The role of adipokines in obesity-associated asthma

By

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Author's declaration

The following tests on patients were carried out by myself and nurses in the asthma clinic at the Royal Brompton Hospital: lung function measurements, exhaled nitric oxide (FE_{NO}) measurement, skin prick testing, methacholine provocation test (PC_{20}), weight, height, waist/hip measurement, bioelectrical impedance analysis (BIA), and skin fold measurements.

Fibreoptic bronchoscopies were performed by me and previous research fellows in the Airways Disease Section at the National Heart and Lung Institute (NHLI), Imperial College London.

The experiments carried out in this PhD were carried out using the latest equipment available to me at the NHLI. The staining of bronchoalveolar lavage cytospins with Oil red O (Chapter 4) and the staining of endobronchial biopsies with perilipin (Chapter 5) was performed by Dr. Jie Zhu. The multiplex assay (Chapter 7) was performed by Dr. Christos Rossios.

Signed……

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Abstract

Obesity is a risk factor for the development of asthma and plays a role in disease control, severity and airway inflammation. The relationship between asthma and adiposity is multifactorial and incorporates *in-utero* conditions, genetic factors, comorbidities and inflammation secondary to excess adipose tissue. Adipocytes produce cytokines, termed adipokines, which play an important role in systemic inflammation and have been correlated to the development of asthma and disease severity. Retrospective analysis of trials using inhaled corticosteroids and an *in vitro* study using alveolar macrophages has associated obesity with corticosteroid insensitivity in asthma.

The first part of this study looked at data taken from a large cohort of well-characterised severe asthmatics and compared patient demographics, disease characteristics, healthcare utilisation and medication according to body mass index (BMI). Lipid laden macrophages (LLMs) in bronchoalveolar lavage fluid (BALF) and perilipin as a marker of lipid droplets in endobronchial biopsies were analysed according to asthma severity and BMI. Finally, peripheral blood mononuclear cells (PBMCs) were used to study the effect of BMI on steroid sensitivity in patients with asthma using an *in vitro* model and the effects of adipokines (leptin, resistin and adiponectin) on the release of pro-inflammatory cytokines and corticosteroid response were assessed.

Severe asthmatics are often overweight (29.3%) or obese (48.3%). Increasing BMI was associated with increasing medication use in terms of short-acting β_2-agonist (SABA) use per day, nebulisers and oral corticosteroid requirements (both maintenance and short burst therapy). In addition, obese severe asthmatics reported greater gastro-oesophageal reflux disease (GORD) and were less likely to be in full

time employment due to their asthma. LLMs are more prevalent in subjects with GORD which may explain the higher lipid laden macrophage index (LLMI) score seen in severe compared to mild/moderate asthmatics. Perilipin was expressed in the airway epithelium and submucosa in approximately half the study population. Greater expression was seen in the submucosa of asthmatic subjects compared to healthy controls. However, no significant correlations were noted in terms of asthma severity or BMI. Leptin, resistin and adiponectin were pro-inflammatory, in terms of CXCL8 release from PBMCs taken from asthmatics and healthy controls. PBMCs taken from severe asthmatics were less responsive to steroid suppression. Adiponectin induced IL-6, IL-10, CCL2, CCL3 and TNF-α release from PBMCs taken from asthmatics and healthy controls and IFN-γ, IL-1RA, CCL2, CCL3 and TNF-α release was suppressed by dexamethasone. Adiponectin induced CCL2 release and demonstrated relative corticosteroid insensitivity at dexamethasone 10^{-7} M in the severe asthma group.

In conclusion, obesity related severe asthma is associated with more symptoms, a significant societal impact and patients are at risk of the many deleterious side effects of corticosteroid use. Leptin, resistin and adiponectin are pro-inflammatory and adiponectin may play an important role in modulating corticosteroid sensitivity.

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Abbreviations

ACT	Asthma control test
ACQ	Asthma control questionnaire
AQLQ	Asthma quality of life questionnaire
ATS	American Thoracic Society
BIA	Bioelectrical impedance analysis
BAL	Bronchoalveolar lavage
BMI	Body Mass Index
BTS	British Thoracic Society
CS	Corticosteroids
DEXA	Dual energy X-ray absorptiometry
ENFUMOSA	European Network For Understanding Mechanisms of Severe Asthma
ERV	Expiratory reserve volume
ERS	European Respiratory Society
ERK	Extracellular signal-regulated kinases
FEV_1	Forced expiratory volume in 1 second
FE_{NO}	Exhaled nitric oxide
FRC	Functional residual capacity
FVC	Forced vital capacity
JNK	c-Jun N-terminal kinase
GINA	Global initiative for Asthma Guideline
GORD	Gastro-oesophageal reflux disease
GR	Glucocorticoid receptor

ICS	Inhaled corticosteroid
IgE	Immunoglobulin E
K_{CO}	Carbon monoxide transfer coefficient
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
MKP	Mitogen-activated protein kinase phosphatase
MRI	Magnetic resonance imaging
MTT	Methylthiazolydiphenyl-tetrazolium bromide
NAEPP	National asthma education and prevention program
NO	Nitric oxide
NF-κB	Nuclear factor-kappa B
OCS	Oral corticosteroids
OGD	Oesophago-gastroduodenoscopy
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PC_{20}	Concentration of methacholine (mg/ml) required to produce a 20% fall in FEV_1 from baseline
PPI	Proton pump inhibitor
RAST	Radioallergosorbent test
RBM	Reticular basement membrane
SABA	Short-acting β_2-adrenergic agonist
SARP	Severe Asthma Research Program
SEM	Standard error of the mean
SPT	Skin prick test

TENOR	The Epidemiology and Natural History of Severe Asthma: Outcomes and Treatment Regimens
TLC	Total lung capacity
TLR	Toll-like receptor
TV	Tidal volume
TNFα	Tumour necrosis factor alpha
WHO	World Health Organisation
WHR	Waist-hip ratio

Chapter 1: Introduction

Chapter 1: Introduction

1.1 Asthma

The Global Initiative for Asthma (GINA, 2015) defined asthma as '*a heterogeneous disease, usually characterised by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable airflow limitation.'*

Asthma represents a spectrum of disorders characterised by airway obstruction. The main features include hyper-responsiveness of the airways to a wide variety of exogenous and endogenous stimuli and a specific pattern of mucosal inflammation involving activated mast cells, eosinophils and T lymphocytes. This is associated with altered responses of structural cells in the airway, including epithelial cells and airway smooth muscle cells (ASMCs) (Dichlberger et al., 2013, B, 2003) leading to changes in the airway wall and remodelling.

Asthma can be divided into extrinsic asthma (atopic), intrinsic asthma (non-atopic), and occupational asthma. Extrinsic asthma is associated with atopy and usually manifests in childhood or early adulthood. Atopy is defined as a genetic predisposition to produce immunoglobulin E (IgE) against common environmental allergens such as grass pollen, animal dander and house dust mite. IgE is a class of antibody, which has only been identified in mammals and is associated with type 1 hypersensitivity reactions and the immune system response to parasitic (helminthic) infection (Amarasekera, 2011). The exact aetiology of intrinsic asthma is unknown. It usually occurs in patients after the age of 40 and is not related to atopy (Reed,

2010). Triggers such as respiratory infections, exercise, stress, air pollution and the inhalation of chemical irritants may cause bronchoconstriction.

An association between asthma and obesity was first reported in adults in the 1980's (Seidell et al., 1986, Negri et al., 1988). The link has been a relatively recent suggestion, underpinned by a marked increase in the prevalence and incidence of both.

1.1.1 An increasing problem

Globally, there are more than 300 million people with asthma and this figure is expected to rise by a further 100 million by 2025 (Masoli et al., 2004). There are approximately 5.4 million people in the UK who are receiving medication for asthma and the number of adults with asthma in the UK has increased by 400,000 since the last audit of UK asthma in 2001 (UK, 2006).

Despite significant advances in therapy over recent years, there are still over 71,000 hospital admissions and 1400 deaths each year in the UK directly attributable to asthma (UK, 2004). It is estimated that approximately 90 % of asthma deaths could have been prevented if the patient, carer or health care professional had acted differently (Asthma UK, 2014). The National Review of Severe Asthma Deaths (NRAD) assessed asthma deaths in the UK over a one year period (Royal College of Physicians, 2014). They found that where body mass index (BMI) data were available, 25% of cases were overweight (BMI 25 to 29.99) and 31% were obese or very obese (BMI ≥30).

There are over 4.1 million GP consultations each year and a GP with 2000 patients will see approximately 85 people with asthma each year - each of these will consult three times on average (Surveys, 1995).

The total cost of asthma to the National Health Service (NHS) is estimated at approximately £1 billion per year, which equates to about 1% of the total NHS budget, with hospital admissions comprising £45.8 million. The costs to the economy include lost productivity accounting for £1.23 billion with additional costs of £161 million in benefits (UK, 2004). A real-world evaluation of the economic costs of persistent asthma in Europe found that the mean total cost per patient was €1,583. This cost ranged from €509 in controlled asthma to €2,281 in uncontrolled disease with increasing BMI and the presence of chronic cough or sputum production significantly increasing the total cost (Accordini et al., 2013).

1.1.2 Severe asthma

The majority of asthmatics can be well controlled using inhaled corticosteroids (ICS) and long-acting β_2-agonists (LABAs). However, approximately 5% have a severe, refractory form of the disease (ATS, 2000). This group of patients is often difficult to treat, require significant attention and utilise 50% of the available NHS resources for asthma. Difficult to control asthma costs the NHS over £680 million a year (UK, 2004) and the average cost per patient with severe asthma is nearly six times that encountered with patients with mild asthma (Serra-Batlles et al., 1998). Furthermore, severe asthmatics are 20 times more likely to be admitted to hospital and 15 times more likely to require access to emergency care (Robinson et al., 2003).

The ERS/ATS (Chung et al., 2014) defined severe asthma as *asthma which requires treatment with guidelines suggested medications for GINA steps 4 – 5 asthma (high dose ICS and LABA or leukotriene modifier/theophylline) for the previous year or systemic CS for ≥50% of the previous year to prevent it from becoming "uncontrolled" or which remains "uncontrolled" despite this therapy.* Uncontrolled asthma is defined as at least one of the following:

1) Poor symptom control: ACQ consistently >1.5, ACT <20 (or "not well controlled" by NAEPP/GINA guidelines)

2) Frequent severe exacerbations: two or more bursts of systemic CS (>3 days each) in the previous year

3) Serious exacerbations: at least one hospitalisation, ICU stay or mechanical ventilation in the previous year

4) Airflow limitation: after appropriate bronchodilator with FEV_1 <80% predicted (in the face of reduced FEV_1/FVC defined as less than the lower limit of normal).

1.2 Obesity

The World Health Organisation (WHO) defines overweight and obesity as abnormal or excessive fat accumulation that presents a risk to health. The WHO has classified obesity as one of the eight principal causes of preventable chronic disease worldwide (WHO, 2005).

Obesity is strongly correlated with an increased risk of cardiovascular disease, stroke, peripheral vascular disease, renal failure, cancer, osteoarthritis, and Type 2 diabetes mellitus (Whitlock et al., 2009, Kopelman, 2000).

The global epidemic of overweight and obesity - "globesity" - is rapidly becoming a major public health problem in many parts of the world. The WHO highlight that approximately 1.6 billion adults (≥15 years) were overweight in 2005 with at least 400 million of these classified as obese. In 2014 this had risen to 1.9 billion (39% of adults ≥18 years) and 600 million (13%) respectively. Their projections for 2015 are that these figures will increase to 2.3 billion and over 700 million, respectively (WHO, 2011).

1.2.1 Obesity in the UK

The prevalence of obesity in England has increased markedly over recent years, placing a major burden on healthcare resources (Office, 2001). Between 1993 and 2009 the proportion of the population who are obese (BMI ≥30) has increased from 13% of men and 16 % of women in 1993 to 22% and 24% in 2009, respectively (figure 1.1). Data from the 2009 Health Survey for England (HSE) revealed that 61.3% of adults (aged 16 or over), and 28.3% of children (aged 2 to 10) in England were overweight or obese. Of these, 23% of adults and 14.4% of children were obese (Health, 2011). The Foresight report, entitled Tackling Obesities: Future Choices project, has predicted that if no action is taken by 2050 approximately 60% of men, 50% of women, and 25 % of children will be obese.

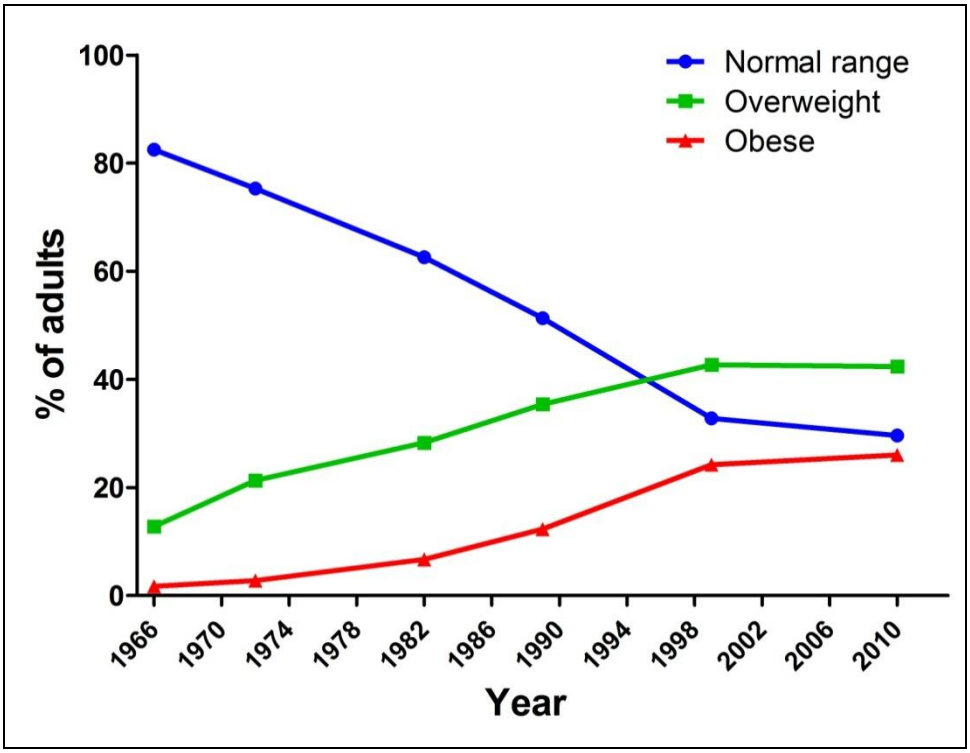


Figure 1.1. Changing prevalence of BMI in the United Kingdom and Northern Ireland (Organisation, 2011).

1.2.2 The impact of obesity

The global obesity epidemic has been referred to as 'arguably the gravest and most poorly controlled public health threat of our time' (Katz, 2005). Adult obesity has roughly doubled since 1980 and it is now a recognised disease and significant risk factor for fatal conditions including ischaemic heart disease, stroke, hypertension, diabetes and some types of cancer (Hughes and McGuire, 1997). The cost of obesity to the NHS is extremely difficult to accurately calculate. Estimates can help establish the economic magnitude of obesity but the overall complexity and associated comorbidities results in problems with validity (Bierl et al., 2013).

The NHS estimates that obesity contributes directly to about 1000 deaths every week. In 2011, 53% of obese men and 44% of obese women suffered with high blood pressure. Compared to 2001/2, hospital admissions relating to obesity rose by over 11 times (11,736 in total) in 2011/12 (NHS, 2013). The National Obesity Observatory (part of Public Health England) estimates that the NHS costs attributable to overweight and obesity are £1 billion and are projected to reach £9.7 billion by 2050. The estimated total wider costs of raised BMI to society were £7.0 billion in 2001 (2.9% of the total NHS spending) and this had increased to £15.8 billion (6%) in 2007. Future predictions show that this figure is set to increase to £37.2 (11.9%) billion by 2025 (Olofsson et al., 2009, Pacheco et al., 2002).

1.3 Asthma and obesity

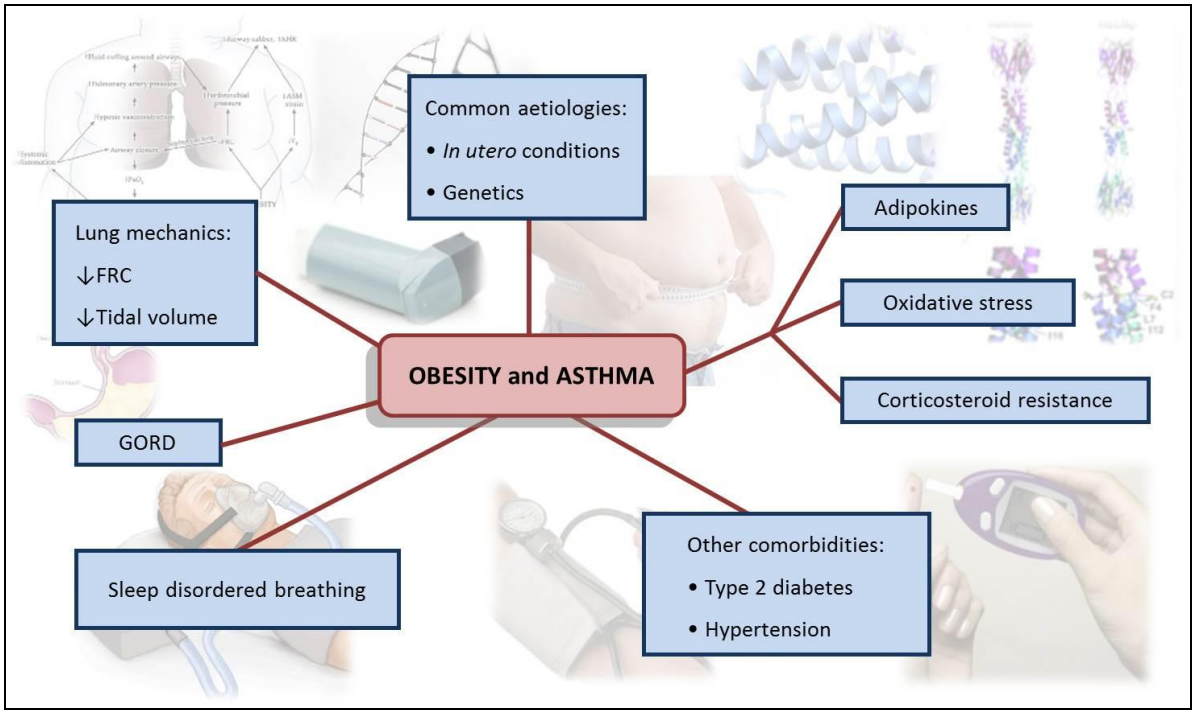
1.3.1 Epidemiology

Over 50 cross-sectional and case-controlled studies have reported on the relationship between asthma and obesity since the 1990's (Lugogo et al., 2010). The vast majority reported a greater prevalence of asthma in the obese. Data from 16 out of 17 prospective studies involving over 200,000 adults and children indicates that obesity antedates a diagnosis of asthma (Shore and Johnston, 2006, Beuther and Sutherland, 2007, Ford, 2005). These studies followed-up several hundred thousand subjects who were initially free of asthma for between two and 21 years. The results indicated that as BMI increases, the relative risk of developing asthma also increases. Additionally, obesity may increase disease severity in subjects who are known to be asthmatic (Akerman et al., 2004, Shore, 2007).

1.3.2 Associations between asthma and obesity

The marked increase in the prevalence and incidence of both asthma and obesity has led to an interest in a potential link between the two. In addition, recent studies have suggested that obese patients with asthma appear to have worse symptoms and a reduced response to their asthma medications (Sutherland et al., 2010, Taylor et al., 2008). Additionally obesity is a risk factor for asthma attacks (Fitzpatrick et al., 2012).

The link between the two appears to be multifactorial and is likely to involve a combination of *in-utero* conditions, genetic factors, comorbidities and inflammation secondary to the excess adipose tissue (figure 1.2) (Shore, 2008).



FRC = functional residual capacity, GORD = gastro-oesophageal reflux disease

Figure 1.2. Proposed mechanisms involved in the relationship between obesity and asthma (Shore, 2008).

A workshop report on obesity and asthma was organised by the American Thoracic Society in May 2010. The expert panel concluded that *there is an urgent need for research to better understand the mechanisms of asthma in the obese, and to develop new therapies specifically targeted to this unique patient population* (Dixon et al., 2010).

1.3.3 Phenotyping asthma and obesity

Asthma can be classified by severity based on a combination of lung function (FEV_1), daytime and nocturnal symptoms, and the frequency of rescue bronchodilator therapy use (Asthma, 2008, Network, 2008, ATS, 2000, Chung et al., 2014). However, this approach fails to take into consideration the heterogeneity of the disease that has been observed in different populations (Wenzel, 2006, Haldar et al., 2008). The identification of specific factors unique to different groups of asthmatics takes into account this heterogeneity and allows a classification system by phenotype. This method of classification may allow targeted management and treatment plans, leading to improved control, reduced exacerbations and potentially avoiding reductions in lung function (Kiley et al., 2007, Wenzel, 2005, Chung and Adcock, 2013).

The Severe Asthma Research Program (SARP) (Moore et al., 2007) was established in 2001 and recruited 800 patients in less than four years. Participants underwent multiple investigations to characterise their asthma. The study set out to determine factors that differentiate severe asthma from those with mild disease. The initial data set revealed that there were differences in the severe asthma phenotype stratified by age of onset with a group of later-onset, less atopic subjects who reported frequent respiratory tract infections (Moore et al., 2007). Cluster analysis

was then applied to the database to identify five unique groups or clusters of individuals with asthma. The third cluster was composed of patients with a higher BMI (58 % with a BMI ≥ 30). This group were mainly older women (71 % female, mean age 50 years) with late onset non-atopic asthma (all older than 23 years, 64 % non-atopic) (Moore et al., 2010).

1.3.4 Lung function in the obese

Obesity leads to a reduction in the elastic properties of the chest wall with an associated reduction in functional residual capacity (FRC). Breathing at lower lung volumes correlates with increased airway responsiveness (Barros et al., 2006). The obese also tend to have lower tidal volumes (TV) and this may lead to a reduced capacity of the bronchodilating effects of tidal volume stretch (Young et al., 2002) (figure 1.3).

In addition, upper airway function is often impaired in obesity. The risk of developing obstructive sleep apnoea (OSA) increases markedly at higher BMIs. This correlation is in part explained by disturbances in the neuromuscular control of the pharyngeal dilatory muscles resulting in a failure to protect the upper airway against high extra-luminal pressures (Horner, 2007) and greater adipose tissue deposition around the soft tissues of the neck and tongue resulting in increased extraluminal pressures (Patil et al., 2007).

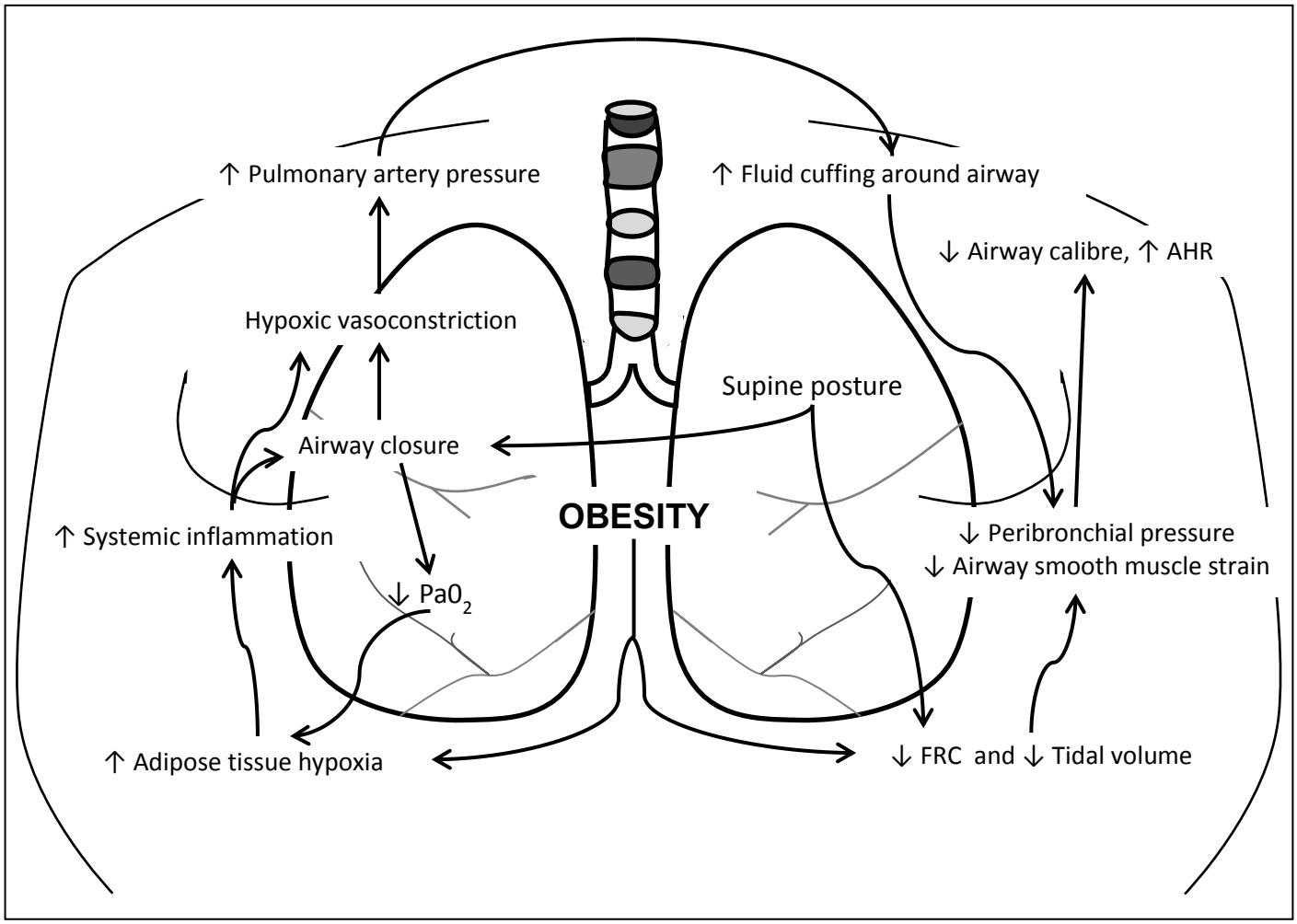


Figure 1.3. Schematic representation showing how changes in lung function in the obese state may lead to asthma symptoms (Shore, 2008). Increasing BMI results in a reduction in functional residual capacity (FRC) and tidal volumes (VT). This leads to decreased airway calibre and increased airway hyper-responsiveness (AHR). Obesity is additionally associated with increased adipose tissue hypoxia (discussed in greater detail in figure 1.4) which contributes to systemic inflammation which in turn causes hypoxic vasoconstriction.

In addition, the combination of obesity with sleep disordered breathing and hypercapnia is well recognised – otherwise known as the obesity-hypoventilation syndrome. There are also links with obstructive sleep apnoea and pulmonary hypertension (with a prevalence of 5 % in people with a body mass index of above 30 kg / m^2) (McQuillan et al., 2001, Mokhlesi and Tulaimat, 2007).

1.3.5 Bariatric Surgery

Studying the effects of bariatric surgery on lung function, markers of lung inflammation, bronchial hyper-responsiveness and medication use can provide an insight into the role of obesity in asthma. Bariatric surgery in obese mild to moderate asthma is associated with a significant reduction in the use of ICS and exhaled nitric oxide as a marker of lung inflammation (Lombardi et al., 2011). In addition weight loss post bariatric surgery has been associated with improvements in asthma control, asthma quality of life and AHR in obese asthmatics (Dixon et al., 2011, Boulet et al., 2012).

1.4 Animal models of obesity and asthma

Murine obesity has been correlated with AHR and inflammation. Different models of obese mice (table 1.1) have been characterised and evaluated; with different phenotypes of obesity exhibiting similar trends with increased AHR and airway inflammation (Shore, 2007).

- ***Ob/Ob mice***

The Ob/Ob mouse is the result of a spontaneous mutation in the leptin gene, which was first noticed in a colony of mice at Jackson Laboratories. Leptin is a satiety hormone (Bluher and Mantzoros, 2015) and these mice eat excessively;

classified as obese after four weeks and by eight weeks they are twice the weight of their wild type controls. The higher body weight is due to high fat and these mice exhibit a low resting metabolic rate, hypothermia, hypoactivity, hyperinsulinaemia, hypertryglyceridaemia, increased serum cortisone, increased cholesterol and triglycerides, and infertility. The repletion of leptin reverses this phenoytype (Shore, 2007).

- ***Db/Db mice***

Db/Db mice are the result of a mutation in the cytoplasmic domain of the long form of the leptin receptor, Ob-Rb (Shore, 2007). This leads to a loss of expression of this isoform and of activation of signal transducer and activator of transcription 3 (STAT-3), an important mediator of the signalling pathway by which leptin manifests its effects on satiety and metabolism. Db/Db mice are similar to Ob/Ob mice and also weigh twice as much as their wild type controls by eight weeks (Shore, 2007).

- ***Cpe^{fat} mice***

Carboxypeptidase E (Cpe) is necessary for the processing of insulin, enkephalins, neurotensin and other neuropeptides (Shore, 2007). Cpe^{fat} mice result from a missense mutation in the enzyme which leads to an inactive protein (due to the replacement of Ser^{202} by Pro^{202}) that is no longer able to cleave and process neuropeptides which mediate eating behaviour and energy expenditure. These mice develop obesity more slowly and weigh 50 % more than wild type controls by 10 weeks and 75 % more by 15 weeks.

- ***Diet induced obesity (DIO)***

Mice fed a lard rich diet where 60 % of their calories are comprised of fat, will become obese. By 20 weeks, their body weight is approximately 25 % greater than their wild type controls and by 32 weeks it is about 45 % greater (Shore, 2007).

Table 1.1. Phenotypes of obese mouse (Shore, 2007).

	Ob/Ob	**Db/Db**	**Cpe^{fat}**	**DIO**
Obesity	Massive	Massive	Moderate	Milder
↑ insulin, cholesterol, sugar	Yes	Yes	Yes	Yes
Small lungs	Yes	Yes	Yes	No
0_3 induced AHR	> Wild type	> Wild type	> Wild type	> Wild type
0_3 induced inflammation	> Wild type	> Wild type	> Wild type	> Wild type

1.5 Adipocytes and inflammation

1.5.1 Adipose tissue, inflammation and the role of adipokines

Obesity and asthma are both characterised by inflammation and a common inflammatory pathway has been suggested as a potential link between the two (Weiss, 2005). In women this link may be stronger given a higher body fat percentage compared to men at any given body weight (Gallagher et al., 1996).

It has become increasingly clear that adipose tissue is not benign and in fact behaves like an endocrine tissue, secreting a wide variety of bioactive cytokines (termed adipokines) into the circulation. These cytokines are produced by the adipocytes themselves and by fat infiltrated immune-inflammatory cells such as macrophages. Visceral obesity produces a chronic, low grade inflammatory state that places the individual at increased risk of the metabolic syndrome, cancer, hypertension, liver disease, type 2 diabetes mellitus and associated cardiovascular complications.

The obese state is characterised by an excess of adipose tissue, which produces a number of pro-inflammatory mediators (table 1.2). Plasma concentrations of IL-6, TNF-α and CRP are significantly raised in the obese state (Festa et al., 2001). Peripheral blood mononuclear cells (PBMCs) from obese subjects are in a proinflammatory state, exhibiting increased nuclear factor-kappa B (NF-κB) binding, a decrease in IκB-β, and an increase in the transcription of proinflammatory genes regulated by NF-κB (Ghanim et al., 2004). Circulating PBMCs in the obese state are exposed to metabolic factors such as dyslipidaemia and inflammatory molecules produced by other organs and tissues. This may lead to a phenotypic shift in macrophages to the more inflammatory classical M1 type (Bories et al., 2012).

Adipose tissue is made up of many different cells. These include mature adipocytes, preadipocytes, mesenchymal cells, stromal cells, macrophages and fibroblasts (Trujillo and Scherer, 2006). The tissue acts as a buffering system for lipids by taking up triacylglycerol (TAG) and inhibiting the release of free fatty acids (FFA) (Mancuso, 2010). In the obese state, adipocytes become saturated with TAG and this leads to an increased circulating level of TAG and FFA. FFAs act as ligands

for Toll-like receptor-4 (TLR4) (Horner, 2007) and this may contribute to increased systemic inflammation.

Table 1.2. Mediators released by adipocytes.

Cytokines	Chemokines	Energy regulating hormones	Acute phase reactants	Other factors
TNF-α	CXCL8	Adiponectin	Serum amyloid A	Acylation stimulating protein
IL-1	CCL2	Leptin	CRP	Angiotensinogen
IL-6	CCL3	Resistin	α1 acid glycoprotein	Complement B, C3 and D
Visfatin	Eotaxin		PAI-1	VEGF
IL-10				IL-1RA
TGFβ				Retinal binding protein 4

1.5.2 Alveolar macrophages, obesity and asthma

Alveolar macrophages (AMs) from overweight and/or obese asthmatics demonstrate enhanced proinflammatory responses (Lugogo et al., 2012). However, the precise mechanism and nature of this inflammation remains unclear.

Lugogo et al performed bronchoscopies and bronchoalveolar lavage (BAL) on 42 asthmatics and 46 healthy control subjects. They found that although BALF differential cell counts were similar in lean and overweight/obese asthmatics (13% of

lean subjects with asthma and 15% of overweight/obese subjects with asthma had a greater than 2% eosinophil count), BALF leptin levels were significantly higher in the overweight/obese female individuals regardless of asthma status. Additional *ex vivo* studies found that AMs taken from overweight/obese asthmatics had an enhanced proinflammatory response to lipopolysaccharide (LPS) and this was enhanced with pre-exposure to leptin; suggesting that leptin may contribute to the pathophysiology of airways disease in the obese state (Lugogo et al., 2012).

A study looking at the effect of bariatric surgery in obese women (Sideleva et al., 2012) found that bronchial epithelial cells taken at baseline and 12 months post-surgery, exhibited greater adiponectin (APN) receptor expression in asthma compared to healthy controls. BALF leptin levels were higher and adiponectin levels lower in the asthmatic group suggesting that both adipokines may be important mediators of airway disease in the obese state.

1.6 Obesity, inflammation and oxidative stress

1.6.1 Obesity and corticosteroid insensitivity

The association between corticosteroid (CS) insensitivity and obesity in asthma is supported by retrospective analysis of clinical trials using ICS and additionally by an *in vitro* study using alveolar macrophages.

Retrospective analysis of four clinical trials using Fluticasone propionate (FP)/Salmeterol and montelukast found an association between BMI and an attenuated response to ICS. Subgroup analysis revealed that improvements in FEV_1, symptom scores, and albuterol use to FP/Salmeterol were significantly greater in the normal and overweight BMI group compared to the obese (Camargo et al., 2010a).

Post hoc analysis of 3073 asthmatics from four double blind placebo controlled studies randomised 894 to receive beclomethasone, 1439 to montelukast and 740 placebo. Analyses was performed according to BMI (BMI <25.0, overweight BMI 25 to 29.99 and obese BMI ≥ 30). The response to ICS decreased with increasing BMI in contrast to montelukast where no difference was seen. In addition, the placebo response for all end points was lower with increasing BMI. This suggests that increasing BMI may influence the response to ICS and the natural history of asthma control (Peters-Golden et al., 2006).

Anderson and Lipworth performed a post hoc analysis of 72 patients with mild to moderate persistent asthma. Patients were divided into two groups (BMI < 25 and ≥ 25) and each group received 4 weeks' treatment with ICS (budesonide 200 μg/day and then 800 μg/day with a washout period). Greater improvements in terms of FE_{NO} and symptom scores were seen in the normal weight group compared to the overweight group for both ICS doses (Anderson and Lipworth, 2012).

An *in vitro* study using PBMCs and BAL cells taken from 45 adults (33 with asthma and 12 healthy subjects) found that dexamethasone (10^{-6} M)-induced MAPK phosphatase (MKP1) suppression was attenuated in mild asthmatics with a BMI ≥25 compared to those <25 (Sutherland et al., 2008a). MKP1 is a signalling pathway that can be used as a marker of the anti-inflammatory action of corticosteroids.

These studies suggest that overweight/obesity is associated with a reduced clinical response to CS therapy in asthma.

1.6.2 Obesity and oxidative stress

Reactive oxygen species (ROS) are oxygen containing molecules that are highly reactive and have been shown to play an important role in the pathogenesis of many pulmonary diseases, as well as many regulatory physiological functions. The effect of ROS depends on the balance between ROS production and the defensive capacity of antioxidant systems (Tkaczyk and Vizek, 2007). Cross-sectional studies have demonstrated increased levels of oxidative stress biomarkers in obese individuals compared to their lean counterparts (Keaney et al., 2003). There are multiple potential sources of increased oxidative stress in obesity, including increased adiposity, co-morbidities or behavioural changes associated with being obese (Holguin and Fitzpatrick, 2010). The systemic inflammation and oxidative stress secondary to adipose tissue may be due to the leptin/adiponectin ratio and other adipokines such as TNF and plasminogen activator inhibitor (PAI-1) (Wu et al., 2009). Chronic oxidative stress in obese subjects has a cumulative effect and may lead to end organ damage. This has been noted in the cardiovascular system through atherosclerosis and in the liver as non-alcoholic hepatic steatosis (Medoff et al., 2009). Weight gain, diabetes and poor glycaemic control are associated with reduced lung function and steeper airway function decline which raises the possibility that obesity-mediated oxidative stress may directly affect the lungs. However, the precise nature of this association remains unclear (Holguin and Fitzpatrick, 2010).

Asthmatic patients have increased systemic oxidative stress compared to non-asthmatics (Bruno et al., 2009). Acute asthma attacks raise the oxidative burden and studies have demonstrated that patients hospitalised for acute asthma exacerbations have a six-fold increase in the levels of urinary metabolites of F2-isoprostanes and total plasma antioxidant capacity is reduced during these episodes

(Filkova et al., 2009). In addition, higher levels of oxidative stress have been noted in asthmatics with greater airways obstruction (Miller et al., 2005).

1.6.3 Adipocytes and inflammation

Adipose tissue acts as a buffering system for lipids by taking up TAG and FFAs. In the obese state adipocytes hypertrophy and may become saturated with TAG leading to increased circulating levels of both TAG and FFAs. FFAs act as a ligand for TLR4 receptors and may contribute to systemic inflammation in this way (figure 1.4). Adipocytes additionally produce adipocytokines, the majority of which are pro-inflammatory. Adipose tissue in the obese state becomes infiltrated with macrophages which correlate with both adipocyte size and BMI. These macrophages aggregate in crown like structures that surround dead adipocytes and scavenging debris.

With the development of obesity, adipocytes hypertrophy. These larger cells outstrip their blood supply and this leads to localised hypoxia and apoptosis. The resultant cellular debris leads to the production of cytokines such as monocyte chemoattractant protein-1. In turn, this results in the recruitment of macrophages and T cells from the peripheral circulation (Beuther et al., 2007). Recruited macrophages produce TNF-α, IL-6 and other cytokines which inhibit the differentiation of preadipocytes and preventing the further uptake of TAG. Existing adipocytes continue to hypertrophy and eventually undergo apoptosis, thereby creating a cycle resulting in increasing macrophage recruitment and cytokine production. These cytokines then spill out into the peripheral circulation thereby contributing to the chronic inflammation seen in obesity (Ford et al., 2005).

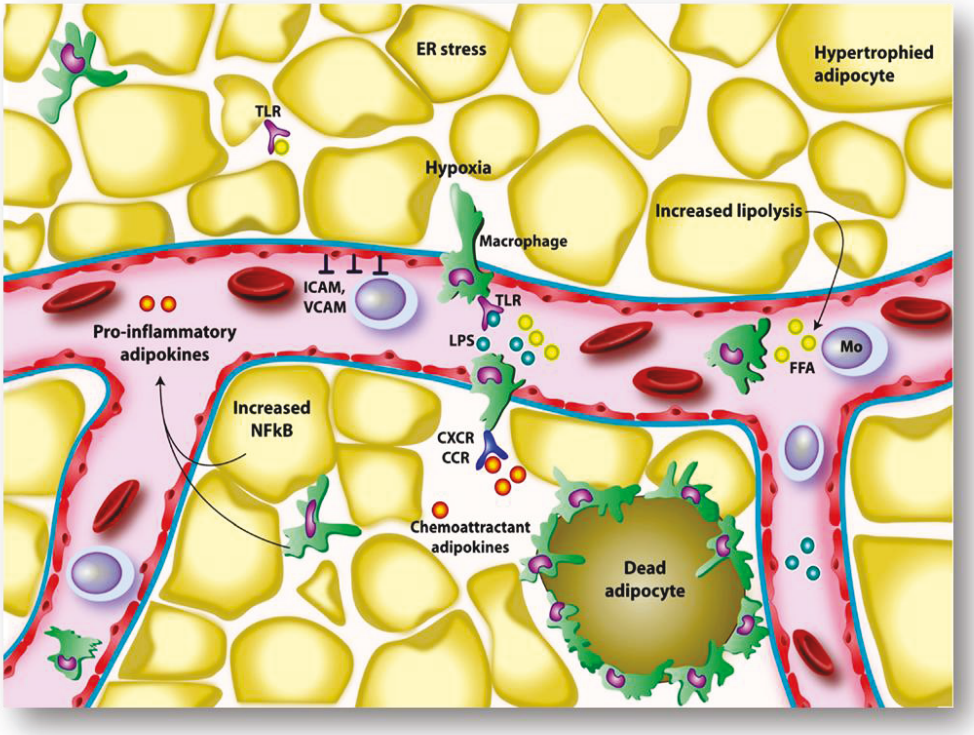


Figure 1.4. Potential mechanisms leading to inflammation in obesity. As adipocytes hypertrophy they outstrip their blood supply leading to localised hypoxia and apoptosis. This results in pro-inflammatory cytokine production and the recruitment of macrophages and T cells from the peripheral circulation. In addition, hypertrophied adipocytes become saturated with triacylglycerol (TAG) and result in raised levels of circulating TAG and free fatty acids (FFAs). FFAs are a ligand for TLR4 and results in a systemic inflammatory response. Adipocytes produce pro-inflammatory adipocytokines such as TNF-α, IL-6, CXCL8, leptin, resistin and adiponectin (Horner, 2007).

1.7 Adipokines: Leptin, adiponectin and resistin

Adipokines are cytokines produced by adipocytes (table 1.2). Many adipokines correlate to the development of asthma and play a role in its severity. Leptin, adiponectin and resistin are adipokines are of particular interest (Sood and Shore, 2013).

1.7.1 Leptin

Leptin is a 16 kDa adipokine that was first identified in 1994 and noted to play a vital role in the regulation of food intake (satiety) and body weight (Zhang et al., 1994). It is pro-inflammatory and serum levels are positively correlated with obesity (Considine et al., 1996). In healthy humans circulating levels are usually below 10 ng/ml, rising to between 10 to 60 ng/ml in obese individuals (Licinio et al., 1997). Obese individuals seem to lose the inhibitory activity that leptin has on food intake and satiety (Iikuni et al., 2008).

Leptin is also a regulator of energy homeostasis, insulin secretion, angiogenesis, bone formation and reproduction (Morton et al., 2006, Emilsson et al., 1997, Sierra-Honigmann et al., 1998, Ducy et al., 2000, Chehab et al., 1996). Decreased plasma leptin levels are linked to impaired immune function (La and Matarese, 2004). The administration of inflammatory stimuli such as LPS, or experimental acute inflammation, results in increased levels of leptin expression in both serum and adipose tissue (Faggioni et al., 1998, Iikuni et al., 2008).

The structure of leptin is similar to that of the IL-6 cytokine family. In mice, six different receptor isoforms (OB-Ra, OB-Rb, Ob-Rc, OB-Rd, OB-Re and Ob-Rf), derived from alternative splicing of RNA transcripts of the *db* gene, have been

identified (Wang et al., 1996). In humans, only OB-Ra, OB-Rb and OB-Rc have been reported (Chua et al., 1997). OB-Rb appears to be the most important and is involved with the satiety effects of leptin and is expressed in high levels in the hypothalamus and at lower levels in many peripheral tissues (De et al., 2007b, Kielar et al., 1998). OB-Rb can also be found on immune cells including sub-populations of T cells, B cells, dendritic cells, monocytes, neutrophils, macrophages, and natural killer (NK) cells (Iikuni et al., 2008).

Once bound to its receptor, leptin activates Janus kinase 2 (JAK2), which becomes autophosphorylated. Subsequent phosphorylation of tyrosine residues on the intracellular domain of the receptor upregulates the binding and activation of signal transducers and activators of transcription (STAT) – including STAT1, STAT3, STAT5, and STAT6. Phosphorylated JAK2 activates other pathways such as mitogen-activated-protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) and Akt (Martin-Romero and Sanchez-Margalet, 2001) (figure 1.5).

PBMCs incubated in the presence of leptin have been shown to phosphorylate both ERK-1 and ERK-2 in a concentration dependent fashion (Martin-Romero and Sanchez-Margalet, 2001). Leptin additionally leads to sustained phosphorylation of p38 MAPK in human PBMCs (Procaccini et al., 2009).

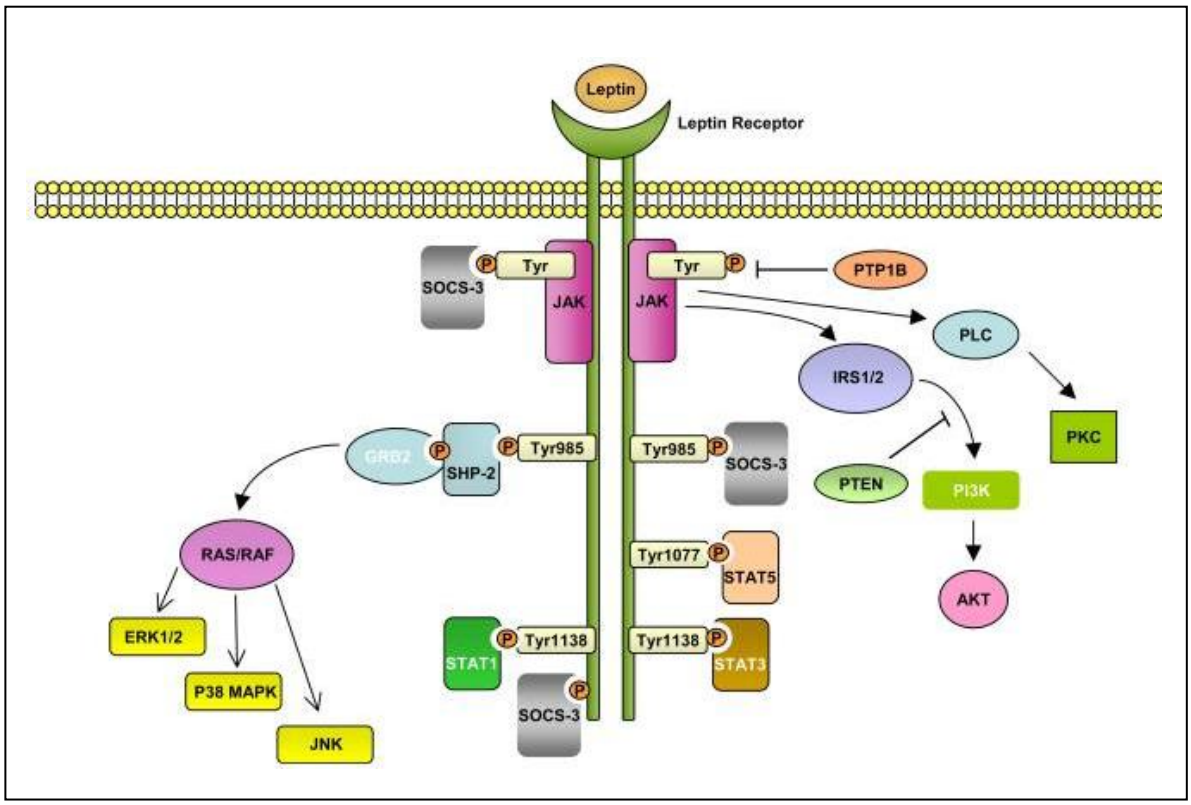


Figure 1.5: The pathways influenced by leptin. Leptin binds to the leptin receptor (OB-Rb), activating Jak2 and resulting in the autophosphorylation of tyrosine residues and phosphorylation of tyrosines. Autophosphorylated Jak2 can phosphorylate insulin receptor substrate 1/2 (IRS1/2) resulting in activation of phosphatidylinositol 3-kinase (PI3K)/Akt pathway. NF-κB activation can occur via Akt activation. Leptin binding to OB-Rb can also activate phospholipase C (PLC) resulting in stimulation of c-jun N-terminal protein kinase (JNK) via protein kinase C (PKC). Tyr^{1077} and Tyr^{1138} bind to STAT5. Tyr^{1138} additionally recruits STAT1 and STAT3. Tyr^{985} binds to the SH2 domain-containing phosphatase 2 (SHP2) resulting in activation of mitogen-activated protein kinase (MAPK) pathways including ERK 1/2, p38 MAPK and JNK (Procaccini et al., 2009).

1.7.2 Leptin and asthma

Shore and colleagues used mini-Alzet pumps to increase blood leptin levels during allergen challenge of sensitised mice (Shore et al., 2005). Leptin augmented the AHR but did not appear to alter eosinophil influx or T_H2 cytokine expression. This suggests that leptin is able to augment AHR through mechanisms that are independent of T_H2 mediated inflammation. The authors also reported that exogenous administration of leptin to lean mice increased their acute ozone induced inflammatory response (Shore et al., 2003).

An *in vitro* study using bronchial epithelial cells from asthmatic subjects found that TGF-β expression of leptin and leptin receptors were significantly lower in mild uncontrolled untreated asthmatics and in severe uncontrolled asthmatics than in healthy controls. These lower levels suggest a dysregulation in the leptin/leptin receptor pathway linked to asthma control and bronchial inflammation. In addition, TGF-β expression was inversely correlated with leptin and leptin receptor expression and positively correlated with reticular basement membrane (RBM) thickness but only when severe uncontrolled asthmatics on ICS and oral steroids were excluded (Bruno et al., 2009).

Serum leptin concentrations are higher in asthmatics compared to healthy controls even after adjusting for BMI (Gurkan et al., 2004). This association appears to be stronger in atopic boys (Guler N. Leptin- does it have any role in childhood asthma? JACI 2004). A raised serum leptin concentration has also been correlated with an increased risk of developing asthma. In women, a serum leptin concentration of >21.9 ng/ml was associated with an increased risk of developing asthma (Sood et

al., 2006). However, other studies have failed to confirm this finding (Jartti et al., 2009, Sutherland et al., 2009).

Sputum leptin concentrations appear to be higher in asthmatics than healthy controls but BMI may be a significant confounding factor (Lessard et al., 2011). However, other studies have not found a correlation between leptin concentrations in asthma in BAL or serum, even after adjusting for obesity (Holguin F, 2011, Sideleva et al., 2012, Jang et al., 2009). There may be an association between serum leptin concentrations and asthma severity. A small case-controlled study found high serum levels were present in women with severe asthma compared to mild/moderate disease. However, BMI values were significantly different between the groups (Tsaroucha et al., 2013). In contrast, another study found no association between serum or BAL leptin concentrations and lung inflammatory biomarkers (Holguin F, 2011).

Overall, there appears to be a stronger correlation between leptin (serum, sputum and BAL) and asthma in children than there does in adults. This may reflect a number of factors such as specific childhood asthma phenotypes, atopic status and the fact that leptin concentrations in children are more reflective of BMI than in adults due to the modifying effects of sex hormones (Sood and Shore, 2013).

1.7.3 Adiponectin

Adiponectin, also known as Acrp30, AdipoQ, APM1, and GBP28, was initially described as an abundant serum protein produced by adipocytes (Scherer et al., 1995, Hu et al., 1996, Nakano et al., 1996). It is a 247-amino acid, 30 kDa adipokine that is produced predominantly by adipocytes and is the most abundant adipokine, accounting for 0.01% of total plasma protein (Hu et al., 1996), circulating in the plasma at concentrations of between 3 and 30 μg/ml (Folco et al., 2009). There are three major oligomeric complexes (including trimeric, hexameric, and high molecular mass form) with distinct biological functions (Okamoto et al., 2006).

In humans, adiponectin is negatively correlated with increasing obesity and levels increase following weight loss (Kern et al., 2003). Furthermore, lower serum adiponectin levels are found in patients with Type 2 Diabetes, the metabolic syndrome and cardiovascular disease compared to healthy controls, matched by BMI (Lara-Castro et al., 2007). Although white adipose tissue is the main site of adiponectin secretion, other cell types are able to produce small quantities. These include brown adipocytes, colonic mucosal cells, fetal tissue (Corbetta et al., 2005), salivary gland epithelial cells (Katsiougiannis et al., 2006), osteoblasts (Berner et al., 2004), myocytes (Pineiro et al., 2005) and myofibroblasts (Matsunaga et al., 2008).

1.7.4 Adiponectin isoforms

Adiponectin contains four different domains based on its primary sequence: an N-terminal signal peptide, a short-hyper variable region, a collagen domain, and a C-terminal globular domain (Sun et al., 2009). There are six isoforms: globular adiponectin (gAPN), full length adiponectin (fAPN), low molecular weight adiponectin (LMW), medium molecular weight adiponectin (MMW), high molecular weight

adiponectin (HMW), and the serum albumin bound LMW form (Alb-LMW). In plasma, adiponectin circulates in LMW and HMW isoforms (Bozza et al., 1997).

fAPN is also known as monomeric adiponectin. Three monomers, via hydrophobic interactions within globular domains, can form a homotrimer/trimeric/LMW form with a molecular weight of about 75-90 kDa (Sun et al., 2009). MMW or hexameric adiponectin is made up from two LMW trimmers by a disulphide bond and the HMW isoform (400 to 600 kDa) is composed of eight or more monomers or two/three hexamers by noncovalent bonds (Giannessi et al., 2007). fAPN may be proteolytically cleaved to a smaller form, globular adiponectin, by leukocyte elastase (Waki et al., 2005). In comparison to other adiponectin isoforms, gAPN is present in small amounts in plasma (Fruebis et al., 2001) and appears to be more extensively biologically active (Nishida et al., 2007). Furthermore, the HMW isoforms have been shown to be more active than LMW isoforms (Song et al., 2009, Pajvani et al., 2003).

1.7.5 Adiponectin receptors

Three adiponectin receptors have been identified: AdipoR1, AdipoR2 and T-Cadherin. (Takemura et al., 2007, Hug et al., 2004, Yamauchi et al., 2007). These receptors are expressed in multiple cell types, including bronchial epithelial cells but their functional role in respiratory disease remains poorly understood (Hug et al., 2004). More recently, calreticulin has been identified as a fourth adiponectin binding protein. Calreticulin is a ubiquitous calcium binding protein of the endoplasmic reticulum (ER) that may provide protection from systemic inflammation through binding adiponectin by promoting the clearance of early apoptotic cells by macrophages (Takemura et al., 2007).

AdipoR1 is present on approximately 1% of T cells, 93% of monocytes, 47% of B cells and 21% of NK cells (Pang and Narendran, 2008). AdipoR2 distribution is similar to AdipoR1 with 2%, 87%, 36% and 19% respectively. AdipoR1 is more prevalent on monocytes but expression decreases following differentiation into mature macrophages whilst AdipoR2 expression remains constant and becomes the predominant adiponectin receptor (Chinetti et al., 2004).

Globular adiponectin primarily binds to AdipoR1 whilst multimeric adiponectin binds to AdipoR2 (Arima and Fukuda, 2011). The expression of AdipoR2 and T-cadherin mRNA is greater in bronchial epithelial cells taken from obese asthmatics in comparison to obese healthy controls (Sideleva et al., 2012).

1.7.6 Adiponectin: cellular action

Adiponectin stimulates AMP-activated protein kinase (AMPK) and this is implicated in its insulin-sensitising properties in liver and muscle (Nawrocki et al., 2006, Yamauchi et al., 2002, Kadowaki and Yamauchi, 2011). The receptors AdipoR1 and R2 regulate glucose and fatty acid metabolism partly through the activation of AMPK, Ca^{2+} and peroxisome proliferator-activated receptor alpha (PPARα) signalling pathways (table 1.6). However, this does not explain the pleiotropic effects of adiponectin. More recently, adiponectin has been shown to decrease cellular ceramide levels through activation of AdipoR1 and R2 and independently of AMPK. Ceramide is a sphingolipid and increased levels have been correlated with atherosclerosis, insulin resistance and heart failure. (Holland et al., 2011).

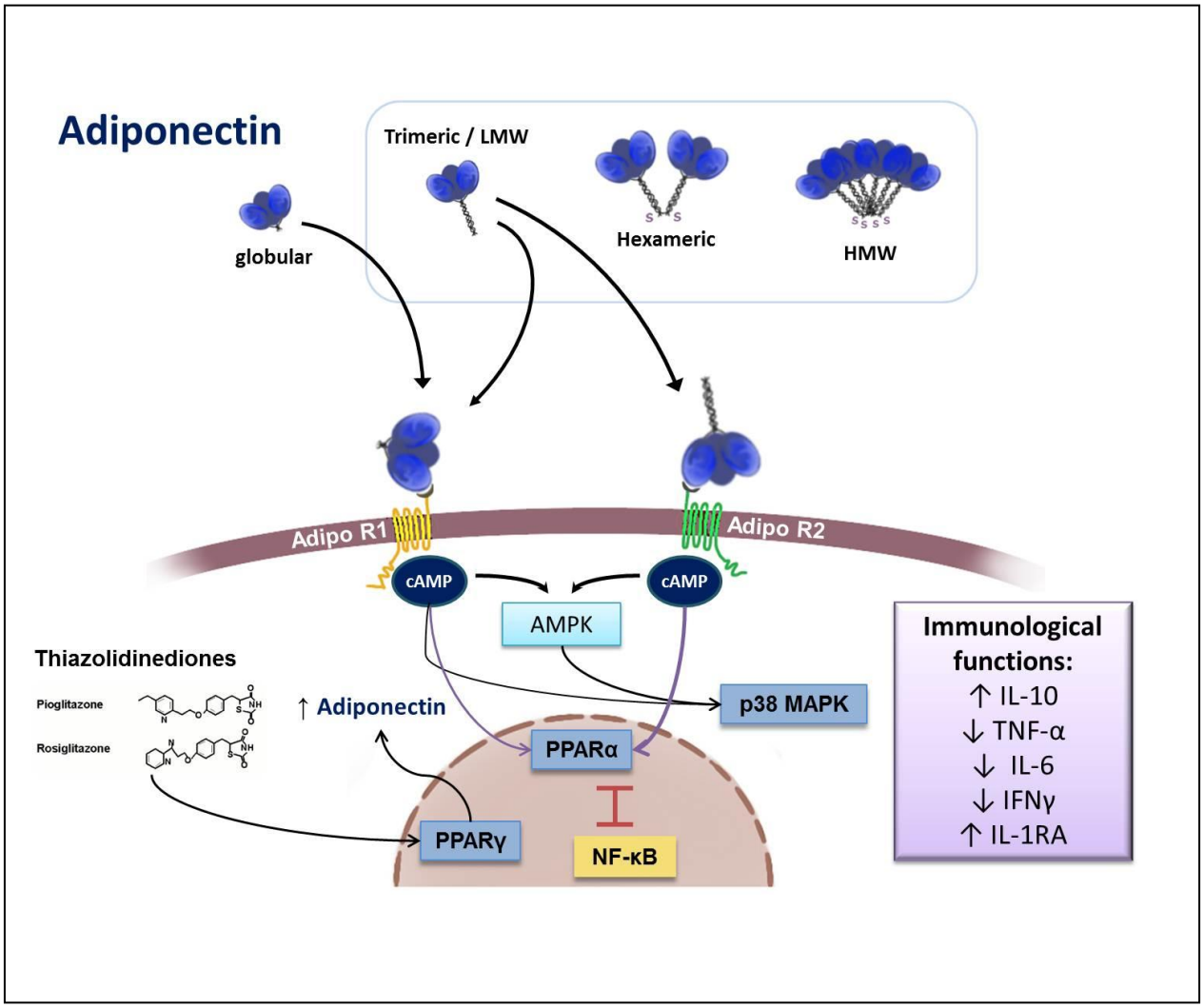


Figure 1.6: Schematic diagram of the pathways influenced by adiponectin. Adiponectin interacts with AdipoR1 and AdipoR2 resulting in the stimulation of peroxisome-proliferator-activated receptor-α (PPARα), AMP-activated protein kinase (AMPK) and p38 mitogen-activated protein kinase (p38 MAPK). Adiponectin's anti-inflammatory actions relate to suppression of the synthesis of tumour-necrosis factor (TNF) and interferon-γ (IFN-γ) and via increased production of interleukin-10 (IL-10) and IL-1 receptor antagonist (IL-1RA). PPARγ activation results in an anti-inflammatory action via inhibition of the transcriptional activation of pro-inflammatory response genes.

1.7.7 Adiponectin and asthma

In a mouse model of acute asthma, adiponectin infusion attenuated airway inflammation and airway hyperresponsiveness (Shore et al., 2006). APN-deficient mice develop spontaneous emphysema and their lung macrophages express higher levels of inflammatory mediators compared to their wild type control group (Summer et al., 2008). Male apolipoprotein E deficient mice fed a high fat diet developed pulmonary hypertension associated with lower APN levels compared to wild type mice. Subsequent treatment with rosiglitazone, a proliferator-activated receptor-γ activator, resulted in raised APN level and complete regression of pulmonary hypertension and pulmonary artery remodelling (Hansmann et al., 2007).

Mice acutely challenged with ovalbumin demonstrate decreased serum levels of APN (Shore et al., 2006) and APN (-/-) mice display more eosinophilic airway inflammation in a chronic model of asthma (Medoff et al., 2009). These findings suggest that adiponectin plays a role in the development and severity of asthma.

T-cadherin (-/-) mice are protected from OVA-induced AHR, increases in BAL inflammatory cells, and induction of IL-13, IL-17 and eotaxin expression. Serum adiponectin levels were higher in the T-cad (-/-) mice, possibly due to a lack of sequestering of adiponectin by endothelial T-cad, and the reduction in AHR may be due to adiponectin acting on alternative signalling pathways (Williams et al., 2012).

In humans, a low total serum adiponectin concentration has been correlated with a greater risk of developing asthma. One study found that a low serum adiponectin predicted asthma in women more strongly than BMI (Sood et al., 2012). Conversely, the same study noted that prevalent asthma did not predict a low future adiponectin concentration. Other studies have noted that low serum adiponectin was

associated with a greater risk of developing asthma in premenopausal women and prepubertal girls (Sood et al., 2008, Nagel et al., 2009). In contrast, other studies have failed to identify a correlation (Kim et al., 2008, Jartti et al., 2009, Sutherland et al., 2009).

Sputum adiponectin concentrations are lower in asthmatics compared to healthy controls (Sood, 2012) and appear to predict the presence of asthma better than serum adiponectin. Other studies have failed to find a difference between BAL adiponectin concentrations between asthmatic patients and healthy controls despite matching or adjusting for BMI (Holguin F, 2011, Jang et al., 2009). A proposed explanation for this difference is that sputum adiponectin concentrations are approximately 40 times more concentrated than that in BAL (Sood and Shore, 2013). With regard to asthma severity, high serum adiponectin concentrations have been correlated with more frequent asthma medication use and higher symptoms in men. Conversely, the same study found beneficial effects in women (Sood et al., 2011).

1.7.8 Adiponectin: inflammatory action

The cellular actions and *in vivo* studies discussed above support the view that adiponectin is an anti-inflammatory cytokine. However, there are some disease states which are associated with raised adiponectin levels. These include rheumatoid arthritis, systemic lupus erythematosis (SLE), type 1 diabetes and inflammatory bowel disease. In these disease states there is a paradox of high levels of adiponectin in the presence of systemic inflammation (Fantuzzi, 2008). Potential explanations for this include the observations that adiponectin isoforms may exert different inflammatory effects and the suggestion that the ratio of different isoforms, such as HMW to total adiponectin, may determine cellular effects (Pajvani et al., 2004).

1.7.8.1 The anti-inflammatory role of adiponectin

In humans, pre-treatment of monocyte-derived macrophages (MDMs) with 30 μg/ml (the upper range of circulating plasma concentrations) adiponectin for 48 hours has been shown to increase tissue inhibitor of metalloproteinase (TIMP) -1 mRNA levels. In addition, the MDMs exhibited increased IL-10 mRNA expression within six hours with a significant increase in IL-10 protein secretion within 24 hours. Pre-treatment of the MDMs with an anti-IL-10 monoclonal antibody indicated that the effect on TIMP-1 appeared to be due to the upregulation of IL-10 (Kumada et al., 2004). Globular adiponectin suppresses TLR4-mediated signalling via IL-10 expression in RAW 264.7 macrophages via a cAMP-dependent pathway and transcriptional and posttranscriptional control of TNF-α expression (Park et al., 2007, Wolf et al., 2004).

The pre-treatment of human macrophages with 10 μg/ml of adiponectin suppressed the LPS induced IκB, JNK and p38 MAPK phosphorylation. In addition, adiponectin inhibited the TNF-α induced IκB, JNK, and p38 MAPK phosphorylation as well as IL-6 induced STAT-3 phosphorylation. These anti-inflammatory effects appear to be independent of IL-10 (Folco et al., 2009).

It has been suggested that macrophages from adipose tissue in lean individuals express markers of M2 macrophages whilst obesity leads to a reduction in these markers and an increase of genes associated with the pro-inflammatory M1 macrophages (Lumeng et al., 2007). Peritoneal macrophages from adiponectin knock-out mice display increased M1 markers and decreased M2 markers. Adiponectin appears to stimulate the expression of M2 markers in human monocyte derived macrophages and regulate macrophage polarisation towards an anti-inflammatory phenotype (Ohashi et al., 2010).

Adiponectin inhibits LPS-induced NF-κB activation and IL-6 production and increases PPARγ2 expression in adipocytes (Ajuwon and Spurlock, 2005). Furthermore, adiponectin attenuates TNF-α and IL-6 production by activated porcine macrophages via suppression of NFκB and ERK1/2 activity (Wulster-Radcliffe et al., 2004). In human aortic endothelial cells (HAEC) adiponectin inhibits TNF-α induced CXCL8 synthesis via PKA dependent NFκB signalling (Kobashi et al., 2005).

1.7.8.2 The pro-inflammatory role of adiponectin

Adiponectin is a potent pro-inflammatory agent for a number of different cell types. Human placenta and adipose tissue release IL-1β, IL-6, TNF-α, and prostaglandin E_2 (PGE_2) in the presence of adiponectin (Lappas et al., 2005). Globular APN induces the release of TNF-α and IL-6 from primary human peripheral

macrophages, in THP-1 human macrophage cell line, and in primary mouse peritoneal macrophages. However, pre-treatment with low dose adiponectin (1 μg/ml) for 24 hours prior to re-stimulation led to tolerance and suppression of TNF-α and IL-6 release. This suggests that globular APN may induce macrophage tolerance although this effect was not seen with higher concentrations of adiponectin (10 μg/ml) (Tsatsanis et al., 2005). Microarray analysis of cDNA from the RNA prepared from human umbilical vein endothelial cells (HUVECs) treated with globular adiponectin (10 μg/ml) using gene expression analysis has revealed that adiponectin increased the expression of 187 genes, including intercellular adhesion molecule-1, E-selectin, vascular cell adhesion molecule-1, and monocyte chemoattractant protein-1. This gene expression is dependent on NF-κB activation which is induced by globular adiponectin (Hattori et al., 2006).

Stromal preadipocytes exposed to adiponectin released PGE_2 and expressed cyclooxygenase-2 (COX-2) (Yokota et al., 2002). Adiponectin induces CXCL8 secretion from human chondrocytes (Gomez et al., 2011).

Adiponectin has been shown to exert a pro-inflammatory effect on human placenta and adipose tissue through the release of proinflammatory cytokines (Lappas et al., 2005). Treatment with the anti-inflammatory ERK1/2 MAPK inhibitor, U0126, BAY 11-7082, and troglitazone (a PPARγ ligand), all suppressed adiponectin (and leptin) induced IL-1, IL-6 and TNFα release from human placenta cells. This suggests that NF-κB, PPARγ, ERK 1/2 and MAPK all appear to be key transcription factors involved in the adiponectin induced pro-inflammatory response.

There are disease states in which adiponectin levels are raised. In SLE, patients with acute lupus nephritis, urine HMW adiponectin levels are raised (Song et

al., 2009) suggesting that HMW adiponectin contributes to the renal inflammation of SLE. In rheumatoid arthritis, serum adiponectin levels are raised and may even correlate to disease severity (Tan et al., 2009, Chen et al., 2012). Adiponectin levels are significantly higher in synovial fluid of patients with osteoarthritis and rheumatoid arthritis and increases the secretion of MMP-3, which may contribute to the breakdown of articular cartilage, in cultured human chondrocytes. These actions were inhibited by the pre-treatment with AMPK inhibitor (compound C), p38 MAPK inhibitor (SB203580), and NF-κB inhibitor (PDTC and TPCK) (Tong et al., 2011). Adiponectin induces phosphorylation of AMPK (Kang et al., 2010), JNK, ERK1/2 (Kang et al., 2010, Koskinen et al., 2011), and p38 MAPK (Koskinen et al., 2011) in primary chondrocytes isolated from osteoarthritis cartilage.

A study in patients with rheumatoid arthritis found that globular adiponectin enhanced ROS production via the phagocytic NADPH oxidase (Chedid et al., 2012). In contrast, production was inhibited by adiponectin. Selective inhibitors of p38 MAPK and ERK1/2 abrogated p47(phox) phosphorylation by adiponectin. This suggests different roles for adiponectin and globular adiponectin on phagocyte ROS production and an imbalance between these isoforms may contribute to chronic inflammation (Chedid et al., 2012).

Adiponectin treatment of CD4+ T cells increased phosphorylation of p38 MAPK and STAT4, and augmented T-box transcription factor (T-bet) expression. Inhibition of p38 with SB203580 abrogated adiponectin induced IFN-γ production. This indicates that adiponectin enhances T_H1 differentiation through the activation of a p38-STAT4-Tbet axis (Cheng et al., 2012).

1.7.9 Adiponectin: tolerance

Short term treatment of macrophages with adiponectin initially induces pro-inflammatory signalling (Cheng et al., 2012, Folco et al., 2009, Park et al., 2007), whereas, prolonged exposure is associated with tolerance. The mechanisms involved may include increased IL-10 expression, suppression of TNF-α mRNA stability and translation, and increased expression of IRAK-M (Zacharioudaki et al., 2009). The pre-treatment of HUVECs with globular adiponectin for 16 hours followed by stimulation with TNFα to induce NF-κB activation produces suppression in a concentration-dependent manner (Hattori et al., 2006).

1.7.10 Prokaryotic or eukaryotic derived adiponectin

There are two main sources of commercially available adiponectin: those produced from either prokaryote or eukaryote cells. Adiponectin expressed in eukaryotic cells appears able to form HMW complexes whilst prokaryotic derived adiponectin forms trimers and hexamers due to inadequate folding of the collagen-like domain (Richards et al., 2006). This difference may have important implications for *in vitro* work given the differences in the inflammatory actions of the various forms of adiponectin.

1.7.11 Resistin

Resistin is part of the resistin-like molecule (RELM) gene family; also known as found in inflammatory zone (FIZZ); (figure 1.7) and four members have been identified to date; RELM-α, RELM-β, RELM-γ and resistin. These cytokines appear to induce inflammation, angiogenesis, and smooth muscle proliferation (Fang

et al., 2012, Sood and Shore, 2013); responses which have led to an interest in asthma.

Resistin (also known as found in inflammatory zone, FIZZ3, and adipocyte secreted factor, ADSF) is a 12.5 kDa adipocytokine originally described in obese mice in 2001 as an exciting link to diabetes ("resistance to insulin"). Resistin is positively correlated with increasing BMI, adipose mass and is viewed as proinflammatory (Al-Suhaimi and Shehzad, 2013). However, whilst resistin levels are raised in obese patients who have had a previous myocardial infarction (Piestrzeniewicz et al., 2008), they are not raised in obese subjects following weight loss (Iqbal et al., 2005). Furthermore, a more recent study failed to identify a correlation between indices of adiposity and serum resistin levels, indicating that adipose tissue does not appear to be responsible for resistin production (Cabrera de Leon et al., 2014). Therefore, the precise relationship between serum resistin and adiposity remains unclear.

Resistin is predominantly expressed in macrophages in humans and to a lesser degree by white adipocytes (Fukuhara et al., 2005). Normal physiological serum levels range from 7 to 22 ng/ml and are raised in inflammatory states including acute coronary syndrome, inflammatory bowel disease, rheumatoid arthritis and sepsis (Fadda et al., 2013). It is produced by PBMCs, macrophages and adipocytes. Resistin expression is increased by TNF-α, IL-1β, IL-6 and LPS. It induces the synthesis and secretion of TNF-α and IL-12 from macrophages through NFκB and MAPK via TLR4. Little is known about its receptors, and the exact biological function and mechanism of action of resistin remains unclear.

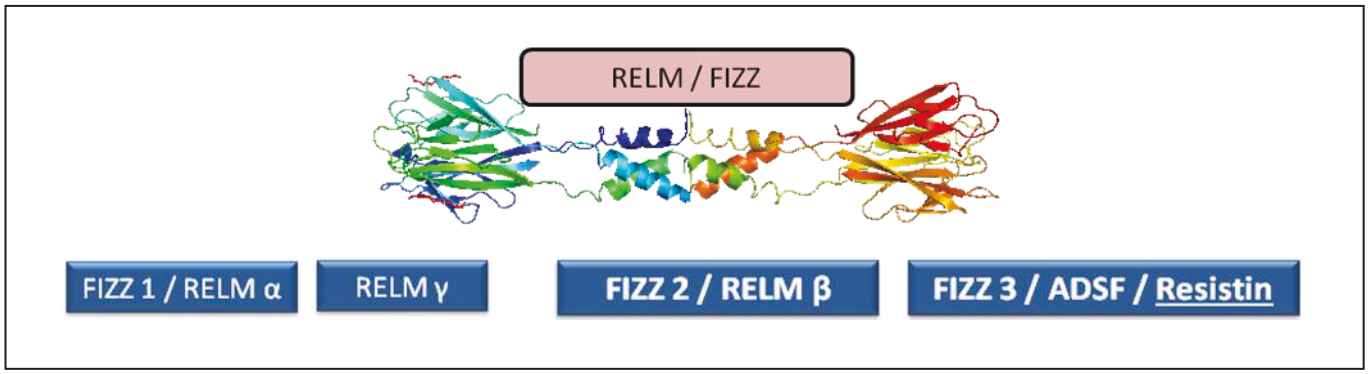


Figure 1.7: The RELM/FIZZ family. Resistin, otherwise known as found in inflammatory zone (FIZZ) has four members; RELM-α, RELM-β, RELM-γ and resistin.

1.7.12 Resistin and asthma

In contrast to adiponectin and leptin, considerably less is known about the relationship between resistin and asthma. There is additionally mixed data regarding resistin levels in serum and asthma. A study looking at serum resistin levels in children with asthma found no difference compared to healthy controls (Arshi et al., 2010). One study in children showed that atopic asthmatics exhibited lower resistin levels than non-atopic asthmatics and controls. In addition, the study noted a negative correlation between serum resistin and eosinophil counts and between serum resistin and total IgE. A positive correlation between serum resistin and methacholine PC_{20} was identified, indicating lower AHR (Kim et al., 2008). Multiple regression analysis revealed that after adjustment for other adiposity measures such as BMI and serum leptin and adiponectin, low serum resistin levels were strongly predictive of asthma. In contrast another study found higher resistin levels, which correlated with disease severity, in a cohort of moderate to severe persistent

asthmatics (Larochelle et al., 2007). High serum resistin levels in non-obese women with newly-diagnosed steroid naive asthma predicted a good anti-inflammatory response to ICS; suggesting that high resistin levels were a marker of a steroid-sensitive phenotype (Leivo-Korpela et al., 2011).

1.8 Leptin, adiponectin and resistin: summary of *in vivo* data

The data for leptin, adiponectin and resistin *in vivo* have mainly focused on serum concentrations and association studies with asthma. There appears to be a positive correlation between serum leptin concentration and asthma; an association that is stronger in childhood. In contrast, low serum adiponectin concentrations appear to predispose to a greater risk of developing asthma and improved asthma symptoms and lower asthma medication use in women. There is a paucity of data regarding serum resistin concentrations and asthma and the correlation remains unclear.

The lacks of clear correlations are likely to reflect a number of factors. Previous studies have not looked at specific asthma phenotypes and have relied on subgroup analysis. Furthermore, adipokines are probably just a component of the correlation between obesity and asthma. Other important factors include genetics, *in utero* conditions, oxidative stress, comorbidities, lung mechanics, sleep disordered breathing and hormonal effects (figure 1.2).

1.9 Adipokines: diurnal variation

A study taking blood samples at 15-minute intervals from six healthy normal weight men, receiving an isocaloric diet, found that circulating adiponectin and the soluble cleaved extracellular part of the leptin receptor (sOB-R) levels, which is the

main binding protein in the serum, exhibited ultradian pulsatility and diurnal variation with a significant nocturnal decline and morning nadir (Lammert et al., 2001, Gavrila et al., 2003).

1.10 Adipokines in the lung

Obese leptin receptor deficient mice (db^-/db^-) exhibit higher concentrations of BAL leptin and reduced adiponectin compared to lean wild type mice (Holguin et al., 2007). BALF taken from obese women prior to undergoing bariatric surgery found higher leptin and lower adiponectin levels in asthmatics compared to healthy controls (Sideleva et al., 2012). Another study in lean and obese asthmatics found that increasing BMI was associated with increased BAL leptin and a small reduction in adiponectin (F et al., 2011).

Epithelial cells isolated from brushings of patients with asthma exhibit higher expression of Adipo R2 and T-cadherin mRNA. Leptin expression, but not leptin receptor, has been shown to decrease following bariatric surgery. BALF adiponectin levels are lower and leptin levels higher in asthmatics compared to healthy controls (Sideleva et al., 2012).

Studies using induced sputum have noted increased adiponectin levels following inhalational challenge (Biagioni et al., 2014) whilst leptin levels are positively correlated with BMI and serum leptin (Lessard et al., 2011).

1.11 White and brown adipose tissue

Mammals have two distinct types of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). In mice and rats the subcutaneous deposit amount to 60-70% of total adipose tissue. The remaining 30-40% is composed of

visceral deposits in the thorax, abdomen and limbs (Cinti, 2009). The coexistence of white and brown adipocytes within the same depots has led to the concept of the adipose organ (Cinti, 2009). In humans, there is greater extension of subcutaneous depots and in females they are more developed in the mammary glands and gluteo-femoral areas. In addition, adipocytes form normal constituents of bone marrow, parotid glands, parathyroid glands, pancreas and synovial membranes.

WAT is composed of white adipocytes (figure 1.8B). These are large spherical cells, 60 to 80μm in diameter with a compressed nucleus, poorly developed organelles, few mitochondria and characterised by a single lipid droplet which accounts for 90% of the cell volume.

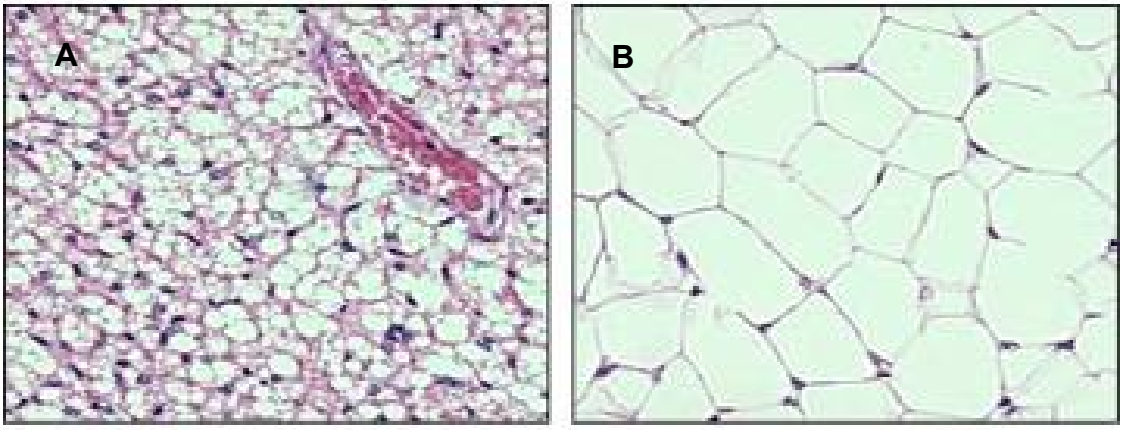


Figure 1.8: Brown (A) and white (B) adipose tissue. Brown adipose tissue contains multiple small lipid droplets, numerous mitochondria which are able to store iron and create the brown appearance**.** White adipose tissue contain a single large lipid droplet (Vieira-de-Abreu et al., 2005).

WAT stores and releases chemical energy in the form of fatty acids which allow animals to have intervals between meals. In addition they play an important role, via the secretion of leptin, in satiety (DiSpirito and Mathis, 2015).

BAT is composed of brown adipocytes (figure 1.8A). In contrast to WAT they are polygonal cells with a diameter of 30 to 50 μm, a central round nucleus and abundant cytoplasm containing numerous characteristic mitochondria and multiple lipid droplets. BAT plays an important role in thermogenesis and weight loss.

Although white and brown adipocytes differ with regard to cell morphology and physiology, they both are able to store and release lipid and express β_3-adrenoreceptors on their cell surface (DiSpirito and Mathis, 2015).

1.12 Lipid-laden macrophages (LLMs)

Preliminary Oil red O staining of BAL, endobronchial biopsies and lung biopsies (figure 1.9) show the presence of lipid in the form of lipid laden macrophages. These cells may form following phagocytosis of lipid material by AMs or from lipid laden blood monocytes that migrate to the airways (Shibuya et al., 1991); and the precise derivation of lipid laden macrophages remains unclear.

Lipid laden macrophages are associated with impaired phagocytosis (Schrijvers et al., 2005), increased predisposition to apoptosis, raised lipid peroxidation and may play a role in the pathophysiology of asthma.

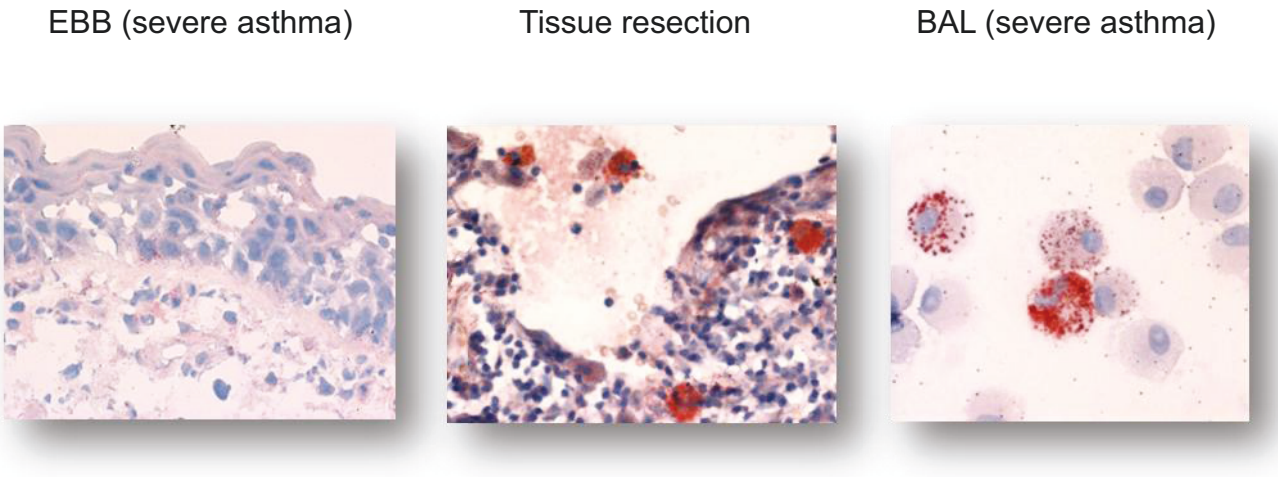


Figure 1.9: Oil red O staining. Oil red O staining in an endobronchial biopsy (EBB), lung tissue resection and bronchoalveolar lavage (BAL) fluid. Fat droplets stain bright red and are weakly positive in epithelial cells and the subepithelial layer in EBB. Lipid laden macrophages can be seen in the tissue resection and BAL pictures (Cowburn et al., 1998).

1.13 Adipose tissue plasticity and adipocyte transdifferentiation

Adult stem cells are able to produce differentiated cells from embryologically unrelated tissues. However, even fully differentiated cells are able to change their phenotype and this is known as transdifferentiation (Tosh and Slack, 2002). Examples of transdifferentiation include the appearance of hepatocytes in the pancreas, myoblasts to adipocytes and Wolffian regeneration.

Adipocytes are able to undergo reversible transdifferentiation. This process occurs when a cell reversibly changes its phenotype under physiological stimuli without passing through a stage of de-differentiation. Adult cells are therefore able to

spontaneously reprogramme their genes to develop different characteristics according to the requirements of their local environment.

In vivo murine experiments suggest that white adipocytes are able to undergo reversible transdifferentiation under particular physiological conditions. In mice treated with a β_3-adrenoreceptor agonist, a subpopulation of unilocular adipocytes were converted to multilocular mitochondria-rich adipocytes; a process consistent with white to brown adipocyte transdifferentiation (Himms-Hagen et al., 2000, Granneman et al., 2005, Barbatelli et al., 2010). Mice subjected to 6°C for 10 days revealed that 80% of adipocytes in dissected adipose tissue were brown adipocytes (Murano et al., 2009). This increase in brown adipocytes corresponds to a decrease in white adipocytes in the absence of cell degeneration or apoptosis (Vitali et al., 2012). This process is attenuated in β_3-adrenoreceptor knockout mice exposed to cold (Jimenez et al., 2003).

Another example of reversible transdifferentiation of adipocytes is the tissue plasticity of mammary glands during pregnancy, lactation and post-lactation stages (Morroni et al., 2004). In virgin females the majority of the mammary gland consists of adipocytes. During pregnancy and lactation the organ is replaced by approximately 90% secretory epithelial cells. This process is reversed following rapid and massive adipogenesis after lactation (Hennighausen and Robinson, 2001, Silberstein, 2001). The transdifferentiation process in this case is supported by the findings that electron microscopy has excluded the presence of dormant adipocytes during lactation and BrdU injections have demonstrated the absence of dormant adipocytes in mammary tissue during post lactation adipogenesis and a low rate of epithelial proliferation during pregnancy (Morroni et al., 2004).

1.14 Hypothesis and aims

The link between obesity and asthma is a relatively recent association and there is an increasing body of research suggesting the link is both bidirectional and multifactorial. I have chosen specific areas that form important components of this relationship and currently remain poorly understood.

1) Obesity-associated severe asthma is a distinct phenotype because obesity-related inflammation contributes to the severity of asthmatic inflammation, remodelling and to the associated corticosteroid insensitivity.

To address this, I aim to analyse a large database of severe asthmatics with regard to multiple variables (including demographic data, medical history, allergen testing, healthcare utilisation and medication, lung function, blood testing and bone density) and look for correlations between obesity and severe asthma. This will enable me to define the phenotypic characteristics of patients with obesity-related compared to non-obese severe asthma in terms of demographics, clinical features, markers of inflammation, healthcare utilisation and lung physiology. This is discussed in Chapter 3.

2) Obesity related severe asthma is associated with increased lipid laden macrophages and intracellular lipid droplets.

Lipid laden macrophages in bronchoalveolar lavage cells will be analysed using Oil Red O staining (Chapter 4) and lipid droplets in endobronchial biopsies will be assessed using perilipin staining (Chapter 5). Their relationship with asthma severity and BMI will be examined.

3) Raised BMI is associated with corticosteroid insensitivity in patients with asthma.

Suppression of LPS-induced cytokine release by dexamethasone from PBMCs taken from asthmatics and healthy controls will be examined and assessed according to BMI (Chapter 6).

4) Adipokines modulate inflammatory cytokine release and corticosteroid insensitivity in severe asthma.

The role of adipokines in obesity related severe asthma through *in vitro* experiments involving peripheral blood mononuclear cell taken from asthmatics and healthy controls is examined in Chapters 6 and 7. The aims are to study the effect of adiponectin, resistin and leptin on PBMCs in terms of inflammatory response, corticosteroid insensitivity and their relationship with BMI.

Chapter 2: Materials and Methods

Chapter 2: Materials and Methods

2.1 Patient cohorts and characterisation

Chapter 3: Obesity-associated asthma: a distinct phenotype

Subjects were included if they fulfilled the ATS definition of refractory asthma (ATS, 2000) and had a recorded body mass index (BMI). Subjects had at least one major and two of the minor criteria listed below.

Major criteria (must show at least one from):

1) Treatment with continuous or near continuous (>50 % of year) oral corticosteroids (OCS)

Or

2) Requirement for treatment with high dose ICS

Minor criteria (at least 2 out of the following):

1) Requirement for daily treatment with a controller medication in addition to ICS e.g. long acting β_2-agonists (LABA), theophylline or leukotriene antagonists

2) Asthma symptoms requiring short acting β_2-agonists (SABA) on a daily or near daily basis

3) Persistent airways obstruction (FEV_1 <80 % predicted, diurnal peak expiratory flow (PEF) variation >20 %)

4) One or more emergency care visits for asthma per year

5) 3 or more steroid “bursts” per year

6) Prompt deterioration with ≤ 25 % reduction in oral CS or ICS

7) Near fatal asthma event in the past

Chapter 4: The relationship between lipid-laden macrophages, asthma and BMI and chapter 5: Asthma, BMI and lipid droplets

All patients underwent a screening visit. Healthy subjects had no smoking history and no history of respiratory or cardiac disease. Asthma was based on a physician diagnosis and patients either had a PC_{20} methacholine concentration causing a 20% fall in FEV_1 of <8mg/ml and/or an FEV_1 reversibility of ≥ 12% of baseline. Severe asthma was defined according to the ATS definition of refractory asthma (ATS, 2000). Patients who did not fulfil the criteria for severe asthma were classified as non-severe asthmatics and they were taking ≤1000mcg of inhaled beclomethasone dipropionate (BDP) or equivalent per day. Patients with chronic cough reported a dry persistent cough for at least 8 weeks duration. All subjects had been investigated with a standard protocol to exclude a diagnosis of asthma or other respiratory disease.

Healthy subjects had no smoking history and no history of respiratory or cardiac disease.

Chapter 6: The impact of leptin, resistin and adiponectin on PBMCs in severe and non-severe asthma and chapter 7: The effect of adiponectin on PBMCs: inflammatory response and corticosteroid insensitivity in severe asthma

Subjects underwent spirometry with reversibility testing, a methacholine challenge test (to assess the degree of bronchial hyper-responsiveness), skin prick tests and IgE levels (to assess atopic status), and measurement of exhaled nitric

oxide (as a non-invasive marker of inflammation). All subjects had blood taken to obtain PBMCs. In addition, asthmatic subjects completed an Asthma Control Questionnaire.

Non-severe asthmatic subjects: mild to moderately severe asthma. The groups were defined as follows, according to their need for treatments (as established in the Asthma Management GINA or BTS guidelines): (i) Mild: intermittent symptoms and need for reliever bronchodilator less than once a day; (ii) moderate asthma: well-controlled asthma with minimal symptoms while on ICS therapy not exceeding 2,000 µg beclomethasone equivalent .

Severe asthmatic subjects: fulfilled the ATS, 2000 criteria for refractory asthma as previously described. (ATS, 2000).

Obese subjects: obesity is defined as a body mass index (BMI) over ≥ 30

Overweight subjects: 25 to 29.99

Normal weight subjects: BMI ≤ 24.99

Exclusion criteria (for all chapters):

- Current smokers, or < 3 years since quitting smoking (< 5 pack/years)
- Less than 4 weeks from an asthma exacerbation
- On steroid-sparing agent or immunosuppressant (e.g. azathioprine, methotrexate and ciclosporin)
- Concomitant anti-IgE therapy
- On anti-platelet or anti-coagulant drugs
- Low platelet count
- Pregnancy or breast-feeding

2.2 Clinical Methods

2.2.1 Ethics

All studies were approved by the Office for Research Ethics Committees Northern Ireland (ORECNI) (10/NIR02/37), the West London 3 and the Brompton, Harefield and NHLI Research Ethics Committees (11/H0706/4 and 08/H0708/29). Subjects provided written informed consent. Clinical investigations were performed in accordance with 'Good Clinical Practice' guidelines.

Healthy subjects, patients with chronic cough, mild/moderate asthmatics and severe asthmatics were recruited at the National Heart and Lung Institute (NHLI) and the Royal Brompton Hospital.

2.2.2 Skin prick testing

Skin prick testing was performed on the anterior forearm of one arm. A lancet was used to breach the surface of the epidermis through a drop of allergen extract on the skin at a 90 degree angle and surplus fluid was blotted with a tissue. After 15 minutes, the sites were examined and the reaction to individual allergens measured using a transparent ruler. Measurements were taken from the wheal produced and not the erythematous reaction. Measurements were recorded in the patient notes. The following allergens were tested: *Dermatophagoides pteronyssimus*, mixed grass pollen, cat dander and *Aspergillus fumigatus*. Histamine (10 mg/ml) and normal saline served as positive and negative controls respectively. A positive reaction occurs when a wheal measures 3 mm in maximum diameter with a negative control or when the wheal is 3 mm greater than the wheal produced by the negative control.

2.2.3 Spirometry

Baseline spirometry was measured in all subjects using a calibrated, dry wedge spirometer (Vitalograph, Buckingham, UK). Assessments were performed according to a standardised protocol. Subjects rested for 15 minutes prior to three measurements of FEV_1 and FVC. The procedure was performed with the subject sitting and they were asked to take a full inspiration, seal his/her lips around the mouthpiece and perform an expiratory manoeuvre as rapidly and forcefully as possible. Expiration was encouraged until a two second plateau phase had been achieved or 6 seconds overall. This was repeated a further two times with a one minute rest between and the best value recorded.

2.2.4 Methacholine provocation test (PC_{20})

Bronchial challenge testing with methacholine was conducted according to the departmental protocol. This incorporated a wash out period of 8 hours for short-acting β_2-adrenergic agonists. Following arrival to the laboratory, subjects were required to rest for 15 minutes prior to an assessment of lung function. Three measurements of FEV_1 at 1 minute intervals were taken and the highest value used for the baseline. Subjects then inhaled five breaths of 0.9% saline nebulised from a breath activated dosimeter by inspiring slowly from functional residual capacity (FRC) to total lung capacity (TLC) over 3 seconds and then breath holding for 6 seconds. Subsequent FEV_1 measurements were then taken at 30 seconds, 90 seconds and 180 seconds post nebulisation. At each time point, three measurements were taken and the highest was used. The concentration of methacholine inspired was doubled until a ≥ 20% fall in FEV_1 from the post saline

value was achieved. The starting concentration of methacholine is 0.03 mg/ml. Patients were given an inhaled β_2-agonist if required.

Patients with severe asthma who had an FEV_1 <1.5 litres or <60% and patients in whom it was felt clinically inappropriate did not undergo a PC_{20}.

2.2.5 Exhaled nitric oxide (NO) measurement

Exhaled NO measurements were taken using the hand-held device NObreath (Bedfont Scientific Ltd, UK). This is a non-invasive test that is easy to perform, requires a warm up time of approximately 60 seconds followed by a baseline measurement of ambient air NO and the breath time, at an exhalation flow rate of 50mls per second, is approximately 12 seconds. The machine concentration range is from 5 to 300 ppb NO with an accuracy and repeatability of ±5 ppb for values ≤50 ppb and ± 10% of measured value >50 ppb. Three successive measurements were performed on each occasion and the highest reading recorded. Ambient air NO was recorded for a second time after each subject was studied.

2.2.6 Measuring obesity

There are a number of different ways to measure body fat percentage and the definition of ‘overweight’ and ‘obesity’ is dependent on which method is used. Each way has particular advantages and disadvantages (see table 2.1) with cost implications meaning that anthropometric measurements are the most used worldwide. The methods used in this study are highlighted in bold and underlined.

Table 2.1. Different methods to measure obesity.

Method	Pros	Cons
Body Mass Index (BMI) Chapters 3, 4, 5, 6, 7	• Simple • Inexpensive • Easy to perform • Standardised cut off points • Well validated	• Inaccurate in specific groups (e.g. body builders, the elderly) • Sex and racial variance (women generally have more fat than men and that Asians have more fat than whites)
Waist hip ratio Chapters 6 and 7	• Easy to perform • Measure of abdominal obesity • Inexpensive	• Not standardised • Difficult to measure • Less accurate in the obese
Hydrostatic weighing	• Accurate	• Time consuming • Not practical - subjects need to be submerged in water.
Bioelectrical impedance analysis (BIA) Chapters 6 and 7	• Easy to perform • Quick results	• Requires calibration • Ratio of water to fat is variable (e.g. dehydration, weight loss, illness) • Reduced precision for high BMI
Air displacement plethysmography (AP)	• Accurate • Good for very high BMI, elderly and children	• Expensive
Dilution method (hydrometry)	• Accurate • Applicable to all subjects	• Variability of body water to fat-free mass ratio e.g. during illness, dehydration, weight loss
Skin fold thickness Chapters 6 and 7	• Inexpensive • Quick to perform, portable	• User dependent • Requires training • Not always reproducible • Difficult to measure in those with a high BMI (e.g. >35)
Dual energy X-ray absorptiometry (DEXA)	• Accurate	• Expensive • Not portable • Time consuming • Cannot distinguish types of fat (visceral vs. subcutaneous) • Most machines cannot handle subjects with a high BMI (>35)
CT or MRI	• Accurate	• Expensive • Non-portable • Not possible in all cases • Radiation risk • Unable to accommodate those with very high BMI
Ultrasound (US)	• Portable • Accurate • Quick to perform	• Expensive • User dependent – requires experience and skill • Not standardised • Inherent artefacts (e.g. fascia)
In vivo neutron activation analysis	• Accurate	• Cost • Inaccessibility

BMI

The body mass index (BMI) is the most basic and widely used method. It is a simple index of weight-for-height and a popular method in classifying overweight and obese individuals and populations. BMI is calculated by the dividing the weight in kg by the square of the height in metres (kg/m^2). A BMI of ≥ 25 is considered to be overweight whilst those ≥ 30 are classified as obese (table 2.2).

BMI is particularly useful because it is standardised, the same for both sexes and for adults of all ages. However, there are some important considerations when using BMI. These include the fact that it does not take body composition, fat distribution and racial variance into account. BMI does not differentiate between adipose tissue and lean mass and therefore results in specific populations (e.g. body builders) may be misleading.

Although BMI does not appear to be a particularly good way to quantify body fat composition, it is widely used as a risk factor for disease and as a marker for mortality. BMI tends to underestimate higher body fat percentages and there is some age dependent variability with overestimation in older subjects and underestimation in younger subjects (Deurenberg et al., 2001, Bohm and Heitmann, 2013).

Table 2.2. The International Classification of adult underweight, overweight and obesity according to BMI (Organisation, 2011).

Classification	BMI (kg/m2)	
	Principal cut-off points	Additional cut-off points
Underweight	<18.5	<18.5
Severe thinness	<16.0	<16.0
Moderate thinness	16.0 – 16.99	16.0 – 16.99
Mild thinness	17.0 – 18.49	17.0 – 18.49
Normal range	18.5 – 24.99	18.5 – 22.99 23.0 – 24.99
Overweight	≥25.0	≥25.0
Pre-obese	25.0 – 29.99	25.00 – 27.49 27.5 – 29.99
Obese	≥30.0	≥30.0
Obese class I	30.0 – 34.99	30.0 – 32.49 32.5 – 34.99
Obese class II	35.0 – 34.99	35.0 – 37.49 37.5 – 39,
Obese class III	≥40.0	≥40.0

Waist-hip ratio (WHR)

WHR is the ratio of the circumference of the waist compared to that of the hips and focuses on central adiposity. A longitudinal study of 1462 middle aged women found subjects with a high WHR were associated with a significantly raised 12 year incidence of myocardial infarction, angina, stroke, and death (Lapidus et al., 1984) (table 2.3). This relationship was stronger than other anthropometric variables (including skin fold measurements and BMI). A 13 year follow up study of 79,254

men aged 54 found that WHR, but not BMI or skin fold measurements, was significantly correlated with stroke, ischaemic heart disease or death from all causes (Larsson et al., 1984).

Table 2.3. Correlating waist hip ratio (WHR) with myocardial infarction, stroke and death – 12 year follow up of 1462 women aged 38 to 60 (Lapidus et al, 1984).

Centiles of waist to hip ratio	End points Age standardised incidence (%)		
	Myocardial infarction	Stroke	Death
0 – 10	0	0	2.9
11 – 20	0.4	0.4	3.2
21 – 40	0.4	0.7	5.4
41 -60	2.4	0.4	3.9
61 - 80	2.4	1.1	6.5
81 - 90	2.2	0	3.6
91 - 95	2.9	1.5	2.9
96 - 100	5.9	4.4	15.5

The WHO advises that waist circumference should be measured at the approximate midpoint between the lower margin of the last palpable rib and the top of the iliac crest whilst hip circumference should be taken around the widest portion of the buttocks (WHO, 2008).

Skinfold measurements

Skinfold measurements can be used as an indirect measurement of body composition. It is simple to perform, inexpensive and repeatable. Two of the most common methods are the Durnin and Wormersley equation (Durnin and Womersley, 1974) and the Jackson and Pollock equation (Jackson and Pollock, 1978). These methods use regression equations to estimate body density using skinfold measurements and age. A further equation is then used to convert body density to body fat percentage. The Durnin and Wormersley method uses skinfold thickness measurements taken at four sites (biceps, triceps, subscapular and supra-iliac areas) on the right side of the body with the subject standing (figure 2.1). Jackson and Pollock equations calculate body density using either the quadratic or log form of the sum of skinfolds, in combination with age, waist and forearm circumference (Jackson and Pollock, 1978).

Measurements are taken to the nearest mm, except for values below 5 mm where the nearest 0.5 mm is used. The Womersley and Durnin (Durnin and Womersley, 1974) method was used. Four skinfold sites were measures (triceps, biceps, subscapular and suprailiac). Body density was then calculated using the log of their sum in one of the equations shown in table 2.4. The density value was then converted to a body fat percentage using the Siri Equation.

1. Bicep
2. Tricep
3. Subscapular
4. Suprailiac

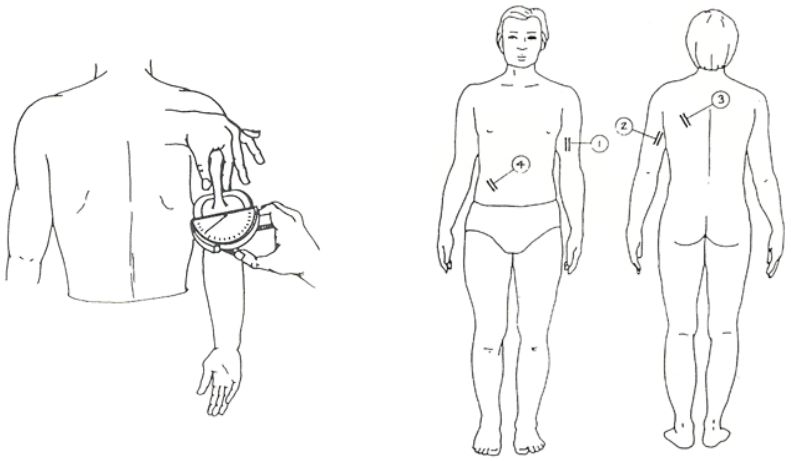


Figure 2.1: Durnin and Womersley Method 1974. Skin fold measurements are taken at four sites (bicep, tricep, subscapular and suprailiac). Body fat percentage can then be estimated using the log of their sum into one of the equations seen in table 2.4.

Table 2.4. Body density equations: Durnin and Womersley (Durnin and Womersley, 1974).

Age (years)	Equations for males	Equations for females
< 17	D = 1.1533 - (0.0643 X L)	D = 1.1369 - (0.0598 X L)
17-19	D = 1.1620 - (0.0630 X L)	D = 1.1549 - (0.0678 X L)
20-29	D = 1.1631 - (0.0632 X L)	D = 1.1599 - (0.0717 X L)
30-39	D = 1.1422 - (0.0544 X L)	D = 1.1423 - (0.0632 X L)
40 -49	D = 1.1620 - (0.0700 X L)	D = 1.1333 - (0.0612 X L)
> 50	D = 1.1715 - (0.0779 X L)	D = 1.1339 - (0.0645 X L

D = predicted density of the body (g/ml), L = log of the total of the 4 skin fold sites (mm). The density can then be converted to body fat percentage using the Siri Equation (% Body Fat = 495/Body Density) – 450) (Patil et al., 2007).

Bioelectrical impedance analysis (BIA)

BIA was introduced in the 1980's as a new method to calculate body composition (Baumgartner et al., 1989). Body fat percentage can be calculated using one or more frequencies introduced via electrodes across the body (usually via the hands or feet). The body resistance, or impedance (voltage drop), of the electrical flow is detected and is determined by the amount of fluid present. This method is non-invasive, portable and convenient. However, it relies on correlation and multiple

regression analysis, with an assumption that body geometry and fat-free mass is constant. Therefore there are concerns regarding repeatability and accuracy in specific situations. These include changes in weight, fluid volumes (for example in diseases such as renal failure and heart failure) and hydration status. In addition, BIA may underestimate body fat percentage in obese individuals (body fat > 30%) (Bohm and Heitmann, 2013).

BIA was measured using a Tanita TBF-300MA composition analyser. Subjects stood on the BIA after taking their shoes and socks off. Feet were free of excess dirt and heels were placed directly on the top of the posterior electrodes, while the front part of the foot was in contact with the anterior electrodes. In the event of calluses, 0.5cc of normal saline or water was placed in the centre of each electrode to ensure that current could freely pass through. A print out provided data on impedance, body fat percentage, fat mass and fat free mass.

Hydrostatic weighing

Hydrostatic weighing is considered the gold standard for measuring body density. It utilises the Archimedes principle to calculate body volume by measuring the difference between a subject's weight submersed in water and that in air. Whole-body density can subsequently be calculated. The subject must be at maximal exhalation whilst submerged in water to minimise the effect of buoyancy from air in the lungs and result in an accurate measurement (Behnke et al., 1995). This is a well-established method with excellent reliability. However, it is time-consuming, often uncomfortable for the subject and not accessible for many different groups (elderly, obese, disabled, chronic illness).

Air displacement plethysmography

Air displacement plethysmography (AP) uses a chamber with a fixed volume of air and determines body volume by measuring the reduction in volume after a subject is introduced. Whole-body density and body composition can subsequently be calculated using validated prediction equations (Biaggi et al., 1999).

Dilution Method (hydrometry)

The dilution method (hydrometry) utilises isotope-labelled liquid for ingestion or injection and then subjects provide body fluid samples which are subsequently analysed for isotope levels. Total body water, fat free mass and body fat mass can then be calculated utilising the dilution of the isotope. This is based on the premise that more of the isotope will be mixed (diluted) when there is a greater amount of lean tissue and lower body fat.

Computerised tomography (CT) and magnetic resonance imaging (MRI)

CT, utilising x-rays, and MRI, using magnetic fields, scans can be used to visualise the fat and lean tissues and measure the amount of visceral fat.

Neutron Activation Analysis (NAA)

NAA is a sensitive analytical technique based on the interaction of a neutron with an atomic nucleus. This results in the transformation of the target nucleus to an unstable isotope or nuclear state and subsequently decays. The amount of energy produced in this process can be measured in the form of gamma rays (Kehayias and Valtuena, 1999). This process can be used to calculate the rate of bone loss, using ^{49}Ca gamma ray measurement with DEXA absorptiometry, and body composition, using ^{40}K gamma ray measurement. ^{40}K is present in equal proportions to total body

potassium which allows it to be used as a surrogate marker and as a measurement of total body cell mass, synonymous with fat free mass.

Dual energy X-ray absorptiometry (DEXA)

DEXA uses x-rays of two different energies to scan the body. One ray is absorbed more readily by fat compared to the other. This allows calculation of body composition and body fat using a computer program to subtract one image from the other.

Techniques used in this study

This study incorporates BMI, bioelectrical impedance, waist-hip ratio and skin fold measurements. These methods were chosen in view of the various pros and cons highlighted above.

2.2.7 Fibreoptic bronchoscopy:

Fibreoptic bronchoscopies were performed under sedation according to standard clinical practice at the Royal Brompton Hospital. All patients underwent a screening visit and those with significant respiratory or inflammatory comorbidities and those requiring treatment for a respiratory tract infection within the preceding four weeks were excluded. Healthy subjects had no smoking history and no history of respiratory or cardiac disease.

BAL was performed from a segment of the right middle lobe (RML). The bronchoscope was wedged and warmed aliquots of buffered normal saline were installed (50 ml x 4, total 200 mls) and immediately aspirated through the bronchoscope by gentle suction.

BAL fluid was centrifuged (500 x *g* for 10 minutes), washed with Hanks' balanced salt solution (HBSS) and the cell pellet re-suspended in culture media (RPMI with 0.5% fetal bovine serum, antibiotics and L-glutamine) to a density of ~ 0.5 x 10^6 macrophages/ml. Cytospins were stained with REASTAIN Quick-Diff kit (Reagena International Oy Ltd, Finland) and a differential cell count was performed. Cytospins were then stained with Oil Red O prior to calculation of the lipid laden macrophage index (LLMI).

Endobronchial biopsies were taken from segmental and subsegmental airways of the right middle and right lower lobe. Biopsies were embedded in optimal cutting temperature medium and then snap-frozen in pre-cooled isopentane in liquid nitrogen and stored at ¯70 °C. Cryostat sections (6 mm) were obtained from biopsies, placed on poly-L-lysine-coated microscope slides and stained with haematoxylin and eosin for measurement of subbasement membrane (SBM) thickness. Slides were additionally stained for CD45+ cells and perilipin.

2.3 Laboratory Methods:

All materials, compounds, buffers and solutions used in this project are listed in the following tables.

Table 2.5. Materials for cell culture.

Material	Supplier
Acid Citrate Dextrose (ACD) (see table 2.7 for recipe)	Sigma-Aldrich, Dorset, UK
Ficoll Paque™ Plus	GE Healthcare, Buckinghamshire
Hank's Buffered Saline Solution (HBSS)	Sigma-Aldrich, Dorset, UK
Heat-inactivated foetal bovine serum (FBS)	PAA Laboratories Ltd, UK
Kimura Stain (see table 2.7 for recipe)	Reagents from Sigma-Aldrich, Dorset, UK
L-Glutamine	Sigma-Aldrich, Dorset, UK
Penicillin-Streptomycin solution	Sigma-Aldrich, Dorset, UK
Percoll™	GE Healthcare, Buckinghamshire, UK
Phosphate-buffered saline (PBS)	Sigma-Aldrich, Dorset, UK
Roswell Park Memorial Institute (RPMI) 1640 medium	Sigma-Aldrich, Dorset, UK
Sodium chloride 0.9% (w/v) IV	Baxter Healthcare, Berkshire, UK
Sterile tissue culture grade water	Sigma-Aldrich, Dorset, UK

Table 2.6. Corticosteroids, cytokines and inhibitors.

Treatment	Source	Stock solution
Dexamethasone (Dex)	Sigma-Aldrich, Dorset, UK	10^{-3} M in sterile water
Lipopolysaccharide (LPS; from Escherichia coli)	Sigma-Aldrich, Dorset, UK	1 mg/ml in PBS
Adiponectin (APN)	Insight Biotechnology Ltd, Wembley, UK	1 mg/ml in DMSO
Leptin	R & D Systems, Abington, UK	1 mg/ml in DMSO
Resistin	Peprotech, London, UK	100 µg/ml in water
Dexamethasone	Sigma-Aldrich, Dorset, UK	10^{-3} in water
GW856553	GlaxoSmithKline, Stevenage, UK	10^{-2} in water
U10126	Sigma-Aldrich, Dorset, UK	10^{-2} in DMSO
SP600125	Sigma-Aldrich, Dorset, UK	10^{-2} in DMSO
PI3K wortmannin	Sigma-Aldrich, Dorset, UK	10^{-3} in DMSO
Perilipin antibody	New England Biolabs, Hitchin, UK	1:100 dilution
Oil Red 0	Sigma-Aldrich, Dorset, UK	Ready to use
Anti CD45 monocolonal mouse anti-human antibody	Dako Ltd, Cambridge, UK	1:100 dilution
Polyclonal rabbit anti-human perilipin protein	New England Biolabs, Hitchin, UK	1:50 dilution
EnVision-alkaline phosphatase (EV-AP)	Dako Ltd, Cambridge, UK	Ready to use
Labelled polymer, AP (prediluted secondary antibodies, rabbit anti-mouse/rabbit) (K4018	Dako Ltd, Cambridge UK	Ready to use
Polyclonal goat anti-mouse rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (P0448)	Dako Ltd, Cambridge UK	Ready to use

Table 2.7. Buffer, solution or stain recipe.

Material	Supplier
Acid citrate dextrose solution (ACD)	5 g glucose; 4.2 g di-sodium hydrogen citrate; made up to 100 ml with deionised water; filtered and sterilised prior to use.
Kimura stain	11 ml Toluidine Blue; 0.5 ml Saponin saturated in 50% (v/v) ethanol; 0.8ml Light Green; 5 ml PBS; filtered and sterilised prior to use.
Stop solution (used for ELISAs)	11ml Sulphuric Acid; made up to 100 mls with deionised water
Wash buffer (for ELISA)	5 PBS tablets diluted in 1000mls of deionised water; 500 µl Tween®20.
Oil Red O	Sigma-Aldrich, Dorset, UK. 0.7% in propylene glycol (Sigma-Aldrich, Dorset)
Perilipin	1:100 dilution in PBS

Table 2.8. Kits and assays.

Material	Supplier
Duoset ELISA Kits (for detection of IL-6 and CXCL8)	R & D Systems, Abingdon, UK
Milliplex® MAP Human Magnetic Cytokine/Chemokine kit	Millipore, Watford, UK

Table 2.9. Equipment and devices used.

Equipment or device	Supplier
96-well MicroWell NUNC plates	Fisher Scientific UK Ltd, Loughborough, UK
Inverted light microscope	Microsystems, Buckinghamshire, UK Leica
SpectraMax microplate reader	Molecular Devices, Sunnyvale, CA, USA

2.3.1 Blood processing and isolation of PBMCs

80 millilitres of peripheral venous blood was collected into a syringe containing acid citrate dextrose (ACD), an anticoagulant. The blood was then divided into four aliquots of 20 ml and diluted with an equal volume of Hanks' Buffered Saline Solution, HBSS (Sigma, Dorset, UK). 25 mls were then carefully layered onto 20 ml of Ficoll-Paque^{TM} PLUS (Amersham plc, Dorset, UK) in a 50 ml Falcon tube. Following centrifugation (30 minutes at 400 x *g* at 21°C), PBMCs were collected from the interface between Ficoll and plasma. They were then washed with HBSS and centrifuged (10 minutes at 400 x *g*). The cell pellet was then resuspended with 1 ml of HBSS and red cell lysis was induced by the addition of 5 ml of cold water added whilst agitating the mixture. This was subsequently diluted with HBSS and a further centrifugation was carried out (10 minutes at 400 x *g*). PBMCs were then resuspended in 1 ml of RPMI culture media (Sigma; 0.5% v/v Foetal bovine serum, 2 mM L-glutamine, 100U penicillin-streptomycin) and counted using a haemocytometer and *Kimura* staining.

2.3.2 Cell viability assay

PBMCs were seeded in 96 well plates and stimulated as described later in this section. Following stimulation, the supernatant was removed and cells subsequently incubated with 200 µl of 1 mg/ml MTT solution in serum-free DMEM at 37°C in 5% $C0_2$/95% air (vol/vol) for 20 minutes. Staining of the cells (green/purple tinge) was confirmed by observation under a light microscope, the MTT solution was removed, and 100 µL of DMSO was then added to each well. The resting solution was then transferred into 96-well microtitre plates and the absorbance at 550 nm was measured with the absorbance microplate reader. The

effect of a treatment on cell viability was determined as a fold change in absorbance compared to the unstimulated control.

2.3.3 PBMC plating and stimulation for steroid/inhibitor combination study experiments

PBMCs were then diluted to a stock solution of 0.5 million per ml using RPMI. 200 µL (100, 000 cells) were placed in each well of a 96 well plate and allowed to settle in an incubator (37^{o}C, 5% $C0_2$, 90% humidity) for 2 hours. Cells were then pre-treated with/without dexamethasone (Dex) for one hour prior to the addition of leptin, resistin or adiponectin (APN) for a further hour prior to the addition of LPS. PBMCs were then incubated overnight (37ºC, 5% $C0_2$, 90% humidity) and supernatants harvested after 20 hours and stored at -20ºC until ready for cytokine analysis.

2.3.4 Cytokine analysis using ELISA

IL-6 and CXCL8 cytokine release levels in supernatant were measured by enzyme-linked immunosorbent assay (ELISA) using DuoSet ELISA kits (R & D Systems, Abingdon, UK) according to the manufacturer's protocol.

ELISA uses an immunodetection method which utilises biotinylated detection antibodies to identify specific antigens in a complex of proteins. This is a sensitive method that allows accurate measurement of the concentration of a specific cytokine in a sample. The technique has been termed a sandwich assay because an antibody-antigen-antibody complex is formed. A 96 well microtitre plate is coated with a 'capture antibody' against the target protein. The antigen in the sample is then immobilised by the capture antibody in the well. A biotin-labelled 'detection antibody' is then added which is also directed against the protein being tested. This antibody

binds to exposed sites on the protein and the biotin tag allows conjugation to streptavidin horseradish peroxidise (HRP) enzyme. Following the addition of a substrate, e.g. tetramethylbenzidine, an enzymatic reaction produces a visible colour change which is detected. The reaction is halted with 'stop solution' (2N H_2SO_4) and the colour intensity, which is proportional to the concentration of the protein tested, can be measured on a microplate reader.

Briefly, 100 µl of capture antibody solution at the working concentration in PBS were added to a 96-well microtitre plate and incubated for 24 hours at room temperature. The capture antibody solution was then removed; wells were washed three times with 300 µl of wash buffer and then dried by blotting the plate several times against a pad of paper towels. 300 µl of Blocking Buffer (1% BSA and 0.05% NaN_3 in PBS) per well were used to block and the plate was incubated for 1 hr at room temperature prior to a second wash step (as described above). A seven-point protein standard with 2-fold serial dilutions was prepared from the stock protein standard in Reagent Diluent buffer (0.1% BSA and 0.05% Tween 20 in PBS). 100 µl of the standards and diluted supernatants were then aliquotted into the wells of the plate. Following an incubation period of 2 hours at room temperature, the wash step was repeated. 100 µl of detection antibody, diluted to the working concentration in Reagent Diluent buffer, was added to each well and the plate incubated for 2 hours at room temperature. Following another wash step, 100 µl of streptavidin-HRP solution, diluted 1/200 in Reagent Diluent buffer, was placed into each well and the plate was incubated and covered, to protect from sunlight, for 20 min at room temperature. Following a final wash step, 100 µl of the substrate solution, prepared by mixing equal volumes of Colour Reagents A (H_2O_2) and B (tetramethylbenzidine), was added to each well and incubated until a blue colour developed. 50 µl of stop

solution (2N H_2SO_4) was added to each well to terminate the substrate reaction. The absorbance of each well was then measured at 450 nm with a 540 nm correction using a microplate reader and a four-parameter logistic standard curve was automatically generated using the microplate reader software. The cytokine concentration of each sample was determined from the standard curve and corrected for the dilution by multiplying by the dilution factor.

2.3.5 Multiplex Assay

Concentrations of six different human cytokines in culture medium were assayed using the Milliplex® MAP kit (table 2.10). Human cytokine/chemokine magnetic bead panels were purchased from Millipore (Watford, Hertfordshire, UK).

Black 96-well plates were coated with 200 µl wash buffer and left for 10 min at room temperature. Wash buffer was decanted and all residual amount of buffer was removed. 25 µl of each standard or quality control were added to the appropriate wells. 25 µl of assay buffer was used for the background (0 pg/ml) standard. Dilutions for a standard curve were prepared (as shown in table 2.11) and pipetted onto the plate in duplicate. 25 µl of assay buffer was added to the sample wells. 25 µl of matrix solution were added to the background, standard and quality control wells. 25 µl were added to the sample well (neat). Finally, 25 µl of mixed magnetic beads were added to all wells. Plates were incubated overnight at 4 ^{o}C with gentle shaking. Plates were washed twice with 200 µl wash buffer per well, using a hand-held magnet. Briefly, the plates were placed on the magnet for a minute to allow complete settling of the magnetic beads. All contents were removed by decanting the plates. Plates were washed twice by removing them from magnet and adding the wash buffer and allow shaking for 30 seconds. Once again the plate was placed on

the magnet for a minute to allow the settling for the magnetic beads before removing all contents of wells. 25 µl of detection antibodies were added to each well and the plate was incubated at room temperature for 1 hour, followed by the addition of 25 µl of Streptavidin-Phycoerythrin to each well for further 30 minutes incubation. Plates were washed as explained before and 150 µl of drive fluid were added to all wells. Beads were re-suspended on a plate shaker for 5 min. The plate was run on a MAGPIX® instrument with a xPONENT software (Austin, TX, USA). The mean fluorescent intensity was analysed using a 5-parameter logistic method for on XLfit software.

Table 2.10. H-CYTOMAG-60K – 6-Plex Panel.

Bead/Analyte Name	Luminex Magnetic Bead Region	Catalog #
Anti-Human IFNγ Bead	25	HCYIFNG-MAG
Anti-Human IL-10	27	HCYIL10-MAG
Anti-Human IL-1RA	42	H1RA-MAG
Anti-Human CCL2	67	HCYMCP1-MAG
Anti-Human CCL3	72	HMIP1A-MAG
Anti-Human TNFα	75	HCYTNFA-MAG

Concentrations of six different human cytokines in culture medium were assayed using the Milliplex® MAP kit. Human cytokine/chemokine magnetic bead panels were purchased from Millipore (Watford, Hertfordshire, UK).

Table 2.11. Preparation of reagents for immunoassay.

H-CYTOMAG-60K	
Antibody-Immobilised beads	Add 60 µl of each antibody bead vial to the mixing bottle and make up to 3 ml with Bead diluent
Quality Controls	Reconstitute Quality controls 1 and 2 with 250 µl of deionised H_2O
Human Cytokine Standard 10,000 – 3.2 pg/ml	Reconstitute human cytokine standard with 250 µl of deionised H_2O for a 10,000 pg/ml concentration for all analytes. Prepare a 6-point standard curve by making a 1:5 serial dilution with assay buffer
10X Wash buffer containing 0.05% Proclin	Dilute 30 ml of 10X wash buffer with 270 ml deionised H_2O
Matrix Solution	RPMI-1640 medium supplemented with 0.5 % (v/v) FBS and 2 mM L-glutamine

2.3.6 Slide staining

The methods for oil red O, perilipin and CD45 staining for BAL cytospins and endobronchial biopsies are discussed in this section. A more detailed description of the immunohistochemistry stain protocols and methods for scoring slides or biopsies can be seen in the methods section of each chapter.

Oil red O staining and lipid laden macrophage index (LLMI) score calculation

A lipid laden macrophage index (LLMI) was used to quantify lipid accumulation in AMs. BAL cytospins were stained with Oil-Red-O (Sigma-Aldrich Ltd, UK) to enable scoring and LLMI calculated by grading the amount of intracellular Oil-Red-O positive particles per 100 AMs. Intracellular lipid was evaluated using a modified version of that proposed by Corwin and Irwin (RW and RS, 1985, Colombo and Hallberg, 1987). Cells were scored from 0 to 4 whereby 0 = no opacification, 1 = up to ¼ opacified, 2 = ¼ to ½ opacified, 3 = ½ to ¾ opacified, and 4 = totally opacified (figure 4.2). The LLMI was calculated by the sum of the scores of 100 cells giving a potential range from 0 to 400. The person who measured the LLMI was not aware of clinical findings.

Perilipin

Endobronchial biopsies were stained for CD45+ cells and for perilipin. Monoclonal mouse anti-human antibodies anti-CD45 (Dako, cat no: M0701), at 1:100 dilution, and polyclonal rabbit anti-human perilipin protein (Cell Signaling Technology, cat no: 3470), at 1:50 dilution, were applied to bronchial biopsy sections for 1 hour.

EnVision-alkaline phosphatase (EV-AP) technique (Dako Ltd, Cambridge, UK) was used to label CD45+ pan leukocyte/inflammatory cells. A modified EnVision

peroxidase staining method (Dako) was used to identify perilipin+ cells as previously described (O'Shaughnessy et al., 1997) for 2 hours.

Irrelevant mouse IgG_1 kappa antibody (MOPC21) and rabbit serum were used to substitute for the primary layer as negative control for staining specificity of CD45 and Perilipin antibodies, respectively.

Histological slides were coded to avoid observer bias. The immunostaining intensity for perilipin expression in the airway epithelium, where it was mostly observed, was semi quantitatively given a score ranging from 0 to 2 (0: negative, 0.5: weak staining, 1: moderate staining and 2: strong staining). An example of the perilipin immunohistochemistry staining in bronchial epithelial cells and scoring can be seen in figure 5.1.

2.2.7 Statistical methods

Chapter 3

Between group comparisons for continuous variables were made using Kruskal-Wallis and for categorical variables using Chi-squared exact testing.

Chapter 4

Categorical variables were reported as percentages and comparisons done using the chi–square or Fisher's exact test. Numerical variables were reported as mean (SD) or median (IQR) and comparisons done using Mann-Whitney test or Kruskal Wallis test. Statistical significance was defined as $p<0.05$. Logistic regression was used to assess the predictive factors for GORD.

Chapter 5

Statistical analysis was performed using StatView (SAS Institute. Inc. and GraphPad Prism 4 (GraphPad Software, Inc.). One way ANOVA followed by the unpaired Student's t-test was used for the analyses of age, serum IgE, lung function and histamine PC_{20} data between groups. In respect of cell counts, these data were non-normally distributed and differences between groups were assessed first using the Kruskal-Wallis test, which, if significant, was followed by Mann-Whitney U-test between groups at baseline and infection. The coefficient of variation (CV = SD/mean $\times$ 100) was used to express the error of repeat counts. A p value of < 0.05 was accepted as indicating a significant difference.

Chapter 6 and 7

GraphPad Prism Version 5.03 was the software for statistical analysis. Repeated measures analysis of variance (ANOVA) with Dunnet multiple comparison test was used for intra-group analysis of i) the effect of cytokine compared to unstimulation and ii) the effect of leptin, resistin, adiponectin, dexamethasone, LPS or in combination compared to control. Kruskal-Wallis test with Dunn's multiple comparison was used to compare results between groups. $p<0.05$ was taken as significant.

Power calculations: studies were exploratory in nature and no data existed for assessing power. For chapters 6 and 7 numbers required were estimated using data from previous studies from the research group. Chapters 4 and 5 were retrospective studies and therefore numbers were dependent on available samples and data. Primary and secondary comparisons were pre-determined to avoid the need for correction given the multiple tests performed.

Chapter 3: Obesity-associated asthma:

a distinct clinical phenotype

Chapter 3: Obesity-associated asthma: a distinct clinical phenotype

Hypothesis and aim

Obesity-associated severe asthma is a distinct phenotype because obesity-related inflammation contributes to the severity of asthmatic inflammation, remodelling and to the associated corticosteroid insensitivity.

To address this, I aim to analyse a large database of severe asthmatics with regard to multiple variables (including demographic data, medical history, allergen testing, healthcare utilisation and medication, lung function, blood testing and bone density) and look for correlations between obesity and severe asthma. This will enable me to define the phenotypic characteristics of patients with obesity-related compared to non-obese severe asthma in terms of demographics, clinical features, markers of inflammation, healthcare utilisation and lung physiology.

3.1 Introduction

Obesity has been identified as a major risk factor for the development of asthma (Dixon et al., 2010). This is supported by large prospective epidemiological studies, almost all of which have noted that obesity antedates a diagnosis of asthma (SA., 2006, Beuther and Sutherland, 2007, Ford, 2005). As BMI increases, the relative risk of developing asthma concurrently increases (Hjellvik et al., 2010). In addition, increasing BMI is associated with a reduced asthma related quality of life, more frequent exacerbations and a greater severity and frequency of respiratory symptoms (Akerman et al., 2004, Shore, 2007, Taylor et al., 2008).

The link between the two appears to be multifactorial and is likely to involve a combination of *in-utero* conditions, genetic factors, comorbidities and inflammation

secondary to excess adipose tissue (Shore, 2008). Obesity-associated asthma may represent a distinct clinical phenotype (Dixon et al., 2010), characterised by later onset, female preponderance and greater symptoms than classic atopic asthma. Cluster analysis of a large cohort of patients noted an obese subgroup in both mild to moderate and refractory asthma. This subgroup was more likely to be female, display greater symptoms and had an absence of eosinophilic airway inflammation. (Haldar et al., 2008).

There is evidence to support the presence of steroid resistance in overweight and obese asthmatics. This stems from post-hoc analyses of clinical trial data, which have associated increasing BMI with a reduced response to ICS (Peters-Golden et al., 2006, Sutherland et al., 2010, Boulet and Franssen, 2007), and by an *in vitro* study on PBMCs, which found that overweight and obese asthmatics exhibited a reduced response to corticosteroids (Sutherland et al., 2008a).

BMI is a simple index of weight-for-height and is calculated by dividing the weight in kg by the square of the height in metres (kg/m^2). A BMI of ≥25 is considered overweight, whilst those ≥30 are classified as obese. It is particularly useful because it is standardised, calculated in the same way for both sexes and for adults of all ages (table 2.2).

The exact role of obesity in the pathophysiology of asthma and the relationship to severe asthma remains unclear. The aim of my study was to explore this relationship in a large well-characterised UK refractory asthma population, derived from the National Difficult Asthma Registry, by comparing the demographics, clinical features, markers of inflammation and healthcare utilisation in patients that were categorised by their BMI.

This chapter led to the following publication:

Gibeon, D., Batuwita, K., Osmond, M., Heaney, L.G., Brightling, C.E., Niven, R., Mansur, A., Chaudhuri, R., Bucknall, C.E., Rowe, A., Guo, Y., Bhavsar, P.K., Chung, K.F., Menzies-Gow, A. Obesity-associated severe asthma represents a distinct clinical phenotype: analysis of the British Thoracic Society Difficult Asthma Registry Patient Cohort according to BMI. Chest 2013; 143, 406-14.

3.2 Methods

The British Thoracic Society (BTS) Research Committee, alongside physicians with a special interest in severe asthma, developed a National Registry for dedicated UK Difficult Asthma Services in 2006. This project aimed to standardise specialist clinical services, to further define and to facilitate research into the assessment and management of difficult asthma. Pilot data from 382 well characterised patients, who fulfilled the ATS definition for refractory asthma, collected from four specialist UK centres, were used to compare patient demographics, disease characteristics and healthcare utilisation (Heaney et al., 2010). At present, there are seven UK dedicated Specialist Difficult Asthma Services submitting data to the registry; two centres in Glasgow and single centres in Belfast, Manchester, Leicester, Birmingham and London. The Registry is hosted online by Dendrite Clinical Systems Ltd and admits password protected anonymised data, after fully informed written consent has been obtained.

Ethical approval for the Registry was obtained from the Office for Research Ethics Committees Northern Ireland (ORECNI) (10/NIR02/37).

Patients at all centres underwent a multi-disciplinary systematic assessment of their asthma. At present there is no standardised method of assessing severe asthmatics in the UK. However, all patients underwent multiple investigations including a thorough medical history and examination, pulmonary function tests, allergy assessment (skin prick testing and/or RAST), blood tests (incorporating serum eosinophil count and IgE), and bone densitometry. Investigations were performed according to protocols at individual centres and lung function % predicted values were centrally calculated. Skin prick testing results were included for

aeroallergens tested at all centres. Although asthma quality of life questionnaires were completed, data was not available at the time of analysis.

Subjects were entered into the Registry in a non-selected manner and were included if they fulfilled the ATS definition of refractory asthma (ATS, 2000) and had a recorded BMI. Patients were then divided into three groups according to their BMI (normal weight 18.5 to 24.99, overweight 25 to 29.99, obese ≥30). The obese group were then subdivided into obese class I (BMI ≥30.0 to 34.99), obese class II (≥35 to 39.99) and obese class III (≥40) for subgroup analysis. These groups are taken from the WHO international classification of underweight, overweight and obesity according to BMI (Organisation, 2011).

3.3 Results

The study population consisted of 666 patients with severe asthma. The median BMI was 29.8 (IQR, 22.5 – 34.0); 48.3% were obese, 29.3% were overweight and 22.4% were of normal weight.

Group data and between BMI comparisons are shown in tables 3.1 to 3.5.

Values are presented in the following manner (% of obese compared to % overweight and % normal weight).

3.3.1 Demographics

There was a predominance of females (65%) and Caucasians (91.1%) in all BMI groups (table 3.1). The median age of asthma diagnosis in the overall cohort was 18 years (IQR 3 – 35) and 43.2% required maintenance oral prednisolone to control or partially control their symptoms. 89.4% had required short burst oral corticosteroid therapy in the preceding year and the median number of courses for those who required short burst therapy was 4 (IQR 2 – 7). There were high medication requirements in terms of leukotriene receptor antagonists (46%), theophylline (39.7%) and nebuliser use (47.9%).

Obese severe asthmatics were less likely to be in full-time employment than overweight or normal weight individuals (34.9% compared to 39.3% and 44.2%, $p<0.005$) and this was more likely to be related to asthma ill health (33.3% compared to 23.6% and 19.6%). The obese group were more likely to have a basic level of education (31.1% compared to 20.7% and 17.0%) and appeared less likely to have achieved an advanced (19.7% compared to 17.0% and 35.8%) or graduate level (9.6% compared to 17.0% and 10.4%). Obese severe asthmatics appeared more

likely to be current smokers or ex-smokers (10.5% and 31.3%, respectively) than their overweight (6.3% and 30.5%) or normal weight (9% and 27.1%) counterparts, although this did not reach statistical significance.

3.3.2 Medical history and atopy

Obese severe asthmatics were more likely to report a history of eczema (31.9% compared to 23.9% and 22.6%), GORD (53.9% compared to 48.1% and 39.7%) and have a documented abnormal pH profile study (pH profile results were available in 57 cases). Nasal polyposis (10.7% compared to 17.4% and 18.4%) and previous nasal surgery were both reported less frequently with increasing BMI (table 3.2).

Increasing BMI was associated with a lower aspergillus sensitisation (either a positive RAST or skin prick test) and a trend towards lower HDM, cat and mixed grass sensitisation (table 3.2). Serum IgE levels were lower in the overweight (median 144 [IQR, 46 – 384]) and obese group (119 [46 – 418]) compared to the normal BMI group (266 [83 – 480]) (table 3.5).

3.3.3 Medications and healthcare utilisation

The use of maintenance oral steroids (48.9% compared to 40.4% and 34.5%, $p<0.01$) increased alongside BMI. The overweight (92%) and obese (91.1%) groups were more likely than the normal (82.4%, $p<0.01$) BMI group to have required a short burst of OCS during the preceding year. Of those requiring OCS courses, the number of courses required was greater in the obese (5 [2-8]) than the overweight (4 [2-6]) and normal (4 [1.3-6]) groups. Nebuliser use, SABA use per day and PPI use (45.4% compared to 30.7% and 29.3%) were all greater with increasing BMI.

Insufficient numbers of patients were on steroid sparing agents or anti-IgE treatment at the time of referral to specialist services for meaningful results to be generated (table 3.3).

No significant difference was noted in the number of previous ICU admissions, emergency admissions or unscheduled visits over the preceding 12 months between BMI groups.

3.3.4 Lung function and radiology

The obese group had a reduced prebronchodilator FVC (80.1% [95% CI, 78.0 – 82.3]) compared to both the overweight (86.7% [83.7 – 89.8]) and normal weight groups (86.2.0% [82.8 – 89.6]). In those subjects where pre/post bronchodilator study data was available, the obese group had a reduced pre- and post-bronchodilator FVC compared to the overweight group. In contrast, the FEV_1/FVC ratio was higher in the obese group (63.5% [61.5 – 65.4]) compared to the overweight group (59.3% [56.6 – 61.9]). The transfer coefficient (Kco) was greater in the obese group (104.5% [95% CI, 102.1 – 106.9]) compared to the normal weight group (97.0% [93.5 – 100.7]). The proportion of CT scans reported as normal by radiologists appeared to increase with BMI (20.9% compared to 15.1% and 13.1%). Central bronchiectasis was reported less often in the obese group (6.7% compared to 12.6% and 17.6%) although this did not achieve statistical significance (Table 4).

3.3.5 Inflammatory markers and bone densitometry

No difference was seen in baseline peripheral blood eosinophil or sputum eosinophil counts between groups. No significant difference in the level of exhaled nitric oxide (FE_{NO}) was seen between groups.

Bone densitometry revealed a trend towards greater T scores at the lumbar spine with increasing BMI. The normal weight group exhibited a lower T score at the femoral neck (T score -1.02 ± 1.21) compared to the overweight or obese group (-0.32 ± 1.13 and +0.074 ± 1.19, respectively).

Table 3.1. Patient demographic data.

	Overall	BMI 18.50 to 24.99	BMI 25.00 to 29.99	BMI ≥ 30.00	p value
	666	**(149, 22.4%)**	**(195, 29.3%)**	**(322, 48.3%)**	
BMI	**29.8 (22.5-34.0)**	**22.8 (18.9-24.9)**	**27.4 (26.3-28.5)**	**34.2 (31.8-38.9)**	
Female (%)	435 (65)	107 (72)	111 (57)	217 (67)	0.009
Race, n (%)					
Caucasian	607 (91.2)	133 (89.3)	173 (89.6)	301 (94.4)	0.31
Asian	36 (5.5)	12 (8.1)	13 (6.7)	11 (3.4)	
African	8 (1.2)	1 (0.7)	3 (1.6)	4 (1.3)	
Other	10 (1.5)	3 (2.0)	4 (2.1)	3 (0.9)	
Age at assessment	45.0 (37.0-54.0)	44.0 (34.0-54.0)	46.0 (38.0-57.0)	45.0 (38.0-54.0)	0.3
Age at diagnosis	18.0 (3.0 - 35.0)	15.0 (3.0 - 35.0)	18.0 (3.0 - 36.0)	18.0 (4.0 - 34.0)	0.94
Smoking status n (%)					
Never	394 (60.9)	92 (63.9)	120 (63.2)	182 (58.1)	0.45
Ex-smoker	195 (30.1)	39 (27.1)	58 (30.5)	98 (31.3)	
Current smoker	58 (9.0)	13 (9.0)	12 (6.3)	33 (10.5)	
Ex or current smoker	253 (39.1)	52 (36.1)	70 (36.8)	131 (41.7)	0.38
Smoking (pack years)	18.6 ± 19.8	14.8 ± 14.0	16.2 ± 19.6	21.2 ± 21.4	0.08
Working status, n (%)					
Full time	246 (38.2)	61 (44.2)	75 (39.3)	110 (34.9)	0.005
Asthma related not working	177 (27.5)	27 (19.6)	45 (23.6)	105 (33.3)	
Other cause for not working	176 (27.3)	38 (27.5)	62 (32.5)	76 (24.1)	
Part time due to asthma	21 (3.3)	4 (2.9)	8 (4.2)	9 (2.9)	
Part time - other cause	24 (3.7)	8 (5.8)	1 (0.5)	15 (4.8)	
Educational achievement, n (%)					
Basic	117 (24.9)	18 (17.0)	28 (20.7)	71 (31.1)	0.0016
Intermediate	165 (35.2)	32 (30.2)	52 (38.5)	81 (35.5)	
Advanced	106 (22.6)	38 (35.8)	23 (17.0)	45 (19.7)	
Graduate	56 (11.9)	11 (10.4)	23 (17.0)	22 (9.6)	
Post-graduate	25 (5.3)	7 (6.6)	9 (6.7)	9 (3.9)	
Family history of asthma (%)	358 (55.2)	80 (54.4)	102 (54.5)	176 (55.9)	0.91
Evidence of poor adherence (%)	86 (13.3)	21 (14.9)	19 (10.1)	46 (14.6)	0.3

Group data (median (IQR)) for all subjects are presented in column 2 followed by data for individual BMI groups. Between group comparisons for continuous variables were made using Kruskal-Wallis and for categorical variables using Chi-squared exact testing.
Educational achievement: *Basic:* Level 1 NVQ, GCSE D-G, Foundation GNVQ, *Intermediate:* Level 2 NVQ, 5 GCSE A*-C, Intermediate GNVQ, *Advanced:* Level 3 NVQ, AS & A level, Advanced GNVQ, Graduate: Level 4 NVQ, Graduate studies, Post Graduate: Level 5 NVQ, Post graduate studies.

Table 3.2. Medical history and allergen testing.

		BMI			
	Overall	**18.50 to 24.99**	**25.00 to 29.99**	**≥ 30.00**	**p value**
	666	**(149, 22.4%)**	**(195, 29.3%)**	**(322, 48.3%)**	
BMI	**29.8 (22.5 - 34.0)**	**22.8 (18.9 - 24.9)**	**27.4 (26.3 - 28.5)**	**34.2 (31.8 - 38.9)**	
Medical History, n (%)					
Prior history of atopy	410 (62.6)	93 (63.7)	116 (61.4)	201 (62.8)	0.9
Perennial rhinitis	225 (34.2)	51 (34.7)	68 (35.6)	106 (33.2)	0.85
Seasonal rhinitis	263 (40.4)	57 (38.5)	75 (39.7)	131 (41.7)	0.78
Eczema	178 (27.3)	33 (22.6)	45 (23.9)	100 (31.9)	0.049
Nasal polyps	94 (14.4)	27 (18.4)	33 (17.4)	34 (10.7)	0.03
Prior nasal surgery	100 (16.3)	26 (17.7)	37 (19.8)	37 (11.7)	0.036
GORD	321 (49.1)	59 (39.7)	91 (48.1)	171 (53.9)	0.018
Prior OGD	27 (4.2)	5 (3.5)	8 (4.3)	14 (4.5)	0.88
Prior pH profile (abnormal profile)	57 (30)	11 (2)	17 (11)	29 (17)	0.036
Aspirin sensitivity	74 (11.7)	20 (14.1)	22 (11.6)	32 (10.7)	0.59
Significant catamenial asthma	33 (8.8)	10 (10.0)	8 (8.7)	15 (7.9)	0.76
Allergen testing, n (%)					
Domestic exposures pets	219 (34.2)	50 (34.7)	59 (31.4)	110 (35.7)	0.61
Aspergillus*	234 (47.3)	65 (58.6)	68 (46.3)	101 (42.6)	0.02
House dust mite*	300 (67.1)	68 (70.8)	90 (69.8)	142 (64.0)	0.37
Cat*	256 (57.4)	59 (62.8)	75 (57.7)	122 (55.5)	0.48
Dog*	237 (53.5)	50 (55.6)	71 (52.6)	116 (53.2)	0.9
Mixed grasses*	238 (54.8)	59 (63.4)	66 (54.1)	113 (51.6)	0.15

Group data (%) for all subjects are presented in column 2 followed by data for individual BMI groups. Between group comparisons were made using Chi-squared exact analysis.
*The data presented are for subjects with either a positive RAST or skin prick test
RAST, radioallergosorbent GORD, gastro-oesophageal reflux disease OGD, oesophagogastroduodenoscopy.

Table 3.3. Medications and healthcare utilisation.

		BMI			
	Overall	**18.50 to 24.99**	**25.00 to 29.99**	**≥ 30.00**	**p value**
	666	**(149, 22.4%)**	**(195, 29.3%)**	**(322, 48.3%)**	
BMI	**29.8 (22.5-34.0)**	**22.8 (18.9-24.9)**	**27.4 (26.3-28.5)**	**34.2 (31.8-38.9)**	
Medications					
PPI use	244 (37.3)	43 (29.3)	58 (30.7)	143 (45.4)	0.0002
Maintenance oral steroids	286 (43.2)	51 (34.5)	78 (40.4)	157 (48.9)	0.009
Oral steroid dose	15 (10 - 20)	10 (10 - 20)	10 (10 - 20)	15 (10 - 22.5)	0.19
Oral corticosteroids (OCS) past year					
Patients requiring rescue steroids	538 (89.4)	112 (82.4)	160 (92.0)	266 (91.1)	0.01
No. rescue courses steroids	4 (2.0 - 7.0)	4 (1.30- 6.0)	4 (2.0 - 6.0)	5 (2.0 - 8.0)	0.01
SABA use per day	8 (4.0 - 10.0)	6 (3.0 - 10.0)	8 (4.0 - 10.0)	8 (4.0 - 10.0)	0.008
Nebuliser use	314 (47.9)	58 (39.7)	89 (46.4)	167 (52.5)	0.03
BDP equivalent dose (µg)	2000 (1600-2000)	2000 (1500-2000)	2000 (1000-2000)	2000 (1600-2000)	0.015
Leukotriene receptor antagonists	303 (46.0)	61 (41.5)	82 (42.7)	160 (50.2)	0.12
Antihistamines	193 (29.4)	45 (30.4)	54 (28.4)	94 (29.6)	0.92
Nasal steroids	184 (27.9)	39 (26.4)	57 (29.7)	88 (27.5)	0.78
Theophylline	262 (39.7)	56 (38.1)	73 (37.8)	133 (41.6)	0.64
Steroid sparing meds	24 of 664	4	6	14	
None	628	142	181	305	
Methotrexate	12	1	2	9	
Ciclosporin	5	3	1	1	
Azathrioprine	5	0	1	4	
Mycophenolate mofetil	1		1		
Other	1		1		
Anti-IgE treatment	15 of 652	4	3	8	0.72
Healthcare utilisation (past year)					
Unscheduled GP/A&E visits	558 (88.4)	132 (90.4)	163 (89.1)	263 (87.1)	0.56
No. unscheduled visits	5 (3 – 8)	4 (2 – 7)	5 (3 – 8)	5 (3 – 8)	0.1
Admissions	319 (48.6)	64 (43.5)	101 (52.1)	154 (48.7)	0.3
No of admissions	2 (1 – 3)	2 (1 – 3)	2 (1 -3)	2 (1 – 3)	0.93
ICU admissions to date	137 (20.9)	28 (19.2)	41 (21.6)	68 (21.4)	0.84
No of ICU admissions to date	1 (1 – 2)	2 (1 – 3)	1 (1 – 2)	1 (1 – 2)	0.51

Group data (median (IQR)) or % are presented in column 2 followed by data for individual BMI groups. Between group comparisons for continuous variables were made using Kruskal-Wallis and for categorical variables using Chi-squared exact analysis.

PPI, proton pump inhibitor SABA, short-acting β_2-agonist BDP, beclomethasone dipropionate GP, general practitioner A&E, accident and emergency ICU, intensive care unit.

Table 3.4. Lung function and FE_{NO}.

		BMI			
	Overall	**18.50 to 24.99**	**25.00 to 29.99**	**≥ 30.00**	**P value**
	666	**(149, 22.4%)**	**(195, 29.3%)**	**(322, 48.3%)**	
BMI	**29.8 (22.5 - 34.0)**	**22.8 (18.9 - 24.9)**	**27.4 (26.3 - 28.5)**	**34.2 (31.8 - 38.9)**	
FE_{NO} ppb	33.0 (15 - 61)	34 (21 - 62.5)	35.5 (15.2 - 85.4)	31 (13.5 - 57.3)	0.11
PreBD FEV_1 (litres)	1.97 (1.9 - 2.0)	2.04 (1.9 - 2.2)	2.00 (1.9 - 2.1)	1.91 (1.8 - 2.0)	0.43
(% predicted)	67.7 (65.8 - 69.7)	69.6 (65.1 - 74.1)	67.8 (64.3 - 71.3)	66.9 (64.2 - 69.5)	0.67
PreBD FVC (litres)	3.10 (3.0 - 3.2)	3.20 (3.0 - 3.4)	3.29 (3.1 - 3.5)	2.94 (2.8 - 3.1)	0.0009
(% predicted)	84.0 (81.9 - 85.0)	86.2 (82.8 - 89.6)	86.7 (83.7 - 89.8)	80.1 (78.0 - 82.3)	0.0004
Subjects with baseline bronchodilator study					
PreBD FEV_1 (litres)	1.90 (1.8 - 2.0)	1.95 (1.8 - 2.1)	1.92 (1.8 - 2.1)	1.86 (1.8 - 2.0)	0.94
(% predicted)	65.5 (63.2 - 67.7)	67.0 (61.9 - 72.1)	65.3 (61.1 - 69.6)	64.8 (61.8 - 67.8)	0.85
PreBD FVC (litres)	3.07 (3.0 - 3.2)	3.12 (2.9 - 3.3)	3.30 (3.1 - 3.5)	2.90 (2.8 - 3.0)	0.01
(% predicted)	82.6 (80.7 - 84.5)	84.5 (80.1 - 88.4)	87.1 (84.0 - 90.8)	78.8 (76.2 - 81.4)	0.002
PostBD FEV_1 (litres)	2.20 (2.1 - 2.3)	2.21 (2.0- 2.4)	2.25 (2.1 - 2.4)	2.16 (2.1 - 2.3)	0.67
(% predicted)	76.0 (73.7 - 78.2)	76.4 (71.4 - 81.3)	76.5 (72.1 - 81.0)	75.4 (72.3 - 78.6)	0.89
BDR (% Δ FEV_1)	19.6 (17.4 - 21.7)	17.3 (13.3 - 21.2)	19.9 (15.8 - 24.0)	20.4 (17.1 - 23.7)	0.34
PostBD FVC (litres)	3.33 (3.2 - 3.4)	3.35 (3.1 - 3.6)	3.53 (3.3 - 3.7)	3.19 (3.1 - 3.3)	0.02
(% predicted)	89.9 (88.2 - 91.7)	91.4 (87.7 - 95.1)	93.5 (89.9 - 97.0)	87.0 (84.7 - 89.3)	0.003
PreBD FEV_1/FVC %	61.8 (60.4 - 63.2)	61.6 (58.4 - 64.8)	59.3 (56.6 - 61.9)	63.5 (61.5 - 65.4)	0.04
PostBD FEV_1/FVC %	65.7 (64.3 - 67.2)	65.1 (61.8 - 68.4)	63.4 (60.8 - 65.9)	67.5 (65.4 - 69.5)	0.052
TLC % pred	103.0 (101.1-104.2)	106.4 (103.1-109.6)	103.6 (100.8-106.3)	100.3 (98.2-102.5)	0.003
RV % pred (n)	124.0 (123.6-131.3)	129.3 (121.2-137.3)	124.2 (117-131.5)	128.7 (123.1-134.3)	0.45
K_{CO} % pred (n)	101.0 (100.3-103.7)	97.0 (93.5-100.7)	101.6 (98.6-104.6)	104.5 (102.1-106.9)	0.002

Group data (mean (95% CI)) for lung function, otherwise (mean ± SD or median (IQR)) for all subjects are presented in column 2 followed by data for individual BMI groups. Between group comparisons for continuous variables were made using Kruskal-Wallis (for posthoc analysis, see text).

FEV_1, forced expiratory volume in 1 second FE_{NO}, exhaled nitric oxide FVC, forced vital capacity K_{CO}, carbon monoxide transfer coefficient BDR, bronchodilator reversibility PreBD, pre-bronchodilator PostBD, post-bronchodilator TLC, total lung capacity RV, residual volume.

Table 3.5. Blood testing and radiology.

		BMI			
	Overall	**18.50 to 24.99**	**25.00 to 29.99**	**≥ 30.00**	**P value**
	666	**(149, 22.4%)**	**(195, 29.3%)**	**(322, 48.3%)**	
BMI	**29.8 (22.5 - 34.0)**	**22.8 (18.9 - 24.9)**	**27.4 (26.3 - 28.5)**	**34.2 (31.8 - 38.9)**	
Blood eos count x 10^9/l (n)	0.28 (0.11 - 0.53) (629)	0.30 (0.1 - 0.67) (141)	0.31 (0.11 - 0.60) (184)	0.26 (0.11 - 0.49) (304)	0.21
Sputum eos count (n)	2.9 (0.5 - 9.9) (174)	4.0 (0.38 - 14.0) (41)	2.8 (0 - 12.1) (49)	2.8 (1.0 - 9.7) (84)	0.92
IgE kU/l (n)	158 (51 - 421) (610)	266 (83 - 480) (133)	144 (46 - 384) (181)	119 (46 - 418) (296)	0.005
Bone density (T-scores) (n)					
L1 to L4	-0.71 ± 1.48 (239)	-1.08 ± 1.25 (50)	-0.73 ± 1.68 (70)	-0.55 ± 1.42 (119)	0.051
Femoral neck	-0.27 ± 1.24 (233)	-1.02 ± 1.21 (48)	-0.32 ± 1.13 (72)	+0.074 ± 1.19 (113)	<0.0001
HRCT (n)					
Reported normal	68, 17.3% (392)	11, 13.1% (84)	19, 15.1% (126)	38, 20.9% (182)	0.21
Abnormal HRCT	344, 81.9% (420)	75, 83.3% (90)	114, 86.4% (132)	155, 78.3% (198)	0.16
Central bronchiectasis	34, 11.1% (306)	12, 17.6% (68)	13, 12.6% (103)	9, 6.7% (135)	0.053
Other bronchiectasis	77, 24.3% (317)	19, 27.9% (68)	28, 26.7% (105)	30, 20.8% (144)	0.42
Bronchial wall thickening	214, 65.4% (327)	44, 62.0% (71)	67, 63.2% (106)	103, 68.7% (150)	0.52
Bronchial dilatation	58, 18.2% (318)	12, 17.9% (67)	21, 20.2% (104)	25, 17.0% (147)	0.81
Ground glass	35, 11.0% (317)	9, 13.0% (69)	11, 10.5% (105)	15, 10.5% (143)	0.84
Air trapping	63,19.7% (319)	12, 17.4% (69)	19, 17.9% (106)	32, 22.2% (144)	0.6
Mucus plugging	31, 9.8% (316)	9, 13.0% (69)	7, 6.8% (103)	15, 10.4% (144)	0.38
Emphysema	15, 4.5% (333)	4, 5.5% (73)	4, 3.7% (109)	7, 4.6% (151)	0.84

Group data (mean (95% CI)) for lung function, otherwise (mean ± SD or median (IQR)) for all subjects are presented in column 2 followed by data for individual BMI groups. Between group comparisons for continuous variables were made using Kruskal-Wallis (for posthoc analysis, see text).

IgE, immunoglobulin E L1, first lumbar vertebra L5, fifth lumbar vertebra HRCT, high resolution computerised tomography.

Note: CT scans were reported at individual centres.

3.3.6 Subdivisions of obesity

The obese group were then subdivided into three groups according to WHO subclasses, used to facilitate international comparisons (Organisation, 2011): class I (BMI 30 to 34.99), class II (35 to 39.99) and class III (≥40). 56.2% of the cohort fell into class I, 23.9% into class II and 19.9% in class III.

Group data and between BMI comparisons are shown in tables 3.6 to 4.1.

Values are presented in the following manner (% of class III compared to % class II and % class I).

Class III were more likely to be female than class II and class I (82.8% compared to 67.5% and 61.9%, $p<0.01$); (table 3.6). In addition, class III were more likely to be current smokers (16.9% compared to 13% and 5.8%, $p=0.013$). No significant differences were seen in terms of medical history or allergen testing. Although no difference was seen regarding maintenance OCS use or the proportion of each group requiring short burst therapy in the preceding year, there was a difference in terms of the number of short burst courses required (6 [4-9] versus 5 [3-7] and 4 [2-7], $p=0.046$). Class III were more likely to be on steroid sparing agents (11.3% versus 3.9% and 2.2%, $p=0.01$) or anti IgE therapy (7.9% versus 1.4% and 1.1%, $p=0.01$). In addition, this group had a greater number of unscheduled GP or A & E visits in the preceding year (6 [4-9.3] versus 5 [4-8] and 4 [4-9.3], $p=0.02$). No significant differences were noted in terms of lung function or inflammatory markers (blood and sputum eosinophilia). Intriguingly, class III had a higher T score for the neck of femur compared to the other two groups (+0.7 ± 1.3 versus +0.5 ± 1.1 and -0.2 ± 1.1, $p=0.005$).

Table 3.6. Patient demographic data (obese cohort).

	Overall	BMI Class I 30 to 34.99	BMI Class II 35 to 39.99	BMI Class III ≥40	P value
	322	**181 (56.2)**	**77 (23.9)**	**64 (19.9)**	
BMI	**34.2 (31.8 - 38.9)**	**32 (31 - 33.1)**	**37.3 (36.1 - 38.9)**	**43.6 (41.1 - 45.3)**	
Female (%)	217 (67)	112 (61.9)	52 (67.5)	53 (82.8)	0.009
Race, n (%)					
Caucasian	301 (94.4)	168 (93.9)	72 (94.7)	61 (95.3)	0.41
Asian	11 (3.4)	7 (3.9)	3 (3.9)	1 (1.6)	
African	4 (1.3)	3 (1.7)	1 (1.3)	0	
Other	3 (0.9)	1 (0.6)	0	2 (3.1)	
Age at assessment	45.0 (38.0 - 54.0)	46 (38.5 - 55)	46 (40 – 52)	42 (34.4 – 48.8)	0.02
Age at diagnosis	18.0 (4.0 - 34.0)	18 (3 – 37.8)	18 (6.3 – 33)	15 (4 – 27.3)	0.39
Smoking status n (%)					
Never	182 (58.1)	104 (60.5)	41 (53.2)	37 (57.8)	0.013
Ex-smoker	98 (31.3)	58 (33.7)	26 (33.8)	14 (21.9)	
Current smoker	33 (10.5)	10 (5.8)	10 (13)	13 (16.9)	
Ex or current smoker	131 (41.7)	68 (39.5)	36 (46.8)	27 (42.2)	
Smoking (pack years)	21.2 ± 21.4	17.4 ± 22.0	25.5 ± 18.5	24.8 ± 4.5	0.013
Working status, n (%)					
Full time	110 (34.9)	77 (43.3)	19 (25)	14 (23)	0.07
Asthma related not working	105 (33.3)	51 (28.7)	29 (38.2)	26 (42.6)	
Other cause for not working	76 (24.1)	40 (22.5)	21 (27.6)	15 (24.6)	
Part time due to asthma	9 (2.9)	3 (1.7)	2 (2.6)	3 (4.9)	
Part time due to other cause	15 (4.8)	7 (3.9)	5 (6.6)	3 (4.9)	
Educational achievement, n (%)					
Basic	71 (31.1)	39 (31.0)	18 (26.5)	14 (31.8)	0.7
Intermediate	81 (35.5)	42 (33.3)	22 (32.4)	17 (38.6)	
Advanced	45 (19.7)	25 (19.8)	11 (16.2)	9 (20.5)	
Graduate	22 (9.6)	15 (11.9)	3 (4.4)	4 (9.1)	
Post-graduate	9 (3.9)	5 (4.0)	4 (5.9)	0	
Family history of asthma (%)	176 (55.9)	94 (53.1)	47 (61.8)	35 (56.5)	0.44
Evidence of poor adherence (%)	46 (14.6)	24 (13.6)	12 (15.8)	10 (16.1)	0.84

Group data (median (IQR)) for all subjects are presented in column 2 followed by data for individual BMI groups. Between group comparisons for continuous variables were made using Kruskal-Wallis and for categorical variables using Chi-squared exact testing.

Educational achievement: *Basic:* Level 1 NVQ, GCSE D-G, Foundation GNVQ**,** *Intermediate:* Level 2 NVQ, 5 GCSE A*-C, Intermediate GNVQ**,** *Advanced:* Level 3 NVQ, AS & A level, Advanced GNVQ**,** Graduate: Level 4 NVQ, Graduate studies**,** Post Graduate: Level 5 NVQ, Post graduate studies.

Table 3.7. Medical history and allergen testing (obese cohort).

		BMI			
	Overall	**Class I 30 to 34.99**	**Class II 35 to 39.99**	**Class III ≥40**	**P value**
	(322, 48.3%)	**181 (56.2)**	**77 (23.9)**	**64 (19.9)**	
BMI	**34.2 (31.8 - 38.9)**	**32 (31 - 33.1)**	**37.3 (36.1 - 38.9)**	**43.6 (41.1 - 45.3)**	
Medical History, n (%)					
Prior history of atopy	201 (62.8)	112 (62.2)	48 (63.2)	41 (64.1)	0.96
Perennial rhinitis	106 (33.2)	59 (33.0)	23 (29.9)	24 (38.1)	0.59
Seasonal rhinitis	131 (41.7)	80 (44.9)	25 (33.8)	26 (41.9)	0.26
Eczema	100 (31.9)	59 (33.7)	25 (33.3)	16 (25.4)	0.46
Nasal polyps	34 (10.7)	20 (11.2)	7 (10)	7 (12.3)	0.89
Prior nasal surgery	37 (11.7)	24 (13.6)	6 (7.9)	7 (11.1)	0.43
GORD	171 (53.9)	94 (53.1)	41 (53.2)	36 (57.1)	0.85
Prior OGD	14 (4.5)	9 (5.1)	5 (6.7)	0 (0)	0.14
Prior pH profile (abnormal profile)	29 (17)	12 (60)	2 (40)	3 (75)	0.56
Aspirin sensitivity	32 (10.7)	19 (11.2)	6 (8.4)	7 (12.1)	0.77
Significant catamenial asthma	18 (8.1)	11 (9.7)	1 (2.6)	3 (7.7)	0.37
Allergen testing, n (%)					
Domestic exposures pets	110 (35.7)	64 (36.6)	25 (34.7)	21 (34.4)	0.94
Aspergillus*	101 (42.6)	60 (44.4)	24 (42.9)	17 (37.0)	0.67
House dust mite*	142 (64.0)	83 (64.8)	33 (63.5)	26 (61.9)	0.94
Cat*	122 (55.5)	71 (54.6)	30 (58.8)	21 (53.8)	0.86
Dog*	116 (53.2)	68 (54.0)	26 (50.0)	22 (42.3)	0.86
Mixed grasses*	113 (51.6)	73 (56.2)	23 (46.9)	17 (42.5)	0.24

Group data (%) for all subjects are presented in column 2 followed by data for individual BMI groups. Between group comparisons were made using χ^2 exact analysis.

*The data presented are for subjects with either a positive RAST or skin prick test.

RAST, radioallergosorbent GORD, gastro-oesophageal reflux disease OGD, oesophago-gastroduodenoscopy.

Table 3.8. Medications and healthcare utilisation (obese cohort).

	Overall	BMI			P value
		Class I	Class II	Class III	
		30 to 34.99	35 to 39.99	≥40	
	(322, 48.3%)	181 (56.2)	77 (23.9)	64 (19.9)	
BMI	34.2 (31.8 - 38.9)	32 (31 - 33.1)	37.3 (36.1 - 38.9)	43.6 (41.1 - 45.3)	
Medications					
PPI use	143 of 315 (45.4)	72 (40.7)	37 (49.3)	34 (54.0)	0.14
Maintenance oral steroids	157 of 321 (48.9)	81 (44.8)	42 (55.3)	34 (53.1)	0.23
Oral steroid dose	15 (10 - 22.5)	15 (10 -20)	15 (10 – 20)	15 (10 – 30)	0.68
Oral corticosteroids past year:					
Patients requiring rescue steroids	266 of 292 (91.1)	152 (91)	60 (89.6)	54 (93.1)	0.78
No. rescue courses steroids	5 (2.0 - 8.0)	4 (2 – 7)	5 (3 – 7)	6 (4 – 9)	0.046
SABA use per day	8 (4.0 - 10.0)	6 (4 – 8)	8 (7.5 – 12)	8 (8 – 10.5)	<0.0001
Nebuliser use	167 (52.5)	81 (45.3)	42 (55.3)	44 (69.8)	0.003
BDP equivalent dose (µg)	2000 (1600- 2000)	2000 (1600–2000)	2000 (1600–2400)	2000 (1600–3000)	0.23
Leukotriene receptor antagonists	160 (50.2)	88 (49.4)	35 (45.5)	37 (57.8)	0.33
Antihistamines	94 (29.6)	51 (28.3)	21 (27.6)	22 (35.5)	0.52
Nasal steroids	88 (27.5)	52 (28.9)	21 (27.3)	15 (23.8)	0.74
Theophylline	133 (41.6)	70 (39.1)	31 (40.3)	32 (50)	0.31
Steroid sparing meds	13	4 (2.2)	3 (3.9)	7 (11.3)	0.01
None	305				
Methotrexate	9	3	2	4	
Ciclosporin	1		1		
Azathrioprine	4	1		3	
Mycophenolate mofetil					
Anti-IgE treatment	8 (2.6)	2 (1.1)	1 (1.4)	5 (7.9)	0.01
Healthcare utilisation (past year)					
Unscheduled GP/A&E visits	263 (87.1)	148 (86.5)	65 (91.5)	50 (83.3)	0.36
No. of unscheduled visits	4 (2 - 7)	4 (4 -9.25)	5 (4 – 8)	6 (4 – 9.25)	0.02
Admissions	154 (48.7)	86 (48.0)	38 (51.4)	30 (47.6)	0.87
No. of admissions	2 (1 – 3)	2 (1 – 3)	2 (1 – 3.25)	2.5 (1 – 4.25)	0.13
ICU admissions to date	68 (21.4)	39 (21.8)	14 (18.7)	15 (23.4)	0.78
No. of ICU admissions	1 Range (1 - 2)	1 (1 – 2)	1 (1 – 2.3)	1 (1 – 1)	0.73

Group data (mean (95% CI)) for lung function, otherwise (mean ± SD or median (IQR)) for all subjects are presented in column 2 followed by data for individual BMI groups. Between group comparisons for continuous variables were made using Kruskal-Wallis (for posthoc analysis, see text).

PPI, proton pump inhibitor SABA, short-acting β_2-agonist BDP, beclomethasone dipropionate GP, general practitioner A&E, accident and emergency ICU, intensive care unit.

Table 3.9. Lung function and FE_{NO} (obese cohort).

	Overall	BMI Class I 30 to 34.99	BMI Class II 35 to 39.99	BMI Class III ≥40	P value
	(322, 48.3%)	**181 (56.2)**	**77 (23.9)**	**64 (19.9)**	
BMI	**34.2 (31.8 - 38.9)**	**32 (31 - 33.1)**	**37.3 (36.1 - 38.9)**	**43.6 (41.1 - 45.3)**	
Lung function and FE_{NO}					
FE_{NO} ppb	31 (13.5 - 57.3)	29 (13 – 48)	26 (13 – 50.3)	32.5 (15 – 62)	0.41
PreBD FEV_1 (litres)	1.91 (1.8 - 2.0)	1.82 (1.3 – 2.4)	1.8 (1.4 – 2.5)	2.1 (1.7 – 2.5)	0.1
(% predicted)	66.9 (64.2 - 69.5)	64 (46 – 83.5)	67.5 (46.5 – 86.8)	70 (55.3 – 81)	0.73
PreBD FVC (litres)	2.94 (2.8 - 3.1)	2.1 (1.5 – 2.74)	2.1 (1.48 – 2.4)	2.1 (1.7 – 2.5)	0.76
(% predicted)	80.1 (78.0 - 82.3)	80 (68 – 94)	80 (68.3 – 93)	78.5 (69.3 – 88)	0.82
Subjects with baseline bronchodilator (BD) study					
PreBD FEV_1 (litres)	1.86 (1.8 - 2.0)	1.82 (1.3 – 2.4)	1.67 (1.28 – 2.1)	1.83 (1.42 – 2.3)	0.6
(% predicted)	64.8 (61.8 - 67.8)	65.5 (46 – 81.5)	59 (43 – 83)	71 (57 – 85.5)	0.42
PreBD FVC (litres)	2.90 (2.8 - 3.0)	2.96 (2.4 – 3.64)	2.74 (2.2 – 3.25)	2.71 (2.3 – 3.38)	0.2
(% predicted)	78.8 (76.2 - 81.4)	80 (67 – 93)	78 (68 – 90)	84 (71.5 – 96.8)	0.5
PostBD FEV_1 (litres)	2.16 (2.1 - 2.3)	2.13 (1.5 – 2.74)	2.1 (1.5 – 2.42)	2.1 (1.7 – 2.55)	0.73
(% predicted)	75.4 (72.3 - 78.6)	74 (44.8- 91.3)	76 (53 – 94)	85 (67 – 95)	0.31
BDR (% Δ FEV_1)	20.4 (17.1 - 23.7)	18.7 (14.8-22.7)	24.4 (15.7-33.1)	20.0 (14.3-25.8)	0.6
PostBD FVC (litres)	3.19 (3.1 - 3.3)	3.27 (2.64 – 3.85)	2.97 (2.42 – 3.76)	2.97 (2.74 – 3.47)	0.35
(% predicted)	87.0 (84.7 - 89.3)	87 (75.5 – 96)	90 (75.3 – 96)	91 (78 – 103)	0.35
PreBD FEV_1/FVC ratio %	63.5 (61.5 - 65.4)	65 (57 – 76.8)	68 (52 – 72)	68.5 (57 – 76.8)	0.26
PostBD FEV_1/FVC ratio %	67.5 (65.4 - 69.5)	67 (57 – 76)	70.5 (60 – 78)	72.5 (60.3 – 81)	0.39
TLC % predicted (n)	100.3 (98.2-102.5)	102 (91 – 111)	99 (88.8 – 109)	98 (83.8 – 108.3)	0.19
RV % predicted (n)	128.7 (123.1-134.3)	127 (99 – 155.5)	122 (103 – 151)	123.5 (88.5–141.8)	0.37
K_{CO} % predicted (n)	104.5 (102.1-106.9)	103 (90.8 – 117.8)	104 (91.8 – 118.3)	105.5 (98.3–117.5)	0.49

Between group comparisons for continuous variables were made using Kruskal-Wallis (for posthoc analysis, see text). Group data (mean (95% CI)) for lung function, otherwise (mean ± SD or median (IQR)) for all subjects are presented in column 2.

FEV_1, forced expiratory volume in 1 second FE_{NO}, exhaled nitric oxide FVC, forced vital capacity K_{CO}, carbon monoxide transfer coefficient BDR, bronchodilator reversibility PreBD, pre-bronchodilator PostBD, post-bronchodilator TLC, total lung capacity RV, residual volume.

Table 3.10. Blood testing and radiology (obese cohort).

	Overall	BMI Class I 30 to 34.99	BMI Class II 35 to 39.99	BMI Class III ≥40	P value
	(322, 48.3%)	**181 (56.2)**	**77 (23.9)**	**64 (19.9)**	
BMI	**34.2 (31.8 - 38.9)**	**32 (31 - 33.1)**	**37.3 (36.1 - 38.9)**	**43.6 (41.1 - 45.3)**	
Blood testing and radiology					
Blood eos count x 10^9/l (n)	0.26 (0.11 - 0.49)	0.27 (0.1 – 0.58)	0.22 (0.1 – 0.37)	0.26 (0.13 – 0.4)	0.33
Sputum eos count (n)	2.8 (1.0 - 9.7) (84)	4 (1 – 10.3)	1.3 (0.2 – 4.3)	1.5 (0 – 11.3)	0.069
IgE kU/l (n)	119 (46–418) (296)	157.5 (55.1–457.5)	115.5 (58.8–270.5)	70.5 (32.5–349.5)	0.18
Bone density (T scores) (n)					
L1 to L4	-0.55 ± 1.4 (119)	-0.68 ± 1.5 (76)	-0.42 ± 1.1 (24)	-0.22 ± 1.4 (19)	0.43
Femoral neck	+0.074 ± 1.2 (113)	-0.2 ± 1.1 (73)	0.5 ± 1.1 (23)	0.7 ± 1.3 (17)	0.005
HRCT					
Reported normal (n)	38, 20.9% (182)	20 (17.1)	7 (17.5)	11 (37.9)	0.039
Abnormal HRCT (n)	155 , 78.3% (198)	99 (84.6)	35 (71.4)	21 (65.6)	0.028
Central bronchiectasis (n)	9, 6.7% (135)	7 (8.4)	1 (3.4)	1 (5)	0.62
Other bronchiectasis (n)	30, 20.8% (144)	20 (22.0)	8 (24.2)	2 (10)	0.42
Bronchial wall thickening (n)	103, 68.7% (150)	68 (70.8)	23 (65.7)	12 (63.2)	0.73
Bronchial dilatation (n)	25, 17.0% (147)	20 (21.3)	3 (8.8)	2 (10.5)	0.18
Ground glass (n)	15, 10.5% (143)	11 (12.0)	3 (9.1)	1 (5.6)	0.69
Air trapping (n)	32, 22.2% (144)	19 (20.9)	7 (20.6)	6 (31.6)	0.57
Mucus plugging (n)	15, 10.4% (144)	9 (9.8)	5 (15.2)	1 (5.3)	0.5
Emphysema (n)	7, 4.6% (151)	6 (6.2)	1 (2.9)	0	0.42

Between group comparisons for continuous variables were made using Kruskal-Wallis (for posthoc analysis, see text).
FEV_1, forced expiratory volume in 1 second, Group data (mean (95% CI)) for lung function, otherwise (mean ± SD or median (IQR)) for all subjects are presented in column 2 followed by FVC, forced vital capacity, K_{CO}, carbon monoxide transfer coefficient.
IgE, immunoglobulin E L1, first lumbar vertebra L5, fifth lumbar vertebra HRCT, high resolution computerised tomography.

3.4 Discussion

This study has found that overweight and obesity are common findings in severe asthma (77.6% of the study population) and these patients display particular characteristics according to BMI. These include greater medication use in terms of SABA use per day, nebulisers and oral steroid requirements (both maintenance and short burst therapy). Additionally, severe asthmatics that are obese reported greater GORD and PPI use but were less likely to have a history of nasal polyposis. Lung function testing revealed reduced FVC, TLC and elevated carbon monoxide transfer coefficient. This group were also less likely to be in full time work due to their asthma symptoms. These factors support the hypothesis that that obesity-associated severe asthma represents a distinct clinical phenotype.

Obesity is frequently encountered in severe asthma and was observed in just under half of this cohort of severe asthmatics taken from the National Registry for dedicated UK Difficult Asthma Services. This is greater than that seen in the general population, almost a quarter of the UK population aged ≥16 (care, 2011). Determining whether obesity is a co-morbidity, affecting pathophysiology and response to therapy, or secondary to disease driven inactivity and/or systemic steroid therapy is often unclear and requires further study.

The severe asthmatics analysed in this cohort were predominantly female and the median BMI was just below the obese range. This compares with other cohorts of severe asthmatics such as TENOR (adult mean 30.4) (Dolan et al., 2004) and is higher than in the ENFUMOSA cohort (male mean 26.5, female mean 27.2) (Group, 2003). The majority (77.6%) of patients in this cohort had a BMI of ≥25 and just under half had a BMI of ≥30.

The overall figure for the cohort of 9% for current smokers is lower than the rates in asthmatics of all severity in the UK (just under 25%) (report, 2005) and in the general population where the proportion of obese adults who smoke is approximately 20% (Healton et al., 2006). Just over 30% of the cohort were ex-smokers which is higher than the figure of 18% (Cerveri et al., 2012) seen in subjects with asthma of all severity in a large cohort and 21% seen in the general population (glance, 2013). The overall difference may reflect the impact of disease severity on patients and greater clinical input from primary and secondary care with regard to smoking cessation. The differences between BMI groups is harder to explain, but may be related to health beliefs and perceptions, or potential concerns regarding further weight gain following smoking cessation.

Severe obese asthmatics were less likely to be in full-time employment and asthma was volunteered as the reason for not working in a third of this group. This is in contrast to the overweight and normal weight groups where 23.6% and 19.6%, respectively, felt their asthma prevented them from working. This observation may be related to increased inactivity and reduced mobility, potentially secondary to greater disease severity. However, the lower levels of educational achievement (33.3% achieving an advanced, graduate or post-graduate level compared to 40.7% and 52.8% respectively) seen in the obese group are also likely to play an important role. The relationship between employment and educational achievement is complex and a lack of information regarding the development of obesity and asthma diagnosis makes it difficult to infer causality.

The view that increasing BMI in severe asthma is associated with CS insensitivity is supported by the findings from this cohort. CS burden was greatest in the obese group, where just fewer than half the patients were on maintenance oral

prednisolone and short burst steroid use was increased. Previous studies have noted that obese asthmatics appear to be less responsive to ICS. A *post hoc* analysis, pooling data from four double-blind, placebo controlled studies, randomising 3073 moderate adult asthmatics to montelukast (1439), beclomethasone (894) or placebo (740) noted that the response to ICS decreased with increasing BMI. Interestingly, the response to placebo was also lower with increasing BMI whilst that to montelukast remained stable. Taken together, this implies that BMI influences placebo and drug response in asthma (Peters-Golden et al., 2006). Another study looked at five clinical trials incorporating 1242 asthmatic patients who were not taking ICS prior to enrolment. Obese patients, particularly those with a BMI ≥40 had a lower probability of achieving well-controlled asthma with FP or FP/salmeterol therapy (Boulet and Franssen, 2007). Other retrospective studies have noted similar findings (Camargo et al., 2010a, Camargo et al., 2010b). There is a paucity of molecular data supporting the link between obesity and corticosteroid insensitivity. One *in vitro* study using PBMCs and BAL cells taken from 45 non-smoking adults (33 with asthma, 12 healthy subjects) found that asthmatics with a higher BMI showed relative corticosteroid insensitivity in terms of attenuated dex ($10^{-6}M$) induced MKP-1 suppression (Sutherland et al., 2008a).

Although the obese group were more likely to be using maintenance oral steroids, there was no significant difference in the dose between groups. This difference may in fact reflect that raised BMI is a consequence of long term steroid use.

In addition to differences in steroid use, SABA use per day was greater in the overweight and obese group and nebulised bronchodilator use increased alongside BMI, suggesting that asthma symptoms increase with increasing BMI. A study of 366

adults with well characterised asthma taking part in the Epidemiological Study on the Genetics and Environments of Asthma, looking at the relationship between BMI and asthma severity by sex, found that asthma severity was significantly correlated with increasing BMI in women but not in men (Varraso et al., 2005). This difference persisted despite adjusting for confounders and the association appeared strongest in women with early menarche. Higher BMI has also been associated with lower Asthma Quality of Life Questionnaire (AQLQ) scores and greater Asthma Control Questionnaire (ACQ) scores (Lavoie et al., 2006).

Although no significant difference was noted between BMI groups in terms of health care utilisation, other studies have noted hospitalisation rates (Rodrigo and Plaza, 2007) and unscheduled doctor visits (Taylor et al., 2008) are greater in obese patients with asthma compared to under/normal weight subjects with asthma.

GORD and PPI use were reported more in the obese group as was an abnormal 24 hour oesophageal pH study. The link between GORD and obesity in the general population remains unclear and a recent meta-analysis of 30 studies found a weak to moderate association. However, sub-analysis of subjects with a BMI greater than 35 revealed that they were six times more likely to suffer with GORD than their normal weight counterparts (Eslick, 2012). GORD was reported by 49.1% of this cohort, which is slightly higher than the 41% reported in severe asthmatics in the SARP cohort (Moore et al., 2007) and in contrast to 12.5% to 30% seen in unselected adult cohorts with a BMI of ≥30 (Nilsson et al., 2003, Locke et al., 1999). GORD in severe asthma is a common finding and is compounded by systemic corticosteroid use. This is particularly relevant given that steroid use in our cohort increased with BMI.

Asthma is classically described as being characterised by type 2 T helper cell (T_H2) mediated allergic inflammation. There appears to be no correlation between obesity and atopy (Loerbroks et al., 2008, von Mutius et al., 2001) or eosinophilia (Lugogo et al., 2010). Obesity has been associated with a lack of both eosinophilic and neutrophilic inflammation in the airways (Todd et al., 2007, Dixon et al., 2010). The lack of eosinophilic inflammation is supported by the similar sputum eosinophil counts and FE_{NO} levels between groups in this cohort, a finding that has been noted in previous studies (Sutherland et al., 2008b). IgE levels and aspergillus sensitisation decreased with BMI despite a lack of difference in a prior history of atopy. These findings fit well with the reduced atopy and lower IgE levels in conjunction with raised BMI that were factors in 2 of 5 clusters in the SARP cohort (Moore et al., 2010). A recent study looking at 131 severe asthmatics, categorised into lean, overweight and obese groups according to BMI found that sputum IL-5 and submucosal eosinophils, but not sputum eosinophils, were greater in the obese group (Desai et al., 2013). This study suggests that eosinophilic inflammation may play an important role in severe obese asthmatics who have until now been labelled as non-eosinophilic and/or paucigranulocytic.

In this cohort, the obese group were more likely to report a history of eczema (31.4%) than the overweight (23.9%) or normal weight (22.6%) group. This is similar to the 33.9% of obese adults in a recent study looking at patients presenting to an allergy clinic who were found to have atopic dermatitis (Silverberg et al., 2012). However, both cohorts relied on self-reporting and this may be a bias in the obese population. These collective findings, in addition to a non-significant difference in FE_{NO} and lower IgE levels noted in the obese group, are discordant, and potentially other skin complaints may have been misreported as eczema. Obesity has been

associated with a reduction in FEV_1 and FVC (Schachter et al., 2001), findings that were also seen in this cohort. In healthy obese adults, the reductions in FEV_1 and FVC are usually small with preservation of FEV_1/FVC ratio (Schachter et al., 2001, Gurkan et al., 2004, Sood et al., 2006). These findings are essentially seen in this cohort of severe asthmatics where the reductions in FEV_1 and FVC and the degree of airflow obstruction were not markedly different between BMI groups.

Obesity is associated with increased intra-abdominal pressure on the diaphragm and the chest wall, leading to lower tidal volumes (TV), functional residual capacity (FRC) and expiratory reserve volume (ERV). The FEV_1/FVC ratio may be normal or elevated if gas trapping and airway closure reduce FVC (Dixon et al., 2010). The small but significant decrease in total lung capacity (TLC) seen in this cohort with increasing BMI has been well documented in previous studies (Jartti et al., 2009, Jang et al., 2009). Despite the small reduction in TLC, absolute levels remained within normal limits and residual volumes (RV) were maintained. These findings have also been shown previously (Tsaroucha et al., 2013, Sood et al., 2006). The mechanism for the reduction in TLC is not completely understood but may reflect the mechanical effects of adipose tissue. This includes limiting the volume for lung expansion, preventing the downward movement of the diaphragm and a reduced chest cavity volume due to adipose deposition in the subpleural space (Jartti et al., 2009). This is supported by an increase in TLC seen in obese subjects following weight loss (Sood, 2012, Sood et al., 2012).

Obesity appears to be associated with greater bone density, particularly at the femoral neck and this was greatest in the most obese subgroup (class III). Previous studies have reported that obesity exerts a protective effect on bone density and that a decrease in body weight correlates with associated bone loss. This correlation has

been noted in men and women, all adult age ranges and at all assessed sites in the skeleton (Zhao et al., 2008, Ravn et al., 1999). This is felt to be secondary to several mechanisms, including greater mechanical stress placed on bone secondary to the greater fat mass, increased levels of oestrogens and adipokine imbalance (Greco et al., 2010). Additionally, adipocytes and osteoblasts originate from the same progenitor, pluripotential mesenchymal stem cells (Akune et al., 2004). Conversely, other studies have suggested that adiposity is not associated with protection against osteoporosis (Janicka et al., 2007). One study found that fat mass negatively correlates with bone mass when the mechanical effect of total body weight is statistically removed (Zhao et al., 2007). Furthermore, even though obesity appears to be associated with increased bone density, the quality of the bone may be reduced and the fracture risk increased (Blum et al., 2003).

Analysis of the subdivisions of the obese cohort revealed that the super-obese (class III) are more likely to be female, current smokers, had greater use of their short-acting β_2-agonist per day and was more likely to be taking steroid sparing medications or anti-IgE therapy. These findings are similar to the initial analysis according to normal weight, overweight and obese groups but suggest that the differences are magnified in the super-obese.

There are several limitations to this study, which should be considered when interpreting these results. BMI is a crude population measurement and does not take account of body composition, fat distribution and racial variance, although these factors are not routinely measured in clinical practice. Increasing BMI is associated with comorbidities that may influence respiratory symptoms such as impaired ventilatory function (reduced FEV_1, FVC, TLC, FRC and ERV), sleep disordered breathing, reduced exercise tolerance and GORD. This may result in a diagnostic

bias in severe asthma. A study looking at 540 individuals with physician diagnosed asthma found that just under a third of those assessed did not have objective evidence of asthma. There were no differences between the obese and normal weight groups in terms of asthma diagnosis suggesting an over diagnosis of asthma was not more likely to occur in obese individuals (Aaron et al., 2008). However, patients in this cohort were subject to a detailed systematic assessment and multidisciplinary decision regarding their asthma diagnosis prior to inclusion, which makes over diagnosis less likely to be a significant factor. This is supported by a mean bronchodilator reversibility for the entire cohort of 19.6% (95% CI, 17.4% to 21.7%), with no significant difference between BMI groups. In addition, whilst our previous study identified differences in severe asthma characteristics between different centres, BMI figures remained similar.

Although the use of BMI is convenient and widely accepted as a measure of adiposity, it may not be the best measure of the effect of adiposity on the lung (Dixon et al., 2010). Central body fat distribution may be a better associated factor with the mechanical effects of obesity on lung function (Collins et al., 1995). The National Registry does not include data regarding exercise habits or levels of physical activity, which prevents any inferences regarding their role on BMI in severe asthma. In addition, the cohort of patients in this study did not have temporal information regarding the development of obesity. This would provide insight into the direction of causality for many of the factors discussed. It has been suggested that early onset asthmatics become obese following a diagnosis of severe asthma, whilst those with late onset disease are more likely to have severe disease resulting from obesity (Holguin et al., 2011). Obesity-associated severe asthma is likely to comprise a spectrum of patients with two distinct ends: severe asthmatics who gain weight

secondary to disease related inactivity and corticosteroid use, and those where obesity predated the diagnosis of asthma, contributing to symptoms and relative corticosteroid insensitivity.

Analysis of this large cohort of severe asthmatics provides evidence that obese severe asthmatics may represent a distinct clinical phenotype within the severe asthma population. Further studies looking at the mechanisms by which obesity affects asthma as well as longitudinal studies designed to explore the temporal role of obesity in asthma will improve our understanding of this group of patients with the ultimate aim of providing targeted therapeutic interventions.

Chapter 4: The relationship between lipid-laden macrophages, asthma and BMI

Chapter 4. The relationship between lipid-laden bronchoalveolar macrophages, asthma and BMI

Hypothesis and aim

Obesity related severe asthma is associated with increased lipid laden macrophages. Lipid laden macrophages in bronchoalveolar lavage cells will be analysed using Oil Red O staining and their relationship with asthma severity and BMI will be examined.

4.1 Introduction

Macrophages were the first immune cell type to be identified in adipose tissue (Sood et al., 2011, Govindaraju et al., 2006) and increasing adiposity is associated with macrophage recruitment and inflammation. Adipose tissue macrophages are thought to clear dead, hypertrophied adipocytes and have been postulated to result in LLMs (Sood et al., 2011). Alveolar macrophages are responsible for the phagocytosis and endocytosis of dying cells and pathogens. They are derived from circulating bone marrow-derived monocytes which then become tissue macrophages. These links suggest that AMs may correlate with obesity and in particular there may be higher numbers of LLMs in the obese state.

AMs were thought to develop from circulating bone marrow-derived monocytes. They have recently been shown to develop from primitive precursors before birth (Geissmann et al., 2010) and the overall pool of AMs is only minimally made up of bone marrow-derived monocytes with two groups of researchers suggesting that there may be a self-maintaining, proliferating pool of macrophages in the lung (Gibson et al., 2001, Chanez et al., 1996).

AMs that become laden with lipid (predominantly cholesterol) are termed pulmonary foam cells (PFCs) or lipid-laden alveolar macrophages (LLAMs). Their formation may be due to endogenous sources, such as with endogenous lipoid pneumonia, or an exogenous source, for example following aspiration (Hadda and Khilnani, 2010). These macrophages are more likely to undergo apoptosis and exhibit impaired phagocytosis (Schrijvers et al., 2005), which may play an important pathogenic role in asthma. Furthermore, markers of lipid peroxidation measured in plasma are increased in asthma subjects (Rahman et al., 1996) whilst erythrocytes and platelets exhibit alterations in membrane fatty acid composition (De et al., 2007a). These factors may lead to increased PFC formation in asthma.

A high fat content diet and the administration of a lipid emulsion in rats has been associated with the formation of LLAMs, leading to the view that the lungs may be a potential route for lipid excretion. However, it remains unclear whether such cells are derived from AMs ingesting lipids in the alveoli or from lipid-laden blood monocytes that migrate to the alveoli (Shibuya et al., 1991). In addition, macrophages have been shown to internalise and degrade surfactant lipids and surfactant protein A (SP-A) *in vitro*, suggesting a role for AMs in surfactant clearance (Wright and Youmans, 1995). Other factors such as obesity and cholesterol may also influence lipid-laden macrophage formation, but their role has not yet been established.

Lipid accumulation in AMs appears to occur primarily through the phagocytosis of external lipoproteins. This process may be secondary to endogenous sources, such as lipoid pneumonia, where the presence of bronchial obstruction, chronic lung infection or a lipid storage disorder results in the accumulation of LLAMs; or an exogenous source, for example following aspiration or

inhalation of lipid (Hadda and Khilnani, 2010). There are other factors which have been associated with an increase in LLAM formation. These include gastro-oesophageal reflux disease (GORD) (Adams et al., 1997, Parameswaran et al., 2000), hypoxia (Bostrom et al., 2006) and iatrogenic causes, whereby drugs such as amiodarone, fluoxetine, and gentamicin containing a cationic amphiphilic structure can induce cellular phospholiposis through a dose-dependent process involving the inhibition of lysosomal phospholipase activity and accumulation of lamellar bodies (Reasor et al., 2006, Jacobson et al., 1997).

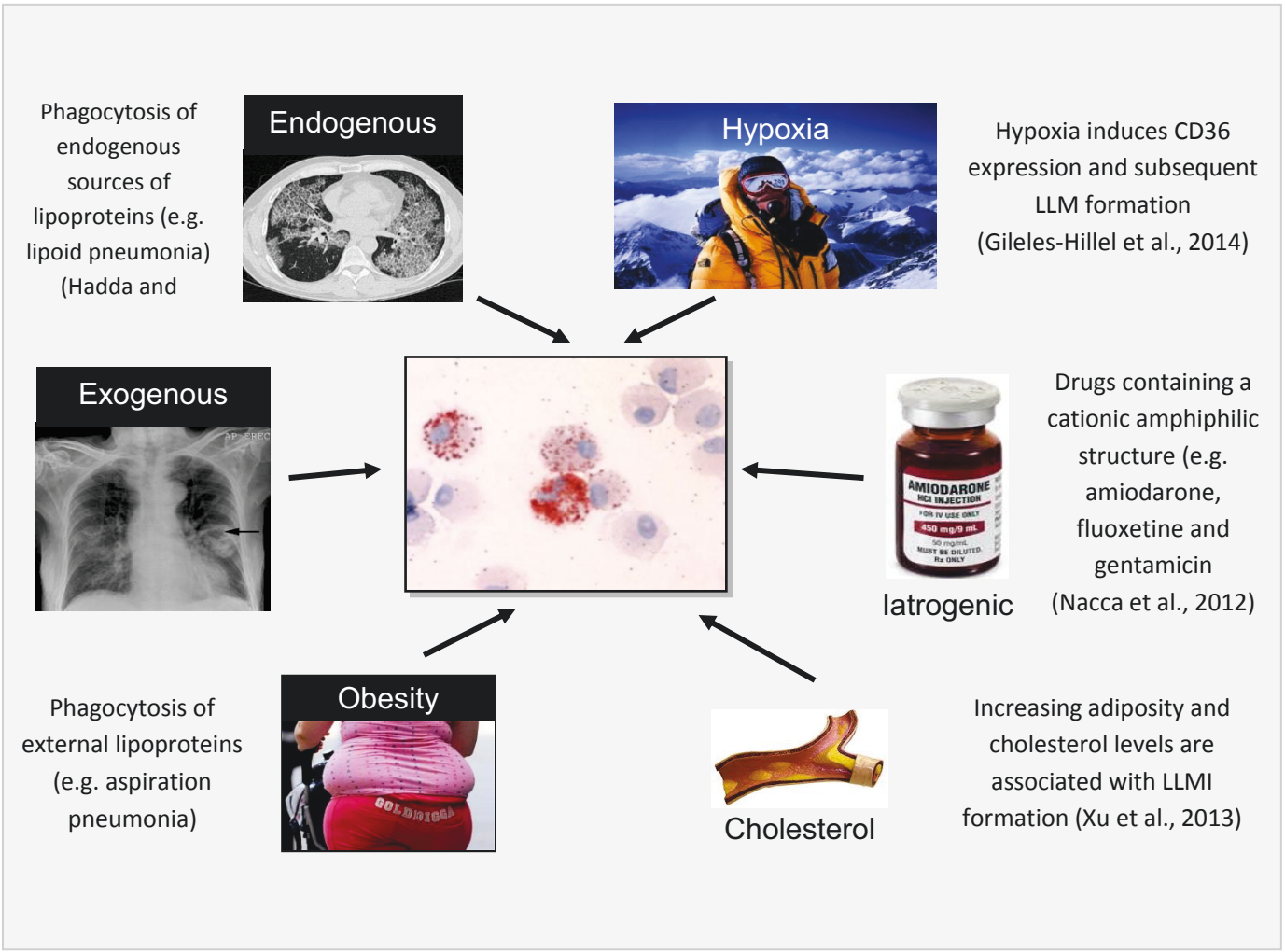


Figure 4.1. Potential factors involved in lipid laden macrophage formation.

The lipid laden macrophage index (LLMI) was first introduced in 1985 by Corwin and Irwin as a marker of aspiration in parenchymal lung disease (Corwin and Irwin, 1985). It is calculated using Oil-Red-O staining in BALF and grading the amount of intracellular Oil-Red-O per 100 AMs (figure 4.2).

LLMI has been identified as a marker of recurrent aspiration in children (Colombo and Hallberg, 1987) and correlates with increased airway neutrophils, suggesting a link with parenchymal inflammation (Sacco et al., 2006). However, other studies have failed to show a correlation (Knauer-Fischer and Ratjen, 1999, Chang et al., 2005) and the use of LLMI as a marker of aspiration and lung inflammation remains unclear.

The utility of the LLMI in adults as a marker of aspiration, inflammation or lung disease has not been investigated. LLMI could provide a novel way of identifying chronic aspiration and assessing the response to reflux treatment. Furthermore, increasing BMI and airway inflammation may result in LLM formation. These factors suggest that LLMI could potentially be a marker of asthma severity and correlated to obesity related severe asthma in particular.

In this chapter, the presence of LLAMs in asthma and their relationship with BMI are examined. The aim of this study was to explore the relationship between the presence of LLAMs and asthma severity in a cohort of patients with mild/moderate and severe asthma. In addition, the influence of obesity, GORD, lung inflammation and the degree of airflow obstruction were examined in terms of intracellular lipid accumulation.

This chapter led to the following publication:

Gibeon D, Zhu J, Sogbesan A, Banya W, Rossios C, Saito J, Rocha JP, Hull JH, Menzies-Gow AN, Bhavsar PK, Chung KF. Lipid-laden bronchoalveolar macrophages in asthma and chronic cough. Respir Med 2014; 108(1); 71-7.

4.2 Methods

4.2.1 Patients

Patients were recruited from our Asthma Clinic and through advertisement, and underwent a screening visit. Those with significant respiratory or inflammatory comorbidities and those requiring treatment for a respiratory tract infection within the preceding four weeks were excluded. All subjects underwent a bronchoscopic procedure with BAL.

Asthma was based on a physician diagnosis and patients either had a PC_{20} methacholine concentration causing a 20% fall in FEV_1 of <8mg/ml and/or an FEV_1 reversibility of ≥ 12% of baseline. Severe asthma was defined according to the ATS definition of refractory asthma (ATS, 2000). Patients who did not fulfil the criteria for severe asthma were classified as non-severe asthmatics and they were taking ≤1000mcg of inhaled beclomethasone dipropionate (BDP) or equivalent per day.

Two control groups were investigated. Healthy subjects had no smoking history and no history of respiratory or cardiac disease. Patients with chronic cough were recruited from a specialist cough clinic at our hospital and all subjects reported a dry persistent cough for at least 8 weeks duration. All subjects had been investigated with a standard protocol to exclude a diagnosis of asthma or other respiratory disease.

Ethical approval was obtained from the West London 3 and the Brompton, Harefield and NHLI Research Ethics Committees (11/H0706/4 and 08/H0708/29).

4.2.2 Fibreoptic bronchoscopy and bronchoalveolar lavage

Sixty-seven patients (21 severe asthma, 19 mild/moderate asthma, 16 patients with chronic cough, and 11 healthy subjects) underwent fibreoptic bronchoscopy with BAL. The methods for this have been discussed in section 2.2.7.

4.2.3 Measurement of lipids in macrophages

We used the LLMI to quantify lipid accumulation in AMs. Slides were stained with Oil-Red-O (Sigma-Aldrich Ltd, UK) to enable scoring and LLMI calculated by grading the amount of intracellular Oil-Red-O positive particles per 100 AMs. Intracellular lipid was evaluated using a modified version of that proposed by Corwin and Irwin (RW and RS, 1985, Colombo and Hallberg, 1987). Cells were scored from 0 to 4 whereby 0 = no opacification, 1 = up to ¼ opacified, 2 = ¼ to ½ opacified, 3 = ½ to ¾ opacified, and 4 = totally opacified (figure 4.2). The LLMI was calculated by the sum of the scores of 100 cells giving a potential range from 0 to 400. The person who measured the LLMI was not aware of clinical findings.

4.2.4 Statistical analysis

Categorical variables were reported as percentages and comparisons done using the chi–square or Fisher's exact test. Numerical variables were reported as mean (SD) or median (IQR) and comparisons done using Mann-Whitney test or Kruskal Wallis test. Statistical significance was defined as $p<0.05$. Logistic regression was used to assess the predictive factors for GORD.

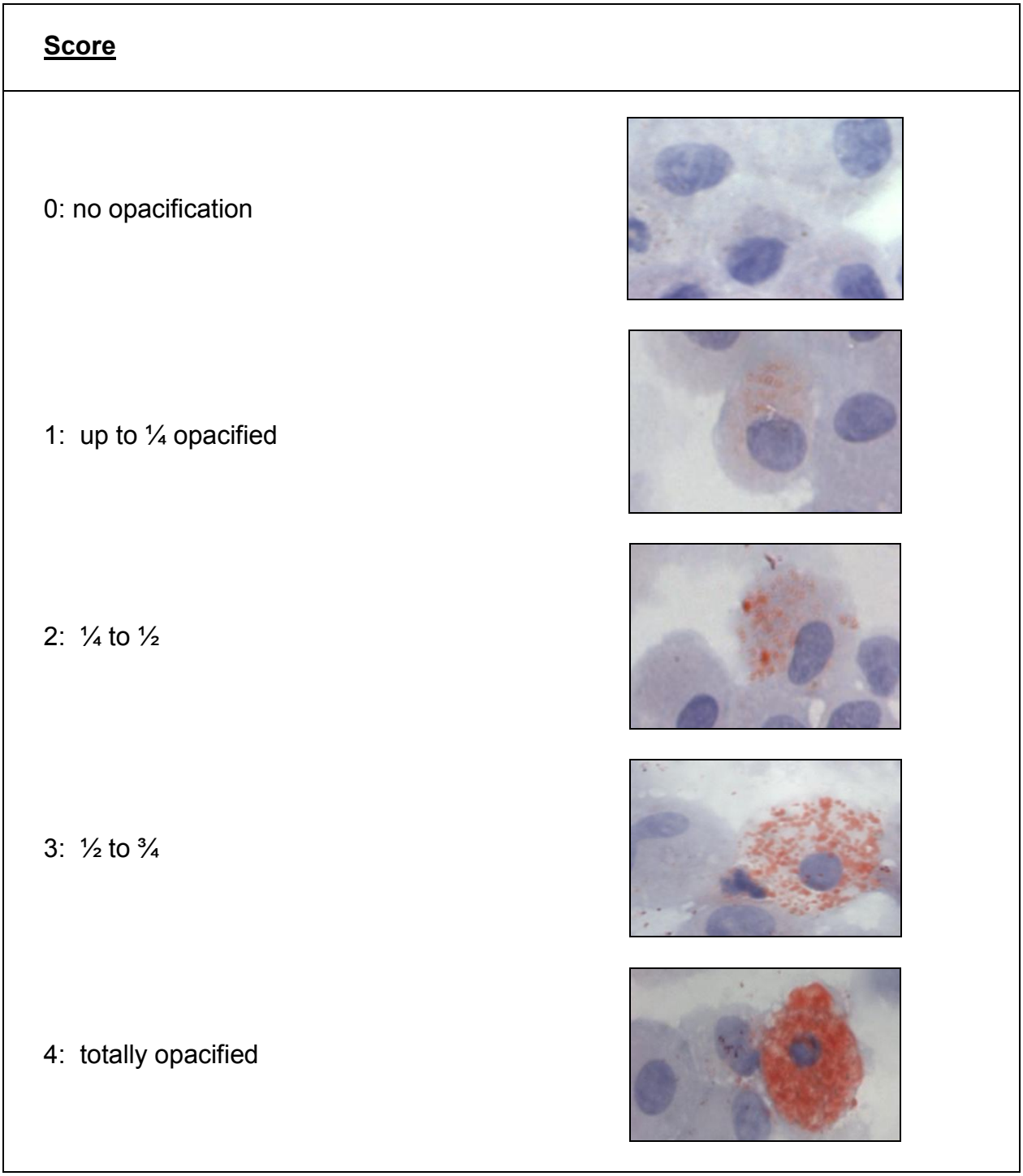


Figure 4.2. LLMI calculation.

Determination of lipid-laden macrophage index (LLMI). BAL cytospins were stained with Oil-Red-O. AMs were graded by the amount of lipid in the cytoplasm staining red. Cells were scored from 0 to 4: 0 = no opacification, 1 = up to ¼ opacified, 2 = ¼ to ½ opacified, 3 = ½ to ¾ opacified, and 4 = totally opacified. The panels show examples of each score. LLMI was reported as the sum of the scores of 100 cells giving a potential score range of 0 to 400.

4.2.5 Determination of lipid-laden macrophage index (LLMI)

BAL cytospins were stained with Oil-Red-O. AMs were graded by the amount of lipid in the cytoplasm staining red. Cells were scored from 0 to 4: 0 = no opacification, 1 = up to ¼ opacified, 2 = ¼ to ½ opacified, 3 = ½ to ¾ opacified, and 4 = totally opacified. The panels show examples of each score. LLMI was reported as the sum of the scores of 100 cells giving a potential score range of 0 to 400.

4.3 Results

4.3.1 Demographics

67 patients (21 severe asthma, 19 mild/moderate asthma, 16 patients with chronic cough, 11 healthy subjects) with BAL specimens were included in the study. Two patients (1 severe and 1 mild/moderate asthma) were excluded from subsequent analysis due to poor quality of the staining and five patients had missing BAL differential cell count data.

Significant differences were seen between groups in terms of inhaled and oral corticosteroid use, FEV_1 % predicted and FEV_1/FVC ratio (table 4.1). 14 out of 21 (66.7%) patients with severe asthma were taking maintenance OCS with a median dose of 10mg a day. This group were also taking high dose ICS (median 1600 µg, IQR [1600 – 4000]) compared to the mild/moderate group (median 200 µg, IQR [0 – 600]). The severe asthma group exhibited greater bronchodilator reversibility and lower PC_{20}. Both asthmatic groups were more likely to be atopic than the chronic cough and healthy control group (81% and 87.5% compared to 33.3%, 27.3% respectively; p<0.0005). Sixteen patients (28.6 %) reported a history of GORD of which 14 were taking medication with proton pump inhibitors. A greater history of GORD and PPI use was noted in the severe asthma (40% and 42.1% respectively) and chronic cough (41.7% and 44.4%) groups compared to the mild/moderate asthma (21.4% and 13.3%) and healthy control (none reported) groups.

BAL differential cell counts were similar between all the groups with an increase in eosinophils noted in the mild/moderate group (p<0.05). A higher neutrophil percentage in BALF was noted in the severe group (median 3.4%, [IQR 0.9 - 7.3]), but this did not reach statistical significance (table 4.3).

Table 4.1. Demographics.

	Healthy subjects	Chronic cough	Mild/moderate asthma	Severe asthma	*P value*
N	11	16	17	21	
Age (years)	35.6 ± 13.9	43.1 ± 12.8	43.7 ± 14.6	43.8 ± 12.5	0.34
M/F	8/3	5/11	11/6	11/10	0.13
BDP eq (µg)	0	0 (0 - 500)	200 (0 - 600)	1600 (1600 - 4000)	<0.0001
Oral steroid (n)	0 (0)	0 (0)	0 (0)	10 (0 - 20) 14	
BMI	26.9 (25.6 - 32.4)	25.5 (23.9 - 27.4)	24.9 (21.1 - 30.3)	28.4 (24.6 - 33.5)	0.33
Smoking					
Current	0	1	0	0	0.45
Ex	1	1	0	3	
Never	10	13	17	18	
FEV_1 %	104.8 (98.2 - 108.0)	87.7 (83.6 - 100.4)	84.9 (69.8 - 88.9)	74.9 (60.6 - 84.0)	<0.0001
FEV_1/FVC %	81.6 (75.6 - 86.1)	79.0 (75.3 - 84.4)	69.9 (67.7 - 74.2)	64.7 (54.5 - 72.9)	<0.0001
BDR (n)	1.4 ± 2.3	NA	13.9 ± 8.2 (14)	22.8 ± 13.8 (17)	0.0002
PC_{20} (n)	>16 (11)	>16 (3)	2.3 ± 2.5 (14)	0.88 ± 0.6 (5)	<0.0001
FE_{NO} (n)	20.1 ± 12.8 (5)	37.9 ± 19.4 (3)	43.5 ± 37.7 (12)	45.3 ± 39.6 (12)	0.6
Atopic (n, %) *	3 (10, 27.3)	4 (12, 33.3)	14 (16, 87.5)	17 (21, 81.0)	0.0005
IgE kU/l	73.5 (21 - 126.3)	58.5 (16 - 137.5)	151.0 (63 - 268.5)	184 (49.5 - 615.5)	0.06
GORD (n, %)	0 (11, 0)	6 (12, 50)	2 (14, 14.3)	8 (19, 42.1)	0.02
PPI (n, %)	0 (11, 0)	4 (9, 44.4)	2 (16, 12.5)	8 (19, 42.1)	0.03

Data are presented as mean ± SD or median (IQR). Between group comparisons for continuous variables were made using Kruskal-Wallis test and for categorical variables using Chi Squared exact testing.

BDP, beclomethasone dipropionate BMI, body mass index BDR, bronchodilator response PPI, proton pump inhibitor.

*The data presented are for subjects with either a positive RAST or skin prick test.

4.3.2 Lipid-laden macrophage index (LLMI) in different groups

LLMI exhibited a wide range in the severe asthma (0 to 246) and chronic cough (0 to 297) groups. The distribution of LLMI by diagnoses can be seen in figure 4.3. The mild/moderate asthmatic group had the lowest LLMI (median, 7; IQR 0.5-37) with a significant difference noted versus the severe asthma (median, 37; IQR 11.5–61, $p<0.05$) and healthy groups (median, 48; IQR 10-61, $p<0.05$). No significant difference was noted between the other groups.

No significant associations were noted between BALF cell differential and the LLMI for the entire cohort or for individual groups. One chronic cough patient with an LLMI of 297 had a BAL neutrophilia of 69% although, in two other cases where the LLMI was greater than 200, there was no neutrophilia.

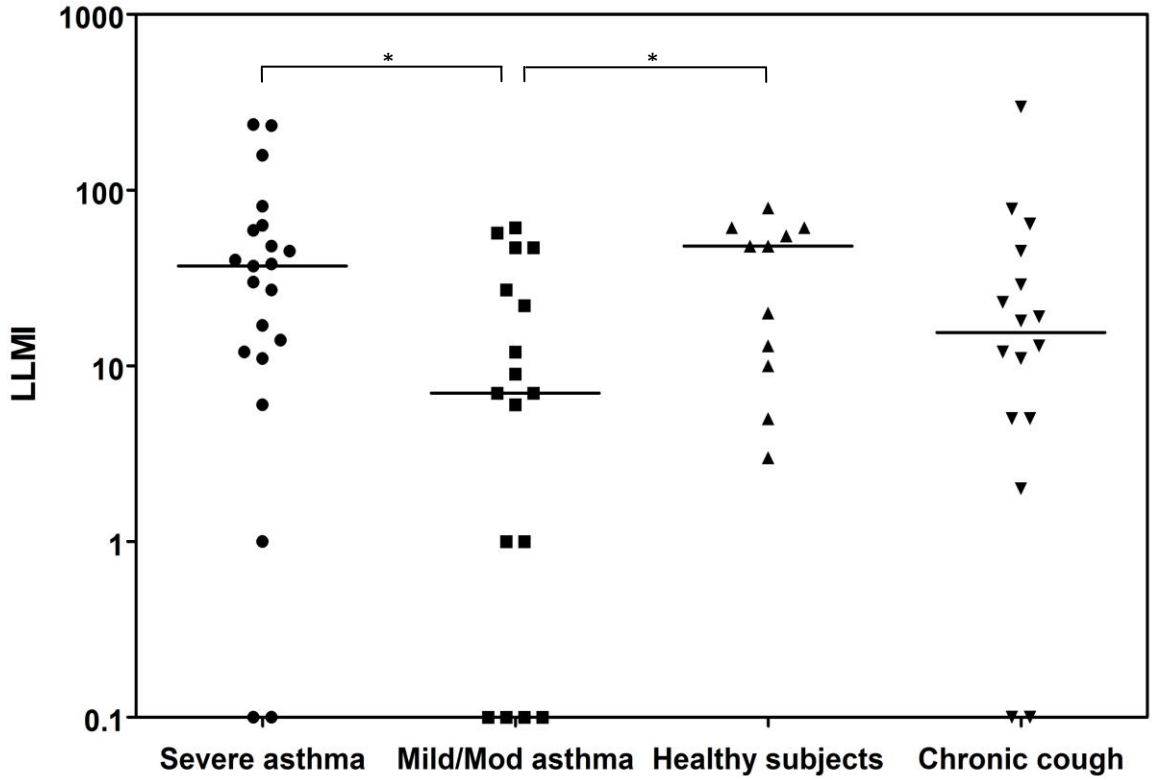


Figure 4.3. LLMI by diagnosis. LLMI scores were calculated for all cohorts. Mild/moderate asthmatic exhibited a significantly lower LLMI score compared to the severe asthmatic and healthy cohorts. Horizontal lines represent median. *p<0.05.

Table 4.2. LLMI by diagnoses.

Diagnosis	No.	Mean of LLMI	Median (IQR) (SD)	Minimum LLMI	Maximum LLMI
Healthy subjects	11	36.6 (26.9)	48 (10 - 61)	3	79
Chronic cough	16	38.8 (72.5)	15.5 (5 - 41)	0	297
Mild/moderate asthma	17	17.9 (21.7)	7 (0.5 - 37)	0	61
Severe asthma	21	55.1 (69.5)	37 (11.5 - 61)	0	246

4.3.3 Lipid-laden macrophage index (LLMI) and BAL differential cell count

BALF differential cell counts were similar between all the groups with a small but significant increase in eosinophils noted in the mild/moderate group (p<0.05). A higher neutrophil percentage was noted in the severe group (median 3.4%, [IQR 0.9 - 7.3]) although this did not reach statistical significance (table 4.3).

Table 4.3. Differential cell counts in BALF.

	Healthy subjects	Chronic cough	Mild/moderate asthma	Severe asthma	*P value*
N	11	15	16	18	
Neutrophils %	1.5 (0 - 1.8)	1.7 (0.9 - 5.2)	1.2 (0.5 - 2.6)	3.4 (0.9 - 7.3)	0.15
Eosinophils %	0.3 (0 - 0.8)	0.2 (0 - 1.0)	1.4 (0.3 - 6.4)	0.6 (0 - 3.8)	0.03
Macrophages %	90.2 (55.8 - 95.8)	90.4 (64.1 - 94.3)	87.0 (73.8 - 93.2)	87.4 (75.5 - 93.3)	0.98
Lymphocytes %	7.5 (2.0 - 39.8)	5.7 (4.0 - 24.7)	5.4 (2.1 - 12.0)	5.6 (2.0 - 12.9)	0.84

4.3.4 Lipid-laden macrophage index (LLMI) and GORD

Six of the top eight subjects with the highest LLMI scores, including the top four, reported a history of GORD. The median LLMI score was higher in the GORD group (median 41.5 [IQR, 11.3 - 138] versus 12.5 [2.3 – 35.3]) compared to those who did not (p<0.05) (figure 4.4). Univariate logistic analysis (Table 4.5) showed a significant association between the presence of GORD and an LLMI score of ≥ 60.

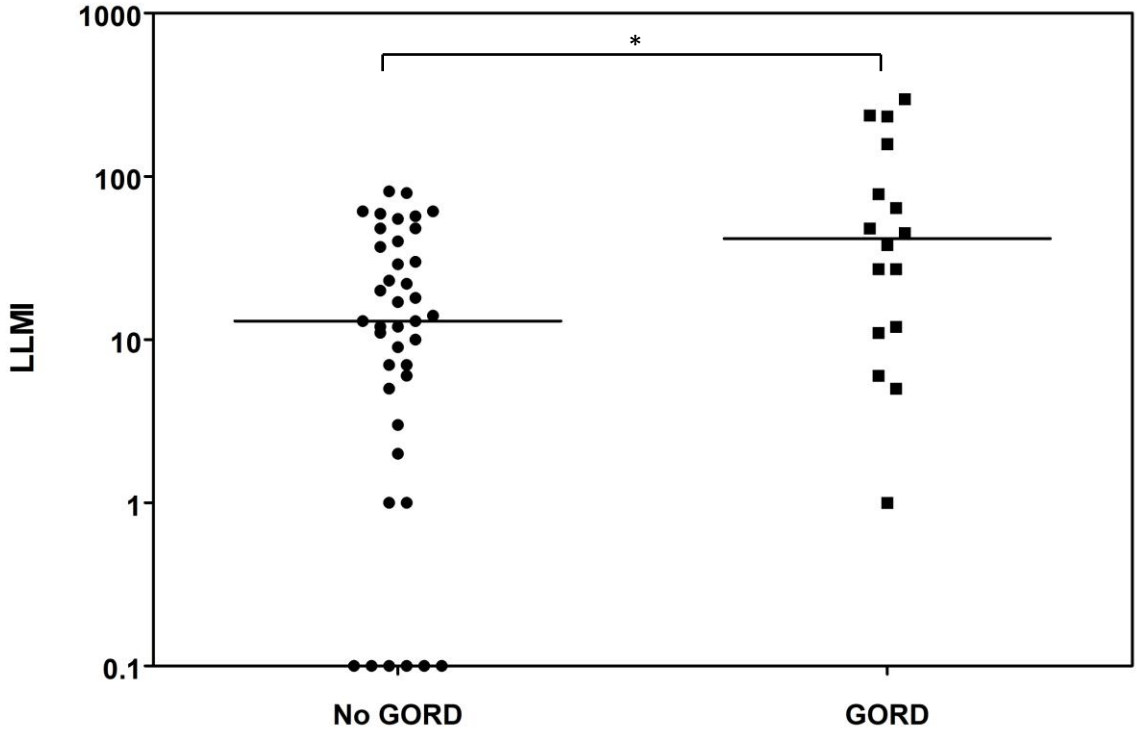


Figure 4.4. LLMI and GORD. Subjects with GORD exhibited a significantly higher LLMI score compared to those without. Horizontal lines represent median. *p<0.05.

Table 4.4. GORD and LLMI.

GORD	No. of patients	Mean of LLMI (SD)	Median (IQR)	Minimum LLMI	Maximum LLMI
Yes	16	80.38 (95.8)	41.5 (11.3 - 138)	1	297
No	40	22.8 (23.7)	13 (0 - 39.3)	0	81

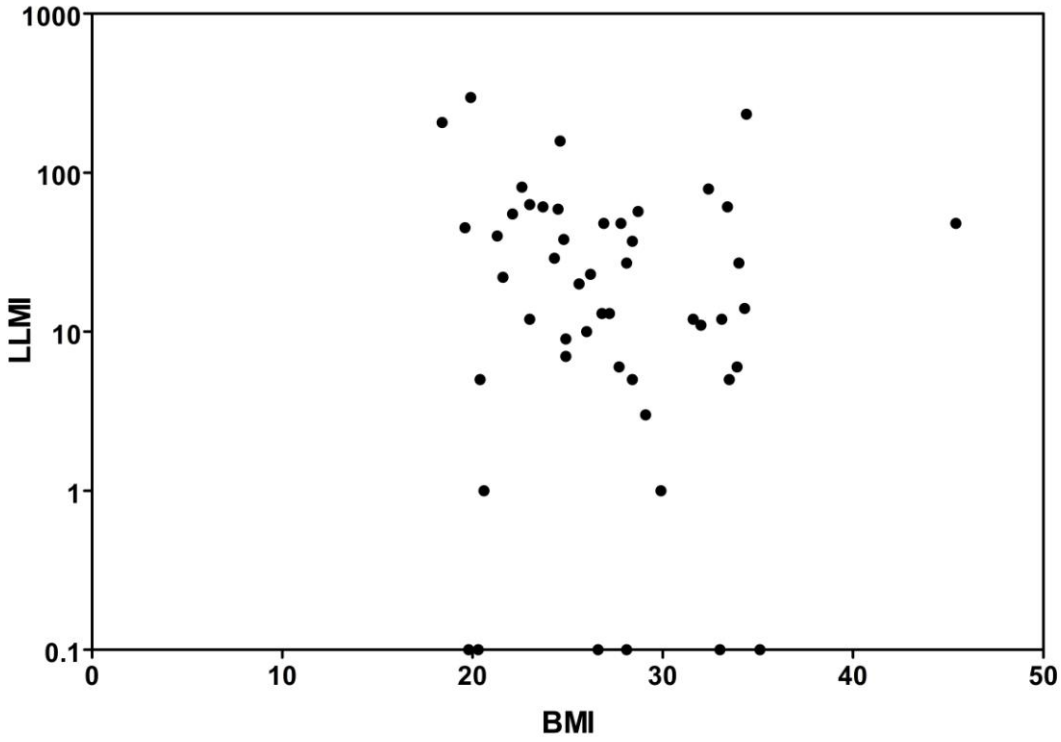


Figure 4.5. LLMI and BMI. No correlation was seen comparing LLMI with BMI.

4.3.5 Other associations

The severe asthma group had a significantly lower FEV_1% predicted and FEV_1/FVC ratio compared to other groups (p<0.0001). However, there was no association between these values and LLMI in individual groups or overall. There were no differences in LLMI according to age, BMI (figure 4.5), gender or atopy.

Table 4.5. Univariate logistic regression of predictive factors for GORD.

Predicting factors	Odds ratio	95% CI	*P* value
Age	1.01	0.97 – 1.05	NS
Gender	1.07	0.34 – 3.39	NS
FEV1 (% pred)	0.99	0.96 – 1.02	NS
BAL eos (%)	1.31	0.28 – 6.02	NS
BAL neut (%)	0.78	0.28 – 2.91	NS
LLMI			
0 – 20	1.0		
21 – 59	2.64	0.64 – 10.91	0.18
≥ 60	8.7	1.79 – 42.3	0.007

CI = confidence interval, NS = non-significant

4.4 Discussion

This study found lower LLMI scores in mild/moderate asthmatics compared to the severe asthmatics and healthy controls. However, analysis of the groups by BMI revealed no significant correlation. Furthermore, analysis of the entire cohort correlating LLMI to BMI did not reveal any significant link. The lack of correlation with BMI may reflect a number of factors; there was not a significant difference in BMI between the groups assessed and in order to see an effect of BMI and LLMI within a particular group, such as severe asthma, a larger cohort with a greater BMI range may be needed. These findings do not support the hypothesis that obesity-related severe asthma is associated with an increased LLMI. This may reflect a number of factors. The sample size and BMI of the groups may not have been sufficiently large or diverse to find significant differences. In addition, GORD influences the LLMI and the use of OCS in the severe asthma cohort may have been a significant factor.

The higher LLMI seen in the severe asthma compared to the mild/moderate cohort may reflect the fact that GORD was reported more in the severe group (42.1% versus 14.3%). Another potential explanation relates to increased levels of oxidised or glycated low-density lipoproteins (LDL) seen in asthma (Shute et al., 1997, Neumeier et al., 2011). This has been suggested as a modulator of inflammation and results in LLM formation via macrophage scavenger receptor recognition (Brown et al., 2013). The use of OCS is another potential confounding factor. 14 (66.7%) of the severe cohort were on maintenance OCS therapy. However, subanalysis of the cohort according to OCS use did not find a significant difference in LLMI scores.

Patients with chronic cough had a lower LLMI compared to the severe asthma group and healthy controls, although this did not reach statistical significance. LLMI has been evaluated as a marker of chronic aspiration in chronic cough. A study of 30 consecutive adults presenting to a tertiary referral centre with chronic cough found that there was no significant difference in LLMI score when compared to 20 asymptomatic adults (Carney et al., 1997).

The higher scores seen in the healthy cohort are harder to explain given that no GORD was reported. This supports the view that lipid accumulation is a normal process including the ingestion of lipids present within the alveolar space, such as lung surfactant (Wright and Youmans, 1995). Another explanation may be related to microaspiration and the fact that none of the healthy cohort were taking PPIs. LLMI scores were highly variable and there was considerable overlap between groups. Despite previous reports associating LLMI with BALF neutrophilia (Sacco et al., 2006) and airflow obstruction (Kazachkov et al., 2001), we did not find an association in this cohort.

There was a correlation between the presence of GORD and LLMI, with greater scores in the symptomatic group. The association between aspiration and LLMI has been previously evaluated in children with some studies reporting a positive correlation (Colombo and Hallberg, 1987, Bauer and Lyrene, 1999) and suggesting that particular cut-off values may be a useful diagnostic test (Furuya et al., 2007). However, other studies have failed to confirm that LLMI, measured in lavage fluid or sputum, is an indicator of GORD (Colombo and Hallberg, 1987, Kitz et al., 2012), highlighting a lack of specificity to detect reflux-related respiratory disease (Rosen et al., 2008). In adults, LLMI has been proposed as a marker for aspiration pneumonia (Adams et al., 1997). In cystic fibrosis patients post lung transplantation,

it may be a useful method of assessing response to Nissen fundoplication (Hayes et al., 2012) and a score of >150 was found to have a sensitivity of 82.3% and a specificity of 76.4% correlating with an abnormal 24-hour pH test in these lung transplant recipients (G et al., 2000).

LLMI measured in macrophages obtained from sputum samples has been correlated to oropharyngeal reflux (Parameswaran et al., 2000). However, other studies have failed to confirm this (Koksal et al., 2005) and the use of LLMI in induced sputum is limited in smokers; where the presence of inclusions from cigarette smoke within the cytoplasm, resulting from the phagocytosis of cigarette smoke particulates, increases the LLMI score (Wilson et al., 2011).

Although this study has identified an association between GORD and LLMI, there are some limitations. We relied on self-reported GORD and did not use objective measures such as validated questionnaires or a 24-hour pH probe. A study incorporating objective measures and selecting a more symptomatic cohort would be better placed to examine this potential association. LLMI calculation is time-consuming and a simpler semi-quantitative method of calculating alveolar lipid content has been suggested (Kazachkov et al., 2001) which appear to produce similar results (Ding et al., 2002). In addition, the repeatability of LLMI scores in individuals is not known. Further studies, incorporating repeat bronchoscopies, are required to establish whether this simpler method could replace the LLMI and provide information on individual variability. We have compared asthma and chronic cough patients to a cohort of healthy subjects. Furthermore, LLMI may fluctuate over time depending on factors such as hyperlipidaemia, BMI and micro-aspiration. LLMI should be correlated to the underlying disease process and co-morbidities that may influence the presence of LLMs.

The role of LLMs in asthma may become more relevant with the potential use of solid lipid microparticles as a controlled release delivery system for inhaled bronchodilators and corticosteroids (Scalia et al., 2012, Mezzena et al., 2009). This method of drug delivery may result in a marked increase in PFCs with impaired phagocytosis.

An accurate measurement of LLMI requires a pathologist with experience in reporting BALF. There may be a diagnostic role for LLMI but this is likely to be in conjunction with other variables and the precise role of the LLMI in the clinical setting requires further investigation.

This is the first study to assess LLMI in asthma. Although no correlation was seen with BALF neutrophils or with the degree of airways obstruction, there was a strong association with GORD.

Chapter 5: Asthma, BMI and lipid droplets

Chapter 5: Asthma, BMI and lipid droplets

Hypothesis and aim

Obesity related severe asthma is associated with increased intracellular lipid droplets.

Lipid droplets in endobronchial biopsies will be assessed using perilipin staining and their relationship with asthma severity and BMI will be examined.

5.1 Introduction

Lipid droplets (LDs) are important cytoplasmic organelles found in almost any type of mammalian cell (Murphy, 2001). They are cytoplasmic structures comprised of an outer monolayer of phospholipids, a neutral lipid-rich core and a unique and variable fatty acid and protein composition depending on the type and activating status of cell (Moreira et al., 2009). LDs are sparse in 'normal healthy' cells and exhibit increased numbers in cells associated with inflammation (Meadows et al., 2005).

A variety of epithelial cell types display LDs within their cytoplasm (Beil et al., 1995, Accioly et al., 2008) and LDs in macrophages and other leukocytes play a role in inflammation and phagocytosis (Melo et al., 2011). During macrophage phagocytosis, the LDs move position to surround phagolysosomes, suggesting that they have an active role. Lipid bodies in mast cells have been shown to be granule-independent organelles with early electron microscopy work on human lung mast cells suggesting that lipid bodies act as a storage site of arachidonic acid. This suggests that LDs contribute to eicosanoid biosynthesis, metabolism and contribute to the release of proinflammatory lipid mediators (Dvorak et al., 1983, Dichlberger et al., 2011).

Perilipins are a class of 42 to 56 kDa polypeptides that localise exclusively to the surface of lipid droplets and are not found in any other subcellular compartment (Blanchette-Mackie et al., 1995). There are five subtypes: perilipin 1 (PLIN1, formerly perilipin), perilipin 2 (PLIN2, formerly adipose differentiation-relate protein), perilipin 3 (PLIN3, formerly tail-interacting protein of 47kDa), perlipin 4 (PLIN4, formerly S3-12), and perilipin 5 (PLIN5, formerly lipid storage droplet protein 5) (Dichlberger et al., 2011). Perilipin 1 is the best characterised and was first described in 1991 as an adipocyte protein that modulates adipocyte lipid metabolism, coating lipid storage droplets to protect them from being broken down by hormone-sensitive lipase (Greenberg et al., 1991, Brasaemle, 2007, Ducharme and Bickel, 2008, Smith and Ordovas, 2012). This action protects and conserves stored triacylglycerol (TAG) and preserves its function as a primary energy reserve (Garcia et al., 2004).

Perilipin 1 has been associated with an increased risk of developing obesity and the complications of obesity (Smith and Ordovas, 2012). The overexpression of perilipin 1 in mice fed a high fat diet protects against adipocyte hypertrophy, the development of obesity, and glucose intolerance (Miyoshi et al., 2010). In contrast, perilipin 1 knockout mice (*plin1*) exhibit resistance to obesity, increased basal lipolysis and reduced isoproterenol-stimulated lipolysis despite similar food consumption when compared to wild type (Tansey et al., 2001).

The number of perilipin positive adipose cells in mammary tissue declines significantly during pregnancy and lactation (Russell et al., 2007). This supports the theory of adipocyte transdifferentiation where, in this case, adipocytes are able to change to secretory epithelial cells and back again following termination of lactation.

In humans, there have been conflicting studies looking at perilipin expression. One study noted that adipose perilipin 1 mRNA and protein levels were higher in non-diabetic obese compared to lean subjects and positively correlated with body fat percentage (Kern et al., 2004). Another study noted that perlipin 1 expression is reduced in severely obese (mean BMI 53), compared to non-obese (mean BMI 25) individuals (Wang et al., 2003). Furthermore, perilipin protein content appears to be reduced in the obese state and associated with higher levels of lipolysis in obese women (Mottagui-Tabar et al., 2003).

The *PLIN1* genotype has also been associated with obesity. Two SNPs have been linked with obesity phenotypes in Caucasian women (Qi et al., 2004) whilst other studies have shown different SNPs are linked with protection from developing obesity (Qi et al., 2005). However, there are likely to be ethnic differences and the SNPs evaluated to date may be a marker of underlying functional variants (Smith and Ordovas, 2012).

The role of LDs and perilipin in asthma is currently unclear. The aims of the study are to investigate the relationship between intracellular LDs, asthma severity and BMI. The presence of perilipin1 and CD45+ staining in endobronchial biopsies will be quantified; perilipin1 is used as a marker of intracellular droplets whilst CD45+ labels pan leukocyte/inflammatory cells. The presence and intensity of staining will then be correlated to asthma severity and BMI.

5.2 Methods

5.2.1 Patients

54 patients (22 severe asthma, 18 mild/moderate asthma, and 14 healthy subjects) who underwent a research fibreoptic bronchoscopy with endobronchial biopsies between 2005 and 2012 were recruited retrospectively. All patients underwent a screening visit and those with significant respiratory or inflammatory comorbidities and those requiring treatment for a respiratory tract infection within the preceding four weeks were excluded. Healthy subjects had no smoking history and no history of respiratory or cardiac disease. Bronchoscopies were performed under sedation according to standard clinical practice at the Royal Brompton Hospital.

Asthma was based on a physician diagnosis and patients either had a methacholine provocation test concentration of <8 mg/ml (concentration of methacholine causing a 20% fall in FEV_1; PC_{20}) and/or and FEV_1 reversibility of ≥12%. Severe asthma was defined according to the ATS definition of refractory asthma (ATS, 2000). Patients who did not fulfil the criteria for severe asthma were classified as mild/moderate asthma and this group were taking ≤1000 mcg of inhaled beclomethasone dipropionate (BDP) or equivalent per day.

Ethical approval was obtained from the West London 3 and the Brompton, Harefield and NHLI Research Ethics Committees (11/H0706/4 and 08/H0708/29).

5.2.2 Endobronchial biopsies

Endobronchial biopsies were taken from segmental and subsegmental airways of the right middle and right lower lobe. Biopsies were embedded in optimal cutting temperature medium and then snap-frozen in pre-cooled isopentane in liquid

nitrogen and stored at -70ºC. Cryostat sections (6 mm) were obtained from biopsies, placed on poly-L-lysine-coated microscope slides and stained with haematoxylin and eosin.

5.2.3 Immunohistochemistry (IHC)

Slides were stained for CD45+ cells and for perilipin. Monoclonal mouse anti-human antibodies anti-CD45 (Dako, cat no: M0701), at 1:100 dilution, and polyclonal rabbit anti-human perilipin protein (Cell Signaling Technology, cat no: 3470), at 1:50 dilution, were applied to bronchial biopsy sections for 1 hour.

EnVision-alkaline phosphatase (EV-AP) technique (Dako Ltd, Cambridge, UK) was used to label CD45+ pan leukocyte/inflammatory cells. A modified EnVision peroxidase staining method (Dako) was used to identify perilipin+ cells as previously described (O'Shaughnessy et al., 1997) for 2 hours.

Irrelevant mouse IgG_1 kappa antibody (MOPC21) and rabbit serum were used to substitute for the primary layer as negative control for staining specificity of CD45 and Perilipin antibodies, respectively.

The immunostaining procedures for detecting the CD45+ and perilipin+ cells

Sections were dewaxed, loaded onto a Techmate 'Horizon' automated immunostainer (LJL Biosystems Inc, USA), and immunostained with anti-CD45 antibody. Sections were then washed and incubated with labelled polymer, AP (prediluted secondary antibodies, rabbit anti-mouse/rabbit) (K4018, DAKO, Carpinteria, USA). After a further wash, bound alkaline phosphatase was detected as a red product following incubation with the chromogen, New Fuchsin substrate system. Then the slides were mounted in AquaPerm mounting medium (Thermo

Electron Corporation Cat No. 484990). Following incubation with anti-perilipin primary antibodies, sections were incubated with polyclonal goat anti-mouse rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (cat. no. P0448; Dako) and subsequently incubated with diaminobenzidine (DAB) liquid and peroxide buffer (Dako) for 30 minutes. Stained antigen sites were detected as a brown product. Slides were counterstained with haematoxylin to provide nuclear morphological detail and then mounted in DPX synthetic resin mountant.

5.2.4 Quantification

Histological slides were coded to avoid observer bias. The immunostaining intensity for perilipin1 expression in the airway epithelium, where it was mostly observed, was semi quantitatively given a score ranging from 0 to 2 (0: negative, 0.5: weak staining, 1: moderate staining and 2: strong staining). An example of the perilipin immunohistochemistry staining in bronchial epithelial cells and scoring can be seen in figure 5.1.

The areas of epithelium and subepithelium, excluding muscle and gland, were assessed quantitatively using an Apple Macintosh computer and Image 1.5 software. CD45+ inflammatory cells infiltrated in both epithelium and subepithelium and subepithelium perilipin+ cells were counted using a Leitz Dialux 20 light microscope (Leitz Wetzlar, West Germany). Two to three bronchial biopsies for each subject were measured and counted in order to take account of within subject variability. The total epithelial and subepithelial areas of two or three biopsies from each bronchoscopy were measured and counted, respectively. Then the total counts were divided by total area to normalise the counts as the number of cells per unit. The data for bronchial biopsy cell counts were expressed as the number of cut cell profiles

with a nucleus visible (i.e. positive cells) per mm^2 of the subepithelium and per 0.1 mm^2 epithelium. The coefficient of variation for repeat counts of cells immunopositive for sub-type markers of inflammatory cells by one observer ranged between 5% and 6%.

5.2.5 Statistical analysis

Statistical analysis was performed using StatView (SAS Institute. Inc. and GraphPad Prism 4 (GraphPad Software, Inc.).

One way ANOVA followed by the unpaired Student's t-test was used for the analyses of age, serum IgE, lung function and histamine PC_{20} data between groups. In respect of cell counts, these data were non-normally distributed and differences between groups were assessed first using the Kruskal-Wallis test, which, if significant, was followed by Mann-Whitney U-test between groups at baseline and infection. The coefficient of variation (CV = SD/mean $\times$ 100) was used to express the error of repeat counts. A p value of < 0.05 was accepted as indicating a significant difference.

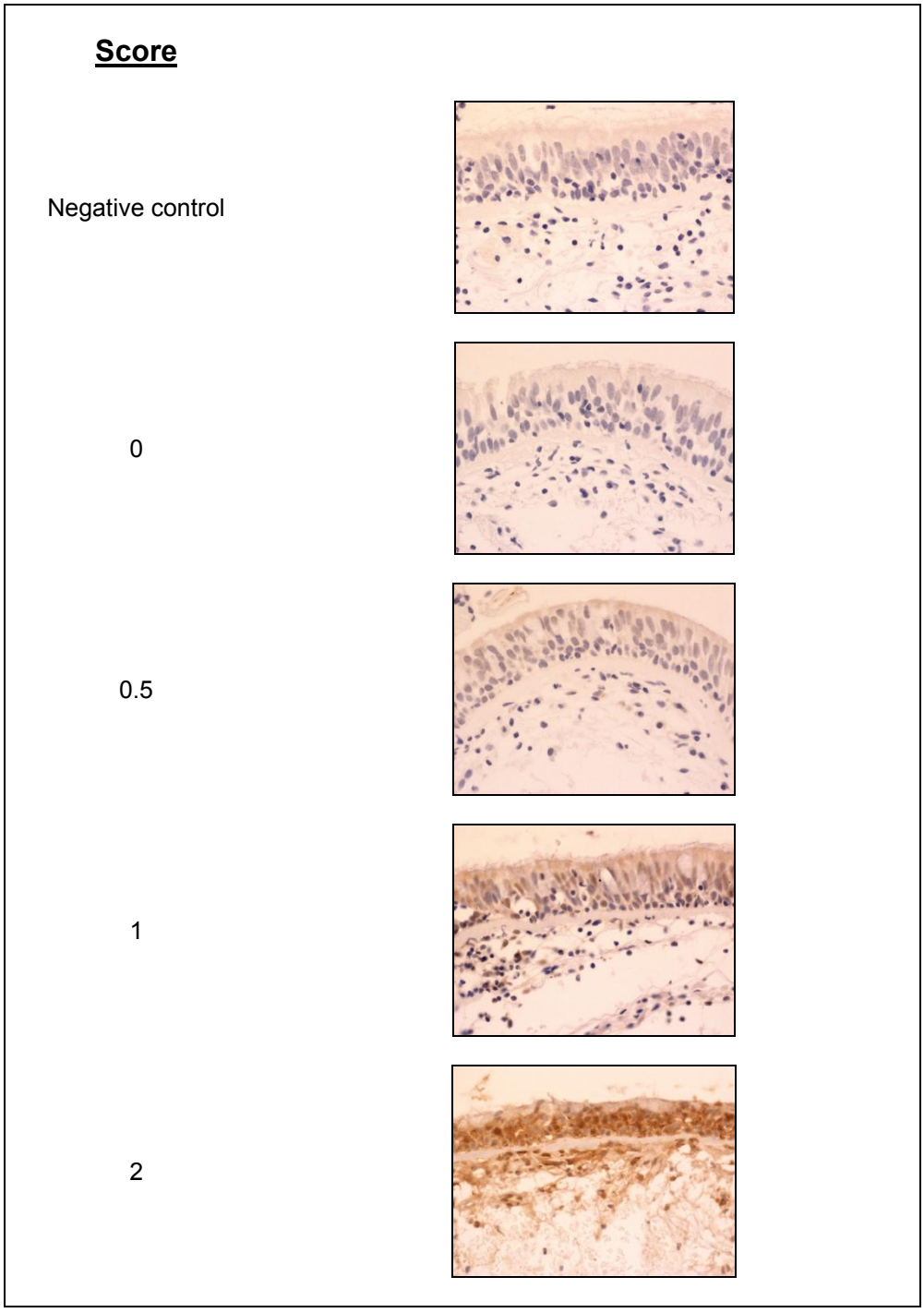


Figure 5.1. Epithelial perilipin1 staining intensity score.

Endobronchial biopsies were stained with polyclonal rabbit anti-human perilipin protein. Epithelial cells were graded by the intensity of epithelial cells staining brown. Cells were scored from 0 to 2: 0 = no opacification, 2 = complete opacification. The panels show examples of each score.

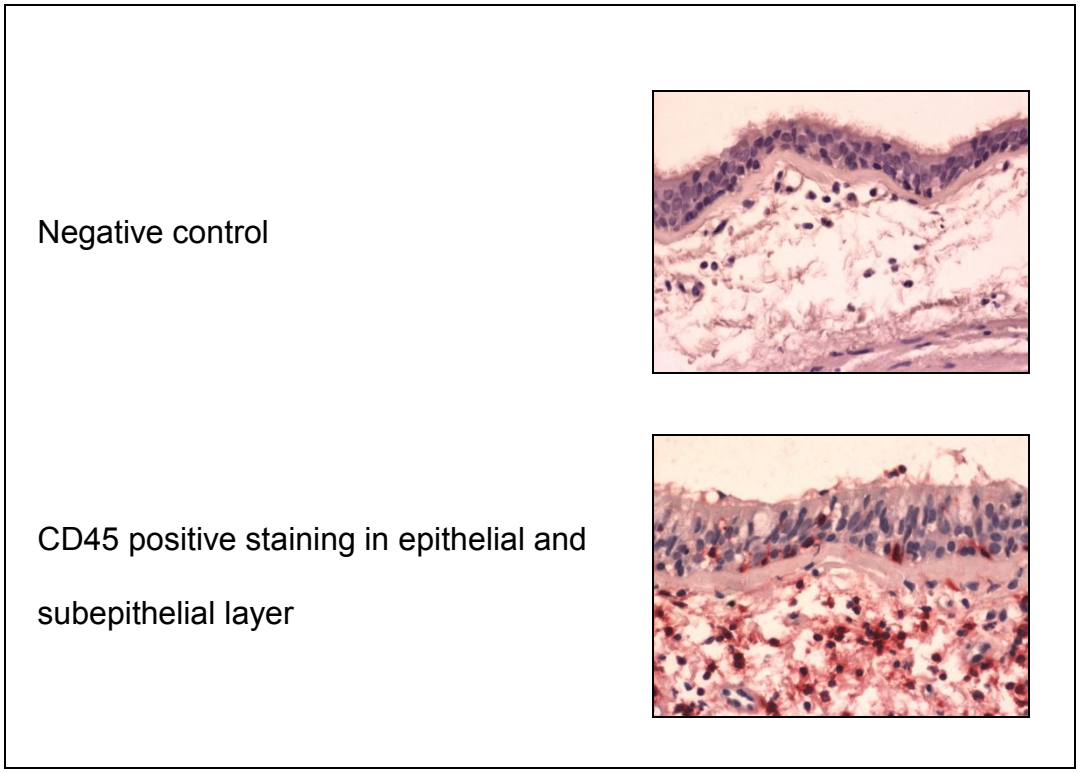


Figure 5.2. CD45 staining.

Endobronchial biopsies were stained with anti-CD45 antibody. The total epithelial and subepithelial areas were measured and counted. Total counts were divided by total area to normalise the counts as the number of cells per unit. CD45+ inflammatory cells were counted in endobronchial biopsies.

5.3 Results

5.3.1 Demographics

Baseline data of patients can be seen in Table 5.1. The severe asthma group were slightly older than the mild/moderate and healthy groups (43.9 yrs versus 40.7 and 35.5, respectively) although this did not reach statistical significance. All three groups had a mean BMI in the overweight category (25 ≤BMI< 30) and similar proportions had never smoked. There were a higher, but statistically non-significant, proportion of females in the severe asthma cohort compared to the other two groups. The severe asthmatic cohort exhibited greater airflow obstruction, bronchodilator reversibility and a lower methacholine PC_{20}. Both asthmatic groups exhibited higher levels of atopy (81.8% and 83.3% in the severe and mild/moderate compared to 28.6% in the healthy group; p=0.0009). In addition, higher serum IgE levels and peripheral blood eosinophil counts were seen in the asthmatic groups. The highest levels of GORD were noted in the severe group (57.1% compared to 18.8% in the mild/moderate and none in the healthy group; p=0.0017). The severe group were taking greater amounts of inhaled (1800µg versus 400µg; p<0.0001) and oral (63.6% versus none; p<0.0001) corticosteroids than the mild/moderate group.

Table 5.1. Demographics, lung function, corticosteroid use and comorbidities.

	Healthy subjects	Mild/moderate asthma	Severe asthma	*p value*
N (%)	14 (26)	18 (33)	22 (41)	
Age (years)	35.5 ± 15.1	40.7 ± 15.0	43.9 ± 14.2	0.2
M/F	9/5	12/6	11/11	0.51
BDP equivalent dose (µg)	0	400 (0 - 850)	1800 (1600 - 2550)	<0.0001
Oral corticosteroid (n)	0	0	14 (63.6)	<0.0001
Oral corticosteroid dose (mg)	0	0	10 (10 - 20)	
BMI	26.8 (23.3 - 32.4)	25.1 (20.3 - 28.1)	28.0 (23.2 - 31.7)	0.31
Never smoked (%)	11 (78.6)	14 (77.8)	20 (90.9)	0.46
FEV_1 (l)	4.0 (3.3 - 4.6)	2.8 (2.4 - 3.3)	2.5 (2.0 - 2.9)	0.0002
FEV_1 %	104.4 (97.7 - 107.6)	84.3 (71.1 - 88.6)	75.1 (62.4 - 85.4)	<0.0001
FEV_1/FVC %	80.5 (75.9 - 85.3)	70.2 (66.6 - 75.5)	68.45 (59.6 - 75.0)	0.0008
BDR (n)	1.1 ± 1.8 (9)	16.1 ± 6.8 (15)	22.8 ± 14.0 (20)	<0.0001
PC_{20} (n)	>16 (14)	0.4 (0.18 - 1.9) (14)	0.89 (0.78 - 0.95) (3)	<0.0001
FE_{NO} (n)	13.5 (9.9 - 28.3) (5)	50.25 (16.4 - 108.7) (10)	56.5 (21.7 - 91) (11)	0.11
Atopic (n, %) *	4 (28.6)	15 (83.3)	18 (81.8)	0.0009
IgE kU/l	82.5 ± 66.1	255.5 ± 302.8	426.4 ± 664.6	0.03
Blood eos count x 10^9/l	0.14 ± 0.08	0.37 ± 0.26	0.34 ± 0.35	0.004
History of GORD (n, %)	0	3 (18.8)	12 (57.1)	0.0017
PPI (n, %)	0	2 (12.5)	12 (57.1)	0.0007

Data are presented as mean ± SD or median (IQR). Between group comparisons for continuous variables were made using Kruskal-Wallis test and for categorical variables using χ^2 exact testing.

BDP, beclomethasone dipropionate BMI, body mass index FEV_1, forced expiratory volume in 1 second FVC, forced vital capacity BDR, bronchodilator response PC_{20}, concentration of methacholine (mg/ml) required to produce a 20% fall in FEV_1 from baseline FE_{NO}, exhaled nitric oxide IgE, immunoglobulin E GORD, gastro-oesophageal reflux disease PPI, proton pump inhibitor.

*The data presented are for subjects with either a positive RAST or skin prick test.

5.3.2 Expression of perilipin

Perilipin1 was expressed weakly or moderately in the airway epithelium of healthy controls and the asthmatic groups. There was also expression in subepithelial inflammatory cells and blood vessel endothelial cells.

26 (50%) of the overall study population had a positive score and expressed perilipin in the airway epithelium. This proportion was consistent with all three subgroups. There were higher scoring individuals in the asthmatic cohort but the median values were not statistically significant (figure 5.4).

Over half the overall study population (53.8%) had a positive score and expressed perilipin in the subepithelial layer. A greater proportion of the asthmatic cohort had positive scores in comparison to the healthy cohort (60% versus 50%) and the subepithelial cells staining positive for perilipin were more prominent with a higher median value (4.2 IQR [0.1 to 83.2] compared to 0.1 [0.1 to 4.5]; p=0.03) (figure 5.5). When asthmatics were subdivided by severity (figure 5.6) no significant difference was seen between the mild/moderate and severe groups (median value 3.5 IQR [0 to 103.2] compared to 4.9 [0 to 91], p=0.8).

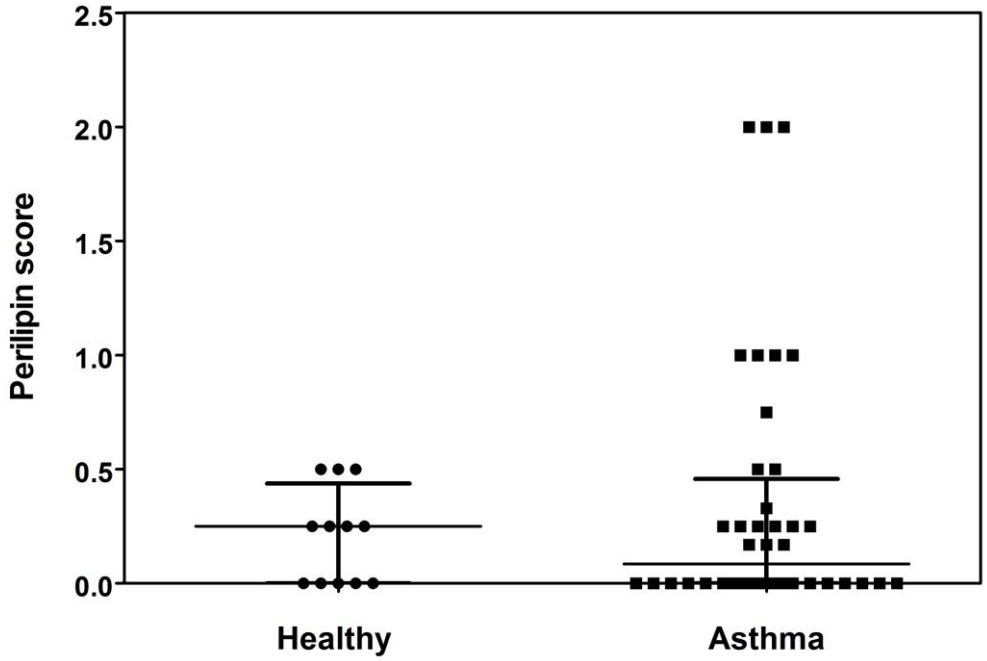


Figure 5.3. Epithelial perilipin score.

Epithelial perilipin score in healthy subjects and asthmatic patients. Median scores and IQR.

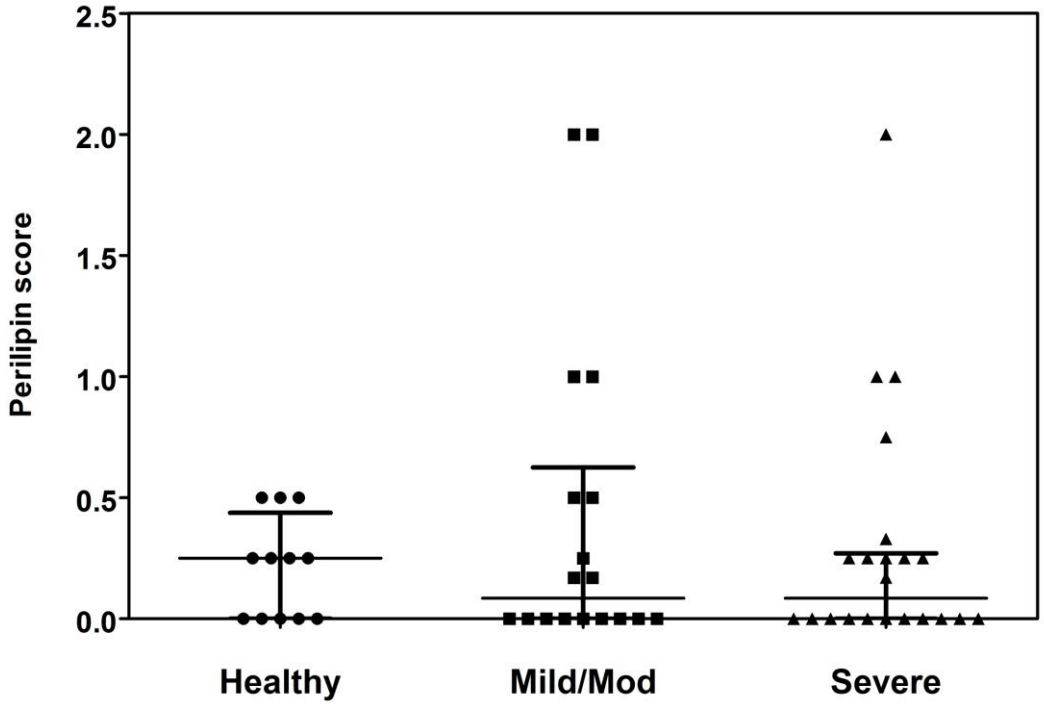


Figure 5.4. Epithelial perilipin score (all groups).

Epithelial perilipin score in healthy subjects, mild to moderate and severe asthmatic patients. Median scores and IQR.

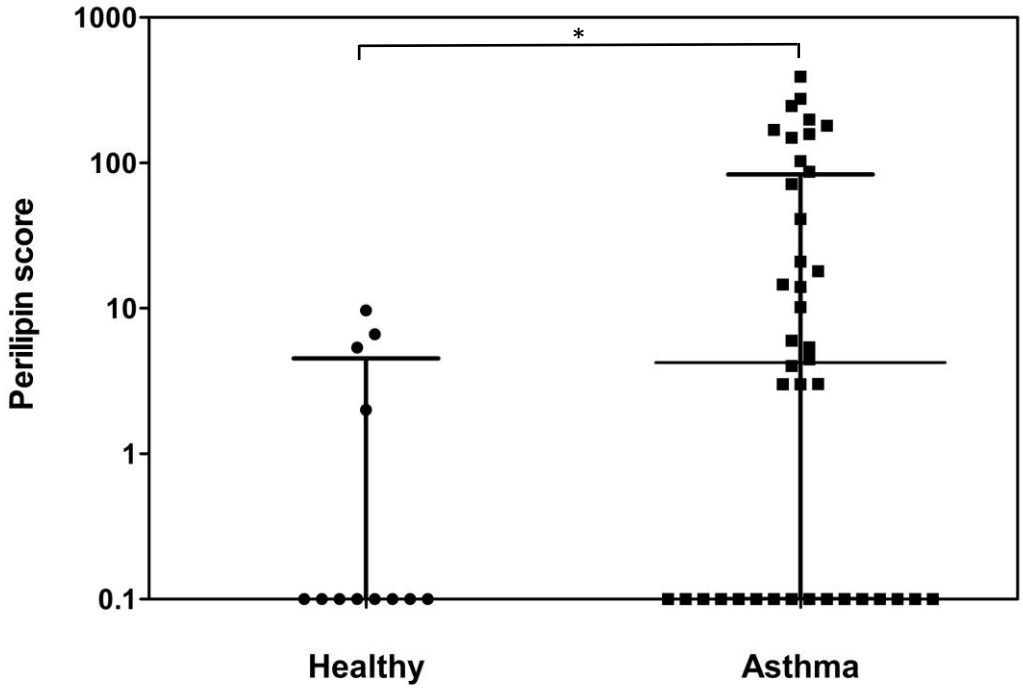


Figure 5.5. Submucosa perilipin score.

Submucosal perilipin score (expressed on $\log_{10}$ scale) in healthy subjects and asthmatic patients. Median scores and IQR. *p<0.05.

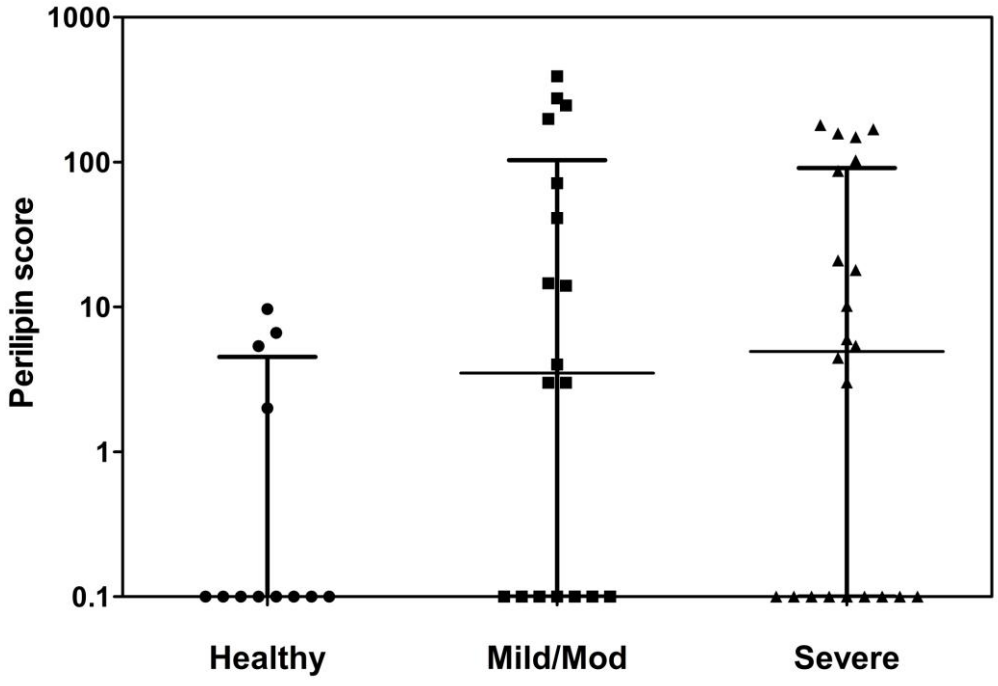


Figure 5.6. Submucosa perilipin score (all groups).

Submucosal perilipin score (expressed on $\log_{10}$ scale) in healthy subjects, mild to moderate and severe asthmatic patients. Median scores and IQR.

Table 5.2. Positive submucosa and epithelial scores for perilipin.

	Healthy Subjects	Mild/Mod Asthma	Severe Asthma	*p* value
	14	18	22	
Perilipin Epithelium n (%)	7 (50)	9 (50)	11 (50)	1.0
Perilipin Submucosa	4 (28.6)	11 (61.1)	13 (59.1)	0.08

Presented as n (%) and between group comparisons were made using χ^2 exact testing.

Table 5.3. Median submucosa and epithelial scores for perilipin and CD45.

	Healthy	Mild/Mod Asthma	Severe Asthma	*p* value
	14	18	22	
Perilipin Ep	0.25 (0 - 0.44)	0.085 (0 - 0.63)	0.085 (0 - 0.27)	0.95
Perilipin Subm	0 (0 - 4.5)	3.5 (0 - 103.2)	4.9 (0 - 91.0)	0.1
CD45+ Ep	56.2 (39.4 - 99.3)	62.2 (36.0 - 110.7)	47.6 (31.1 - 105.5)	0.95
CD45+ Subm	664.2 (445.6 - 975.6)	495.0 (202.3 - 965.3)	755.5 (256.0 - 1022)	0.41

Group data (median (IQR). Between group comparisons were made using Kruskal-Wallis.

5.3.3 Expression of CD45+

CD45+ expression in epithelial cells (figure 5.7) was similar in the asthmatic (60.7 IQR [32.8 to 106.5]) and healthy group (56.2 IQR [39.4 to 99.3], *p*=0.9). In addition, no significant difference was seen when the asthmatic group was divided into the mild/moderate (62.2 IQR [36.0 to 110.7]) and severe (47.6 IQR [31.1 to 105.5], p 0.9) groups (table 5.3 and figure 5.9).

The submucosa layer exhibited similar values in the asthmatic and healthy group in terms of CD45+ expression (555.4 IQR [230.3 to 1015] compared to 664.2 IQR [445 to 975.6] respectively, p 0.39). No significant difference was seen when the asthmatic group was divided by severity (755.5 IQR [256 to 1022]; *p*=0.35) (table 5.3 and figure 5.10).

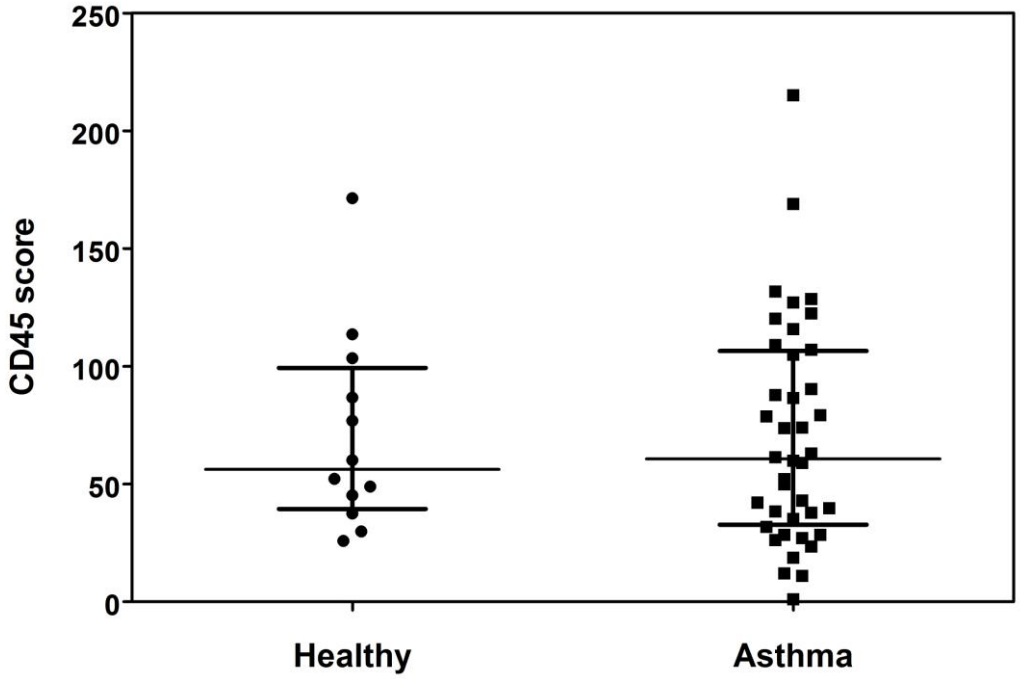


Figure 5.7. Epithelial CD45 score.

Epithelial CD45 score in healthy subjects and asthmatic patients. Median scores and IQR.

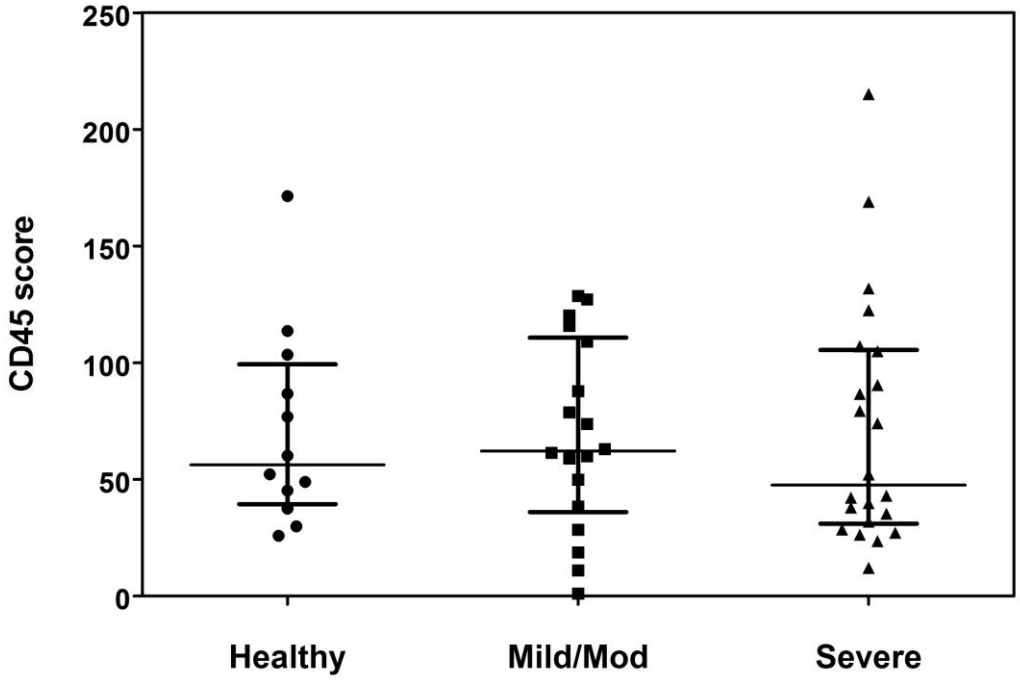


Figure 5.8. Epithelial CD45 score (all groups).

Epithelial CD45 score in healthy subjects, mild to moderate and severe asthmatic patients. Median scores and IQR.

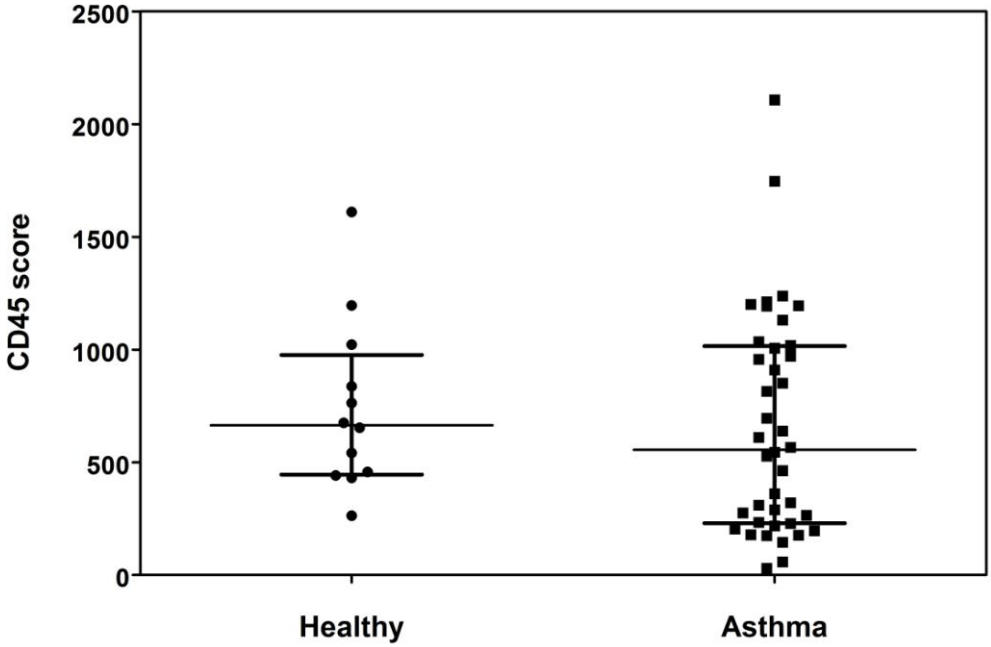


Figure 5.9. Submucosa CD45 score.

Submucosal CD45 score in healthy subjects and asthmatic patients. Median scores and IQR.

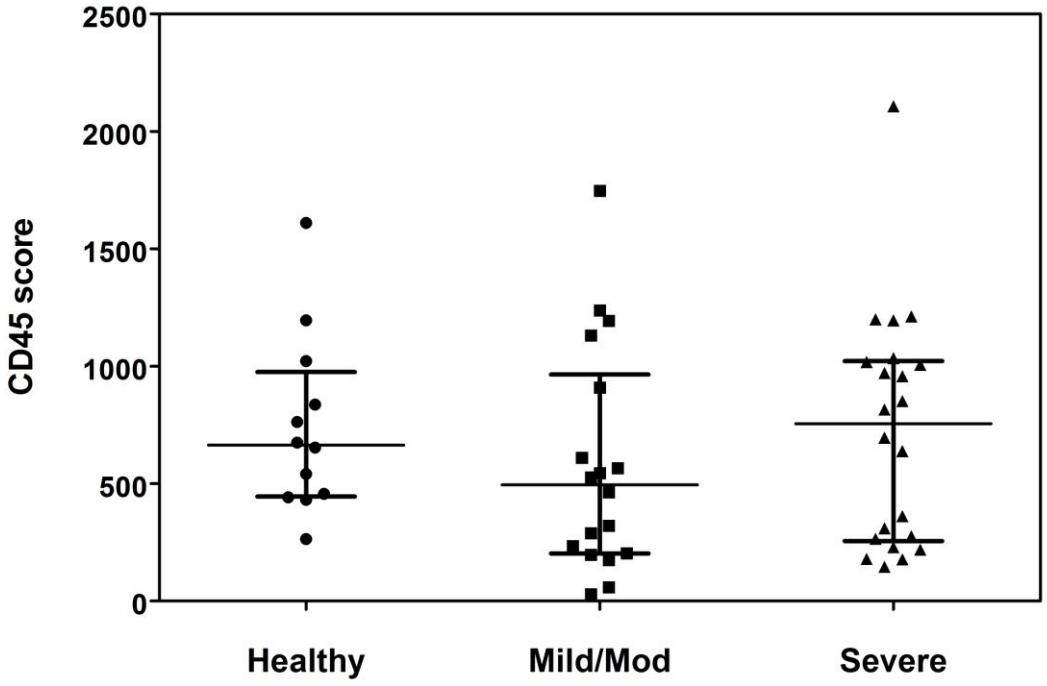


Figure 5.10. Submucosa CD45 score (all groups).

Submucosal CD45 score in healthy subjects, mild to moderate and severe asthmatic patients. Median scores and IQR.

5.3.4 Perilipin, CD45+ and BMI

No significant relationship was noted between epithelial or submucosal perilipin and CD45+ expression and BMI.

Univariate logistic analysis

Univariate logistic analysis (table 5.4) did not demonstrate significant associations between a positive epithelial or subepithelial perilipin score and other variables. A trend was noted between subepithelial perilipin score and the presence of GORD with an OR of 3.73 (p=0.05).

Table 5.4. Univariate Logistic Regression of predictive factors for positive epithelial perilipin score.

Predicting factors	Odds ratio	95% CI	*P* value
Asthma	0.71	0.19 – 2.63	0.61
GORD	2.03	0.59 – 7.05	0.26
FEV_1 (% pred)	1.01	0.98 – 1.04	0.34
FEV_1/FVC ratio	1.05	0.99 – 1.11	0.08
BMI	1.02	0.92 – 1.13	0.75
Gender	1.34	0.44 – 4.1	0.61
Atopy	0.78	0.24 – 2.54	0.68
ICS	1.58	0.5 – 5.0	0.43

GORD, gastro-oesophageal reflux disease FEV1, forced expiratory volume in 1 second FVC, forced vital capacity BMI, body mass index ICS, inhaled corticosteroid.

CI = confidence interval.

Table 5.5. Univariate Logistic Regression of predictive factors for positive subepithelial perilipin score.

Predicting factors	Odds ratio	95% CI	*P* value
Asthma	2.08	0.78 – 11.65	0.11
GORD	3.73	0.98 – 14.2	0.05
FEV_1 (% pred)	1.00	0.97 – 1.03	0.86
FEV_1/FVC ratio	1.00	0.96 – 1.06	0.81
BMI	1.04	0.94 – 1.16	0.46
Gender	1.52	0.5 – 4.64	0.46
Atopy	1.25	0.38 – 4.07	0.71
ICS	2.54	0.79 – 8.2	0.12

GORD, gastro-oesophageal reflux disease FEV_1, forced expiratory volume in 1 second FVC, forced vital capacity BMI, body mass index ICS, inhaled corticosteroid.

CI = confidence interval

5.3 Discussion

This study found that perilipin expression was a common finding in epithelial cells and the submucosa. Although the asthmatic groups exhibited greater perilipin expression in the submucosa compared to the healthy cohort, no significant correlations were noted in terms of asthma severity or BMI. This is in contrast to the hypothesis and may reflect the fact the fact that the cohort was not diverse enough in terms of BMI to establish significant differences, that direct measures of LD identification and location were not used and the fact that LDs may reflect inflammation rather than just adiposity.

The perilipin expression in endobronchial biopsies taken in this study is indicative of the presence of intracellular LDs and is a novel finding in airway epithelial cells. The observation that approximately half of all samples stained positive for perilipin in the epithelial layer, regardless of the presence of asthma, indicates that lipid bodies are likely to be an important and integral part of cellular function in the airway lining.

LDs have been noted within the cytoplasm of a variety of epithelial cells (Beil et al., 1995, Accioly et al., 2008), they are a common finding in the cytoplasm of most eukaryotic cells and their presence in macrophages has been correlated with inflammation and phagocytosis (Melo et al., 2011). The exact role of LDs in epithelial cells remains unclear. They may function as a storage site for arachidonic acid, represent intracellular loci involved in eicosanoid synthesis (Plotkowski et al., 2008) and play a role in the release of pro-inflammatory mediators (Dichlberger et al., 2011, Melo et al., 2013). The trend towards a correlation between a positive perilipin

score in the subepithelial layer and the presence of asthma or GORD on univariate logistic regression may reflect these points.

Macrophage LD formation is increased with *in vitro* bacterial (Peyron et al., 2008), parasitic (D'Avila et al., 2011) and viral infection (Melo and Dvorak, 2012, Samsa et al., 2009). LDs appear to be dynamic organelles, able to relocate in the cytoplasm and interact with phagosomes, presumably aiding the phagocytic process (Melo et al., 2006). Extrapolating this data, it would seem reasonable to hypothesise that LDs may aid the survival and integrity of airway epithelial cells and play an important role in host defence. Further studies incorporating cellular function and cytokine release with reference to LD content in bronchial epithelial cells would enable these theories to be tested in airways disease.

The higher perilipin expression seen in the subepithelial layer in the asthmatic compared to the healthy cohort may reflect underlying airway inflammation with greater release of eicosanoid mediators and phagocytic activity. However, whilst this study confirms that there is perilipin expression in the subepithelial layer, it does not provide information regarding which particular cell types are involved. Further work incorporating cell surface markers for specific cell types would allow further assessment in this area.

Previous studies have noted that obesity is associated with excess lipid storage and LDs in adipose and non-adipose tissue (Walther and Farese, 2012). In turn the excess LDs correlate with a wide range of factors including obesity, fatty liver, coronary artery disease and infectious diseases such as tuberculosis or hepatitis C virus (McDonough et al., 2009). Given the known correlation with obesity and other associated disease states, it was surprising that no correlation between

the expression of perilipin in either the epithelial or subepithelial layer and BMI was seen in this study. This may be explained by the use of perilipin as a marker for LDs rather than more direct measures such as electron microscopy or a combination to confirm that the perilipin is detecting LDs as intended. Alternatively, airway epithelial cells and subepithelial cells may behave differently and LDs may be more of a marker of inflammation and phagocytic function rather than obesity.

There are some important limitations to consider with this study. A greater proportion of the severe cohort (50%) were female compared to the mild/moderate and healthy cohorts (33% and 36% respectively). However, analysis of the overall data according to gender did not reveal a significant difference in epithelial or subepithelial perilipin scores. The severe cohort had an older median age than the other two cohorts and although this did not reach statistical significance, it is possible that increasing age may play an important role in lipid body development, number and perilipin expression. No significant difference was seen in CD45+ expression between the groups. This is in contrast to previous studies which noted increased subepithelial CD45+ expression in asthmatic patients compared to healthy subjects (Zhu et al., 2014). Although no significant relationship was noted between perilipin and BMI, the study population exhibited a narrow BMI range and further work expanding these measurements would be better placed to examine this relationship. This study incorporated univariate logistic regression analysis but a study incorporating a greater number of samples would allow multivariate analysis to be used which may provide a different perspective to the data.

The quality of the endobronchial biopsy and integrity of the epithelium may influence the staining and scoring of perilipin and CD45. Future work incorporating a quality control score or review would prevent this being a significant factor. CD45,

also known as leukocyte common antigen, provides an overall marker of inflammation but further work incorporating specific stains for eosinophils or neutrophils may allow greater insight into asthma specific inflammatory patterns.

Although perilipin expression is essentially synonymous with the presence of LDs, the endobronchial biopsies in this study were not assessed to confirm this correlation. The concurrent use of electron microscopy would enable this to be confirmed and could be used in future studies. Furthermore, the function of the lipid bodies was not addressed in this study. Further work looking at how lipid bodies relate to cellular function in terms of cytokine release and phagocytosis would provide greater insight into their role in healthy subjects and asthmatics.

Chapter 6: The impact of leptin, resistin and adiponectin on PBMCs in severe and non-severe asthma

Chapter 6: The impact of leptin, resistin and adiponectin on PBMCs in severe and non-severe asthma

Hypothesis and aims

Adipokines modulate inflammatory cytokine release and corticosteroid insensitivity in severe asthma. In this chapter, the effect of adipokines (leptin, adiponectin and resistin) on PMBCs are examined in terms of inflammatory response, corticosteroid sensitivity and their relationship with BMI through *in vitro* experiments involving peripheral blood mononuclear cell taken from asthmatics and healthy controls. Adiponectin is regarded as anti-inflammatory whilst leptin and resistin are both pro-inflammatory adipokines.

6.1 Introduction

Adipocytes produce a number of pro- and anti-inflammatory cytokines, termed adipokines, which have multiple roles and play an important part in the inflammatory response (table 1.2). The excess adipose tissue seen in the obese state is associated with greater production of pro-inflammatory adipokines: serum concentrations of IL-6, TNF-α and CRP are all raised in obesity (Festa et al., 2001).

PBMCs taken from obese subjects are in a proinflammatory state, exhibiting increased intranuclear NF-κB binding and an increase in the transcription of NF-κB regulated proinflammatory genes (Ghanim et al., 2004). In addition, exposure to relatively high levels of dyslipidaemia and inflammatory molecules, produced by other organs, and tissues may result in a phenotypic shift in macrophages to the more inflammatory classical M1 type (Bories et al., 2012).

Severe asthma has been associated with relative corticosteroid insensitivity (Bhavsar et al., 2008). There is additionally an association between corticosteroid insensitivity and obesity in asthma (Gibeon et al., 2013). This is supported by retrospective analysis of clinical trials using ICS (Peters-Golden et al., 2006, Camargo et al., 2010a, Anderson and Lipworth, 2012) and by *in vitro* work (Sutherland et al., 2008a). Sutherland and colleagues found that dexamethasone induced MKP1 suppression in PBMCs taken from mild asthmatics was attenuated in subjects with a BMI ≥25 compared to those <25.

Leptin is produced mainly by white adipocytes, is positively correlated with increasing BMI and is viewed as proinflammatory (Kelesidis et al., 2010, Considine et al., 1996). Circulating levels in healthy humans are usually below 10 ng/ml, rising to between 10 to 60 ng/ml in obese individuals (Licinio et al., 1997). Serum levels increase following the administration of inflammatory stimuli such as lipopolysaccharide (LPS), or in experimental acute inflammation (Faggioni et al., 1998, Iikuni et al., 2008). Decreased levels are linked to impaired immune function (La and Matarese, 2004) and humans with congenital leptin deficiency exhibit a higher mortality incidence related to infection (Ozata et al., 1999). This correlation appears to be multifactorial and related to both direct and indirect modulation of immune pathways with leptin playing an important role in innate immunity control (Procaccini et al., 2015). Leptin up-regulates phagocytic function (Mancuso et al., 2002), induces proinflammatory cytokine secretion (including TNF-α, IL-6 and IL-12) (Loffreda et al., 1998), stimulates the proliferation and activity of monocytes *in vitro*, induces neutrophils chemotaxis (Zarkesh-Esfahani et al., 2001) and plays an important role in the development, survival and activation of NK cells (Zhao et al., 2003).

Three leptin receptors have been identified in humans: OB-Ra, OB-Rb and OB-Rc (Chua et al., 1997). OB-Rb appears to be the most important and is expressed in high levels in the hypothalamus (De et al., 2007b) and has been identified on macrophages, monocytes, natural killer (NK) cells, neutrophils, dendritic cells and subpopulations of T and B cells (Iikuni et al., 2008).

Leptin's pro inflammatory activity appears to be via Jak2, MAPK, ERK, PI3K, Akt and STAT1, 3, 5 and 6 (Martin-Romero and Sanchez-Margalet, 2001). PBMCs pre-treated with leptin exhibit increased phosphorylation of both ERK-1 and ERK-2 in a concentration dependent manner (Martin-Romero and Sanchez-Margalet, 2001) and additionally a sustained phosphorylation of p38 MAPK (Procaccini et al., 2009).

Adiponectin is generally viewed as an anti-inflammatory adipokine. It is produced predominantly by adipocytes and is the most abundant adipokine, accounting for 0.01% of total plasma protein (Hu et al., 1996). Adiponectin plasma concentrations range between 3 and 30 µg/ml (Folco et al., 2009). It is negatively correlated with increasing BMI (Lara-Castro et al., 2007) and levels increase with weight loss (Kern et al., 2003). Other cell types, including brown adipocytes, colonic mucosa cells, foetal tissue (Corbetta et al., 2005), salivary gland epithelial cells (Katsiougiannis et al., 2006), osteoblasts (Berner et al., 2004), myocytes (Pineiro et al., 2005) and myofibroblasts (Matsunaga et al., 2008) are able to produce small quantities,

There are six isoforms of adiponectin. These include globular adiponectin (gAPN), full length adiponectin (fAPN or monomeric), low molecular weight adiponectin (LMW or trimeric), medium weight adiponectin (MMW), high molecular weight adiponectin (HMW), and the serum albumin bound LMW form (Alb-LMW).

Monomers can form trimeric isoforms via hydrophobic interactions and subsequently other isoforms via disulphide bonds.

Four adiponectin receptors have been identified: Adipo R1, Adipo R2, T-Cadherin, and calreticulin (Takemura et al., 2007, Hug et al., 2004, Yamauchi et al., 2007). Adipo R1 and R2 is expressed in multiple cell types, including bronchial epithelium, T cells, monocytes, B cells and NK cells (Pang and Narendran, 2008).

In mice, adiponectin attenuates airway inflammation and AHR (Shore et al., 2006) whilst $APN^{-/-}$ mice display more eosinophilic airway inflammation in a chronic model of asthma (Medoff et al., 2009). Pre-treatment of human monocyte derived macrophages (MDMs) (Kumada et al., 2004) and AMs (Ohashi et al., 2010) with adiponectin has been shown to significantly increase IL-10 secretion and suppress LPS induced inflammation via IκB (Folco et al., 2009). Furthermore, adiponectin appears to stimulate the expression of M2 markers in human MDMs, resulting in macrophage polarisation towards an anti-inflammatory phenotype (Ohashi et al., 2010).

6.2 Methods

Subjects underwent spirometry with reversibility testing, a methacholine challenge test (to assess the degree of bronchial hyper-responsiveness), skin prick tests and IgE levels (to assess atopic status), and measurement of exhaled nitric oxide (as a non-invasive marker of inflammation). All subjects had blood taken to obtain PBMCs. In addition, asthmatic subjects completed an Asthma Control Questionnaire. A full description of the methods can be seen in Chapter 2.

Adipokine availability meant that some experiments were conducted at different time intervals. Therefore sub-groups of the overall cohort (table 6.1) were used for leptin (6.2), resistin (6.3) and adiponectin (6.4). CXCL8 was chosen as the primary cytokine response. CXCL8 induces the migration of neutrophils, monocytes and eosinophils to sites of infection, injury or inflammation (Govindaraju et al., 2006) with increased levels in sputum (Gibson et al., 2001), BAL fluid (Chanez et al., 1996) and endobronchial biopsies (Shute et al., 1997) taken from asthmatic patients.

6.3 Results

6.3.1 Overall demographics

Baseline data for all subjects recruited are presented in table 6.1. All groups were of a similar age (mean 44.7 years for healthy subjects versus 44.1 years and 48.7 years for mild/moderate and severe asthma subjects respectively) and smoking history. The severe group had a greater proportion of female subjects than the mild/moderate asthma or healthy cohort (71.4% versus 42% and 39%, p=0.039). Although all groups were of a similar BMI, the severe group had a higher body fat % calculated by BEI (35.6% IQR [21.1 – 44.6] versus 25.5% [14.1 – 34.6] and 27.1 [13.0 – 40.5], p=0.044).

As expected, the severe group were on higher doses of ICS and just under half (46.4%) were taking maintenance OCS. They were also more likely than the mild/moderate group to be receiving additional therapies such as LTRA (50% versus 15.8%, p=0.0001) and aminophylline (42.9% versus 0%, p>0.0001).

FEV_1, FVC and the FEV_1/FVC ratio were reduced in the asthmatic groups and to a greater degree in the severe cohort. Greater bronchodilator reversibility was seen in the severe compared to the mild/moderate group. The asthmatic groups were more likely to be atopic (71.4% severe, 84.2% mild/moderate and 30.4% healthy, p=0.0007) and report GORD symptoms (35.7%, 10.5% versus 8.7%, p=0.037). Asthma related quality of life in terms of ACQ (2.2 [1.6 – 3.1] compared to (1.0 [0.4 – 1.4], p<0.0001) and AQLQ (total AQLQ 4.7 [4.5 – 6.0] compared to 6.2 [5.8 – 6.3], p=0.005) were significantly worse in the severe compared to the mild/moderate cohort.

Table 6.1: Demographics of subjects recruited.

	Healthy subjects	Mild/mod asthma	Severe asthma	*P value*
N	23	19	28	
Age (yrs)	44.7 ± 10.7	44.1 ± 14.9	48.7 ± 12.2	0.34
M/F	14/9	11/8	8/20	0.039
Smoking History				
Never	20	18	24	0.6
Ex	3	1	4	
Height	172.5 (160.5 - 179.3)	174 (166 - 180)	164.5 (161.3 - 172.8)	0.06
Weight	76.4 (63.6 - 101.1)	76.5 (66.1 - 89.2)	73.8 (64.1 - 93.4)	0.87
BMI	27.3 (20.8 - 33.1)	25.3 (21.1 - 30.0)	26.8 (22.6 - 34.9)	0.26
BEI fat %	27.1 (13.0 - 40.5)	25.5 (14.1 - 34.6)	35.6 (21.1 - 44.6)	0.044
Skin fold fat %	34.8 (23.8 - 40.1)	33.4 (22.5 - 35.7)	34.3 (27.8 - 40.0)	0.39
waist/hip ratio	0.89 (0.87 - 1.0)	0.87 (0.81 - 0.99)	0.90 (0.81 - 0.99)	0.71
BDP equiv dose (µg)	0	400 (200 – 1000)	2000 (1600 – 4000)	<0.0001
Oral corticosteroid (%)	0	0	13 (46.4)	<0.0001
OCS dose (mg)	0	0	10 (9 – 20)	<0.0001
Aminophylline (%)	0	0	12 (42.9)	<0.0001
LTRA (%)	0	3 (15.8)	14 (50)	0.0001
FEV_1 (l)	3.4 (2.6 - 3.8)	2.7 (2.0 - 3.5)	1.6 (1.3 - 2.4)	<0.0001
FEV_1 %	98.8 (91.7 - 105.1)	82.4 (71.7 - 89.0)	72.2 (41.9 - 72.2)	<0.0001
FVC	4.2 (3.0 - 4.7)	3.9 (3.3 - 4.3)	2.6 (2.2 - 3.5)	0.0003
FVC %	100.4 (91.8 - 106.7)	95.7 (90 - 107.4)	79.6 (67.5 - 88.6)	0.0001
FEV_1/FVC %	81.9 (78.6 - 85.8)	72.9 (65.2 - 73.6)	65.7 (54.4 - 72.9)	<0.0001
BDR (n)	1 (-2 - 4) (11)	13.1 (9.9 - 19.1) (14)	20.3 (12.3 - 27.6) (24)	<0.0001
PC_{20} (n)	>16 (23)	0.96 (0.43 - 5.7) (15)	1.0 (0.4 - 1.6) (2)	<0.0001
Atopic (n, %) *	7 (30.4)	16 (84.2)	20 (71.4)	0.0007
IgE kU/l	64 (23 – 89)	221 (127 – 367)	108 (75 – 301)	0.038
Blood eos count x 10^9/l	0.1 (0.1 – 0.2)	0.3 (0.2 – 0.5)	0.3 (0.1 – 0.6)	0.047
GORD (n, %)	2 (8.7)	2 (10.5)	10 (35.7)	0.037
PPI (n, %)	0	2 (10.5)	8 (28.6)	0.013
ACQ		1 (0.43 – 1.43)	2.2 (1.57 – 3.11)	<0.0001
AQLQ				
Activities		6.3 (5.8 – 6.6)	5 (4 – 6.2)	0.0038
Symptoms		5.8 (5.5 – 6.4)	4.9 (4 – 5.9)	0.0058
Emotional		6.4 (5.5 – 6.8)	5.4 (4.7 – 6.4)	0.017
Environmental		6 (5.5 – 6.5)	5.4 (4.3 – 5.9)	0.028
Total		6.2 (5.8 – 6.3)	4.7 (4.5 – 6.0)	0.005

Data are presented as mean ± SD or median (IQR). Between group comparisons for continuous variables were made using Kruskal-Wallis test and for categorical variables using χ^2 exact testing.
BMI, body mass index BEI, bioelectrical impedance BDP, beclomethasone dipropionate OCS, oral corticosteroid LTRA, leukotriene receptor antagonist FEV_1, forced expiratory volume in 1 second FVC, forced vital capacity BDR, bronchodilator response PC_{20}, concentration of methacholine (mg/ml) required to produce a 20% fall in FEV_1 from baseline GORD, gastro-oesophageal reflux disease PPI, proton pump inhibitor OCS, oral corticosteroid ACQ, asthma control questionnaire AQLQ, asthma quality of life questionnaire.
*The data presented are for subjects with either a positive RAST or skin prick test.

Table 6.2: Demographics of subjects recruited for leptin studies.

	Healthy subjects	Mild/moderate asthma	Severe asthma	*P value*
N	16	10	12	
Age (years)	42 (36 - 49.3)	44 (33.3 - 56)	49 (42.3 - 58.5)	0.36
M/F	11/5	7/3	5/7	0.27
Smoking History				
Never	12	9	11	0.41
Ex	4	1	1	
Height	174 (166.3 - 179.8)	179 (172.3 - 182.8)	164 (160.3 - 175.8)	0.16
Weight	70.3 (61.7 - 101.8)	73 (64 - 83.5)	86 (74.6 - 108.2)	0.16
BMI	24.3 (20 - 32.3)	22.6 (21 - 26.2)	30.7 (25 - 34.9)	0.03
BEI fat %	19.4 (12.6 - 34.1)	18.1 (13.4 - 26.5)	38.8 (21.1 - 46.3)	0.05
Skin fold fat %	32.8 (22.5 - 39.1)	28.1 (21.7 - 35.8)	37.2 (30.7 - 42.2)	0.12
waist/hip ratio	0.89 (0.8 - 1)	0.87 (0.82 - 0.98)	0.9 (0.84 - 0.93)	0.85
BDP equivalent dose (µg)	0	600 (0 - 1000)	1800 (1600 - 3600)	<0.0001
Oral corticosteroid (%)	0	0	7 (58.3)	
Oral corticosteroid dose (mg)	0	0	7.8 (0 - 13.8)	0.0001
Aminophylline (%)	0	0	6 (50)	0.0004
LTRA (%)				
FEV_1 (l)	3.4 (3.2 - 4.2)	3.0 (2.2 - 3.6)	1.9 (1.6 - 2.7)	0.0006
FEV_1 %	98.6 (88.5 - 106.3)	83.7 (70.9 - 88.0)	68.1 (58.3 - 72.0)	<0.0001
FVC	4.2 (3.8 - 5.3)	4.0 (3.6 - 4.6)	2.8 (2.2 - 3.6)	0.02
FVC %	100.9 (92.2 - 107.1)	92.6 (87.4 - 101.5)	79.6 (73 - 86.0)	0.01
FEV_1/FVC %	81.9 (78.7 - 85.5)	73.3 (67.5 - 78.1)	70.5 (64.9 - 73.7)	<0.0001
Atopic (n, %) *	6 (37.5)	7 (70)	9 (75)	0.09
IgE kU/l	64 (27 – 228)	212 (29 – 519)	114 (90 – 207)	0.3
Blood eos count $x10^9$/l	0.1 (0.1 – 0.2)	0.3 (0.1 – 0.6)	0.3 (0.1 – 0.5)	0.29

Data are presented as mean ± SD or median (IQR).

Between group comparisons for continuous variables were made using Kruskal-Wallis test and for categorical variables using χ^2 exact testing

BMI, body mass index BEI, bioelectrical impedance BDP, beclomethasone dipropionate LTRA, leukotriene receptor antagonist FEV_1, forced expiratory volume in 1 second FVC, forced vital capacity IgE, immunoglobulin E.

*The data presented are for subjects with either a positive RAST or skin prick test.

6.3.2 Subjects recruited for leptin studies

Baseline data of patients recruited for the leptin studies is presented in table 6.2. Similar demographics were seen compared to the overall cohort (table 6.1) However, differences were noted in terms of BMI (30.7 [25 – 34.9] in the severe group compared to 22.6 [21 – 26.2] in the mild/moderate and 24.3 [20 – 32.3] in the healthy groups, p=0.03)

6.3.3 Effect of leptin on PBMC viability

To establish whether leptin alters PBMC viability, cells were pre-treated with leptin, LPS, Dex (10^{-6} M) or a combination for 20 hours prior to performing the MTT assay. Leptin did not significantly influence the MTT signal, in isolation or in combination with Dex (10^{-6} M), LPS or both (figure 6.1).

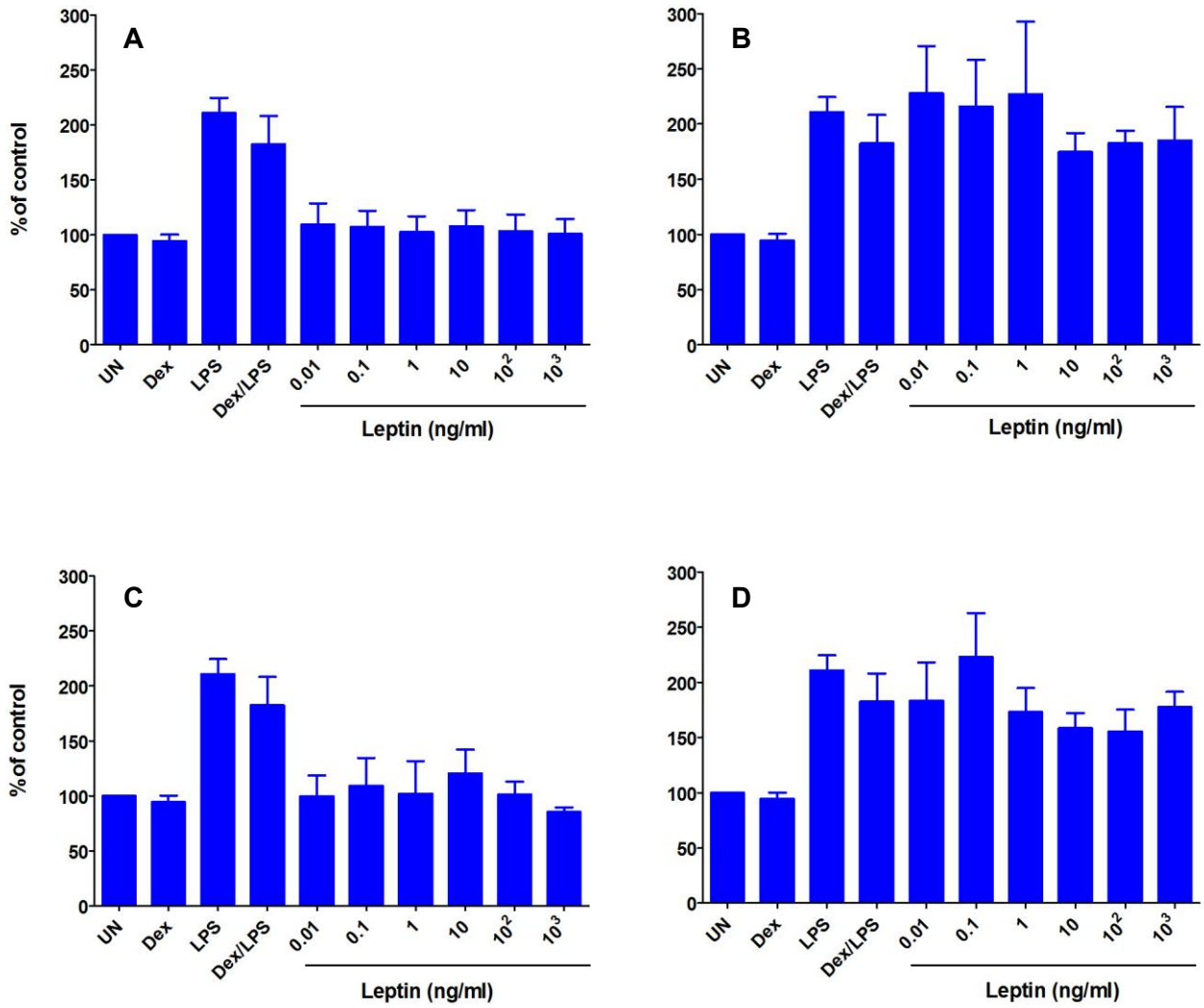


Figure 6.1: Leptin MTT assay. PBMCs were treated with (A) Leptin (10 pg/ml to 1 µg/ml), (B) Leptin and LPS (0.5 ng/ml), (C) Leptin and Dex (10^{-6} M), or (D) a combination of Leptin, LPS (0.5 ng/ml) and Dex (10^{-6} M) for 20 hours prior to collection of supernatant (n=4).

6.3.4 Effect of leptin, LPS and Dex (10^{-6} M) on CXCL8 release from PBMCs

To examine the effects of leptin on CXCL8 release from PBMCs and the interaction of LPS and Dex, cells were pre-treated with increasing concentrations of leptin and Dex (10^{-6} M) ± LPS for 20 hours prior to collecting supernatant. Leptin induced CXCL8 release at the highest concentration of 1 μg/ml in severe, mild/moderate asthma and healthy subjects (3.9, 2.8 and 3.5 fold increase when normalised to untreated cells, respectively). At concentrations between 100 pg/ml to 100 ng/ml there was no significant increase in CXL8 release compared to untreated PBMCs (figure 6.2).

CXCL8 release in response to LPS was lower in the severe cohort compared to mild/moderate asthmatics and healthy subjects (13248 pg/ml [SD 6402] versus 20036 [12505] and 17425 [10610], respectively, p=0.34) although this did not reach statistical significance. Leptin did not enhance the release of CXCL8 induced by LPS.

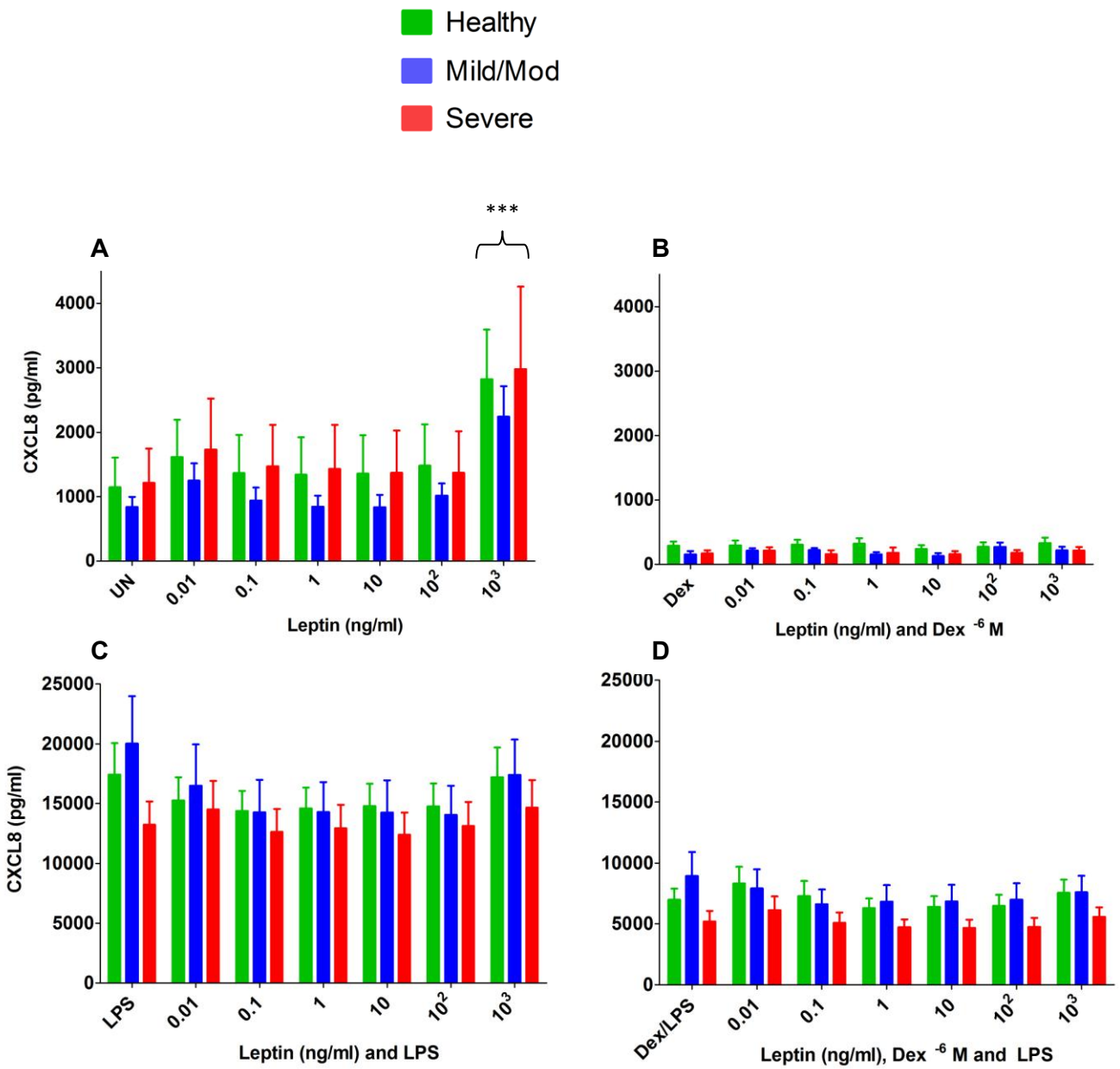


Figure 6.2: The effect of leptin, LPS, Dexamethasone, or in combination on CXCL8 release from PBMCs. Healthy (n=16), Mild/Moderate asthma (n=10), Severe asthma (n=12). PBMCs treated with (A) Leptin (10 pg/ml to 1µg/ml), (B) Leptin and Dex (10^{-6} M), (C) Leptin and LPS (0.5 ng/ml), or (D) a combination of Leptin, Dex (10^{-6} M) and LPS (0.5 ng/ml) for 20 hours prior to collection of supernatant. CXCL8 release was measured by ELISA. Data represent mean ± SEM. *** $p<0.001$ vs. control.

6.3.5 Subjects recruited for resistin studies

Baseline data of patients is presented in Table 6.3. Similar demographics were seen compared to the overall cohort (table 6.1) However, differences were noted in terms of weight and BMI (23.6.7 [20.8 – 25.4] in the mild/moderate group compared to 30.8 [21 – 35.8] in the healthy and 32.7 [27.9 – 37.9] in the severe cohorts, *p*=0.03).

Table 6.3: Demographics of subjects recruited for resistin studies.

	Healthy subjects	Mild/moderate asthma	Severe asthma	*P value*
N	10	7	10	
Age (years)	45 (39.5 – 53.5)	55 (50 - 59)	49 (44.8 – 59.5)	0.29
M/F	8/2	3/4	4/6	0.15
Smoking History				
Never	7	7	9	0.2
Ex	3	0	1	
Height	178.5 (168.5 – 182.3)	173 (160 - 177)	164 (160.8 - 177)	0.3
Weight	98.3 (69.5 - 107)	66 (56 – 78.3)	86.2 (76.6 - 115)	0.036
BMI	30.8 (21 – 35.8)	23.6 (20.8 – 25.4)	32.7 (27.9 – 37.9)	0.033
BEI fat %	30.4 (12.5 – 40.5)	25.5 (16.4 – 32.4)	40.9 (23.8 - 47)	0.19
Skin fold fat %	37 (23 – 40.9)	33.5 (29.7 – 35.3)	38.5 (35.4 - 43)	0.18
Waist/hip ratio	0.98 (0.9 – 1.1)	0.84 (0.74 - 0.99)	0.9 (0.84 - 0.99)	0.23
BDP equivalent dose (µg)	0	400 (0 - 1000)	1800 (1600 - 2800)	<0.0001
Oral corticosteroid (%)	0	0	5 (50)	0.005
Oral corticosteroid dose (mg)	0	0	10 (8.8 -22.5)	0.007
Aminophylline (%)	0	0	4 (40)	0.0019
LTRA (%)	0	1 (14.3)	7 (70)	0.0016
FEV_1 (l)	3.5 (3 - 4.4)	2.5 (1.6 – 2.9)	1.9 (1.5 - 2.6)	0.0022
FEV_1 %	104.8 (91.7 – 107.6)	78.4 (71.5 - 86.0)	64.8 (56.9 - 71.4)	0.0002
FVC	4.6 (3.4 - 5.6)	3.7 (2.5 - 4.1)	2.8 (2.2 - 3.6)	0.019
FVC %	104.4 (94 - 108.1)	92.6 (90 - 103.4)	77.5 (70.5 - 83.9)	0.013
FEV_1/FVC %	82.8 (77.1 - 86.1)	70.2 (63.7 – 73.5)	71.7 (63.9 - 74.9)	0.0009
Atopic (n, %) *	3 (30)	4 (57)	8 (80)	0.079
IgE kU/l	63.5 (38.3 – 80.5)	189 (18 – 358)	108 (76.5 – 156)	0.22
Blood eos count x 10^9/l	0.1 (0.1 – 0.2)	0.3 (0.2 – 0.5)	0.2 (0.1 – 0.3)	0.16

Data are presented as mean ± SD or median (IQR).

Between group comparisons for continuous variables were made using Kruskal-Wallis test and for categorical variables using χ2 exact testing.

BMI, body mass index BEI, bioelectrical impedance BDP, beclomethasone dipropionate LTRA, leukotriene receptor antagonist
FEV_1, forced expiratory volume in 1 second FVC, forced vital capacity IgE, immunoglobulin E.

*The data presented are for subjects with either a positive RAST or skin prick test.

6.3.6 Effect of resistin on PBMC viability

To establish whether resistin alters PBMC viability, cells were pre-treated with resistin, LPS, Dex (10^{-6} M) or a combination for 20 hours prior to MTT assay. Resistin did not significantly influence the MTT signal, in isolation or in combination with Dex (10^{-6} M), LPS or both (figure 6.4). This indicates that resistin did not affect PBMC viability.

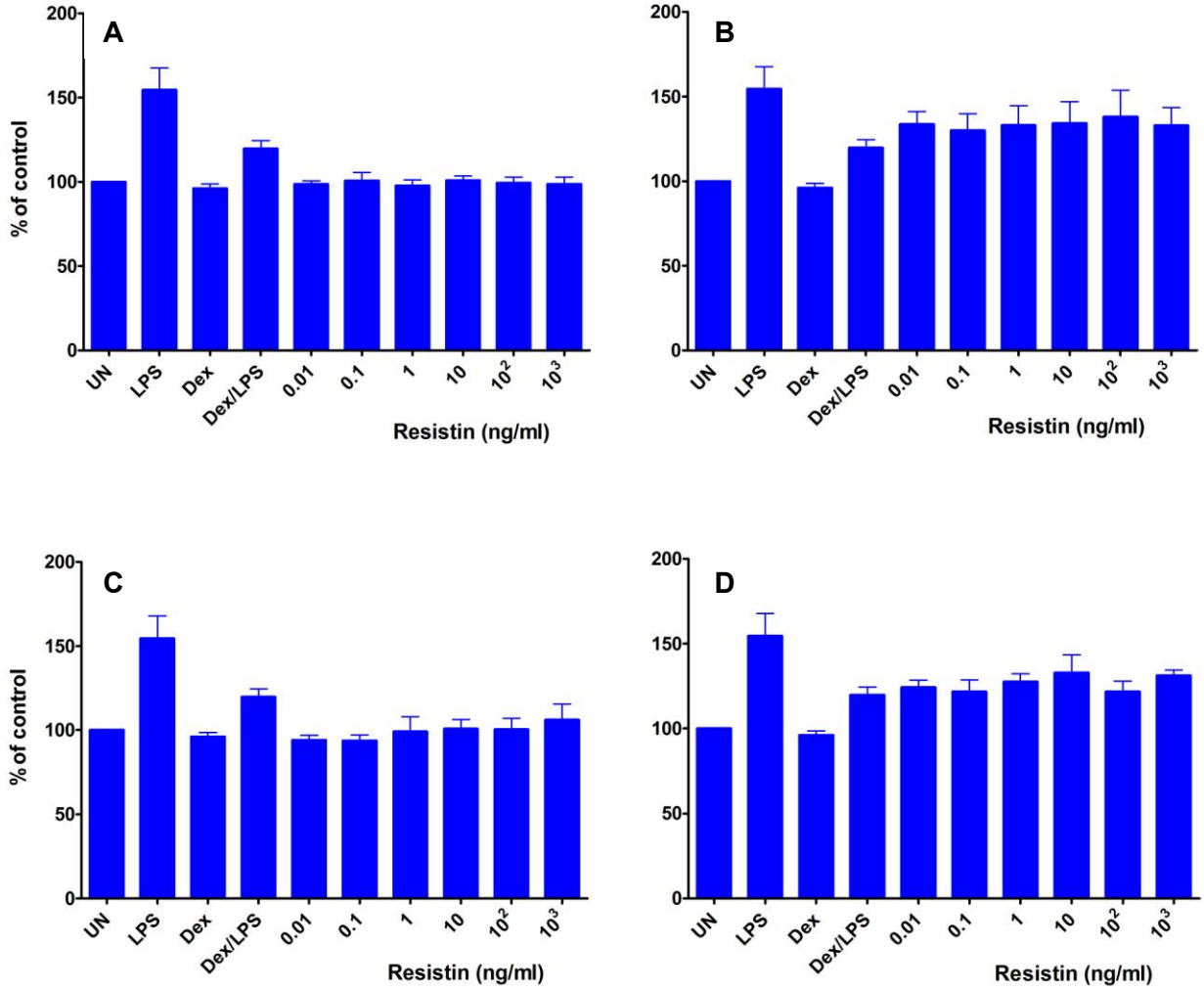


Figure 6.3: Resistin MTT assay. PBMCs treated with (A) Resistin (10 pg/ml to 1µg/ml), (B) Resistin and LPS (0.5 ng/ml), (C) Resistin and Dex (10^{-6} M), or (D) a combination of Resistin, LPS and Dex (10^{-6} M) for 20 hours prior to collection of supernatant.

6.3.7 Effect of resistin, LPS and Dex (10^{-6} M) on CXCL8 release from PBMCs

To examine the effects of resistin on CXCL8 release from PBMCs and the interaction of LPS and Dex, cells were pre-treated with increasing concentrations of resistin and Dex (10^{-6} M) ± LPS for 20 hours prior to collecting supernatant.

CXCL8 release was greater at the two highest concentrations of resistin (100 ng/ml and 1 µg/ml) in all groups. The mild/moderate asthmatics exhibited lower CXCL8 release, in terms of absolute and normalised data, compared to the healthy and severe cohorts at concentrations of resistin ranging from 10 ng/ml to 1 µg/ml (figure 6.4, panel A).

Release of CXCL8 in response to LPS was not significantly different between groups (11676 pg/ml [SD 8152] for the severe cohort versus 13917 [6866] mild/moderate and 20244 [4025] healthy subjects, p=0.2) (figure 6.4, panel C). The pre-treatment of PBMCs with resistin was not associated with an increase in CXCL8 release following stimulation with LPS (figure 6.4, panel D).

PBMCs in the presence of Dex (10^{-6} M) released a similar amount of CXCL8 in all groups (90.4 [88.2] for the severe cohort versus 122.5 [92.6] mild/moderate and 156.8 [135.9] healthy subjects, p=0.74). In the presence of the highest concentrations of resistin (100 ng/ml and 1 µg/ml), the mild/moderate cohort exhibited the lowest CXCL8 release. At the highest concentration of resistin (1 µg/ml) PBMCs in all cohorts released greater amounts of CXCL8 compared to control (Dex 10^{-6} M). However, only the severe cohort had a significant increase (p=0.012) (figure 6.4, panel B).

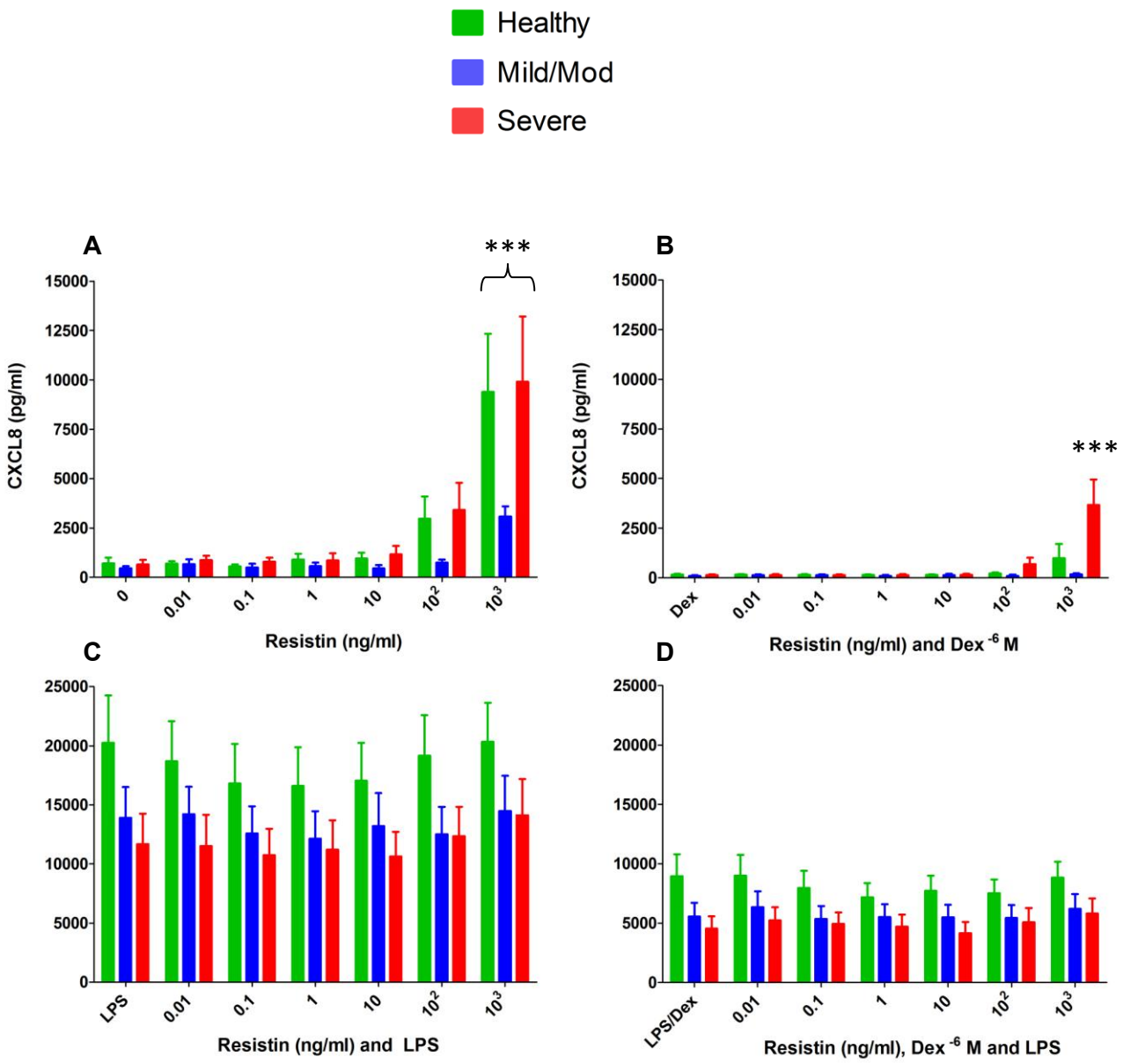


Figure 6.4: Effect of resistin, LPS, Dexamethasone, or in combination on CXCL8 release from PBMCs. Healthy (n=10), Mild/Moderate asthma (n=7), Severe asthma (n=10). PBMCs treated with (A) Resistin (10 pg/ml to 1 µg/ml), (B) Resistin and Dex (10^{-6} M), (C) Resistin and LPS (0.5ng/ml), or (D) a combination of Resistin, Dex (10^{-6} M) and LPS (0.5 ng/ml) for 20 hours prior to collection of supernatant. CXCL8 release was measured by ELISA. Data represent mean ± SEM. *** $p<0.001$ vs. control.

6.3.8 Subjects recruited for adiponectin studies

Baseline data of patients is presented in Table 6.4. Similar demographics were seen in the subgroup of patients taken from all recruited subjects compared to the overall cohort (table 6.1). As expected, significant differences were noted in terms of asthma medication requirements, lung function, and asthma symptoms in the severe asthma compared to the mild to moderate cohort.

Table 6.4: Demographics of subjects recruited for adiponectin studies.

	Healthy subjects	Mild/mod asthma	Severe asthma	*P value*
N	11	12	14	
Age (yrs)	43 (36 – 48)	51 (35.5 – 58.8)	56 (45 – 61)	0.14
M/F	5/6	4/8	3/11	0.44
Smoking History				
Never	9	12	12	0.32
Ex	2	0	2	
Height	172.0 (155 - 180)	169 (161.5 - 175.3)	164.5 (160 - 169.8)	1
Weight	70.5 (61.1 - 87.5)	77.0 (60.0 - 88.8)	66.5 (61.9 - 94.1)	0.41
BMI	25.3 (20.5 - 32.5)	26.6 (21.9 - 30.5)	24.5 (22.5 - 35.4)	0.79
BEI fat %	25.4 (13 - 40.2)	31.6 (23.8 - 38.7)	37.7 (27.2 - 46.6)	0.14
Skin fold fat %	35.0 (28.5 - 39.3)	33.4 (29 - 37.1)	35.9 (25.7 - 40.9)	0.78
waist/hip ratio	0.88 (0.79 - 0.96)	0.84 (0.75 - 0.98)	0.91 (0.84 - 0.96)	0.45
BDP equiv dose (μg)	0	400 (250 - 700)	2400 (2000 - 2800)	<0.0001
Oral corticosteroid (%)	0	0	5 (35.7)	0.0087
OCS dose (mg)	0	0	14.5	
Aminophylline (%)	0	0	7 (50)	0.0008
LTRA (%)	0	1 (8.3)	7 (50)	0.0042
FEV_1 (l)	3.3 (2.3 - 4.2)	2.3 (1.7 - 2.9)	1.5 (1.1 - 1.8)	0.0001
FEV_1 %	100.5 (95.9 - 107.5)	82.1 (71.6 - 88.3)	61.4 (40.1 - 72.2)	<0.0001
FVC	4.1 (2.7 - 5.3)	3.6 (2.6 - 4.1)	2.6 (2.1 -3.3)	0.005
FVC %	13.5 (97.7 - 107.1)	98.9 (90.2 - 109.9)	79.1 (64.3 - 88.1)	0.0024
FEV_1/FVC %	82.8 (78.3 - 85.5)	70.6 (64.0 - 73.5)	59.4 (49.2 - 70.1)	<0.0001
BDR (n)	1 (-2 - 4.0)	15.3 (9.9 - 19.1)	19.6 (12.2 - 27.7)	<0.0001
PC_{20} (n)	>16	0.9 (0.2 - 1.9)	*	
Atopic (n, %) *	3 (27.3)	11 (91.7)	10 (71.4)	0.0044
IgE kU/l	64 (25.5 - 376)	316.5 (162.5 - 399.5)	94.5 (65.5 - 532.3)	0.23
Blood eos count $x10^9$/l	0.25 (0.1 – 0.33)	0.3 (0.2 – 0.4)	0.2 (0.1- 0.43)	0.38
History of GORD (n, %)	0	2 (16.7)	10 (71.4)	0.0003
PPI (n, %)	0	2 (16.7)	6 (42.9)	0.031
ACQ		8.5 (4 – 10)	14 (10.5 – 21.5)	0.038
AQLQ				
Activities		6.3 (6 – 6.6)	4.8 (3.8 – 6.3)	0.056
Symptoms		5.8 (5.6 – 6.3)	4.5 (3.8 – 6.1)	0.029
Emotional		6.4 (5.2 – 6.6)	5.4 (4.90 – 6.6)	0.15
Environmental		6 (5.5 – 6.5)	5.3 (4.4 – 6.4)	0.15
Total		6.1 (5.9 – 6.3)	4.7 (4.4 – 6.3)	0.091

Data are presented as mean ± SD or median (IQR). Between group comparisons for continuous variables were made using Kruskal-Wallis test and for categorical variables using χ^2 exact testing.

BMI, body mass index BEI, bioelectrical impedance BDP, beclomethasone dipropionate LTRA, leukotriene receptor antagonist FEV_1, forced expiratory volume in 1 second FVC, forced vital capacity IgE, immunoglobulin E GORD, gastro-oesophageal reflux disease PPI, proton pump inhibitor ACQ, asthma control questionnaire AQLQ, asthma quality of life questionnaire.

*The data presented are for subjects with either a positive RAST or skin prick test.

6.3.9 Adiponectin: concentration response

A concentration response for adiponectin (100 pg/ml to 10 μg/ml) can be seen in figure 6.5. Adiponectin was pro-inflammatory in a concentration dependent manner. The mild/moderate cohort had a lower baseline response to LPS compared to the severe and healthy cohorts (4455 [791] compared to 5089 [3865] and 6460 [3291] respectively, p=0.74) although this was not statistically significant.

A similar pattern was seen when the cohort was subdivided into healthy, mild/moderate and severe asthma groups (figure 6.5, panels B, C and D). Concentrations of adiponectin including and above 10 ng/ml led to a greater release of CXCL8 in the absence of stimulation with LPS.

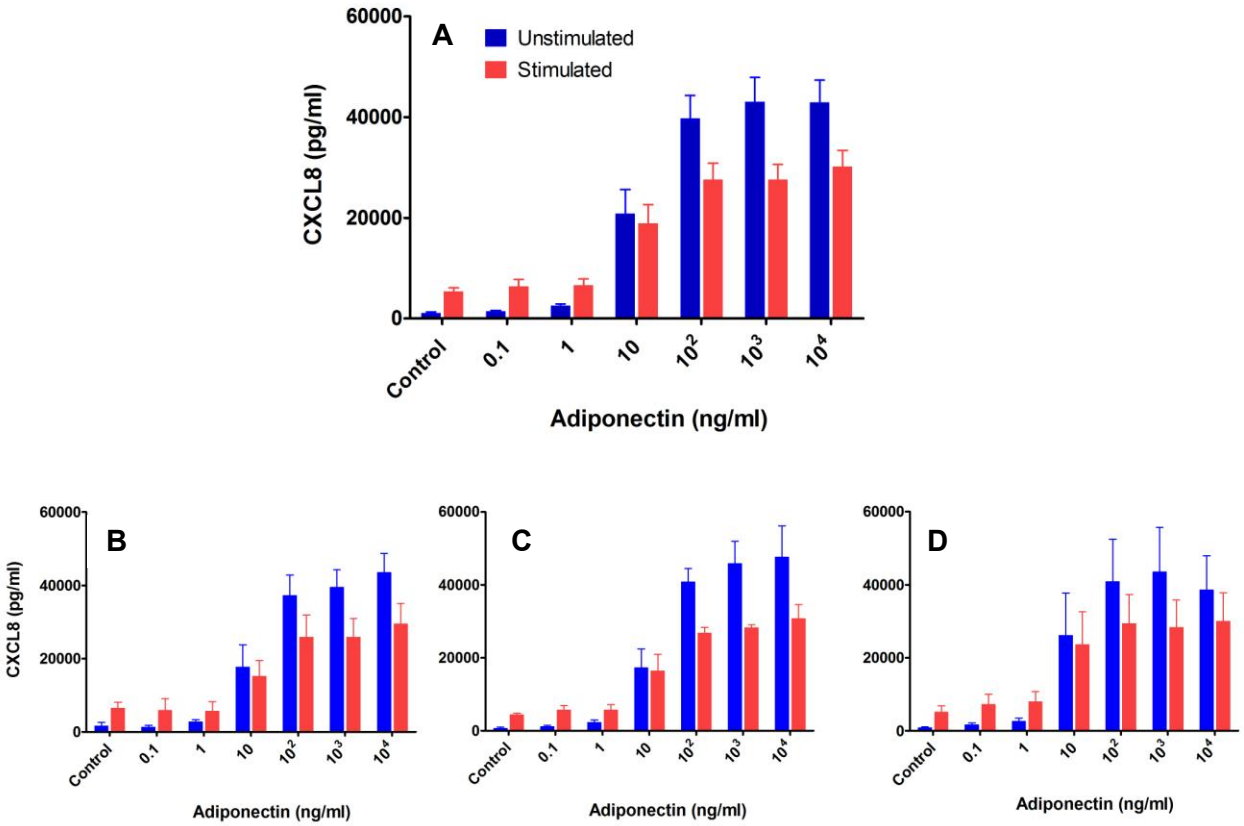


Figure 6.5: Release of CXCL8 as a function of concentration of adiponectin in the presence or absence of LPS. Healthy (n=4), Mild/Moderate asthma (n=4), Severe asthma (n=10). PBMCs treated with adiponectin (100 pg/ml to 10 µg/ml) for 18 hours prior to stimulation with LPS (0.5 ng/ml) for 20 hours in (A), the overall cohort, (B) healthy subjects, (C) mild/moderate asthma, and (D) severe asthma.

6.3.10 Prokaryote versus eukaryote derived adiponectin

A comparison between the effects of prokaryote derived adiponectin (Escherichia coli; E. coli) and eukaryote derived adiponectin (human embryonic kidney; HEK) in terms of CXCL8 release is presented in figure 6.6. Both samples of adiponectin were pro-inflammatory in a concentration-dependent manner with no significant difference noted at adiponectin concentrations of 100 pg/ml to 10 µg/ml. Similar response in terms of CXCL8 release was noted in PBMCs pre-treated with adiponectin prior to LPS stimulation. No significant differences were seen for the treatment groups at all concentrations of adiponectin (100 pg/ml to 10 µg/ml).

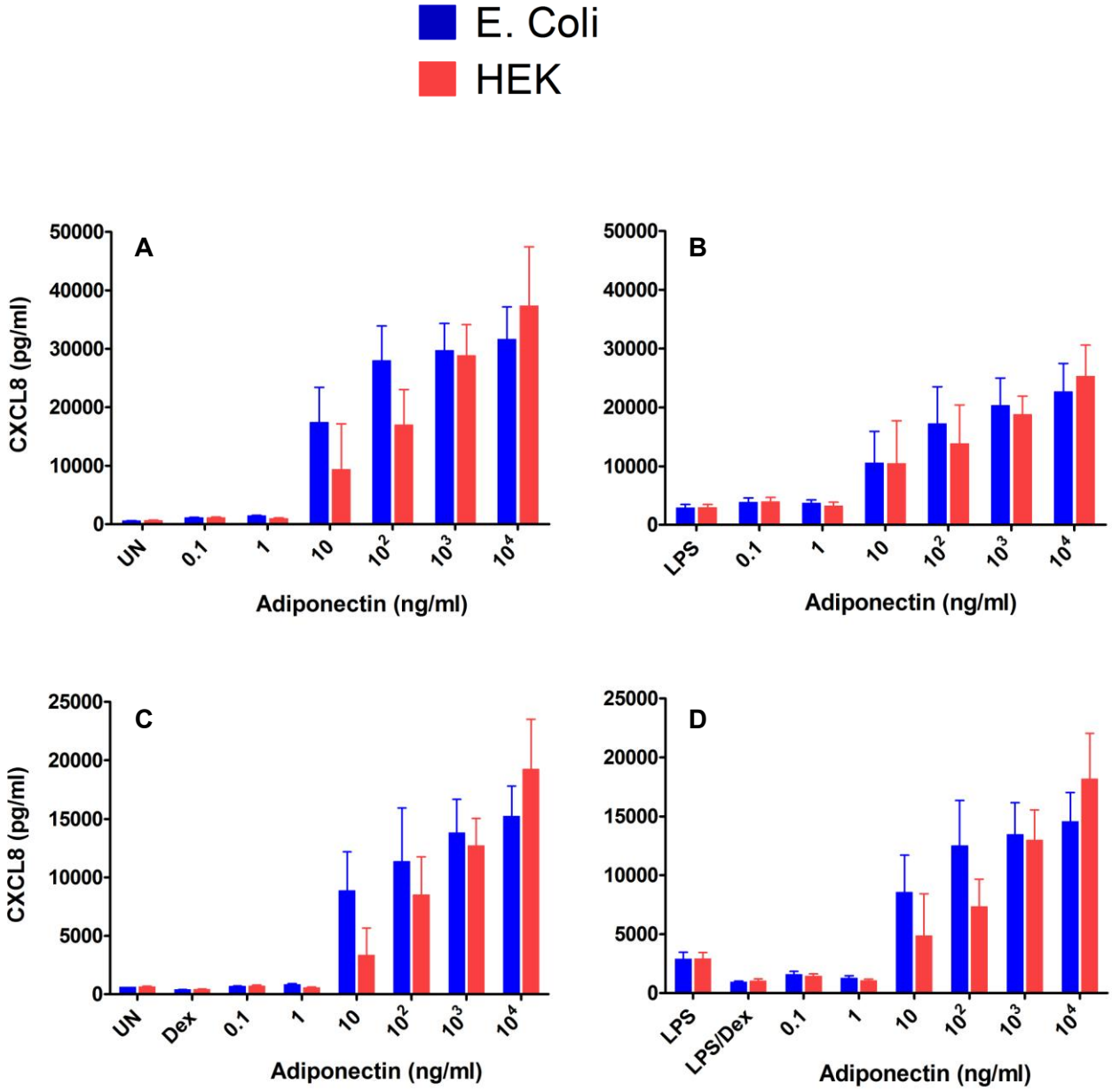


Figure 6.6: Prokaryote (E.Coli) and eukaryote (HEK) derived adiponectin.

Healthy (n=3). PBMCs treated with (A) adiponectin (100 pg/ml to 10 µg/ml) for 18 hours, (B) adiponectin prior to stimulation with LPS (0.5 ng/ml) for 20 hours, (C) adiponectin and Dex (10^{-6} M), or (D) adiponectin, Dex (10^{-6} M) and LPS (0.5 ng/ml). CXCL8 release was measured by ELISA. Data represent mean ± SEM.

6.3.11 Adiponectin: time course

A time course using 100 ng/ml of adiponectin or 0.5 ng/ml LPS and in combination can be seen in figure 6.9. PBMCs were left for between 0.5 and 40 hours prior to the collection of supernatant and measurement of CXCL8. Adiponectin and LPS induced a similar response of CXCL8 release over the 40 hour time course. There is a time-dependent increase in CXCL8 release from PBMCs reaching significance at 4 hours post stimulation with maximal response achieved at 16 hours for all combinations of stimuli. This was maintained over 40 hours and adiponectin did not attenuate LPS induced CXCL8 release. From these data, a 20 hour time course was chosen for subsequent studies to analyse the effects of these inflammatory stimuli on cells taken from patients with severe and non-severe asthma.

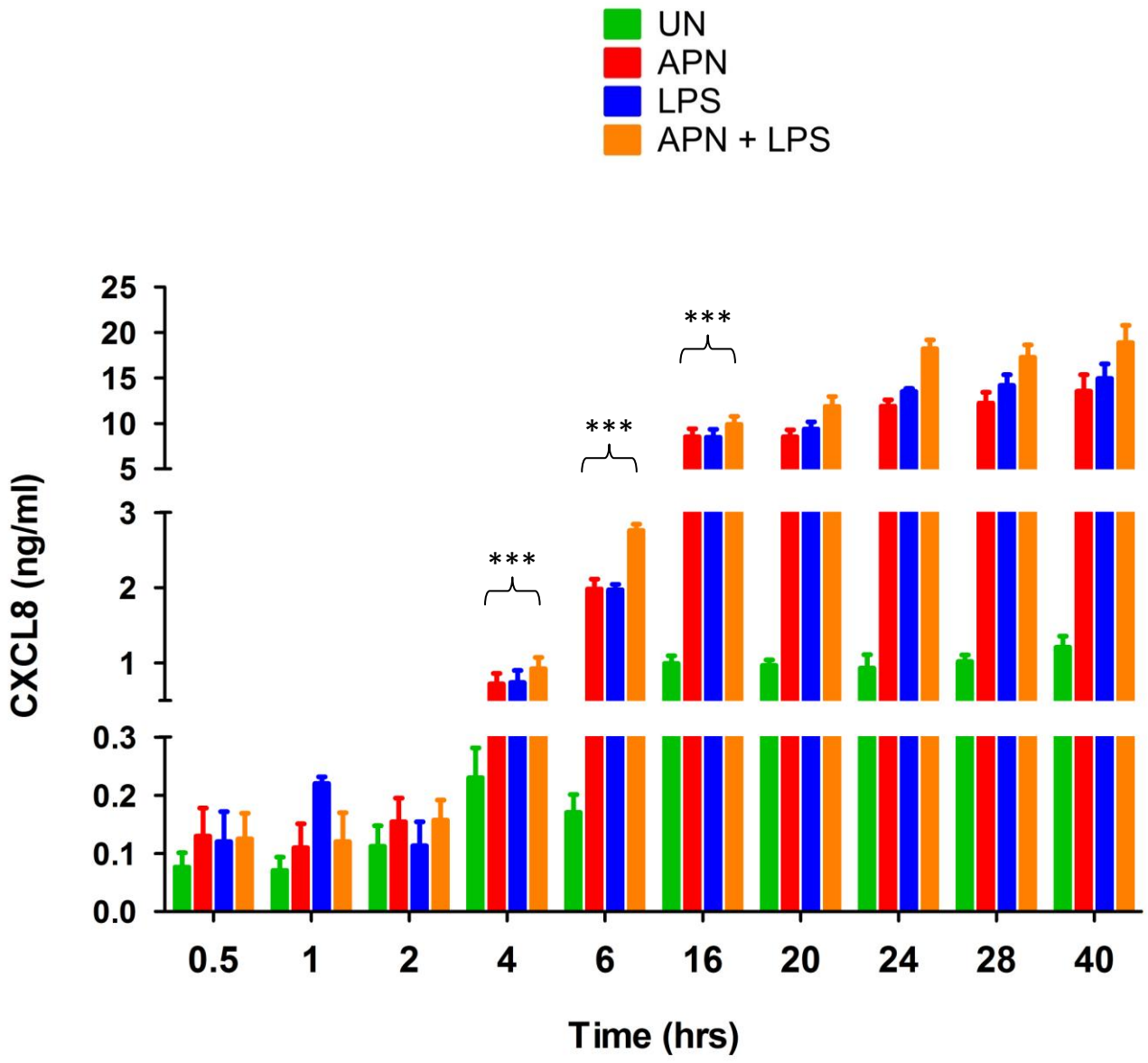


Figure 6.7: Adiponectin time course.

Severe asthmatics (n=4). PBMCs treated with adiponectin (red bar; 100 ng/ml), LPS (blue bar; 0.5 ng/ml) or both in combination (orange bar) for 0.5 to 40 hours prior to collection of supernatant. CXCL8 release was measured by ELISA. Data represent mean ± SEM. *** $p<0.0001$ vs. control.

6.3.12 Effect of adiponectin on PBMC viability

To establish whether adiponectin alters PBMC viability; cells were pre-treated with increasing concentrations of adiponectin, LPS (0.5 ng/ml), Dex (10^{-6} M) or all in combination for 20 hours prior to the MTT assay. Adiponectin did not significantly influence the MTT signal, in isolation or in combination with Dex (10^{-6} M), LPS or both (figure 6.8).

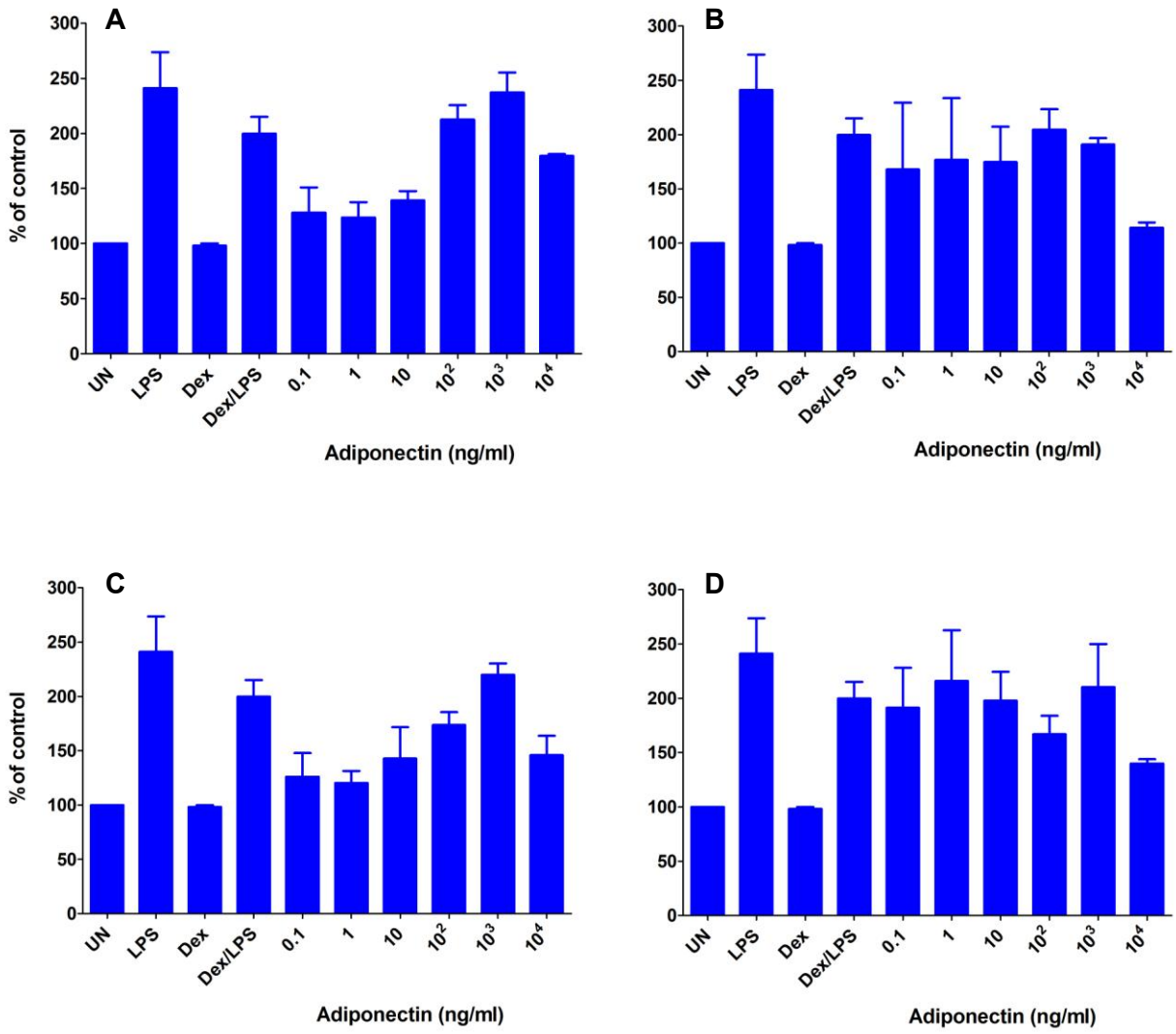


Figure 6.8: Adiponectin MTT assay. PBMCs treated with (A) adiponectin (100 pg/ml to 10µg/ml), (B) adiponectin and LPS (0.5 ng/ml), (C) adiponectin and Dex (10^{-6} M), or (D) a combination of adiponectin, LPS (0.5 ng/ml) and Dex (10^{-6} M) for 20 hours prior to collection of supernatant. CXCL8 release was measured by ELISA. Data represent mean ± SEM.

6.3.13 Effect of adiponectin on CXCL8 release from PBMCs

Adiponectin induced a similar amount of CXCL8 release from PBMCs taken from healthy subjects, mild/moderate and severe asthmatics (8736 [SD 9134], 8673 [9763] and 9088 [5843] respectively, p=0.72). The mild/moderate cohort exhibited greater suppression of CXCL8 release compared to the healthy and severe asthma groups for Dex concentrations of 10^{-7} M (780.7 [SD 225] compared to 2327 [1450] and 4188 [4166] respectively, p=0.054) and 10^{-6} M. (1184 [1102] compared to 5993 [1807] and 4008 [1071], p=0.094) (figure 6.9).

The pre-treatment of PBMCs with adiponectin did not significantly alter CXCL8 release following stimulation with LPS or the response to increasing concentrations of Dex (10^{-10} to 10^{-6} M).

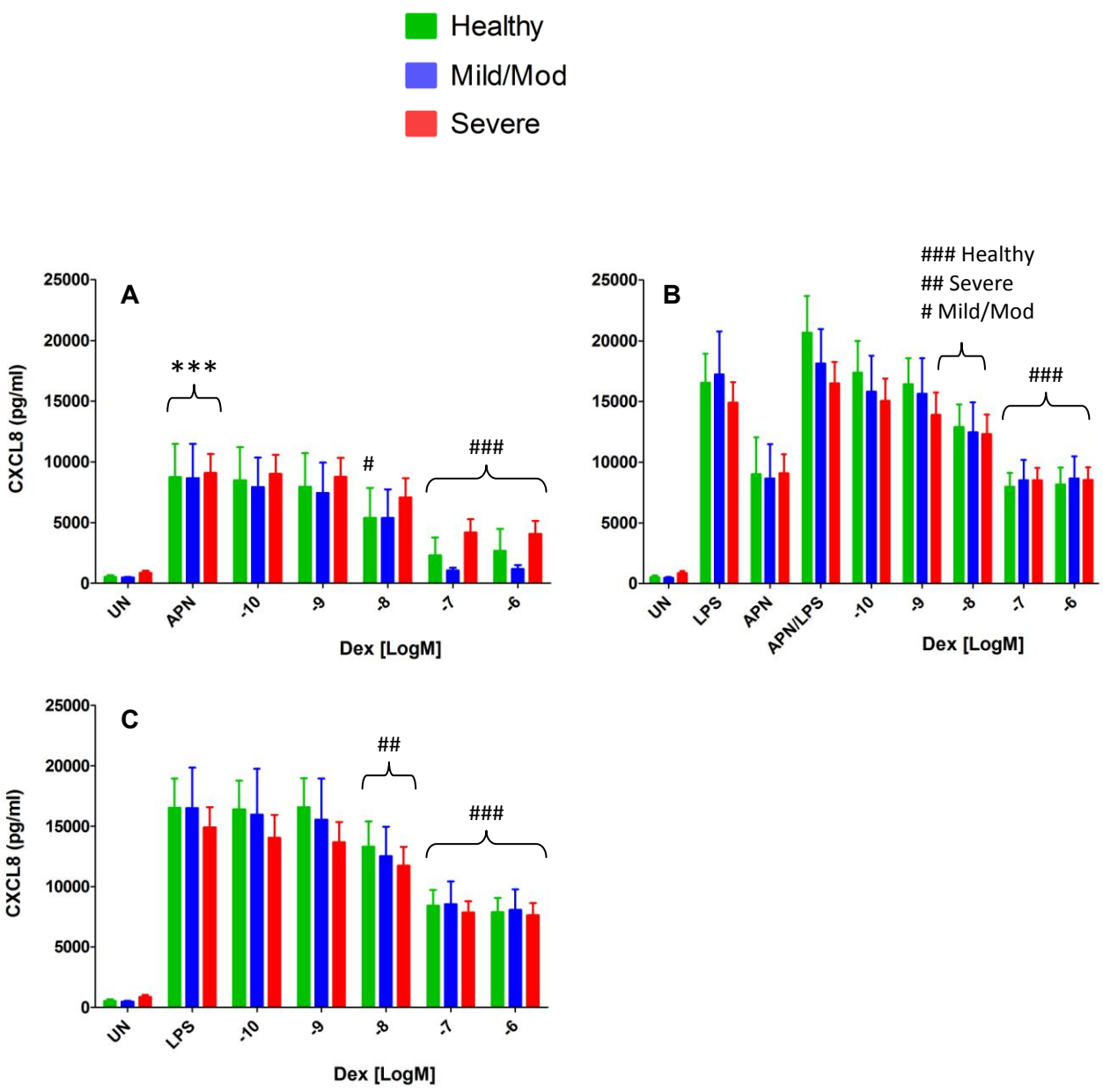


Figure 6.9: Effect of adiponectin, LPS, Dexamethasone, or in combination on CXCL8 release from PBMCs. Healthy (n=11), Mild/Moderate asthma (n=12), Severe asthma (n=14). PBMCs treated with (A) adiponectin (100 ng/ml) ± Dex (10^{-10} to 10^{-6} M) for 21 hours, (B) adiponectin (100 ng/ml) ± Dex (10^{-10} to 10^{-6} M) for 1 hour and stimulated with LPS (0.5 ng/ml) for 20 hours, (C) Dex (10^{-10} to 10^{-6} M) for 1 hour and stimulated with LPS for 20 hours. CXCL8 release was measured by ELISA. Data represent mean ± SEM. *** p<0.0001 for APN vs. UN; # p<0.05, ## p<0.01, ### p<0.0001 for APN and Dex vs. APN, LPS and Dex vs. LPS or LPS and Dex vs. LPS.

6.3.14 Effect of adiponectin on CXCL8 release from PBMCs according to BMI.

Data displayed in figure 6.9 was then subdivided for each cohort by BMI, separating subjects into two groups: obese with a BMI ≥ 30 and a normal BMI ≤ 25 (figure 6.10). No significant difference was seen in terms of CXCL8 release according to BMI in response to adiponectin.

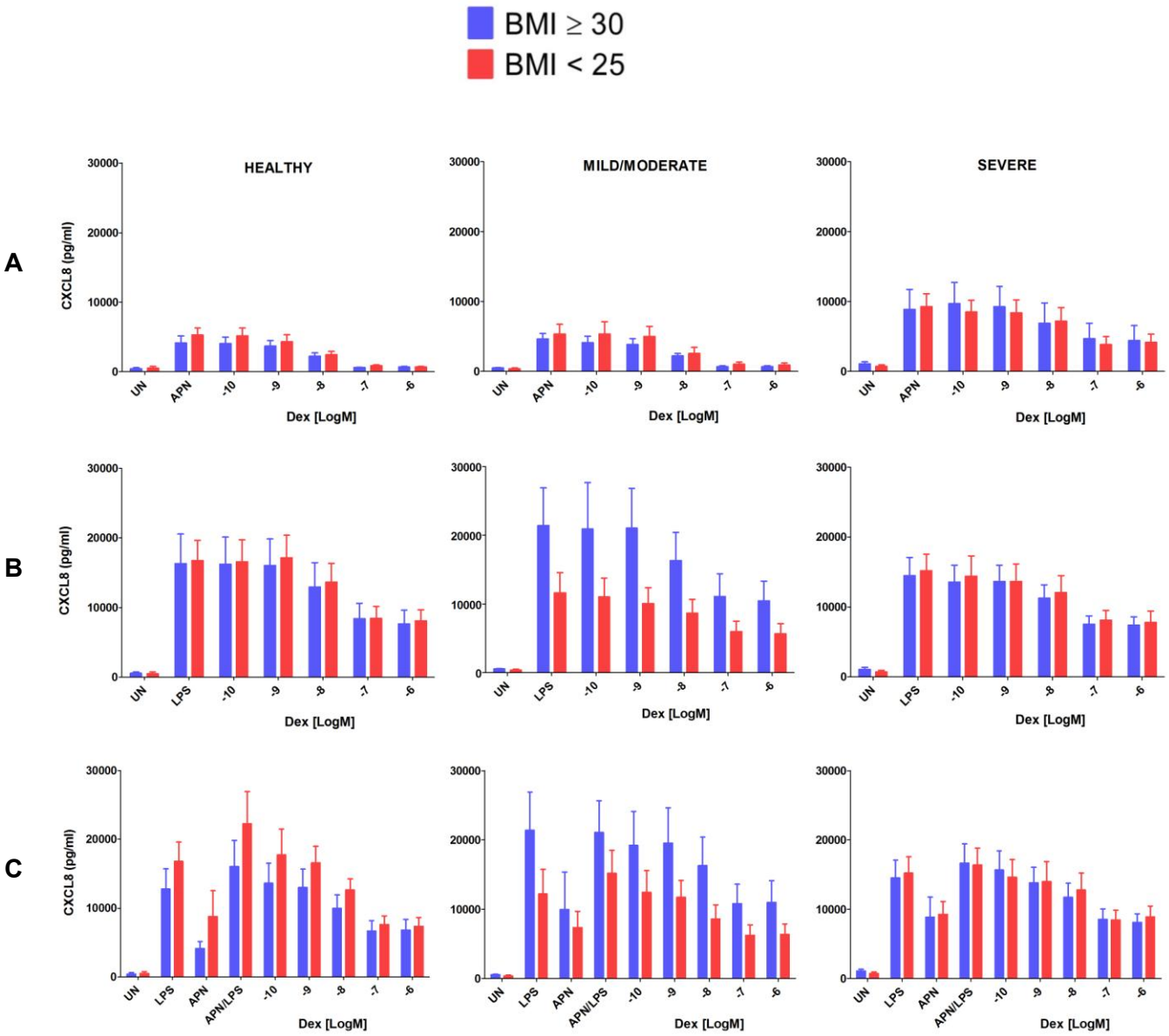


Figure 6.10: Effect of adiponectin, LPS, Dexamethasone, or in combination on CXCL8 release from PBMCs according to BMI. BMI ≥30: Healthy (n=5), Mild/Mod (n=6), Severe (n=6); BMI <25: Healthy (n=6), Mild/Mod (n=6), Severe (n=8). PBMCs treated with (A) adiponectin (100 ng/ml) with/without Dex (10^{-10} to 10^{-6} M) for 21 hours, (B) Dex (10^{-10} to 10^{-6} M) for 1 hour and stimulated with LPS (0.5 ng/ml) for 20 hours, (C) adiponectin (100 ng/ml) with/without Dex (10^{-10} to 10^{-6} M) for 1 hour and stimulated with LPS (0.5 ng/ml) for 20 hours. CXCL8 release was measured by ELISA.

6.3.15 Suppressive effect of dexamethasone on CXCL8 release in PBMCs pre-treated with adiponectin and/or LPS.

Normalised data can be seen for adiponectin, adiponectin/LPS and LPS alone for each cohort (figure 6.11).

The severe cohort (figure 6.11, panel C) exhibits a similar inhibition of CXCL8 with increasing concentrations of Dex (10^{-10} to 10^{-6} M) for PBMCs treated with APN, APN/LPS and LPS alone. Approximately 50% suppression was seen at a concentration of 10^{-6} M. Although the inhibition curve for LPS alone was similar in all three cohorts (figure 6.13, panel B), differences were seen with adiponectin alone (figure 6.13, panel A) and in combination with LPS (figure 6.13, panel C) between the groups. Both the healthy and mild/moderate cohort exhibited a greater % suppression of CXCL8 for Dex at all concentrations but significantly different from 10^{-10} to 10^{-6} M for APN and APN/LPS and from 10^{-8} to 10^{-6} M for LPS alone (figure 6.11). At Dex concentrations 10^{-7} and 10^{-6} M there was an approximate 85% suppression of CXCL8 release. APN and LPS in combination appeared to increase the inhibition of CXCL8 at Dex concentrations of 10^{-9} to 10^{-7} M in the healthy and mild/moderate cohorts; although this did not achieve statistical significance.

Whilst the severe cohort did not exhibit a difference between the treatments, the healthy and mild/moderate cohorts revealed greater Dex-induced suppression of CXCL8 release in PBMCs stimulated with APN compared to APN/LPS and LPS alone. The asthmatic cohorts did not exhibit significant differences comparing stimulation with LPS and APN/LPS. However, significant differences were seen in the healthy cohort at Dex (10^{-8} M) (mean 29.3% suppression versus 36%

respectively, p=0.0029), Dex (10^{-7} M) (46.3% versus 59.4%, p=0.0014) and Dex (10^{-6} M) (49.9% versus 61.4%, p=0.003).

Figure 6.12 displays the same data as figure 6.11 but divided by BMI into subjects with a BMI ≥ 30 and classified as obese and those with a normal BMI < 25. No significant differences were seen in the three cohorts when divided by BMI.

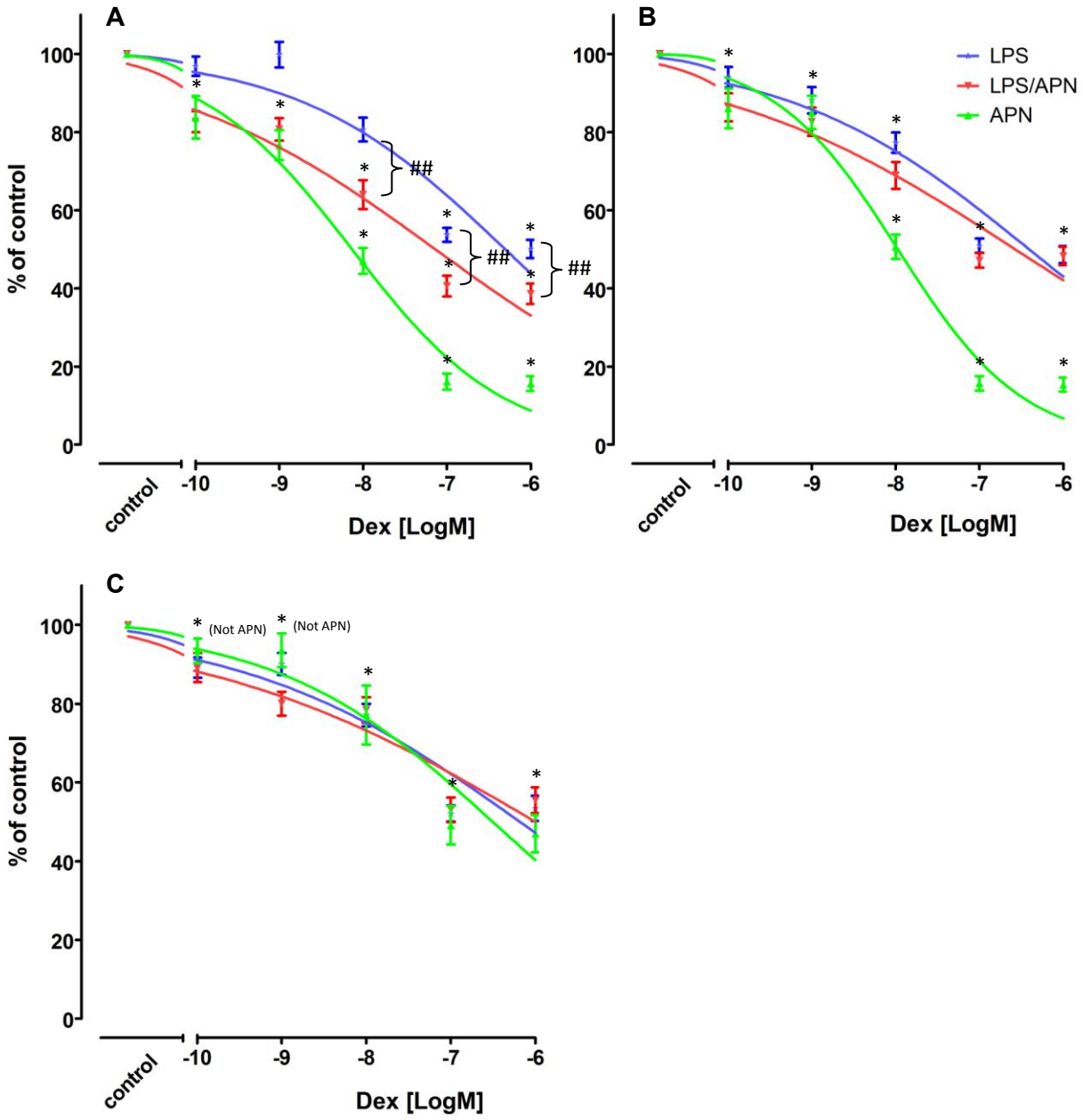


Figure 6.11: Suppressive effect of dexamethasone on CXCL8 release in PBMCs of healthy subjects, mild/moderate and severe asthmatics. (A) Healthy (n=10), (B) Mild/Mod (n=12), (C) Severe (n=14). PBMCs treated with adiponectin (100 ng/ml) with/without Dex (10^{-10} to 10^{-6} M) for 2 hours prior to stimulation with LPS (0.5 ng/ml) for 20 hours. CXCL8 release was measured by ELISA. Data represent mean ± SEM. ** p<0.0001 versus control, ## p<0.005 LPS versus APN/LPS.*

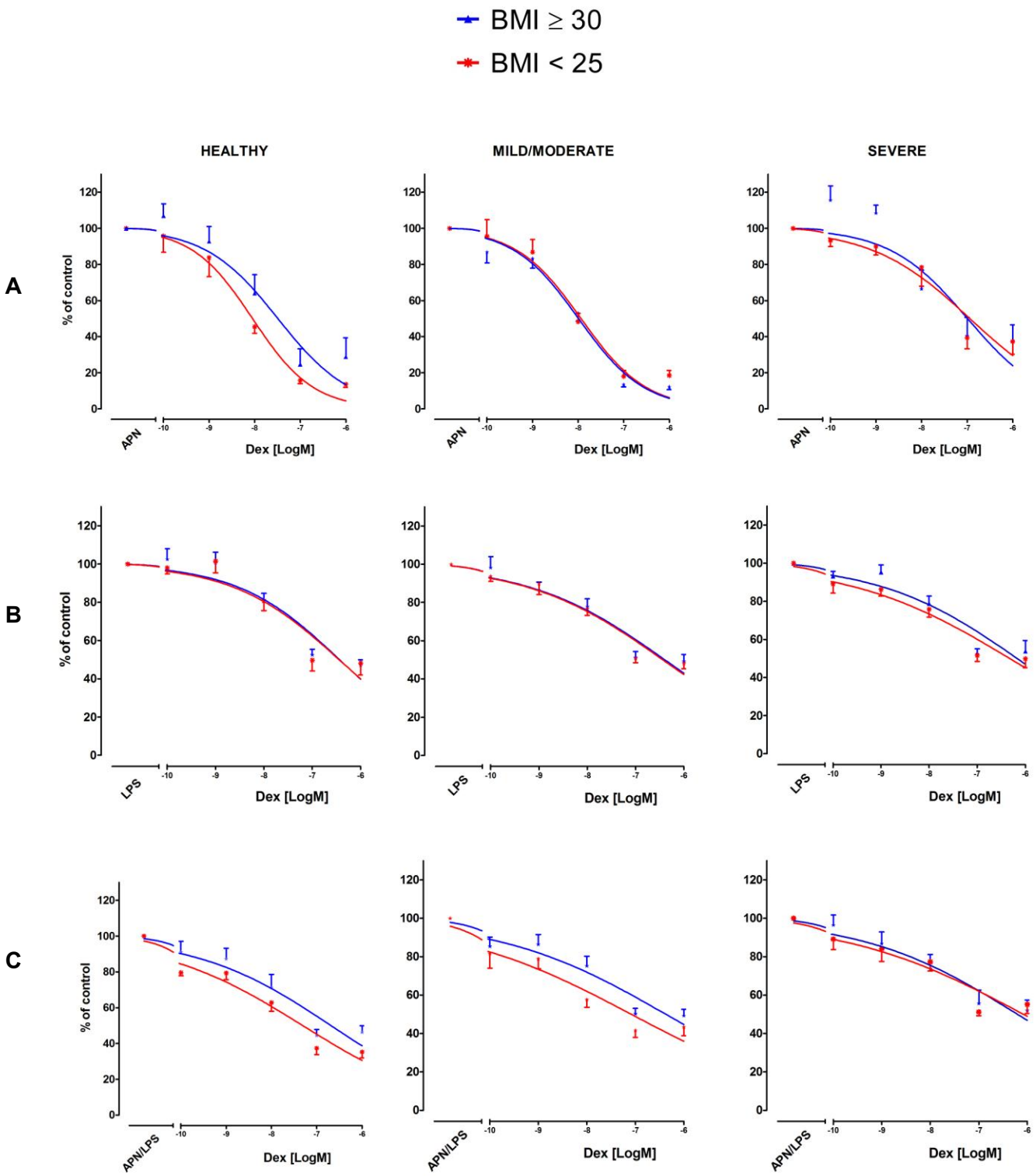


Figure 6.12: Effect of adiponectin, LPS, Dexamethasone, or in combination on CXCL8 release from PBMCs according to BMI. BMI ≥30: Healthy (n=5), Mild/Mod (n=6), Severe (n=6); BMI <25: Healthy (n=6), Mild/Mod (n=6), Severe (n=8). PBMCs treated with (A) adiponectin (100 ng/ml) with/without Dex (10^{-10} to 10^{-6} M) for 21 hours, (B) Dex (10^{-10} to 10^{-6} M) for 1 hour and stimulated with LPS (0.5 ng/ml) for 20 hours, (C) adiponectin (100 ng/ml) with/without Dex (10^{-10} to 10^{-6} M) for 1 hour and stimulated with LPS (0.5 ng/ml) for 20 hours. CXCL8 release was measured by ELISA.

6.3.16 Suppressive effect of dexamethasone on CXCL8 release in PBMCs pre-treated with adiponectin.

Figure 6.13 shows the same data represented in figure 6.11 but by treatment (adiponectin, adiponectin/LPS or LPS alone) allowing comparisons to be made between cohorts. No significant differences were seen in CXCL8 release in response to stimulation with LPS or with adiponectin and LPS in combination. However, in the latter there appears to be a difference with the severe group less responsive to suppression of CXCL8 by Dex.

PBMCs treated with adiponectin alone were less responsive to Dex-induced suppression of CXCL8 release in the severe cohort with significant differences when compared to the mild/moderate and healthy cohorts at concentrations of 10^{-6}, 10^{-7} and 10^{-8} M.

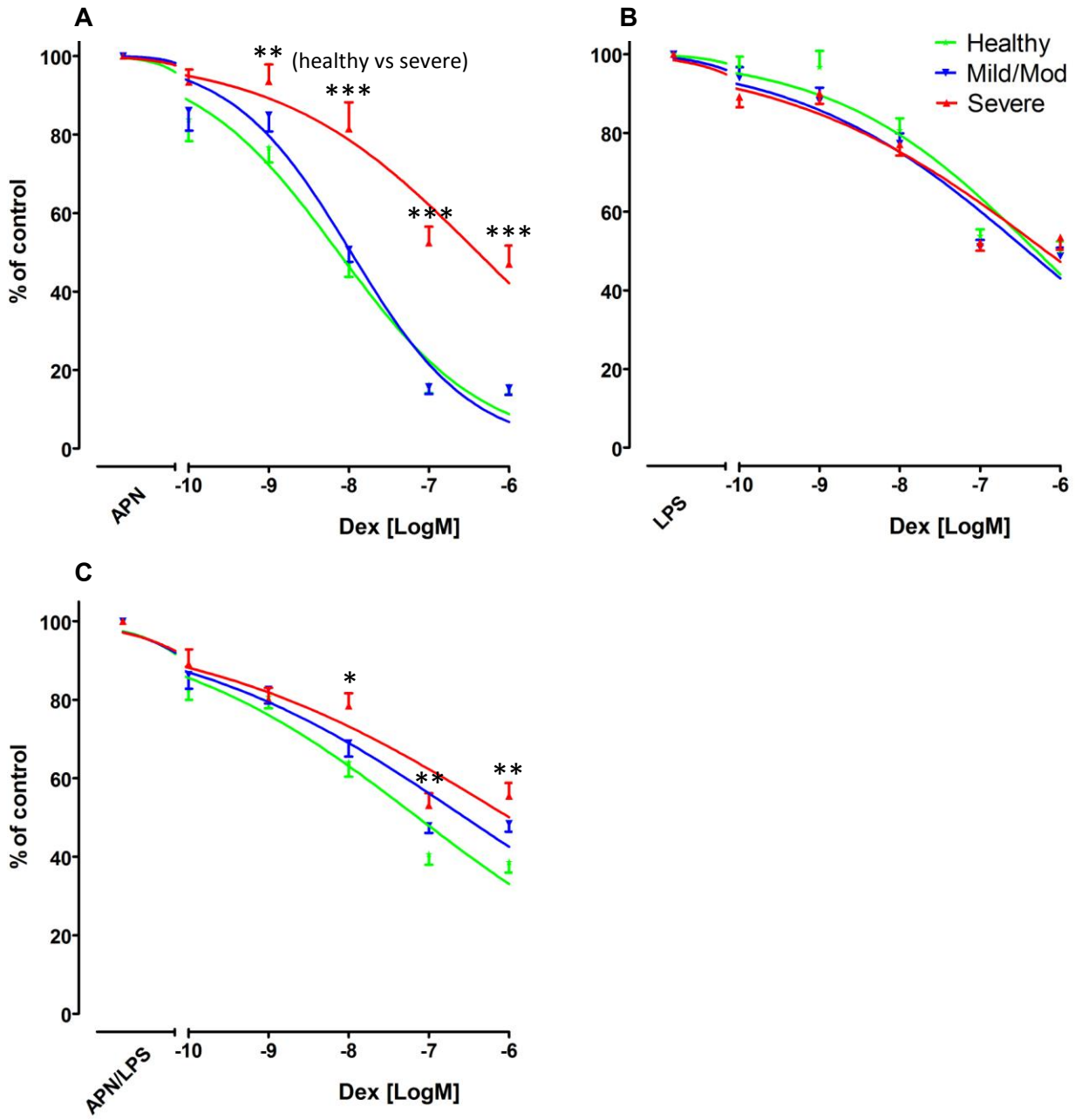


Figure 6.13: Suppressive effect of dexamethasone on CXCL8 release in PBMCs pre-treated with adiponectin, LPS, Dexamethasone, or in combination. Healthy (n=10), Mild/Mod (n=12), Severe (n=14). PBMCs treated with (A) adiponectin (100 ng/ml) with/without Dex (10^{-10} to 10^{-6} M) for 21 hours, (B) Dex (10^{-10} to 10^{-6} M) for 1 hour and stimulated with LPS (0.5 ng/ml) for 20 hours, (C) adiponectin (100 ng/ml) with/without Dex (10^{-10} to 10^{-6} M) for 1 hour and stimulated with LPS (0.5 ng/ml) for 20 hours. CXCL8 release was measured by ELISA. Data represent mean ± SEM. * *p<0.05, ***p<0.01, *** p<0.0001* severe vs. mild/moderate and healthy cohort.

6.3.17 Effect of PI3K, JNK, ERK and p38 MAPK inhibitor on CXCL8 release from PBMCs treated with adiponectin.

To investigate the cellular pathways involved in the action of adiponectin, PBMCs were pre-treated with PI3K, JNK, ERK and p38 MAPK inhibitors for 2 hours prior to the addition of adiponectin (figure 6.14).

Adiponectin induced CXCL8 release from PBMCs was inhibited with GW-856553 (a selective p38α/β inhibitor), with maximal suppression by 94% at 10^{-6} M (p<0.0001). U10126 (an inhibitor of MEK1/2-mediated ERK1/2 phosphorylation) inhibited CXCL8 release with 63% suppression at 10^{-6} M (p=0.0007). SP600125 (a JNK inhibitor that competes with ATP to inhibit the phosphorylation of c-Jun) and wortmannin (a PI3K inhibitor) did not affect CXLC8 release at concentrations up to 10^{-6} M. 45% suppression was seen at 10^{-5} M for wortmannin PI3K (p=0.0009); although this may represent off target effects at high concentration (figure 6.14).

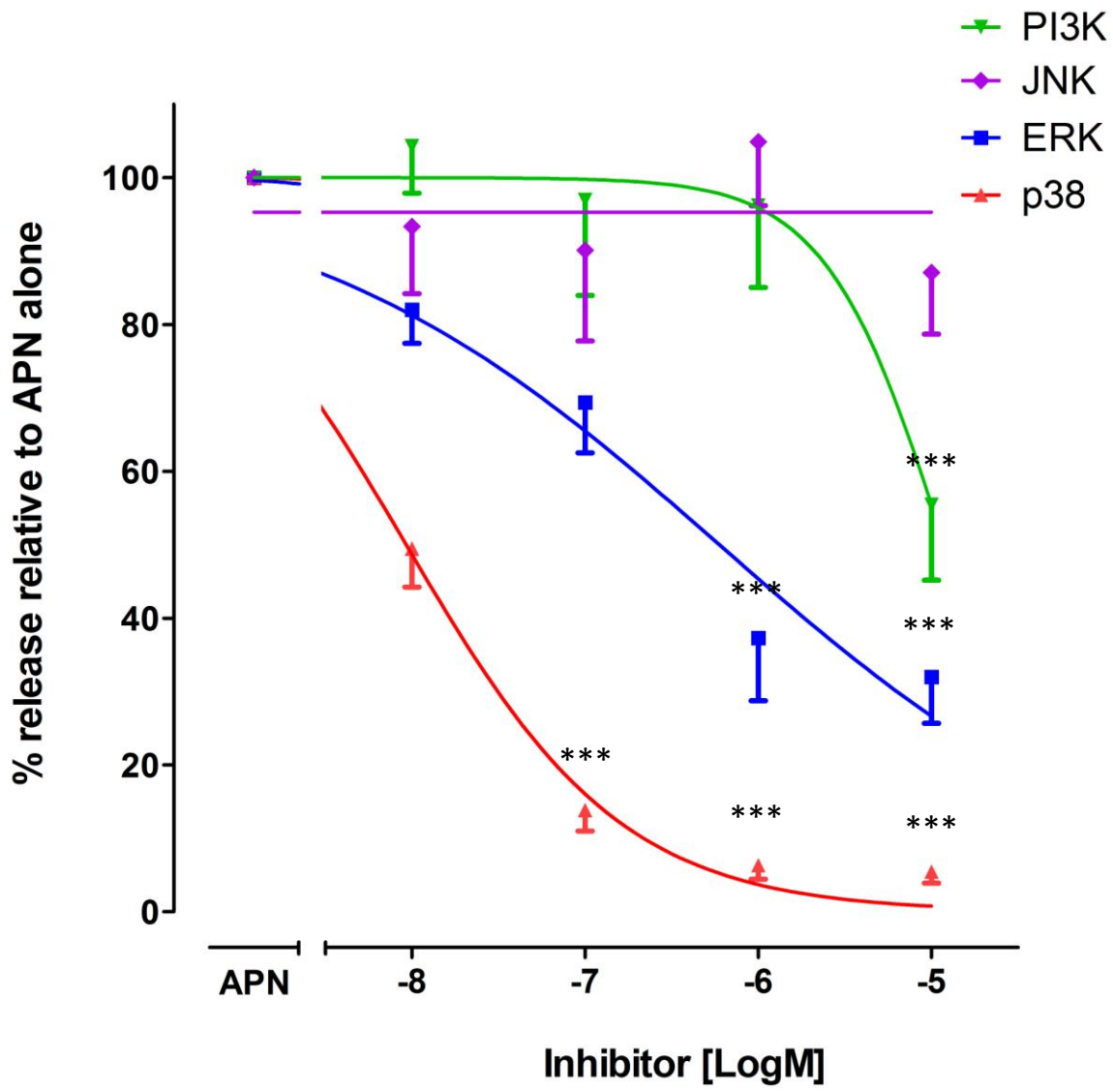


Figure 6.14: Effect of PI3K, JNK, ERK and p38 MAPK inhibitor on CXCL8 release from PBMCs treated with adiponectin. PI3K (green) n= 13; JNK (purple) n= 5; ERK (blue) n=5; p38 (red) n= 12. Cells were pre-treated with increasing concentrations (10^{-5} to 10^{-8} M) of the various inhibitors: GW-856553, U10126, SP600125 or PI3K wortmannin for 2 hours prior to treatment with adiponectin (100 ng/ml) for 20 hours. CXCL8 release was measured by ELISA. Data represent mean ± SEM. ***p<0.0001 versus APN.

6.4 Discussion

Leptin and resistin were pro-inflammatory, in terms of CXCL8 release from PBMCs taken from healthy subjects, mild/moderate and severe asthma when used at a concentration of 1 μg/ml. However, no significant effects were seen in terms of LPS induced CXCL8 release or corticosteroid sensitivity. Adiponectin exhibited a pro-inflammatory effect at a concentration of 100 ng/ml and adiponectin-treated PBMCs taken from severe asthmatics were less responsive to steroid mediated suppression, suggesting a pro-inflammatory effect in this group. These findings follow the hypothesis that adipokines modulate inflammatory cytokine release and, in the case of adiponectin, they augment corticosteroid sensitivity.

The severe asthmatics in this study were predominantly female (71%) and 46% had a BMI in the obese range (median BMI 34.4). The majority were atopic and just over half reported GORD. This compares to other large cohorts of severe asthma such as TENOR (The Epidemiology and Natural History of Severe Asthma: Outcomes and Treatment Regimes), where the adult mean BMI was 30.4 and 71.2% were female, and SARP (Severe Asthma Research Program) where 41% of severe asthmatics had GORD (Moore et al., 2007). The majority of both asthmatic cohorts (84.2% in the mild/moderate and 71.4% in the severe cohort) were atopic which also compares well with SARP (81% - mild, 85% - moderate, 71% - severe). Just over 30% of the healthy population were atopic which is similar to figures of about 33% seen in previous studies of healthy cohorts (Strachan et al., 1997).

Leptin is pro-inflammatory and serum levels are positively correlated with obesity. It has been shown to cause AHR in mice (Shore et al., 2005) and induces phosphorylation of ERK-1, ERK-2 (Martin-Romero and Sanchez-Margalet, 2001) and

p38 MAPK in human PBMCs (Procaccini et al., 2009). In this study, leptin induced CXCL8 release only at the highest concentration of 1 μg/ml. This is in excess of the upper limit of physiological levels seen in humans (usually below 10 ng/ml and rising to between 10 ng/ml and 60 ng/ml in the obese state) (Licinio et al., 1997). Local leptin concentrations *in vivo* are unknown and the pro-inflammatory effect seen at 1 μg/ml may reflect the high concentration exposure or potentially off target effects. No significant effects of leptin on LPS induced CXCL8 release or on steroid sensitivity were seen in this study. Other studies have noted pro-inflammatory effects of leptin. Faggioni et al found that LPS induced leptin induction, dependent of IL-1β, in mice (Faggioni et al., 1998). Shen et al found that leptin enhanced TNF-α production, with a concentration dependent increase in MAPK activity, in LPS stimulated Kupffer cells (Shen et al., 2005, Faggioni et al., 2000). Martín-Romero and Sánchez-Margalet found that PBMCs incubated with leptin activated PI3K and MAPK (Martin-Romero and Sanchez-Margalet, 2001) and Procaccini et al. noted sustained phosphorylated of p38 MAPK in PBMCs pre-treated with leptin (Procaccini et al., 2009). Furthermore, leptin has been shown to have a stimulatory effect on human circulating monocytes, inducing TNF-α and IL-6 production (Santos-Alvarez et al., 1999). In subsequent studies, the same authors noted that PBMCs taken from patients with HIV displayed increased leptin receptor expression, suggesting a role of leptin in the immune response (Sanchez-Margalet et al., 2002).

Resistin is also positively correlated with obesity (Al-Suhaimi and Shehzad, 2013) and regarded as pro-inflammatory. I found that resistin was pro-inflammatory in all three cohorts at the highest concentration of 1 μg/ml and in the severe asthma and healthy cohorts for the second highest concentration of 100 ng/ml. These are both well above the normal physiological levels in serum of between 7 and 22 ng/ml

(Fadda et al., 2013). Previous studies have shown that resistin induces the secretion of TNF-α and IL-12 from macrophages through NFκB and MAPK via TLR4 (Silswal et al., 2005). There is a paucity of data regarding the cellular action in asthma and the majority of previous studies looking at resistin and asthma have focused on serum levels and disease, disease severity or response to corticosteroids. Whilst some studies have noted that low serum levels are predictive of developing asthma (Kim et al., 2008), others noted higher levels correlate to disease severity (Larochelle et al., 2007) and may even predict a good anti-inflammatory response to corticosteroid therapy (Leivo-Korpela et al., 2011). Although this study did not find that resistin influenced the inhibitory action of Dex, only CXCL8 release was analysed. Furthermore, other cell types such as AMs may be more representative.

There are a number of limitations and considerations with both the leptin and resistin work. Only CXCL8 was assessed for both these cytokines and further work looking at a combination of cytokines may explore their inflammatory action on PBMCs in greater detail. The use of Western blotting to evaluate protein expression would also be useful to explore the effects of these cytokines on PBMCs. This study did not show a significant difference in CXCL8 levels for leptin and resistin when subdividing for obesity. This may reflect the low numbers available for comparison.

Adiponectin is an abundant serum protein, accounting for 0.01% of total plasma proteins, and is generally viewed as an anti-inflammatory cytokine (Hu et al., 1996). However, this study has found a pro-inflammatory effect in terms of CXCL8 release from PBMCs. Adiponectin induced CXCL8 release from PBMCs at a concentration of 100 ng/ml. This is below the physiological levels of adiponectin seen *in vivo* of between 3 and 30 μg/ml (Folco et al., 2009).

The pro-inflammatory effects of adiponectin seen are likely to reflect a combination of factors. The predominant APN receptors found in PBMCs are adipoR1 and adipoR2. Other *in vitro* studies have noted that adiponectin induces IL-6 and CXCL8 from hepatocytes (Suzuki et al., 2011); via ERK1/ERK2, p38 MAPK, NF-κB and STAT3 signalling pathways (Kuerschner et al., 2008).

Adiponectin has been shown to be raised in some disease states such as rheumatoid arthritis, SLE, type 1 diabetes and inflammatory bowel disease; suggesting a potential pro-inflammatory role (Fantuzzi, 2008). In addition, previous studies have shown that adiponectin induces CXCL8 release from human chondrocytes (Gomez et al., 2011), human placenta and adipose tissue (Lappas et al., 2005) and induces TNF-α and IL-6 release from primary human peripheral macrophages (Tsatsanis et al., 2005).

In addition, some studies have suggested that it is the length of time that cells are exposed to adiponectin that alters the cellular response. Cheng and colleagues noted that adiponectin induced a pro-inflammatory action in macrophages that preceded or mediated tolerance to further pro-inflammatory stimuli through mechanisms that remain unclear (Cheng et al., 2012). Tsatsanis and colleagues demonstrated that although globular adiponectin induced TNF-α and IL-6 release from primary human peripheral macrophages, their pre-treatment with low concentration of adiponectin for 24 hours prior to re-stimulation led to tolerance and suppression of TNF-α and IL-6 release (Tsatsanis et al., 2005). These studies suggest that short term *in vitro* experiments, such as those in this study, involving acute exposure to adiponectin are not representative of the chronic exposure encountered *in vivo*. Furthermore, the adiponectin used in this work is prokaryote derived and predominantly globular which has been suggested to be more

biologically active and pro-inflammatory than the other adiponectin isoforms (Richards et al., 2006).

Adiponectin treated PBMCs taken from severe asthmatics were less responsive to steroid suppression, suggesting an exaggerated pro-inflammatory effect in this group. In contrast, PBMCs treated with adiponectin and LPS exhibited greater suppression of CXCL8 compared to LPS alone for Dex (10^{-8} to 10^{-6} M) in the healthy cohort. This suggests that APN potentiated the steroid response; a phenomenon not seen in the asthmatic groups. The reasons for this difference are not clear but may represent an anti-inflammatory action of adiponectin in the healthy population that is not present in asthma or diminishes with increasing disease severity. This is in contrast to population studies looking at circulating or lung levels of adiponectin and clinical disease outcome (Sood and Shore, 2013).

This study did not find any significant difference in Dex suppression of LPS induced CXCL8 release between the cohorts. This is in contrast to a previous study which found PBMCs taken from patients with severe asthma exhibited diminished corticosteroid sensitivity when compared to non-severe asthma (Hew et al., 2006).

Furthermore, I did not see a difference in the effect of adiponectin on CXCL8 release from PBMCs when subdividing by BMI (comparing normal, BMI <25 with obesity BMI ≥30). This is surprising given that adiponectin levels negatively correlate with increasing adiposity and is in contrast to previous studies which have noted corticosteroid insensitivity in obesity-associated asthma. This stems from the post-hoc analysis of clinical trial data, which have associated increasing BMI with a reduced response to ICS (Peters-Golden et al., 2006, Sutherland et al., 2008a, Boulet and Franssen, 2007) and by an *in-vitro* study on PBMCs, which found

overweight and obese asthmatics exhibited a reduced response to corticosteroids (Sutherland et al., 2008a).

The pre-treatment of PBMCs with p38 MAPK and ERK inhibitors markedly attenuated adiponectin induced CXLC8 release indicating the involvement of these pathways. Previous studies looking at human chondrocytes found that p38 MAPK and ERK1/2 inhibition attenuated inflammatory pathways in terms of MMP-3 release and phosphorylation of AMPK (Tong et al., 2011, Kang et al., 2010). Other studies have noted that p38 MAPK and ERK1/2 are key signalling pathways involved in adiponectin induced inflammation in human placenta, adipose tissue (Lappas et al., 2005), T cells (Cheng et al., 2012) and human phagocytes (Chedid et al., 2012).

There are some important limitations and considerations relating to this study. Adiponectin is present in different oligomeric complexes that are likely to impact on its function. Different isoforms exert different effects and it may in fact be their relative proportions or ratio that influences the inflammatory response (Pajvani et al., 2004). The adiponectin used in this study is fAPN, known as monomeric adiponectin. Although present *in vivo*, this is not representative of physiological adiponectin which contains a mix of the different adiponectin isoforms. The monomeric form has been reported as being more biologically active compared to other isoforms (Nishida et al., 2007). Repeating this work using a physiological mix of adiponectin would allow for these differences and potentially be more representative.

The derivation and manufacturing method of globular adiponectin may also play an important role in inflammatory activity. There are two commercially available sources of adiponectin; eukaryote or prokaryote derived. In the latter, E.coli inclusion bodies are used in the process and therefore there is the possibility of LPS

contamination. In addition, eukaryote derived adiponectin appears to be able to form HMW complexes whilst its prokaryote counterpart has inadequate folding of the collagen-like domain, meaning it tends to be in globular, trimeric or hexameric forms (Richards et al., 2006). This is a particularly significant factor given that different isoforms of adiponectin appear to have differing inflammatory actions. This study has compared HEK versus E.coli derived adiponectin (figure 6.6) and found no significant difference in terms of CXCL8 release in the presence of dexamethasone and stimulated with LPS. However, this was only with three matched subjects and only looked the release of one cytokine. Repeating this with additional cytokines and in a higher number of subjects would be useful to address this point.

Serum adiponectin levels were not assessed in this study. Serum levels at the time blood was taken from subjects could have been correlated with the cellular outcome and in addition, serum adiponectin displays diurnal variation and tolerance. However, blood for this study was taken at similar times in the morning. Repeating the study with measurement of serum adiponectin levels at the same time as PBMC extraction would allow greater interpretation of the role of adiponectin in asthma.

The adipokines assessed did not augment LPS-induced CXCL8 release from PBMCs. LPS is ubiquitous in the environment, is a potent inflammatory stimulant, can induce airway inflammation and exacerbate allergic responses – factors which led to LPS being chosen as a positive control for this study. The fact that all three adipokines were pro-inflammatory in terms of CXCL8 release from PBMCs meant that the inclusion of LPS in these experiments became largely redundant.

Finally, PBMCs were used for this study and they may not be representative of the effects of adiponectin in asthma. Repeating this work using AMs, ASMCs and

bronchial epithelial cells would provide greater insight into the effects of adiponectin in asthma.

In summary, leptin and resistin were pro-inflammatory at the highest concentration tested which is supraphysiological. Adiponectin was pro-inflammatory at a concentration below physiological concentrations and adiponectin treated PBMCs taken from severe asthmatics exhibited an attenuated corticosteroid response in terms of CXCL8 release compared to mild/moderate asthmatics and healthy subjects. However, no correlations were seen in relation to BMI. Adiponectin augments the steroid response of PBMCs taken from healthy subjects in cells stimulated with LPS. Further investigation is required to establish the mechanism of this link and establish the role of adiponectin in asthma severity.

Chapter 7: The effect of adiponectin on PBMCs: inflammatory response and corticosteroid insensitivity in severe asthma

Chapter 7: The effect of adiponectin on PBMCs: inflammatory response and corticosteroid insensitivity in severe asthma

Hypothesis and aim

Adiponectin modulates inflammatory cytokine release and corticosteroid insensitivity in severe asthma. I will examine the role of adiponectin in obesity-related severe asthma through *in vitro* experiments involving peripheral blood mononuclear cell taken from asthmatics and healthy controls. The aims are to study the effect of adiponectin on PBMCs in terms of inflammatory response, corticosteroid insensitivity and their relationship with BMI.

7.1 Introduction

In general, adiponectin is viewed as an anti-inflammatory cytokine that is predominantly produced by adipocytes and is negatively correlated with obesity. Previous studies have shown that it exerts an anti-inflammatory action by inducing IL-10 and IL-1RA release and by decreasing the release of pro-inflammatory cytokines such as IL-6, IFN- γ and TNF-α (Wolf et al., 2004, Folco et al., 2009, Tsatsanis et al., 2005). MDMs pre-treated with adiponectin exhibit increased IL-10 expression (Kumada et al., 2004) and adiponectin inhibits macrophage TNF-α and IL-6 mediated inflammation (Folco et al., 2009). In contrast, adiponectin has been shown to induce IL-1β, IL-6 and TNF-α release from human placenta and adipose tissue (Lappas et al., 2005). Furthermore, the pre-treatment of primary human macrophages induces the release of TNF-α and IL-6 (Lappas et al., 2005).

Adiponectin has been discussed in Chapter 1 and the Introduction to Chapter 6. This study is a continuation from Chapter 6 and uses the same patient cohort and samples. Although adiponectin is largely viewed as anti-inflammatory, results from Chapter 6 showed a pro-inflammatory effect, in terms of CXCL8 release from

PBMCs. In view of this and with the aim of clarifying whether adiponectin is beneficial in asthma, this study sets out to look at seven more cytokines (IL-6, IFN-γ, IL-10, IL-1RA, CCL2, CCL3 and TNF-α). These cytokines were chosen in view of the known cellular effects of adiponectin (figure 1.6). Adiponectin interacts with monocyte AdipoR1 and AdipoR2 receptors resulting in the suppression of IL-6, IFN-γ, CCL2, CCL3 (Neumeier et al., 2011) and TNF-α, whilst increasing the production of IL-10 and IL-1RA (Wolf et al., 2004).

IL-6 was measured in all of the original samples used for CXCL8 analysis and the remaining cytokines were measured using a multiplex assay. Two Dex concentrations (10^{-7} and 10^{-8} M) were chosen to look at steroid response for use with the multiplex assay.

LPS was used to stimulate PBMCs for each cytokine assessed both in isolation and in combination with adiponectin. LPS is present within the cell wall of Gram-negative bacteria and acts as a strong stimulator of the innate immune reponse. It is known to activate monocytes via TLR4 resulting in the release of inflammatory cytokines. Our study group has previously used LPS to investigate relative corticosteroid insensitivity in asthma (Hew et al., 2006, Bhavsar et al., 2008). The use of LPS enabled a baseline response to be measured and comparison to the effects of adiponectin.

7.2 Method

To examine the effects of adiponectin on cytokine release from PBMCs and the interaction of LPS, cells were pre-treated with adiponectin with/without Dex 10^{-7} and 10^{-8} M for one hour prior to stimulation with LPS (10 ng/ml) and supernatant collected after 20 hours. A multiplex cytokine assay was used to assess the concentrations of six different cytokines. IL-6 was measured in all original samples using a DuoSet ELISA kit (R & D Systems, Abingdon, UK) according to the manufacturer's protocol as detailed in chapter 2.

7.3 Results

7.3.1 Demographics

Baseline data of patients can be seen in table 7.1. Similar demographics were seen in the subgroup of patients taken from all recruited subjects compared to the overall cohort (table 6.1). Significant differences were noted in terms of asthma medication requirements, lung function, and asthma symptoms in the severe asthma compared to the mild to moderate cohort. A more detailed description can be found in Chapter 6.

Table 7.1 : Demographics for subjects recruited for adiponectin studies.

	Healthy subjects	Mild/mod asthma	Severe asthma	*P value*
N	11	12	14	
Age (yrs)	43 (36 – 48)	51 (35.5 – 58.8)	56 (45 – 61)	0.14
M/F	5/6	4/8	3/11	0.44
Smoking History				
Never	9	12	12	0.32
Ex	2	0	2	
Height	172.0 (155 - 180)	169 (161.5 - 175.3)	164.5 (160 - 169.8)	1
Weight	70.5 (61.1 - 87.5)	77.0 (60.0 - 88.8)	66.5 (61.9 - 94.1)	0.41
BMI	25.3 (20.5 - 32.5)	26.6 (21.9 - 30.5)	24.5 (22.5 - 35.4)	0.79
BEI fat %	25.4 (13 - 40.2)	31.6 (23.8 - 38.7)	37.7 (27.2 - 46.6)	0.14
Skin fold fat %	35.0 (28.5 - 39.3)	33.4 (29 - 37.1)	35.9 (25.7 - 40.9)	0.78
waist/hip ratio	0.88 (0.79 - 0.96)	0.84 (0.75 - 0.98)	0.91 (0.84 - 0.96)	0.45
BDP equiv dose (μg)	0	400 (250 - 700)	2400 (2000 - 2800)	<0.0001
Oral corticosteroid (%)	0	0	5 (35.7)	0.0087
OCS dose (mg)	0	0	14.5	
Aminophylline (%)	0	0	7 (50)	0.0008
LTRA (%)	0	1 (8.3)	7 (50)	0.0042
FEV_1 (l)	3.3 (2.3 - 4.2)	2.3 (1.7 - 2.9)	1.5 (1.1 - 1.8)	0.0001
FEV_1 %	100.5 (95.9 - 107.5)	82.1 (71.6 - 88.3)	61.4 (40.1 - 72.2)	<0.0001
FVC	4.1 (2.7 - 5.3)	3.6 (2.6 - 4.1)	2.6 (2.1 -3.3)	0.005
FVC %	13.5 (97.7 - 107.1)	98.9 (90.2 - 109.9)	79.1 (64.3 - 88.1)	0.0024
FEV1/FVC %	82.8 (78.3 - 85.5)	70.6 (64.0 - 73.5)	59.4 (49.2 - 70.1)	<0.0001
BDR (n)	1 (-2 - 4.0)	15.3 (9.9 - 19.1)	19.6 (12.2 - 27.7)	<0.0001
PC_{20} (n)	>16	0.9 (0.2 - 1.9)	*	
Atopic (n, %) *	3 (27.3)	11 (91.7)	10 (71.4)	0.0044
IgE kU/l	64 (25.5 - 376)	316.5 (162.5 - 399.5)	94.5 (65.5 - 532.3)	0.23
Eos	0.25 (0.1 – 0.33)	0.3 (0.2 – 0.4)	0.2 (0.1- 0.43)	0.38
History of GORD (n, %)	0	2 (16.7)	10 (71.4)	0.0003
PPI (n, %)	0	2 (16.7)	6 (42.9)	0.031
ACQ		8.5 (4 – 10)	14 (10.5 – 21.5)	0.038
AQLQ				
Activities		6.3 (6 – 6.6)	4.8 (3.8 – 6.3)	0.056
Symptoms		5.8 (5.6 – 6.3)	4.5 (3.8 – 6.1)	0.029
Emotional		6.4 (5.2 – 6.6)	5.4 (4.90 – 6.6)	0.15
Environmental		6 (5.5 – 6.5)	5.3 (4.4 – 6.4)	0.15
Total		6.1 (5.9 – 6.3)	4.7 (4.4 – 6.3)	0.091

Data are presented as mean ± SD or median (IQR). Between group comparisons for continuous variables were made using Kruskal-Wallis test and for categorical variables using χ^2 exact testing.

BMI, body mass index BEI, bioelectrical impedance BDP, beclomethasone dipropionate LTRA, leukotriene receptor antagonist FEV_1, forced expiratory volume in 1 second FVC, forced vital capacity IgE, immunoglobulin E GORD, gastro-oesophageal reflux disease PPI, proton pump inhibitor ACQ, asthma control questionnaire AQLQ, asthma quality of life questionnaire.

7.3.2 Effect of adiponectin on IL-6 release from PBMCs

Similar amounts of IL-6 release were seen in all three cohorts from untreated PBMCs (7.6 pg/ml [6.3], 8.9 [5.7], 8.5 [4.7]) for healthy, mild/moderate and severe asthmatic cohorts, respectively, p=0.89). LPS induced a lower but non statistically significant amount of IL-6 release in the mild/moderate cohort (9676 [3923]) compared to the healthy and severe asthma cohorts (14893 [9494] and 12358 [7628], p=0.37). Adiponectin appeared to induce a higher amount of IL-6 release from PBMCs taken from severe asthmatics (2883 [2896]) compared to the mild/moderate asthmatic and healthy cohorts (1245 [1050] and 1027 [680]). However, this did not achieve statistical significance (p=0.49).

All cohorts exhibited Dex-induced suppression of IL-6 release in a concentration-dependent manner (figure 7.1). Significant decreases were seen at Dex 10^{-7} and 10^{-6} M. The pre-treatment of PBMCs with adiponectin did not significantly change IL-6 release following stimulation with LPS or the response to increasing concentrations of Dex 10^{-10} to 10^{-6} M.

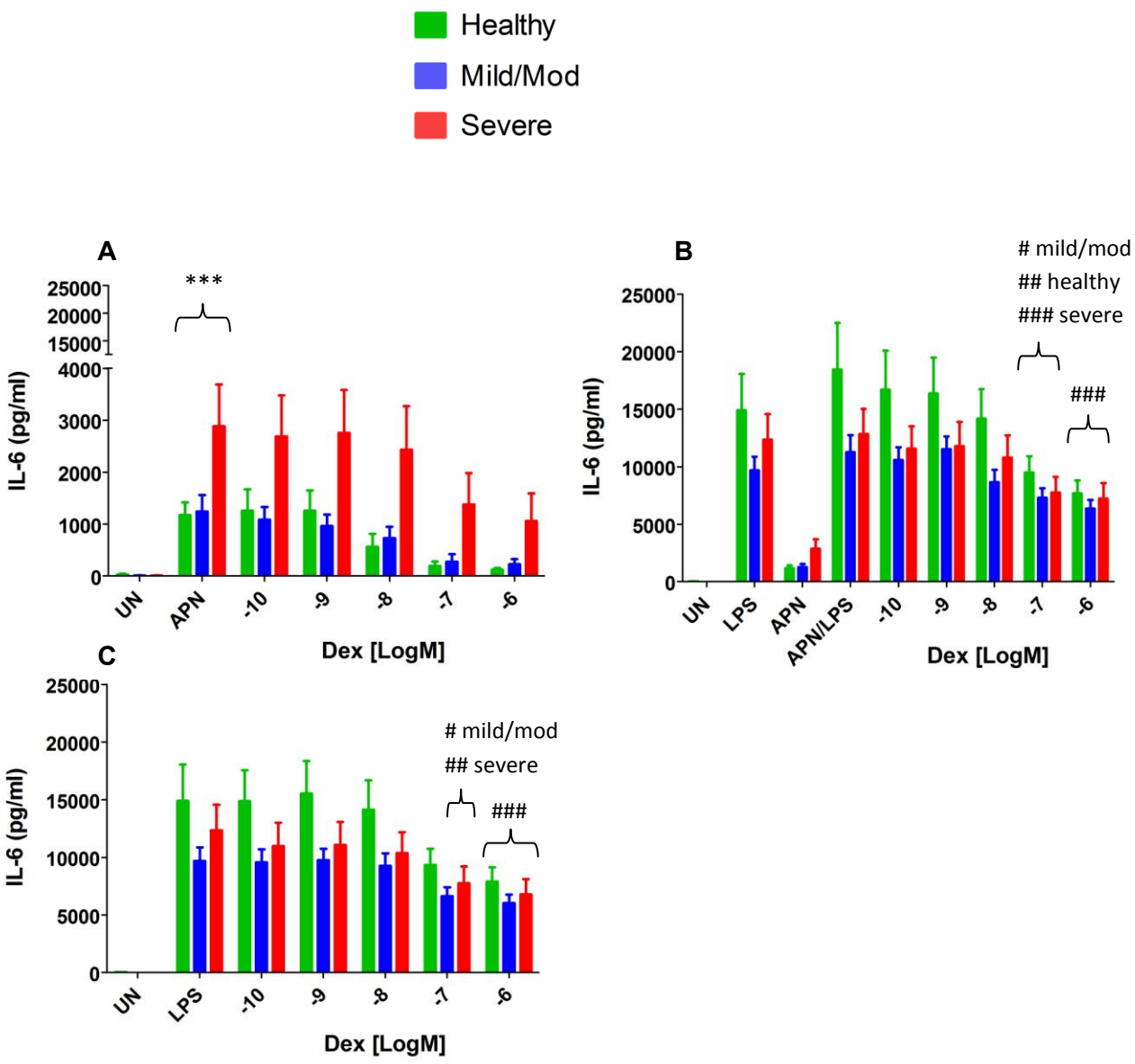


Figure 7.1: Effect of adiponectin, LPS, Dexamethasone, or in combination on IL-6 release from PBMCs. Healthy (n=11), Mild/Moderate asthma (n=12), Severe asthma (n=14). PBMCs treated with (A) adiponectin (100 ng/ml) with/without Dex (10^{-10} to 10^{-6} M) for 21 hours, (B) adiponectin (100 ng/ml) with/without Dex (10^{-10} to 10^{-6} M) for 1 hour and stimulated with LPS for 20 hours, (C) Dex (10^{-10} to 10^{-6} M) for 1 hour and stimulated with LPS for 20 hours. CXCL8 release was measured by ELISA. Data represent mean ± SEM. *** $p<0.0001$ vs control for APN vs UN; # $p<0.05$, ## $p<0.01$, ### $p<0.0001$ for APN and Dex vs APN or LPS and Dex vs. LPS.

7.3.3 Suppressive effect of dexamethasone on IL-6 release in PBMCs pre-treated with adiponectin and/or LPS

Normalised data can be seen for adiponectin, adiponectin/LPS and LPS alone for each cohort in figure 7.2. Figure 7.3 displays the same data but grouping the treatments by cohort to allow direct comparison.

All the cohorts exhibited similar patterns of IL-6 inhibition with increasing concentrations of Dex 10^{-10} to 10^{-6} M for PBMCs treated with adiponectin, adiponectin/LPS and LPS alone. For the adiponectin treated PBMCs approximately 80% and 85% inhibition was seen at a concentration of Dex 10^{-7} and 10^{-6} M respectively (p<0.0001). However, only the mild/moderate cohort exhibited a significant inhibition in IL-6 release at dexamethasone 10^{-8} M (p<0.001). No significant differences were seen comparing treatment types by cohort. Similar levels of Dex-induced inhibition of IL-6 release were seen in both the adiponectin/LPS and LPS alone groups with approximately 40% suppression at dexamethasone 10^{-7} M and 50% at 10^{-6} M. A significant difference in inhibition of IL-6 release was noted comparing LPS versus APN/LPS-stimulated PBMCs for Dex 10^{-8} M in the mild/moderate cohort (p=0.012) with the addition of adiponectin resulting in greater inhibition (99% of control versus 72% respectively). This difference was not seen in the other cohorts.

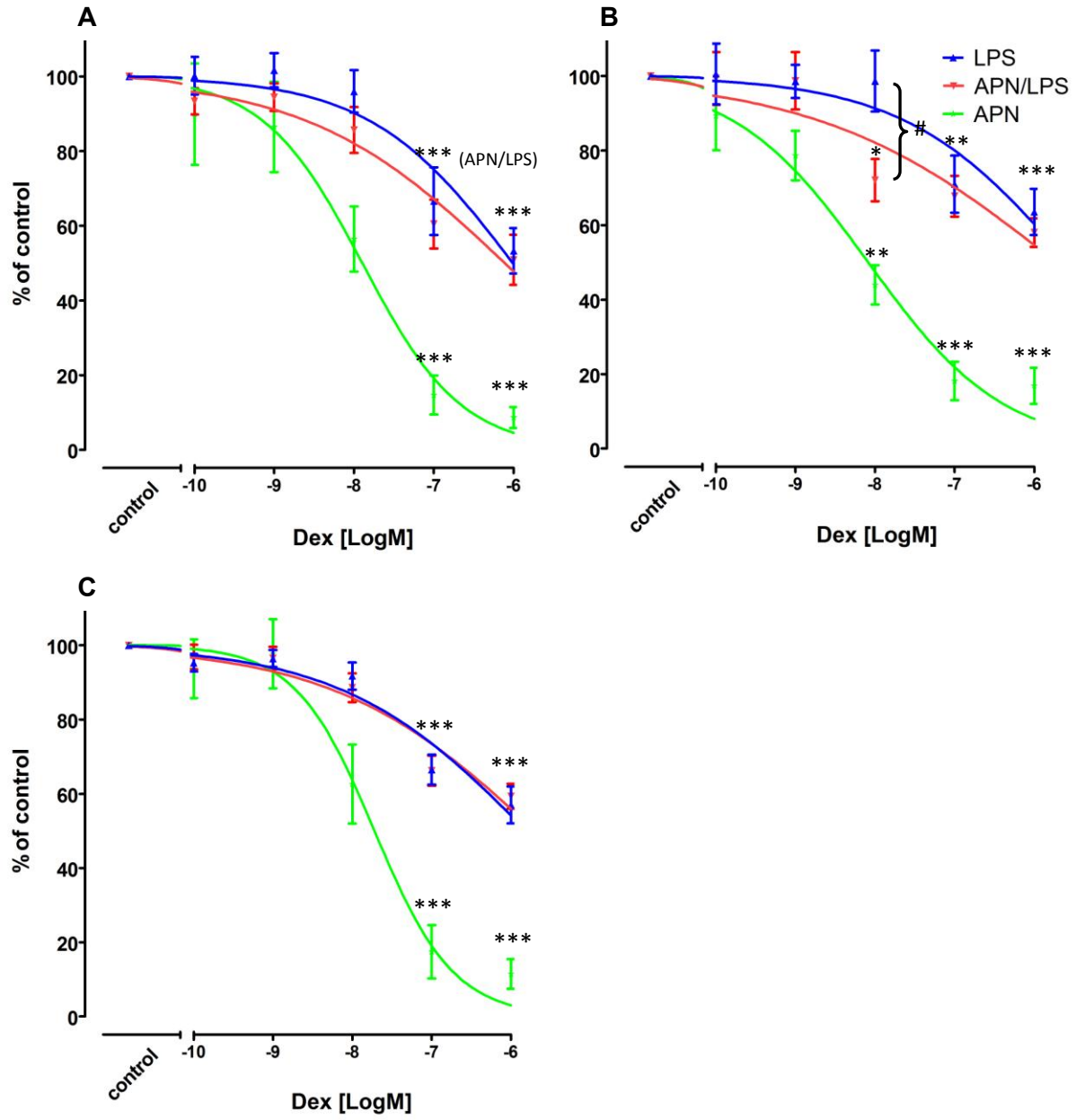


Figure 7.2: Suppressive effect of dexamethasone on IL-6 release in PBMCs of healthy subjects, mild/moderate and severe asthmatics. (A) Healthy (n=9), (B) Mild/Mod (n=11), (C) Severe (n=13). PBMCs treated with adiponectin (100 ng/ml) with/without Dex (10^{-10} to 10^{-6} M) for 2 hours prior to stimulation with LPS (0.5 ng/ml) for 20 hours. IL-6 release was measured by ELISA. Data represent mean ± SEM. *# p<0.05 LPS versus APN/LPS, * p<0.05, **p<0.01, *** p<0.0001 versus control.*

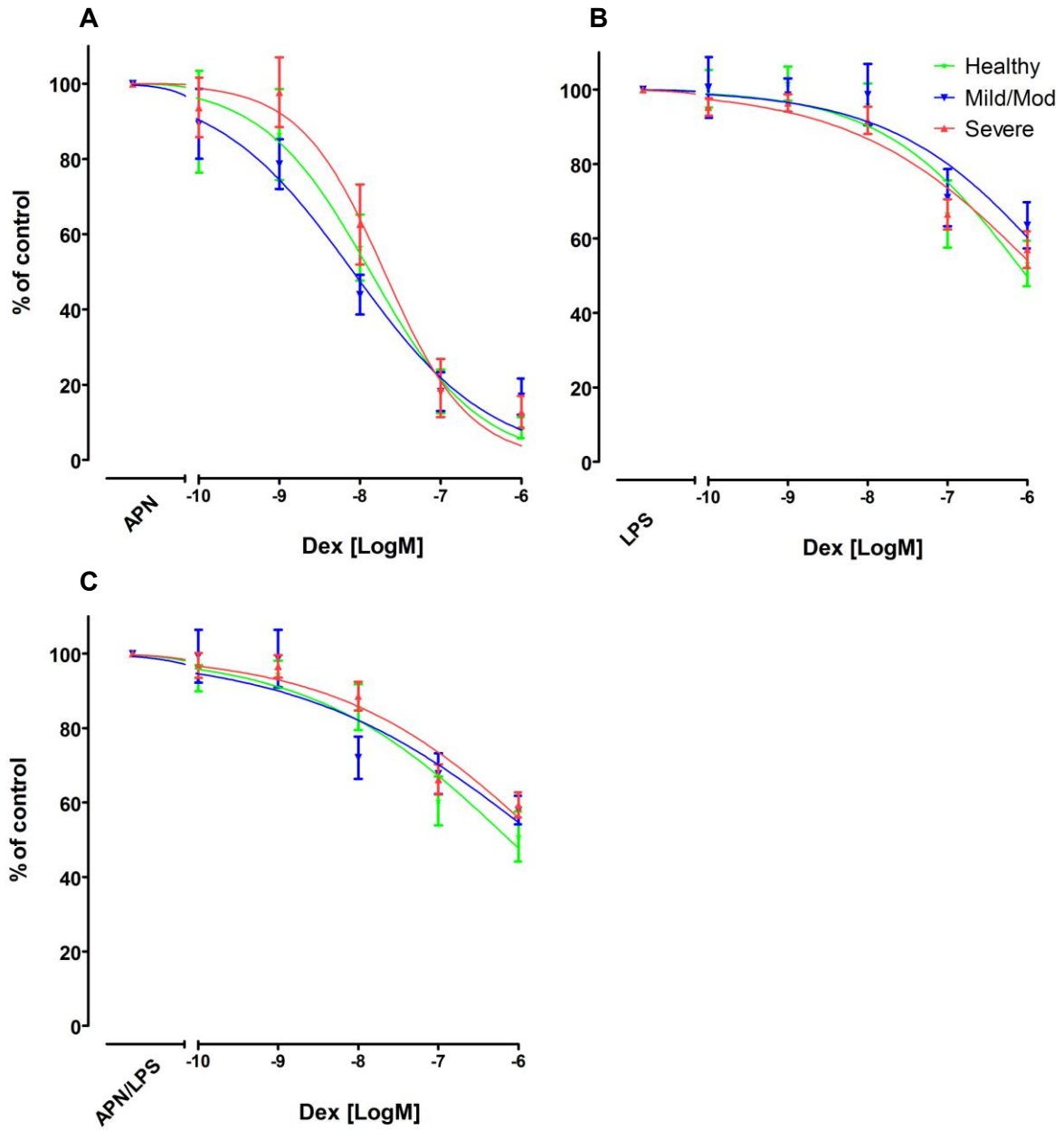


Figure 7.3: Suppressive effect of dexamethasone on IL-6 release in PBMCs pre-treated with adiponectin, LPS, Dexamethasone, or in combination. Healthy (n=9), Mild/Mod (n=11), Severe (n=13). PBMCs treated with (A) adiponectin (100 ng/ml) with/without Dex (10^{-10} to 10^{-6} M) for 21 hours, (B) Dex (10^{-10} to 10^{-6} M) for 1 hour and stimulated with LPS (0.5 ng/ml) for 20 hours, (C) adiponectin (100 ng/ml) with/without Dex (10^{-10} to 10^{-6} M) for 1 hour and stimulated with LPS (0.5 ng/ml) for 20 hours. IL-6 release was measured by ELISA. Data represent mean ± SEM.

7.3.4 Effect of adiponectin on LPS-induced cytokines in PBMCs

There was less spontaneous release of IFN-γ in PBMCs from healthy subjects compared to both asthmatic groups (0.33 pg/ml [1.1] compared to 2.79 [2.1] for the mild/moderate cohort and 3.0 [1.7] for the severe cohort; p=0.0014) (figure 7.4). Adiponectin induced a similar amount of IFN-γ in both asthma cohorts but a significantly lower amount in the healthy group (0.92 pg/ml [2.0] compared to 5.0 [1.9] for the mild/moderate and 4.9 [1.8] for the severe cohort; p=0.0009). LPS and APN/LPS induced significant increases in IFN-γ in all three cohorts. However, no significant differences were seen between the groups.

Spontaneous release of IL-10 was lower in the healthy cohort (3.4 [1.9]) compared to the mild/moderate and severe asthma cohorts (4.8 [1.7] and 5.1 [1.5], respectively; p=0.038) (figure 7.5). LPS and APN/LPS induced significant increases in IL-10 in all three cohorts. However, no significant differences were seen between the groups. Adiponectin induced a significant increase in IL-10 release in all cohorts (healthy 139 [110], mild/moderate 92.8 [93] and severe 180.4 [183]; p<0.05).

Spontaneous IL1-RA release was lower in the mild/moderate (117 [81.7]) versus the severe asthma and healthy cohorts (170 [108] and 188 [148] respectively) but this did not reach statistical significance (p=0.34) (figure 7.6). APN alone induced a non-significant rise in IL1-RA release whilst LPS and APN/LPS induced significant IL1-RA release. However, no differences were noted between groups.

Similar spontaneous release of CCL2 was noted from untreated PBMCs taken from all cohorts (figure 7.7). Adiponectin, LPS and APN/LPS treatment led to significant increases in CCL2 release in all cohorts. LPS and Dex 10^{-7} M induced less CCL2 in the mild/moderate cohort compared to the healthy cohort (p=0.042).

Spontaneous CCL3 release appeared to be higher in the healthy cohort (709 ± 1541) compared to the mild/moderate and asthmatic cohorts (100 [241] and 58 [98]) (figure 7.8) but this did not reach statistical significance (p=0.11). All three cohorts exhibited a similar level of CCL3 release following stimulation with LPS, LPS/APN or APN alone. No significant difference was seen comparing groups.

All three cohorts exhibited a similar amount of spontaneous TNF-α release (healthy 17.4 pg/ml [13.0], mild/moderate 23.0 [14.6] and severe 26.0 [22.4], p=0.48) (figure7.9). LPS and APN/LPS induced significant increases in TNF-α release but no significant difference was noted comparing the cohorts. APN alone induced greater TNF-α from the severe (777 [699]) compared to mild/moderate asthma cohort (390 [409]) and the healthy cohort (191 [128]) although this did not achieve statistical significance (p=0.076).

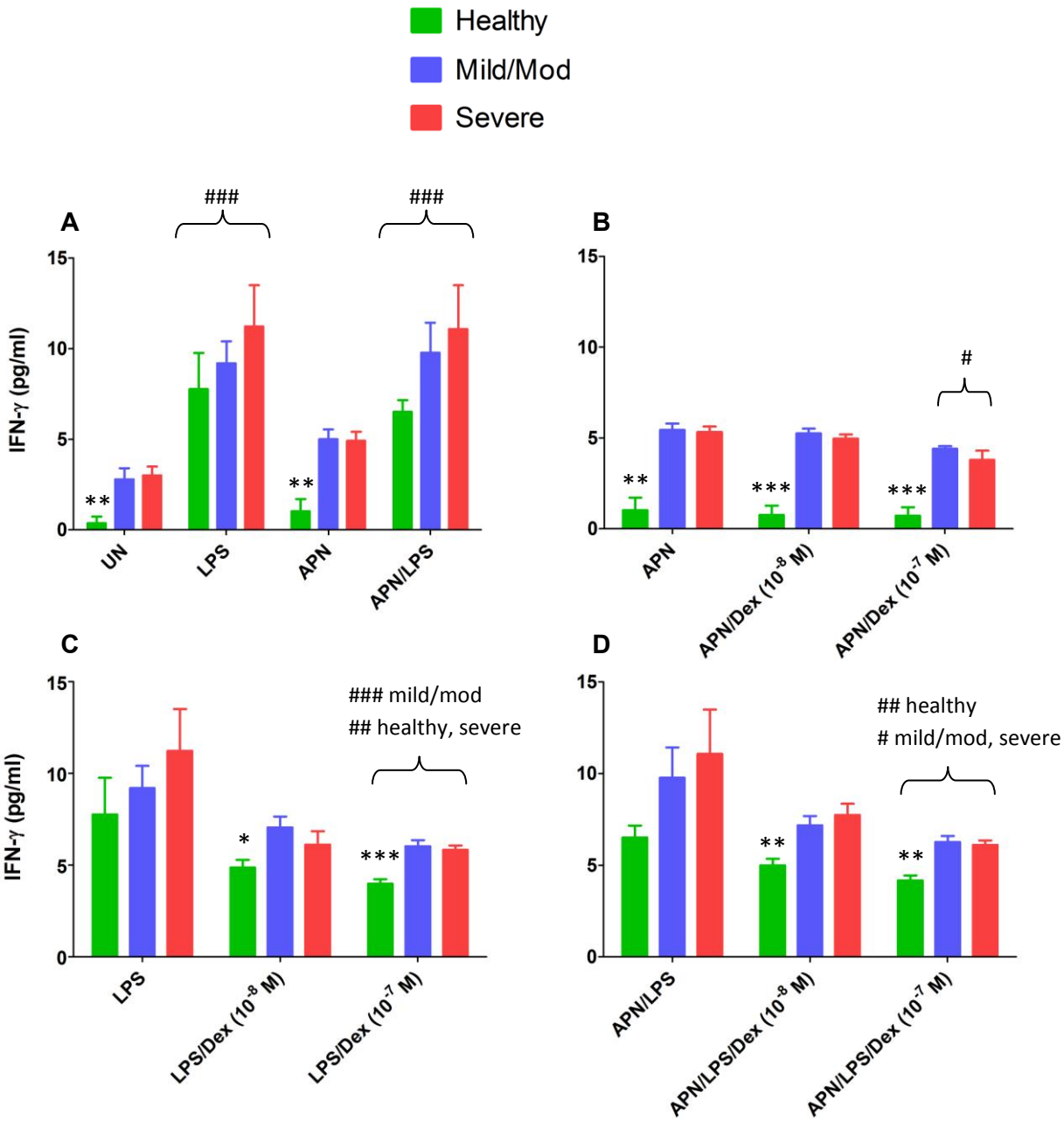


Figure 7.4: Effect of adiponectin, LPS, dexamethasone, or in combination on IFN-γ release from PBMCs. Healthy (n=10), Mild/Moderate asthma (n=12), Severe asthma (n=13). PBMCs treated with (A) adiponectin (100 ng/ml), LPS (0.5 ng/ml) or in combination (B) adiponectin (100 ng/ml) with/without Dex (10^{-8} and 10^{-7} M), (C) LPS (0.5 ng/ml) with/without Dex (10^{-8} and 10^{-7} M), (D) adiponectin (100 ng/ml) and LPS (0.5 ng/ml) with/without Dex (10^{-8} and 10^{-7} M). IFN-γ release was measured by multiplex assay. Data represent mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001 comparing groups; # p<0.05, ## p<0.01, ### p<0.0001 for APN and Dex vs. APN, LPS and Dex vs. LPS or LPS and Dex vs. LPS.

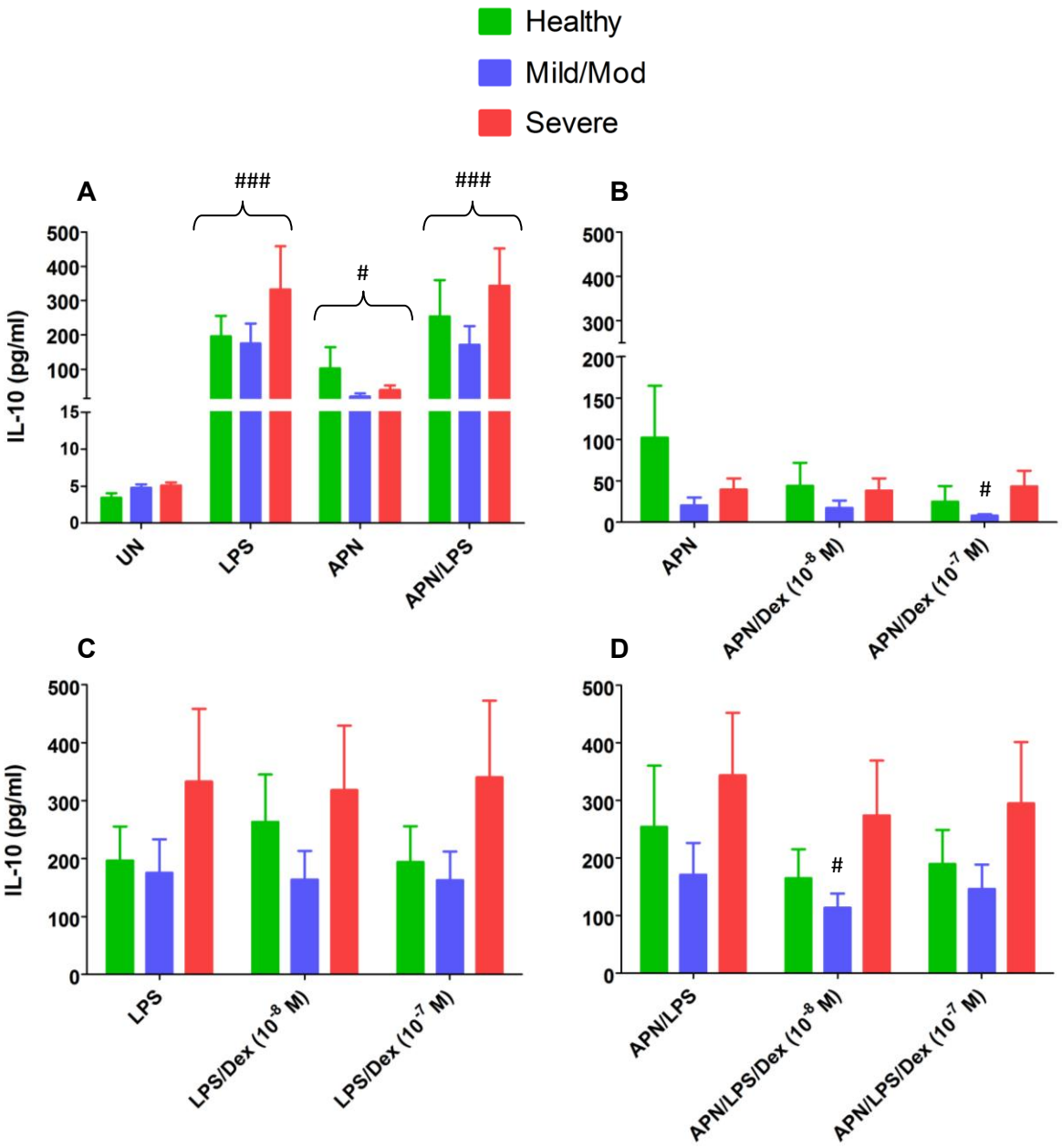


Figure 7.5: Effect of adiponectin, LPS, dexamethasone, or in combination on IL-10 release from PBMCs. Healthy (n=10), Mild/Moderate asthma (n=12), Severe asthma (n=13). PBMCs treated with (A) adiponectin (100 ng/ml), LPS (0.5 ng/ml) or in combination (B) adiponectin (100 ng/ml) with/without Dex (10^{-8} and 10^{-7} M), (C) LPS (0.5 ng/ml) with/without Dex (10^{-8} and 10^{-7} M), (D) adiponectin (100 ng/ml) and LPS (0.5 ng/ml) with/without Dex (10^{-8} and 10^{-7} M). IL-10 release was measured by multiplex assay. Data represent mean ± SEM. # p<0.05, ### p<0.0001 for APN and Dex vs. APN, LPS and Dex vs. LPS or LPS and Dex vs. LPS.

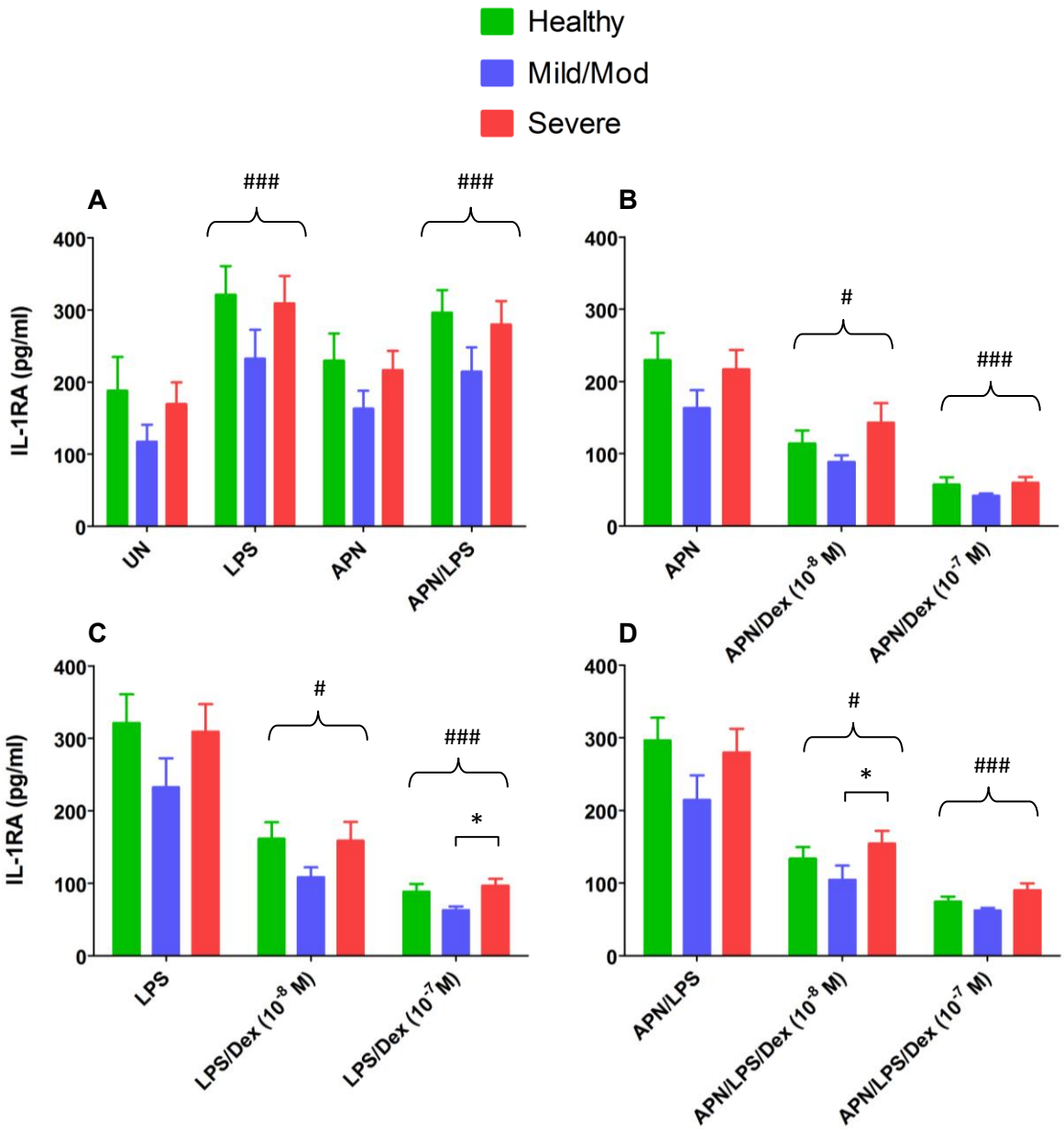


Figure 7.6: Effect of adiponectin, LPS, dexamethasone, or in combination on IL-1RA release from PBMCs. Healthy (n=10), Mild/Moderate asthma (n=12), Severe asthma (n=13). PBMCs treated with (A) adiponectin (100 ng/ml), LPS (0.5 ng/ml) or in combination (B) adiponectin (100 ng/ml) with/without Dex (10^{-8} and 10^{-7} M), (C) LPS (0.5 ng/ml) with/without Dex (10^{-8} and 10^{-7} M), (D) adiponectin (100 ng/ml) and LPS (0.5 ng/ml) with/without Dex (10^{-8} and 10^{-7} M). IL-1RA release was measured by multiplex assay. Data represent mean ± SEM. * p<0.05 comparing groups; # p<0.05, ## p<0.01, ### p<0.0001 for APN and Dex vs. APN, LPS and Dex vs. LPS or LPS and Dex vs. LPS.

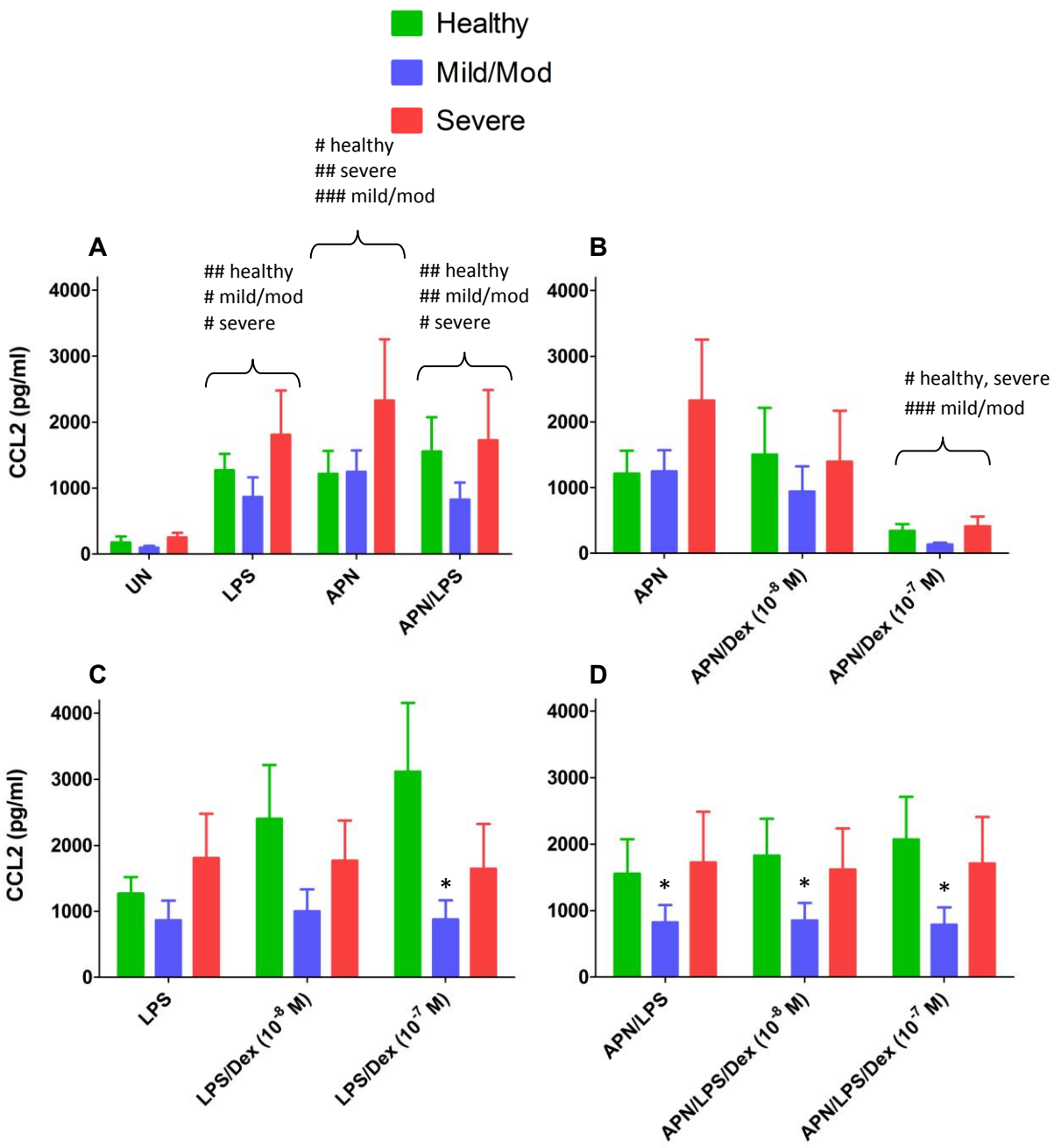


Figure 7.7: Effect of adiponectin, LPS, dexamethasone, or in combination on CCL2 release from PBMCs. Healthy (n=10), Mild/Moderate asthma (n=12), Severe asthma (n=13). PBMCs treated with (A) adiponectin (100 ng/ml), LPS (0.5 ng/ml) or in combination (B) adiponectin (100 ng/ml) with/without Dex (10^{-8} and 10^{-7} M), (C) LPS (0.5 ng/ml) with/without Dex (10^{-8} and 10^{-7} M), (D) adiponectin (100 ng/ml) and LPS (0.5 ng/ml) with/without Dex (10^{-8} and 10^{-7} M). CCL2 release was measured by multiplex assay. Data represent mean ± SEM. * p<0.05 comparing groups; # p<0.05, ## p<0.01, ### p<0.0001 for APN and Dex vs. APN, LPS and Dex vs. LPS or LPS and Dex vs. LPS.

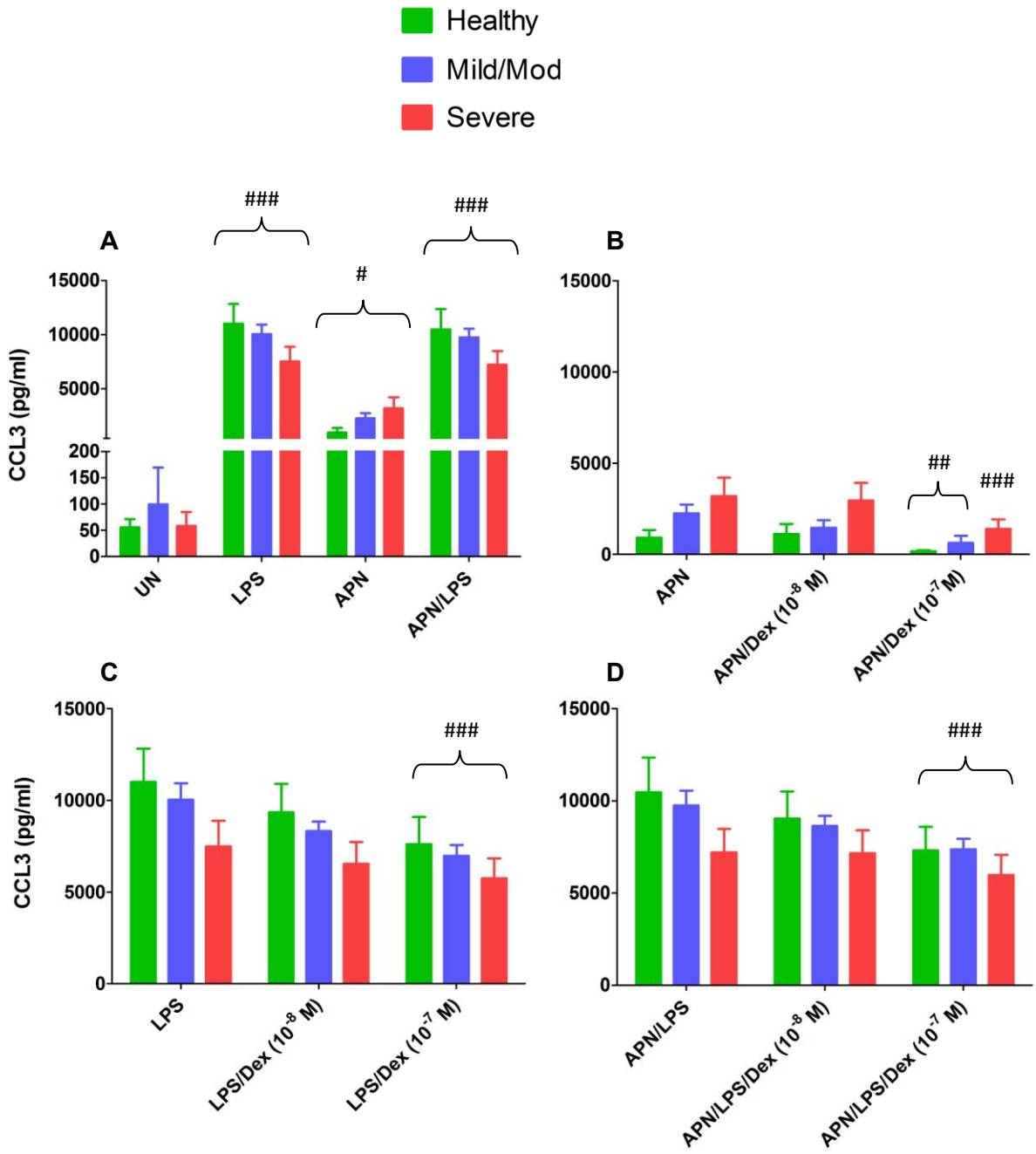


Figure 7.8: Effect of adiponectin, LPS, dexamethasone, or in combination on CCL3 release from PBMCs. Healthy (n=10), Mild/Moderate asthma (n=12), Severe asthma (n=13). PBMCs treated with (A) adiponectin (100 ng/ml), LPS (0.5 ng/ml) or in combination (B) adiponectin (100 ng/ml) with/without Dex (10^{-8} and 10^{-7} M), (C) LPS (0.5 ng/ml) with/without Dex (10^{-8} and 10^{-7} M), (D) adiponectin (100 ng/ml) and LPS (0.5 ng/ml) with/without Dex (10^{-8} and 10^{-7} M). CCL3 release was measured by multiplex assay. Data represent mean ± SEM. # p<0.05, ## p<0.01, ### p<0.0001 for APN and Dex vs. APN, LPS and Dex vs. LPS or LPS and Dex vs. LPS.

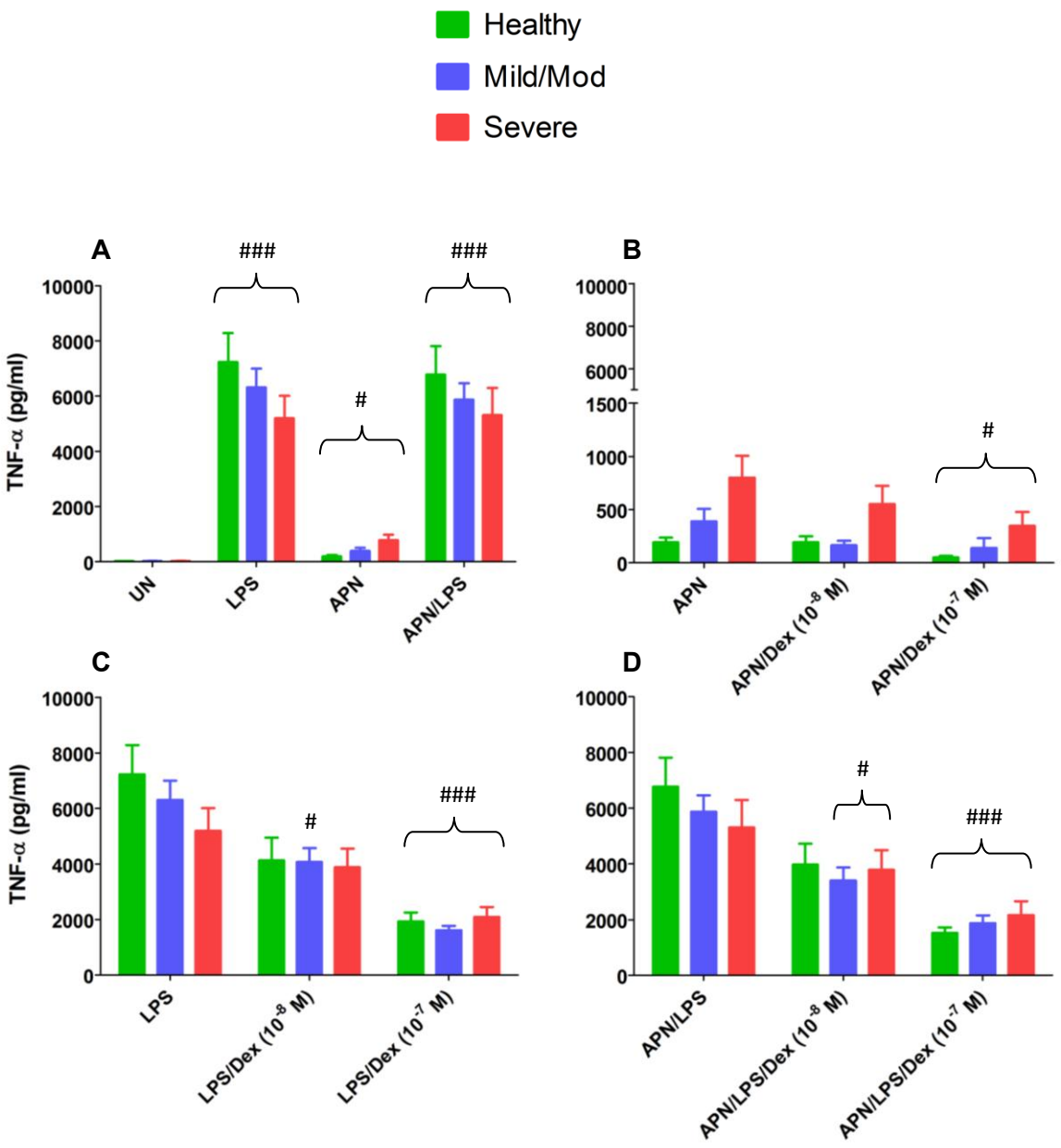


Figure 7.9: Effect of adiponectin, LPS, dexamethasone, or in combination on TNF-α release from PBMCs. Healthy (n=10), Mild/Moderate asthma (n=12), Severe asthma (n=13). PBMCs treated with (A) adiponectin (100 ng/ml), LPS (0.5 ng/ml) or in combination (B) adiponectin (100 ng/ml) with/without Dex (10^{-8} and 10^{-7} M), (C) LPS (0.5 ng/ml) with/without Dex (10^{-8} and 10^{-7} M), (D) adiponectin (100 ng/ml) and LPS (0.5 ng/ml) with/without Dex (10^{-8} and 10^{-7} M). TNF-α release was measured by multiplex assay. Data represent mean ± SEM. # p<0.05, ### p<0.0001 for APN and Dex vs. APN, LPS and Dex vs. LPS or LPS and Dex vs. LPS.

7.3.5 Suppressive effect of dexamethasone on cytokine release induced by adiponectin, LPS or in combination in PBMCs.

PBMCs from all three groups exhibited suppression of IFN-γ release in the presence of Dex 10^{-7} M and 10^{-8} M following stimulation with LPS with significant reductions in IFN-γ release were noted at 10^{-7} M (figure 7.4). Healthy controls demonstrated greater suppression of IFN-γ release at Dex 10^{-7} M (4.0 [0.8] versus 6 [1.1] for mild/moderate and 5.8 [0.8] for severe; p=0.003) and 10^{-8} M (4.9 [1.2] versus 7.1 [2.1] and 6.1 [2.7]; p=0.019) compared to the asthmatic cohorts. Dex 10^{-7} M caused a reduction in IFN-γ release from asthmatic PBMCs. All cohorts exhibited a significant reduction in IFN-γ for Dex 10^{-7} M in PBMCs pre-treated with LPS and APN. PBMCs from healthy subjects demonstrated a greater suppression of IFN-γ release for both Dex 10^{-7} M (3.3 [1.9] versus 6.3 [1.1] for mild/moderate and 6.1 [0.9] for severe; p=0.0002) and 10^{-8} M (4.5 [1.9] compared versus 7.2 [1.7] for mild/moderate and 7.7 [2.2] for severe; p=0.0004) compared to the asthmatic cohorts.

Stimulation with APN alone induced a greater, but not statistically significant, release of IL-10 in healthy subjects compared to the asthmatic cohorts. The mild/moderate cohort exhibited a significant suppression in IL-10 release for Dex (10^{-7} M) following stimulation with APN and for 10^{-8} M for PBMCs stimulated with APN/LPS (figure 7.5).

Significant suppression in IL-1RA release was noted in all cohorts for all treatments at Dex 10^{-7} and 10^{-8} M (figure 7.6). Whilst the overall appearances were similar for all cohorts, significant differences were noted comparing PBMCs stimulated with LPS at Dex 10^{-7} M taken from mild/moderate compared to severe

asthmatics (63 [18.1] versus 97 [34.3]; p=0.01). Similarly, PMBCs taken from mild/moderate asthmatics stimulated with APN/LPS at Dex 10^{-8} M exhibited a significantly lower amount of IL-1RA release compared to the severe asthma cohort (104.5 [67.5] versus 154 [63.2]; p=0.05).

CCL2 release appeared to be lower in the mild/moderate compared to the severe asthma and healthy cohorts following stimulation with LPS (figure 7.7). A statistically significant difference was noted for PBMCs pre-treated with Dex 10^{-7} M (646 [630] for the mild/moderate cohort versus 3117 [3291] for the healthy cohort; p=0.027). APN induced a similar amount of CCL2 in the healthy and mild/moderate asthma cohort (mean 1213 pg/ml and 1245 pg/ml, respectively). A higher amount was seen in the severe asthma cohort (1756 pg/ml) although this was not statistically significant. All cohorts exhibited suppression of CCL2 release at Dex 10^{-7} M and although this appeared to be greater for the mild/moderate cohort (130 [102] versus 410 [512] for severe and 339 [322] for healthy cohorts) it did not reach statistical significance. CCL2 release following stimulation with APN/LPS was lower in the mild/moderate cohort (826 [890] compared to 1034 [1231] for the severe asthma and 1556 [1643] for the healthy cohort). Significant differences were noted for both Dex concentrations at 10^{-7} M (791 [895] compared to 1711 [2515] severe asthma and 2072 [2033] for the healthy cohort; p=0.048) and 10^{-8} M (851 [920] compared to 1619 [2234] and 1825 [1764]; p=0.046).

All the cohorts exhibited a significant reduction in CCL3 release in the presence of Dex 10^{-7} M following stimulation with LPS but no differences were noted between cohorts (figure 7.8). Stimulation with APN appeared to result in greater CCL3 release in the asthmatic cohorts (2251 [1685] mild/moderate and 3197 [3508] severe asthma) compared to the healthy group (917 [1219]), however this did not

reach statistical significance (p=0.09). Pre-treatment with Dex 10^{-7} M resulted in significant inhibition of CCL3 release for all cohorts. Although there appeared to be less release in the healthy (177 [146] compared to 637 [1316] mild/moderate and 1414 [1876] severe) compared to the asthmatic cohorts, this was not statistically significant. Stimulation with APN/LPS resulted in similar levels of CCL3 release with all cohorts exhibiting a significant reduction when pre-treated with Dex 10^{-7} M.

Stimulation with LPS and APN/LPS produced similar levels of TNF-α release from PBMCs in all cohorts (figure 7.9). The addition of Dex 10^{-7} M (all cohorts) and 10^{-8} M (mild/moderate only for LPS; mild/moderate and severe cohorts for APN/LPS) resulted in significant decreases in TNF-α release. Stimulation with APN alone resulted in greater, but non significant, release of TNF-α in the severe cohort (801 [710] compared to 390 [409] mild/moderate and 191 [128] healthy; p=0.1). Similar findings were noted for PBMCs pre-treated with Dex 10^{-7} (348 [452] compared to 138 [320] mild/moderate and 49 [45] healthy; p=0.15) and Dex 10^{-8} M (551 [627] compared to 165 [138] mild/moderate and 191 [172] healthy; p=0.24).

7.4 Discussion:

The original hypothesis was that adiponectin would modulate inflammatory cytokine release and corticosteroid insensitivity in severe asthma. I found that adiponectin induced IL-6, IL-10, CCL2, CCL3 and TNF-α release from PBMCs taken from healthy controls, mild/moderate and severe asthmatics. Baseline release of IFN-γ and IL-10 were significantly lower in healthy subjects compared to the asthmatic groups. Adiponectin induced IFN-γ, IL-1RA, CCL2, CCL3 and TNF-α release was suppressed by Dex. The pre-treatment with adiponectin did not affect LPS induced cytokine release. The results follow the hypothesis in terms of inflammatory cytokine release but not in terms of corticosteroid sensitivity.

These findings are supported by previous studies. Wolf and colleagues found that adiponectin induced IL-10 production in primary human monocytes, MDMs and dendritic cells (Wolf et al., 2004). The pre-treatment of MDMs with adiponectin has been shown to result in significant increases in IL-10 secretion (Kumada et al., 2004). Other studies have found that adiponectin stimulates IL-6 and TNFα release from human placenta (Lappas et al., 2005) and from primary human blood macrophages (Tsatsanis et al., 2005, Song et al., 2009). In cultured astrocytes, globular adiponectin induced secretion of IL-6 and CCL2 with NF-κB, p38 MAPK and ERK1/2 pathways playing important roles (Wan et al., 2014).

Adiponectin induced more IFN-γ in the asthmatic cohorts compared to healthy controls. Previous studies have noted raised IFN-γ in asthmatics compared to healthy controls (ten Hacken et al., 1998). Although this study did not look at the mechanism of adiponectin induced IFN-γ production, results from the previous chapter noted that adiponectin-induced CXCL8 release was attenuated by p38

MAPK and ERK inhibition. Cheng and colleagues noted that inhibition of p38 MAPK abrogates adiponectin induced IFN-γ production (Cheng et al., 2012).

The pro-inflammatory effects of adiponectin seen in this study follow the suggestion that adiponectin may behave this way in particular diseases (Fantuzzi, 2008). In rheumatoid arthritis (Li et al., 2015), SLE (De Sanctis et al., 2009), type 1 diabetes (Schalkwijk et al., 2006) and inflammatory bowel disease (Karmiris et al., 2006) high levels of adiponectin and inflammation have been reported. The reasons behind this apparent paradox remain unclear; it is possible that circulating levels may not accurately reflect interstitial levels.

Baseline release of IFN-γ and IL-10 from PBMCs was significantly lower in the healthy compared to the asthmatic cohorts. This is in contrast to a previous study in PBMCs where no significant differences were noted comparing PBMCs taken from healthy controls, non-severe and severe asthmatics (Hew et al., 2006). The same study noted that patients with severe asthma demonstrated relative corticosteroid insensitivity for LPS induction of IL-1β, CCL3 and borderline significance for IL-6 at Dex 10^{-6} M – findings that were not replicated in this study. These differences may reflect the fact that Dex 10^{-7} and 10^{-8} M were assessed in this study whereas Hew and colleagues used Dex at the higher concentration of 10^{-6} M.

Analysis of normalised data revealed that only adiponectin induced CCL2 release demonstrated a significant difference in terms of Dex (10^{-7} M) mediated suppression; demonstrating relative corticosteroid insensitivity in the severe compared to the mild/moderate asthma cohort. BALF CCL2 has been shown to increase following an allergen challenge, implying a role in the regulation of cell trafficking in asthma in response to allergen exposure (Holgate et al., 1997).

This is the first time corticosteroid sensitivity *in vitro* has been assessed in response to stimulation with adiponectin. Fallo and colleagues demonstrated that intravenous hydrocortisone inhibited adiponectin production for healthy volunteers *in vivo* (Fallo et al., 2004). This work is supported by *in vitro* work demonstrating that the treatment of 3T3-L1 adipocytes with 100 nM Dex for 16 hours suppressed adiponectin gene expression by between 50 and 85% (Fasshauer et al., 2002). In contrast, Uchida and colleagues demonstrated that plasma levels of adiponectin were significantly increased in patients with IgA nephropathy following pulsed methylprednisolone (Uchida et al., 2006). The authors concluded that this discrepancy may be due to the anti-inflammatory action of pulsed glucocorticoid therapy in terms of IL-6 production, which is a potent inhibitor of adiponectin. They acknowledged that their findings may reflect a disease specific effect. These are important findings in relation to this study, particularly in view of the fact that 83 % of the mild/moderate and 100 % of the severe cohort were receiving ICS and that 35.7 % of the severe asthma cohort were taking maintenance OCS therapy in addition to ICS. Future work incorporating the measurement of serum adiponectin levels in severe asthmatic patients before and after commencing OCS therapy would allow this relationship to be further explored.

The pro-inflammatory effect of adiponectin, in terms of IL-6 release, appears to be very steroid sensitive for mild/moderate, severe asthmatics and healthy controls. This is in contrast for CXCL8, where the severe asthma cohort exhibited a reduced steroid response in terms of CXCL8 release following stimulation with adiponectin compared to the other groups, no significant differences were seen for IL-6. Dex suppressed adiponectin induced INF-γ, IL-1RA, CCL3 and TNF-α release. For IL-10, no significant suppression was noted other than the mild/moderate group

at Dex 10^{-7} M and CCL2 exhibited significant suppression for all groups at 10^{-7} M. These findings are consistent with the action of adiponectin partly investigated in the previous chapter.

There are some important limitations and considerations relating to this study. As previously discussed, adiponectin is present in different isoforms that are likely to exert different effects and their relative proportions or ratio may influence the inflammatory response (Pajvani et al., 2004). Furthermore the two main commercially available types of adiponectin, prokaryotic and eukaryotic derived, may behave differently – in part relating to potential LPS contamination and the formation of the more biologically active globular adiponectin (Richards et al., 2006). The monomeric adiponectin used in this study may not be representative of physiological adiponectin and has in fact been previously shown to be more biologically active (Nishida et al., 2007). Repeating this work using a physiological mix of adiponectin would allow for these differences and potentially be more representative. As previously noted, serum adiponectin was not assessed with this work and knowledge of their levels would have allowed correlation with the cellular effect. There is additionally likely to be a gender effect on adiponectin levels and activity with greater serum levels in adolescent girls compared to boys, a difference that persists to adulthood (Bottner et al., 2004). Furthermore previous studies analysing serum adiponectin levels in relation to asthma have noted no correlation in peri/postpubertal boys (Kim et al., 2008), potentially a harmful effect on men (Sutherland et al., 2009) but beneficial effects for peri/postpubertal girls (Nagel et al., 2009) and premenopausal women (Sood and Shore, 2013) (Sood et al., 2008). This is a relevant factor in this study where 79% of the severe cohort, 67% of the mild/moderate cohort and 55% of the healthy cohort were female.

In summary, adiponectin was pro-inflammatory and adiponectin treated PBMCs released more IFN-γ and IL-10 in the asthmatic cohorts compared to healthy controls. An attenuated corticosteroid response in terms of CCL2 release was seen in the severe asthma compared to the mild/moderate asthma cohort. Further investigation is required to establish the mechanism of these links and establish the role of adiponectin in asthma severity.

Chapter 8: General Discussion

This thesis forms part of the ever growing and important research into the link between obesity and asthma. Approximately 8% of the adult UK population has asthma and the BMI of the UK population has significantly increased since the 1960s, when less than 20% were overweight or obese, to a figure of approximately 70% (Asthma UK, 2006, Department of Health, 2011). This has a significant role in asthma in terms of asthma control, healthcare utilisation, comorbid disease, corticosteroid burden and quality of life. A greater understanding of this relationship will allow us to treat this often difficult to control group and pave the way for further mechanistic work that may lead to targeted therapies.

My thesis has identified that overweight and obesity are common findings in severe asthma (77.6% of the study population). Severe asthmatics with a BMI in the obese range were noted to have greater medication use in terms of SABA use per day, nebulisers and oral steroid requirements (both maintenance and short burst therapy). These findings are in keeping with previous cohorts of severe asthmatics such as SARP, where cluster analysis identified a unique group with a higher BMI (58 % with a BMI ≥ 30) and predominantly older females (71% female, mean age 50 years) (Moore et al., 2010). In addition, I found that GORD was reported to be more frequent in the obese group and obese severe asthmatics were less likely to be in full time employment due to their asthma. This suggests that obesity-related severe asthma is associated with more severe symptoms, a significant societal impact and patients are at risk of developing many deleterious side effects associated with corticosteroid use. The role of adipocytokines, lipid laded macrophages and lipid droplets in asthma remains poorly understood. I have found that leptin, resistin and adiponectin are pro-inflammatory and adiponectin appears to have a role in corticosteroid sensitivity in severe asthma. The identification of perilipin expression in

epithelial cells and the submucosa is a novel finding that suggests lipid droplets may correlate with airway inflammation.

Obesity in severe asthma can be a comorbidity, affecting pathophysiology and response to therapy, or secondary to disease driven inactivity and/or systemic steroid therapy. Two recent studies have noted a genetic link between obesity and asthma which suggest obesity is associated with the development of asthma. Genomewide association studies (GWAS) have identified 32 independent loci associated with BMI with an estimated 250 common variant loci yet to be discovered (Speliotes et al., 2010). A study looking at 4835 children and the 32 established independent loci, used Mendelian randomisation to investigate the causal effect of BMI, fat mass and lean mass on childhood asthma. A weighted allele score was strongly associated with BMI, fat mass, lean mass and childhood asthma. This strongly suggests that a greater BMI, fat mass and lean mass in mid childhood increases the risk of developing asthma (Granell R, 2013).

High fat diet and raised BMI appear to be risk factors for the development of intracellular lipid droplets and lipid laden macrophages. This study noted a higher lipid laden macrophage (LLMI) scores in severe compared to the mild/moderate asthmatics. This is likely to reflect the greater levels of GORD seen in the severe asthma group but may relate to disease severity and the higher BMI encountered in severe asthma.

I found that perilipin expression is a common finding in epithelial cells and the submucosa. Higher perilipin expression was seen in the subepithelial layer in asthmatic subjects compared to healthy controls, which may reflect underlying airway inflammation associated with a greater release of eicosanoid mediators and

phagocytic activity. The presence of lipid droplets in airway epithelial cells, as suggested by perilipin expression, is a novel finding. These droplets may play an important inflammatory role and augment macrophage function.

The mechanistic relationship between asthma and raised BMI is bidirectional. It is increasingly clear that adipose cells play an important role in systemic inflammation. Adipocytes produce inflammatory cytokines, termed adipokines, which correlate to the development of asthma and play a role in its severity. This study has looked at three adipokines (resistin, leptin and adiponectin) and subsequently examined adiponectin in greater detail in terms of inducing cytokine release and its role in corticosteroid insensitivity *in vitro*. Leptin and resistin were pro-inflammatory in terms of CXCL8 release from PBMCs but no significant effects were seen in relation to asthma severity or corticosteroid sensitivity. Adiponectin was pro-inflammatory *in vitro* and potentiated the steroid response in healthy subjects and mild/moderate asthmatics but not in severe asthma. This indicates that PBMCs taken from severe asthmatics exhibit an attenuated response to adiponectin. These are novel findings in asthma.

Previous studies have assessed serum levels of adipokines and noted correlations with asthma in terms of disease presence, severity and corticosteroid sensitivity. However, these findings were present in particular age groups and appear to be gender related. There is a paucity of data at the cellular level in terms of the inflammatory effects of adipokines and how this relates to asthma, disease severity and BMI.

There are some important overall limitations to consider:

1) The recruitment of obese mild/moderate asthmatics and severe asthmatics with a normal BMI was challenging and further work incorporating larger numbers of subjects in these groups would enhance this study. This would allow comparisons between obese and non-obese subgroups of mild/moderate and severe asthma; providing further insights into the role of obesity on the inflammatory response and inhibitory effect of corticosteroids on PBMCs in the presence of adipokines.

2) A total of eight cytokines were assessed in the adiponectin study and initially only CXCL8 release was analysed. Future work incorporating other adipocytokines would provide further information on the actions of adiponectin and the potential role in corticosteroid insensitivity.

3) The use of PBMCs does not allow the identification of particular cell types that are responsible for the effects seen. For example, the cellular studies could be repeated using alveolar macrophages. The work incorporating lipid laden macrophages and lipid droplets did not assess particular and potentially key aspects such as how they impact macrophage phagocytosis and the location of lipid droplets.

The results and discussion presented in here indicate a number of directions for future research, either in the form of new work or to clarify existing controversies:

1) **Asthma as a cause or effect of obesity.** A longitudinal study to look at the role of obesity in asthma and disease severity. Analysis and extended follow up of a large cohort of patients to look at BMI prior to at the point of and

subsequent to a diagnosis of asthma. This would need to be a large study via primary care over a prolonged period of time. Additionally, intervention studies incorporating bariatric surgery to induce weight loss provide further information in to the role of obesity in asthma. Weight loss in obese asthmatic patients who underwent bariatric surgery has been associated with significant improvements in asthma control, asthma quality of life and AHR (Dixon et al., 2011, Boulet et al., 2012). Incorporating the cellular studies from this thesis for patients before and after bariatric surgery would provide an interesting insight into the mechanistic basis of these factors.

2) **The effects of adipokines in asthma.** i) extending this study to examine the effects of other adipokines (table 1.2) and other cell types such as monocyte derived macrophages, airway macrophages, airway smooth muscle cells, airway epithelial cells and adipocytes ii) measurement of adipokines in serum and bronchoalveolar lavage fluid and protein expression of adipokines via western blot – correlate to asthma, disease severity and to serum levels and cellular function

3) **Assessment of different types of adiponectin.** A comparative study incorporating prokaryote and eukaryote derived adiponectin alongside 'physiological' adiponectin (a mixture of different adiponectin isoforms).

4) **Mechanisms underlying the formation and effects of lipid droplets in asthma.** The use of electron or light microscopy to assess location and quantify lipid droplets could be correlated to asthma, disease severity and cellular function. This could be extended to different cell types including airway epithelial cells, airway smooth muscle cells and airway macrophages.

Macrophage function could be correlated with lipid droplet presence using fluorescently-labelled polystyrene beads or labelled *H. influenzae* or *S. Aureus*.

In conclusion, my thesis has shown that obesity is often encountered in severe asthma with important correlations in terms of disease severity, symptoms and corticosteroid use. This is the first time that the effect of adipokines on inflammation and corticosteroid response in PBMCs taken from severe asthmatics has been assessed. Leptin, resistin and adiponectin are pro-inflammatory and adiponectin appears to play a role in corticosteroid sensitivity. Lipid laden macrophages and lipid droplets are frequently found in asthma and their role in disease severity and obesity requires further research.

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