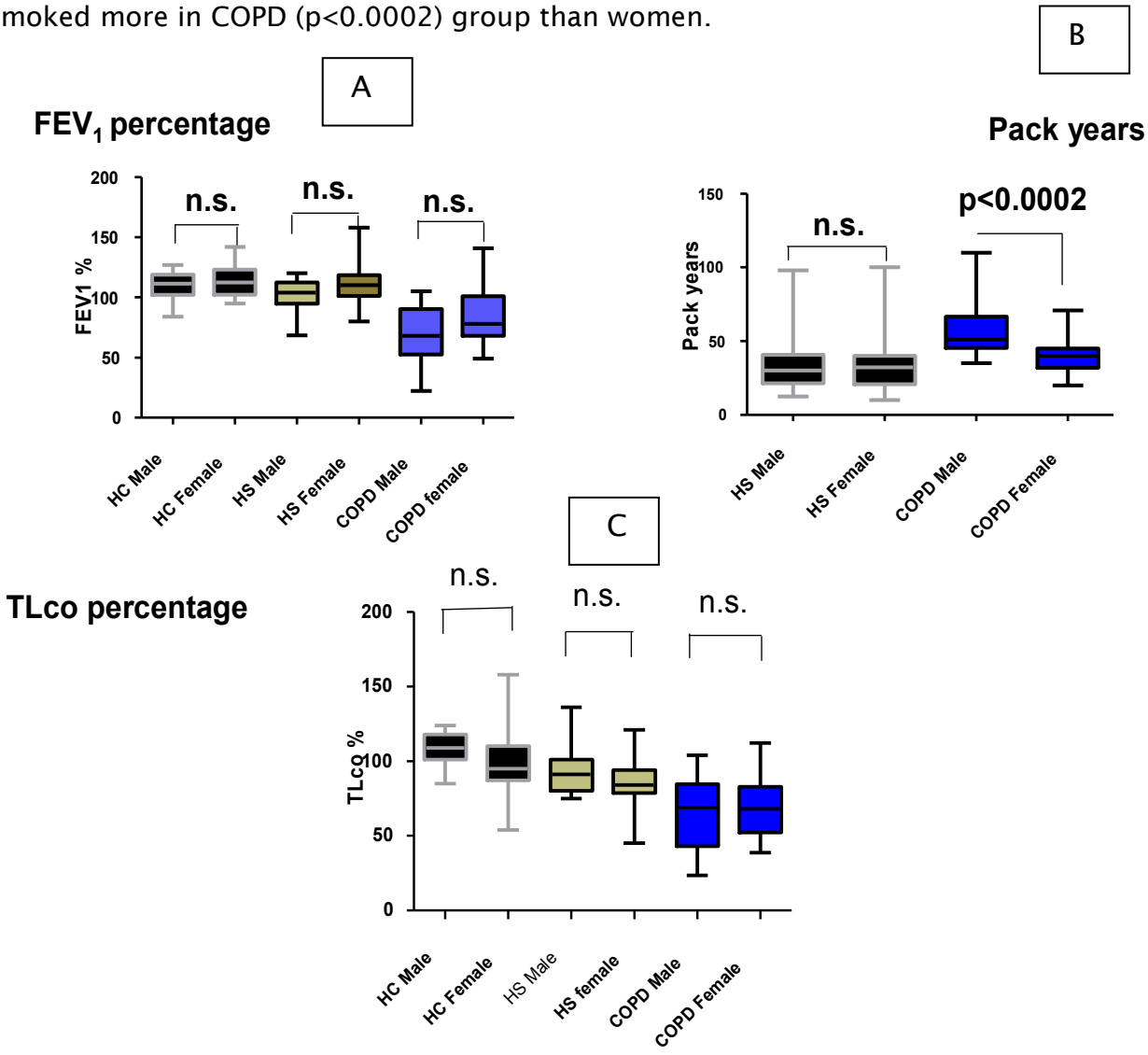


Figure 6.1 FEV1 percentage (A), pack years (B) and transfer factor (C) in non-smoker controls (HC) (A and C) Healthy smokers(HS) and COPD (A, Band C).

The data are represented as median and quartiles.FEV_1:Forced expiratory volume in 1 second, TLco:Transfer factor for carbon monoxide, n.s.:not significant.

6.3.2. Measurement of blood inflammatory cells

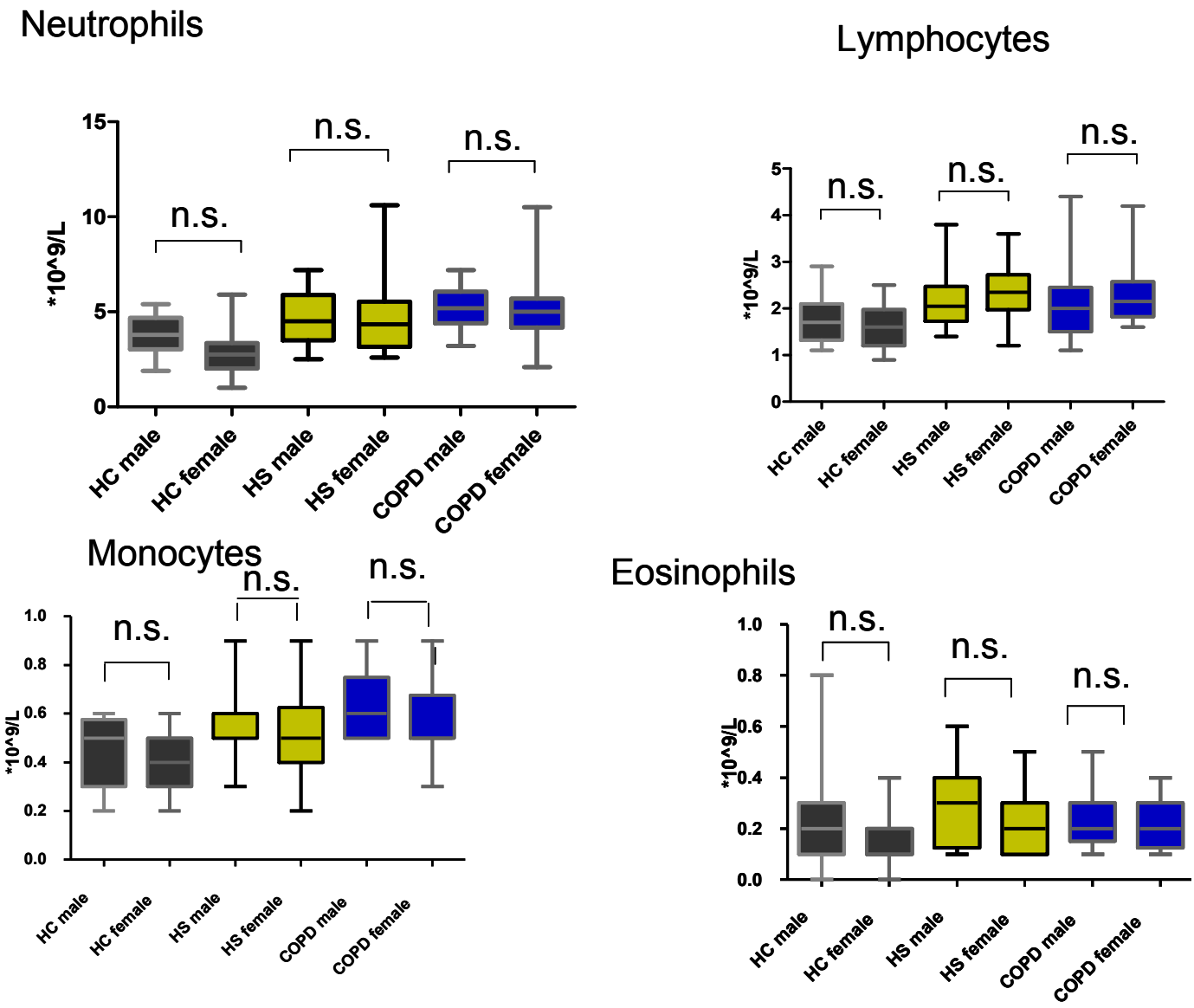


Figure 6.4 Inflammatory cells.The data are represented as median and quartiles.

HC: Healthy non smoker controls, HS:Healthy smokers, COPD:Chronic obstructive pulmonary disease, n.s.:not significant.

Blood inflammatory cells play an important role in smokers and COPD. Inflammatory cells like neutrophils, lymphocytes, monocytes, and eosinophils were measured in blood. There was no difference in neutrophils, lymphocytes,

monocytes, and eosinophils between men and women in non-smokers, healthy smokers or in COPD subjects.

6.3.3. Measurement of inflammatory marker C - reactive protein (CRP)

CRP is a clinical marker of inflammation and is widely used in clinical practice, which helps in diagnosis or in follow up after treatment. CRP was measured in the hosipital laboratory. The data was compared between men and women by Mann-Whitney U test. There was no difference in CRP levels between men and women in any of the group.

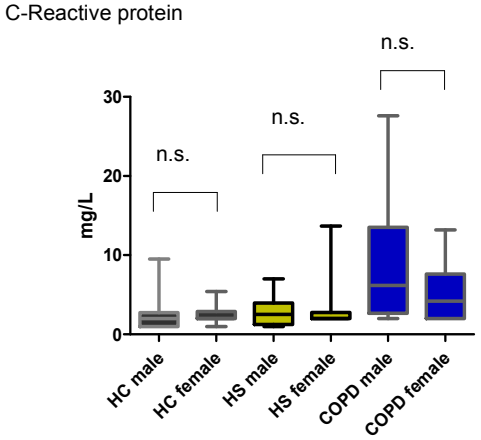


Figure 6.5 C-Reactive Protein (CRP) levels in non-smoker controls (HC), healthy smokers (HS) and COPD men and women.
The data are represented as median and quartiles.

6.3.4. Measurement of features of MeS in men and women

Metabolic syndrome features are central obesity measured by waist circumference or waist/hip ratio, fasting glucose, triglycerides, HDL and blood pressure. IDF criteria was used in this study to diagnose MeS.

6.3.5. Measurement of waist circumference and waist/hip ratio in non-smokers, healthy smokers and COPD men, and women

Central obesity is one of the key features of MeS. Waist circumference is the measure for central obesity. Waist/hip ratio is a more accurate measure for central obesity. According to the International Diabetic Federation waist circumference over 80cms in women and 94cms in men and waist/hip ratio >1 is considered to be abnormal.

In my study the analysis showed significant difference in waist circumference in non-smokers (Fig 6.4 A) ($p<0.002$) and in healthy smokers ($p<0.006$). Men tend to have more central obesity than women in my study, however, there was no difference in waist circumference in COPD men and women. There was a difference throughout groups in waist/hip ratio (Fig 6.4 B). Men again tend to have increased central obesity in non-smokers ($p<0.0001$), healthy smokers ($p<0.0002$) and in COPD subjects ($p<0.007$).

6.3.6. Measurement of triglycerides, HDL and blood glucose

The normal range for fasting triglyceride was 0-2.0mmol/L and HDL was 1.2-1.8mmol/L. There was a difference in HDL levels between men and women in non-smokers ($p<0.01$) and healthy smokers group ($p<0.02$), however COPD subjects showed no difference.

There was a borderline difference in triglyceride levels in non-smokers ($p<0.057$). Blood glucose was high in non-smokers male ($p<0.05$) and healthy smokers male ($p<0.02$, Fig 6.5).Triglyceride were no different except for a borderline significance in the non-smoker group ($p<0.057$). Fasting blood glucose was high in non-smoker males ($p<0.05$) and in healthy smoker males ($p<0.02$).

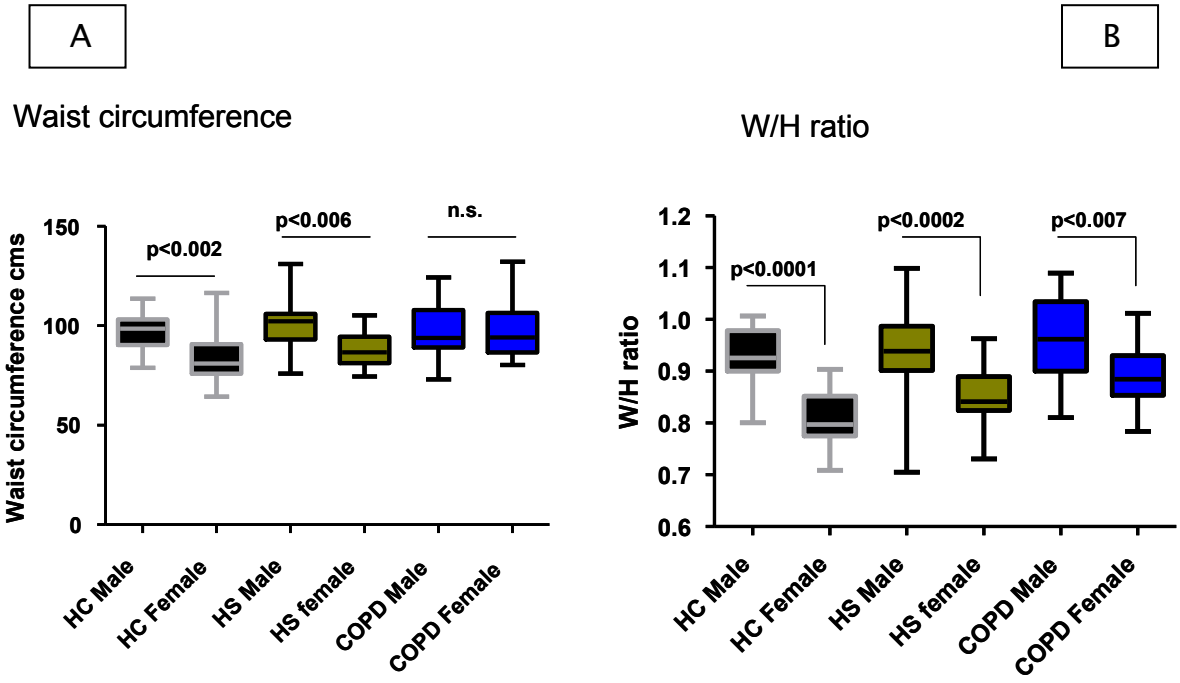


Figure 6.6 (A) waist circumference (cms), (B) waist/hip ratio in healthy smoker controls (HC), healthy smokers (HS) and COPD men and women.

The data are represented as median and quartiles.

HC:Healthy non smoker controls, HS:Healthy smokers, COPD:Chronic obstructive pulmonary disease, n.s.:not significant, w/h:waist/ hip

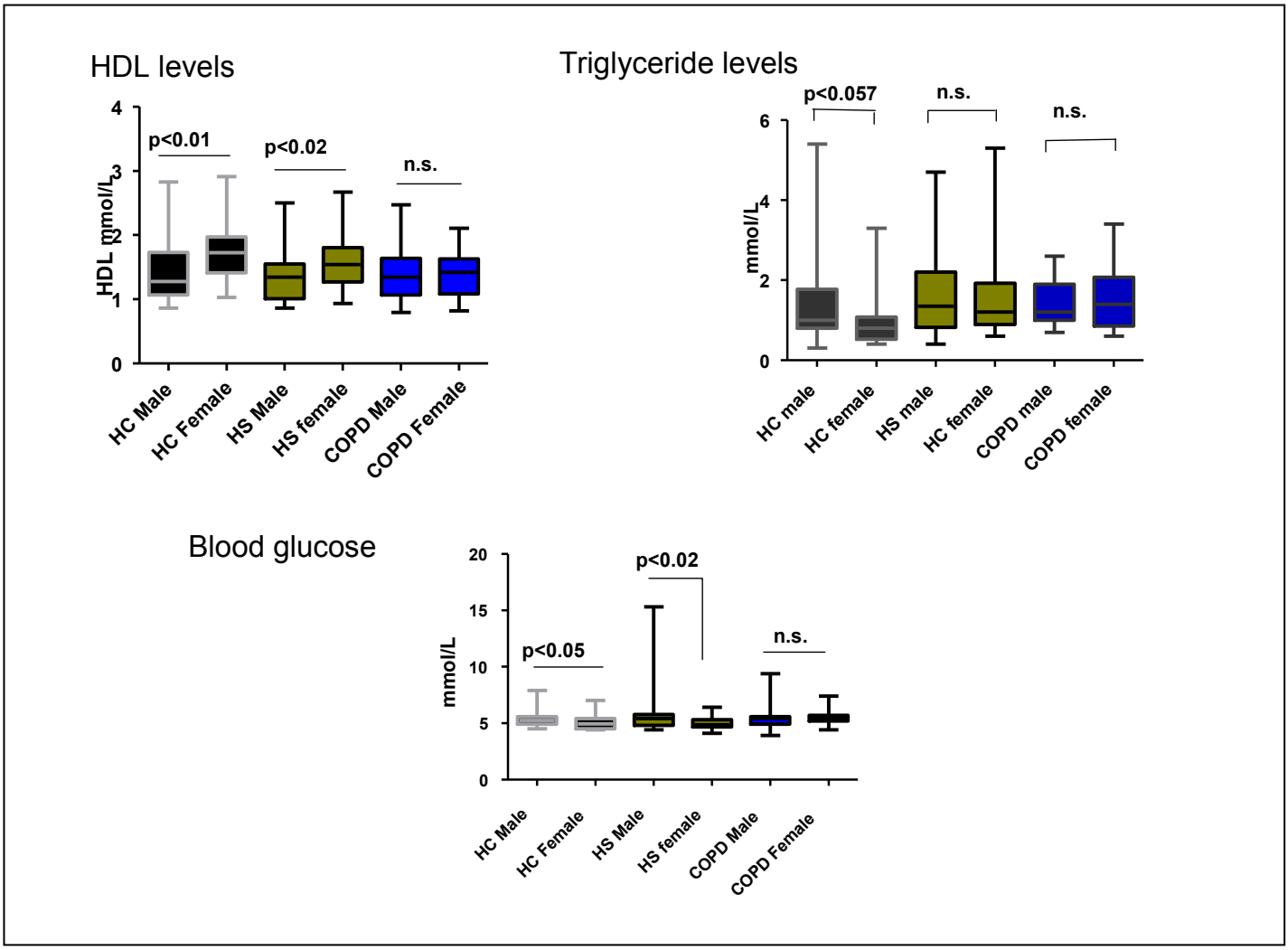


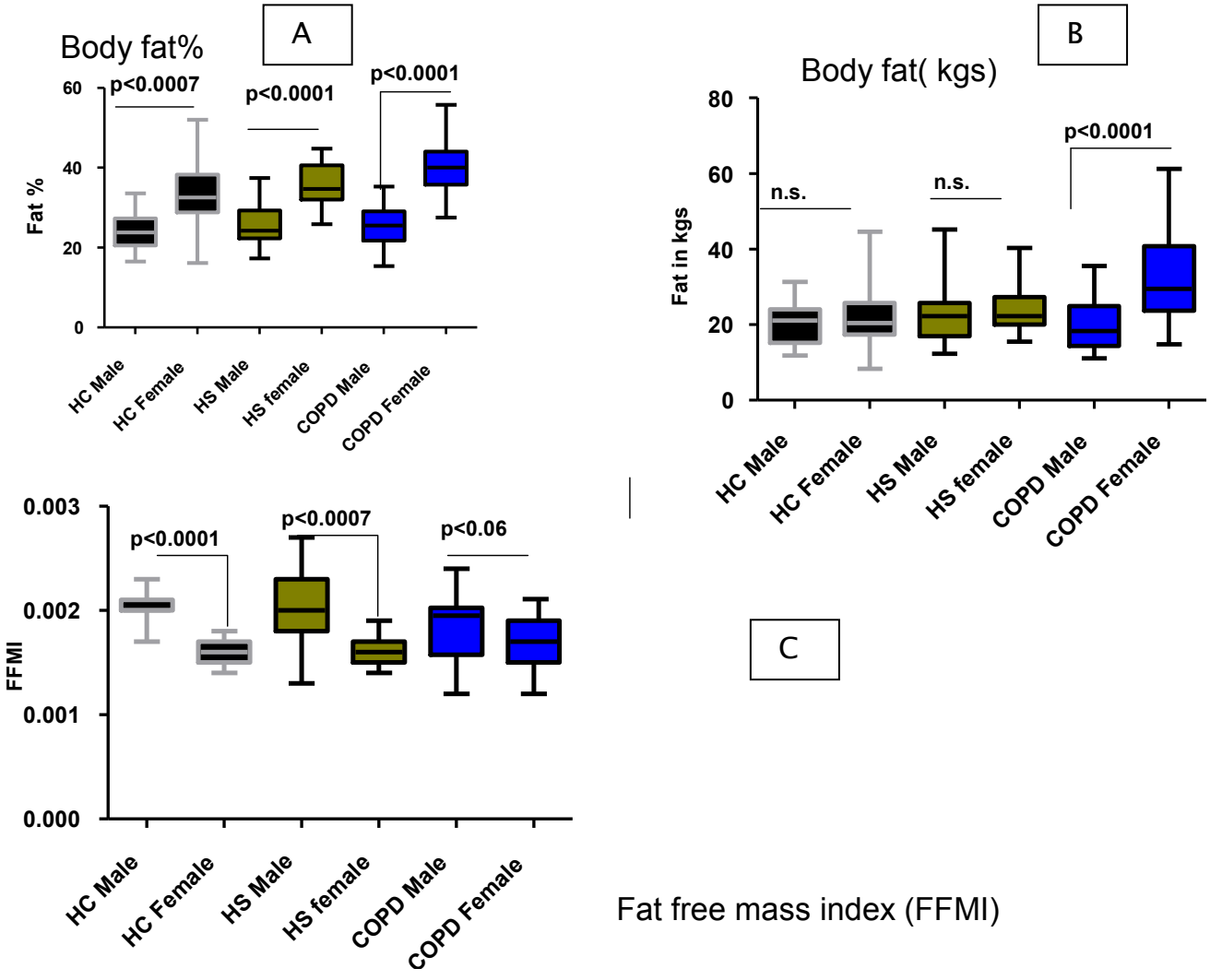
Figure 6.7 Fasting HDL, triglyceride, and blood glucose in non-smoker controls (HC), healthy smokers (HS) and COPD men and women.

Data are represented as median and quartiles <0.05 was considered significant. HDL:High density lipoprotein, HC:Healthy non smoker controls, HS:Healthy smokers, COPD:Chronic obstructive pulmonary disease, mmol/L:Millimoles per liter, n.s.:not significant.

6.3.7. Body composition measurements

Body composition measurement includes fat in percentage and kilogram, lean mass or fat free mass, total body water and BMI. The important measure is fat and fat free mass. More accurate is the fat free mass index (FFMI) which takes into account the person's muscle mass and relates to their height. This is a better measure than BMI. In this study, body fat and FFMI was compared in

non-smokers, healthy smokers and COPD men and women. Women in the non-smokers group (p<0.0007), healthy smokers group (p<0.0001) and COPD group (p<0.0001) were all fatter (fat %) than their counterparts and there was a difference in fat in kgs in COPD women. The FFMI was significantly different in all three groups, more so in non-smokers (p<0.0001) and healthy smokers group (p<0.0007).



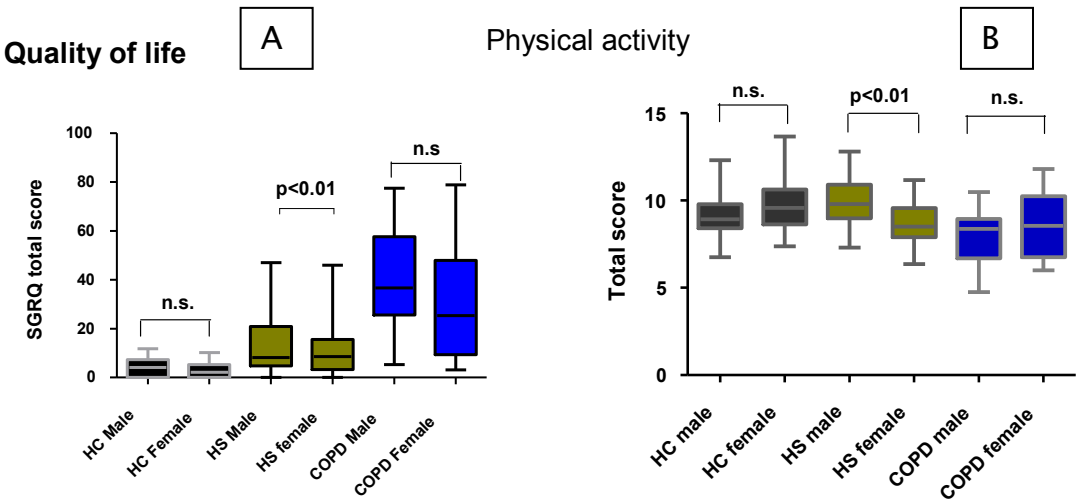
Fat free mass index (FFMI)

The data are represented as median and quartiles. Women had more body fat % in all the groups.

FFMI: Fat free mass index, HC: Healthy non-smoker controls, HS: Healthy smokers, COPD: Chronic obstructive pulmonary disease.

6.3.8. Measurement of quality of life (SGRQ) and physical activity (Baecke's habitual physical activity) questionnaire

Quality of life (QOL) was difficult to measure as it has many components. Physiological and psychological factors could contribute to this complex state of mind. One of the validated questionnaires to measure quality of life in smokers is the St George's Respiratory Questionnaire. In this study, the measure of QOL was collected by means of completing the questionnaire by all subjects. There was a significant difference between healthy smokers men and women ($p<0.01$). However, there was a trend in poor QOL in COPD women. Reduced physical activity plays an important part in the development of obesity and MeS. A total of 131 (HC, M=24, F=20, HS, M=24, F=22, COPD, M=22, F=19) completed the SGRQ and 120 completed physical activity questionnaire (HC, M=20, F=24, HS, M=23, F=18, COPD, M=21, F=14).



The data are represented as median and quartiles.

SGRQ: St George's Respiratory Questionnaire, HC: Healthy non-smoker controls, HS: Healthy smokers, COPD: Chronic obstructive pulmonary disease, n.s.: not significant.

6.3.9. Gender differences in smokers

In the prelimary, analysis there was no difference in prevalence of MeS in smokers and COPD. This finding leads to the assumption that smoking rather than COPD per se is associated with features of MeS. Keeping this in mind, I decided to group all smokers (HS + COPD smokers) together. The aim was to test the hypothesis that there were differences in markers of inflammation, body composition, QOL, physical activity between men and women smokers with features of MeS.

6.3.10. Measurement of FEV_1, pack years and TLco in men and women smokers

The total number of smokers was 87 in the study. They were sub divided into men and women (Male=45, Female=42). The subjects were compared for differences in FEV_1 percentage, pack years (total number of pack of cigarettes per day number of years smoked). The results are in Fig 6.8 A, B and C. Men had more reduced FEV_1 percentage ($p<0.04$*) and men smoked more cigarettes than women ($p<0.01$**). Difference in percentage of TLco was analysed between men and women (TLco is a measure for emphysema in the lungs) and found no difference between men and women smokers.

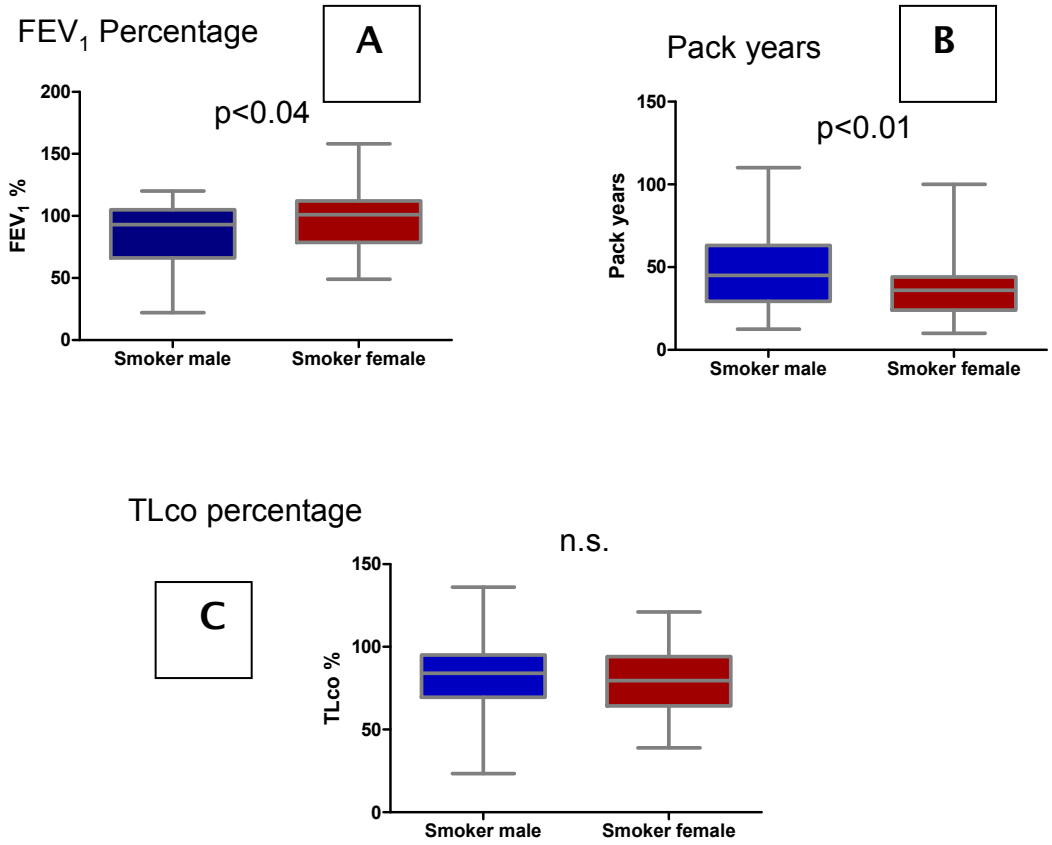


Figure 6.10 FEV1 percentage (A), pack years (B) and TLco percentage (C) between men and women smokers.

There was a significant difference in FEV_1 percentage ($p<0.04$*) and pack years ($p<0.01$**). There was no difference in TLco percentage.*t-test, ** Mann-Whitney-U test.

FEV_1: Forced expiratory volume in 1 second, TLco: Transfer factor for carbon monoxide.

6.3.11. Blood inflammatory cells

Neutrophils, lymphocytes, monocytes, and eosinophils were measured in blood. They are expressed as *10^9/L. When compared between men and women smokers there was no difference. Independent t-test for normally distributed data and Mann-Whitney-U test was used to analyse the data. The data are expressed as median and quartiles.

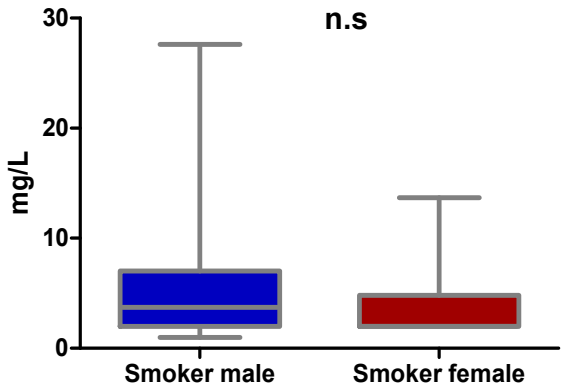


Figure 6.11 C-Reactive Protein (CRP) measurement in men and women smokers.
The data are shown as medians and quartiles.
mg/L: Milligram per litre, n.s.: not significant.

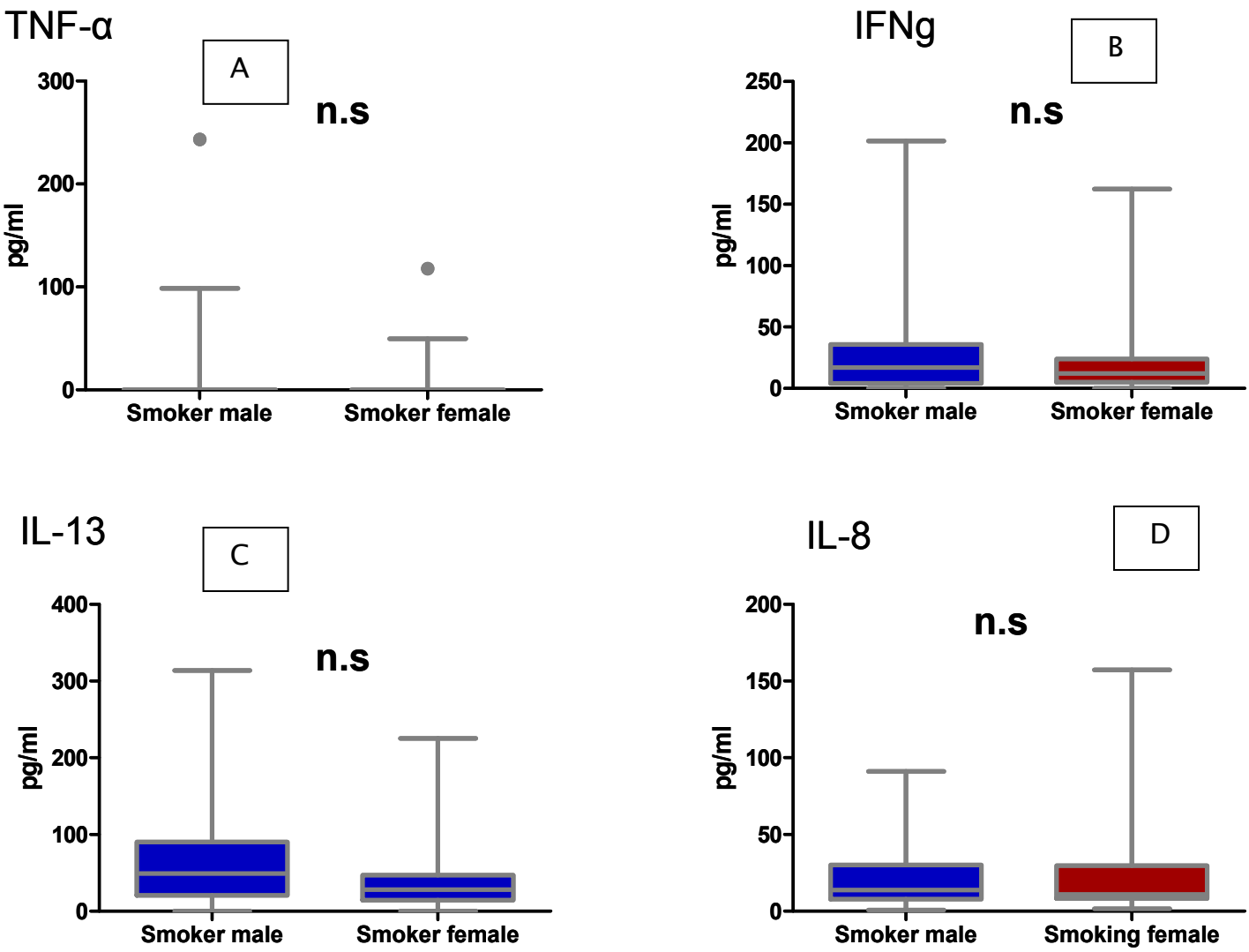
6.3.12. Measurement of inflammatory marker in smokers

C - reactive protein is a helpful inflammatory marker to understand the extent of inflammation in clinical practice. In my study, blood CRP was measured in all subjects. Differences in men and women smokers were analysed and the results showed no significant difference in CRP levels between them (Fig 6.10).

6.3.13. Inflammatory cytokines and apolipoprotein1 (apo1) in smokers men and women

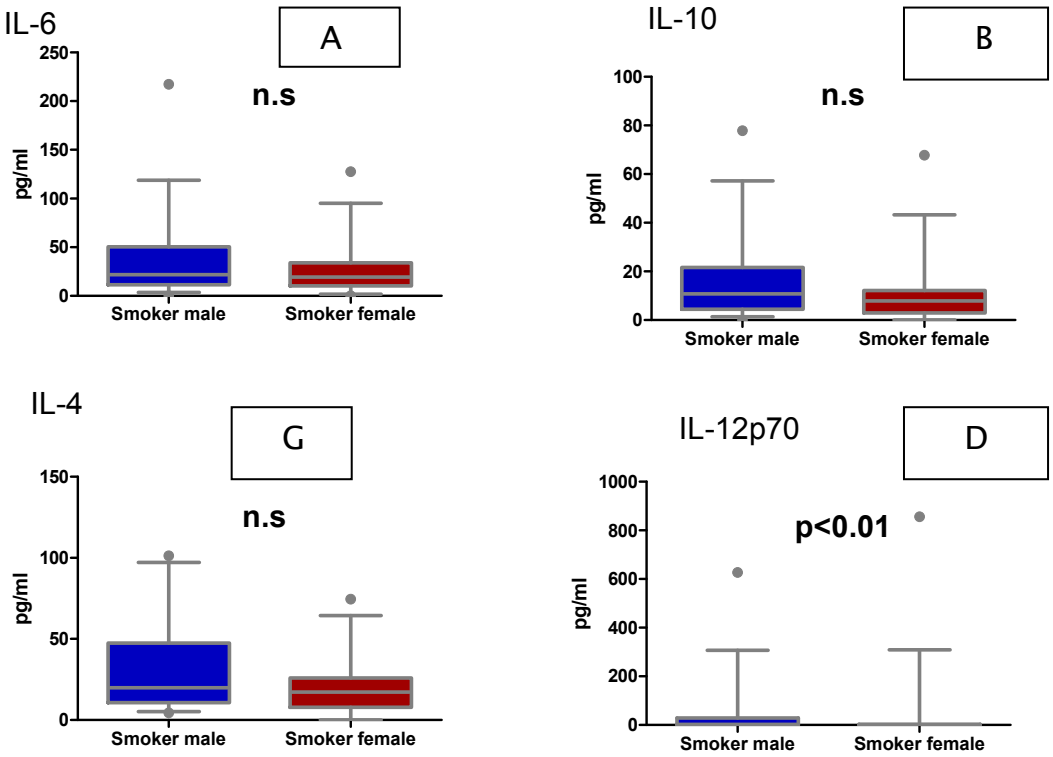
Inflammatory mediators play an important role in the disease process. The important mediators measured in my study were TNF-α, IFNg, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, and IL-12p70. APO-1 antigen is a transmembrane glycoprotein, which is involved in apoptosis within the immune system and cell death, was also measured in study.

The inflammatory mediators were measured my multiplex Luminex and apo1 by ELISA methods (Fig 6.11). A total of 67 samples were analysed (men were=33 and women were=34). Differences between men and women were analysed and found that there was no difference in TNF-□, IFNg, IL-13, IL-8, IL-6, IL-10, IL-4, IL-2, and IL-5 levels in plasma. There was a significant difference in IL-12p70 ($p<0.01$) and apo1 ($p<0.006$) between men and women smokers (Fig 6.11).



The data are represented as median and quartiles.

TNF-α: Tumor necrosis factor alpha, IFNg: Inteferone gamma, IL: Interlukin, pg/L:picogram per litre, n.s :not significant.


IL-6
A
n.s
pg/ml
250
200
150
100
50
0
Smoker male
Smoker female
IL-10
B
n.s
pg/ml
100
80
60
40
20
0
Smoker male
Smoker female
IL-4
G
n.s
pg/ml
150
100
50
0
Smoker male
Smoker female
IL-12p70
D
p<0.01
pg/ml
1000
800
600
400
200
0
Smoker male
Smoker female

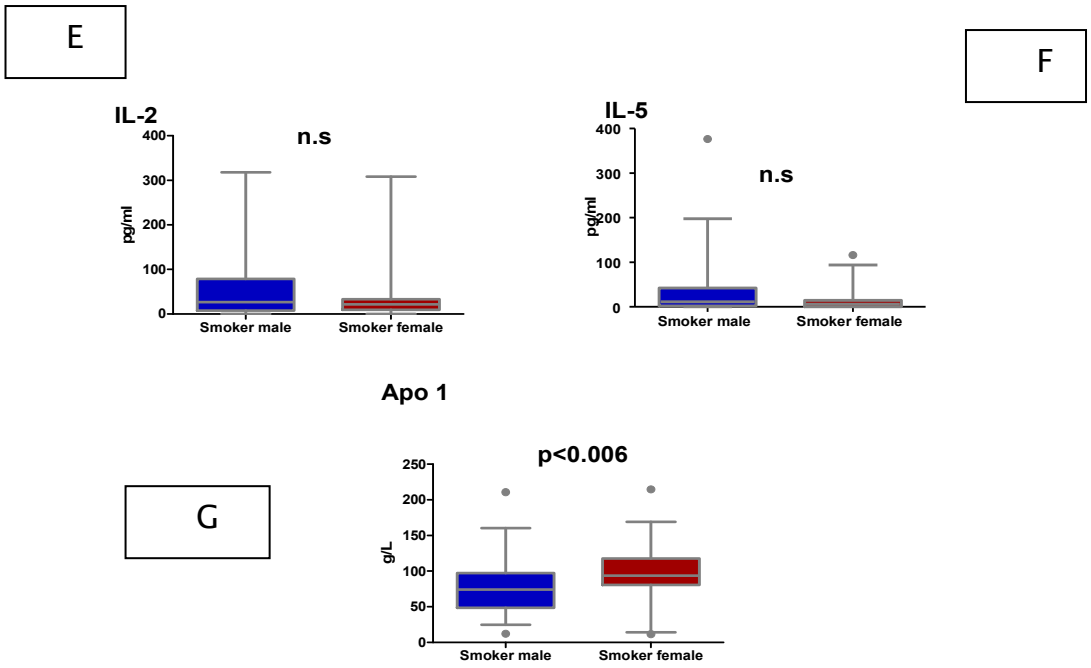


Figure 6.13 Plasma inflammatory mediators measured by Luminex in male and female smokers, (A) IL-6, (B) IL-10, (C) IL-4, (D) IL-12p70,(E) IL-2 (F) IL-5 and (G) Apo-1.

IL: Interlukin, Apo: Apolipoprotein. g/L: grams per litre, pg/L: Picogram per litre.

6.3.14. Prevalence and features of metabolic syndrome in men and women

The prevalence of MeS was analysed in men and women smokers. The classification is based on IDF criteria. Central obesity is the key for the subjects to have MeS and any of the other two features from the list of increased triglycerides, low HDL, high blood glucose, and increased blood pressure must be present. There was a prevalence of 44.4% in men and 23.52% in women. Chi-square test was applied for the analysis. There was an increased trend but the differences were not statistically significant (Fig 6.13)

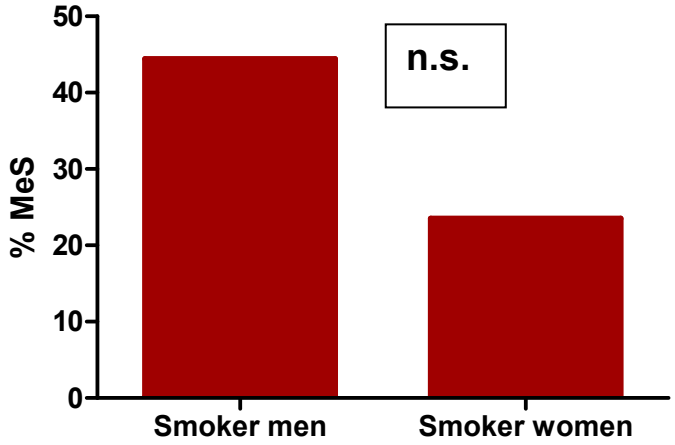


Figure 6.14 Prevalence of metabolic syndrome

Prevalence of metabolic syndrome between men and women in all smokers (healthy smokers and COPD smokers combined). Men (n=45) and women (n=42) were analysed by chi-square test and represented in bar diagram. The Y-axis is the percentage of MeS and diagnosis on the X-axis.

% MeS: Percentage of metabolic syndrome, n.s.: not significant.

6.3.15. Comparison between features of MeS in men and women smokers

Men and women smokers were compared for features of MeS, which were waist circumference, fasting glucose, triglycerides, HDL, and systolic and diastolic pressure. There was no difference in fasting glucose (Fig 6.14 A), triglycerides (Fig B) However, men tend to have less HDL levels compared to women (Fig C, $p<0.02$).

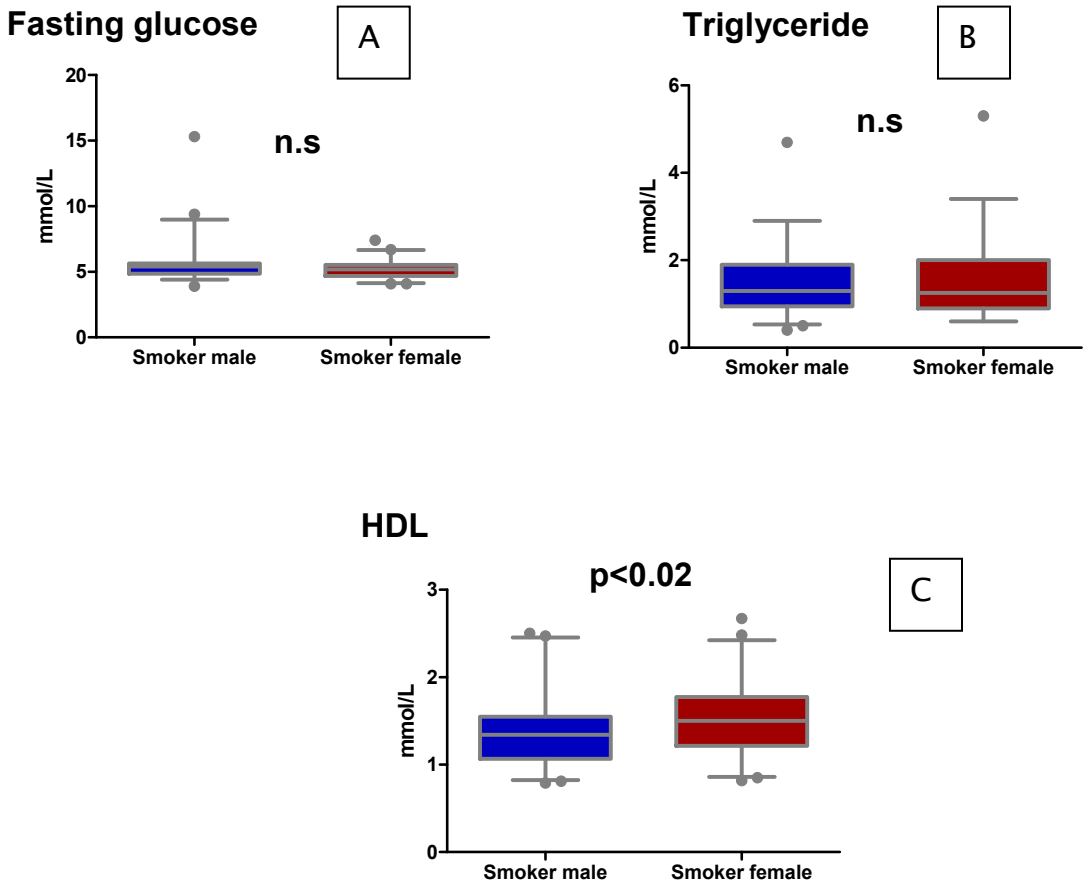


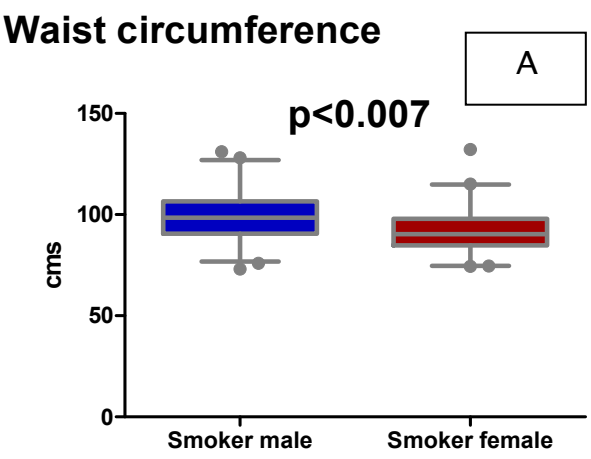
Figure 6.15 Fasting glucose, (B) triglyceride (C) HDL levels in men and women smokers.

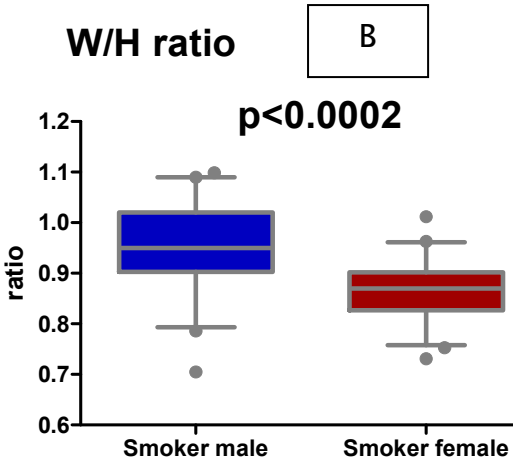
The data are represented as median and quartiles.

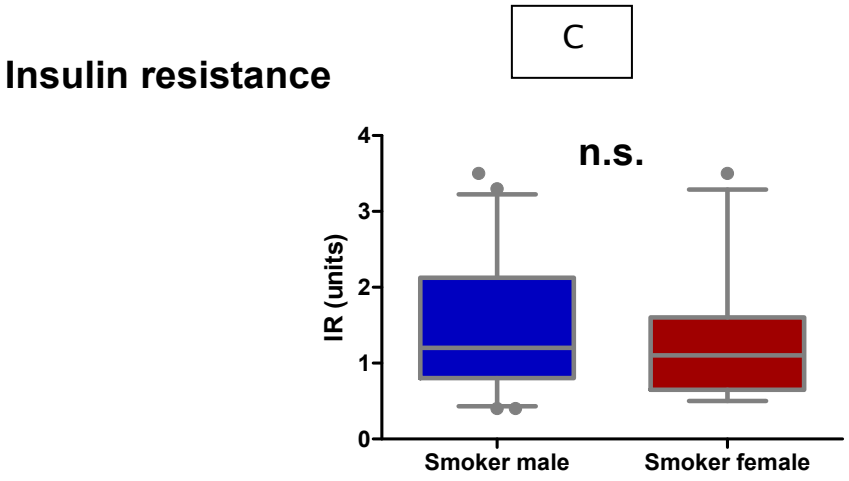
HDL: High-density lipoprotein, mmol/L: millimoles per litre.

6.3.16. Measure of waist circumference, W/H ratio, insulin resistance and mean blood pressure

Waist circumference and waist to hip ratio are the measure to diagnose central obesity. Insulin resistance is associated with central obesity in people with MeS. Waist circumference and w/h ratio was measured in all smokers. Insulin resistance (M=42, F=33) was measured by HOMA-R. There was a difference in waist circumference ($p<0.007$), w/h ratio ($p<0.0002$), systolic BP ($p<0.0003$) and diastolic BP ($p<0.0002$) shown in Fig 6.15. There was no difference in insulin resistance between men and women smokers.


Waist circumference
A
p<0.007
150
100
50
0
cms
Smoker male
Smoker female


W/H ratio
B
p<0.0002
1.2
1.1
1.0
0.9
0.8
0.7
0.6
ratio
Smoker male
Smoker female


Insulin resistance
C
n.s.
4
3
2
1
0
IR (units)
Smoker male
Smoker female

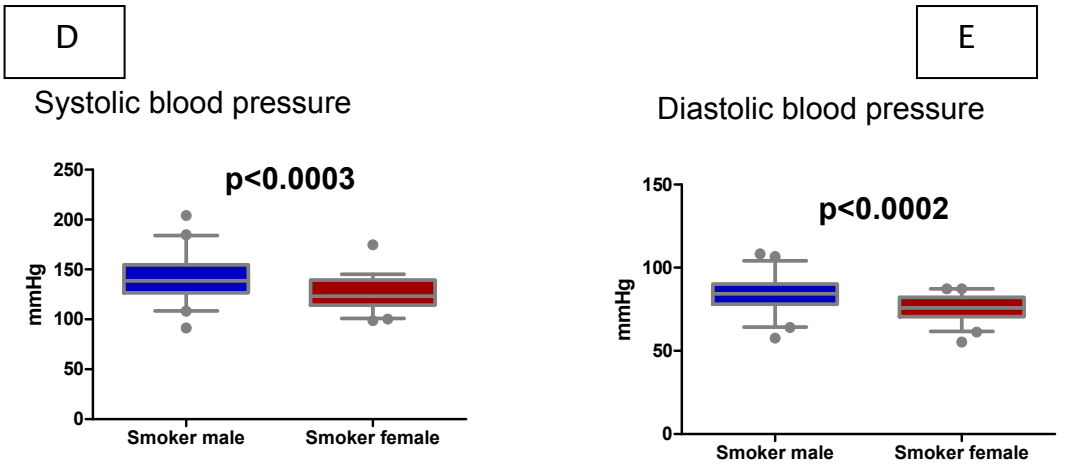


Figure 6.16 Features of MeS, (A) waist circumference (p<0.007), (B) waist/hip (w/h) ratio, (C) insulin resistance, (D) systolic blood pressure and (E) diastolic blood pressure in male and female smokers.

The data are represented as median and quartiles.

6.3.17. Body composition measurements in men and women smokers

Anthropometric measurements or body composition includes measurement of total body fat, fat free mass. Fat free mass can be measured more accurately by calculating fat free mass index (FFMI). In this study it was measured by bioelectrical impedance analysis (BIA). The principal and methods are explained in chapter 2 (section 2.3.2). All subjects were measured in minimal clothing. The analysis between men and women showed that, women were more fat than men in the study (Fig A and B, p<0.003 and p<0.0006) and men had more fat free mass (Fig C and D, p<0.0001 and p<0.0003) than women.

Figure 6.17 Anthropometric measurements in men and women smokers, (A) Fat % and (B) fat in kilogram, (C) lean mass in kg and (D) fat free mass index

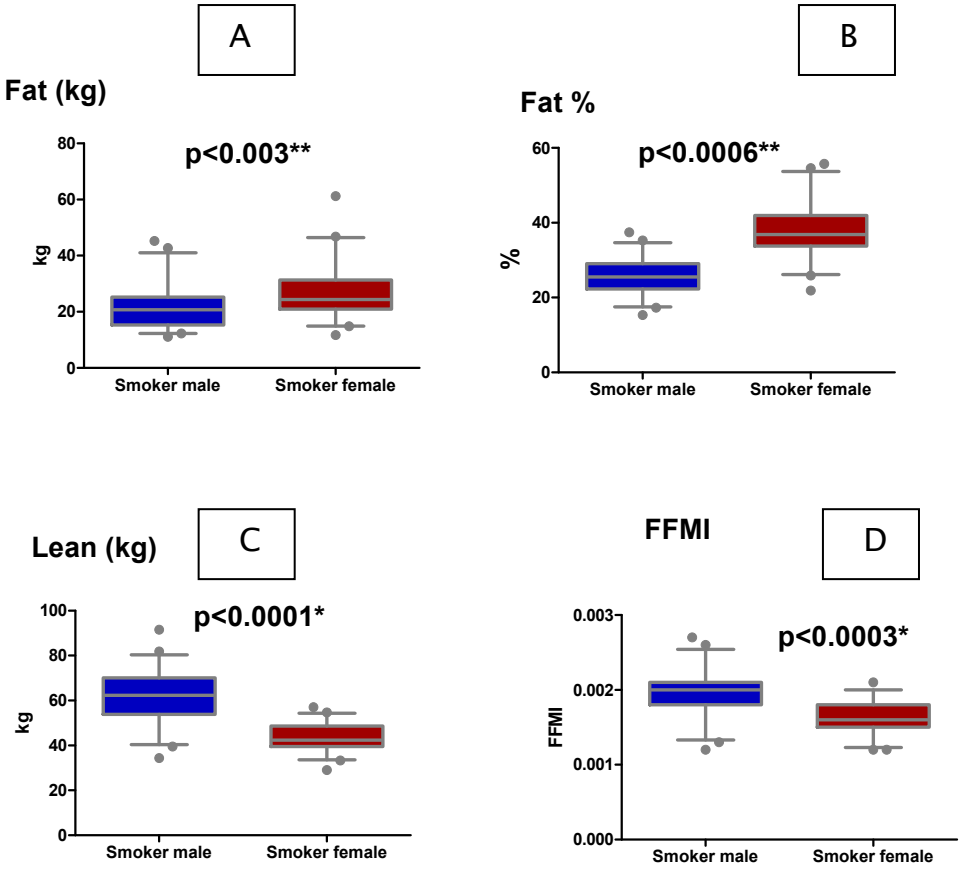


Figure 6.18 Anthropometric measurements in men and women smokers, (A) Fat % and (B) fat in kilogram, (C) lean mass in kg and (D) fat free mass

FFMI: Fat free mass Index. kg:Kilogram, %:Percentage

6.4. Discussion

This study for the first time, has demonstrated gender differences in smokers with features of MeS. The main findings were that the prevalence of MeS was not significantly different; however, there was a trend towards an increase in men in all three groups. Men demonstrated increased central obesity measured by w/h ratio. HDL levels were high in women in non-smoker controls and smokers without COPD group, however there was no difference in triglyceride levels. Fasting blood glucose was increased in men in non-smoker controls and healthy smokers group, however there was no such difference in COPD subjects. The body composition was different in men and women, Total percentage body fat was increased in women in all the groups and there was a difference in fat free mass index. The quality of life as measured by SGRQ and physical activity as measured by Baecke's physical activity questionnaire were found to be different in only in healthy smokers. I found that men smoked more than women and their FEV_1 was significantly reduced, however, there was no difference in TLco. Men demonstrated increase in blood pressure compared to women smokers. Blood inflammatory markers, measured from peripheral blood, neutrophils, lymphocytes, monocytes and eosinophils and inflammatory cytokines (except IL-12p70) showed no difference between men and women. Interestingly there was a difference in apolipoprotein-1 and this finding suggests that there is an autoimmune response in smokers. These differences in men and women smokers are discussed in detailed below.

When features of metabolic syndrome were analysed there was an increasing trend in men. There were many important differences in the individual features of MeS. Men had larger waist circumference than women. W/h ratio increased in men in all groups, suggesting that men smokers have more central obesity than women. This could be one of the reasons for men to have reduced FEV_1. It was shown before that w/h ratio is a better predictor of FEV_1 in men[169]. This also supports the idea that the gender difference in the form of central obesity is one mechanism of impairment in lung function due to weight gain[403, 404]. This has an impact on the decline in lung function[169]. The second observation was that men had a higher glucose concentration than women and may suggest a higher prevalence of pre diabetes in the cohort of men smokers. Women tend to have higher HDL cholesterol concentration than men, which may be due to consequences of lesser smoking habits. Women in

my study who smoke had higher fat mass in smokers and COPD groups, which probably confirm that smoking does not reduce fat mass in everyone and there are susceptible smokers who gain weight through increased fat mass. Interestingly I found that women had a decreased measurement for quality of life compared to men. However, there was no difference in physical activity to explain the reasons behind such a reduced quality of life. Again, this could be due to the many reasons, one of which could be due to excess weight and excess fat mass which affects their behaviour like depression.

Women in my study smoked less than men, which may offer the reason for the more diametric reduction in FEV_1 in men. Smoking varies by social class and is highly interrelated to behaviour, gender, age and ethnicity[405]. Historically smoking in men was associated with a strong sense of social acceptance and identity. However, in the present environment there are more women smokers than men. One arguable explanation for this is that women think that it reduces appetite and stops them putting on weight. When smoking pack years was measured in my study there were men who smoked more than women in the COPD group. However, in the smokers without COPD group there was no difference in the amount of cigarettes smoked between men and women. There is an increasing appreciation of gender differences in smokers with COPD; however, there is less data concerning low lung function as a risk factor might better contribute to the development of MeS in men and women. In the current study women had more preserved FEV_1 compared to men. The general view is that the prevalence of COPD is higher in men historically because of heavy smoking. However, there are more deleterious effect of smoking in women which has partly contributed to the recent increase in the prevalence rate of COPD in women[391, 394, 406, 407]. Burrows *et al* suggested that a low initial reduction in lung function would predict subsequent rapid decline in FEV_1 in smokers and called it 'horse racing effect' found only in men[408]. Other measure of lung function decline and a reflection of small airways disease is the measurement of transfer factor (TLco). TLco is clinically useful for the diagnosis of pulmonary vascular and interstitial lung disease and also importantly to diagnose emphysema in smokers with airway disease[409-411]. Age and change in weight predicts the rate of decline of TLco[412], however, there is no significant change in young adults male and female until age of 40 years and suggests that TLco may be constant until this period and begins to decline later[412] and is independent of smoking status. In my study the measure of TLco

showed no difference in men and women. In a study undertaken by Anthonisen *et al,* they concluded that the lung function loss is similar in both sexes if they continue to smoke[413]. However, in an another study it was shown that an increase in the number of cigarettes smoked resulted in a steeper FEV_1 decline in men but not in women[414].

Inflammatory cells play a crucial role in the development of airway disease. . However, in the present study there was no difference in CRP measurements between men and women smokers with and without MeS. There is enough evidence to show that there are raised neutrophils in smokers with COPD[79] and increased eosinophils in asthmatic airways[415]. Increased neutrophil count is related to risk of death in cardiovascular disease[416]. There is also ongoing subclinical inflammation which is associated with features of MeS[417] and atherosclerosis. Most of the studies in the literature measured CRP as a marker of inflammation. In my study many other inflammatory cells were measured including blood neutrophils, monocytes, lymphocytes and eosinophils and found that in smokers and COPD there was no difference in blood neutrophils, monocytes, lymphocytes and eosinophils between men and women. However, there was a decreasing trend in female smokers with COPD subjects for neutrophils, monocytes and eosinophils. C-reactive protein is an acute phase protein released in the body during an inflammatory state and it is used as a clinical marker of inflammation[418]. Mild chronic elevations in CRP concentrations even within the clinically normal range independently predicts future cardiovascular events[419, 420]. Smoking is associated with elevated CRP[421] and it may be due to the tissue damaging effect of smoking. The inflammation persists even after quitting smoking after many years[422, 423]. CRP is also elevated in smokers with COPD[355] and in the cohort I studied there was a linear increase in CRP levels in smokers with and without COPD. However, when this was analysed independently in men and women there was no significant difference. CRP predicts the development of features of MeS independently of the levels of adiposity and insulin resistance in women[418]

There was a significant difference in IL-12p70 measurement between men and women. Inflammatory mediators are released in the airway epithelium in smokers due to the influx of inflammatory cells. These are also represented in the systemic circulation and could be measured in the peripheral blood. Some of the inflammatory markers are TNF-α, interferon gamma (IFNg) and interleukins. TNF-α is a pro inflammatory mediator and also a neutrophilic

chemoattractant[424] which is raised in smokers with and without COPD. Plasma TNF-α was measured by Luminex method in the study and found that it was no different in men and women and no difference in smokers with and without features of MeS. IL-2 is a key inducer of Th1 associated inflammatory response which protects against intracellular infections and cancer but its involvement in autoimmune tissue destruction is important[425]. Human Th2 cells interact with dendritic cells which effectively induce bioactive IL-12p70 and cause T cells to revert to Th0/Th1 phenotype[425]. The measurement of IL-2 was no different in men and women smokers in previous studies.

In summary, I found that men smoke more than women I speculate that the body fat distribution would explain the link between low FEV_1 and development of features of MeS. High waist hip ratio is associated with impaired glucose metabolism and hyperlipidemia. In addition it has been well established in the general population that FEV_1 and FVC are predictors of overall mortality[426-428]. There were no major differences in inflammatory markers. The prevalence of MeS was no significantly different, however, men were shown to have obesity that is more central, and women had more total body fat. Women had reduced QOL in general, however, there was no difference in QOL and physical activity between men and women with MeS.

7.0 Chapter-Predictors of lung function decline in a follow up study in smokers and COPD

7.1. Introduction

Understanding lung function is essential, as the physiology of the lungs is more complex. This complexity of the mechanism involved is still more difficult to understand in the lung, which has a disease. The rate of decline as a marker of disease progression in COPD was developed many years ago by Fletcher and Peto[53]. However, this decline is not uniform across all smokers. Decline is seen more rapidly in susceptible smokers who develop chronic obstructive pulmonary disease (COPD) which is a preventable and treatable disease of the airways. In this disease, there is a progressive decline in forced expiratory volume in 1 second (FEV_1) which is partially reversible in the early stages of the disease. Lung function decline is important not only to measure disease process but also an indicator of premature mortality in smokers[429]. Smoking cessation is seen so far only means of reducing or stopping the rapid decline in FEV_1[430].

There could be more than one reason for the decline in lung function. The main contributor is the duration and quantity of cigarette smoked. There is a correlation between duration of smoking and FEV_1 decline[431]. Other factors which could contribute to decline include genetic predisposition, air pollution, occupational exposure[432] and susceptibility with gender. Women suffer from early COPD changes than men. There are few physiological reasons for the susceptibility in women for the disease. Firstly it is said that the women have smaller lung than men and require less exposure to nicotine to produce negative effect[433] and the other was that the manner in which women smoke and it is understood that women tend to inhale deeper and hold the breath for a longer time[398]. There are relatively few measures one could take to reduce the decline in lung function. The only proven one so far is to encourage smoking cessation. The other possible measure according to Judith Garcia-Aymerich *et al* is moderate to high level of regular exercise could reduce lung function decline and risk of developing COPD in smokers[432].

FEV_1 is associated with the diagnosing the airway disease there are other important indices, which could contribute to the disease process. Anthonisen *et al* and Burrows *et al* suggested that other prognostic factors which contributes to the disease for e.g. age, active smoking, symptoms, poor bronchodilator response, functional reserve capacity, exercise tolerance[54, 55]. Smoking also contributes to airway inflammation, which could be measured in

sputum. Airway obstruction and chronic expectoration are associated with increased number of sputum neutrophils[147, 359] which is associated with accelerated decline in lung function.

The purpose of this study was to understand the FEV_1 decline in this cohort of smokers and COPD. The subjects in my study underwent baseline spirometry and a repeat spirometry between 5-6 years. Data was compared to the base line to calculate the annual decline. The other aim of the study was to evaluate the association with age, pack years, sputum neutrophils, HRCT that predicts FEV_1 decline in subjects who were followed annually for the study.

7.2. Methodology

7.3. Subject characterisation

In total 102 were followed up once and 70 were followed up once more after a further year. Out of 70 subjects, there were 15 healthy controls, 21 healthy smokers, 10 COPD GOLD stages 0&I, 14 COPD GOLD II & III and 10 subjects with diagnosis of emphysema on HRCT but no obstruction on lung function testing. The study design is explained in chapter 2 (section study design 6). The final analysis was done on 64 well-characterised subjects (table 7.1).

All participants underwent medical examination, skin prick testing, pulmonary function testing, and induce sputum. All subjects had their lung functions done in the morning to avoid any inconsistency in the measurement. They had bronchodilator reversibility with 400µg of inhaled salbutamol and subjects with ≥ 12% reversibility were excluded from the study. Bloods were taken, centrifuged and stored for future analysis. All subjects who were followed previously had HRCT and densitometry measurements done and analysed by then research fellow for his study titled' Differential gene expression study in health smokers and COPD. Bronchial brushings and biopsies were taken as a part of the study. As with my previous predecessor, pre-bronchodilator FEV_1 was used for his analysis .Henceforth in the follow-up study I used the same to compare with the base line measurement.

Subject characteristics

	Non-smoker controls	Healthy smoker	GOLD stage 0 and 1	GOLD stage II and III	Ex-smokers
No of Subjects	15	21	10	14	4
M/F	6/9	8/13	7/3	10/4	2/2
Age	60.2 ± 7.5	52.7 ± 10	56.8 ± 6.2	61.1 ± 6	59.3 ± 7.5
Pack years	N/A	36.6± 14.4	43.6± 18.4	55.4± 18.4	56.7± 25.2
Baseline FEV_1 (%)	105.3 ± 13	102.1±11.6	97.6± 12.4	63.1± 14.6	87.5± 37.2
Annual FEV_1 change (%)	-0.75 ± 1.3	-0.15 ± 1.3	-1.73 ± 1.3	-2.5 ± 2.6	1.64 ± 1.8

Table 7.1 Subject characterisation.

The data are represented as mean and standard deviation. FEV_1: Forced expiratory volume in 1 second, M/F: Male/Female, N/A: Not applicable, %: Percentage.

7.4. Statistical analysis

The outcome variable used was annual percentage change in pre bronchodilator FEV_1 from baseline for each subject. This was calculated from the baseline visit to the final visit for each subject. The variables investigated univariately for their ability to predict annual percentage change from baseline were, 1) the percentage volume of lung tissue < -950HU in density on inspiratory CT. 2) baseline FEV_1, 3) Pack years, the baseline pack years was used as this appeared to be more reliable. 4) Age at final visit. 5) gender 6) baseline percentage neutrophils in sputum. 7) baseline absolute count of neutrophils per gram. 8) baseline count of CD8+ve cells per square mm of submucosa from the bronchial biopsy.

The results were obtained from the univariate analysis data for 70 subjects. As there were only two variables that reached the 5% level of significance, and these were both neutrophil variables, multivariate analysis was not performed. Baseline percentage neutrophils in sputum was the best predictor of decline in FEV_1, and this variable explained around 10% of the variability in the model. This is a reasonably high amount of variability for a single predictor to explain. The rest of the variables showed no significance with the FEV_1 decline.

7.5. Results

7.5.1. Association of FEV1 decline with clinical and pathological correlates of the rate of decline variables in all smokers with and without COPD.

A number of clinical variables for e.g. age, pack years, HRCT, sputum neutrophils, CD8+ cells were examined to understand which of these independently predicts the FEV_1 decline in the subjects. The following section will discuss individually the above variables.

7.5.2. Correlation of FEV1 with the HRCT densitometry measurement

A total of 64 subjects underwent high resolution CT scan and an experienced radiologist reported the scans. An expert in the field did the densitometry measurement by using a programme, which quantified HRCT density

parameters. Three parameters were measured, percentage (%) area of low attenuation, mean lung density and ratio of expiration to inspiration. For the analysis, the percentage of volume of lung tissues was used to correlate with decline in FEV_1 (Fig 7.1). There was a weak correlation and only 3 % of the variation was explained and the correlation was not significant.

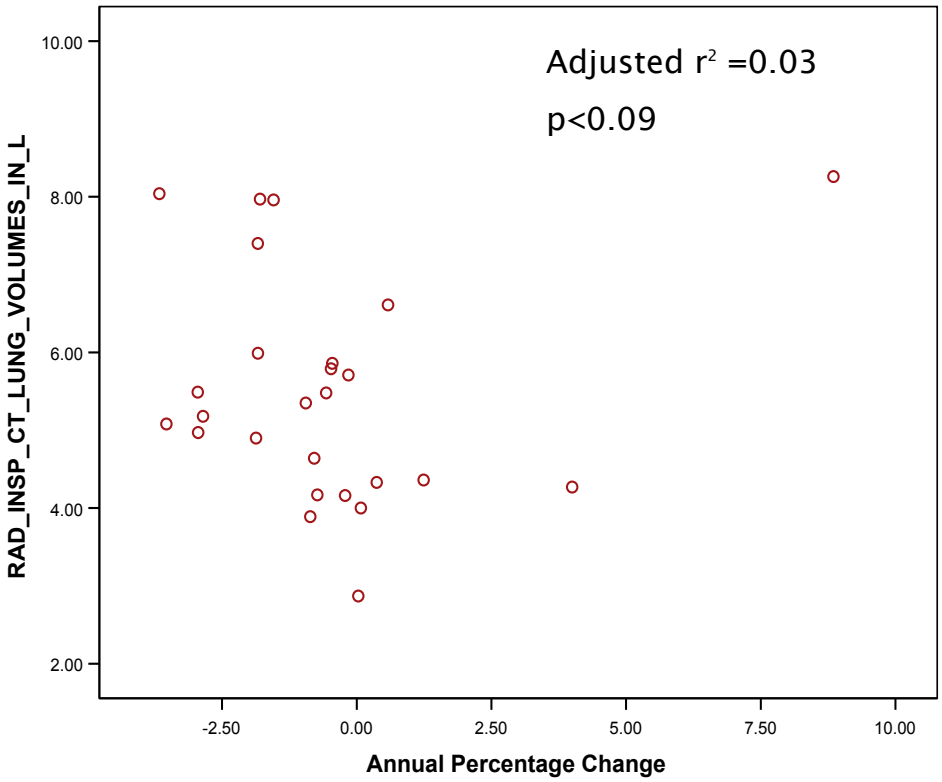


Figure 7.1 Annual percentage change in FEV1 decline (X-axis) to inspiratory lung volumes (Y-axis)

FEV_1: Forced expiratory volume in 1 second.

7.5.3. Association of FEV_1 decline with age

Age is an important factor for normal lung function decline. In this study, the association with age was measured against FEV_1 decline. The decline was calculated annually and the age at the final visit was considered for the analysis. A total of 64 subjects were analysed and the linear regression analysis showed (Adjusted r^2=0.010 & p<0.1) that age does not independently predicts the decline in FEV_1 (Fig 7.2) when controlled for gender and smoking.

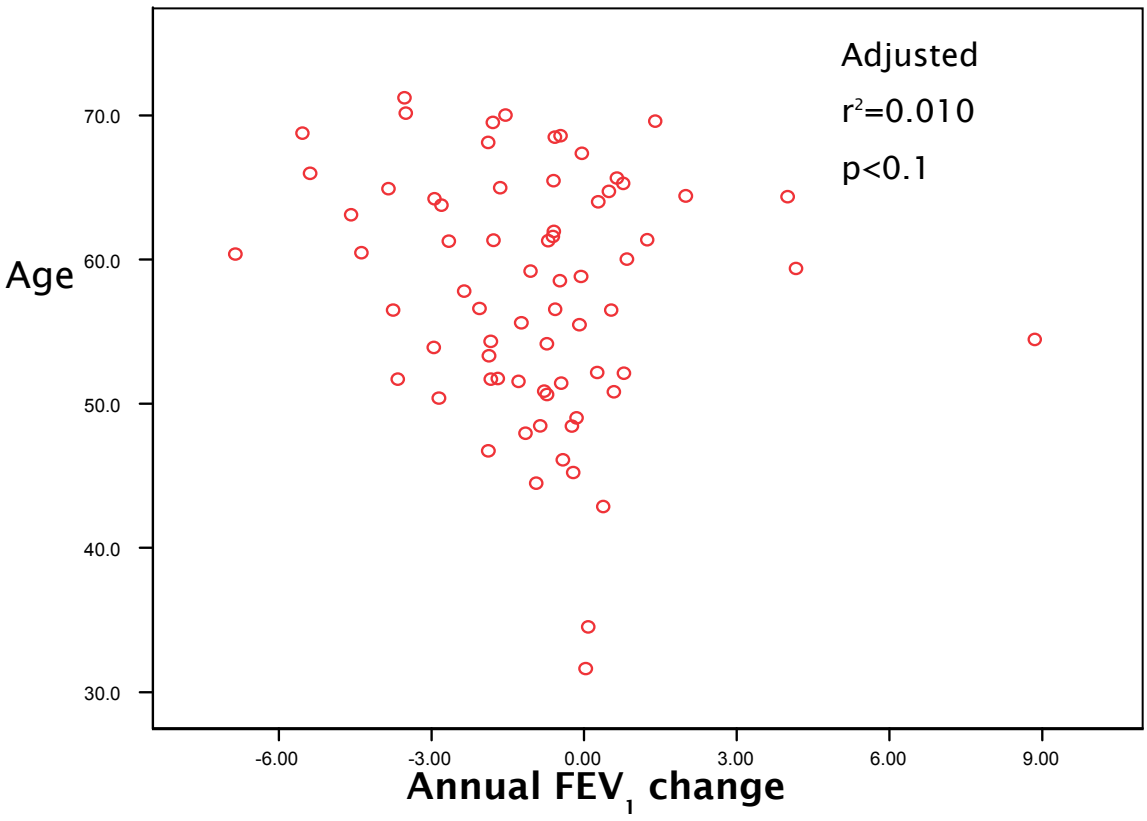
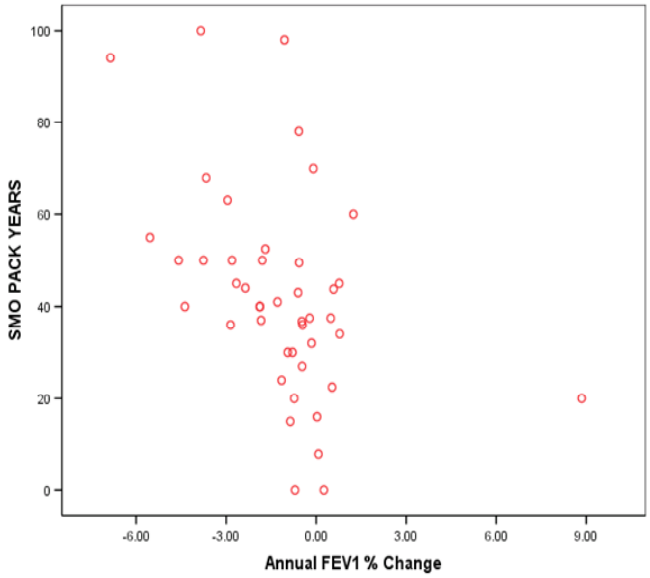


Figure 7.2 Annual percentage change in forced expiratory volume in 1 second (FEV1) decline in the X-axis and age in the Y-axis.

linear regression analysis was not significant (R^2 adjusted was 0.010 and $p<0.1$).

7.5.4. Association of FEV_1 decline and pack years

Pack years was calculated by using the formula, number of cigarette (20 cigarette = I packs) smoked per day times number of years of smoked. A total of 64 subjects were then analysed for the FEV_1 decline. Linear regression analysis was done and found that pack years was not independently associated with decline (Adjusted r^2 was 0.001 and $p<0.3$, Fig 7.3).



Adjusted r^2 =0.001
p<0.3

Figure 7.3 Smoking pack years and annual percentage change in FEV1 decline.
There was no statistical difference in the linear regression analysis (Adjusted r^2 was 0.001 and p<0.3). FEV_1: Forced expiratory volume in 1 second.

7.5.5. Association between neutrophils in sputum and FEV_1 decline

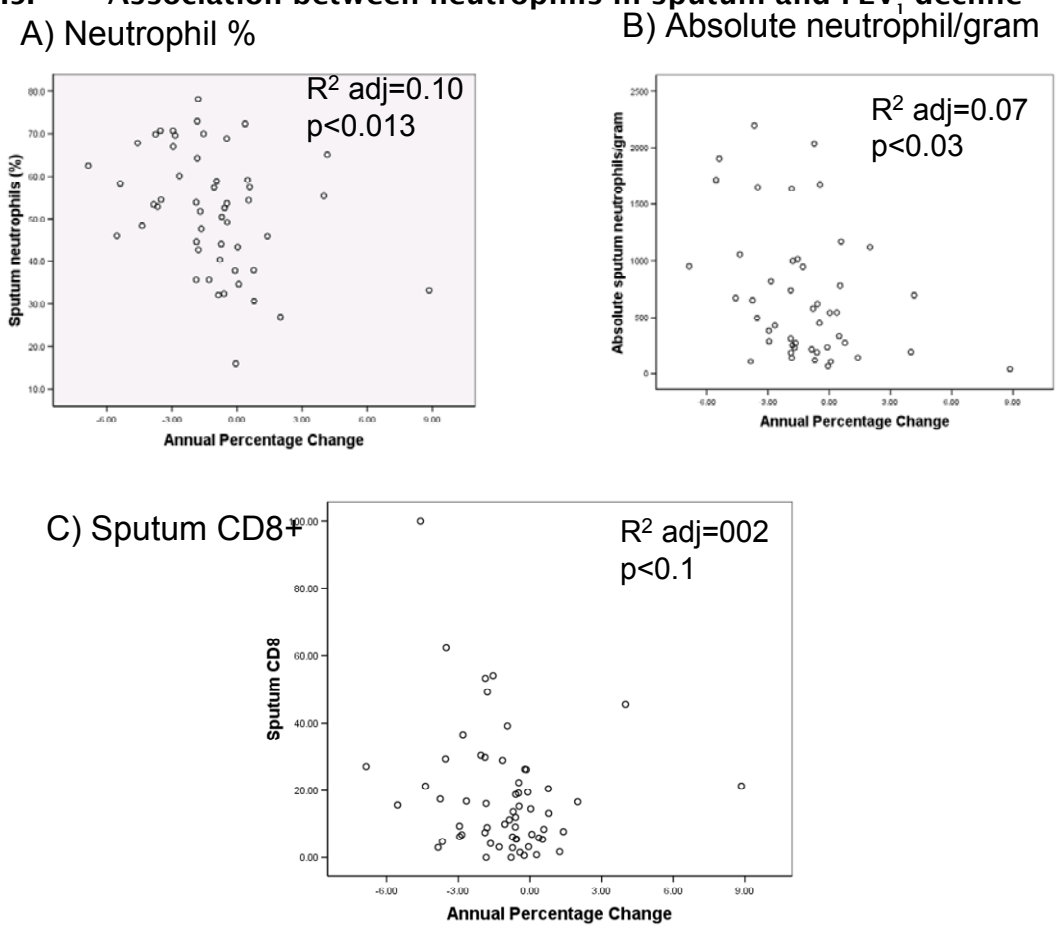


Figure 7.4 A) Percentage of neutrophils in sputum and annual percentage change in FEV1 decline. B) absolute neutrophils/gram in sputum and annual percentage change in FEV1 decline. C) sputum CD8+ cells and annual percentage change in FEV1 decline.

Sputum was processed (chapter 2 section 2.4.7) in 51 subjects who could produce sufficient sputum and analysed for inflammatory cell neutrophils. There was an increase in sputum neutrophils in smokers and COPD. In the linear regression analysis there was a significant difference in neutrophil percentage in sputum (Fig 7.4A, adjustedr^2 was 0.10 and $p<0.013$) when controlled for smoking, age and gender. There was a difference in absolute neutrophil count in the sputum (Fig B, R^2 adjusted was 0.07 and $p<0.03$). This suggests that sputum neutrophils predicts FEV_1 decline in smokers and COPD. However, there was no difference in CD8+ cells in the bronchial biopsies of the subjects (Fig C, adjusted r^2 was 0.02 and $p<0.1$

7.6. Discussion

The aim of this study was to understand and explore the determinants of FEV_1 decline in our study population. This was a longitudinal study of cohort diagnosed as either healthy non-smoker controls or smokers with or without COPD. The main findings in this study was an increase in sputum neutrophils both absolute and percentage in smokers and COPD. In the linear regression analysis there was a significant difference in neutrophil percentage in sputum when controlled for smoking, age and gender. There was also a difference in absolute neutrophil count in the sputum, which suggests that sputum neutrophils predicts FEV_1 decline in smokers and COPD. The other important variables like smoking, age and HRCT densitometry measurements did not independently predict FEV_1 decline in this cohort. The findings are discussed in detail below.

COPD is a chronic disease of the airways, which is caused by smoking. There is no single criterion by which the severity of the disease could be adequately described. The most commonly used determinant to diagnose disease severity is FEV_1. This is considered the most important predictor of mortality besides age in COPD patients[434, 435]. There are determinants that are associated with predicted FEV_1 which are smoking, age, gender, respiratory symptoms, exercise capacity. However, the results are not the same in every study. Weaver *et al* found FEV_1 was associated with exercise capacity and not with dyspnea[436]. In our study, the annual rate of decline was not determined by age and there was a poor correlation between age and annual rate of FEV_1 decline. This may be because of the relatively younger population in the study.

Long-term smokers develop COPD. Airway inflammation is the important in pathogenesis of COPD which leads to alveolar destruction and airway remodelling[338]. The toxic substances in the cigarette smoke promote an inflammatory response in the lung which is characterised by increase in the number and activity of inflammatory cells e.g. neutrophils and macrophages[437]. There are inflammatory cells e.g. neutrophils, monocytes and lymphocytes which play a major role in COPD. There is enough evidence to prove that neutrophils are raised in the sputum in patients with[79] COPD. Sputum neutrophils were measured in my follow up study, which showed that both percentage neutrophils and absolute neutrophil count were independent

determinants of annual FEV_1 decline. However, there was no correlation with CD8+ cells in the bronchial biopsies.

Cigarette smoking is the major cause of reduced lung function which is an important characteristic of COPD[438, 439]. Other factors, which are associated with accelerated lung function decline in general population, are gender, body weight, and respiratory symptoms[168, 393, 440, 441].

In 1976, Fletcher et al. published a monograph summarizing the results of an 8-year observational study of the relationship between cigarette smoking, chronic expectoration, and the development of irreversible airflow obstruction[53]. According to this study, COPD consisted of two components, chronic bronchitis, and emphysema. However, there was no mention of airway obstruction at that time. The clinical features were described as either 'pink puffer' who were patients with COPD and reduced BMI (cachexia) or blue blotters who were obese or overweight and clinically suffered from respiratory failure. The study also showed that a subgroup of smokers had an accelerated rate of decline in FEV_1. This finding of accelerated decline in FEV_1 led to subsequent investigations into apparent susceptibility factors for cigarette smoke and therapies that could slow down the decline in FEV_1[442].

In my study, smoking pack years was analysed in the linear regression model and found that degree of smoking was not independently contributing to FEV_1 decline in our subjects. Smoking is the major cause of COPD in both men and women[443, 444]. In the Six Cities Study, a cross-sectional analysis showed that current smokers had a lower FEV_1 compared to ex-smoker[393]. Interestingly both ex and current smokers had identical pack years history and the decline of FEV_1 was proportional to the number of cigarette smoked each day which imply that heavy smokers will regain more lung function than light smokers after smoking cessation[445]. There is a also evidence to show that increase in the number of cigarettes smoked results in steeper FEV_1 decline in men, however, it is not same in women[414]. The gender differences is controversial as Xu *et al* concluded from his 24yrs longitudinal follow up study that the female smokers overall tend to have faster decline in FEV_1 than male smokers. The observations were consistent among ex-smokers and continued smokers[393]

In the linear regression analysis, I found that percentage of volume of lung tissue showed a weak correlation with FEV_1 decline when controlled for age, gender. COPD is a chronic disease, which consists of chronic bronchitis and emphysema. Chronic bronchitis is a measure of productive cough and

duration of the cough with preserved lung function test. This was previously categorised as GOLD stage 0 COPD, however, this has been withdrawn from the current guidelines. Emphysema is the disease of the small airways and is diagnosed by High Resolution Computed Tomography scan (HRCT). HRCT is the most sensitive method of diagnosing different subtypes of emphysema in the lung both visual and quantitatively. These HRCT findings are well correlated with the pathological extend of the disease[446]. The results were further compared and classified by densitometric measurement. In my study three parameters were measured by densitometry, percentage (%) area of low attenuation, mean lung density, and ratio of expiration to inspiration. For the analysis for this study, the percentage of volume of lung tissues was used to correlate with decline in FEV_1

There are longitudinal studies, which have tried to explore the treatment options to reduce or stop the accelerated decline in FEV_1 in patients with COPD. The recent UPLIFT (Understanding Potential Long-Term Impacts on Function with Tiotropium) study had a primary objective to determine whether tiotropium reduces the rate of lung function decline as measured by FEV_1. The results of this study showed no difference at the end of 4 years in rate of decline compared to the placebo arm of the study[447]. There are many other studies which have shown similar results[448, 449]. However, only intervention that has disease modifying therapy is smoking cessation as shown in the Lung Health Study[450] . The Lung Health Study assessed effects of changes in the smoking habits on FEV_1 decline in COPD patients and found that women who quit smoking had an average improvement in FEV_1 which was two and half times as great as that in men[451].

The limitation of this study was the number of subjects who were followed up was half of the actual number recruited for the main study. There were various reasons for the poor follow up, a) some patients had moved houses, b) two of them were deceased due to lung cancer and c) some were unable take time off their work. d) The data analysed was pre bronchodilator, as I had no post bronchodilator data from the original cohort, e) the lung function machine used for the follow up study was different from the machine used for the original study which could induce variations.

In summary, I conclude that age, smoking pack years, densitometric measurement for assessing percentage lung volume, CD8+ cells do not independently determine FEV_1 decline in smokers and COPD. However, sputum

neutrophils was found to independently determine FEV_1 decline in stable smokers and COPD subjects. Future work should include a longitudinal study with a larger cohort to identify other clinical biomarkers for the decline in FEV_1.

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8.0 Chapter-Main conclusions and general discussion

The main conclusions of my study were:

1. Prevalence of MeS was higher in smokers with and without COPD compared to non-smoker controls, however, there was no difference between smokers with and without COPD, suggesting the effect is due to smoking rather than the disease. Subjects with features of MeS demonstrated increased reduction in FEV_1 compared to subjects without MeS in smokers. However, TLco was reduced in smokers with MeS when compared with non-smokers with MeS.

2. Inflammatory marker CRP and inflammatory cell neutrophils, monocytes, lymphocytes and eosinophils were raised in smokers. However, CRP, neutrophils, monocytes, lymphocytes, and inflammatory cytokines for e.g. TNF-□, IFNg did not show any difference between smokers with and without MeS suggesting that inflammation play no role in the development of MeS in smokers.

3. Insulin resistance was shown to be the main cause for the development of MeS in smokers and non-smokers in the study. Physical activity and QOL did play a role in this process.

4. There were many gender differences in smokers. Men smoked more and showed increased reduction in FEV_1 compared to women. There was an increase in central obesity in men and total body fat in women. Men showed a trend towards an increase in prevalence of MeS than women. QOL was reduced in women smokers and were physically less active compared to their counterpart. The inflammatory cytokine IL-12 was reduced and apolipoprotein 1 was increased in women.

5. The determinants of the annual FEV_1 decline were sputum neutrophils in the study, however, age, pack years, HRCT densitometry measurement, CD8+ cells in the biopsies did not show the same affect on FEV_1 decline.

My first hypothesis was that smokers have an increased prevalence of MeS and this was found to be correct in my study. My other hypothesis was that there is an association between MeS and COPD. One explanation would be that COPD is a systemic inflammatory disease and, as such, contributes to the development of features of MeS. The other explanation would be that MeS precedes the development of and is a risk factor for COPD. However, I found that COPD was not associated with a clear increase in MeS, although there was a trend towards a higher prevalence of MeS in patients with COPD. As my final major hypothesis, in a prospective study I wanted to identify factors which could predict progression of lung function decline. Of all the factors that I assessed only sputum neutrophilia was found to be a predictive factor for the change in FEV_1.

The recruitment for the study done by word of mouth and subjects came from all occupations. No particular group was targeted for recruitment to avoid any potential bias. I therefore believe that the findings of the study are representative of the general population. All subjects were Caucasians as ethnicity plays an important role in the development of features of MeS.

My first step was to explore the prevalence of MeS in my cohort. Subjects were carefully classified as non-smoker controls who were life long non-smokers, smokers without COPD who had well preserved lung function without any symptoms of bronchitis and the third group was smokers with COPD. COPD subjects were diagnosed according to GOLD criteria and the subjects in the study were from COPD stage0 (currently referred to as chronic bronchitis) to COPD stage III. In order to diagnose the features of MeS, the IDF and NCEP ATP III criteria were used. These classifications were well recognised and commonly used in clinical practice across the globe. The recruitment was done across Wessex, mainly from Southampton city.

The prevalence of features of MeS is on the rise around the world. The current estimated prevalence of MeS is around 16%[363]. The prevalence varies with ethnicity and with various chronic diseases. The first analysis in my study showed that there was an increase in prevalence of features of MeS in smokers with and without COPD when compared with non-smoker controls. There was a trend towards an increase in MeS from healthy smokers to COPD. However, the prevalence of MeS in smokers with and without COPD was no different. This was in contrast to a paper which suggested that the prevalence of features of MeS is high in COPD patients[236] . There were many limitations to that study.

Firstly, the study had no healthy smokers and the subjects were recruited from the cardio-pulmonary rehabilitation programme. Secondly, there was no information about the smoking status.

The important message from this first observation in my study was that there was an increase in the prevalence of MeS, which is due to something, in common for both smokers with and without COPD, i.e. cigarette smoke. The conclusion at that stage was that smoking causes features of MeS and that it was not due to the systemic effect of COPD. It was then important to understand various pathways which may be responsible for causing the development of MeS in my subjects. All smokers (healthy smokers and COPD smokers) were then grouped together to understand different determinants triggered by smoking causing lung damage and to understand the relationship with features of MeS. The analysis showed that smokers with MeS and smokers without MeS differed only in respect of the prevalence of individual features of MeS (as would be expected) but were no different in respect of a series of pro-inflammatory markers except for IL-12. However, insulin resistance was seen as being a driving force in MeS and correlated with smoking history. Furthermore, FEV_1 was reduced in smokers with MeS which was an interesting observation.

Smoking is the major contributor for up to 87% of emphysema, chronic bronchitis[452] and lung cancers, it is estimated that there are over 430,000 annual deaths from cancer. Smoking affects the fetus, children, and adolescents who are vulnerable early in life by maternal smoking either during pregnancy or because of environmental tobacco smoke (passive smoke). Some studies indicate that an increased susceptibility to the effects of smoking and exposure to environmental tobacco smoke may exist among those with specific genetic polymorphisms[453]. Several smoking behaviours may account for increased dependence and higher measures of smoke-related toxins experienced among smokers especially African Americans. Although African American smokers generally smoke fewer cigarettes per day, and take less puff per cigarette than white smokers, they tend to inhale deeper[454]. They like to smoke mentholated cigarettes, which have higher cotinine and tar levels. By doing this they also inhale 30% more nicotine which will result in 2 hour longer clearance rate in the blood[455-457].

Cigarette smoke has immediate or acute effects and long-term chronic changes on the pulmonary and extrapulmonary tissues in the body. In

comparison with the chronic effects there are fewer studies which have looked at the immediate effect like inflammation and lung function[458]. In chronic smoking the inflammatory cells like neutrophils increased in peripheral blood and broncho alveolar fluid[459-461], however, in acute smoke exposure they are both increased[462] and unchanged in neutrophil numbers in the bronchoalveolar fluid[463]. In my study, there was an increase in peripheral blood neutrophils, monocytes, lymphocytes in smokers. Interestingly there was an increase in eosinophils counts in smokers regardless of whether smokers were non-atopic and had no symptoms or infection in 6 weeks prior to recruitment. One could think that these subjects may be steroid sensitive smokers and if they develop, COPD in later stages may benefit from anti-inflammatory drugs. However, some studies show that peripheral blood eosinophils are reduced in acute smoke exposure[464]. These inflammatory cells were also analysed between men and women smokers in my study to understand the phenotypic nature, but found that there were no differences between them. Other inflammatory markers for e.g. CD19 positive B cells and the total number lymphocytes were also decreased during acute exposure to cigarette smoke[464, 465]. However, in the chronic smoke exposure like that in my study showed increased lymphocytes in the peripheral blood in smokers.

The role of inflammatory mediators is important in smokers with airway disease and most of the studies show an increase release in IL-8 from various cell types after exposure to smoke[466, 467]. There are inconsistent results showing increase in TNF-□, IL-1â and ICAM being increased when exposed to cigarette smoke[468, 469]. It is worthy of note that some of the studies have shown that cigarette smoke exposure depresses the release of LTB_4, interferon gamma and IL-2[470, 471]. I measured the inflammatory cytokines for e.g. TNF-□, IFNg and interleukins (IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, and IL-13) in smokers and non-smokers. Surprisingly there was no difference between the groups. One could speculate that these inflammatory cytokines may not be raised in stable state and that looking in the airways would show more differences. Smoking also inhibits fibroblast function, which is one of important cells in the repair process in the airway epithelium. The ongoing inflammation and chronic injury and repair process caused by smoking contribute to the development of COPD[472] in susceptible smokers.

C-reactive protein is an important inflammatory marker in many diseases. Smoking is associated with long term elevation of CRP[473]. One of the markers of

systemic inflammation which is constantly raised in COPD is also CRP[139] when compared with healthy controls. The levels of CRP in my study showed an increase across the groups, and COPD subjects in particular showed maximum raise. This emphasises that COPD subjects even in stable state show ongoing systemic inflammation. CRP was also analysed in men and women smokers in my study and found that there was no difference in their levels between them. Regression analysis was done and when adjusted for smoking, age, and gender, CRP was shown to be a significant and independent predictor of FEV_1 reduction.

Smoking causes systemic effects and is a risk factor for many diseases for e.g. cardiovascular disease, several forms of cancers, COPD and others[474]. While smoking habits have declined among adult men in the western world, it is still increasing among young adolescent men and women[475]. Most women know that smoking could cause chronic bronchitis and emphysema, cancers and heart disease. Smoking can also cause early wrinkling of the skin, and contributing to infertility and menopause and osteoporosis[476, 477]. In my study smokers, men and women were analysed for various clinical determinants to understand the gender differences. As discussed earlier there were no differences in blood inflammatory cells for e.g. neutrophils, monocytes, lymphocytes, and eosinophils. There were also no differences in blood CRP levels. The inflammatory mediators in my study were not markedly raised except for IL-12p70 which is a bioactive cytokine produced by IL-4 which is Th2 specific, which revert to a IFNg producing Th0/Th1 phenotype when they interact with dendritic cells. IL-12p70, which helps in cell mediated immunity, was raised in men compared to women in my study. Men smoked more than women in my study and the most important finding was that FEV_1 was reduced more in men than in women. This could be because men smoked more than women. However, there was no difference in transfer factor between the two genders.

Body composition and physical activity are two important determinants which could also potentially affect the quality of life in smokers. Waist circumference and w/h ratio have been used to estimate the abdominal fat accumulation as a part of MeS [478-480]. W/h ratio was reported to be associated with cardiovascular risk factors[481, 482]. Differences in body composition in men and women are quite common. In my study, there was an increase in waist circumference in men compared to women. There was also a difference in w/h

ratio between men and women. However, the percentage body fat and fat weight were raised in women. This is an indication that men have obesity that is more central and women have generalised body fat accumulation. This is an important finding for the reason that men with increased central obesity also had reduced FEV_1. However, these findings did not change their physical activity score, as measured by Baecke's physical activity questionnaire. Similarly, one would expect a reduction in quality of life because of increased central obesity in men and total body fat in women, however there was no difference in QOL as measured by SGRQ between them.

Metabolic syndrome has evolved into an increasingly recognised and important clinical entity. There are many differences in the measurements of features of MeS as many factors determine the prevalence. Some of them are more important than the others because of variation, for e.g. age, ethnicity, and gender. Over the past decade, progress has been made in the understanding of the pathways, which link obesity to insulin resistance, and other aspects of metabolic syndrome. It was important in my study to use a common definition, which was accepted, hence, the classification of the features of MeS was done according to IDF recommendation. According to IDF, MeS was defined by ≥ 3 criteria of the IDF definition and central obesity being the key features for the diagnosis. In my study, the prevalence of features of MeS was high in smokers when compared with non-smoker controls. Both smoking and MeS are independent risk factors for cardiovascular disease and type II diabetes and previous studies have reported increased rates of MeS in adult smokers[483, 484]. However, this study is unique in exploring the relationship between smoking and MeS with respect to lung function changes, inflammation, body composition, insulin resistance, gender, physical activity, and QOL.

Impaired lung function as indicated by reduced FVC or reduced FEV_1 is a risk factor for cardiovascular events and death independent of smoking[485]. The association of FEV_1 and features of MeS was measured in my study and found that smokers with features of MeS had reduced FEV_1 and interestingly there was a difference in FEV_1/FVC ratio between smokers and non-smokers with MeS. The transfer factor showed similar results. These results suggest that in smokers, the development of features of MeS is caused in relation to reduction in FEV_1 and FEV_1/FVC ratio. However, the fact that COPD patients who have MeS have no greater reduction in lung function when compared to COPD patients

without MeS suggest that, once COPD is established, the reduction in FEV_1 play little or no role.

Smoking causes inflammation and it is the key feature in the development of airway disease and features of MeS. In my study inflammatory cells for e.g. neutrophils, monocytes and lymphocytes were raised in smokers. However, when analysing smokers with and without MeS there was no association between inflammatory cells except for lymphocytes, which showed a strong positive correlation with triglyceride levels. The possible cause of the development of MeS is not inflammation alone but it may play a minor role in the disease progression.

Anthropometric measurements are important in many chronic diseases, which are responsible for the mortality and morbidity of that particular disease. Smoking is well known to cause an increase in abdominal fat or central obesity[486]. In my study, I have confirmed this. In addition, I found that the fat mass was increased in smokers with MeS and it was more pronounced in women then in men. The conclusion from these results is that smoking increases central obesity in men more so than in women and this could be one of the explanations for the reduced FEV_1 in men. The tendency in women is to accumulate generalised body fat, which could be a smoking-related effect on the endocrine hormones in the body.

Increased fat mass provides a focal point for the immunological and metabolic events leading to MeS[487]. It is proposed that increased enzyme aromatase activity results in reduced testosterone and increased estradiol concentration resulting in decrease in muscle mass and increase in total fat mass in elderly men and this increase in fat mass contributes to oxidative stress, inflammation and increase in aromatise levels which will be responsible for the development of MeS[488]. Another hypothesis is that increased visceral fat releases higher amounts of non-esterified fatty acids which promote the development of dyslipidemia and in turn MeS[489].

Insulin resistance is one of the key features, which drives features of MeS. Smoking is associated with a continuous inflammatory response and Bolton *et al* found that insulin resistance was linked to circulating IL-6[490] in COPD patients. Fasting insulin was measured in my study and found that it was significantly elevated in smokers with MeS. It was also noted that there was an increase in insulin resistance in smokers with MeS. Surprisingly similar results were found in non-smoker controls with MeS. It is not clear from my study that

insulin resistance in smokers was due to smoking alone or with other associated factors for e.g. diet and physical activity. Hypoxemia induces glucose intolerance in healthy individuals, however, the results are conflicting in COPD and other severe diseases[491-493]. Since physical inactivity and excess weight are the main underlying contributors to the development of metabolic syndrome, getting more exercise and losing weight can help reduce or prevent the complications associated with this condition. Increased activity alone can improve insulin levels. A brisk 30-minute walk a day can result in weight loss, improved blood pressure, improved cholesterol levels, and a reduced risk of developing diabetes. Physical activity and dietary management are the first line therapies in overweight and obese patients with MeS. The major benefits of these life style changes are improvement in dyslipidemia, insulin sensitivity, glucose tolerance, and blood pressure[494]. These life change modification is very important as I discovered that smokers have poor quality of life. It was shown in my study that women who smoke have a worse quality of life score. It was also shown that it does not matter whether they have MeS or not. Women in my study were shown to be less physically active compared to men.

There is no specific drug so far indicated for the treatment of the metabolic syndrome. This is due, in part, to lack of understanding of pathogenesis, the heterogeneity of diagnostic criteria and uncertainty of which one to treat first. Therefore, the current approach is to correct the individual risk factors for e.g. smoking cessation. In the XENDOS trial, the use of orlistat (an intestinal lipase inhibitor indicated for the treatment of obesity) plus lifestyle modification reduced the incidence of type 2 diabetes in obese patients by 37% more than lifestyle alone[495]. In addition to antihypertensive agents, established therapies include those directed towards individual aspects of metabolic syndrome like the statins to reduce the cardio vascular morbidity and mortality in patients with type 2 diabetes or other components of metabolic syndrome[496]. The use of fibrates which are peroxisome proliferators activated receptor gamma agonists, reduce major coronary events in patients with low HDL cholesterol levels[497], these fibrates also improve insulin sensitivity, thereby improving glucose utilization.

In the second part of my study, I measured the annual rate of decline in smokers and COPD. The aim of the study was to understand and determine the independent predictors of FEV_1 decline. FEV_1 is a widely used outcome measure of disease severity. The natural history of COPD is characterised by an

accelerated decline in FEV_1[53]. The classification of severity of COPD is based on FEV_1 .The one and only proven intervention to reduce the rate of decline is by smoking cessation[498]. There is a normal decline of 30ml//year in a healthy individual, however in susceptible smokers it is 60ml/year[499]. A number of predictors of lung function have been studied including lower respiratory illness[500] and low starting Kco[501]. The rate of decline is also associated with airway reactivity, exacerbations, and inflammation[499]. In my study sputum neutrophils (both percentage and absolute neutrophil) counts independently predicted FEV_1 decline but not any change in TLco. I examined various clinical and pathological variables, which could predict the decline. The first variable was age and the linear regression analysis showed that age did not independently predict the rate of decline. Smoking pack years was shown in the past to be associated with decline, however, in my study it did not independently predict the decline. HRCT and densitometry tools were used to analyse the extent of lung damage. The percentage of lung volume measured by HRCT also did not predict the decline.

Cross sectional and longitudinal studies have also been performed to evaluate the effects of smoking and smoking cessation on age related FEV_1 decline[502] and smokers have been shown to decline more rapidly than non-smokers. Men who quit smoking had reduced rate of decline than continued smokers.

Study limitations

1. The subjects were mostly recruited in and around city of Southampton. The study took place at the Southampton General Hospital, which is located in an impoverished area of the city. The socioeconomic status was not documented, however I believe they represented the general population. It is possible that a large proportion of the subjects came from the local community. This could introduce a potential bias to the findings of the study.

2. All COPD smokers were mild to moderate in severity and they were stable at the time of the recruitment. The reason for recruiting mild to moderate COPD subjects was because of the reason that co morbidities is associated with severe and very severe disease which could be clinically difficult to interpret whether diagnose of features of MeS is due to smoking/COPD or due to the influence of other co-morbid conditions like cardiovascular abnormalities. This will not enable us to understand what exact happens in an uncontrolled environment. It was difficult to recruite subjects with severe disease as most of them were on inhaled corticosteroids or oral prednisolone and other medications which could influence the outcome of the study.

3. The other limitation was that the study was slightly underpowered. With more numbers in the study group could shed more light on to the prevalence of MeS in COPD and the role of inflammatory markers in MeS. The Physical activity questionnaire measured was subjective and I feel that better measurement should be used to explore the activity levels and energy expenditure as this plays an important role on lung function and on the features of MeS. The future study should include socio-economic status, dietary factors, and intake of alcohol which are known to contribute to obesity in health and disease which I have not documented in my study.

8.1. Future work

My study demonstrated that smoking is a major factor for the development of MeS, however MeS does not per se cause or occur has a consequence of COPD. The prevalence of MeS was high in COPD subjects, however it was not statistically significant compared to healthy smokers. It may be worth visiting this issue in a larger cohort of subjects with COPD. I was unable to identify a set of inflammatory mediators which could explain the pathogenesis of MeS in smokers. Future studies should explore this in sputum and in biopsies of smokers with MeS. I have also shown that gender differences are important as they showed many differences in the disease process. Hence, future work should try to seek an explanation in a larger cohort for this at a pathophysiological level and understand the interactions between arterial disease, insulin resistance, blood pressure, lipids, and glucose at the molecular level to identify different pathways which could be a target for future drug discovery. My findings have potential clinical implications. No studies have been conducted to see whether smoking cessation results in an improvement in MeS; such studies are, therefore, needed and to understand whether reversal of MeS might exists and it may contribute to this improvement in COPD. However, even in the absence of such data, patients should be strongly encouraged to stop smoking, as this is the only known intervention so far to reduce the progression of COPD. There is also a need to understand other pathways for e.g. oxidative stress in smokers to explore any association between smoking and MeS.

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