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Obesity & Asthma:

The role of innate immunity, adipokines and regulatory T cells

**A thesis submitted to Swansea University in fulfilment of the requirement
for the degree of Doctor of Medicine**

by

Michael Pynn, MB ChB, MRCP (UK)

2013



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“If you are going through hell, keep going”

Sir Winston Churchill (1874-1965)

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Abstract

Introduction: Obesity and asthma are associated but the mechanism is poorly understood. Enhanced systemic inflammation may underlie the obesity-asthma paradigm. Although there is good mechanistic data that obesity augments the immune response as well as promoting immune dysregulation by reducing regulatory T cell numbers, there is little work relating this to obesity and asthma.

Methods: A case-control study examined 6 groups of pre-menopausal women (n=84): non-obese, overweight and obese individuals with and without asthma. Measures of adiposity and lung function were taken and peripheral blood collected during the first 7 days of the menstrual cycle. Innate immune parameters measured included: full blood count and differential; chemiluminescence recorded whole blood reactive oxygen species; neutrophil related cytokines; neutrophil and monocyte activation markers by flow cytometry, and LPS induced whole blood cytokine responses. Insulin resistance, adipokine levels and free fatty acid levels were recorded. Dendritic cell and lymphocyte subtypes including FoxP3⁺ regulatory T cells (Tregs) were quantified by flow cytometry and PHA-induced cytokine responses measured in whole blood.

Results: Obesity and asthma appeared to have synergistic effects with regards to circulating neutrophil count, plasma IL-6 and leptin with obese asthmatics having the highest levels. Reactive oxygen species production followed a similar trend. Increasing BMI within asthmatics was associated with a reduction in eosinophils and myeloid dendritic cells, and increased PHA-induced IFN γ . Obesity across the entire study group was associated with increased neutrophil counts and neutrophil related cytokines, reduced FoxP3⁺ Tregs and increased PHA-induced IL-17 response.

Conclusions: Systemic changes in immunity occur in obesity and asthma; some of these are additive. Within asthmatics, obesity is associated with responses suggesting T helper 1 (Th1) rather than Th2 bias. Obesity-associated systemic changes in immunity might encourage a loss of immune tolerance. These findings suggest that obesity might mediate its effects in asthma through systemic inflammatory mechanisms.

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Acknowledgements

A heartfelt thanks to my supervisors Gwyneth Davies and Cathy Thornton for the endless encouragement (when faced with what at times can only be described as unending pessimism), patience, advice and tuition, without which this thesis could never have been written.

A special thanks to Owen Bodger for his guidance with regards to statistical analysis of the data.

I would also like to mention people who tutored and supported me through the somewhat foreign world of an immunology laboratory: Aled Bryant, Ruth Jones, Rachel Smith and Wendy Francis.

Thanks to Steve Luzio for undertaking the FFA, glucose and insulin measurements and Rachel Still for performing the total IgE analysis.

A special mention must go to the ladies at Slimming World, who kindly agreed to allow me to visit their clubs, and showed a warm welcome whenever I attended, as well as keeping me nourished by providing a seemingly inexhaustible repertoire of healthy snacks.

To my wife, for all the sleepless nights you have endured with our newborn son so I could stay up and write this, for all the practical support including proof reading and formatting and for not divorcing me at times when it must have been very tempting: thank-you.

To my son Noah, your daddy is not surgically attached to a lap top and I am sorry we haven't spent more time together in the last year. Thank you for putting all the frustrations and stresses to the back of my mind with a simple smile. One day, if you are really naughty, I might make you read this!

The biggest thanks must go to the patients, for giving up their free time to take part in this study. Their ability to show up to clinic with a contagious optimism, in the face of what can be a distressing and disabling condition, put the challenge of completing this thesis into perspective and provided a constant motivation to try and further our understanding in this area.

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Abbreviations

ABMU HB	Abertawe Bro Morgannwg University Health Board
AAM	Alternatively activated macrophages
AGE	Advanced glycosylation end products
β 2 agonist	Beta-2 adreno-receptor agonist
BAL	Bronchoalveolar lavage
BDCA	Blood dendritic cell antigens
BHR	Bronchial hyper-responsiveness
BMI	Body mass index
CCL	Chemokine ligand
COPD	Chronic obstructive pulmonary disease
CCR	Chemokine receptor
CI	Confidence interval
CM	Central memory T cells
CRP	C-reactive protein
CXCL12	C-X-C chemokine ligand
CXCR4	C-X-C chemokine receptor
DEXA	Dual energy x-ray absorptiometry
ECM	Extracellular matrix
EM	Effector memory
EGF	Epithelial growth factor
ELISA	Enzyme linked immunosorbant assay
FEF25-75	Forced Expiratory Flow at 25%-75% of expired vital capacity
FeNO	Exhaled nitric oxide
FEV1	Forced expiratory volume in 1 second
FFA	Free fatty acids
FoxP3	Forkhead box P3
FSC	Forward scatter
FVC	Forced vital capacity
GATA-3	Trans-acting T-cell-specific transcription factor
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte /macrophage colony stimulating factor
GORD	Gastro oesophageal reflux disease
gp130	Glycoprotein 130
HDAC2	Histone deactylase 2
iNKT	Invariant natural killer T cells
ICS	Inhaled corticosteroids
IL	Interleukin
IFN γ	Interferon gamma
IR	Insulin resistance
IRF4	Interferon regulatory factor 4
LPS	Lipopolysaccharide
LT	Leukotrienes
mDC	Myeloid dendritic cell
MFI	Mean fluorescence intensity
MHC	Major-histocompatibility complex
MNC	Mononuclear cells
MPO	Myeloperoxidase
NF κ B	Nuclear factor- κ B
NK cells	Natural killer cells

NLRs	NOD-like receptors
NW	Normal weight
OB	Obese
OSA	Obstructive sleep apnoea
OW	Over weight
pDC	Plasmacytoid dendritic cell
PAMPS	Pathogen associated molecular patterns
PCOS	Polycystic ovary syndrome
PEFR	Peak expiratory flow rate
PMA	Phorbol myristate acetate
PMT	Photomultiplier tubes
PG	Prostaglandin
PHA	Phytohaemagglutinin-L
PRR	Pattern recognition receptors
RBC	Red blood cells
ROR γ T	Retinoic-acid-related orphan nuclear receptor gamma T
ROS	Reactive oxygen species
sgp130	Soluble gp130
sIL6R	Soluble interleukin 6 receptor
SCF	Stem cell factor
SOD	Super oxide dismutase
SSC	Side scatter
STAT	Signal transducer and activation of transcription
TAOS	Total antioxidant status
TARC	Thymus and activation regulated chemokine
TBARS	Thiobarbituric acid reactive substances
TCR	T cell receptor
Tc	Cytotoxic T cells
TGF β	Transforming growth factor β
Th cells	T helper cells
TNF α	Tumour necrosis factor alpha
TLRs	Toll-like receptors
TRECS	T cell receptor excision circles
TSLP	Thymic stromal lipoprotein
Tregs	Regulatory T cells
VEGF	Vascular endothelial growth factor
WCC	White cell (leukocyte) count
WHO	World Health Organisation
WHR	Waist-hip ratio

Chapter 1

Introduction

1.1 Overview

Asthma is one of the most important and common chronic diseases in the UK affecting 1 in 9 adults [1]. It is a chronic inflammatory disease of the conducting airways characterised by variable airflow obstruction, inflammation and bronchial hyper-responsiveness (BHR). The pathogenesis is far from well understood but involves complex gene-environment interactions. Atopy is the greatest risk factor for asthma development and is defined as a genetic pre-disposition towards a type I hypersensitivity reaction against common environmental antigens (allergens), manifested clinically by epithelial inflammation. However, aeroallergen sensitisation is only estimated to contribute towards 30% of the disease, suggesting that the remainder is an inflammatory response to an as yet unidentified trigger [2].

Whilst atopic asthma has been traditionally thought of as a T-helper 2 (Th2) mediated disease, it is increasingly recognised that changes in innate immune system priming and behaviour determine the resultant adaptive immune response. The hygiene hypothesis for allergy suggests that a lack of early childhood exposure to microbial triggers: pathogen associated molecular patterns (PAMPs), primes the innate immune system to promote a Th2 biased adaptive immune response [3]. Dendritic cells are important primers of the adaptive immune response and may play an important role in asthma pathogenesis [4]. Whilst Th1/Th2 skewing may play a role in atopic disease, it is increasingly recognised that other Th subsets are important [5], including regulatory T cells (Tregs) [6].

The heterogeneity of asthma is further highlighted by the existence of distinct clinical phenotypes. A phenotype is defined as “the visible characteristics of an organism resulting from the interaction between its genetic makeup and the environment” [7]. Although it has been apparent to clinicians for many years that different clinical presentations exist within the syndrome of asthma, recent cluster analyses have more clearly defined these entities [8]. These phenotypes differ in their clinical features in terms of age of onset, sex predominance, degree of symptoms and response to treatment. This is likely to reflect different underlying pathophysiological processes, with the type of airway inflammation and its concordance with patient symptoms varying between phenotypes [8]. Such distinct pathophysiological mechanisms underpinning different asthma phenotypes now termed “endotypes”, warrant further exploration as this could lead to the development of more personalised and effective treatments [9].

Globally there is an obesity epidemic with 1.6 billion individuals affected in 2006 [10]; the situation is predicted to escalate and it is estimated that by 2050 up to 60% of adults in the UK will be obese [11]. Asthma is a common co-morbidity amongst the morbidly obese with a comparable prevalence to more traditionally obesity-related disorders such as diabetes [12]. Cross-sectional and subsequently longitudinal studies have repeatedly shown that obesity is associated with increased asthma prevalence [13] and incidence [14] and the relationship appears to be stronger in women [13, 15]. Two cluster analyses have identified an obese female predominant phenotype characterised by an absence of eosinophilic airway inflammation [8, 16] and in keeping with this, studies suggests that these individuals have a poor response to inhaled corticosteroid (ICS), the cornerstone of traditional asthma therapy [17, 18]. Therefore the underlying pathophysiology needs to be determined in order to adequately manage this increasingly common disease phenotype.

The mechanism which underpins the obesity-asthma association is not well understood. Obesity has a number of systemic effects which could be of relevance to asthma and is associated with co-morbidities including gastro-oesophageal reflux disease and obstructive sleep apnoea which could mimic or exacerbate this disease [19]. However, over diagnosis of asthma is not a greater issue in the obese than the wider population [20]. In the obese state, adipose tissue becomes infiltrated with pro-inflammatory macrophages, and systemic changes are seen in the numbers and activation of cells derived from the innate arm of the immune system [21]. Hormones (adipokines) released by adipose tissue, the most studied being leptin can also impact on innate and adaptive immunity and more specifically regulatory T cells [22] adipokines may also have more direct effects on airway function [23]. Studies in humans to date suggest that whilst leptin may be associated with asthma this appears to be independent of BMI [24].

The idea that obesity therefore mediates its effects on asthma through systemic inflammation is an appealing one, but work to date, whilst finding evidence of changes in systemic immunity with obesity and asthma, have shown them to co-exist rather than interact in a synergistic fashion [25]. The larger studies have not always used stringent asthma definitions [26], a potential limitation in a disease with a high rate of mis-diagnosis albeit independent of BMI [20]. In many cases these studies have not been able to control for a large number of confounders which is particularly important in asthma where disease activity will fluctuate, and in obesity which can be associated with a vast number of co-morbidities. The effects of sex hormones have also not been taken into account, which is of particular importance in a phenotype with a preponderance for pre-menopausal women. Small well-designed studies have tried to address this question and, for the limited number of parameters examined, have not shown evidence that systemic immunity plays a role [25]. However in perhaps the most tightly controlled of these studies, the majority of patients were exacerbating at the time of blood sampling and therefore work is needed to focus on these individuals during periods of disease stability [25].

What seems clear from clinical data is that whatever the underlying pathophysiology, at least within the airways, it is not typical eosinophilic inflammation as evident in atopic disease when measured using sputum cell counts [25] or exhaled nitric oxide (FeNO) [27-29]. There is good mechanistic data that innate immune function is altered in obesity [21], including systemic changes in numbers and activation markers of neutrophils [30] and the monocyte/macrophage compartment [31] which could have relevance in asthma. Furthermore, the response of innate cells to danger signals such as PAMPs, including lipopolysaccharide (LPS) could also be enhanced in the obese [32] and to the candidate's knowledge innate immunity has not been examined in obese asthmatics. Dendritic cells could also play a role and early work suggests that obesity might modify numbers and function of these cells [33]. In addition, Tregs may also be down regulated in obesity and its related diseases [34] previous work to date suggests that this area of immune regulation is important in asthma pathogenesis, although studies have been limited by a lack of surface markers. These areas of immunity have not been addressed in this asthma subpopulation.

1.2 Study aims

The aim of this work is to perform a case control study in normal, overweight and obese premenopausal women with and without asthma confirmed by objective criteria. It will attempt to control as far as possible for potentially confounding co-morbidities including the effects of cyclical hormonal changes. Whilst the participants are stable and free of exacerbation, the study will examine whether detectable changes in systemic immunity can be observed in the obese asthmatics compared to the other groups with a specific focus on the following areas which have not been examined to date.

1.2.1 Study hypotheses

- 1: Systemic changes in innate immunity are important in the obese asthma phenotype.
- 2: Obesity in asthma is associated with changes in adaptive immunity including a reduction in circulatory Tregs.
- 3: Changes in systemic immunity seen in obese asthmatics are associated with changes in adipokine levels.

1.2.2 Research objectives

- To examine the innate immune system in obese females with and without asthma looking at whether there are changes in the number or percentage of circulating leukocytes, markers of neutrophil and monocyte activation, and the cytokine response to an inflammatory stimulus in the form of LPS.
- To study systemic markers of long term oxidative stress (TBARS and TAOS) in these individuals as well as acute reactive oxygen species (ROS) response to a non-specific inflammatory stimulus.
- To study metabolic parameters which might impact on immunity including; adipokines encompassing those in which there has been very little work; insulin resistance and free fatty acids.
- To measure changes in dendritic cell populations.
- To examine changes in adaptive immunity including the percentage of circulating Tregs.

Chapter 2

Background

2.1: Asthma overview

Asthma is a chronic, complex disorder of the airways. Salter, a London physician, in 1860 described a condition characterised by "Paroxysmal dyspnoea of a peculiar character with intervals of healthy respiration between attacks" [35]. There are no gold standard criteria for asthma and the diagnosis is a clinical one. Central to more modern definitions are the presence of chronic airways inflammation, recurrent and variable symptoms and airflow obstruction that is reversible either spontaneously or with treatment [36]. Asthma is a disorder of the conducting airways (bronchi and bronchioles) within the lungs, therefore affecting approximately the first 15 generations of these but the disease can spread proximally and distally with time [37]. Inflammation is a cardinal feature; the release of potent mediators causes constriction of the airway smooth muscle and airway wall oedema with thickening of up to 300%, accounting for the characteristic variable airflow obstruction seen. As this continues chronically, changes to the structure of the airway wall are seen in a process called remodelling [38].

Asthma is common with approximately 1 in every 9 adults in England and Wales affected [1]. Mortality rates from the disease are continuing to fall but it is estimated that 15 individuals per million die from the condition. Atopy is the greatest risk factor for asthma development, However it is estimated that aeroallergen sensitization contributes to only 30% of the disease [2]. This observation suggests that the vast majority of asthma results from inflammatory response to as yet unidentified triggers.

The aetiology of asthma is not well understood but it is clear that many sufferers have a genetic susceptibility which interacts with environmental factors at critical stages in early life (Table 2 taken from Pynn et al [39]), resulting in disease expression. Early twin studies provided evidence that genetics were important in asthma development with the observation that concordance rates are significantly higher in monozygotic than dizygotic twins [40]. Some studies estimate the heritability of asthma to be as high as 60-70%, particularly in pre-school children [41, 42]. No single gene has been identified that can explain the majority of asthma cases or determine severity, however linkage analysis and more recently, genome wide association studies have suggested that more than 100 genes might contribute to asthma risk with the impact of each being relatively small [43]. Such gene-environmental interactions result in inappropriate activation of the immune defences within the lung resulting in perpetual inflammation.

Agent	Examples	Study	Findings
Allergens	House dust mite	<i>Prospective</i>	<ul style="list-style-type: none"> • Sensitization increases asthma risk • Early childhood exposure increases asthma risk • Minimal threshold level of allergen exposure [44]
	Animal allergens: Cat/Dog	<i>Prospective cohort</i>	<ul style="list-style-type: none"> • Exposure decreases sensitization to other aeroallergens • No protective effect on asthma [45]
Pollutants	Nitrogen dioxide (NO ₂)	<i>Prospective</i>	<ul style="list-style-type: none"> • Proximity to roads - elevated NO₂, ↑ asthma risk [46]
	Diesel exhaust particles	<i>Mechanistic</i>	<ul style="list-style-type: none"> • Diesel exhaust particles promote dendritic cell maturation [47] • Diesel exhaust particles cause airway epithelial activation and pro-inflammatory cytokine release [48]
Viral infections		<i>Prospective cohort</i>	<ul style="list-style-type: none"> • Increased infant viral infections - ↓ risk of asthma and atopy [49]
Smoking	Active smoking	<i>Prospective cohort</i>	<ul style="list-style-type: none"> • Smoking - ↑ risk of asthma development [50]
	Second hand smoking	<i>Prospective cohort</i>	<ul style="list-style-type: none"> • Prenatal maternal smoking - ↑ asthma risk [51] • Adult passive smoking - ↑ doctor diagnosed asthma [52]
Medication use	Antibiotic use in childhood	<i>Meta-analysis of prospective and retrospective studies</i>	<ul style="list-style-type: none"> • Childhood antibiotic use in first year of life - ↑ asthma risk [53]
	Hormonal replacement therapy	<i>Prospective</i>	<ul style="list-style-type: none"> • HRT use - ↑ asthma incidence [54]
Obesity		<i>Prospective</i>	<ul style="list-style-type: none"> • Dose dependent effect between BMI and asthma risk [14] • Weight loss studies improve disease control [55]
Early menarche		<i>Cross-sectional Longitudinal</i>	<ul style="list-style-type: none"> • Early menarche - ↑ asthma risk [56]
Peri-natal	Maternal diet	<i>Prospective cohort</i>	<ul style="list-style-type: none"> • ↑ Maternal vitamin E - ↓ child wheeze in second year of life [57] • ↓ Maternal vitamin D - ↑ wheeze [58]
	Prematurity	<i>Retrospective meta-analyses</i>	<ul style="list-style-type: none"> • Prematurity - higher asthma risk [59]
	Breast feeding	<i>Cross-sectional</i>	<ul style="list-style-type: none"> • Breast feeding - ↓ non atopic wheeze, no effect on atopic wheeze [60]

Table 2.1: Summary of the main environmental factors implicated in the aetiology of asthma. The findings listed here are taken from some of the main studies examining this area. The papers listed are predominantly observational and data in many of these areas is conflicting, reflecting the complex nature of this area.

2.2: The immune system and the lung

The immune system consists of physical barriers, specialised cells and molecules which protect the body from harmful environmental organisms (pathogens). This system is nowhere more pertinent than in the lung, which with a surface area of $>100\text{m}^2$ comes into contact with $>10,000$ litres of inhaled air per day. The immune system can be divided into two arms. The innate immune system is evolutionarily ancient and provides an immediate response to potentially harmful pathogens. It recognises generic molecules (pathogen associated molecular patterns (PAMPs)) found in various types of micro-organisms through a limited number of germ-line encoded receptors termed pattern recognition receptors (PRR) [61]. The adaptive immune system provides a temporally delayed response which is highly specific, recognising peptides unique to a particular pathogen by the use of an almost infinite number of randomly generated, clonally expressed receptors [62]. Upon stimulation, clonal expansion of these cells results in immunological memory, enabling a much faster specific response on subsequent encounter of the same antigen. The two arms of the immune system therefore complement each others' strengths and weaknesses; the innate immune system with its fast yet non-specific response which can lead to collateral tissue damage and the acquired immune system with its highly specific response conferring memory and limiting neighbouring tissue damage at the expense of a temporal delay.

2.2.1 Innate immunity

The innate immune system encompasses physical and chemical barriers preventing the entry of noxious substances across surface epithelia. Via the use of PRRs, it produces non-specific responses in the form of inflammation and activates the adaptive immune system. PRRs detect the principal components of pathogens; pathogen associated molecular patterns (PAMPs). There are a number of families of PRRs expressed by cells of the innate immune system; these include those families that consist of trans-membrane receptors - Toll like receptors (TLRs) and C-type lectin receptors (CLRs), and those with cytoplasmic receptors such as nucleotide-binding oligomerisation domain receptors (NLRs) and retinoic acid-inducible gene (RIG) like receptors (RLRs) [63]. The most widely described family of PRRs are the TLRs. TLRs were originally identified as being important in the dorsal-ventral patterning of the fruit fly, *Drosophila melanogaster*, but were subsequently found to be important in recognising a variety of PAMPs [63]. In humans 11 TLRs have been identified to date [64]. Activation of these receptors initiates an extensive signal transduction cascade leading to activation of nuclear transcription factor κB (NF κB), and expression of pro-inflammatory cytokines including tumour necrosis factor alpha (TNF α), interleukin 1 β (IL-1 β), IL-6 and IL-8 [65, 66].

2.2.1 (i) The airways' epithelium

At the most superficial level, surface barriers exist at environmental interfaces preventing pathogens entering the body. The conducting airways of the lung are lined by a stratified epithelium which is

bound together by tight junctions: complexes consisting of interacting proteins and receptors which prevent noxious molecules from penetrating the epithelium [67]. Mucosal epithelia are wet surfaces lined with mucus, providing further protection [68]. The airway epithelium consists of ciliated columnar epithelial cells and secretory cells (Clara and Goblet cells) [69]. The secretory cells continuously produce mucin, a heavily glycosylated protein which forms a gelatinous layer which is removed through the continuous beating movement of cilia located on the columnar epithelia cells [70]. In the larger airways, sub-mucosal glands also contribute towards this mucous layer. The mucous layer forms a physical barrier which is up to 10µm thick; this glycoprotein rich substance prevents microbes recognising and binding to surface epithelial glycoproteins and contains antimicrobial products (lysozymes, defensins and IgA) and immunomodulatory molecules (cytokines) [69]. Diseases of abnormal mucous composition such as cystic fibrosis [71], and impaired mucous clearance, such as primary ciliary dyskinesias [72], result in chronic infection and inflammation within the airways leading to irreversible dilatation (bronchiectasis), illustrating the importance of this basic defence mechanism. The epithelium is also bombarded constantly with noxious substances, mediating their tissue damage through the generation of free radicals, and is well equipped to deal with such insults utilising antioxidant enzymes and free radical traps [73].

In addition to providing a mechanical and chemical barrier, the airway epithelium expresses TLRs [64]. Activation of these receptors on the epithelial surface results in the expression of inflammatory cytokines including TNF α and IL-8 [65, 66], placing the epithelium physically and functionally in the ideal position to coordinate an inflammatory response to noxious stimuli.

2.2.1 (ii) The inflammatory response

Inflammation is a protective response which promotes both the removal of pathogens and tissue healing [74]. It is characterised clinically by pain, swelling, erythema, heat and loss of tissue function. These clinical manifestations reflect increased vascular permeability and inflammatory cell infiltrate at the tissue level. Cytokines including TNF α , IL-1 β and IL-6 orchestrate the inflammatory response and their production is regulated at a transcriptional level through the activation of PRRs. However, IL-1 β goes through a two step process involving the synthesis of a pro-IL-1 β form of the molecule in response to TLR signalling which is then cleaved to produce the active cytokine. Cleavage takes place via a complex containing the enzyme caspase 1, referred to as the inflammasome [75].

The inflammatory response involves the influx of a number of white blood cells, or leukocytes, derived from myeloid progenitors (Figure 2.1) which form part of the innate immune system.

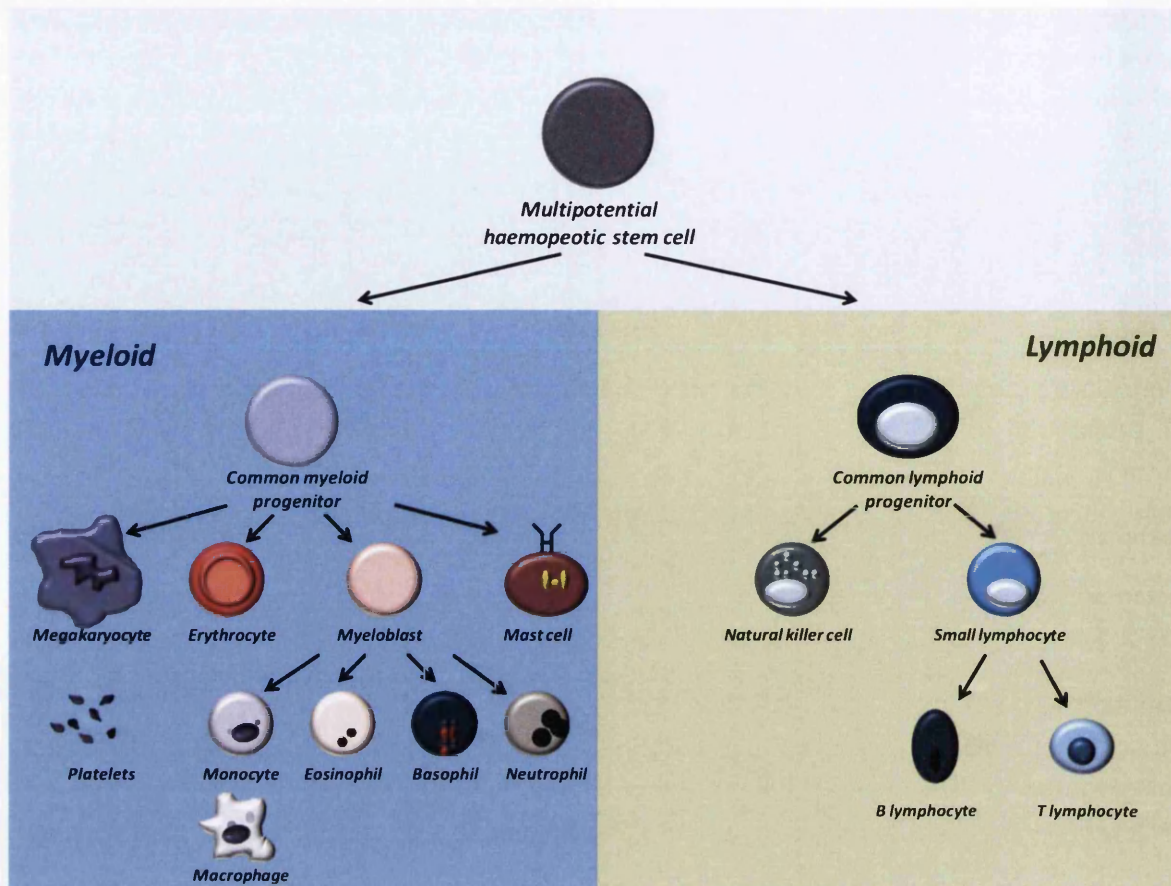


Figure 2.1: Schematic representation of haematopoiesis. Multi-potential haemopoetic stem cells differentiate into common myeloid and lymphoid progenitors. Cells of the innate immune system are typically of myeloid lineage (NK cells being the exception to this), whilst lymphocytes of the adaptive immune system develop from lymphoid progenitors. B lymphocytes subsequently mature in the bone marrow whilst T lymphocytes mature in the thymus.

Macrophages are the most abundant immunologically active cell in the lungs. They are phagocytes and upon activation via PRRs serve to eradicate the lung of noxious substances, pathogens and debris. As well as resident macrophages in the lung, others are recruited from the blood from circulating pro-inflammatory CD16⁺ monocytes [76]. This mature subset of monocytes accounting for 5-8% of all those circulating, is responsible for cytokine production in acute (e.g. sepsis) and chronic inflammatory (e.g. tuberculosis) processes [77]. Two classes of macrophage reside in the lung and develop according to the cytokine environment to which they are exposed. Exposure to interferon gamma (IFN γ), predominantly produced by T helper 1 cells (Th1) (see section 2.2.4), results in the development of pro-inflammatory M1 macrophages, which are efficient at phagocytosis and antigen presentation [78]. M1 macrophages are important in the response to intracellular bacteria including *Mycobacterium tuberculosis*. The T helper 2 (Th2) cytokines IL-4 and IL-13 promote the development of an Alternatively Activated Macrophage (AAM) or M2 macrophage which has traditionally been thought to have anti-inflammatory properties, but may also have a role in defence against parasites [79].

Eosinophils are granulocytes and contain highly toxic granular proteins which are released upon their activation [80]. IL-5 along with IL-3 and granulocyte/macrophage colony stimulating factor (GM-CSF) are important in promoting recruitment of eosinophil progenitor cells from the bone marrow and their maturation [81]. Eosinophils are important in Th2 related immunity, they respond to helminth infections [82] and are also important in the pathophysiology of atopic conditions including atopic eczema [83], allergic rhinitis [84], and asthma (see section below (2.3.1 (ii))). Upon activation they release a plethora of active mediators including major basic protein, cationic protein and eosinophil peroxidase, as well as a number of cytokines and chemokines. Basophils are also important in the response to helminth infections and can function as antigen presenting cells initiating Th2 responses [85].

Mast cells, named “mastzellen” - meaning well fed cells reflecting their stuffed cytoplasm - by Erlich in 1876, reside in connective tissue in the skin and at mucosal surfaces including the lung; they do not circulate in the blood [86]. Mast cells differentiate in the bone marrow in response to stem cell factor (SCF) and Th2 cytokines IL-4, IL-5 and IL-9 [87]. Upon activation mast cells release granules which containing preformed mediators including histamine, and newly synthesised arachidonic acid metabolites, including prostaglandins (PGD₂) and leukotrienes (LTC₄), as well as cytokines (TNF- α , IL-4, IL-5, IL-6, IL-1 β and IL-13) [86].

Neutrophils are one of the most abundant cells of the innate immune response and have strong phagocytic and antimicrobial properties; they also recognise pathogens using PRRs. In addition, they are able to generate reactive oxygen species (ROS), which can damage DNA, proteins and lipoproteins and they arrive at sites of inflammation within a few hours [88]. The blood neutrophil count is tightly regulated by a number of cytokines including G-CSF, IL-17 and IL-23 [89] (see section 5.1.1 for more details). IL-23 is a cytokine produced by macrophages and dendritic cells in response to an inflammatory stimulus via NF κ B and induces IL-17 expression by Th17 cells (see section 2.2.3 (ii)) [90]. IL-17 in turn is a potent inducer of G-CSF production which promotes neutrophil differentiation at the level of the bone marrow [91]. Humans deficient in G-CSF develop profound neutropenia [92].

ROS are molecules which contain unpaired electrons and react vigorously with other chemical compounds altering their structure and function [93]. ROS are generated as part of normal metabolism and are also produced by cells including neutrophils and monocytes as part of the innate immune response. Oxidative stress arises due to an imbalance between ROS production and counteracting antioxidants and can cause oxidative injury resulting in further inflammation [94].

2.2.2 Dendritic cells

Whilst the inflammatory response is immediate, it is also non-specific and if allowed to continue in an unregulated manner would result in widespread tissue damage. A more specific and targeted response by the adaptive immune system not only confers memory but also limits collateral

damage. However, this requires cells which are activated quickly via PRR but then are able to present a specific peptide (antigen) to the adaptive immune system. Dendritic cells (DCs) are ideally placed for this role, being present in areas of the body in contact with the external environment, including the lungs and the gut. These cells, located just beneath the epithelial cell layer, are able to extend their finger-like processes through the epithelial junctions to directly sample the microenvironment [95]. Knowledge of the anatomical distribution of DCs in humans is incomplete. Conventionally they are divided into pro-inflammatory myeloid dendritic cells (mDCs), which consist of type I mDCs, type II mDCs and plasmacytoid dendritic cells (pDCs) and can be differentiated by cell surface markers (Table 6.2) [96]. Myeloid dendritic cells constitute 0.5%-1.0% of mononuclear cells within the circulation and share a common lineage with macrophages and monocytes, whereas pDCs represent <0.3% of circulating mononuclear cells and express lymphoid development markers [97]. DCs also form part of the innate immune response and in the airways, are activated by inhaled substances through a variety of PRRs including TLRs [98]. Upon activation they are able to take up antigen, migrate to lymph nodes, and present antigen in association with Major Histocompatibility Complex (MHC) class I and II molecules thus activating the adaptive immune response [99]. They are also able to polarise the type of adaptive response generated through the release of instructive cytokines including IL-12, IL-10, transforming growth factor β (TGF- β) and IL-6, which influence the type of T cell polarisation seen [100] (see section 2.2.3 (i)). DCs therefore bridge the two arms of the immune system.

2.2.3 Adaptive Immunity

The adaptive immune system involves B and T lymphocytes, derived from lymphoid progenitors (Figure 2.1). These cells circulate through the blood and lymphatics to lymph nodes which they enter through high endothelial venules [101]. Here they encounter antigen presenting cells, including DCs which ordinarily reside in tissue. Unlike cells of the innate immune system they express highly specific receptors capable of recognising a discrete antigen in the context of MHC class I or class II.

2.2.3 (i) T lymphocytes

T cells are produced by the bone marrow but mature in the thymus as thymocytes. T cells express a T-cell Receptor (TCR) which is a heterodimer consisting of 2 subunits. The majority of T cells express an $\alpha\beta$ receptor; however some, which are usually resident in mucosal tissue, express $\gamma\delta$ receptors. Whilst in the thymus, DNA encoding the TCR undergoes somatic rearrangement [102], resulting in a vast repertoire of cells, each expressing a different TCR capable of recognising a discrete antigen. Conventional T cells recognise such peptides presented in the cleft of MHC molecules. Within the thymus, developing thymocytes encounter self antigen presented within MHC on thymic epithelial cells: thymocytes incapable of recognising any self antigen presented in MHC die (negative selection) whilst those which react too strongly to self antigen are also deleted (positive selection) [103]. T cells can be divided broadly into two major subsets according to expression of different surface glycoproteins; cluster of differentiation 8 (CD8) expressing T cells and CD4 expressing T cells. [104].

CD8⁺, or cytotoxic T (Tc) cells recognise antigen presented in the cleft of MHC class I molecules which are expressed on all nucleated cells within the body [104]. Ordinarily these molecules express self antigen generated through protein synthesis within the cell, however upon infection with a virus they will begin to express foreign antigen which is recognised by a specific Tc cell. Through the production of mediators such as IFN γ , perforin and granzyme, they destroy virally infected cells [105] and also respond to cancerous cells, such that novel treatments are being developed to augment their activity in malignancies [106].

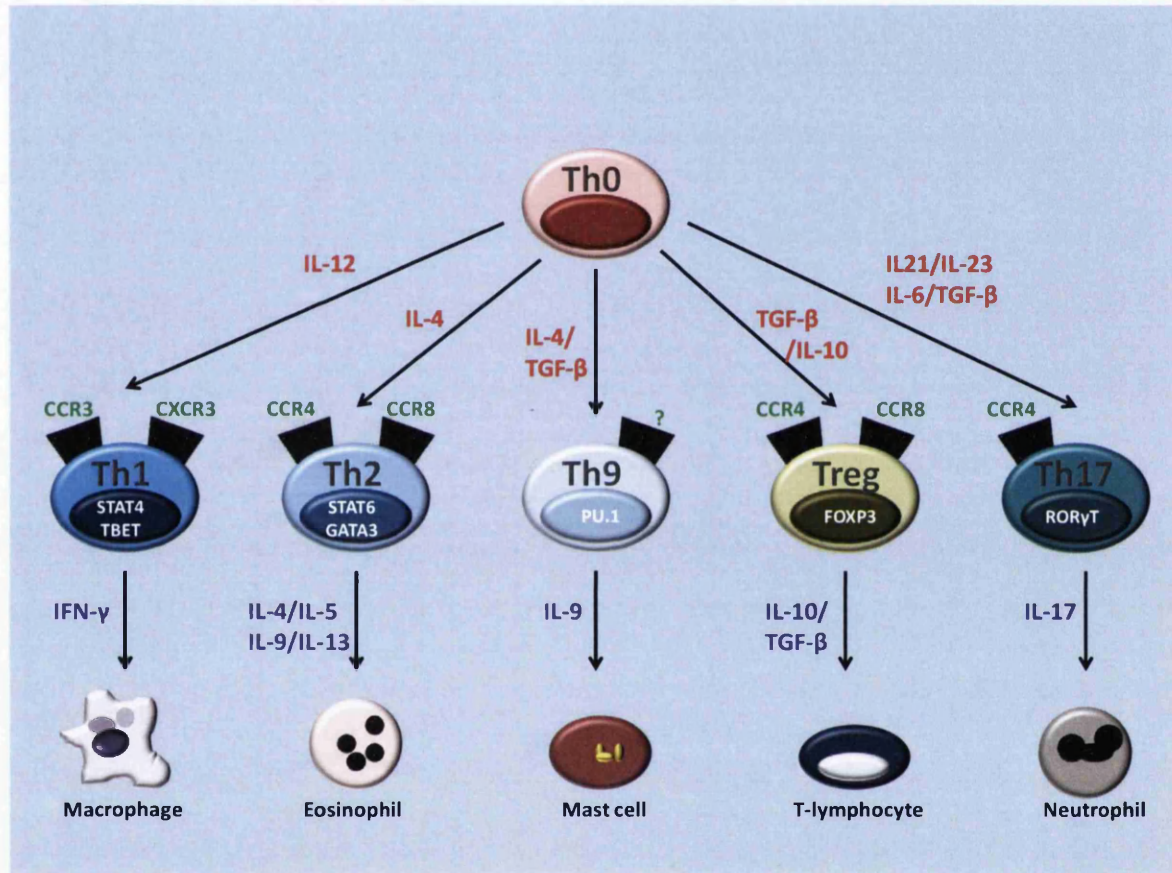


Figure 2.2: Schematic of CD4⁺ T cell subsets. The local cytokine milieu (shown in red) leads to the activation of specific transcription factors (shown in white) which result in the differentiation of different CD4⁺ subsets. These subsets express different CC-chemokine receptors (CCR, shown in green) and can be identified by their cytokine signature (shown in purple). These cytokines act on target cells (bottom row) which influence the type of inflammatory response seen.

CD4⁺, or T helper (Th) cells recognise antigen bound to HLA class II molecules expressed on professional antigen presenting cells, although some exceptions to this have been noted [107]. Class II molecules present proteins from bacterial, fungal and helminth infections, degraded in endosomal compartments. CD4⁺ T cells lack any phagocytic or cytotoxic activity; rather they “help” other components of the immune system. They are further subcategorized according to their cytokine

expression (Figure 2.2); initially two main subsets were identified in animal models [108] and humans [109]. T helper 1 cells (Th1) differentiate in response to IL-12 produced by DCs [100] which causes up-regulation of the transcription factor T-bet [110]. They secrete IFN γ which acts on macrophages to increase their phagocytic capacity, important in responding to bacterial infections. IL-4 stimulates T helper 2 (Th2) differentiation through the activation of transcription factors signal transducer and activation of transcription 6 (STAT6) and trans-acting T-cell-specific transcription factor 3 (GATA3). Th2 cells produce the cytokines IL-4, IL-5, IL-6, IL-9 and IL-13[110], a profile important in stimulating B cells to produce IgE and promoting maturation of eosinophils during helminth infections [111].

In recent years, further novel subsets, again defined by their cytokine expression, have been identified. T helper 17 (Th17) cells discovered in 2005 [112] produce IL-17 [113], a cytokine important in neutrophilic inflammation [114]. The factors favouring Th17 differentiation remain unclear (section 7.1.2), however IL-21, IL-23, IL-6, IL-1 β and TGF- β play a role in promoting the expression of the retinoic-acid-related orphan nuclear receptor gamma T (ROR γ T) [115]. Th17 cells are helpful in the clearance of extracellular pathogens including fungi and bacteria [116]. However, over-expression of these cells and IL-17 have been linked to a number of autoimmune conditions including inflammatory bowel disease, psoriasis and systemic lupus erythematosus as well as asthma [117].

T lymphocytes with regulatory or immunosuppressive properties, termed regulatory T cells (Tregs), are regarded as the principal mediators of immune tolerance, influencing cells of the innate and adaptive immune systems [118]. Naturally occurring Tregs exist to prevent an inappropriate response to self or harmless antigen and have thus been implicated in many disease states including type I diabetes [119]. Previously Tregs have been identified by the surface antigen CD25 (the alpha chain of the IL-2 receptor) however the majority of T lymphocytes will express this surface marker upon activation [120]. A more specific marker, the transcription factor forkhead box P3 (FoxP3) has been identified and is postulated to have a critical role in Treg development [121]. The IL-7 receptor CD127, is also down-regulated in Tregs, and has been used in their identification [122]. Within the literature there is sufficient data to suggest that the use of CD25, CD127 and FoxP3 will identify most of the naturally occurring Treg population. Treg populations can also be induced during the course of the immune response and these include Tr1 and Th3 cells. Tr1 lymphocytes are identified by their production of IL-10 [123] and the absence of FoxP3 expression [124], whilst Th3 cells produce TGF- β in abundance [125]. Tregs exert their effects through a variety of mechanisms (see section 7.1.2 and Figure 7.1 for details). They produce IL-10, an immunoregulatory cytokine which suppresses effector T cell responses [126] as well as promoting Treg cell development [127]. Tregs also express high levels of the IL-2 receptor CD25, and along with other effector T cells require stimulation with this cytokine for proliferation and survival [128]. Tregs do not produce IL-2 and they limit the proliferation of other effector T cells by competing for this cytokine [129, 130]. Tregs can also cause cytolysis of effector T cells [131] as well as raising the threshold for dendritic cell activation of T cells [132, 133].

T-helper 9 cells (Th9) characterised by production of IL-9 [134] (Figure 1.2). Th9 cells differentiate in response to TGF- β , which causes upregulation of the transcription factor PU-1, and IL-4 which up-regulates the transcription factors STAT6 and subsequently GATA3 and interferon regulatory factor 4 (IRF4), whilst suppressing FOXP3 expression [135]. Through IL-9 production, enhanced by IL-25

exposure [136], Th9 cells stimulate proliferation of mast cells and may have a role in autoimmune and allergic diseases [137].

2.2.3 (ii) Innate-like T cell subsets

The rapid but non-specific innate immune system primes the slower adaptive response, however some T cells exist, which react rapidly to noxious stimuli and promote early recruitment of innate cells as well as functional polarisation of the adaptive response. These innate-like T cells include Natural Killer T (NKT) cells and $\gamma\delta$ T cells. These differ from conventional T cells in the type of TCR they express and in that they are not MHC restricted. NKT cells express an invariant TCR (V α 24/V β 11) and are often termed invariant NKT (iNKT) cells. Unlike conventional T cells, those of iNKT cells recognise glycolipids presented by the MHC class I-like molecule, CD1d rather than peptides presented by MHC. The TCR used by these cells is highly conserved between species suggesting it functions more as a PRR. In keeping with this, activation of iNKT results in an early rapid cytokine response forming an important bridge between the innate and adaptive immune systems and leads to the production of an array of cytokines, including IFN γ and IL-4, which subsequently drive the adaptive response [138]. As discussed in 1.2.3 (i) T cells typically express $\alpha\beta$ TCR or the less common $\gamma\delta$ TCR. Like iNKT cells, $\gamma\delta$ T cells also recognise non-peptide antigen and are not MHC restricted. They have a tropism towards epithelial surfaces where they reside and have a large number of roles including protection against pathogens, tumour surveillance, and modulation of the innate and adaptive immune responses [139].

2.2.3 (iii) B lymphocytes

B lymphocytes differentiate and mature in the bone marrow. These cells express immunoglobulin as a surface receptor and as with the TCR, DNA coding for this undergoes somatic rearrangement, leading to numerous B cells each capable of recognising a specific antigen [140]. Unlike T cells, B lymphocytes recognise the protein (antigen) in its natural state without any antigen processing/presentation. Upon recognition of antigen, B cells proliferate and undergo somatic hypermutation whereby the immunoglobulin produced is mutated slightly to increase specificity/affinity of binding [140]. Whilst the bulk of these B cells will die once the threat is removed, two critical populations survive: plasma cells and memory cells. Plasma cells produce and secrete vast quantities of immunoglobulin, and memory cells ensure a faster antigen-specific response on re-exposure to the same antigen [141].

Immunoglobulins or antibodies are Y-shaped protein structures with a variable region capable of recognising specific antigen (Fab) and a constant region (Fc) which is recognised by plasma membrane receptors on innate cells [142]. The specific antibody produced has a number of effects useful in immune defence including agglutination of pathogens and activation of the complement system which is a series of proteins synthesised by the liver and involved in the innate immune defence system. Antibodies also coat pathogens (opsinisation) via the F_{ab} end of the molecule, and then bind via the F_c region to cells of the innate immune system to facilitate efficient phagocytosis [143].

2.3 Overview of asthma immunology

Central to the definition of asthma is the concept of chronic inflammation predominantly affecting the conducting airways, although it can spread more distally [37]. It is a multi-cellular process involving components of the innate (eosinophils, neutrophils, mast cells, dendritic cells) and the adaptive immune systems, namely CD4⁺ T cells and B cells. The two arms of the immune system interact in a bidirectional manner perpetuating a chronic inflammatory process (Figure 2.3 (taken from Pynn et al [39])) which in the smaller airways involves mainly the mucosa, spreading to the sub-mucosa in the larger airways [144].

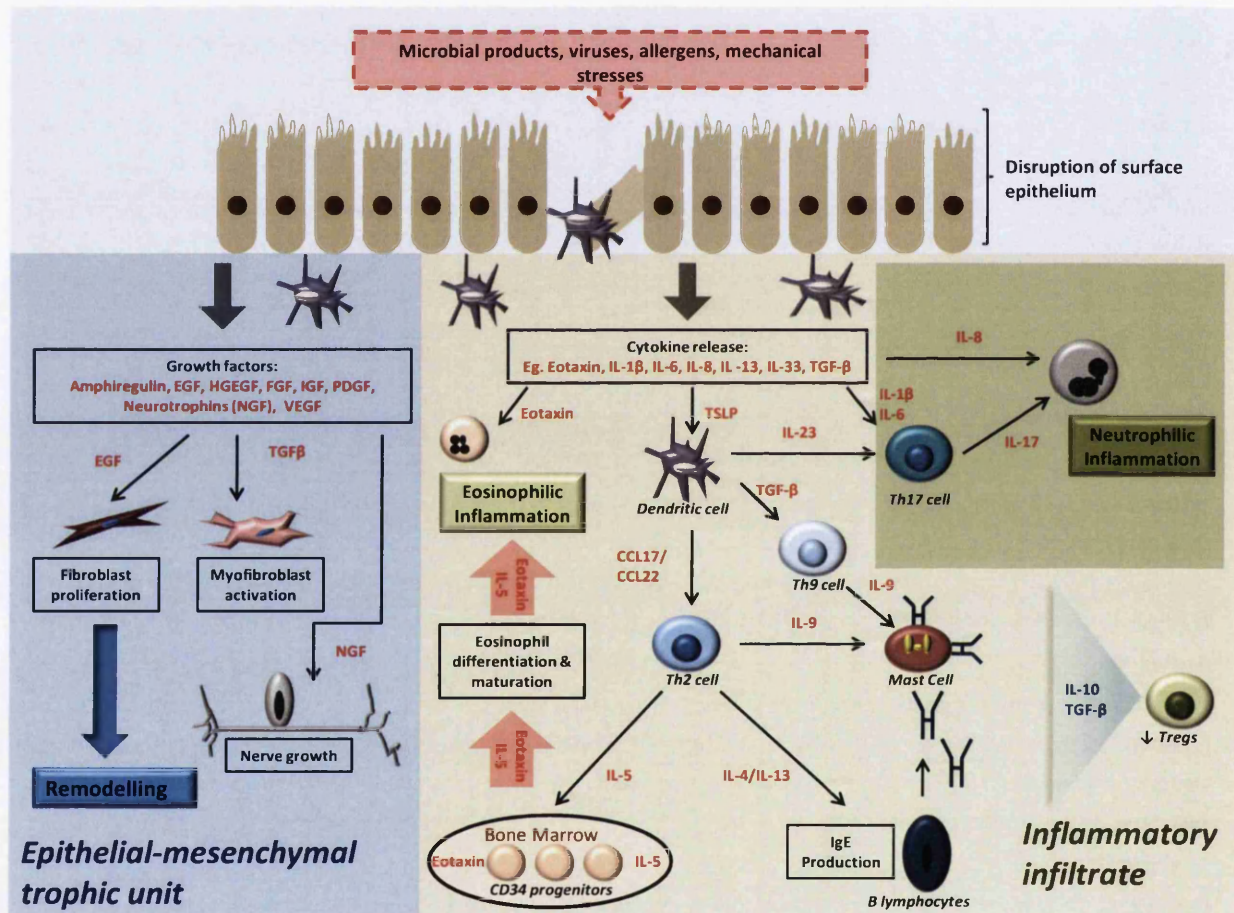


Figure 2.3: Schematic representation of the epithelial-mesenchymal trophic unit and inflammatory cells important in the pathogenesis of asthma. The epithelium releases a number of growth factors important in the coordination of airway remodelling. Cytokines are also released which promote migration and activation of various inflammatory cells. Th2 cells are pivotal to orchestrating eosinophilic inflammation and IgE production; Th9 cells might play a role in local IL-9 generation. IL-17, produced by Th17 cells, and IL-8 could have roles in the development of neutrophilic disease. Regulatory T cells (Tregs) promote immunological tolerance and are decreased in number and function in asthmatics.

2.3.1 Innate immune activation

2.3.1 (i) The lung epithelium

In asthma the physical barrier of the airway epithelium is compromised and renders the airway susceptible to further injury from allergens, pathogens, pollutants and other insults. The asthmatic airway epithelium is deficient in anti-oxidant defences [73], tight junctions are disrupted [145] and there are signs of epithelial shedding [146] with defective wound repair [147]. Changes in the epithelium may be instrumental to the development of the disease rather than a consequence of chronic inflammation [148].

Asthma is characterised pathologically by airway wall thickening, smooth muscle hypertrophy, goblet cell hyperplasia, mucus hypersecretion, basement membrane thickening and neoangiogenesis [110]. These structural changes are all encompassed by the term remodelling and occur due to the release of a number of growth factors, many from the epithelium upon insult, which stimulate the mesenchymal tissue. These growth factors include TGF- β , epithelial growth factor (EGF), vascular endothelial growth factor (VEGF) and neurotrophins [148]. The interstitium within the airway wall is dynamic, involving a balance between extracellular matrix (ECM) synthesis and degradation. In asthma this balance is tipped towards increased ECM deposition, and resident airway fibroblasts are the primary source of this. TGF- β , produced by the epithelium and eosinophils, along with cytokines such as IL-13, promote the transition of airway resident fibroblasts into myofibroblasts which deposit ECM resulting in sub-epithelial fibrosis [149]. TGF- β is also important in the proliferation of airway smooth muscle cells which further contributes to the airway wall thickening [150], whilst VEGF promotes neoangiogenesis [151]. Mucus production by the epithelium is also enhanced as a result of goblet cell hyperplasia and an increase in the size of submucosal glands [152]. The constituents of the mucus are altered and increased amounts of highly viscous mucins such as 5AC and 5B are observed [153]. Th2 cytokines such as IL-4, IL-9, and IL-13 are important in driving mucin gene expression and goblet cell metaplasia [154].

Through the activation of PRRs on the airway epithelium a number of chemokines and cytokines are produced which mediate the local inflammatory response (Figure 2.3). These include thymic stromal lipoprotein (TSLP) which promotes dendritic cell maturation, upregulating the expression of OX40 which interacts with its ligand OX40L on CD4⁺ T cells and is instrumental in the differentiation of Th2 lymphocytes [155]. The epithelium also releases chemokines including C-C chemokine ligand 17 ((CCL17), or thymus and activation regulated chemokine (TARC)), and CCL22 which via their action on CCR4, promote Th2 accumulation (Figure 2.3). IL-8 and CCL11 (eotaxin) release result in the accumulation of neutrophils and eosinophils respectively, whilst IL-33, an alarmin, alerts the immune system to stress and expands the Th2 response [156].

2.3.1 (ii) The inflammatory response

Eosinophilic inflammation is considered to be the hallmark of atopic asthma and the quantification of eosinophils in sputum is useful in predicting steroid responsiveness and therefore guiding therapy or measuring treatment compliance [157]. Increased numbers of eosinophils are released from the bone marrow in response to cytokines, including IL-5 produced by Th2 cells and to a lesser extent by

mast cells. The eosinophils “home in” to the airway in response to chemokines released locally by the epithelium including CCL5 (or Regulation on activation normal T cell expressed and secreted (RANTES)), CCL7 (previously named monocyte specific chemokine 3 (MCP-3)), and CCL11 [158] which act on CCR3 [159]. Through the release of toxic mediators, eosinophils cause significant tissue destruction and contribute towards bronchial hyperresponsiveness (BHR). Eosinophils also release potent bronchoconstrictors including the lipid derived LTC₄, as well as producing cytokines fundamental to the remodelling process such as TGF-β [160]. Corticosteroid treatment induces eosinophil apoptosis as well as inhibiting their response to survival signals from IL-5 and GM-CSF [161].

Mast cells promote bronchoconstriction in asthma through the release of pre-synthesised mediators including LT C₄, D₄ and E₄ and prostaglandin D₂. Mast cells degranulate in response to cross linking: IgE bound to specific F_c receptors at the surface of mast cells bind specific antigen via the F_{ab} region inducing cross-linking [86]. Mast cells also release a number of Th2 cytokines, including IL-5 and IL-13, which further fuel the inflammatory response and the presence of mast cells in airway smooth muscle has been linked with BHR [162]. Mast cells accumulate within the airways of asthmatics due to the release of SCF by epithelial cells which acts on mast/stem cell growth factor receptor (or cKIT receptor) expressed on the mast cell surface [163], as well as IL-9 produced by Th2 and Th9 cells.

Neutrophils can contribute towards airway inflammation by generating reactive oxygen species (ROS) and releasing proteases; this is important in the pathogenesis of lung conditions including adult respiratory distress syndrome [164], chronic obstructive pulmonary disease (COPD) [165] and severe asthma (see section 5). Elevated blood neutrophil counts are associated with certain asthma phenotypes characterised by chronic cough and sputum production [166]. Refractory asthma patients with persistent airways obstruction have also been shown to have higher levels of sputum neutrophils than those with reversible airways disease who have a sputum eosinophilia [167]. Furthermore sputum neutrophil levels are highest in those with severe disease compared with mild disease [168] and negatively correlate with lung function markers of airflow obstruction [169]. Neutrophils may be recruited to the airways by the chemoattractant IL-8, with sputum neutrophils correlating with IL-8 levels in non-smoking adults with persistent asthma [170]. IL-17 production by Th17 cells may also contribute to neutrophil recruitment with increased IL-17 being reported in the sputum of asthmatic patients and correlating with IL-8 levels and sputum neutrophils [171].

Asthma is characterised by marked mucosal infiltration by macrophages which have many of the phenotypical characteristics of blood monocytes [172] and sufferers also have higher levels of circulating CD14⁺CD16⁺ monocytes [173]. These observations coupled with the fact that local macrophage proliferation does not appear to contribute to the increase numbers seen suggests that these cells are recruited from the circulation [174]. IL-6 and TNFα, produced by monocytes/macrophages also have a direct impact on asthma. Severe asthmatics have higher levels of TNFα in BAL fluid, which promotes neutrophilic inflammation [175]. Circulating IL-6 levels are increased in atopic asthma and its presence in sputum inversely correlates with FEV1 [176].

2.3.1 (iii) Oxidative stress

ROS generation is an important mediator of airway inflammation in COPD, smoking asthmatics and severe asthma. Asthma is associated with enhanced systemic oxidative stress in adults and children, evidenced by higher lipid peroxidation products, higher protein carbonyls, and higher superoxide production by isolated leukocytes [177, 178]. This background oxidative stress is further increased during exacerbations [179]. Asthma is also associated with enhanced oxidative stress locally in the airways as evidenced by increased exhaled 8-isoprostane in asthmatics compared to controls, with levels correlating with disease severity [180].

2.3.2 Dendritic cells

Dendritic cells (DCs) have an important role in asthma and are exquisitely placed beneath the respiratory epithelium to take up antigen, including allergens, and migrate to local lymph nodes where they present it on MHC molecules to antigen specific T cells. The production of TSLP by the lung epithelium, in response to ligation of PRRs, results in maturation of myeloid DCs (mDCs) priming them to promote a Th2 driven response. This is achieved by upregulation of OX40L which interacts with OX40 on undifferentiated CD4⁺ T cells (Th0) cells in local lymph nodes promoting Th2 differentiation [181]. Not only do DCs prime the immune response to allergen but they also perpetuate the inflammatory response by being the predominant source of CCL17 and CCL22 following TSLP exposure [181]. GM-CSF release by epithelial cells also promotes DC maturation resulting in a Th2 response [182] and is the mechanism by which diesel fumes and cigarette smoke may cause Th2 inflammation [183]. mDCs in animal models appear to be particularly important in mediating airway inflammation and have been shown to promote Th2 responses [184] whilst plasmacytoid dendritic cells may promote immunological tolerance to inhaled antigen [185]. Studies in humans have shown that during allergen challenges circulating DC levels decrease during the following 24 hours [186] and this corresponds to a rise in numbers in sputum, again emphasising their importance in antigen presentation [187] (see section 6.1.3 for more details).

2.3.3 Adaptive immunity

Inappropriate activation of the innate immune system leads to antigen presentation to T cells within local lymph nodes and a T cell effector response which propagates further airway inflammation. Historically, asthma has been thought of as Th2 mediated disease and certainly atopic disease has been characterised by the presence of increased numbers of Th2 cells within the airways [188]. Furthermore, the number of Th2 cells present correlates with disease severity [189]. The signature cytokines released by these cells have a number of effects relevant to disease pathogenesis. IL-4 is involved in immunoglobulin class switching in B cells leading to IgE expression important in the process of allergen sensitisation. IL-5 is fundamental to eosinophil differentiation and survival (see section 2.3.1 (ii)); IL-9 promotes mast cell survival and IL-13 causes many of the features of BHR [190]. Although the Th2 response plays a role in atopic/eosinophilic disease other phenotypes of asthma seen are not explained by this mechanism.

Other CD4⁺ T cells have also been implicated in the disease process. Deficiency in Tregs number or function may result in an inappropriate inflammatory response to harmless antigen as seen in asthma. Tregs exert some of their effects via IL-10, and in asthma and allergy IL-10 is postulated to maintain immune homeostasis at environmental interfaces including the lung [191]. TGF- β is also produced by Tregs but plays a more complex role as it has both anti-inflammatory and pro-fibrotic actions [192]; mice deficient in TGF- β have enhanced airway inflammation compared to wild-types [193]. Studies examining the number of Tregs in the peripheral blood of asthmatics have been contradictory (Table 7.1) although this is likely to be due to the use of CD25, expressed on all activated T cell populations, without FoxP3 as the Treg marker [120]. In 2 paediatric studies using FoxP3, a reduction in FoxP3 mRNA [194] and FoxP3⁺ Tregs [195] was noted in peripheral blood and lavage samples of asthmatics compared to controls. In one of these papers by Hartl et al, restoration in FoxP3mRNA levels and Treg function occurred following 4 weeks inhaled corticosteroid (ICS) use [194].

Interestingly, whilst Tregs number and function appear to be down regulated in asthma, bronchial biopsies of asthmatic individuals are infiltrated with Th17 cells [196]. Associated with this, increased IL-17 levels have been found in the sputum of asthmatics correlating with neutrophil numbers [171]. Sputum neutrophilia and IL-17 are associated with steroid resistant disease. It is conceivable that there may be an imbalance in the differentiation of Tregs and Th17 cells in severe neutrophilic asthma. A paediatric study looking at Th17 and Treg expression in lavage fluid and peripheral blood of asthmatics (on ICS) vs. controls showed that the proportion of circulating CD4⁺CD25⁺FoxP3⁺ Tregs (expressed as a percentage of CD4⁺ cells) was significantly reduced in asthma compared with controls, yet the proportion of Th17 cells increased, suggesting a Th17/Treg imbalance [195]. In adults similar findings have been reported, with moderate to severe asthmatics showing increased Th17 cells and decreased CD4⁺CD25⁺ Tregs peripherally [197].

The discovery of Th9 producing cells has led to interest as to whether these may contribute towards asthma pathogenesis since IL-9 is found in abundance in bronchial lavage samples of patients with atopic asthma [198]. However evidence that Th9 cells are specifically involved in asthma is still lacking.

CD8⁺ cytotoxic T cells may also play a role in the asthmatic airway with increased numbers within the airways of those affected [199, 200]. Whether their presence is beneficial or detrimental is still debated. Animal models show enhanced airway inflammation and remodelling on depletion of CD8 α ⁺ cells suggesting that their presence may be protective [201, 202], however such studies are limited by the lack of specificity of CD8 α ⁺ as a marker of cytotoxic T cells. Contrary to these findings, more specific studies involving transfer of CD8 $\alpha\beta$ T cells to sensitised animals showed worsening of eosinophilic inflammation and BHR [203, 204]. In keeping with this, studies of patients who die from acute asthma show increased levels of CD8⁺ T cells within the airways, again suggesting they have a detrimental effect [205].

B lymphocytes are important in the process of sensitisation, producing allergen specific IgE. This process requires the presence of IL-4 and IL-13 along with allergen presentation by Th2 cells and co-stimulation with CD40 and CD40L. IgE binds to high affinity receptors Fc ϵ RI on mast cells and basophils and low affinity Fc ϵ RIII (CD23) on B cells, eosinophils and macrophages [206]. Cross linking of these cells causes degranulation, releasing pro-inflammatory mediators (see section 2.3.1 (ii)).

Whilst the description given here is particularly pertinent to atopic asthma, it is becomingly increasingly clear that different phenotypes of the disease exist with distinct clinical and inflammatory correlates.

2.4. Asthma: a clinical syndrome encompassing different phenotypes and endotypes

Most of the current understanding of asthma mechanisms is centred on an allergen driven process. In animals and humans, exposure to an allergen within the airways results in chronic Th2 inflammation. However a different phenotype of disease which lacks an allergic component, referred to as intrinsic asthma, can have a very similar pathological appearance despite such different clinical presentations [207].

In terms of clinical presentations of asthma or “phenotypes”, several recent cluster analyses highlighted that within the clinical syndrome, there are discrete populations of individuals each of which have different clinical characteristics in terms of the disease onset, lung function, inflammatory characteristics and treatment response, illustrating marked heterogeneity. A British study examining asthma patients within primary and secondary care identified 3 clusters of patients within the primary care setting [8]. Cluster 1 consisted of those with early onset, atopic disease and evidence of airways inflammation and dysfunction. Cluster 2 described a group of obese female patients with asthma symptoms and no evidence of eosinophilic inflammation, whilst cluster 3 displayed a benign phenotype of asthma, with little evidence of active disease in terms of symptoms, eosinophilic airway inflammation, airflow obstruction or BHR. In secondary care clusters 1 and 2 presented with the same phenotypes as primary care whilst cluster 3 consisted of a group of patients with early onset severe symptoms and little evidence of eosinophilic inflammation. Cluster 4 consisted of a male predominant late onset disease with few symptoms but marked eosinophilic inflammation.

A European study looking at data from two cohorts (European and French cohorts), identified 4 clusters of individuals. Two consisted of patients with active symptoms and were differentiated by age of onset and two groups with inactive disease differentiated by age of onset and presence of atopy [208].

Severe or refractory asthma encompasses 5-10% of individuals whose disease remains poorly controlled despite treatment [209]. The Severe Asthma Research Programme (SARP) has identified 5 phenotypes [16]. Clusters 1 and 2 had early onset atopic disease with normal or near normal lung function. Cluster 1 used fewer controller medications with less health care utilisation, whereas medication use and healthcare utilisation were increased in cluster 2. In terms of biomarkers cluster 2 had high levels of IgE. Cluster 3 described a female predominant obese phenotype with later onset disease of shorter duration. Despite this, cluster 3 had moderately impaired lung function at baseline and reported a higher burden of symptoms, greater medication usage (including oral steroids) and

HDU attendance which appeared disproportionate to their lung function. Cluster 3 had the lowest degree of BHR and lowest IgE counts. Cluster 4 consisted of early onset atopic disease and cluster 5 consisted of late onset non-atopic disease. Both clusters 4 and 5 had daily symptoms, use of multiple medications and a high chance of previous HDU attendances. Both of these groups had very abnormal lung function despite the use of multiple medications. Although cluster 4 had impaired lung function despite high levels of treatment, it was still reversible with beta-2 adreno-receptor (β_2) agonist. This cluster also had high levels of IgE, marked BHR and eosinophilic inflammation. Cluster 5 had the most severely impaired lung function which did not reverse well with a β_2 agonist and exhibited marked BHR. Cluster 5 also had low levels of IgE and a greater degree of neutrophilic inflammation on sputum cell counts.

Whilst the methodology between the cluster analyses performed to date varies, there are some common phenotypes seen within the different study populations. These include an early onset atopic disease allergic type and two later onset phenotypes including non-atopic eosinophilic and a late onset obese female group. In addition, the SARP work has highlighted a neutrophilic phenotype. Such observations have fuelled a long standing debate as to whether asthma is a distinct disease or a syndrome encompassing multiple disease processes which all have some common clinical features referred to by the practicing physician as “asthma” [170]. It is therefore unsurprising that finding common genetics and environmental determinants has been such a challenge. Furthermore, using blanket approaches in terms of treatment is unlikely to optimally manage what may be multiple discrete disease processes. To address this issue there has been a move towards describing different asthma “endotypes”, where an endotype is the underlying pathophysiological processes leading to the disease [9]. Such an approach should lead to more tailored therapies, which may tackle these more refractory phenotypes.

2.5 Obesity: a growing problem

Globally there is an obesity epidemic with approximately 1.6 billion people worldwide classified as overweight and 400 million as obese [10]. Obesity is now the most common metabolic disorder worldwide and this new threat to health is ever expanding; in Wales alone 57% of adults are classified as overweight and 22% obese (Welsh Health Survey 2008). In the UK it has been projected that by the year 2050 60% of men, 50% of women and 25% of children will be obese [11].

2.5.1 Defining obesity on an international scale

Obesity is defined as “abnormal or excessive fat accumulation that may impair health”. The word obesity is derived from Latin - ob means “over” and esus, the past participle of edere “to eat”; translating to “have over eaten” [210].

Body Mass Index (kg/m ²)	Category
18.5-24.9	Normal weight
25-29.9	Overweight
30-34.9	Obesity class I
35-39.9	Obesity class II
>40	Obesity class III

$$\text{Body mass index (kg/m}^2\text{)} = \frac{\text{Weight (kg)}}{\text{(Height (m))}^2}$$

Figure 2.4: Calculation of body mass index.

Table 2.2: Classification of body mass index.

Body mass index (BMI) (Figure 2.4) was previously termed the “Quetelet index” after its founder, the father of social sciences: Lambert Adolphe Jacques Quetelet and was first described in 1832 [211]. Quetelet observed that body weight was proportional to the square root of body height in lean individuals. Obesity is defined by the World Health Organisation as a BMI >30kg/m² and may be further subcategorised into 3 classes (Table 2.2). Due to its simplicity of use it has been adopted on an international scale and referred to in many clinical guidelines [212]. However it has the limitation of inferring percentage body fat from weight leading to poor sensitivity and specificity in certain populations [213], and resulting in some studies using more direct measures of body fat composition or body fat distribution (see section 3.1)

2.6 Obesity and asthma: evidence behind the association

2.6.1 Epidemiological studies

Asthma prevalence is following a similar rate of growth to obesity [214], leading to speculation that there may be a direct relationship between the two conditions. The notion that obesity and asthma may be linked came from cross-sectional studies published in the 1990s which showed that the risk of being diagnosed with asthma increased with BMI, particularly in women, in a dose dependent manner. A British study of 8,960 adults demonstrated that the odds ratio (OR) for asthma was 1.51 in women with a BMI of 25-30kg/m² and 1.84 for those with BMI >30kg/m² [215]. Cross-sectional studies cannot determine the direction of causality and some have argued that the manifestations and treatments of asthma (corticosteroids) may increase the subsequent obesity risk. However, longitudinal studies have helped define the temporal relationship and demonstrate that obesity significantly increased the risk of a future asthma diagnosis, again in a dose dependent manner [216]. A meta-analysis of 7 large prospective studies (summarised in Table 2.5, adapted from Beuther et al [14]) has confirmed these observations [14]. Compared to a BMI <25kg/m², overweight or obese individuals had higher odds of developing asthma (OR 1.51). Interestingly, some of the individual studies have only found this association in women [15, 217]. In the context of severely obese individuals such as those undergoing bariatric surgery, it may be surprising to note that asthma is a very common co-morbidity with a similar prevalence to type II diabetes. A study by Belle et al on 2559 bariatric patients noted that the prevalence of asthma was between 21.2-32.7% amongst the various weight groups which was comparable to diabetes (31-41.8%) [12].

Study	Population	Number of participants	Incident asthma cases	Follow up (years)	Measures	Adjusted OR and 95% CI (BMI >30 vs. <25kg/m ²)
Camargo et al [216]	Nurses' Health Study	n= 85,911 (all women)	1586	4	Asthma diagnosis (self reported) BMI (self reported)	2.7 (2.3-3.1)
Chen [15]	Canadian National Health Population Survey	n = 9,149 Men = 4266 Women = 4883	202	2	Asthma diagnosis (self reported) BMI (self reported)	Men 1.0 Women 1.9 (1.1-3.4)
Ford et al [218]	US. National Health & Nutrition Examination Survey	n = 9,456 Men = 3,621 Women = 5,925	346	10	Asthma diagnosis (self reported) BMI (measured)	Men: 1.5 (0.9-2.6) Women: 1.4 (1.0-1.9)
Gunbjorn dottir et al [219]	European Community Respiratory Health Survey	n = 16,191 Men = 7604 Women = 8587	623	7.9	Asthma diagnosis (self reported) BMI (self reported)	Men 2.1 (1.4-3.2) Women 1.6 (1.1- 2.1)
Huovinen et al [220]	Finnish same sex twin study	n = 10,597 Men=4,449 Women = 5,552	130	9	Asthma diagnosis (self reported and national register) BMI (self reported)	Men 3.5 (1.6-7.7) Women 2.3 (0.9-6.1)
Nystad [221]	Norwegian Health Survey	n = 135,405 Men = 66,723 Women = 68,682	4218	21	Asthma diagnosis (self reported) BMI (measured)	Men 1.8 (1.4-2.3) Women 2.0 (1.7-2.4)
Romieu et al [222]	E3N French cohort Study	n = 67229 (all women)	372	3 years	Asthma diagnosis (self reported) BMI (self reported)	2.2 (1.4-3.2) ¹

Table 2.3: Summary of key longitudinal studies examining the obesity-asthma association. Table adapted from Beuther et al. OR reported as odds of asthma diagnosis in obese (BMI≥30kg/m²) vs. normal weight (<25kg/m²).

1: In this study OR comparing BMI ≥27kg/m² vs. the reference BMI category (20.2-21.4kg/m²).

In summary, obesity is a global issue and is rapidly increasing in prevalence, such that by 2050 50% of patients will be obese. A range of adult population studies have consistently demonstrated that obesity is a major risk factor for asthma development and increases risk in a dose dependent manner.

2.6.2 Obesity and atopy

Whilst there are a number of studies that suggest that obesity increases subsequent asthma risk, the impact of obesity on atopy is less clear. Atopy is an important risk factor for the development of asthma. Whether obesity is associated with the broader syndrome of atopy is less evident. Some cross-sectional studies have shown an association between obesity and atopy in adults especially women [223], and some exclusively in females [224], whilst others have not shown an association [225]. The difference in findings between studies is likely to reflect the varying definitions of atopy, with some using clinical history of an atopic condition and others skin prick tests or RAST tests to a varying panel of potential allergens (see section 4.1.1 and Table 4.1 for details of studies). Whilst there is still debate as to whether obesity is associated with risk of atopy, what does appear clear from a large number of cross-sectional studies is that obesity increases risk of asthma in non-atopic individuals (see section 4.1.1 and Table 4.2 for a summary of the studies in this area).

2.6.3 Weight loss studies

Further evidence that obesity is associated with asthma development and severity comes from weight loss studies. To date, 16 studies have examined this relationship and all have demonstrated consistent improvements in markers of asthma control irrespective of whether weight reduction was achieved by medical or surgical methods.

In four studies weight loss was achieved by medical methods (low calorie diet/weight loss programmes) (Table 2.6, adapted from Eneli et al [55]). Each of these studies were small (n=10-58), and although a physician diagnosis of asthma was required in all, a stringent objective definition of this was only specified in two. The percentage weight reduction achieved during the follow up period (range 8 weeks to 1 year) was between 8-19%. Benefits of weight loss observed included improvement in standard spirometry, post bronchodilator forced expiratory volume in 1 second (FEV1), peak flow rate (PEFR) variability, exacerbation rates, symptom scores and medication use. The surgical studies were larger (n= 33-893 patients (Table 2.7, adapted from Eneli et al [55])), achieved higher degrees of weight loss (27-40%) within a longer follow up period (range 1-6 years) and resulted in more marked improvements in surrogate markers of asthma control. These included an 82-84% reduction in medication use with some individuals being able to discontinue asthma therapy.

Whilst the medical and surgical weight loss studies to date do suggest consistent improvement in markers of asthma control, many of the studies lacked objective definitions of disease identification, relying on previous physician diagnosis, self-reported diagnosis and medication use. In many of the surgical weight loss studies the asthma related parameters were measured as secondary outcomes and not the primary endpoint. Furthermore, many of the markers of asthma control within the surgical studies were more subjective, such as medication usage or symptom resolution. Such limitations leave these studies open to misdiagnosis of asthma (a considerable issue in this clinical syndrome, see section 2.7.1) and potentially incorrectly attributing improvements in obesity related symptoms to amelioration of asthma control.

Study	Subjects	Diagnosis	1° or 2° outcome	Intervention	Outcome measures	Key findings
Aaron et al [20]	Women = 58 Men = 0 Asthma = 24 Mean weight = 115kg	Physician diagnosis	2°	900kCal diet: - Over 6 weeks if BMI>30 - Over 12 weeks if BMI >35	3 and 6 months: - Full lung function - Methacholine challenge SGRQ	Mean weight loss 20kg (19%) For every 10% weight loss: - Improvement in FEV1 by 73ml - Improvement in FVC by 93ml - Improvement in respiratory symptoms - No change in BHR
Hakala et al [226]	Women = 11 Men = 3 Asthma = 14 Mean BMI = 37kg/m ²	Physician diagnosis	1°	VLCD 1760kj/day 8 weeks	2 weeks prior to diet and after 8 weeks - Twice daily PEFR monitoring - Full lung function - Raw - ABG - Dyspnoea score - Rescue med use	Mean reduction in BMI 5.1kg/m ² Reduced PEFR variability from 5.5- to 4.5% Improvement in FEV1, FVC, Raw No change in ABG Improvement in dyspnoea score (6.5) Reduced rescue medication use (by 0.2 doses)
Johnson et al [227]	Women = 8 Men = 2 Asthma = 10 Mean weight = 110Kg	Moderate persistent asthma 12% reversibility in past 2 years	1°	Alternate day calorie restriction 8 weeks	- Asthma control questionnaires - Serum glucose, insulin, lipids - Leptin - CRP, TNF, BDNF - Markers of oxidative stress - PEFR - Spirometry with reversibility	8% reduction in weight Improved asthma control Improvement in PEFR by 14.4% No change in spirometry Improvement in post bronchodilator FEV1 by 10.5% Reduction in TNF- α , BDNP, leptin and markers of oxidative stress
Stenius et al [228]	Total = 38 Asthma = 38 Mean weight = 98kg	Positive bronchodilator response >15% or diurnal variation >15%	1°	Randomised control trial Treatment group: - VLCD (1760kj/day) 8 weeks	- Follow up 1 year - Morning PEFR - Spirometry - Asthma symptoms - Number of exacerbations - Steroid use - Quality of life	14.5% reduction in body weight Improvement in FEV1 by 7.2% and FVC by 8.6% Reduction in exacerbations (median 4 in control group vs. 1 in treatment group)

Table 2.4: Summary of key medical weight loss studies. ABG-arterial blood gas, FVC-forced vital capacity; Raw-airways resistance; SGRQ-St George's respiratory questionnaire; VLCD-very low calorie diet.

More recently a study of 44 bariatric patients (23 asthmatics and 21 non asthmatic) with a much more stringent asthma definition, was performed [229]. All individuals were non smokers or had a <20 pack year history. Asthma was defined by evidence of significant reversible airways disease or BHR to methacholine. Weight loss was associated with a significant improvement in asthma control and BHR. The improvement in BHR was only seen in those with normal IgE levels. Weight loss was associated with a non-significant reduction in bronchoalveolar lavage (BAL) neutrophils and eosinophils but a significant increase in BAL lymphocyte count. BAL and serum adiponectin increased significantly with weight loss. Weight loss was associated with a paradoxical increase in the amount of IL-5, IL-6, IL-13, TNF α and IL-17 produced on blood CD4+ T cell stimulation with anti-CD3 and anti-CD28 antibodies.

In summary weight loss appears to be an effective treatment for the obese asthma phenotype, however studies to date have marked limitations and the mechanisms remain to be elucidated.

Study	Subjects	Diagnosis	1 ^o or 2 ^o outcome	Intervention	Outcome measures	Key findings
Ahroni et al [230]	Women = 161 Men = 34 Asthma = 24 Mean BMI 46 kg/m ²	Self reported	2 ^o	LAGB Follow up 1 year	Medication use Improvement in asthma symptoms Quality of life	Mean BMI post surgery = 32.3kg/m ² 4/17 patients on medication able to discontinue, 10/17 able to reduce dose 79.2% "asthma much better"
Dhabuwala et al [231]	Women = 126 Men = 31 Asthma = 34 Mean BMI 45 kg/m ²	History of asthma Medication use	2 ^o	SRGB Median follow up 2.5 years	Reported improvement/resolution of comorbidity	Mean BMI post surgery = 28 kg/m ² 50% resolution of asthma 14% improvement 84% reduced medication use
Dixon et al [232]	Women = 334 Men = 46 Asthma = 32 Mean BMI 46kg/m ²	Physician diagnosis	1 ^o	LAGB Follow up 1 year	Symptom questionnaire	Mean BMI post surgery = 32.9kg/m ² Reduction in asthma score by 30.2 points Decreased daily med usage (82%) 34% resolution of symptoms
Dixon et al [229]	Women =40 Men =4 Asthma = 23 Mean BMI 51.7kg/m ²	Positive methacholine challenge or 12% bronchodilator response	1 ^o	Follow up 1 year	BHR Spirometry Asthma control Medication use Adipokines BAL cell counts	Mean BMI post surgery = 37.5kg/m ² Decreased BHR Improved asthma control Increased sputum cell counts Increased cytokine production by CD4 cells
Hall et al [233]	Women =288 Men = 22 Asthma = 12 Weight 110-115kg	History of asthma Medication use	2 ^o	Vertical Band Gastric Bypass (VBGB) Follow up 3 years	Medication use	Median weight post surgery 76-93kg Discontinuation of meds 50%
Macgregor et al [234]	Women = 32 Men = 8 Asthma = 40 Mean BMI = 46kg/m ²	History of asthma Medication use	1 ^o	VBGB Mean follow up 4 years	Patient reported intensity of treatment and frequency of attacks	Mean BMI post surgery = 30kg/m ² Complete remission in 48%
Murr et al [235]	Women = 48 Men = 14 Asthma = 6 Mean weight 125 kg	History of asthma Medication use	2 ^o	VBGB & BPD-DS Mean follow up 30 months	Medication use	Mean weight loss 44kg at 1 year Decreased medication use (100%)
Narbro [236]	Women = 893 Men =401 Asthma = unreported Mean BMI 41kg/m ²	History of asthma Medication use	2 ^o	Gastric banding Gastric bypass VBGP Follow up 6 years	Cost of asthma medications	Weight loss 16% No reduction in cost of asthma medication used
O'Brien [237]	Women = 603 Men = 106 Asthma = 33 Mean BMI = 45kg/m ²	Medication use	2 ^o	LAGB Follow up 1 year	Asthma severity score Medication use Hospitalisations	Mean BMI post surgery = 31kg/m ² Asthma resolved in 30% Medications discontinued in 60% No hospitalisations or oral steroids needed
Simard et al [238]	Women = 279 Men = 119 Asthma = 34 Mean BMI = 50kg/m ²	History of asthma Medication use	1 ^o	BPD-DS Follow up 2 years	Asthma severity	Mean BMI post surgery = 30kg/m ² 79% improved asthma severity
Spivak et al [239]	Women = 147 Men = 16 Asthma = 11 Mean BMI = 45kg/m ²	History of asthma Medication use	2 ^o	LAGB Follow up 3 years	Asthma severity	Mean BMI post surgery = 35kg/m ² Resolution of symptoms in 82%
Sugerman et al [240]	Total = 33 Asthma = 1 Mean BMI 52kg/m ²	History of asthma	2 ^o	Gastric bypass	Asthma severity	Resolution in single case

Table 2.5: Summary of key surgical weight loss studies. BPD-DS - biliopancreatic diversion with duodenal switch; LAGB - Laparoscopic adjustable band; SRGPB - silastic ring gastric bypass; VBGP - vertical band gastroplasty.

2.6.4 Asthma and obesity: a distinct phenotype?

2.6.4 (i) Chronic disease

Obesity has not only been associated with a higher risk of asthma development but might also modify the disease phenotype. Obese asthmatic children are more symptomatic and have higher numbers of emergency hospital attendances [241]. A study of adult asthmatics using a health plan found that obese patients experienced worse disease control and were more likely to require hospital admission for asthma [242]. Similarly obese individuals in a US asthma survey reported more continuous symptoms, missing more work days, using more medication and having a higher risk of developing severe persistent disease than normal weight individuals [243].

Obesity may also impair response to drug therapy. Two papers have pooled the results from double blinded randomised control trials (RCTs) of inhaled corticosteroids (ICS) to examine treatment efficacy according to patient weight [17, 18]. In one analysis of 5 RCTs looking at the effects of inhaled fluticasone vs. fluticasone with salmeterol, obese patients were less likely to achieve control than non-obese, particularly those with BMI $\geq 40\text{kg/m}^2$ [17]. The second, examining the effects of beclomethasone vs. monteleukast, also noted that obese patients were less likely to achieve control on ICS although no difference was seen in terms of response to the leukotriene receptor antagonist [18]. In keeping with this, retrospective analysis of data from 1,265 patients with well defined asthma revealed that this reduced benefit from ICS was associated with a smaller improvement in airway inflammation as measured by nitric oxide and lung function [244]. The mechanism behind reduced steroid effectiveness has not been elucidated, however steroid resistance (shown by dexamethasone-induced mitogen-induced protein kinase phosphatase-1 expression) has been demonstrated *in vitro* using mononuclear cells isolated from obese asthmatic subjects [245]. Reduced treatment efficacy is also reflected in worse asthma control and quality of life [242, 246]. Consistent with these findings two studies looking at acute exacerbations have noted that obese patients (children and adults) with asthma presenting to the emergency department are on higher doses of therapy [241, 247].

2.6.4 (ii) Acute exacerbations

Whilst obesity appears to adversely affect chronic disease control, its impact on acute exacerbations is less clear with several studies addressing this issue yielding conflicting results. However, there is some evidence that in severe asthma obesity may affect treatment response and hospitalisation/stay in the paediatric population and in female adults.

A large multicentre prospective study of 572 individuals presenting to the accident and emergency (A&E) department with all grades of asthma severity found that BMI did not affect severity, hospitalisations, requirement for intubation or number of departmental visits [248]. The groups were comparable in terms of bronchodilator response. Even when analysis was restricted to severe exacerbations no effect of BMI was seen. Similarly, a prospective study of 90 obese and non-obese adult asthmatics attending an emergency department in Cleveland, found no difference in exacerbation severity, response to β_2 agonist, or admission rates. Interestingly, the obese individuals had a significantly higher PEF at presentation [249].

In contrast, two studies have shown BMI to have an effect in obese female adults presenting with severe asthma, and in acute severe episodes requiring intensive therapy unit (ITU) admission in paediatrics. A prospective study examining severe asthma exacerbations presenting to the A&E department found that BMI was positively associated with an increased stay in the department and higher rates of hospitalisation in women but not men [247]. The obese women showed a significantly smaller improvement in PEFr in response to treatment (systemic steroids and nebulisers) compared to those of normal weight. The study authors also noted that the clinical decisions made were due primarily to increased symptoms and wheeze, and not due to differences in spirometry, with baseline PEFr and FEV1 being higher in the overweight/obese group. The authors speculated that the decision to admit may be due to higher levels of baseline dyspnoea although it was interesting to note that at the end of treatment there was no difference in baseline dyspnoea between the obese and normal weight individuals. In keeping with this, data from a non A&E study also suggests that obese female asthmatics have worse exacerbations as evidenced by a higher rate of hospital attendance [216]. In the paediatric population a single retrospective study of 209 children admitted to ITU with acute severe asthma found that obesity was associated with higher numbers of ITU admissions and hospital stay due to a slower rate of improvement in the obese children [250]. In this study, sex did not affect the primary outcomes.

In summary, obesity does affect chronic disease control and treatment response and in some individuals may affect acute treatment response. A recent cluster analysis examining asthma patients on a single primary care database and two secondary care registers suggests that obese asthma may be a distinct phenotype characterised not only by poor asthma control and treatment response but also by female predominance and absence of eosinophilic airway inflammation [8]. Furthermore an American cluster analysis (SARP) also identified an obese, predominantly female phenotype with a high degree of symptoms and only moderate airflow obstruction. They noted these individuals were on complex treatment regimes with 17 % requiring regular corticosteroids [16].

2.7 Obesity and asthma: potential mechanisms

There is a wealth of epidemiological data that strongly suggests that obesity modifies asthma risk and disease phenotype. A number of explanations (summarised in Figure 2.5, taken from Pynn et al [39]) have been proposed for this apparent association. Each of these potential mechanisms will be considered in turn.

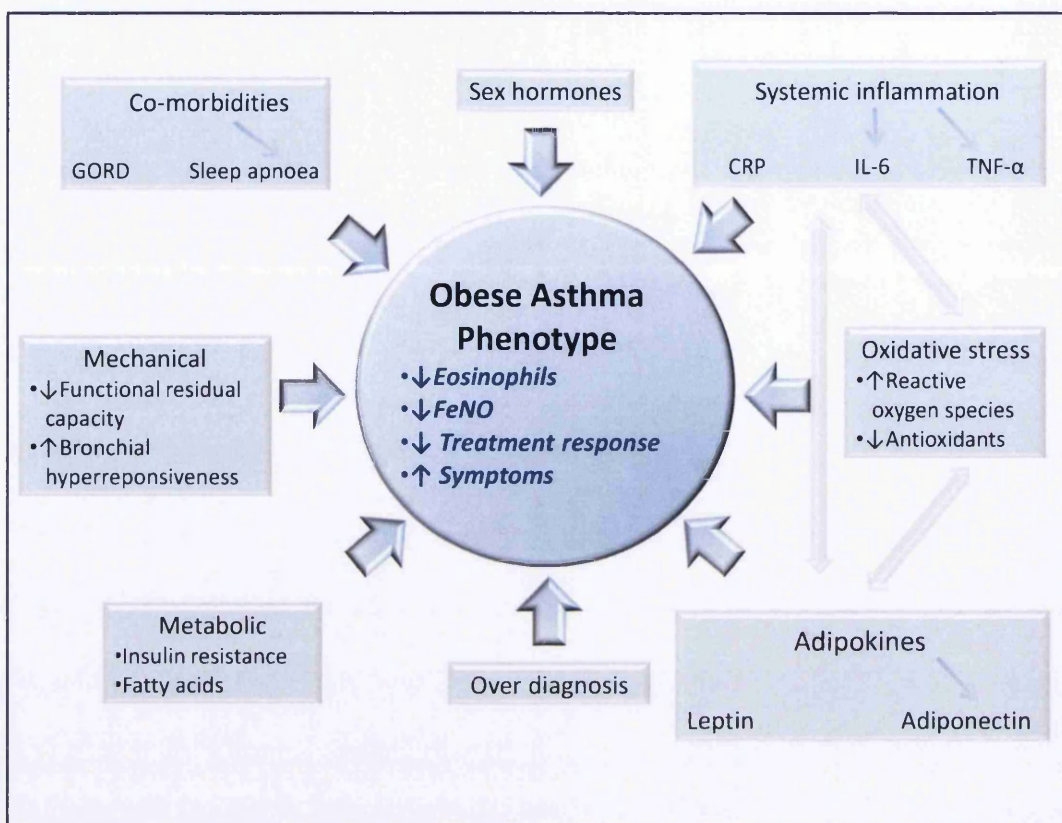


Figure 2.5: Diagram illustrating the potential mechanisms linking obesity and asthma. Some of these factors may interact with each other: adipokines are known to have many immunomodulatory effects and might promote reactive oxygen species generation; metabolic factors such as insulin resistance may have a part in systemic inflammation; whilst fatty acids might moderate inflammation via TLR signalling.

2.7.1 Misclassification bias: over-diagnosis

A potential explanation for the apparent obesity-asthma association is over-diagnosis. It is logical to suppose that the augmented metabolic and physical requirements needed to transport a heavy load would increase dyspnoea in obese individuals and lead to over diagnosis of a respiratory pathology. However, a Canadian study of 540 self-reported physician diagnosed cases showed that over-diagnosis of asthma whilst very high was no more common in obese individuals [20]. Patients with a physician diagnosis of asthma were identified by random digit dialling. Lung function and reversibility testing were then used to confirm the diagnosis and in those who did not have evidence of reversible airflow obstruction, the authors performed bronchial provocation tests. If these were again negative the patients were weaned off the medication and the tests repeated. Overall the rate of misdiagnosis amongst the asthmatics identified was high; however this was statistically no more

likely in the obese group with 31.8% incorrectly diagnosed versus 28.7% in the non-obese group. A paediatric study reported similar findings [251].

2.7.2 Co-morbidities

In 1892, the physician, Sir William Osler first postulated a link between gastro-oesophageal reflux disease (GORD) and asthma by advising patients that they should take their meal at noon to avoid nocturnal symptoms [252]. Gastro-oesophageal reflux symptoms are 4-5 times more common in asthmatics [253] and the condition is associated with BHR and symptoms of wheeze [254]. This is thought to be due to vagal nerve stimulation or microaspiration [255]. With the observation that the incidence of reflux increases with BMI even amongst those of normal weight, it is logical to propose that obesity may potentiate asthma through its effects on reflux [256]. A Dutch study examined 136 patients with persistent asthma symptoms, despite high dose ICS or oral corticosteroids and grouped them according to BMI. Obese patients had less signs of airway inflammation as quantified by exhaled nitric oxide (FeNO) and sputum eosinophils, and a statistically significant higher prevalence of reflux disease (65.5% vs. 44.9%) diagnosed by 24 hour pH monitoring or by a dependency on proton pump inhibitors [28]. However, large population studies found that the association between obesity and asthma persisted despite adjusting for the presence of GORD, suggesting that if GORD does contribute towards the obesity-asthma relationship it is not the only mechanism [219, 257]. More recently a study of 402 patients with inadequately controlled asthma despite high dose ICS showed that whilst obese patients were more likely to self-report reflux symptoms, pH monitoring showed the incidence of proximal reflux was no higher in these individuals than in lean asthmatics [258]. In addition the authors noted that reflux defined either by patient symptoms or pH monitoring did not correlate with lung function or asthma symptom scores. Finally, the weight loss studies performed to date have shown that although weight reduction was associated with significant improvement in reflux symptoms and asthma, the two parameters did not correlate [234, 239]. Improvement in reflux did not predict improvement in asthma symptoms, suggesting that it is not instrumental in disease development or control.

The prevalence of obstructive sleep apnoea (OSA) increases with BMI and is also more prevalent with increasing asthma severity independently of BMI [259]. The mechanisms by which obstructive sleep apnoea worsens asthma control are not fully elucidated but may include vagal nerve stimulation, upper airway inflammation exacerbating lower airway disease or changes in bronchial muscle tone. Studies suggest that treating OSA improves asthma symptoms although it does not appear to improve more objective measures such as spirometry or BHR [260]. In a study of severe asthmatics, symptoms of OSA were more common in the obese and were associated with worse asthma control [258]. However in other paediatric and adult studies, the association between obesity and asthma, whilst attenuated, remains significant even after adjustment for symptoms or signs of OSA [219, 261]. This suggests that whilst OSA may contribute to poor asthma control in the obese it is unlikely to be the sole explanation for the association.

2.7.3 Mechanical effects

Obesity is associated with marked changes in lung volumes. During normal tidal breathing an individual exhales to a volume referred to as the functional residual capacity (FRC). This volume is determined by a balance between the inward elastic recoil of the lungs, the weight of the chest wall, and the outward recoil of the thoracic cage. In obesity this balance is tipped due to increasing weight of the chest wall resulting in a decrease in FRC. King and colleagues examined a cohort of 276 randomly selected adults and demonstrated that obesity correlated negatively with FRC and airways resistance. However, in males the degree of airway narrowing was disproportionate to that expected from the reduction observed in lung volumes [262]. It was estimated that the reduction in lung volumes only accounts for 10% of the increased airways resistance seen, so although a decrease in FRC may contribute to the disease phenotype it does not fully account for the airway obstruction observed.

Obese individuals, breathing at lower tidal volumes [263], might suffer loss of the protective effect of breathing related airway distension, promoting increased BHR [264]. When normal weight non-asthmatics deep breathe, this protects them from developing bronchoconstriction to a noxious stimulus such as methacholine, whereas this protective effect appears to be lost in the obese [265]. Small airway closure is also a factor which compounds airway narrowing in the obese, especially when supine [266].

Although obesity may be associated with changes which promote airflow obstruction and BHR, this has not been found universally. Some studies have demonstrated no correlation between obesity and airflow obstruction [267], whilst others have shown a positive association but only in women [268]. Chinn et al examined the prevalence of BHR in 11,277 individuals and found that BMI was associated positively with BHR, although only statistically significantly in men [269]. Similarly a prospective male cohort study found that both low and high BMI were associated with BHR [270]. Three further studies have found a significant association in both sexes [271-273] whereas other studies, whilst finding a correlation between obesity and asthma diagnosis, have not observed a relationship with BHR [267, 274].

2.7.4: Metabolic effects

2.7.4 (i) Insulin resistance

Obesity is associated with a significant increase in insulin resistance (IR) and subsequent risk of type II diabetes. A study of bariatric patients undergoing surgery showed that the prevalence of asthma and diabetes were broadly similar (21.2 - 32.7% vs. 31.0 - 41.8% respectively) [12]. In addition, hyperinsulinaemia as seen in IR can cause airway smooth muscle contraction. The literature is inconsistent with two paediatric [275, 276] and one adult study [277] showing an association between IR and asthma whilst another adult study found no association [225] (see section 4.1.3 (i) for details). To date, studies examining this important area in both children and adults have lacked robust definitions of asthma relying on self reported diagnosis or typical symptoms. They have also not controlled for potential confounders including steroid treatment and in some cases used

surrogate clinical markers for insulin resistance. Such deficiencies may explain the conflicting results reported.

2.7.4 (ii) Fatty acids

Obesity is also associated with elevated circulating levels of free fatty acids (FFA), which have a number of immunomodulatory effects. For example they can activate the innate immune response through ligation of PRRs including TLR2 and TLR4 [278, 279] resulting in the downstream release of pro-inflammatory cytokines [280]. Very little work has been done in this area but one study has suggested that even a single high fat meal can activate the innate immune system promoting sputum neutrophilia in asthmatics through the activation of TLRs [281].

2.7.4 (iii) Adipokines

Adipokines are cytokine-like hormones produced within adipose tissue. Their principal role is thought to be in regulating metabolism, however they also have profound effects on various components of the immune system. In the obese state, their levels are altered dramatically, promoting a pro-inflammatory milieu. The origins, functions and effects of adipokines are summarised briefly here with more detail in section 4.1.3.3.

Leptin is produced predominantly by adipocytes [282], has a similar structure to IL-6, promotes satiety and regulates energy expenditure [283, 284]. Despite these effects obese individuals have high concentrations of this adipokine, suggesting the possibility of relative leptin resistance [285-287]. It has a plethora of immunomodulatory effects which could have relevance in an inflammatory disorder such as asthma (summarised in Table 4.3) and impacts on multiple cell types from the innate and acquired arms of the immune system [288, 289] including monocytes [290], neutrophils [291], eosinophils [292], NK cells [293, 294], DCs [295] and Tregs [296].

Adiponectin is an insulin sensitising hormone and levels decrease in obesity [297]. This adipokine has anti-inflammatory effects including induction of IL-10 and IL-1 receptor antagonist expression by adipose macrophages [298]. Resistin, a less well studied adipokine is insulin desensitising and expression is elevated in obesity. As with leptin it has a number of pro-inflammatory effects [299]. Visfatin, also known as nicotinamide phosphoribosyltransferase, is produced by a number of cell types including adipocytes, lymphocytes, monocytes, neutrophils and pneumocytes [300]. It has insulin mimetic and pro-inflammatory effects with circulating levels elevated in obesity [295, 300]. Conversely ghrelin, a gut rather than adipose tissue derived hormone, reduced in the obese state, has been shown to counteract the effects of leptin on monocytes/macrophages [301].

The association between adipokines and asthma are discussed in detail in section 4.1.3 (iii), however these are summarised here. Murine models examining the effects of leptin showed its promise as a potential mediator for airway disease with infusion augmenting allergen induced BHR without eosinophil influx or Th2 responses [23]. Human studies have yet to show convincing evidence that leptin is behind the obesity asthma association (see section 4.1.3.3 and Table 4.4 for details of the studies). Only a single paediatric study has found higher leptin levels in overweight asthmatics

compared to normal weight asthmatics and controls, suggesting a role for leptin in the pathogenesis of obesity related asthma [302]. However, other paediatric studies which have shown an association have found this to be independent of BMI [303-305]. Similarly, whilst some adults studies find an association between leptin levels and asthma diagnosis, especially in pre-menopausal women [24, 306], this again has been independent of BMI. Furthermore one paediatric [307] and two adult studies [308, 309] found no association between leptin and asthma. Many of the studies have been population based and therefore relied upon self-reported or physician diagnosis with only a handful using more objective diagnostic criteria (Table 4.4). Overall the current evidence suggests that if leptin does play a role in asthma it appears to be independent of BMI.

With regards to adiponectin, low levels were associated with increased prevalence of symptoms of atopic dermatitis, asthma and eczema in one paediatric study [305]. In adults, as with leptin, this adipokine may play a role in the pathogenesis of disease in pre-menopausal women. A large cross sectional study has suggested that high levels may be protective against current asthma in pre-menopausal women, although this effect was independent of BMI [310] and a follow up longitudinal study suggested low levels were a better predictor of subsequent asthma development in the pre-menopausal women studied [311].

Studies on the remaining adipokines have been few in number. A single paediatric study found that resistin levels were significantly lower in the atopic asthmatics studied compared to non-atopic asthmatics and healthy controls [307], whilst one adult cohort study found the converse with levels elevated in asthma, correlating with disease severity, and independent of BMI [312]. Visfatin is thought to have pro-inflammatory effects yet interestingly, a solitary paediatric case-control study found significantly lower levels in the asthmatics studied compared to healthy controls [313]. Ghrelin is thought to have anti-inflammatory effects and an adult study suggests levels may be reduced during asthma exacerbations [306].

In summary the bulk of the work to date has examined the relationship between leptin, adiponectin and asthma. Results have been conflicting, but those studies showing an association have predominantly been those of pre-menopausal women, have been independent of BMI and have shown leptin and adiponectin to have polar associations. Given that obesity is associated with changes in levels of multiple adipokines, further research is needed to examine the potential role of the others with relationship to asthma.

2.7.5: Differences between sexes: hormones and body fat distribution

2.7.5 (i) Epidemiological data

The impact of sex on the obesity-asthma relationship is not clear. Some studies suggest a relationship solely in women whilst others have found the opposite. A paediatric study reported that girls who became overweight or obese by the age of 11 were more likely to develop new asthma-like symptoms and to have increased BHR [314]. This association was not seen in males and was most marked if menarche was before 11 years. Similarly, Gold et al found that a high baseline BMI or

significant increase in BMI in girls aged 6-14 was associated with a higher risk of developing asthma during follow up (median 5 years), with a less convincing relationship in boys [315]. Longitudinal studies in adults have also found that increased BMI at baseline is associated with a higher incidence of subsequent asthma but only in women [15, 316]. In a Canadian cross-sectional study, a single unit increase in BMI in women was associated with a 6% increase in asthma risk versus only 3% in men [317]. A British cohort study noted a stronger association again in women [217]. In a cluster analysis of a primary care and two secondary care cohorts, Halder et al identified a specific asthma phenotype, which was obese, female, and characterised by an absence of eosinophilic inflammation [8]. However, in a larger longitudinal Norwegian study, although increased BMI was associated with future asthma risk of asthma no differences between sexes was found [221].

2.7.5 (ii) Possible mechanisms for the underlying sex association

Hormonal influence

It is conceivable that any sex difference in the obesity-asthma association could be related to sex hormone levels. Observational data suggests that these hormones may impact on asthma. The pre-pubertal incidence of asthma is higher in boys, then through puberty the incidence increases in girls suggesting that female hormones have an important role [318]. After the menopause, the administration of oestrogen in the form of hormone replacement therapy, is also associated with increased asthma risk [54]. Other markers of hormonal abnormalities such as menstrual irregularity [319] and infertility [320] are also risk factors for asthma diagnosis and medication use, respectively.

Female sex hormone levels are altered with increasing adiposity. Circulating androgens are converted to oestrogen by aromatase, an enzyme located in adipose tissue which also expresses both oestrogen receptors ($ER\alpha$, $ER\beta$). Not only might obese women have higher levels of oestrogen but they may be exposed for longer periods of time as it is well established that menarche occurs earlier in obese individuals [321].

Mechanistically oestrogen has been shown to increase peripheral blood mononuclear cell production of Th2 cytokines (IL-4 and IL-13) important in asthma pathogenesis [322]. Progesterone also has a number of effects which may be relevant to asthma including up-regulation of β_2 receptors on lymphocytes [323], and administration of exogenous progesterone increases the bronchodilatory effects of isoprenaline in pigs [324]. Weight loss increases progesterone levels and β_2 receptor density in women [325] and clinically augments the bronchodilator effects of noradrenaline and terbutaline [326].

Differences in body fat composition

Most studies examining the obesity-asthma association use BMI as an adiposity measure, but this does not account for differences in muscle mass, particularly in men (see section 3.1). Using BMI as a surrogate marker may misclassify some men with higher muscle mass as obese. To address this hypothesis, McLachlan et al measured body fat percentage and BMI and found a correlation between adiposity and asthma in females but not in men [268]. There was a positive correlation

between percentage fat composition and BMI in both sexes however this association was markedly reduced in men. Mean body fat composition was significantly higher in women than men but mean BMI was not significantly different between the groups, suggesting that for a given BMI, body fat composition was higher in the women. The authors concluded that either the relationship between obesity and asthma was confined to women or that the association is not seen in men because they would require a much higher BMI to achieve the same body fat composition.

Another potential explanation may be differences in body fat distribution. The ability to mobilise substances from fatty tissue varies with location in the body. For example with leptin, secretion from subcutaneous fatty tissue is 2-3 fold greater in comparison to visceral tissue and levels correlate more closely with adiposity in females than males [327, 328].

Differences in fatty acid metabolism

Free fatty acids have important effects on innate immunity (see section 2.7.4). Post-prandial deposition of fatty acids differs between the sexes with females storing fat in the femoral gluteal region [329] and males in visceral fatty tissue [330]. Upon lipolysis, mobilised FFAs from visceral tissue pass through the portal venous system and into the liver stimulating TLR receptors on Kupfer cells (hepatic macrophages) resulting in the release of pro-inflammatory cytokines such as IL-6; this does not occur when FFA are mobilised from subcutaneous sites [331]. Such a mechanism would suggest that men would be more susceptible to obesity related inflammation, and therefore, this is unlikely to be responsible for the association seen in asthma.

2.7.6 Systemic inflammation

Another possible mechanism is that the systemic inflammatory state associated with obesity impacts on inflammation within the lungs. White adipose tissue functions as a source of energy storage and contains many different cell types, the most abundant being adipocytes. However, leukocytes are also present, particularly macrophages [332], as well as immunoregulatory cells including Tregs [333] and the relative abundance of these are altered with obesity, promoting a pro-inflammatory state. A number of inflammatory cytokines are therefore elevated in obese individuals and decline with weight loss, including IL-6, IL-8, TNF α and the acute phase marker C-reactive protein (CRP) [334, 335]. The production of immunomodulatory adipokines is also altered in the obese state in favour of a pro-inflammatory profile (see section 2.7.4 (iii)).

The reasons as to why nutrition impacts on immunity remains largely unresolved but may be in part due to the fact that some of the key regulators of metabolism are also involved in controlling the inflammatory response. This would seem logical as the inflammatory response requires large amounts of energy for many of its processes. Some of the hormones, including adipokines, over expressed in the obese state have direct effects on the immune system (see section 2.7.4). Other possible explanations for the low grade inflammation observed is that the obese state inadvertently triggering PRRs in the innate immune system as seen with FFA (see section 2.7.4). Therefore some of the effects of obesity on aspects of both arms of the immune system will be considered.

2.7.6 (i) Innate immunity and obesity

Monocytes and macrophages

In the obese state adipose tissue becomes infiltrated with pro-inflammatory M1 macrophages and the level of infiltration correlates with BMI; these macrophages are a source of circulating inflammatory cytokines including IL-6 and TNF α [332] (see section 6.1.1 for more details). Conversely, lean individuals express M2 macrophages within this tissue which express lower levels of pro-inflammatory cytokines [336]. The source of the M1 macrophages is not certain but adipose tissue necrosis may be a driving factor, or these cells may be recruited from the higher numbers of circulating pro-inflammatory CD14⁺CD16⁺ monocytes [31]. Systemic activation of the monocyte/macrophage compartment could have relevance in asthma (see sections 2.3.1 (ii) and 6.1.1 for details).

Neutrophils

The role of neutrophils in obesity related inflammation is not well understood, however studies suggest that obesity is associated with changes in numbers and activation of circulating neutrophils which could be of relevance in asthma (see section 4.1.2 for details). Higher circulating numbers have been documented in the morbidly obese, with a marked reduction following weight loss from bariatric surgery [30]. Obesity may also increase activation of these cells with differences in the surface expression of adhesion molecules (CD62L and CD11b) in those undergoing bariatric surgery compared to normal controls [31]. Other markers of neutrophil activation are elevated in the morbidly obese including calprotectin, a cytoplasmic bacteriostatic protein, and myeloperoxidase, one of the enzymes responsible for ROS generation [337] (see section 5.1.2 for more details). Neutrophils are associated with severe asthma (see section 2.3.1 (ii) and chapter 5) and therefore systemic activation of these cells in obesity may be important.

Pattern recognition receptor signalling

The obese state can lead to activation of the innate immune system through effects on PRRs and this may be of relevance in the pathogenesis of asthma (see section 6.1.2 (ii) for details). TLR4, the most studied of the PRRs, is activated by the PAMP, LPS which is a constituent of the cell wall in Gram negative bacteria. Elevated levels of LPS are found in the blood of overweight and obese individuals [338] and even after a single high fat meal, concentrations of LPS can increase sufficiently to activate innate immune cells [339]. Whilst each PRR is activated by a distinct PAMP, many PRRs including some of the TLRs [279] and NLRs can be activated by saturated FFAs [340] which are elevated in obesity. Recent studies suggest that the cytokine response in whole blood leukocytes upon stimulation with LPS may also be amplified in the obese [32]. Similarly atopic asthmatics have enhanced cytokine response on LPS stimulation of blood mononuclear cells (MNCs) [341] and therefore should obesity augment this response further this could promote a pro-inflammatory milieu in these individuals.

Oxidative stress

Obese individuals have increased levels of biomarkers of oxidative stress compared to their lean counterparts [342] and this is likely to be secondary to multiple factors (see section 5.1.3 and Table 5.3). Oxidative stress results in tissue and organ damage and is thought to be important in the pathogenesis of several obesity related disorders including fatty liver disease and atherosclerosis [343]. It is therefore possible that this enhanced oxidative stress could also cause end organ disease in the lungs, promoting airway inflammation and asthma.

2.7.6 (ii) Dendritic cells and obesity

Dendritic cells, as professional antigen presenting cells, are able to direct the immune response to an antigen or allergen (section 2.2.2) and therefore play an important role in asthma (sections 2.3.2 and 6.1.3). Dendritic cells express leptin receptor, and incubation with leptin promotes their survival and expression of cytokines promoting a Th1 response, as well as downregulating immunoregulatory cytokines such as IL-10 [295]. Little work has been done on this area in humans, but a single study suggests that obesity in post menopausal women is associated with an increase in blood mDCs compared to normal weight individuals [33]. If obesity does modify DC survival and function, encouraging mDC development with a cytokine profile promoting a pro-inflammatory Th1 response, then this could be of relevance in asthma.

2.7.6 (iii) Adaptive immunity and obesity

Regulatory T cells

Obesity has been linked with autoimmune conditions characterised by impaired Treg cell responses [22, 344]. Neutralisation of leptin results in increased Treg proliferation and FoxP3 production within Tregs in murine models [345]. Leptin and leptin receptor deficiency produce similar findings [345, 346]. Whilst it is acknowledged that obesity is characterised by leptin resistance, murine models have shown that whilst there is leptin resistance related to eating behaviour and sympathetic activity in fat, there is no leptin resistance related to renal sympathetic activity suggesting the possibility that it can be selective [347]. In addition to leptin, IL-6 which is increased in obesity, also down regulates Tregs differentiation [348] (see section 7.1.2). Normal adipose tissue can be a site of Treg cell accumulation [333]. In humans, obesity is associated with a depletion in Treg numbers within visceral adipose tissue with a corresponding increase in pro-inflammatory macrophages and a skew towards a Th1 bias [34, 333]. It has been suggested that high levels of leptin associated with obesity might mediate these effects [349]. Little work has been done on whether this depletion in Treg numbers within adipose tissue extends systemically to the blood and other body organs.

Changes in Treg populations are important in the context of other obesity related pathologies including non-alcoholic steatohepatitis [350]. Tregs in asthma may be down regulated in number or

function systemically in the blood or locally in the airways [194] although studies have been contradictory (See Table 7.1 for full description of studies in this area). This may be due in part to a high percentage of the studies using non-specific Treg markers including CD25 rather than the more specific marker FoxP3 (see section 7.1.2 (iv)) However, if down-regulation of Tregs is important in asthma pathogenesis and obesity is associated with depletion in Treg numbers systemically, then this could provide a mechanism for the association between obesity and asthma.

Changes in other T helper populations

Obesity may be associated with changes in other CD4⁺ T cell populations of relevance to asthma. In murine models, leptin promotes Th1 responses of peripheral blood lymphocytes to allogeneic mononuclear cells and suppresses Th2 responses [288]. In murine models, obesity is associated with a Th17 bias [351] and, in keeping with this, obese females have higher levels of IL-17 than normal weight individuals [352] (see section 7.1.2 (v)). Given that Th17 cells in murine models promote steroid resistant neutrophilic airway inflammation [353] and that serum IL-17 levels in human asthmatics correlate with disease severity [354] (see section 7.1.2 (iv)), an obesity-related Th17 bias might promote a neutrophilic steroid-resistant asthma phenotype.

2.8 Immunity, obesity and asthma: current evidence

2.8.1 Systemic immunity

Although asthma is an inflammatory disease of the airways it is associated with systemic changes in the immune system (see section 4.1.2). There is evidence to suggest that low grade systemic inflammation has a detrimental effect on lung function with high sensitivity CRP (hsCRP), an acute phase reactant produced by hepatocytes upon stimulation by IL-6, correlating with worse lung function in young adults [355, 356]. Only a small number of studies in humans have examined systemic inflammation in obese asthmatics and these have focussed on traditional markers of inflammation, measuring CRP, although some have looked at circulating cytokine levels.

Van Veen et al studied 136 adults with persistent symptoms despite high dose ICS or oral steroids, grouped according to BMI, into obese and non-obese [28]. All had demonstrated reversible airways disease or BHR. They noted hsCRP was increased significantly in the obese patients however there was no control group and therefore it couldn't be determined whether this was more marked in asthmatics or simply an obesity related phenomena. Blood eosinophils, the only other systemic marker of inflammation measured, did not differ between groups. Similarly a study of 6000 adults found that CRP correlated positively with BMI but was not associated with a self reported asthma diagnosis after controlling for confounders such as smoking history [357]. This is in contrast to another large cross-sectional study of 1289 individuals which found that hsCRP was elevated in non-atopic physician diagnosed asthma but not atopic asthma. Interestingly, increasing BMI positively correlated with hsCRP, and BMI was associated with both atopic and non-atopic asthma. However, after adjusting for hsCRP the association between obesity and atopic asthma remained stable, whilst the association between obesity and non-atopic asthma was no longer significant suggesting that

the pathophysiology of obesity related asthma may differ between atopic and non-atopic individuals and that systemic inflammation, as measured by hsCRP, may be important in obesity related asthma in non-atopic individuals [358].

Sutherland et al investigated systemic inflammation in 40 premenopausal female asthmatics (20 obese, 20 non-obese) vs. 40 premenopausal controls (20 obese, 20 non-obese) [25]. Serum/plasma biomarkers measured included IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, TNF α , IFN γ , MCP-1, hsCRP and leptin. Whilst asthma and obesity were both associated with increased systemic inflammatory markers these were largely independent of one another. They found significantly higher circulating levels of IL-4, IL-6, CRP and leptin in the obese subjects. CRP and leptin were higher in the asthmatics vs. non-asthmatics but were independent of BMI. None of the markers were higher in the obese asthmatics than the other groups. This led the authors to conclude either systemic inflammation was not responsible for the association or that this involved other areas of immunity such as innate immune system activation which had not been fully explored in their study. This paper was very strict in its inclusion criteria and diagnosis of asthmatics and controlled well for potential confounders. However it still had limitations; they tried to withdraw ICS from all participants prior to sampling but 28/33 participants lost control of their asthma during this process and were sampled at this point. Therefore this was not a study of asthmatics in a stable state and asthma exacerbations may have masked any differences in phenotype.

More recently a study of 120 children examined 60 asthmatics (30 obese and 30 non obese) and 60 controls (30 obese and 30 non-obese) [359] in whom asthma diagnosis was made by a primary care physician. The authors found significantly higher IL-4 and IL-13 in the blood of the non-obese vs. obese asthmatics consistent with a greater Th2 skewed response in the non-obese asthmatics. Obese asthmatics had higher levels of cytokines associated with neutrophilic inflammation including TNF α and IL-6 compared to normal weight asthmatics, although these levels did not significantly differ between obese asthmatics and obese controls. Using flow cytometric analysis of intracellular IFN γ and IL-4, Th1 and Th2 cell responses were measured after phorbol myristate 13- acetate (PMA) and tetanus toxoid stimulation. The authors observed that the Th1 response in the obese asthmatics was significantly higher than non-obese asthmatics but did not differ from obese controls. This was accompanied by a significantly higher Th2 response in the non-obese asthmatics. The IFN- γ /IL-4 ratio correlated positively with serum leptin levels. The authors concluded that obese asthma was associated with Th1 rather than Th2 skewing and that leptin may be important in driving this.

Studies thus far have been relatively few in number but have shown evidence of changes in systemic immunity with obesity and in some cases in asthma but these have been largely independent of one another.

2.8.2 Systemic oxidative stress

A study by Sood and colleagues examined markers of systemic oxidative stress in 2,865 individuals taking part in the Coronary Artery Risk Development in Young Adults (CARDIA) study [360]; 8.1% of participants had a physician diagnosis of asthma. Increased plasma F2-isoprostanes was associated with BMI in women but not in men. Asthma diagnosis was also associated with markers of higher oxidative stress but this did not persist after adjusting for sex and BMI differences. The authors

concluded that systemic oxidative stress as measured by F2-Isoprostanes may not explain the obesity-asthma association, although the work had weaknesses: there was no baseline information about asthma control or severity and a reported “physician diagnosis of asthma” rather than a more objective measure was used. A further study of plasma 8 isoprostane in 67 non-smoking asthmatics and 33 controls found that whilst levels increased with asthma they did not change significantly across BMI categories [361]. Therefore there has been no convincing evidence published that obesity in asthma is associated with a marked increase in systemic oxidative stress compared to non-asthmatics.

2.8.3 Airway inflammation

Work has also been undertaken focussing on whether obesity impacts on inflammation locally within the airways and studies have assessed this in a number of ways. These include measuring sputum cell counts and cytokines within sputum supernatants, surrogate markers of eosinophilic inflammation including FeNO, and exhaled biomarkers of oxidative stress.

2.8.3 (i) Sputum analysis

Current evidence suggests that obese asthma unlike atopic asthma is not associated with eosinophilic inflammation and increased neutrophilic inflammation has been suggested. A study of 136 patients with persistent asthma showed that obesity was associated with a significantly lower sputum eosinophil count [28]. In a cluster analysis of a single asthma primary care cohort and two secondary care cohorts, the obese female asthma phenotype was characterised by an absence of eosinophilic airway inflammation [8]. A case control study examining 80 obese and non-obese women found no difference in the percentage of sputum eosinophils between the obese and non-obese asthmatics studied [25]. However, the obese patients had the highest mean percentage of sputum neutrophils, although this did not reach statistical significance. Sputum supernatants were also examined and it was noted that IL-1 β , IL-6, IL-6 and IL-8 levels were significantly higher in asthmatics. Although IL-5 and IL-6 were highest in the obese asthmatics these were not significantly higher than the non-obese asthmatics. A retrospective study examined 727 adult sputum samples collected for the assessment of chronic cough, COPD and asthma [362]. Of these, 163 individuals had asthma and each BMI category was associated with an increasing sputum neutrophil count however this was not statistically significant.

2.8.3 (ii) Exhaled nitric oxide

Other indirect measures of eosinophilic inflammation have added further support to the suggestion that obesity in asthma is not associated with eosinophilic but rather neutrophilic airway inflammation. Exhaled nitric oxide (FeNO) is being used increasingly to measure eosinophilic airway inflammation in asthma with levels raised in atopic disease [363]. Large population studies of adults have not shown a relationship between BMI [364] or body fat[365] and FeNO overall. However

smaller studies have suggested that obesity may be associated with declining FeNO in well defined asthma [27, 28].

Study	Design	Participants	Number	Outcome
de Winter-de-grot et al 2005 [366]	Cohort	Healthy adults	n=24 Males = 10 Females = 14	FeNO correlated positively with BMI.
Kim et al 2011 [367]	Cohort	Healthy adults	n = 117	No correlation between BMI and FeNO.
McLachlan et al 2007 [365]	Cohort study	Birth cohort study 1037 individuals, Dunedin 1972-73	n =925 Males = 487 Females = 438	No correlation between body fat composition and FeNO in men or women. FeNO higher in asthmatics. FeNO lower in cigarette users.
Olin et al, 2006[364]	Cross-sectional study	Random adult population sample	n=2200 Males = 1,098 Females = 1,111	No association between BMI and FeNO. Height, age, atopy, asthma symptoms, ICS use positively correlated with FeNO.
Berg et al 2011 [29]	Cohort study	Randomly selected Swedish cohort.	n=2,187 Males = 1,074 Females =1,133	In obese: FeNO lower in those with history of wheeze. In non-obese: FeNO higher in those with history of wheeze or atopy. In wheezing population negative correlation between BMI, WHR, percentage body fat and FeNO.
Komakula et al 2007[27]	Case control study	Moderate to severe asthma Controls: hospital workers	n=114 Cases= 67 Controls = 47	BMI, leptin/adiponectin ratio negatively associated with FeNO in asthmatics. No association in control group.
Leung et al 2004 [368]	Case control	Asthmatic children 7-18 years. Healthy controls	N=115 Cases = 92 Controls = 23	Higher FeNO in asthmatics than controls. No correlation between obesity and FeNO.
Van Veen et al 2008 [28]	Cohort study	Asthma patients with persistent symptoms despite high dose ICS/oral steroids	136 adults Female = 96 Male 40	BMI negatively correlated with FeNO (r=-.30 p<0.01).

Table 2.6: Summary of the main studies examining FeNO and BMI in asthma.

The observation that FeNO is lower in obese individuals with asthma is in keeping with the hypothesis that this is a non-eosinophilic disease. It has led some to argue that obesity does not mediate its effects in asthma through airway inflammation but other diseases such as cystic fibrosis, associated with profound airway inflammation, are also associated with low levels of FeNO [369]. It has been hypothesised that in environments of high oxidative stress the nitric oxide might be oxidised before leaving the airways and therefore low FeNO may still reflect airway inflammation and high oxidative stress, albeit non-eosinophilic [370].

2.8.3 (iii) Airway oxidative stress

Studies examining oxidative stress locally within the airways have also proven inconclusive. Komakula et al found that exhaled F₂-isoprostane increased with BMI within their asthmatic group and negatively correlated with FeNO. This is in keeping with the concept that obesity in asthma is associated with enhanced oxidative stress locally in the airways and this may oxidise the nitric oxide accounting for the reduction in levels often reported [27]. However obese asthmatics did not have higher levels of F₂ isoprostane than obese non-asthmatics. Similarly Holguin and colleagues found a correlation between exhaled F₂ isoprostane and BMI in their 67 asthmatics studied but again asthmatics did not have significantly higher levels than control subjects [361].

Chapter 3

Materials and methods

3.1 Study design

This was an observational case control study designed to test whether the obesity-asthma relationship could be explained by systemic changes in metabolic parameters, innate immune function, dendritic cells or adaptive immunity. The study population consisted of Caucasian asthmatic and non-asthmatic pre-menopausal women (age 18-50 years) of varying body mass index.

Only women were chosen as the relationship between obesity and asthma is more consistent in this group. Furthermore, this would control for any differences in body fat distribution and hormonal impact (see section 2.7.5). Pre-menopausal women were studied since asthma incidence is higher in this group [54] and the menopause affects the levels of circulating pro and anti-inflammatory cytokines [371]. Subjects and controls were divided into 3 groups on the basis of body mass index (BMI), resulting in 6 groups in total (Figure 3.1).

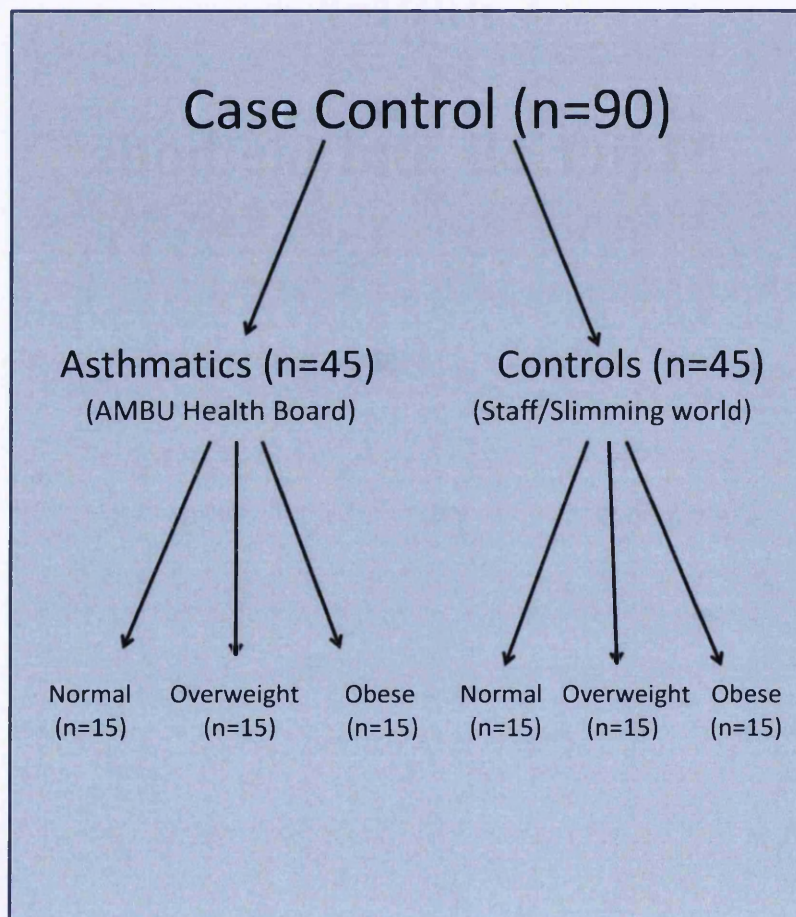


Figure 3.1: Outline of study design.

3.1.1 Measures of obesity

BMI was chosen as a marker of adiposity due to its simplicity of use and adoption on an international scale as well as in many clinical guidelines[212].

According to the World Health Organisation (WHO), normal weight is defined as BMI 18.5-25kg/m², overweight as a BMI \geq 25kg/m² and $<$ 30kg/m² and obesity as a BMI \geq 30kg/m² [212]. Underweight patients were excluded as this group also has been reported to have an increased risk of asthma symptoms, BHR and asthma diagnosis compared to normal weight individuals and therefore may represent a different phenotype to normal weight individuals in the context of asthma [372, 373]. However, whilst participants were classified by BMI, this is only a surrogate marker of adiposity, making the assumption that excessive weight for given height is due to increased fat composition and thus failing to distinguish between lean mass and body fat. Therefore other measures were also taken, including body fat composition measured by biometric impedance, waist circumference (WC) and waist-hip ratio (WHR).

3.1.1 (i) Body fat composition

In the context of body fat composition, the WHO defines obesity as a body fat percentage of $>$ 25% in men and $>$ 35% in women [374]. Body fat composition can be estimated using two different models. The simpler two-compartment model partitions the body into fat mass and fat free mass, the latter consisting of water, protein and minerals [375]. The model assumes that the proportions of water, protein and mineral remains constant, therefore in certain states such as pregnancy, periods of growth, dehydration or sudden weight reduction, it may be inaccurate. The four component method is more precise and involves the measurement of total body mass, body volume, body water and bone mineral density. However the technique is cumbersome and expensive and therefore is not routinely used in population based studies [376].

A number of techniques based on the two compartment model have been developed. Biometric impedance measures resistance to a small electrical current passed through the body. It estimates total body water from which fat free mass is calculated based on assumption that 73% of this is made up of water. Initially the method involved using electrodes attached to the arms and legs, but this has since been replaced by leg to leg impedance measurements, which have similar performance characteristics especially when age, gender and WHR is taken into account [377]. This technique is limited by its inability to differentiate between intracellular and extracellular fluid compartments and therefore values derived are affected by hydration status. Dilution techniques involve estimating total body water and therefore fat free mass by administering radio labelled hydrogen or oxygen [378]. However these measures have the similar limitations. Other methods used include underwater weighing and air displacement plethysmography which measure body volume and fat mass by measuring the amount of water or air displaced [379], but are cumbersome to perform. Dual energy x-ray absorptiometry (DEXA) provides estimates of bone mineral density, fat free mass and fat mass. The technique requires radiation exposure and its estimates of fat mass decrease in accuracy with increasing trunk thickness [380]. Biometric impedance measurements

were chosen for this study as they correlate well with measurements by DEXA scanning in obese hydrated subjects, whilst avoiding the whole body irradiation associated with the latter technique [381].

3.1.1 (ii) Body fat distribution

Central to the definition of obesity is the concept of excessive fat deposition in adipose tissue. Adipose tissue is distributed anatomically in two major areas: subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT). Visceral adiposity is characterised by increased tissue around intra-abdominal organs and this distinct pattern of fat deposition has been linked with specific pathologies including insulin resistance and diabetes [382]. Anthropometric techniques can provide an estimate of visceral and subcutaneous fat deposition which cannot be derived from BMI. Waist circumference reflects visceral fat whilst hip circumference reflects subcutaneous fat. A high WHR correlates with an increased in visceral adipose tissue to subcutaneous adipose tissue ratio on CT imaging [383]. A ratio <0.85 in women and <0.9 in men is defined as normal and in the elderly WHR predicts mortality more accurately than BMI or waist circumference [384]. However whilst anthropometric measures are cheap and non-invasive they are observer dependent and difficult to reproduce [385]. More accurate methods for estimating visceral adiposity have been developed. DEXA can be used to estimate visceral adipose tissue deposition but its accuracy decreases with increasing obesity due to difficult to accurately discern the waste circumference on the trunk [380].

3.1.1 (iii) BMI vs. other measures of adiposity

Studies have shown that BMI does not always correlate well with other measures of adiposity in certain populations, especially men or the elderly. A population based study of 13,601 adults in the United States measured the performance BMI in the diagnosis of obesity, as defined by excess body fat ($>25\%$ in men and 35% in women). They found that whilst BMI estimated 21% of men and 31% of women to be obese, body fat percentage measured using biometric impedance showed this figure to be significantly higher (50 and 62% respectively) [213]. In this study BMI was very specific but insensitive at diagnosing obesity by body fat composition in those with $BMI > 30 \text{ kg/m}^2$. In those with BMI of $25\text{-}30 \text{ kg/m}^2$ (overweight category), BMI was more sensitive but not specific due to its inability to discriminate between body fat and lean mass, especially in male and those over 60 years of age. However of relevance to this current work, BMI correlated very well with body fat percentage in women and a cut off of $\geq 25 \text{ kg/m}^2$ in the overweight range was sensitive and specific in diagnosing obesity as defined by body fat composition. A large meta-analysis of 25 studies comparing BMI vs. body fat percentage using a variety of different methods encompassing 31,968 patients reported similar findings [386]. Based on the relatively good performance of BMI in women, patients were categorised using this variable to enable easy comparison with previously published works but body fat composition was also measured along with WHR as a marker of visceral adiposity.

3.2 Ethical approval

Ethical approval was sought from the South West Wales Research Ethics Committee (REC reference number 10/WMW02/4) and written informed consent obtained from all volunteers and patients (Appendix I). A written information sheet, approved by the local research ethics committee, was given to all interested patients (Appendix II) and controls (Appendix III).

3.3 Identification of potential participants

Asthmatic patients were identified from a number of local healthcare providers:

- Local tertiary asthma clinic (Singleton Hospital, Abertawe Bro Morgannwg University Health Board (ABMU HB)).
- Surrounding secondary care clinics (ABMU HB; Neath Port Talbot, Morriston and Bridgend hospitals).
- Participating GP surgeries (Ty'r Felin surgery, Gorseinon).

Patients recruited from hospitals were initially identified by clinic letter and case note review against the study inclusion/exclusion criteria (Table 3.1). Primary care patients were identified by review of the practice data-base. Telephone contact was made with those asthmatics fulfilling the criteria and they were invited to a clinic appointment in the specialist asthma service.

Control patients were recruited from a number of sources:

- Staff, ABMU HB
- Staff, Swansea University
- Women attending local Slimming World groups

Hospital and university staff were identified following response to an electronic advert, prepared with the aid of "Involving People" and approved by the local ethics committee. Involving people is part of the National Institute for Social Care and Health Clinical Research centre, and encourages public involvement in research. Through this organisation, lay people were involved in the design of the advert and information leaflets. Slimming World agreed to the lead researcher attending local classes and providing a 3 minute presentation on the study and distributing posters. Those considering taking part completed a standardised questionnaire recording basic demographics, co-morbidities, medication history, smoking history, presence of atopy, Epworth score and a validated Modified Bronchial Symptoms Questionnaire (Appendix IV) [387].

3.4 Study participants: recruitment

All asthmatic subjects fulfilling the inclusion criteria attended an appointment at a tertiary asthma service. Asthma diagnosis was confirmed by a respiratory physician. This required consistent symptoms and demonstrable significant reversible airways obstruction to a beta 2 agonist (12% reversibility in FEV1), or significant PEFV variability or a positive methacholine challenge test (Table 3.1). A challenge test was said to show significant BHR if a dose of <8µmol caused a >20% reduction in FEV1.

Inclusion criteria	Exclusion criteria
Asthma: <ol style="list-style-type: none"> 1. Obstructive spirometry with 12% reversibility to a beta agonist 2. Peak flow with 20% diurnal variation 3. Positive methacholine challenge test 	Amenorrhoea: <ol style="list-style-type: none"> 1. Postmenopausal 2. Hysterectomy 3. Depot contraceptive 4. Mirena coil
Female	Diabetes
Pre-menopausal	Current smokers or >10 pack year history
Obese: BMI >30Kg/m ²	Systemic inflammatory disorder
Overweight: BMI 25-30Kg/m ²	
Normal weight: BMI <25 Kg/m ²	
	Other cardio-pulmonary disease
	Immunosuppression/systemic steroids

Table 3.1: Summary of the inclusion and exclusion criteria.

A number of potential confounding factors were exclusion criteria (Table 3.1). Since samples from patients were timed by the menstrual cycle (see section 3.5), any condition which rendered participants amenorrhoeic dictated exclusion. The diagnosis of diabetes was based on measurement of fasting glucose. Diagnosis of systemic inflammatory disorder or other cardiopulmonary diseases was based on interview and review of case notes in the asthmatic patients studied. In addition to these criteria, control subjects were not included if they had any respiratory symptoms determined by completion of a Modified Bronchial Symptom Questionnaire or history of clinical atopy [387]. All

patients were either non-smokers or ex-smokers with <10 pack year history, having ceased more than 6 months previously. Patients who were on maintenance oral steroids or steroid sparing agents were excluded.

All asthmatic patients were assessed in clinic and their asthma control established. In those with very good control, therapy was stepped down to the lowest level to maintain this, in keeping with British Thoracic Society (BTS) guidelines.

3.5 Baseline measurements and sample collection

All asthmatics were considered stable if they had no exacerbations, oral corticosteroid therapy, or respiratory tract infection in the preceding 6 weeks. All participants were asked to attend during the first 7 days of their menstrual cycle, determined using the date of onset of menstruation between the hours of 07.00-09.00. This was to control for the effects of female sex hormones which can influence both asthma control (see section 2.7.5) and some of the parameters of interest including circulating neutrophils [388], lymphocyte subsets [389] and regulatory T cells [390].

3.5.1 Clinical phenotype

Asthmatics were asked to complete a modified European Respiratory Health Survey [391] (Appendix V) and the following information recorded:

- Age of onset
- Markers of symptom control in last 12 months
- Medication usage
- Emergency contact with healthcare providers (GP/clinic attendances, hospital and ITU admissions) in the preceding 12 months
- Days off work
- Co-morbidities
- Epworth score
- Details of menstrual cycle

Acute disease control at the time of recruitment was assessed using the Juniper Asthma Control Questionnaire (Appendix VI) [392]. Disease severity was graded according to the Global Initiative for Asthma (GINA) criteria [393] (Appendix VII) and also quantified by recording symptom severity, medication usage and degree of emergency contact with services in the previous 12 months.

3.5.2 Spirometry

Spirometry was performed on all participants using a portable dry spirometer (Vitalograph) calibrated on the day of use. All asthmatics were asked to withhold medication for the preceding 24 hours to control for any effects of long acting bronchodilators or immediate effects of inhaled corticosteroids (ICS). The best of 3 measurements was taken according to a standardised protocol [394] and expressed as percentage of the age, gender and stature predicted values. The following spirometric measurements were recorded: Forced Expiratory Volume in 1 second (FEV1), Forced Vital Capacity (FVC), FEV1/FVC ratio, Forced Expiratory Flow at 25%-75% of expired vital capacity (FEF25-75) and Peak Expiratory Flow (PEF).

3.5.3 Adiposity measures

On the morning of attendance the following measures were taken with the subject wearing light clothing without footwear:

- Height; to 0.1cm by stadiometer (Leicester; Chasmors, UK)
- Weight; to 0.1kg and body mass index calculated
- Body fat percentage to 0.1% (Body composition analyser, Tanita SC 240 MA; Tanita UK)
- Waist and hip measurements to 0.1cm and ratio calculated

Waist and hip measurements were taken with the subject relaxed with their hands by their sides and feet relaxed at the end of tidal expiration. Waist measurements were taken at the midpoint between the last palpable rib and the iliac crests, and the hip measurement around the widest portion at the level of the buttocks [395]. This provides an estimate of visceral adiposity and has been shown to correlate with Computerised Tomography indices [383], whilst being less invasive, and not subjecting the volunteers to ionising radiation. A limit of anthropometric measurements is the difficulty in reproducibility [385], to address this issue, measurements were performed by the same study investigator and repeated until the difference between measurements was <1cm [396].

Body fat composition was estimated using biometric impedance using a leg to leg medically approved light weight portable analyser. A limitation of biometric impedance analysis is that it measures total body water to calculate fat and fails to take into account hydration status (see section 3.1). To control for this issue all participants were asked to remain starved from midnight, including water consumption and prior to attendance at the research clinic between 07.00-09.00 am.

Fasting blood was then collected using lithium heparinised tubes and gel and clot activator tubes (Vacuette®, Greiner Bio-One) for the following investigation:

- Full blood count analysis
- Flow cytometry to quantify major lymphocyte subtypes, markers of CD4⁺ maturation, CD4⁺ cells including Tregs, dendritic cell subsets, and surface markers of neutrophil and monocyte activation.
- Luminol enhanced chemiluminescence to measure reactive oxygen species (ROS) generation of participants' blood in response to Phorbol Myristate Acetate (PMA) stimulation.
- Whole blood cultures stimulated with lipopolysaccharide (LPS) +/- interferon gamma (IFN γ) or phytohaemagglutinin-L (PHA) for cytokine analysis of supernatants, using enzyme linked immunosorbant assay (ELISA).
- Mononuclear cell (MNC) isolation for cryopreservation.
- Plasma and serum archived for subsequent analysis of circulating adipokines, cytokines, insulin, glucose, free fatty acids, total and specific IgE total antioxidant status and thiobarbituric acid reactive substances (TBARS).

3.6 Full blood count analysis

Full blood count was measured using a CELL-DYN Ruby analyser (Abbott Diagnostics Germany). This provided haemoglobin (Hb), red blood count (RBC), mean corpuscular volume (MCV), white cell count (WBC) and differential and platelet count (PLT). A CELL-DYN Ruby is a multi-parameter automated haematology analyser designed for *in vitro* diagnostic use in clinical laboratories and utilises the technique of flow cytometry (see section 3.7). It contains a vertically polarised 10mW helium-neon laser. The sample is hydrodynamically focussed and passes through the laser beam generating forward and side scatter. Two angles of forward scatter are measured (0° and 10°) whilst the orthogonal scatter is passed through a beam splitter into two portions. One portion of the light is directed straight to a 90° photomultiplier tubes (PMT) whilst the other portion is directed through a horizontal polariser (depolarised) to a 90°D PMT. The PMTs convert the light into an electronic current. All 4 scatter signals are measured in a process termed as MAPSS™ (Multi-Angle Polarised Scatter Separation). In terms of leukocyte count the 0° is used to provide a total WBC, whilst a combination of the 4 scatter signals is used to provide a WBC differential; expressing the percentage of each subpopulation (lymphocytes, neutrophils, eosinophils, basophils and monocytes (Figure 3.2). The absolute numbers of leukocyte subpopulations (expressed as *10⁹/l) are determined by multiplying the total leukocyte count by the percentage of each population.

Before each patient sample was analysed a set of three quality controls containing samples with known quantities of each blood parameter (low, normal and high) were run to ensure that the machine was calibrated and functioning correctly. The sample was then agitated gently at room temperature to ensure mixture and then run through the analyser. All samples were run manually.

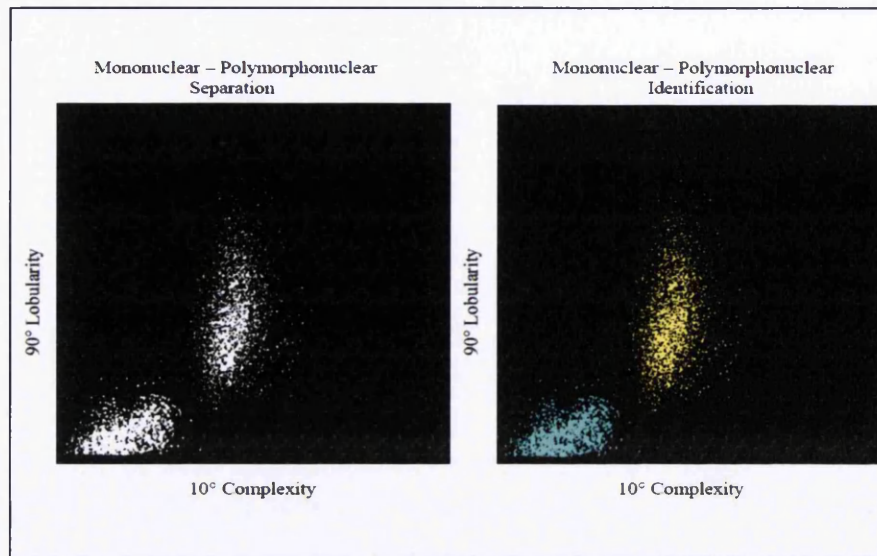
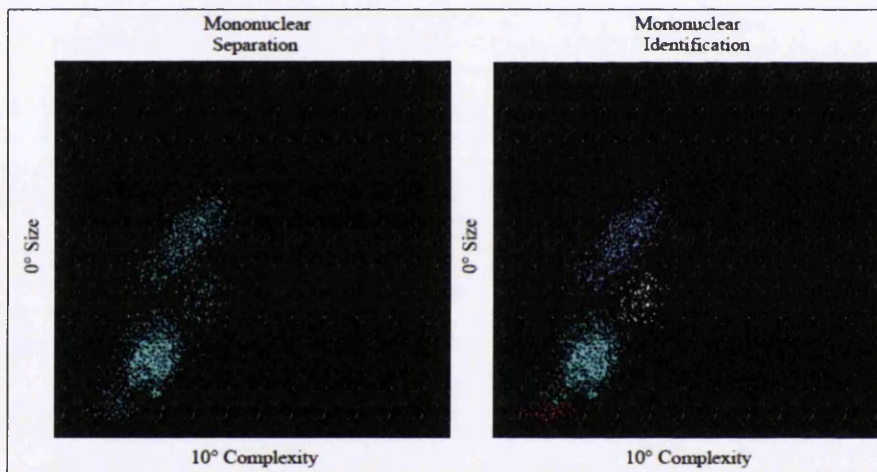
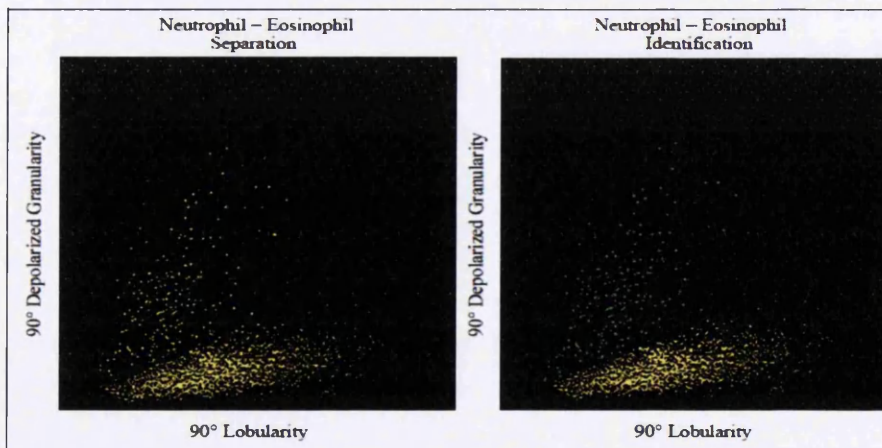
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Figure 3.2: Generation of leukocyte differential using an automated haematology analyser.

A: Leukocytes are initially divided into mononuclear cells (blue) and granulocytes (yellow) using 10° FSC (cell complexity) and 90° SSC (lobularity).

B: By gating on the mononuclear cell population, the respective constituents are displayed using 0° and 10° FSC into basophils (purple), monocytes (pink) and lymphocytes (blue). The red population below the lymphocytes contain cell fragments

C: The subpopulations of granulocytes, eosinophils (green) and neutrophils (yellow), are shown by plotting 90° (lobularity) and 90° D (granularity).

3.7 Whole blood flow cytometry

3.7.1 Principles of flow cytometry

Although the basic principles of flow cytometry can be traced back to the 1900s, the 1970s saw the advent of commercially available cytometers and the beginning of their widespread use in the laboratory setting. With the outbreak of the acquired immunodeficiency disease (AIDS) pandemic in the 1980's the use of flow cytometers became routine in pathology laboratories [397].

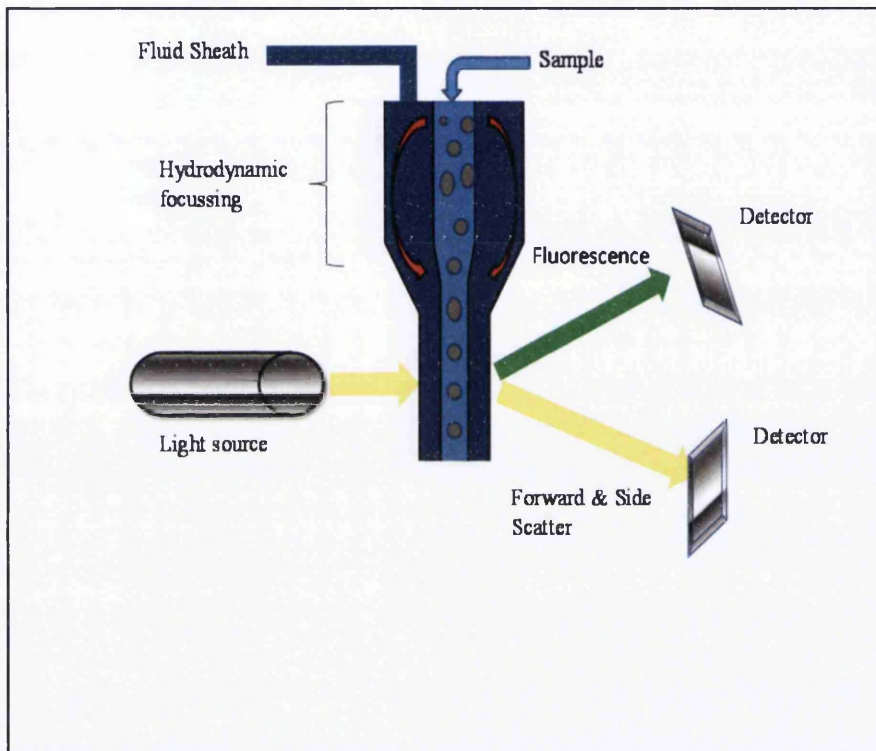


Figure 3.3: Schematic diagram of flow cytometer. Cells are brought into single file by hydrodynamic focussing and passed through a laser. Light is scattered according to size and complexity onto detectors.

Flow cytometry enables the analysis of multiple characteristics of single cells or any particle at a high rate (thousands of events per second). Fundamental to this process is the ability of the fluidic component of the analyser to focus samples into a single stream of cells (hydrodynamic focussing) which pass through one or more high energy light sources, each of a single wave length. The light is then scattered in a forward direction according to the size of the cells, and in a sideways direction according to cell complexity. Additionally fluorochromes/fluorescent dyes either alone or when conjugated to antibodies can bind to specific proteins on the cell surface or within the cell. When these fluorochromes pass through a light source of a certain wavelength (excitation wavelength), light of a different wavelength is emitted (fluorescence emission wavelength) which can be quantified by detectors. Multiple fluorochromes with similar excitation wavelengths but differing

emission wavelengths can be used to measure multiple properties of a cell simultaneously [398]. This enables multiple research applications including the identification of specific cell types by staining against surface and intracellular markers, as well as measuring cytokine and other functional read outs[399]. The technique is also used in the clinical setting, providing CD4⁺ T cell counts for patients with Human Immunodeficiency Virus (HIV) and accurate phenotyping of lymphoproliferative diseases [398]. The basic flow cytometer consists of fluidics, optics (including light source), electronics (detectors) and a computer (Figure 3.3).

3.7.1 (i) Fluidics and hydrodynamic focussing

The principles of hydrodynamic focussing can be traced back to Bernoulli's experiments looking at the inverse relationship between pressure and velocity of fluid flowing through a constricted tube [400]. He noted that as fluid flowed through a narrowed section of tubing the fluid velocity increased at that region with a corresponding reduction in hydrostatic pressure. A colleague and friend, Euler, noted that the velocity of flow in a stream follows a parabolic distribution with the fastest flow in the centre of the stream relative to the periphery [401]. Based on Bernoulli's work it can be appreciated that the pressure within the more rapidly moving centre of the stream must be lower and therefore particles introduced into a stream will slowly move towards the centre following the pressure gradient. Following on from this, Reynolds showed that laminar flow of fluid is dependent on a number of variables including the velocity and density of the fluid and diameter of the tube [402]. As a tube suddenly narrows an area of turbulence will occur at the interface around the outside of the stream and particles flowing through the centre of the stream will be unaffected.

Using the principles outlined, Crossland-Taylor developed the first hydrodynamic focussing chamber [403]. Sheath fluid enters the outer jacket of the chamber and a faster moving central jet carrying the sample passes through the centre. The pressure gradient between the two causes the particles of interest to move to the centre of the jet. By balancing the pressures and flow of the central jet and outer sheath of fluid and forcing the stream through a narrow nozzle, it is possible to focus the jet to a submicron width and bring cells or other particles of interest into single file.

3.7.1 (ii) Optics

The optical system consists of a light source and components needed to direct and focus the source onto the fine stream of cells as well as detectors and mirrors needed to focus light emissions onto the appropriate detectors.

Flow cytometers contain one or more lasers, which produce light at very specific wave-lengths. In the BD FACSAria™ I (BD Biosciences USA) used in this study, there are 3 lasers with emissions of 407nm (violet), 488nm (blue) and 633nm (red). As cells pass through the laser source, light is scattered by the cells and picked up by detectors positioned at 180° and 90° to the source. Light scattered in the forward direction (forward scatter (FSC)), analogous to casting a shadow, correlates with cell size. Light scattered perpendicularly (orthogonal or side scatter (SSC)) corresponds to the

complexity of the cell including cytoplasmic granularity [404]. By measuring these two parameters discrete cell populations can be identified.

3.7.1 (iii) Immunofluorescence

Fundamental to the principles of modern flow cytometry is the technique of immunofluorescence which involves labelling external or internal cellular structures of interest with a molecule which fluoresces. These chemicals, termed fluorochromes, have electrons within their structure that upon exposure to certain wavelengths (excitation spectra) become excited to a higher energy state. Upon returning to their resting energy level light of a separate wavelength is emitted (emission spectra). This difference between the maximum of the excitation spectrum (Ex_{max}) and of the maximum emission spectrum (Em_{max}) is termed "Stoke's Shift" after the Irish physicist [405]. As the cytometer contains lasers which only produce a very narrow spectrum of light, the fluorochromes used must be excited by the same spectra whilst having emission spectra of wavelengths distinct from the light source. Modern machines have multiple lasers; this enables excitation of a broader range of fluorochromes, but in order to simultaneously measure several features of a cell, each fluorochrome must still have discrete emission spectra. The principle fluorochromes used in the following analyses are summarised in Table 3.2.

Fluorochrome	Ex_{max} (nm)	Em_{max} (nm)
APC (Allophycocyanin)	633	660
APC-eFluor780	633	780
Alexa Fluor 488	488	519
Alexa Fluor700	633	719
eFluor450	407	455
FITC (Fluorescein isothiocyanate)	488	519
PE (R phycoerythrin)	488	578
PerCP-Cy5.5(Peridinin chlorophyll protein Cy5.5 conjugate)	488	695

Table 3.2: Commonly used fluorochromes and their respective absorption and emission spectra.

As all the light from the excitation of different fluorochromes is emitted simultaneously, it must be separated into its various components for detection. This is achieved with the use of dichroic mirrors and filters. Dichroic mirrors permit light below a certain wavelength to pass through to a detector whilst reflecting light above the same length. Filters are also used to narrow the spectra of light falling on each detector. Short pass detectors allow only light below a specified wave-length to pass through whilst their counterparts, long pass filters, only allow light above a specified wavelength

through. Band pass filters allow a specified band of wavelengths to pass through. Through the sequence of mirrors and filters, light of specific wavelengths from each of multiple fluorochromes can be separated and detected individually allowing multiple components or properties of a cell to be measured simultaneously.

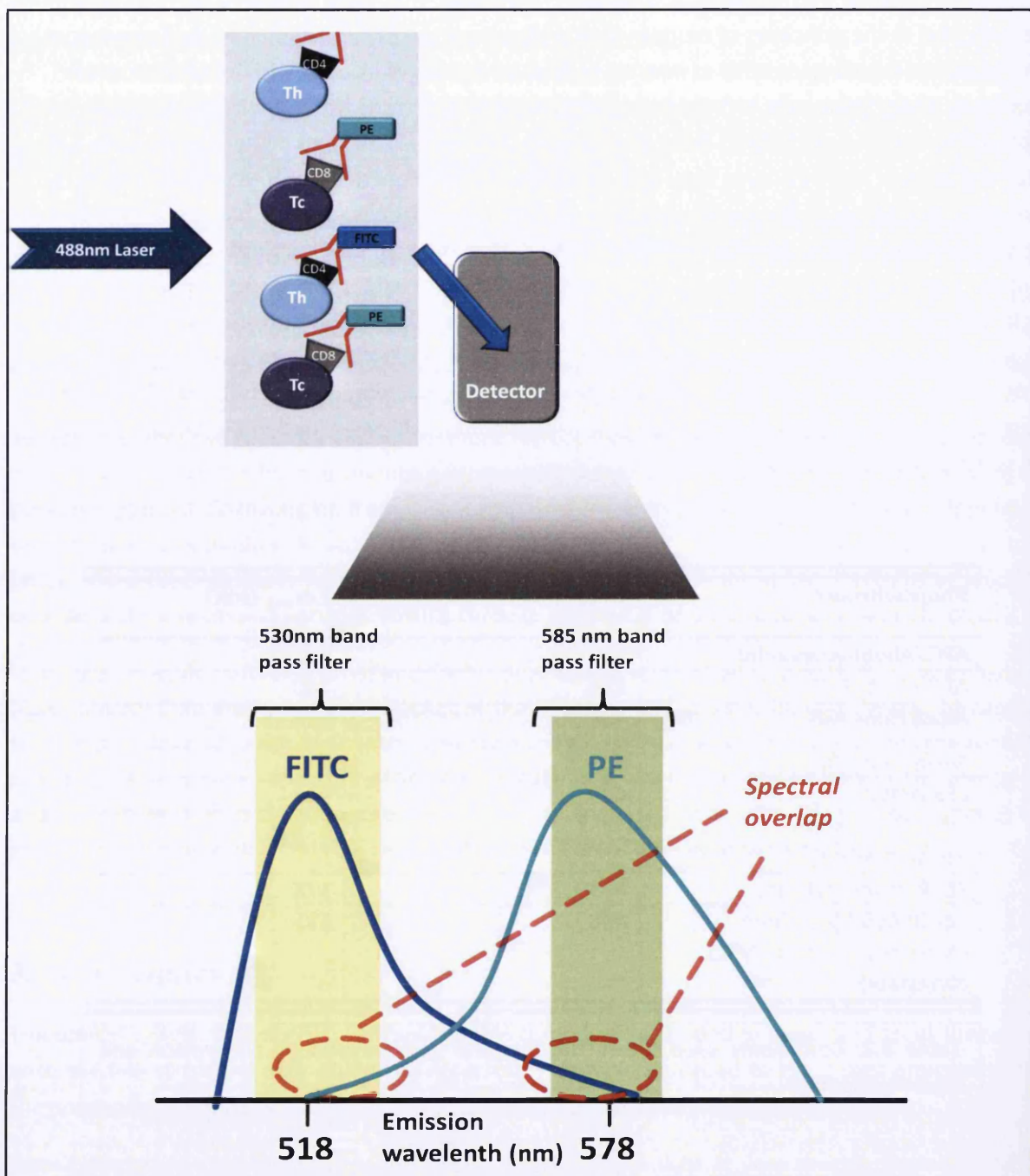


Figure 3.4: Illustration of spectral overlap between the fluorochromes FITC and PE. Whole blood is stained with CD4-FITC and CD8-PE. On passing through the 488nm laser these fluorochromes are excited producing their respective emission spectra. Despite the use of band filters there is still some overlapping of emission spectra. This is addressed using compensation

Although the use of mirrors and filters is effective at isolating discrete emission spectra from fluorochromes, it still relies on the assumption that there is no overlap in spectra between them. Unfortunately many fluorochromes have some breadth to their emission spectra meaning that emissions may overlap with one another: “spectral overlap” (Figure 3.4). Although filters go some way to addressing this issue it is often necessary to subtract a percentage of the observed emission from one fluorochrome on the basis that it is coming from another fluorochrome, a process termed compensation. Such compensation historically was performed prior to data acquisition, however with modern software it can now be performed retrospectively [406].

3.7.1 (iv) Electronics

The light emitted, having passed through filters and mirrors, falls on photomultiplier tubes (PMT), or photodiodes that convert the incident spectra of light into an electronic impulse. The impulse is amplified and converted into a digital signal for subsequent analysis by the computer software. The sensitivity each PMT can be altered by adjusting the voltage supplied to it.

3.7.2 Sample preparation for whole blood flow cytometry

Quantification of lymphocyte, dendritic cell subtypes and neutrophil/monocyte activation markers was performed by multi-parametric flow cytometry using the BD FACSAria™ I (BD Biosciences). All immunophenotyping was done on whole blood using the whole blood lysis method, where red cells are removed after staining with antibody by targeted red blood cell lysis, leaving the leukocytes for analysis. This technique compares favourably with performing flow cytometry on isolated MNCs separated by density gradient centrifugation [407].

3.7.2 (i) Surface staining

Predetermined volumes of monoclonal antibody were added to 100µl of whole blood and the sample vortexed before incubation in the dark on ice for 30 minutes. A list of the antibodies used is summarised in Table 3.3. The samples were then treated with 3mls of red blood cell lysis solution (FACS lysing solution BD Biosciences) and incubated in the dark at room temperature for 10 minutes. Cells were collected by centrifugation (4°C, 515 x g for 7 minutes) and the supernatant discarded before washing in FACS buffer (PBS with 0.2% BSA and 0.05% sodium azide). The tubes were centrifuged, supernatant removed and then the samples for detecting T cell subsets, dendritic cells and neutrophil/monocyte activation markers were fixed using 200µl FACS fix (BD Biosciences, USA), unless intracellular staining was required.

Antigen	Fluorochrome	Cell expression	Clone	Source
CD1c (BDCA-1)	PE	Myeloid dendritic cells		Miltenyl Biotec: dendritic cell enumeration cocktail
CD3	e450	Pan T lymphocytes	UCHT1	eBioscience, UK
CD4	e450	CD4 ⁺ T lymphocytes	OKT4	eBioscience, UK
	FITC		S3.5	Life technologies (Caltag), UK
	Alexa Fluor 488		OKT4	eBioscience, UK
CD8	PE	CD8 ⁺ T cells	OKT8	eBioscience, UK
CD11b (MAC1- α)	APC	Activation epitope of neutrophils and monocytes	CRBM1/5	eBioscience, UK
CD62L (L-selectin)	PE	Low expression after shedding on chronically activated neutrophils and monocytes	DREG-56	eBioscience, UK
CD14	PerCP-Cy5.5	High expression on monocytes	61D3	eBioscience, UK
	PE-Cy5			Miltenyl Biotec: dendritic cell enumeration cocktail
CD15	e450	High expression on neutrophils	H198	eBioscience, UK
CD16	APC	High expression on NK cells and activated monocytes	CB16	eBioscience, UK
	FITC		3G8	BD Biosciences (Pharmingen), UK
CD19	Alexa Fluor 700	B lymphocytes	HIB19	eBioscience, UK
	PE-Cy5			Miltenyl Biotec: dendritic cell enumeration cocktail
CD25	APC	Tregs, activated T lymphocytes	BC96	eBioscience
CD45RA	FITC	High expression on naive T cells and terminally differentiated T cells	H1100	eBioscience, UK
CD56	APC	High expression on NK cells	MEM188	eBioscience, UK
CD127	PerCP-Cy55	Low expression on Tregs	RDR5	eBioscience, UK
CD141 (BDCA3)	APC	Type 2 myeloid dendritic cells		Miltenyl Biotec: dendritic cell enumeration cocktail
CD197 (CCR7)	APC	High expression on central and effector memory T cells.	3D12	eBioscience, UK
CD303 (BDCA-2)	FITC	Plasmacytoid dendritic cells		Miltenyl Biotec: dendritic cell enumeration cocktail
FoxP3	PE	Tregs (intracellular)	PCH101	eBioscience, UK
HLADR	FITC	High expression on activated monocytes	G46-6	BD Biosciences (Pharmingen), UK
Mouse IgG1 isotype control	APC/ PerCP-Cy5.5		P3.6.2.8 .1	eBioscience, UK
	APC/FITC			Miltenyl Biotec: dendritic cell enumeration cocktail
Mouse IgG2a isotype control	PE		20102	R&D systems
				Miltenyl Biotec: dendritic cell enumeration cocktail
Mouse IgG2b isotype control	FITC		eBMG2b	eBioscience, UK
Rat IgG2a isotype control	APC/PE		eBR2a	eBioscience, UK

Table 3.3: Monoclonal antibodies and fluorochromes used.

3.7.2 (ii) Intracellular staining

After surface staining for CD4, CD25 and CD127, 1ml of freshly prepared fixation/permeabilisation buffer (eBiosciences) was added to relevant samples followed by incubation on ice for 1 hour (this time was pre-optimised in preliminary investigations). These samples were then washed twice with 2mls of permeabilisation buffer (eBiosciences). Antibody to FoxP3 was added to sample suspended in 100µl permeabilisation buffer and then incubated at room temperature in the dark for 30 minutes. The samples were then washed twice, with 2mls of permeabilisation buffer before being fixed with 200µl FACS fix (BD Biosciences, USA). All samples were analysed within 24 hours of processing.

3.7.3 Gating

One of the principle uses of flow cytometry is to analyse specific sub-populations of cells (e.g. CD4⁺ T cells) within a mixed sample e.g. whole blood, containing all forms of leukocytes. Gates can be applied to the acquired cells, allowing interrogation of discrete populations which may be based on their morphological appearance using scatter plots [408] (e.g. monocytes vs. lymphocytes), or by gating on surface markers using fluorescence (immunophenotyping) [409]. An example of a gating strategy for major lymphocyte subsets is outlined in Figure 3.5. The gating strategy for each of the populations of interest is shown in their respective results chapters.

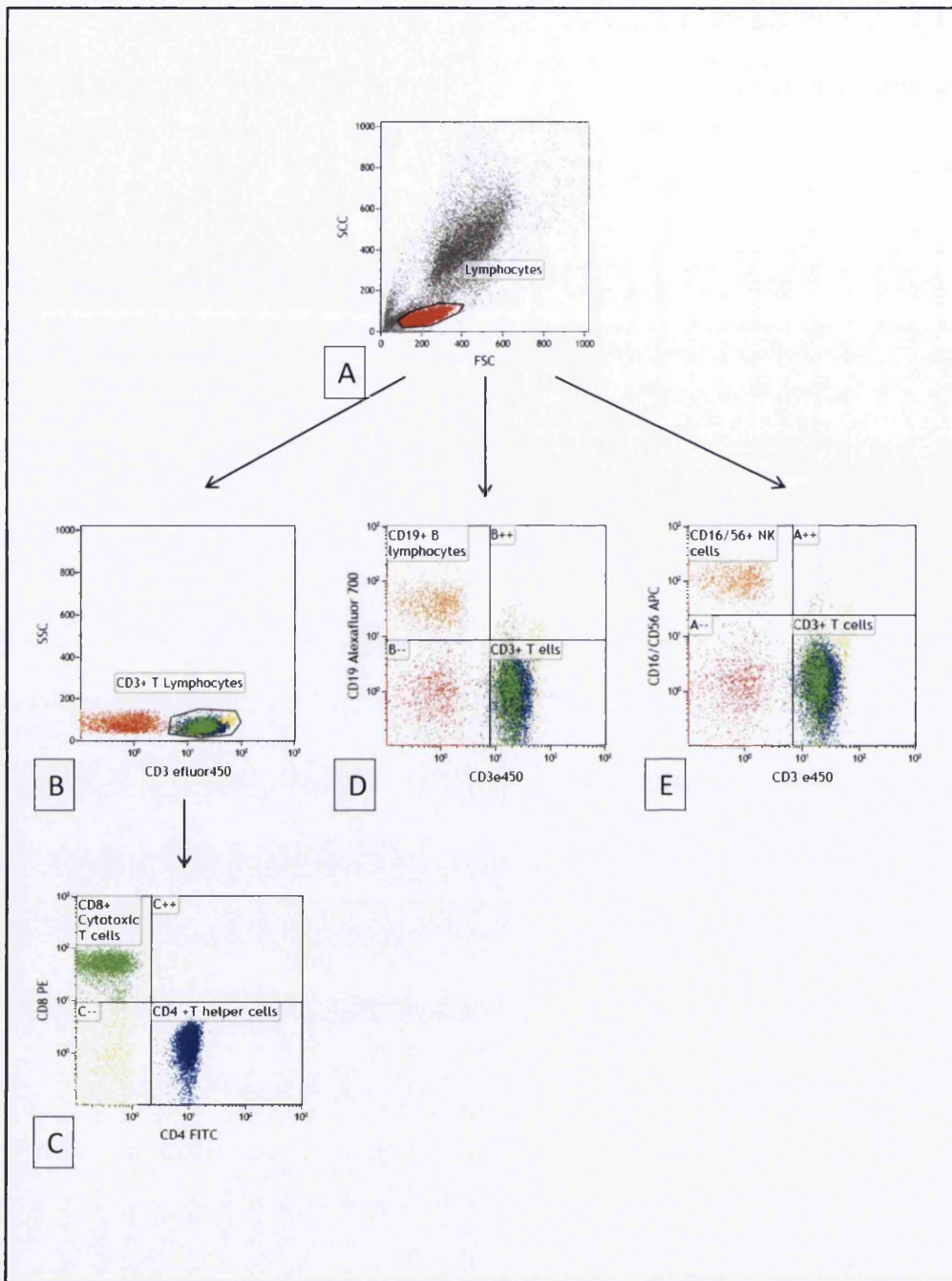


Figure 3.5: Gating strategy for phenotyping of major lymphocyte subsets.

A: Using forward scatter (FSC) and side scatter (SSC), lymphocyte population identified.

B: Gating on lymphocytes, CD3⁺ T lymphocytes identified.

C: Gating on CD3⁺ T lymphocytes, CD4⁺ and CD8⁺ populations identified.

D: Gating on lymphocytes, CD19⁺ B lymphocytes identified.

E : Gating on lymphocytes CD16⁺CD56⁺ positive NK cells identified.

3.8 Measurement of oxidative stress

Oxidative stress arises due to an imbalance between ROS production and counteracting antioxidants and can cause oxidative injury resulting in further inflammation [94]. Oxidative stress can either be measured directly using electron spin resonance, or by measuring products of oxidation [410].

3.8.1 Reactive oxygen species by luminol enhanced chemiluminescence

Phagocytosis is an important aspect of innate immune defence. During this process, phagocytes suddenly increase their oxygen and glucose requirements in a process referred to as the “respiratory burst” [411]. This results in the generation of oxygen containing compounds which kill the phagocytosed pathogen (oxygen dependent intracellular killing) [412]. When phagocytes detect pathogen through the use of PRRs, the activity of the pentose-phosphate pathway escalates, increasing glucose consumption, resulting in NADPH production. The enzyme NADPH oxidase catalyses the reduction of oxygen forming superoxide radicals [413] which are then acted upon by superoxide dismutase, producing hydrogen peroxide. Through the action of myeloperoxidase (MPO) contained in granulocytes, a highly toxic hypochlorite is formed [414]. A number of MPO independent mechanisms also exist to produce reactive oxygen species (ROS) (Figure 3.6). Reactive oxygen species have a number of effects which are important in the killing of pathogens including DNA cleavage and modification, protein denaturation and lipid peroxidation.

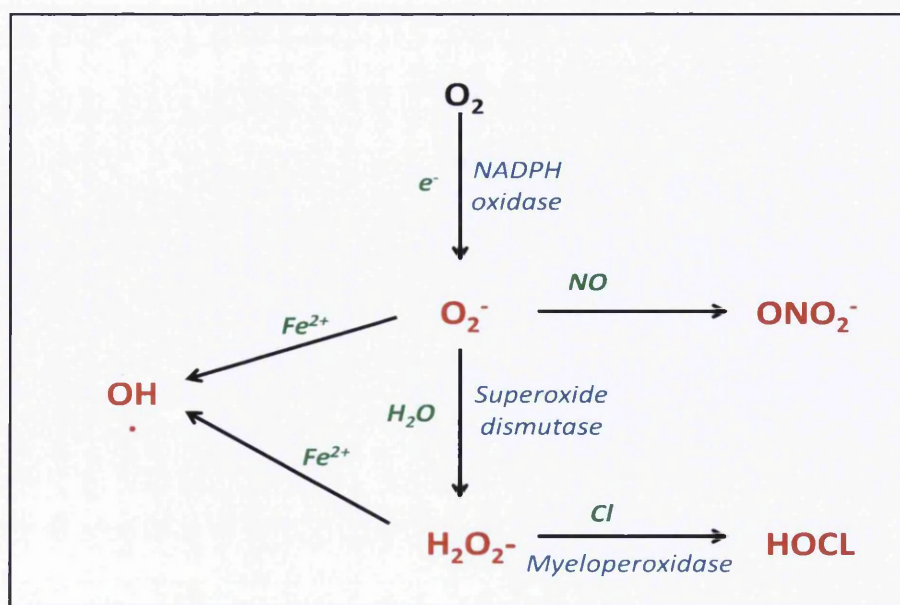


Figure 3.6: Generation of reactive oxygen species by phagocytes. In response to pathogens or stress phagocytes generate superoxide ROS (shown in red) through the activity of NADPH. By reacting with other agents (green) in the presence of enzymes (blue), further ROS are generated.

Enhanced chemiluminescence is an effective method for measuring the release of respiratory burst products. ROS excite the chemical luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), which reacts in its oxidised form and releases a photon of light (chemiluminescence) as it returns to its resting state [415] (Figure 3.7). The amount of light released over a period of time can be quantified as a measure of the respiratory burst. Phorbol myristate acetate (PMA) was chosen to activate the neutrophil respiratory burst in the presence of luminol. PMA activates protein kinase C which stimulates NADPH oxidase in neutrophils [416].

Whole blood (diluted 1:10 in PBS; 25µl) was added to Krebs buffer (50µl) (Appendix VIII), luminol (25µl)(Sigma) and 1µM PMA (25µl)(Sigma). A control well in the absence of PMA was always included. The method was performed in triplicate in a white 96 well white plate (Greiner Bio-one, Germany). Once the activator PMA was added the plate was immediately placed into a pre-warmed (37°C)POLAR Star™ Omega plate reader (BMG Labtech, Germany). The plate reader was set at maximum gain (4095) to obtain chemiluminescence readings 20 times over 40 minutes. The maximum light units produced was measured to quantify ROS generation.

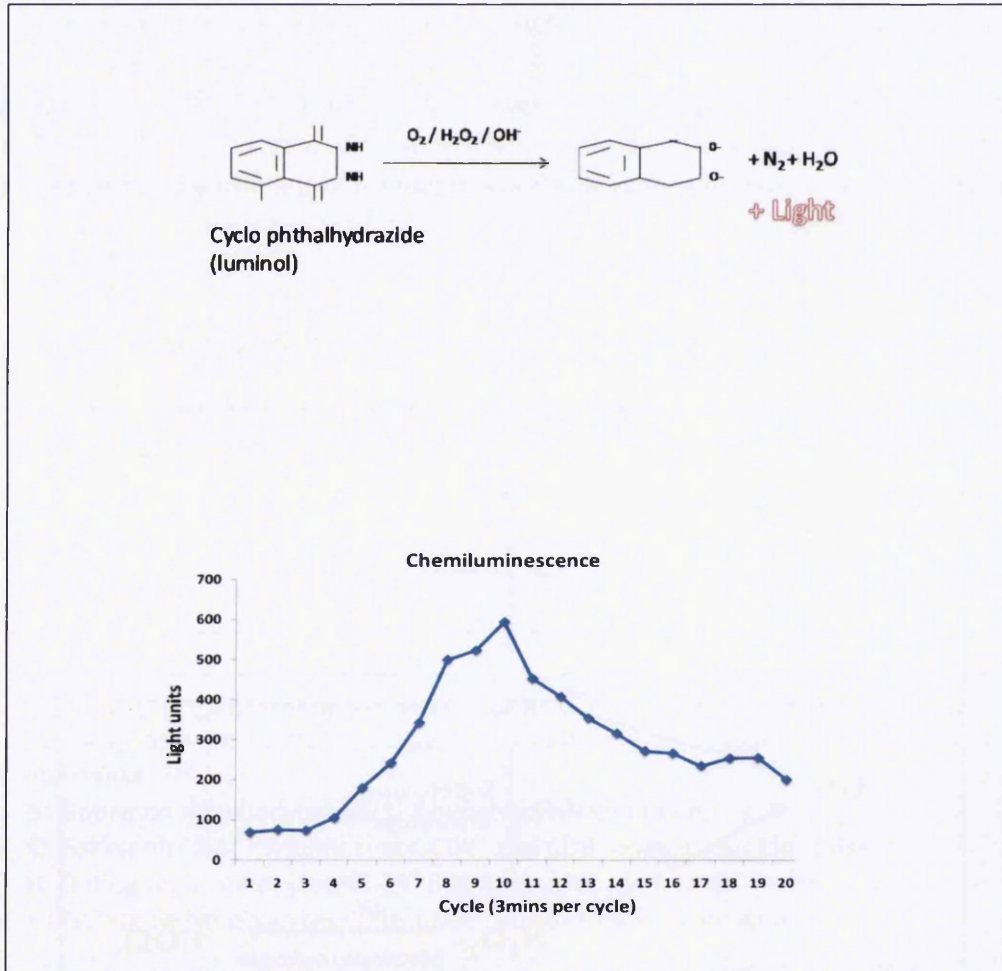


Figure 3.7: Typical output for luminescence measurement of ROS generation.
The peak light produced was used to quantify ROS generation.

3.8.2 Thiobarbituric acid reactive substances

Lipid peroxidation is a consequence of high oxidative stress, thus the resultant products can be measured as a surrogate marker of the chronic state of this. Lipid peroxidation products of polyunsaturated fats are unstable and decompose to form a group of compounds which include carbonyl compounds such as malondialdehyde (MDA). The thiobarbituric acid reactive substances (TBARS) assay is a well established technique for measuring these products in plasma [417]. Under high temperatures (90-100°C) in acidic conditions MDA reacts with TBA to form MDA-TBA adduct which can be measured colourimetrically at 530-540nm.

A commercially available TBARS assay was used (Cayman chemicals) and standards, samples, and reagents bought to room temperature. Each measurement was performed in duplicate. 5ml vials were labelled with an identification number (for samples) or the standard concentration. A colour reagent was mixed containing 530mg of TBA mixed with 50ml of TBA acetic acid solution and 50mls of sodium hydroxide. 100ul of sample or appropriate standard concentration was added to the appropriate labelled vial. 100ul of sodium dodecyl solution (SDS) was added to each vial and the vials mixed. 4ml of colour reagent was added to each sample and then the vials were capped and placed upright in boiling water for 1 hour. They were then placed in an ice bath for 10 minutes to stop the reaction. Following this the vials were centrifuged (1600 x g at 4°C) for 10 minutes. The vials were allowed to come to room temperature over 30 minutes and then 150ul of each sample was added to clear plate and absorbance measured at 530-540nm using a POLAR Star™ Omega plate reader (BMG Labtech, Germany). The concentration of MDA in the sample was determined by plotting the mean absorbance against a standard curve of known MDA concentrations. All measurements were performed in duplicate

3.8.3 Plasma total antioxidant status

Oxidative stress occurs due to an imbalance between oxidant production and antioxidant defences [94]. There are two broad types of antioxidants in the body: enzymatic and non-enzymatic. Enzymes such as superoxide dismutase (SOD) located in the mitochondria convert superoxide radicals to hydrogen peroxide which can then be converted to water by other enzymes including glutathione peroxidase, glutathione S-transferase or catalase. Non-enzymatic antioxidants include vitamins (A, B1, B6, B12, C and E), minerals (zinc, copper and selenium) and mitochondrial proteins [418]. Blood has a pivotal role in maintaining redox balance within the body, containing antioxidant defences, distributing them to different body sites. Plasma total anti-oxidant status (TAOS) is the net effect of different compounds previously described as well as systemic interactions.

Plasma TAOS was measured by a photometric micro-assay previously described by Sampson et al[419]. The TAOS of plasma from each patient was quantified by its capacity to inhibit the peroxidase-mediated formation of the 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS⁺) radical. In the assay, the relative inhibition of ABTS⁺ formation in the presence of plasma is proportional to the antioxidant capacity of the sample. Therefore, there are two arms to the assay, a control arm and test arm. In the control arm phosphate buffered saline (PBS) is used instead of plasma. The assay was performed in a 96 well ELISA plate using 2.5 µl of plasma (in triplicate). In the

control arm phosphate buffered saline (PBS) was used instead of plasma. A reaction mixture made up of (final concentrations) 20 µl ABTS (20 mmol/l)(Sigma), 20 µl horseradish peroxidase (30 mU/ml) (Life technologies, UK), and 40 µl PBS (pH 7.4) was added to each well. The reaction was started by the addition of 20 µl hydrogen peroxide (final concentration 0.1 mmol/l) (Life technologies, UK). The plate was then covered in foil and incubated for 12 min at 37°C and then the absorbance measured at 405nm (POLAR Star™ Omega plate reader (BMG Labtech, Germany). The increase in absorbance due to the accumulation of ABTS⁺ in the test sample was read along with the control. The difference in absorbance (control absorbance minus test absorbance) was divided by the control absorbance and expressed as a percentage. This represents the percentage inhibition of the reaction. Plasma TAOS is inversely related to oxidative stress: the higher the oxidative stress, the lower the TAOS.

3.9 Whole blood cultures

Whole blood was cultured with ligands to activate the innate (LPS) and adaptive, mainly T cell, (PHA) compartment, and the cytokine responses to these stimuli in the various study groups were measured using ELISA (section 3.11).

LPS is a component of the cell wall of Gram negative bacteria and is crucial to its structural integrity[420]. LPS contains a hydrophobic domain (Lipid A) which is a potent endotoxin, able to trigger the release of pro-inflammatory cytokines via the LPS receptor complex, which includes receptor Toll-like receptor 4 (TLR-4), CD14 and MD2. Numerous cell types express components of this complex, including monocytes and macrophages, and the cytokine response to LPS can be used to measure the functional response of the innate immune compartment [421]. IFN γ was added as it can augment LPS response by macrophages [422, 423] particularly the production of the Th1 polarising cytokine IL-12p70 [424]. PHA is a lectin extract from the red kidney bean (*Phaseolus vulgaris*), with strong mitogenic properties [425]. PHA is a tetramer of 4 non-covalently bonded subunits of which there are 2 subtypes termed erythrocyte active (E) and lymphocyte active (L). PHA-L contains only L subunits which have a high affinity for lymphocyte surface receptors but little for those expressed by erythrocytes and therefore can be used to stimulate lymphocyte proliferation in cell cultures [426].

All culture work was undertaken in a class II tissue culture cabinet to maintain sterility. The media used for culture preparation was RPMI 1640/Glutamax (Life Technologies, Paisley, UK), which was supplemented with 50mM of 2-mercaptoethanol (ME; Life Technologies). This culture media (600µl) was added to each of 12 tubes (for 6 treatments in duplicate) and then 200µl of whole anticoagulated blood was added (Figure 3.8). ME is readily oxidised to a disulphide and is used to protect enzymes and other proteins from oxidative damage during culture. IFN γ (10ng/ml); Miltenyl Biotec) was added to 4 tubes (tubes in duplicate) and the cultures incubated for 90 minutes at 37°C in 5% CO₂-in-air. Following this, LPS (10ng/ml; Ultrapure, Life technologies, UK) and PHA-L (5ug/ml; Sigma) were added to the appropriate tubes and incubated at 37°C in 5% CO₂-in-air. Blood incubated with LPS+/- IFN γ or PHA was incubated for 24 and 48 hours, respectively. After incubation the tubes were centrifuged for 7 minutes at 4°C, 515 x g and cell free supernatants removed for storage at -20°C until analysis.

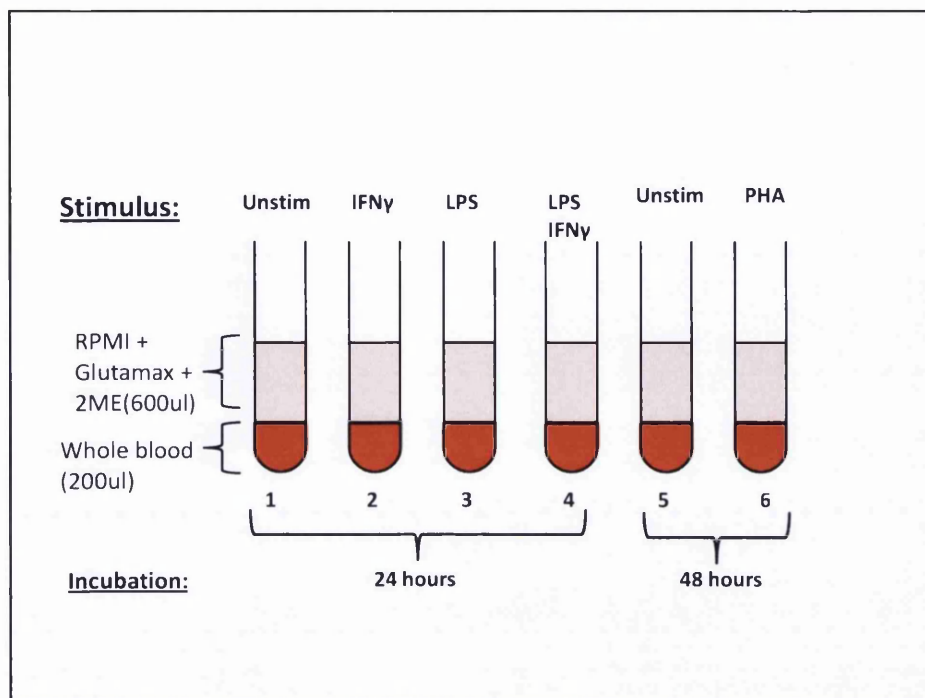


Figure 3.8: Schematic diagram of whole blood cultures. Ligands were added to stimulate innate effector cells (LPS/IFN γ) and T lymphocytes (PHA). They were then incubated for 24 and 48 hours respectively. The experiments were performed in duplicate.

3.10 Isolation of plasma and mononuclear cells

Mononuclear cells can be separated from whole anticoagulated blood by density gradient centrifugation (Figure 3.9). Blood (10ml) was gently layered onto an equal volume of Histopaque 1077 (Sigma, UK) in a 50ml Falcon tube (Greiner Bio-one, Germany) and centrifuged at 805 x g for 20 minutes (no brake). The plasma was then removed, filtered (0.2 μ m polyethersulfone filter; Sigma, UK) and stored at -20°C until analysis. The layer of mononuclear cells beneath the plasma was placed into a 30ml Universal tube (Greiner Bio-one, Germany), re-suspended in RPMI 1640/Glutamax (Life Technologies) and centrifuged at 515 x g for 10 minutes. Following centrifugation the supernatant was discarded and the cell pellet re-suspended in RPMI 1640/Glutamax before being centrifuged at 515 x g for 7 minutes at 4°C. The supernatant was discarded and cells were gently re-suspended in 20ml of cryowash CTL and counted on a disposable haemocytometer (C-Chip; details of supplier). The cells were then cryopreserved using the CTL-Cryo ABC kit (CTL™ Europe). Cryo A and B were warmed to room temperature and mixed together in a ratio of 4:1. Cells were suspended in Cryo C at a concentration of 20 x 10⁶/ml and an equal volume of the Cryo AB mixture was added. The cells were then placed in a freezing container (Nalgene Mr Frosty, Sigma UK) containing isopropyl alcohol,

which when placed in a -80°C freezer enables a critical and repeatable cooling rate of $1^{\circ}\text{C}/\text{minute}$. The vials were placed in the -80°C freezer for at least 24 hours before being transferred to liquid nitrogen.

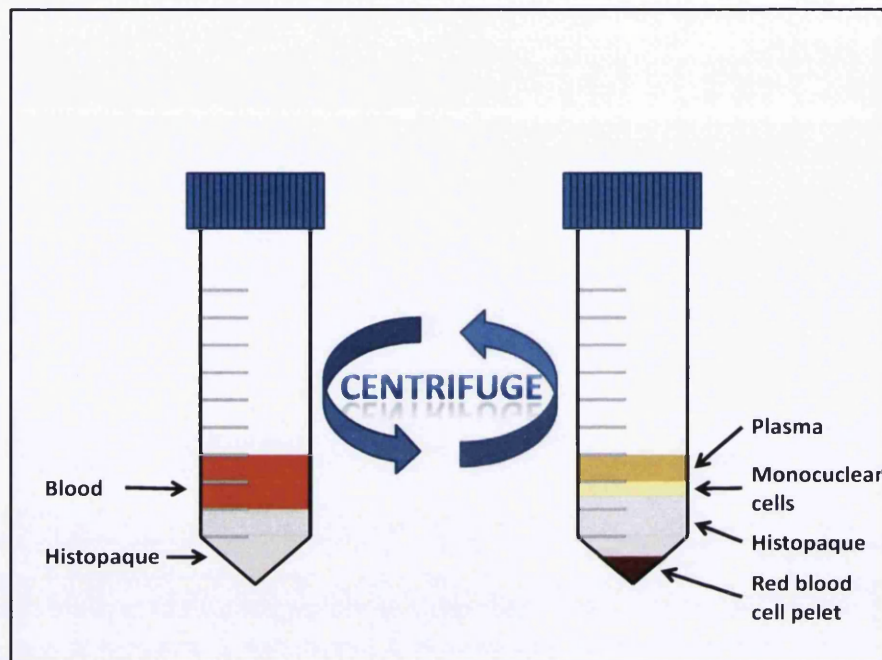


Figure 3.9: Schematic diagram of plasma and mononuclear cell isolation by density centrifugation.

3.11 Cytokine analysis

3.11.1 Principles of ELISA assay

Circulating plasma cytokines and the cytokine response from whole blood cultures were quantified using a sandwich ELISA. This technique was developed by Peter Perlmann and enables the accurate quantification of a substance in a wet media [427]. There are several different variations in this technique including indirect, sandwich, and competitive methods. The sandwich technique was used throughout this study and the basic steps are outlined in Figure 3.10.

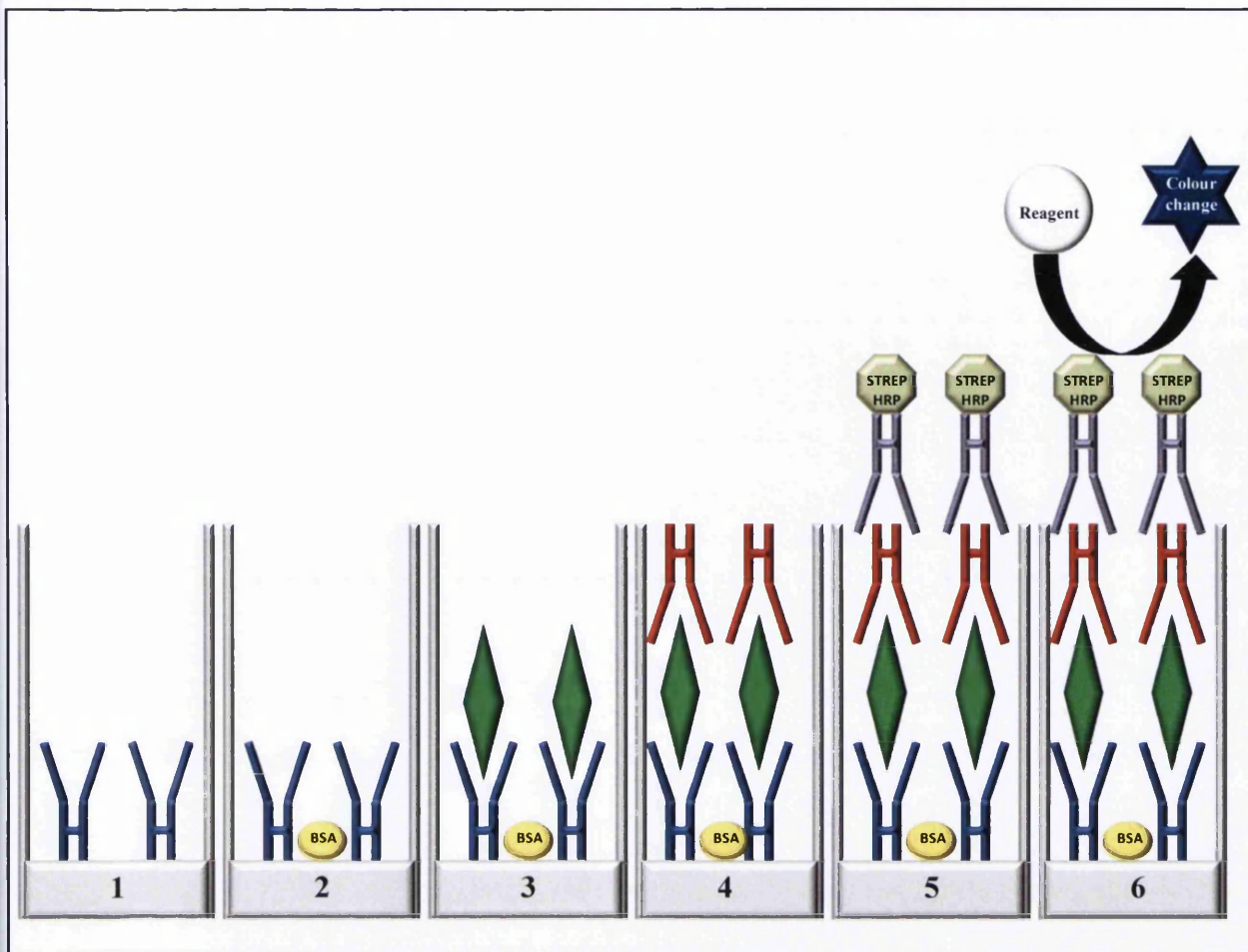


Figure 3.10: Schematic diagram of sandwich ELISA.

- 1: Plate wells are coated with specific capture antibody (blue).
- 2: Non-specific binding is prevented by adding a blocker buffer (BSA).
- 3: Sample is added and the specific antigen (green) binds to the capture antibody.
- 4: Antigen specific detection antibody (red) is added and binds at a different site to capture antibody.
- 5: An enzyme linked secondary antibody (Streptavidin horseradish peroxidise (Strep HRP)) is added which binds to the Fc region of the detection antibody.
- 6: Substrate is added which when acted upon by the enzyme produces a quantifiable colour change.

The advantage of this method is that it enables the very accurate quantification of a specific protein in an impure sample. A plate is coated with a highly specific capture antibody which will bind to a portion of the protein of interest. After incubation, the plate is washed to remove any excess antibody and then a blocking buffer is added to prevent any of the subsequent sample from binding non-specifically to the plate. Again the plate is washed to remove excess buffer before the sample is added and incubated. The protein of interest within the sample will bind to the specific capture antibody and is retained on the plate whilst the remainder of the sample is washed away. A further specific detection antibody is added which also recognises the protein of interest but binds to a different site. This antibody has biotin attached to it which enable the binding of a streptavidin-enzyme (in this case horse radish peroxidase (HRP)) complex. The plate is washed a final time before substrate solution is added. The clear substrate is catalysed by the enzyme (HRP) and changes colour. The degree of colour change is proportional to the amount of cytokine bound to the plate.

The absolute concentration can be quantified by adding a standard curve included on the plate which contains a dilution series of known amounts of the protein of interest.

3.11.2 ELISA method

A number of different commercially available cytokine kits were used as listed in Table 3.4; all used the sandwich ELISA methodology with minor differences in the protocol. For the assay of culture supernatants ½ area ELISA plates were used allowing half the volume recommended for standard ELISA plates to be used.

Cytokine	Sample	Sensitivity (pg/ml)	Source
GCSF	Plasma	39.063	Quantikine, R&D systems, Europe.
GMCSF	Plasma	7.813	Quantikine, R&D systems, Europe.
IL-1β	Culture supernatants	3.906	Duo Set, R&D Systems, Europe.
IL-6	Culture supernatants	9.375	Duo Set, R&D Systems, Europe.
	Plasma	0.156	Quantikine, R&D systems, Europe.
IL-8	Culture supernatants	15.625	Duo Set, R&D Systems, Europe.
	Plasma	1.0	Quantikine, R&D systems, Europe.
IL-9	Culture supernatants	1.563	Ready-SET-Go, eBioscience, UK
IL-10	Culture supernatants	7.813	Opt EIA, BD Biosciences, USA
IL-12p70	Culture supernatants	7.813	Ready-SET-Go, eBioscience, UK
IL-13	Culture supernatants	7.813	Ready-SET-Go, eBioscience, UK
IL-17A	Culture supernatants	7.813	Ready-SET-Go, eBioscience, UK
	Plasma	1.563	Platinum, eBioscience, UK
IL-23	Culture supernatants	125	Duo Set, R&D Systems, Europe.
	Plasma	39.063	Quantikine, R&D systems, Europe.
IFN-γ	Culture supernatants	7.813	Ready-SET-Go, eBioscience, UK
sCD14	Plasma	62.5	Duo Set, R&D Systems, Europe.
sgp130	Plasma	156.25	Duo Set, R&D Systems, Europe.
sIL-6R	Plasma	156.25	Duo Set, R&D Systems, Europe.
TGF-β	Plasma		Duo Set, R&D Systems, Europe.
TNF-α	Culture supernatants	7.813	Opt EIA, BD Biosciences, USA

Table 3.4: List of cytokines assayed, ELISA kits used and their sensitivities.

An example of a typical protocol from R&D Systems DuoSet is outlined as follows:

- a. Dilution of capture antibody (1:180) into coating buffer (PBS) and 50µl added to each well (Costar ½ area 96 well plate; Sigma) and incubated overnight at 4°C.
- b. Coating antibody tipped off, 150µl of blocking buffer added (1% Bovine Serum Albumin (BSA); Sigma UK) and incubated at room temperature for 1 hour.
- c. Blocking buffer tipped off and washed 3 times with wash buffer (PBS with 0.05% Tween-20; Sigma UK).
- d. Standard curve added in duplicate (50µl per well); created from stock with 1 in 2 dilutions in assay buffer (1%BSA in PBS) to give 7 points and a blank (buffer only).

- e. Samples diluted in assay buffer at dilution determined from pre-optimisation studies and added in duplicate (50µl per well). Plate incubated at room temperature for 2 hours.
- f. Plate washed 4 times with wash buffer.
- g. Dilution of biotinylated detection antibody (1:180) into assay buffer and 50µl added per well. Plate incubated for a further 2 hours.
- h. Plate washed 4 times with wash buffer.
- i. Dilution of enzyme reagent; streptavidin-horseradish peroxidase (Strep HRP) (1:200) into assay buffer and 50µl added per well. Plate incubated for 20 minutes.
- j. Plate washed 6 times with wash buffer.
- k. Addition of substrate chromogen (tetramethylbenzidine (TMB), 50 µl/well; prepared according to the manufacturers' instructions; BD biosciences).
- l. Blue colour allowed to develop.
- m. The reaction was stopped with 1M H₂SO₄ (50µl/well).
- n. Subsequent colour intensity (yellow) was recorded as the optical density at 450nm using a POLAR star Omega™ plate reader (BMG Labtech, Germany).
- o. A standard curve was plotted from the known concentrations of standard and the mean absorbance measured (Figure 3.11). Using the equation derived from the standard curve, concentrations of cytokine in each sample were calculated and means taken.

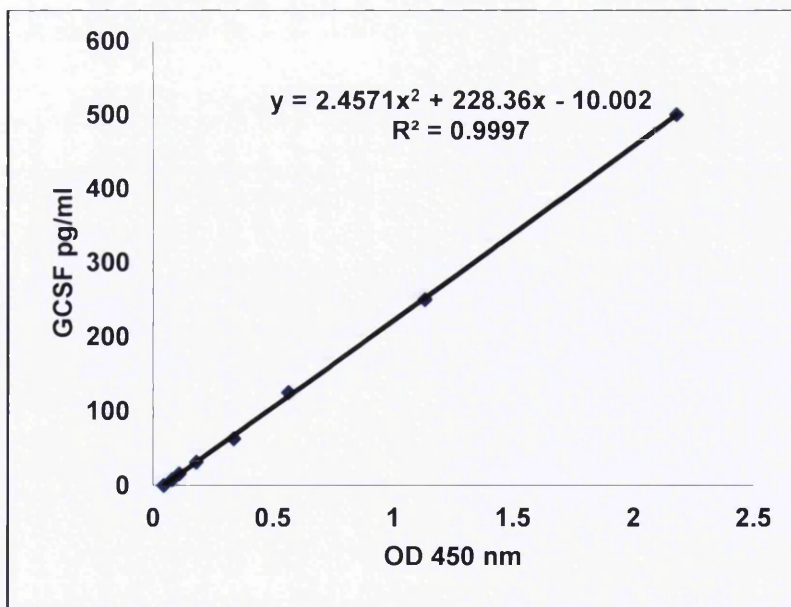


Figure 3.11: Example of a standard curve. Known quantities of the cytokine are added to the plate in a ½ dilution series. The optical density (OD) is plotted against the dilution series and a line of best fit drawn. The equation derived from this line is used to calculate the concentrations of the cytokine in the samples.

3.12 Measuring metabolic parameters

3.12.1 Measuring serum adipokines with a Luminex xMAP assay

Quantification of adipokines was done using Luminex xMAP technology [428]. Central to the technique is the use of 5.6nm polystyrene microspheres which are dyed with differing amounts of red and infrared fluorophores, enabling the creation of up to 100 different microsphere sets. Each microsphere set can then be coupled with a different detection antibody enabling the measurement of multiple analytes simultaneously. However, a limitation of this technique is that all analytes of interest must be measured at the same dilution. The array reader applies the principles of flow-cytometry using a fluidics system to align the beads into single file before passing them through a green (532nm) and red (635nm) laser. The red laser excites the fluorophore dyed microspheres to determine their colour or "region" enabling identification of the bound antibody and thereby the analyte. The green laser excites the bound streptavidin-phycoerythrin (SA-PE) for quantification of the assay signal strength and, with the use of a standard curve, the concentration of the protein of interest.

Measurement of serum adipokines was undertaken using a Bio-Plex assay (Bio-Rad, USA). Leptin, ghrelin, resistin and visfatin were measured simultaneously in a 4-plex; adiponectin was measured alone due to its abundance and the dilution required for use in the assay. Experimental procedures for the assay were carried out at room temperature. Antibody-coupled beads were first incubated with antigen standards or serum samples for 1 hr. After washing using a handheld magnetic washer to remove unbound materials biotinylated detection antibodies were added and incubated for 30 minutes. After washing away the unbound biotinylated antibodies, the beads were incubated with SA-PE for 10 minutes. Following removal of excess SA-PE, the beads were passed through the Bio-Plex™ 200 System array reader (Bio-Rad). Data analysis was performed using Bio-Plex Manager™ software version 4.1.1.

3.12.2 Serum glucose

Serum glucose was measured by an automated ILab 300 analyser (Instrumentation Laboratory UK Ltd). The analyser determines glucose concentration using the principles of a glucose oxidase assay (Figure 3.12). Glucose is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase. The hydrogen peroxide generated reacts with reduced o-Dianisidine in the presence of peroxidase to form a brown coloured oxidised product. The oxidized o-Dianisidine reacts with sulphuric acid to form a more stable pink coloured product. The intensity of the pink colour measured at 540 nm is proportional to the original glucose concentration.

3.12.3 Plasma insulin

Plasma insulin was measured using a commercially available immuno-chemiluminescent assay (Invitron Ltd, UK). The assay is a two-site sandwich immunoassay, utilising an insulin specific solid

phase antibody immobilised on micro-titre wells, and a soluble antibody labelled with an acridinium ester. Labelled antibody and samples, controls, or calibrators are incubated simultaneously in antibody-coated wells. This incubation leads to insulin being bound to the micro-well test plate between the two different antibody-molecules ("sandwiched"). Excess labelled antibody and other components from the sample are removed by washing. The bound luminescence is quantified by a micro-titre plate reading luminometer (Centro, Berthold Technologies), capable of in situ reagent addition. The assay used a calibrator provided as part of the kit and blank to generate a standard curve against which the plasma insulin levels in the samples could be determined.

3.12.4 Free fatty acids

Serum free fatty acids (FFA) were analysed using a commercially available assay (Wako NEFA-C kit, Alpha Labs, UK). The Wako enzymatic method for determination of NEFA relies upon the acylation of coenzyme A by the fatty acids in the presence of added acyl-CoA synthetase (ACS). The acyl-CoA produced is oxidised by added acyl-CoA oxidase resulting in the generation of hydrogen peroxide. Hydrogen peroxide in the presence of peroxidase permits the oxidative condensation of 3-methyl-N-ethyl-N-(2-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple coloured adduct which can be measured colorimetrically at 550 nm (Lab 300plus, Instrumentation Laboratories). The assay contains a calibrator against and blank to generate a standard curve.

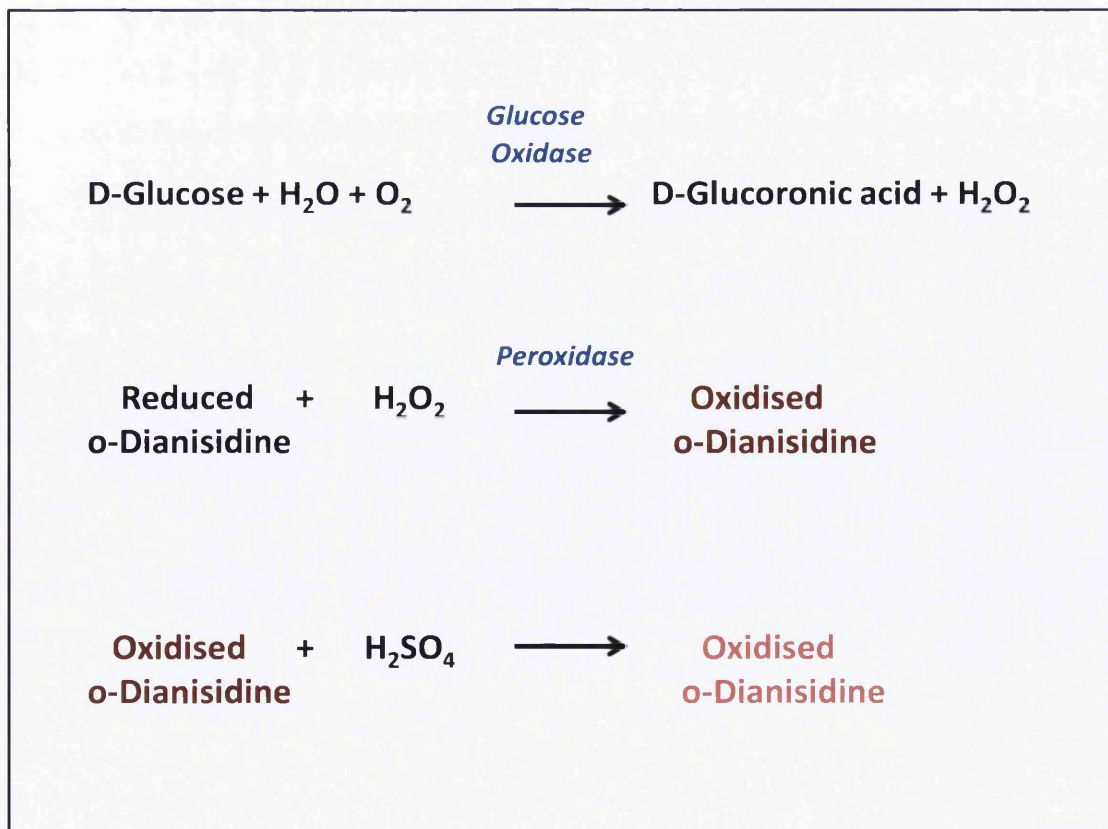


Figure 3.12: Glucose oxidase assay. Glucose from a patient sample is oxidised generating hydrogen peroxide as a by-product, which in turn oxidises o-Dianisidine producing a coloured compound. The intensity of the colour can be measured which will correlate with the concentration of glucose in the sample of interest.

3.13 Serum total and specific IgE

Serum samples were collected in serum separator tubes (Vacurette®, Greiner Bio-one) and centrifuged at 90 minutes after collection (2500 rpm, 4°C for 10 minutes). Serum total IgE (iu/ml) was measured using sandwich ELISA assay (Elecsys IgE assay, Roche Diagnostics, UK) at Morriston Hospital Department of Chemical Pathology. The principle is very similar to the sandwich ELISA method described previously except the antibody used is attached to magnetic particles which are separated by a magnet to allow quantification.

- a. 1st incubation: IgE from 10 µl sample, a biotinylated monoclonal IgE-specific antibody and a monoclonal IgE-specific antibody labelled with a ruthenium complex form a sandwich complex.
- b. 2nd incubation: After addition of streptavidin labelled micro-particles, the complex produced is bound to the solid phase via biotin-streptavidin interaction.
- c. The reaction mixture is aspirated into the measuring cell where the micro-particles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell.
- d. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.
- e. Results are determined via a calibration curve. This curve is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

Serum IgE specific antibodies against 6 allergens (cat dander, dog dander, house dust mite, mixed moulds, mixed trees and mixed grasses) were measured at Morriston Hospital Department of Haematology and Immunology, using an ImmunoCAP Fluorezyme assay. (Thermo Fisher). This assay captures IgE specific antibodies present in serum, with anti-IgE antibodies immobilised on a solid phase, known as 'ImmunoCAP': a sponge with a very large surface area. After washing, enzyme-conjugated antibodies directed against human IgE are added, and bind to the allergen specific IgE molecules captured from the serum sample. Following incubation, unbound enzyme anti-IgE conjugate is washed away and the bound complex is subsequently incubated with a substrate. The action of the enzyme on the substrate forms a fluorescent product and once developed, the reaction of enzyme upon substrate is stopped by the addition of a stop solution. The fluorescence measured is proportional to the concentration of allergen specific IgE in each serum sample. Within every batch of samples, standards, controls and/or calibrators are run. A curve of fluorescence values against the known IgE concentration from the standards. The fluorescence values measured for patient samples are then interpolated in this graph in order to derive the values of total serum IgE concentration present.

3.14 Statistical analysis

This was an observational study examining multiple immunological parameters. By adopting stringent inclusion criteria we sought to exclude many confounders, although in doing so limited the number of subjects suitable for the study dramatically. However, the sample size chosen (n=90) compares favorably with previous studies in this area (n=80-120) [25, 359, 429]. Furthermore, the study design enables the examination of associations between obesity and immune markers and asthma and immune markers as well as studying the associations with obesity within asthmatics.

The database was compiled using SPSS 19.0 software. Each of the variables of interest was examined visually using a histogram and the Kolmogorov-Smirnov (KS) goodness of fit test was performed to verify whether they were normally distributed. A variable was said to be suitable for parametric testing if the distribution approximated normality on visual inspection and passed the KS test ($p > 0.200$). Where necessary, positively skewed data were logarithmically transformed before analysis and the resultant distribution re-inspected and a repeat KS test performed. Continuous data that was not normally distributed after logarithmic transformation was analysed using non-parametric tests: Mann Whitney U test between two categories or Kruskal Wallis analysis between 3 or more categories. For normally distributed variables, differences between groups were analysed using an unpaired t-test or 1 way analysis of variance (ANOVA). Associations between normally distributed continuous variables were examined using a Pearson's correlation coefficient. Potential confounders were identified based on biological plausibility and evidence of correlation with the dependent variable of interest. A general linear model was used to look for the association between continuous covariates, categorical factors and the dependent variable of interest. Potential confounding covariates or factors were added sequentially to the model to see if they affected the significance of the association between the parameters of interest.

Relationships between factor variables were analysed using a Chi-Squared test of association. In some cases the cell counts fell below 5. However, in sample sizes over 40 where cell counts are greater than 1 the chi squared test is still valid [430, 431]. In some cases (e.g. plasma IL-17 levels), the dependent variable of interest was continuous however the majority of values were below the sensitivity of the assay and therefore reported a zero. In such cases the dependent variable was dichotomised into detectable vs. non-detectable and analysed using a chi-squared analysis.

Chapter 4

Clinical data, circulating major cell types and metabolic parameters

4.1 Introduction

In this chapter the clinical data from the study population will be examined to determine whether there are discernible differences in the clinical features of obese female asthma patients as compared to their normal weight counterparts. Full blood count and leukocyte differential data will be presented to explore whether shifts in systemic inflammation and immunity might be detectable at the level of major leukocyte cell types. Metabolic parameters will be described including markers of insulin resistance and fasting free fatty acids (FFA) levels to determine whether there are significant metabolic changes associated with this disease phenotype. Finally levels of adipokines in these individuals will be presented including those for which there is little existing clinical data in the literature yet some possible mechanistic role in the pathogenesis of an inflammatory airway disease.

4.1.1 Obese asthma: a distinct phenotype?

Obesity is associated with a higher risk of asthma development and also modifies the disease phenotype (see section 2.6.4). Obese children and adults with asthma have more severe symptoms and utilise more health care resources [241, 242]. The response to inhaled corticosteroids (ICS), the cornerstone of current asthma therapy, is reduced in this group of individuals [17, 18] resulting in patients presenting to secondary care on higher doses of therapy [241, 247]. Unsurprisingly this reduction in treatment efficacy is associated with poor disease control and quality of life [246]. Cluster analysis examining adult asthma patients in primary and secondary care has shown that this tends to be a female predominant, very symptomatic phenotype with no demonstrable eosinophilic airway inflammation [8, 16]. The jury is out as to whether this poor response to treatment reflects a total absence of airway inflammation or whether obesity via systemic effects switches the type of airway inflammation seen.

Atopy is an important risk factor for the development of asthma. Whilst there are a number of studies that suggest that obesity increases subsequent asthma risk (Table 2.5), the impact of obesity on the broader diagnosis of atopy is less clear. The studies examining this area to date are summarised in Table 4.1. A number of large cross-sectional studies have shown an association between obesity and atopy in adults [432, 433], with some finding this to be more marked in women [223]. However other population studies have not found a relationship [225]. The picture in children is as confusing: some studies have shown an association between BMI and atopy but only in females [224, 434], whilst others have not [435, 436].

The reason behind the conflicting findings with regards to obesity and atopy are likely to reflect differences in the study populations used: some investigators used the wider population and others targeted specific groups (attendees to allergy or difficult asthma clinics). Furthermore the definition of atopy has not been universal with the technique used to measure atopy (skin prick vs. RAST vs. clinical history) and the range of allergens screened for varying hugely between studies. In summary the association between obesity and atopy is still not certain. If there is an effect, it is likely to be modest and more marked in females.

Study design	Population	Definition of atopy	Outcome	Reference
Cross-sectional study	1459 Taiwanese students, 13.2-15.5 years.	Positive skin prick to ≥ 1 of a panel of 6 allergens.	\uparrow odds of atopy (OR 1.77) in girls within highest BMI quintile. No association in boys.	[224]
Cross-sectional cohort study	7370 children, aged 14-17 years, mixed ethnicity.	Positive skin prick test to ≥ 1 of a panel of 12 allergens.	No association with atopy. No difference in sexes.	[435]
Meta-analysis of 7 cross-sectional studies	5993 caucasian children, aged 7-12 years.	Positive skin prick test to ≥ 1 of a panel of 8 allergens.	Positive association between BMI and atopy in girls. No association in boys.	[434]
Longitudinal study	536 school children followed at 4, 8 and 10 years.	Physician diagnosed atopic asthma, eczema or allergic rhinitis.	No association with atopic conditions.	[436]
Cross-sectional study	4773 adults >20 years.	Positive RAST test to ≥ 1 of 15 allergens .	No association with atopy.	[225]
Cross-sectional study	1,997 residents in Canadian town 18-79 years.	Positive skin prick test to ≥ 1 of 4 allergens.	\uparrow odds of atopy in the obese (OR 1.5). Higher odds in women than men.	[223]
Cohort study	2090 adults, >18 years presenting to allergy clinic.	Atopy = Positive skin prick to one of 10 allergens. Atopic disease = condition + positive skin prick test.	No association between obesity and atopy. Increased odds of atopic dermatitis (OR 1.43) or atopic asthma (OR 1.98) with obesity.	[433]
Case control study	798 Chinese adults 266 cases 532 matched controls.	1: Clinical diagnosis of atopic asthma, allergic rhinitis, atopic eczema or food allergy. 2: At least 1 positive skin prick to panel of 16 common allergens .	Increased odd of atopic disease with obesity (OR 3.2). No sex differences. Only significant for rhinitis and eczema	[432]

Table 4.1: Summary of studies examining the association between obesity and atopy in children and adults. The studies are presented in chronological order.

In the context of obesity and asthma although a small study of patients presenting to an allergy clinic found obesity to be associated with an increased odds of atopic asthma but not non-atopic disease

[433], the majority of large population based studies have shown obesity to be associated with asthma more in non-atopic than atopic individuals (Table 4.2, adapted from Sideleva et al [437]).

Study design	Population	OR for asthma in atopic and non-atopic obese individuals	Reference
Cross-sectional	Canadian adults, n=86,144	↑Asthma in non-atopic obese women (OR 2.5) vs. atopic obese women (OR 1.6).	[317]
Cross-sectional	Swedish adults, n=570	↑Asthma in obese with history of allergic rhinoconjunctivitis (OR 1.53) vs. obese without history (OR 1.34).	[438]
Cross-sectional	Danish adults, n=3609	↑Asthma non-topic asthma (OR 1.31) and atopic asthma (OR 1.38) with obesity.	[439]
Cross-sectional	Canadian adults, n=1997	↑Asthma in non-atopic obese (OR 2.01), no significant association in atopic obese.	[440]
Cross-sectional	US adult cohort, n=4773	↑Asthma in non atopic obese (OR 2.5) vs. atopic obese (OR 2.04)	[225]
Cross-sectional	US paediatric cohort (2-19 years), n=16,074	↑Asthma in non atopic obese (OR 2.46) vs. atopic obese (OR 1.34)	[441]

Table 4.2: Summary of studies examining the association between obesity and asthma in atopic and non-atopic individuals. The studies are presented in chronological order.

4.1.2 Systemic inflammation in asthma and obesity

Although asthma is an inflammatory disease of the airways, there is evidence to suggest that it is associated with changes systemically in the immune system. Mechanistically many of the inflammatory cell types involved in the airway are recruited from the bone marrow via the circulation.

A longitudinal study of male adults published in 1984 demonstrated that blood leukocyte count was inversely associated with markers of lung function at baseline including FEV1 and FVC. Increasing leukocyte count over 10 years was associated with decline in these parameters after adjustment for age, height, smoking history and baseline leukocyte count [442]. Schwartz et al in 1993 examined the basic leukocyte subsets in 6,913 adults taking part in the first US National Healthy Nutrition and Examination Survey [443]. They noted that “physician diagnosed asthma” was associated with a significantly higher peripheral eosinophil count whilst “chronic bronchitis” was associated with an increased neutrophil count. Furthermore, specific symptoms were associated with a different blood count picture with chronic cough and sputum production correlating with both eosinophil and neutrophil counts whilst significant dyspnoea was associated positively with neutrophil count. The findings persisted despite adjustment for age, race, sex and cigarette smoking. Expanding on this, in 2001 Lewis et al in a British cross-sectional study examined other blood cell types and their relationship to respiratory symptoms as well as atopy, lung function and BHR. They also noted a

correlation between eosinophil count, symptoms and diagnosis of asthma, atopy, IgE levels and airways hyper-responsiveness [444]. Basophil levels showed similar trends to eosinophils. Neutrophil counts were again associated with symptoms of dyspnoea as well as sputum production, and diagnosis of asthma, especially in the older individuals. In addition monocyte count positively correlated with dyspnoea, cough, sputum production and reduced FEV1.

A French cross-sectional study of well characterised asthmatics by Nadif and colleagues, reported that blood counts were associated with specific asthma phenotypes [166]. They dichotomised eosinophil and neutrophil counts into high and low and created 4 “inflammatory patterns” based on the various permutations of high and low cell counts. High blood eosinophil count ($>250/\text{mm}^3$) across the asthmatic population studied was associated with lower FEV1, higher IgE and more active disease, whilst high neutrophil count ($>5000/\text{mm}^3$) was associated with more significant dyspnoea. Amongst asthmatics with a high eosinophil count, a co-existing high neutrophil count was associated with more nocturnal symptoms and higher asthma symptom scores than those with a high eosinophil and low neutrophil count. In non-smokers “COPD-like” symptoms (chronic cough with sputum production and significant dyspnoea), particularly chronic sputum production, were more prevalent in those with a high neutrophil count. A more recently published cluster analysis looking at adult asthma also confirmed that blood counts were associated with the different phenotypes. Neutrophil count was highest in adult onset active disease and lowest in inactive or mild childhood onset allergic disease, whilst eosinophil counts higher in actively treated allergic childhood onset eosinophilic disease and low in inactive/mild adult onset disease [208].

Obesity is also associated with chronic low grade systemic inflammation detectable by changes in leukocytes and their basic subsets. In keeping with this, a study of 477 bariatric patients undergoing laparoscopic band surgery showed obesity to be associated with a higher number of circulating total leukocytes which was due to increased lymphocytes and neutrophils. Weight loss at 2 years was associated with a significant reduction in total leukocytes, neutrophils and lymphocytes [30]. In asthmatics within the airways, sputum neutrophil levels are associated with increased asthma severity [168] and negatively correlate with lung function and specifically markers of airflow obstruction [169].

Given that obesity is associated with increased circulating neutrophil counts and that neutrophilia in asthma is associated with a more severe and treatment refractory phenotype, it is plausible that obesity skews the asthma phenotype to a neutrophilic one. A recently published study examined 246 atopic patients with and without asthma attending an immunology clinic in New York. In all of the individuals, a higher peripheral blood total leukocyte count with obesity compared to normal weight was due predominantly to increased neutrophils [445]. However, there was no healthy control arm in this study and so one cannot be certain as to whether this was just an obesity effect as seen in the bariatric studies or specific to the atopic patients.

Whilst it is clear that asthma and obesity are both associated with changes in the relative abundance of circulating leukocyte subsets, there is little work looking at the differential effects of BMI on this in asthmatic versus non-asthmatic individuals. Therefore peripheral blood count analysis was performed to determine whether obesity in the context of asthma is associated with changes in the relative proportions of leukocyte subsets at a systemic level.

4.1.3 The metabolic effects of obesity: the missing link?

4.1.3 (i) Insulin resistance

Rising BMI is associated with insulin resistance (IR) and subsequent type II diabetes risk. The prevalence of asthma and type II diabetes in the morbidly obese are broadly similar [12] leading to speculation that insulin resistance may modify asthma risk. Mechanistically, pro-inflammatory mediators and adipokines observed in obesity are known to be involved in the pathogenesis of IR [446], and therefore hypothetically, the aetiology of another inflammatory disease such as asthma in obese individuals could follow a common pathway. In addition IR is associated with a compensatory hyperinsulinaemia and *in vitro* studies suggest that this may have a direct effect of airway smooth muscle enhancing contraction [447]. Several studies have examined this hypothesis in paediatric and adult populations with conflicting results. Two paediatric [275, 276] and one adult study [277] have shown an association between IR and asthma whilst another adult study has not [225]. However most of the studies have significant limitations specifically with regards to the clarification of an asthma diagnosis which will be highlighted below.

A study of children attending an obesity management centre observed an increased prevalence of IR calculated by a homeostasis model assessment (HOMA), in obese “physician diagnosed” asthmatic children compared to obese controls [275]. A more recent cross-sectional study of school aged children noted that parent reported asthmatics were likely to have higher levels of triglycerides and IR, evidenced by the presence of acanthosis nigricans, than those who did not have asthma [276]. This observation was independent of BMI but this study lacked a robust definition of IR using a clinical sign which is not specific in obese individuals. Furthermore both of these studies lacked a robust definition of asthma and neither controlled for the effects of steroid treatment which is well known to be associated with IR. A large adult cross-sectional study of 4773 US individuals found no association between IR and self-reported asthma [225]. However a prospective Danish adult population study of 3441 individuals observed IR was a greater risk factor than BMI for the development of wheezing and asthma like symptoms [277]. Common to both of these studies was the lack of a robust definition of asthma.

4.1.3 (ii) Fatty acids

Free fatty acids (FFAs), elevated in the obese state, have a number of immunomodulatory effects of possible relevance in asthma. Saturated FFAs can activate the innate immune response through ligation of pattern recognition receptors including Toll-like receptors (TLR) 2 and 4 [278, 279], resulting in upregulation of the transcription factor nuclear factor- κ B (NF κ B) [448] and the release of pro-inflammatory cytokines such as TNF- α and IL-6 [280]. Monounsaturated fatty acids inhibit this pathway [449]. Chronically high intake of fatty acids can lead to recruitment of circulating innate cells including neutrophils [450] and such diets have been associated with bronchial hyper-responsiveness [451] and asthma risk [452]. Excess FFAs could therefore explain the asthma obesity association especially the promotion of neutrophilic inflammation but there is little work in this area to date.

4.1.3 (iii) Adipokines

Leptin

Obesity may modify its effects on asthma, through the production of adipokines; hormones produced by adipocytes with a number of metabolic and immunomodulatory effects. Leptin is the most well described of these; it has a similar structure to IL-6, promotes satiety [282] and regulates energy expenditure [283, 284]. Serum concentrations are markedly increased in obesity suggesting the possibility of relative leptin resistance [285-287].

Leptin has effects on several cell types from the innate and acquired arms of the immune system which would be of relevance in asthma [288, 289] (Table 4.3). Consistent with its immunomodulatory effects, leptin levels have been shown to be increased during the acute inflammatory response seen in sepsis syndrome [453]. A sexual dimorphism exists with regards to body fat composition with women carrying more fat subcutaneously and men viscerally. This leads to a different adipokine profile as leptin is released in greater quantities from subcutaneous tissue resulting in higher levels in females than males [327]. If adipokines were to play a role in asthma, gender differences in fat distribution and adipokine profiles could explain the female predominant obese asthma phenotype.

Innate Immunity	
Neutrophil	<ul style="list-style-type: none"> ↑ Survival [291] ↑ Chemotaxis [454] ↑ Neutrophilic airway inflammation in murine models [455]
Monocytes/Macrophages	<ul style="list-style-type: none"> ↑ Surface expression of activation markers (CD69, CD25, CD38, CD71, HLADR, CD11b) [290] ↑ Production of pro-inflammatory cytokines (TNF-α, IL-6) via NFκB ↑ Reactive oxygen species production [456] ↑ Leukotriene synthesis in alveolar macrophages [457]
Eosinophil	<ul style="list-style-type: none"> ↑ Surface expression of adhesion molecules (ICAM-1, Cd18) [292] ↑ Chemotaxis [292] ↑ Production of pro-inflammatory cytokines (IL-6, IL-8, MCP-1) [292]
NK cells	<ul style="list-style-type: none"> ↑ Proliferation [294] ↑ Differentiation ↑ Activation
Dendritic cells	<ul style="list-style-type: none"> ↑ Survival [295] ↑ Priming of Th1 response
Adaptive immunity	
T cells	<ul style="list-style-type: none"> ↑ Activation [458] ↑ Proliferation [458] ↑ Th1 polarisation [288] ↓ Treg proliferation [345] ↑ Th17 response [459]

Table 4.3: Mechanistic effects of leptin on cellular components of the innate and adaptive immune system.

Study design	Population	Asthma definition	Outcome	Reference
Case-control	102 steroid naive asthmatic children. 33 controls.	Physician diagnosed asthma.	↑ leptin in asthmatics, independent of BMI. Only significant in males.	[303]
Longitudinal study	138 children followed up over 12 years.	Physician diagnosed asthma.	↑ leptin in overweight asthmatics vs. normal weight asthmatics and control group.	[302]
Case-control	23 children with new diagnosis of asthma. 20 controls.	Physician diagnosed asthma	Newly diagnosed asthmatics had higher leptin levels than controls, independent of BMI. Leptin levels dropped to levels comparable with controls after 4 weeks of ICS.	[304]
Case-control study	186 asthmatic children. 54 controls.	BHR to methacholine or 12 % reversibility to β_2 agonist.	No difference between asthmatics and controls. Leptin negatively correlated with FEV1 and FEF25-75% in asthmatics.	[307]
Cross-sectional	462 children.	Parental reported asthma.	↑ leptin in asthmatics vs. non asthmatics, independent of BMI. More marked in girls. More marked in non-atopics.	[305]
Interventional weight loss study	84 post-pubertal obese asthmatics.	Physician diagnosed asthma.	↓ leptin levels with weight loss predictive of improvement in lung function.	[460]
Cross-sectional study	5876 adults.	Self reported asthma.	↑ leptin levels in asthmatics, independent of BMI. Stronger association in premenopausal women.	[24]
Longitudinal study	Prospective cohort study: 2620 adults.	Physician diagnosed asthma.	Weak association between leptin and asthma. Did not persist after adjusting for covariates.	[309]
Case-control study	35 steroid naive female asthmatics. 32 female controls	12% reversibility to β_2 agonist or diurnal variability in PEF >20%.	No difference in leptin levels between asthmatics and controls. Leptin correlated positively with asthma symptom score and negatively with FVC (%) and FEV1 (%).	[308]
Case-control study	37 stable female post menopausal asthmatics. 32 female asthmatics during exacerbation.	Physician diagnosis.	Stable asthmatics: ↑ leptin levels vs. controls. Severe asthmatics: ↑ leptin levels vs. mild-moderate. Atopic asthmatics higher leptin vs. non-atopics. Leptin negatively correlated with FEV1 (%) and MEF25-75 (%). ↑ leptin levels in exacerbation.	[306]

Table 4.4: Summary of paediatric and adult studies examining the association between leptin and asthma. Studies are listed in date order.

In murine models , leptin infusion augments allergen induced airway hyper-responsiveness but without eosinophil influx or Th2 responses, suggesting it causes effects by an alternative inflammatory pathway [23]. There are similar findings in non-allergen induced airways inflammation models [461]. Despite the immunological effects of leptin and the promise in animal models of airways disease, human studies have not shown convincing evidence that leptin underlies the obesity asthma association (Table 4.4). A single paediatric study which examined leptin levels in normal weight and overweight children with and without physician diagnosed asthma found that the overweight asthmatics had significantly higher levels of leptin than normal weight asthmatics, overweight and normal weight controls [302]. However all other paediatrics and adult studies which have shown an association between leptin and asthma seem to suggest that its effects are independent of BMI. Some have suggested that leptin levels increase with severity of asthma and may correlate negatively with lung function.

Adiponectin

Adiponectin is an insulin sensitising hormone and levels decrease with obesity [297]. It has anti-inflammatory effects including induction of IL-10 and IL-1 receptor antagonist expression by adipose macrophages [298]. It also inhibits production of pro-inflammatory cytokines (IL-6 and TNF α) by macrophages [462]. In mice, administration of adiponectin attenuates bronchial hyper-responsiveness, Th2 cytokine expression and neutrophilia [463]. *In vitro* studies suggest that adiponectin has direct effects on the bronchial epithelium promoting proliferation and wound repair [464] .

In children low adiponectin levels have been associated with increased prevalence of symptoms of atopic dermatitis, asthma and eczema [305]. A large cross sectional study in adults has suggested that high levels of adiponectin may be protective against current asthma in pre-menopausal women, although this effect was independent of BMI [310]. However two other studies, one longitudinal [309], one cross-sectional [465], have not shown an association between this adipokine and asthma. Leptin to adiponectin ratios have been associated with more severe disease in female asthmatics and with periods of exacerbation independent of BMI [306]. A very recent longitudinal cohort study of 1450 women found that low levels of adiponectin were predictive of developing subsequent asthma in those that were pre-menopausal and was a stronger predictor than BMI. This was most marked in smokers[311].

Resistin, Visfatin and Ghrelin

Resistin is an insulin desensitising adipokine elevated in obesity [299]. It has pro-inflammatory effects including the up regulation of TNF α production by macrophages through activation of NF κ B [466]. A single paediatric study found that resistin levels were significantly lower in atopic asthmatics studied compared to non-atopic asthmatics and healthy controls. Furthermore, levels correlated positively with methacholine PC20 and negatively with blood eosinophil counts and IgE levels [307]. A single adult cohort study found that resistin is elevated in asthma and correlates with disease

severity, independent of BMI [312]. More recently it has been suggested that high baseline levels may predict steroid responsiveness in steroid naive patients [308].

Visfatin also known as nicotinamide phosphoribosyltransferase, is produced by a number of cell types including adipocytes, lymphocytes, monocytes, neutrophils and pneumocytes [300]. It has an insulin mimetic effect and circulating levels are increased in obesity [300]. It has a number of pro-inflammatory effects including activation of NF κ B resulting in the production of inflammatory cytokines (TNF α , IL-1 β and IL-6), inhibition of neutrophil apoptosis, increased ROS generation and promotion of B cell maturation [300]. Interestingly a single paediatric case-control study found significantly lower levels of this adipokine in the asthmatics studied compared to healthy controls. However no differences were seen between BMI groups within the asthmatics studied [313]. No studies to date have examined whether there are changes in this adipokine in adult patients.

Ghrelin, a gut rather than adipose tissue derived hormone, which is reduced in the obese state has been shown to counteract the effects of leptin on monocytes/macrophages [301]. Circulating levels are inversely correlated with IgE in obese children suggesting a mechanistic role in atopic asthma [467]. In adults a single study has suggested that ghrelin levels may be reduced during asthma exacerbations [306].

In summary most studies to date have focussed on leptin and adiponectin and their potential role in the pathophysiology of asthma. Results are conflicting and whilst some mechanistic and population studies suggest a potential association between these adipokines and asthma, this appears to be independent of BMI. Very little work has been done on the other adipokines, but what little there is suggests that these may be associated with asthma.

4.2 Methods

A detailed description of the recruitment process is described in chapter 3 but will be summarised here.

4.2.1 Study population

Pre-menopausal women with and without asthma were recruited. Subjects and controls were divided into 3 groups on the basis of body mass index (BMI), giving 6 groups in total (Chapter 3 Figure 3.1). According to the WHO definition, normal weight is defined as BMI 18.5-25kg/m², overweight as a BMI \geq 25kg/m² and $<$ 30kg/m² and obesity as a BMI \geq 30kg/m².

Asthmatic patients were recruited from a number of local healthcare providers:

- Local tertiary asthma clinic (Singleton Hospital, ABMU Health Board (HB))
- Surrounding secondary care clinics (ABMU HB hospitals in Neath Port Talbot, Morriston and Bridgend)
- Participating GP surgeries (Ty'r Felin surgery, Gorseinon)

After case note review, all asthmatic subjects fulfilling the inclusion/exclusion criteria (Table 3.1) attended an appointment at a tertiary asthma service. Asthma diagnosis was confirmed by a respiratory physician. The diagnosis required consistent symptoms and demonstrable significant reversible airways obstruction to a β 2 agonist (12%), significant PEFV variability or if this was not present, a positive bronchial provocation test. Disease severity was graded according to GINA criteria. In those with very good disease control, therapy was stepped down to the lowest level to maintain this, in keeping with British Thoracic Society (BTS) guidelines. All asthmatics were considered stable if they had no exacerbations, oral steroid therapy or respiratory tract infection in the preceding 4 weeks.

Asthmatics were asked to complete a modified European Respiratory Health Survey [391] (Appendix V) and the following information recorded; age of onset; markers of symptom control in last 12 months; medication; emergency contact with healthcare providers; days off work, co-morbidities; Epworth score; details of menstrual cycle.

Control patients were recruited from a number of sources:

- Staff, ABMU HB
- Staff, Swansea University
- Women attending local Slimming world groups

Those considering taking part completed a standardised questionnaire recording basic demographics, co-morbidities, medication history, smoking history, presence of clinical atopy (history of atopic, asthma, eczema or rhinitis), Epworth score, details of menstrual cycle and a validated bronchial symptoms questionnaire (Appendix IV) [387]. Control subjects were not included if they had any respiratory symptoms determined by completion of a modified bronchial symptom

questionnaire, history of clinical atopy, current smoking history or were ex-smokers with >10 pack year history.

4.2.2 Clinical measures

Participants were asked to attend during the first 7 days of their menstrual cycle, determined using the date of onset of menstruation, in the fasted state (from midnight) between the hours of 07.00-09.00am. Acute disease control at the time of recruitment in the asthmatics was assessed using the Juniper Asthma Control Questionnaire (Appendix VI) [392]. Chronic disease control was graded using the GINA criteria (Appendix VII) [393].

Spirometry was performed using a portable dry spirometer (Vitalograph) calibrated on the day of use. All asthmatics were asked to withhold medication for the preceding 24 hours to control for any effects of long acting bronchodilators or immediate effects of ICS. The best of 3 measurements were taken according to a standardised protocol [394] and expressed as percentage of the age, gender and stature predicted values. The following spirometric measurements were recorded: Forced Expiratory Volume in 1 second (FEV₁), Forced Vital Capacity (FVC), FEV₁/FVC ratio, Forced Expiratory Flow at 25%-75% of expired vital capacity (FEF₂₅₋₇₅) and Peak Expiratory Flow (PEF).

Following this measures of adiposity were taken:

- Height; to 0.1cm by staedimeter (Leicester; Chasmores, UK)
- Weight; to 0.1kg and body mass index calculated
- Body fat percentage to 0.1% (Body composition analyser, Tanita SC 240 MA; Tanita UK)
- Waist and hip measurements to 0.1cm and ratio calculated

Waist and hip measurements were taken with the subject relaxed with their hands by their sides and feet relaxed at the end of tidal expiration. Waist measurements are taken at the midpoint between the last palpable rib and the iliac crests, and the hip measurement around the widest portion at the level of the buttocks. Body fat composition was estimated using biometric impedance.

Fasting blood was collected into lithium heparin tubes and gel and clot activator tubes for serum for the following investigations:

- Full blood count analysis using a CELL-DYN Ruby (Abbott Diagnostics, Germany).
- Serum archived for circulating adipokines, insulin, glucose and free fatty acids.

4.2.3 Measurement of serum parameters (adipokines, insulin, glucose and free fatty acids, total and specific IgE)

Serum samples were collected in Vacuette® tubes (gel and clot activator tubes) and centrifuged 90 minutes after collection (2500 rpm, 4°C for 10 minutes). Measurement of serum adipokines was done using a bioplex (multiplex) assay (Biorad USA). The principles behind this assay and the protocol used are summarised in section 3.12.1. On the first run of samples it was noted that leptin

and resistin levels were higher than the range of the assay when diluted at $\frac{1}{4}$ whilst many of the visfatin and ghrelin levels were below the detectable range. Due to resource limitations it was only possible to repeat the assay once and therefore leptin and resistin levels were repeated at 1/20 dilution and the visfatin and ghrelin levels were dichotomised into detectable versus not detectable.

Serum glucose was measured by an automated ILab 300 analyser (Instrumentation Laboratory UK Ltd). The analyser determines glucose concentration using the principle of a glucose oxidase assay (see section 3.12.2). Plasma insulin was also measured using a commercially available immunoassay (Life Technologies, UK) (section 3.12.3). Insulin resistance (IR) and beta cell function was calculated using the homeostatic model assessment (HOMA). This is a mathematical equation which describes glucose regulation based on physiological studies [468, 469]. A computer programme (HOMA calculator version 2.2) was used to derive the values which is now the accepted standard [470].

Serum free fatty acids (FFA) were analysed using a commercially available assay (Wako NEFA-C kit, Alpha Labs, UK) (section 3.12.4). In addition serum total IgE (Ku/L) was measured using a sandwich ELISA (Elecsys IgE assay, Roche Diagnostics, UK) by the Department of Chemical Pathology at Morrision Hospital (section 3.13). In the asthmatics specific IgE antibodies were measured against 6 common allergens (cat dander, dog dander, house dust mite, mixed moulds, mixed trees and mixed grasses). Evidence of atopy was defined as positive specific IgE ($>0.35\text{kU/l}$) to one or more of this panel.

4.2.4 Statistical analysis

Each the variables measured was examined visually using a histogram for evidence of deviation from normality and the Kolmogorov-Smirnov (KS) goodness of fit test was performed to verify the distribution. Positively skewed data were logarithmically transformed before analysis. Differences in normally distributed variables between groups were analysed using an unpaired t-test or 1 way analysis of variance (ANOVA). Associations between normally distributed continuous variables were examined using a Pearson's correlation coefficient. Potential confounders were added as a factors or covariates sequentially to a general linear model to see if they affected the significance of the association between the parameters of interest. Data that was not normally distributed after logarithmic transformation was analysed using non-parametric tests (Mann Whitney U and Kruskal Wallis tests). Categorical variables were analysed using a chi-squared test of association.

4.3 Results

4.3.1 Demographics

In total, 84 female participants who fulfilled the inclusion criteria for the study were recruited. The target number of 90 was not reached because of difficulties in recruitment due to a number of factors. Exclusion of any significant smoking history or recent/current oral steroid use reduced a large number of potential patients in secondary care, as did the exclusion of patients with diabetes or other cardio-respiratory disease, especially within the obese subgroup. In addition many women were taking hormonal treatments which rendered them amenorrhoeic and therefore they could not be sampled at the required time within the menstrual cycle. Coordinating sampling around this whilst avoiding any recent episode of infection or oral steroid use (within 6 weeks) further added to the recruitment challenge.

The basic demographics are summarised in Table 4.5. All the parameters listed approximated a normal distribution and passed the KS test apart from day of sampling and Epworth score ($p=0.133$ and $p=0.02$ respectively). Day of sampling was logarithmically transformed for analysis and Epworth score was analysed using a non-parametric (Kruskal Wallis) test. In addition, categorical data (number of patients on oral contraceptives) was analysed using a chi-squared test.

Although all women recruited were pre-menopausal, age did differ significantly across the 6 study groups, but did not between asthmatics and controls ($p=0.298$). Across the whole study group obese individuals (OB) were significantly older (mean 38.0 years) than normal weight (NW) (mean 33.2 years) ($p=0.033$) and overweight individuals (OW) (mean 33.1 years), ($p=0.08$). However each BMI category was well matched in terms of age between asthmatics and non-asthmatics (Table 4.5).

The day of the menstrual cycle on which fasting blood and measurements were performed did not differ between groups ($p=0.961$). Patients with a history of sleep apnoea or Epworth score ≥ 11 were excluded from the study. There was no significant difference in Epworth score across the 6 categories.

Every effort was made to exclude patients with significant cardio-respiratory disease, diabetes or other systemic inflammatory diseases. As expected, despite these efforts the obese patients had more co-morbidities than the other groups (Table 4.5). One patient in the NW controls had anxiety and one OW control was on dietary modification for raised cholesterol. Six OB controls had minor co-morbidities (anxiety =2, hypothyroidism=2, 1 irritable bowel syndrome (IBS) = 1, hypertension with raised cholesterol =1). In the asthmatics 2 OW patients (depression =1 and reflux =1) and 4 OB asthmatics (Gastro-oesophageal reflux disease (GORD) =3, depression =1, and hypothyroidism =1 (who also had GORD)) had co-morbidities. However across the entire study group asthmatics did not have a higher number of comorbidities than controls ($p=0.625$). Obese individuals across the study group had significantly more co-morbidities ($n=10$) than OW ($n=3$) and NW individuals ($n=1$) ($p=0.007$). When comparing asthmatics and controls each BMI category was well matched in terms of the number of comorbidities (Table 4.5).

	Controls			Asthmatics			p value
	NW n=15	OW n=13	OB n=15	NW n=14	OW n=12	OB n=15	
Age (yrs) Mean (SD)	33.27 (7.38)	33.38 (9.34)	39.27 (8.30)	33.21 (9.38)	29.17 (8.80)	36.8 (9.00)	All 6 groups: p=0.015 ⁽¹⁾ NW: A vs. C: p=0.987 OW: A vs. C: p=0.258 OB: A vs. C: p=0.440
Cycle day sampled Geometric mean (SD)	4.80 (1.94)	4.23 (1.16)	4.27 (1.87)	4.36 (2.27)	4.33 (1.72)	4.40 (2.06)	All 6 groups p=0.961
Weight (kg) Geometric mean (SD)	59.14 (6.18)	72.23 (7.70)	94.27 (18.4)	58.81 (5.13)	72.13 (4.58)	100.79 (25.4)	NW: A vs. C: p=0.458 OW: A vs. C: p=0.968 OB: A vs. C: 0.403
BMI (Kg/m²) Geometric mean (SD)	21.61 (2.03)	27.53 (1.81)	36.09 (6.21)	22.46 (1.60)	27.34 (1.34)	38.84 (9.17)	NW: A vs. C p=0.264 ⁽²⁾ OW: A vs. C p=0.781 ⁽²⁾ OB: A vs. C p=0.278 ⁽²⁾
Body fat (%) Mean (SD)	26.27 (4.32)	36.31 (3.38)	45.07 (4.73)	28.12 (4.73)	35.91 (4.32)	45.85 (7.07)	NW: A vs. C p=0.283 ⁽²⁾ OW: A vs. C p=0.801 ⁽²⁾ OB: A vs. C p=0.731 ⁽²⁾
WHR (%) Mean (SD)	76.8 (4.04)	81.0 (5.32)	87.1 (5.74)	80.2 (8.57)	81.5 (5.77)	87.5 (7.5)	NW: A vs. C: p=0.191 ⁽²⁾ OW: A vs. C p=0.821 ⁽²⁾ OB: A vs. C p=0.878 ⁽²⁾
Epworth score Median (SD)	1.05 (3.27)	3.23 (3.93)	2.72 (2.97)	2.71 (1.76)	3.15 (3.94)	2.74 (2.67)	All 6 groups: p=0.303
Co-morbidities (n)	1	1	6	0	2	4	All 6 groups: p=0.041 ⁽³⁾ NW: A vs. C: p=.326 OW: A vs. C: p= 0.490 OB: A vs. C: p=0.439
Contraceptive pill (n)	3	3	2	3	6	2	All 6 groups p=0.245
Other medication (n)	1	1	5	0	2	7	All 6 groups: p=0.009 ⁽⁴⁾ NW: A vs. C: p=0.326 OW: A vs. C: p=0.469 OB: A vs. C: p=0.709

Table 4.5: Basic demographic data of asthmatics and controls involved in the study according to BMI category.

Normally distributed data are expressed as mean and standard deviation (SD). Parameters not normally distributed are expressed as geometric mean and standard deviation and were logarithmically transformed before analysis.

1. Obese participants were significantly older than the other groups; however BMI categories were well matched in terms of age between controls (C) and asthmatics (A).
2. BMI categories were well matched in terms of BMI, body fat composition and WHR between asthmatics and controls.
3. Chi-squared analysis found that obese individuals had a greater number of comorbidities than the OW and NW. However BMI categories were well matched in terms of comorbidities between asthmatics and controls.
4. Chi-squared analysis found that obese individuals were on a greater number of medications than the OW and NW. However BMI categories were well matched in terms of medications between asthmatics and controls.

The number of patients on medication in addition to the asthma medications is listed in Table 4.5. Across the study group a similar number of asthmatics (n=9) were on additional medications to controls (n=7) (p=0.508). The obese participants were on more medications (n=12) than OW (n=3) and NW groups (n=1) (p=0.01). One NW control was on a selective serotonin reuptake inhibitor (SSRI) and one OW control took orlistat. In the OB controls, two were on levothyroxine, two were on SSRIs and one patient took an antihypertensive and statin. Of the asthmatics one OW asthmatic took a proton pump inhibitor (PPI) and antihistamine and one took a tricyclic antidepressant. In the obese asthmatics three patients were on PPIs, one patient took orlistat, two patients took antihistamines and one an SSRI. However the BMI categories were well matched in terms of medication use between asthmatics and controls (Table 4.5). There was no significant difference in the percentage of patients on the combined oral contraceptive pill.

4.3.2 Adiposity measures

Weight and BMI did not pass the KS test (p=0.036 and p=0.067 respectively) and so data was logarithmically transformed for analysis. Each BMI group was well matched between asthmatics and controls (Table 4.5). Body fat composition and waist-hip ratio did pass the KS test (p=0.944, p=0.98) and did not differ significantly between asthmatics and controls across any of the BMI groups (Table 4.5). There was a very strong correlation between BMI and body fat composition (r=0.882 p<0.001) and also between BMI and waist to hip ratio (WHR) (r=0.572, p<0.001) (Figure 4.1).

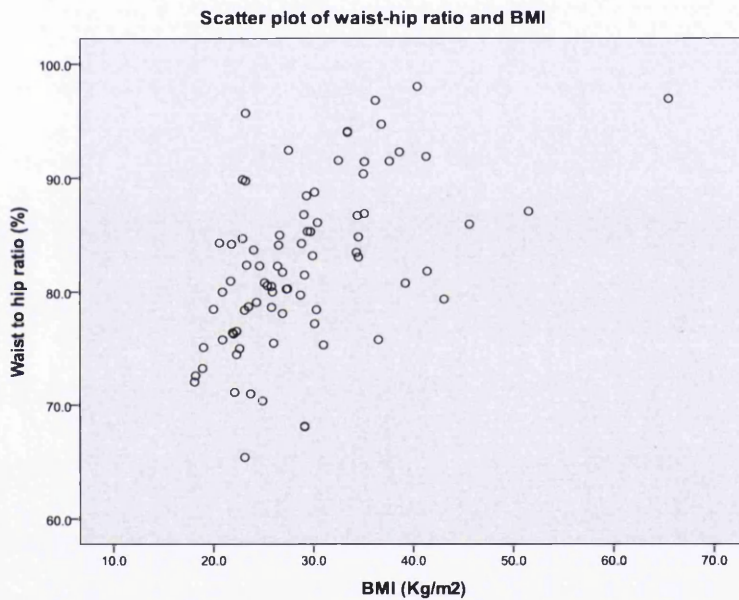
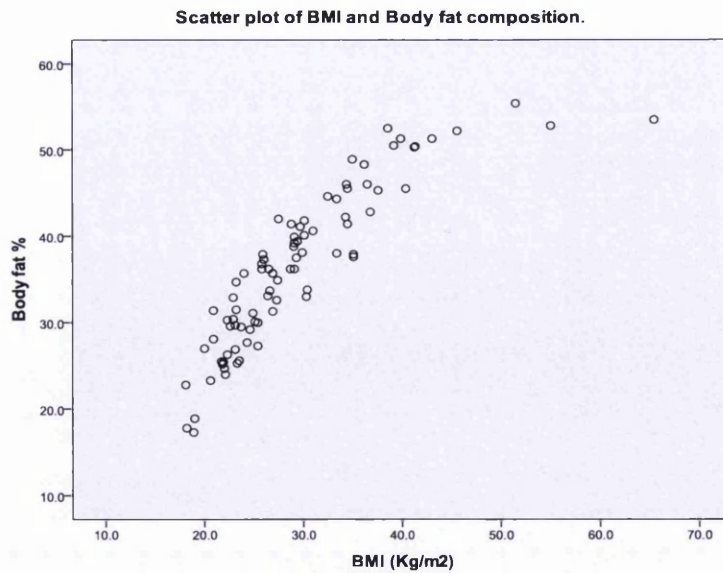


Figure 4.1: Scatter plots of BMI and A: percentage body fat, B: waist-to hip ratio.

4.3.3 Spirometry

All measures taken from spirometry approximated a normal distribution, passing the KS test and are summarised in Table 3.6. When examining the 6 groups of individuals there were significant differences in all of the spirometric values measured. FEV1 expressed as a percentage predicted (%) according to age, sex and height significantly differed across the 6 groups, being significantly lower in asthmatics (mean =79.54%) than controls (mean =100.30%) ($p=0.003$). FEV1(%) did not significantly

vary with BMI category or BMI as expressed as continuous variable across the entire cohort ($p=0.299$; $r=-0.128$, $p=0.248$) or within the control and asthmatic groups individually ($p=0.268$; $r=0.013$, $p=0.771$ and $p=0.214$; $r=-0.163$, $p=0.136$ respectively). No correlations between percentage body fat (%), WHR and FEV1 (%), were seen across the entire study group or in the asthmatics and controls separately.

FVC expressed as a percentage predicted (%) also significantly differed between the 6 groups. Asthmatics had significantly lower FVC (%) than controls and FVC (%) decreased significantly with increasing BMI category across ($p=0.019$) but not with BMI as a continuous variable across the entire study group ($r=-0.168$, $p=0.129$). When the asthmatics and controls were analysed individually no significant association between FVC (%) and BMI category or continuous BMI was seen in the asthmatics ($p=0.090$, $r=-0.280$, $p=0.081$) or controls ($p=0.090$; $r=0.017$, $p=0.913$). In addition no associations were seen between FVC(%) and percentage body fat, WHR and across the entire study group or in the asthmatics and controls separately.

FEV1/FVC ratio, a marker of airflow obstruction, significantly differed across the 6 groups and this with significantly lower levels in the asthmatics (mean =76.68%) than controls (88.87%) (Table 3.6). There was no significant associations with this parameter and BMI categories or continuous BMI over the entire study group ($p=0.79$; $r=-0.563$, $p=0.569$) or when controls ($p=0.258$; $r=-0.196$, $p=0.208$) and asthmatics ($p=0.958$; $r=0.081$, $p=0.613$) were examined individually. In addition no correlations was seen for FEV1/FVC ratio with WHR and percentage body fat across the entire group or in asthmatics/controls separately.

PEFR expressed as a percentage predicted (%) was significantly lower in the asthmatics (mean =78.72%) than the controls (mean= 97.26%) ($p<0.001$). However, again no association with BMI category or continuous BMI were seen across the entire study population ($p=0.560$; $r=-0.26$, $p=0.816$) or when controls and asthmatic groups were analysed individually ($p=0.199$; $r=-0.196$, $p=0.208$ and $p=0.551$; $r=0.081$, $p=0.613$ respectively). WHR and percentage body fat were not significantly correlated with this variable.

MEF25-75 expressed as a percentage predicted (%) and a surrogate marker of small airways disease, was also significantly lower in asthmatics (mean =57.38%) than control subjects (mean =90.40%) ($p<0.001$). No association was seen with BMI category BMI as a continuous variable across the entire cohort ($p=0.985$; $r=-0.26$, $p=0.569$) or when control and asthmatics groups were analysed individually (($p=0.407$; $r=-0.45$, $p=0.782$) and ($p=0.877$; $r=0.149$, $p=0.340$) respectively).

	Controls			Asthmatics			p value
	NW n=15	OW n=13	OB n=15	NW n=14	OW n=12	OB n=15	
FEV1 (L) Mean (SD)	3.16 (0.31)	2.80 (0.36)	2.85 (0.56)	2.49 (0.60)	2.39 (0.83)	2.17 (0.72)	6 groups: p<0.001 A vs. C: p<0.001 BMI: p= 0.156
FEV1 (%) Mean (SD)	102.40 (8.98)	95.46 (14.72)	102.40 (11.06)	86.43 (15.33)	77.09 (25.49)	74.9 (18.34)	6 groups: p<0.001 A vs. C: p=0.003 BMI: p=0.299
FVC (L) Mean (SD)	3.69 (0.39)	3.16 (0.44)	3.24 (0.67)	3.23 (0.56)	3.09 (0.82)	2.79 (0.65)	6 groups: p=0.007 A vs. C: p=0.014 BMI: p=0.019
FVC (%) Mean (SD)	102.87 (10.04)	92.77 (14.60)	101.73 (13.46)	97.86 (12.17)	87.18 (20.89)	85.50 (13.92)	6 groups: p=0.004 A vs. C: p=0.006 BMI: p=0.042
FEV1/FVC (%) Mean (SD)	91.53 (13.28)	88.54 (3.21)	86.20 (5.75)	76.05 (11.40)	77.58 (16.19)	76.54 (13.15)	6 groups: p<0.001 A vs. C: p<0.001 BMI: p=0.709
PEF (L/min) Mean (SD)	407.13 (44.90)	374.46 (58.45)	400.93 (86.90)	343.86 (74.00)	322.25 (97.19)	309.60 (119.40)	6 groups: p=0.008 A vs. C: p=0.012 BMI: p=0.499
PEF (%) Mean (SD)	96.60 (12.15)	92.08 (14.13)	102.40 (17.87)	84.29 (17.06)	75.73 (23.65)	75.73 (24.81)	6 groups: p=0.001 A vs. C: p<0.001 BMI: p=0.560
MEF25-75 (L/s) Mean (SD)	3.39 (0.69)	3.38 (0.56)	3.42 (0.90)	2.24 (0.94)	2.51 (1.26)	2.07 (0.96)	6 groups: p<0.001 A vs. C: p<0.001 BMI: 0.747
MEF25-75 (%) Mean (SD)	87.67 (16.12)	87.69 (17.77)	95.47 (19.76)	59.00 (22.47)	59.0 (31.76)	54.68 (23.81)	6 groups: p<0.001 A vs. C: p<0.001 BMI: p=0.985

Table 4.6: Spirometry measurements of asthmatics and controls according to BMI category.

Data were normally distributed and therefore expressed as mean and standard deviation (SD). Differences between all 6 groups and between BMI categories across the entire study group were analysed using a one-way ANOVA and comparisons between asthmatics (A) and controls (C) made with an unpaired t-test.

4.3.4 Clinical characteristics of the asthmatics studied

The variables regarding age of asthma onset and asthma duration passed the KS test and are shown in Table 4.7. The mean age of onset was 12.8 years and did not significantly differ between BMI groups ($p=0.517$). Similarly, mean asthma duration across the study was 21.7 years and did not differ significantly between groups ($p=0.771$). Percentage reversibility in FEV1 to β_2 agonist did not pass the KS test ($p=0.008$) and so was logarithmically transformed before analysis. By definition, all participants had at least 12% reversibility to a β_2 agonist (with the exception of 3 cases, 2 of which had significant diurnal variation on peak flow and the 3rd had a positive methacholine challenge test). The degree of reversibility observed did not significantly vary between BMI groups ($p=0.843$). The proportion of patients with a history of clinical atopy or a positive specific IgE to at ≥ 1 of a panel of 6 airborne allergens was high across the asthmatics but did not vary across the 3 BMI groups.

	NW n=14	OW n=12	OB n=15	p value
Age of onset (yrs) (Mean)	12.4	9.08	13.8	0.517 ⁽¹⁾
Duration (yrs) (Mean)	20.6	20.4	23.6	0.771 ⁽¹⁾
Reversibility (%) (Geometric mean)	19.64	22.24	22.4	0.843 ⁽²⁾
Atopy history (n)	12	9	12	0.833 ⁽⁴⁾
Atopy according to specific IgE panel (n)	9	9	10	0.827 ⁽⁴⁾
Asthma severity: GINA grading (n)				
Grade 1: Intermittent	2	1	2	0.742 ⁽⁴⁾
Grade 2: Mild persistent	2	2	1	
Grade 3: Moderate persistent	7	3	7	
Grade 4: Severe persistent	3	6	5	
β_2 agonist use (puffs per month) (Geometric mean)	44	66	97	0.175 ⁽²⁾
Inhaled corticosteroid dose (mcg) (Beclomethasone dipropionate equivalent) (Median)	1600	1600	1600	0.822 ⁽³⁾
LABA use (n)	10	12	12	0.137
Monteleukast use (n)	4	7	7	0.344 ⁽⁴⁾
Theophylline use (n)	1	4	4	0.244 ⁽⁴⁾
Asthma control score (0-6) (Geometric mean)	1.74	2.22	2.29	0.435 ⁽²⁾
Emergency hospital attendances in last year (Median)	0	0	0	0.875 ⁽³⁾
Emergency GP attendances in last year (Median)	2	2	2	0.638 ⁽³⁾

Table 4.7: Clinical characteristics of the asthmatics studied.

1. Normally distributed are expressed as a mean and analysed using a one way ANOVA.
2. Positive skewed data are expressed as a geometric mean and were logarithmically transformed before analysis by ANOVA.
3. Multimodal data are expressed as a median and analysed using Kruskal Wallis analysis.
4. Proportional data are represented as an absolute number and analysed using a chi-squared test.

A high proportion of patients, n=31/41 had moderate or severe persistent disease (GINA grade 3/4) with only a small number recruited from primary care practices having intermittent or mild disease. However the 3 BMI categories were matched in terms of the proportion of patients with each disease grading (p=0.742).

β_2 agonist use did not pass the KS test (p=0.026) and so was logarithmically transformed for analysis. Whilst the obese group had higher β_2 agonist use (geometric mean 97 puffs per month) than the normal weight individuals (44 puffs per month) this was not significant (p=0.110). When BMI was examined as a continuous variable there was a positive association with β_2 agonist use (r=0.330, p=0.035). Inhaled corticosteroid dose expressed as micrograms (mcg) of beclomethasone dipropionate equivalent, did not pass the KS test (p=0.045). As the majority of patients were recruited from a tertiary care asthma service it was not surprising to note that the median dose used was high at 1600mcg. However this did not differ between BMI groups (p=0.822). Similarly there was no significant difference in the proportion of patients using a long acting beta-2 agonist, leukotriene receptor blocker or theophylline (Table 4.7). Asthma control score passed the KS test (p=0.253). There was no clinically significant difference in asthma control score (>0.5) between the groups (Table 4.7). Similarly asthma control score did not correlate with BMI as a continuous variable (r=0.233, p=0.141) or other adiposity measures.

4.3.5 Haematology analysis data

Haematology analysis data across the 6 subgroups are summarised in Table 4.8. Total leukocyte count (p=0.064), eosinophil count (p<0.001) and basophil count (p=0.001) and did not pass the KS test as data was positively skewed, and so was logarithmically transformed for statistical analysis.

Leukocyte counts differed significantly across the 6 groups (p=0.001). Asthmatic patients had a significantly higher count than control subjects (p<0.001) when the categories were compared as a whole and when each BMI category was compared individually (NW asthma vs. NW control p=0.010, OW asthma vs. OW control p=0.034, OB asthma vs. OB control p=0.064). Leukocyte count increased significantly with BMI category across the entire study population (p=0.039) and with continuous BMI (r=0.288, p=0.008). When asthmatics (r=0.245, p=0.012) and controls (r=0.314, p=0.0410) were analysed separately significant correlations between BMI and leukocyte count were seen in both groups. Across the entire study group age was not associated with leukocyte count. Within the asthmatics, asthma duration, asthma control score, and spirometric values did not correlate with leukocyte count. Using a general linear model it was possible to see that leukocyte count had independent associations with BMI and asthma (p=0.011 and p<0.001 respectively, $R^2 = 0.228$). Obese asthmatics had the highest white blood cell count which was higher than obese controls (p=0.053) and normal weight asthmatics (p=0.113) (Figure 4.2) although this was not significant. Across the entire cohort percentage body fat and WHR were also significantly correlated with leukocyte count (r=0.219, p=0.046 and r=0.224, p=0.043 respectively).

	Controls			Asthmatics			p value
	NW n=15	OW n=13	OB n=15	NW n=14	OW n=12	OB n=15	
Red blood cells ($\times 10^9/l$) Mean (SD)	4.53 (0.44)	4.73 (0.32)	4.79 (0.28)	4.70 (0.24)	4.68 (0.40)	4.53 (0.37)	6 groups: p=0.229 A vs.C: p=0.443 BMI: 0.618
Leukocyte count⁽¹⁾ ($\times 10^9/l$) Geometric mean (SD)	5.08 (1.05)	5.39 (1.44)	6.13 (1.54)	6.34 (1.29)	6.73 (1.72)	7.23 (1.98)	6 groups p=0.001 A vs.C: p<0.001 BMI: p=0.039
Neutrophil count ($\times 10^9/l$) Mean (SD)	2.60 (0.86)	2.82 (0.79)	3.37 (0.75)	3.22 (0.83)	3.52 (1.05)	4.38 (1.83)	6 groups: p=0.001 A vs.C: p=0.003 BMI: p=0.005
Lymphocyte count ($\times 10^9/l$) Mean (SD)	2.13 (0.48)	2.23 (0.82)	2.39 (0.92)	2.36 (0.74)	2.49 (0.79)	2.41 (0.50)	6 groups p=0.785 A vs. C: p=0.277 BMI: p=0.670
Monocyte count ($\times 10^9/l$) Mean (SD)	0.30 (0.09)	0.32 (0.09)	0.30 (0.10)	0.39 (0.09)	0.44 (0.21)	0.34 (0.13)	6 groups: p=0.025 A vs. C: p=0.05 BMI: p=0.276
Eosinophil count⁽¹⁾ ($\times 10^9/l$) Geometric mean (SD)	0.10 (0.03)	0.12 (0.06)	0.14 (0.17)	0.32 (0.24)	0.20 (0.22)	0.18 (0.17)	6 groups: p<0.001 A vs.C: p<0.001 BMI: p=0.560
Basophil count⁽¹⁾ ($\times 10^9/l$) Geometric mean (SD)	0.07 (0.16)	0.07 (0.03)	0.08 (0.23)	0.08 (0.03)	0.09 (0.17)	0.08 (0.14)	6 groups: p=0.891 A vs.C: p=0.390 BMI: p=0.983
Platelets ($\times 10^9/l$) Mean (SD)	158.90 (78.36)	163.00 (103.10)	175.20 (32.72)	173.18 (71.42)	169.21 (62.83)	162.32 (141.52)	6 groups: p=0.993 A vs. C: 0.924 BMI: p=0.990

Table 4.8: Haematology analysis data in asthmatics and controls according to BMI category. Data unless otherwise indicated are displayed as mean and standard deviation (SD). Parameters not normally distributed were logarithmically transformed before analysis and expressed as geometric mean and standard deviation (SD).

Comparison across the 6 study groups made using one way ANOVA. Differences between asthmatics (A) and controls (C) determined using unpaired t-test. Comparison of the 3 BMI categories across the entire study group made using one way ANOVA.

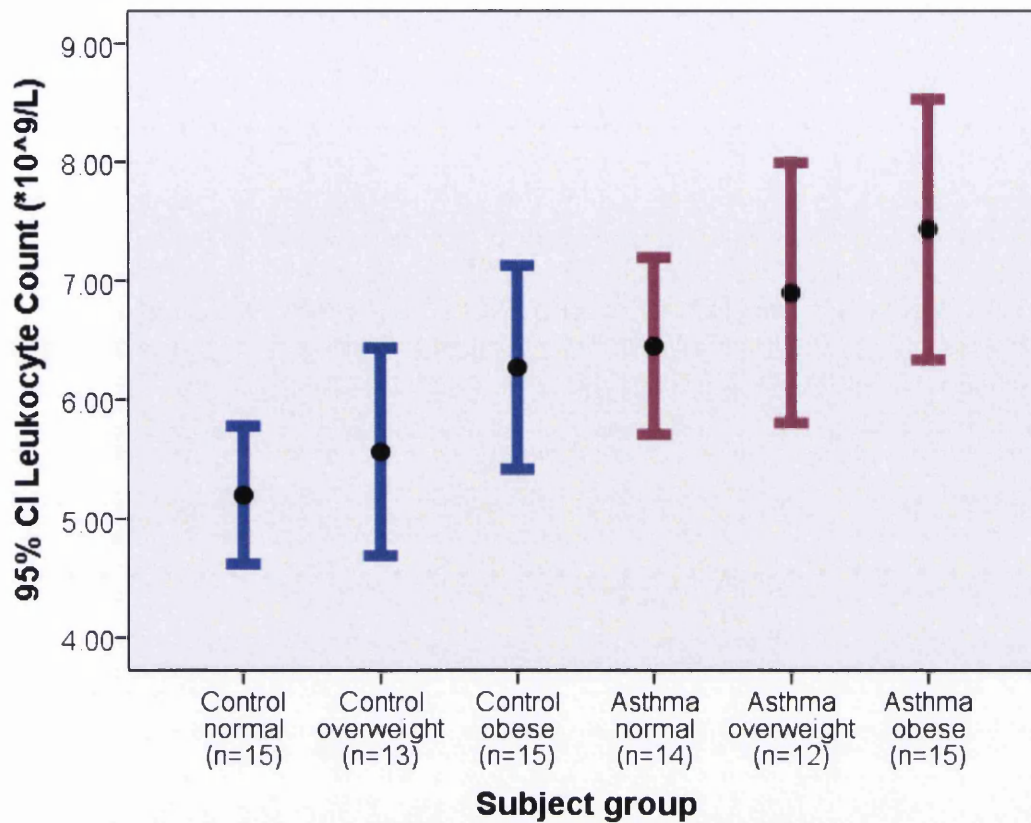


Figure 4.2: Total peripheral blood leukocyte count in asthmatics and controls according to BMI category.

Data shown as mean and error bars. Leukocyte count increased with each BMI group and with asthma such that obese asthmatics had the highest leukocyte count and this was greater than normal weight asthmatics ($p=0.113$) and obese controls ($p=0.053$), although this was not statistically significant.

Neutrophil count differed significantly across the 6 groups ($p=0.010$) (Table 4.8). Across the study group, neutrophil levels increased with increasing BMI category ($p=0.005$) and with BMI as a continuous variable ($r=0.363$, $p=0.001$). Neutrophils also correlated positively with body fat (%) ($r=0.283$, $p=0.009$) and WHR ($r=0.262$, $p=0.017$). Levels were significantly higher in asthmatics (mean = $3.73 \times 10^9/l$) than controls (mean = $2.93 \times 10^9/l$) ($p=0.034$) when each group was compared as a whole. When each of the BMI categories was compared individually, asthmatics had higher levels than controls although this was only significant in the obese category (NW asthma vs. NW control $p=0.058$, OW asthma vs. OW control $p=0.078$, OB asthma vs. OB control $p=0.050$). When asthmatics and controls were analysed separately, neutrophils correlated positively with BMI in both groups (asthmatics: $r=0.335$, $p=0.032$, controls: $r=0.410$, $p=0.006$). Using a general linear model it was possible to see that neutrophil count was independently associated with BMI and asthma status ($p=0.001$ and $p=0.003$ respectively, $R^2 = 0.222$). Obese asthmatic patients had the highest mean

neutrophil count (Figure 4.3) which was significantly higher than normal weight asthmatics ($p=0.020$) and obese controls ($p=0.050$). Across all of the individuals age was not associated with neutrophil count. Within the asthmatics, asthma duration, asthma control score, and spirometric values did not correlate with neutrophil levels.

Given recent interest in fatty acids and neutrophils levels a correlation between these parameters was considered but none was seen ($r=-0.139$ $p=0.207$). In the asthmatics studied inhaled corticosteroid (ICS) use was not significantly associated with neutrophil count ($r=0.279$, $p=0.078$) and within this group the association between BMI and neutrophil count persisted despite adjusting for ICS dose and FFA levels (BMI $p=0.050$, ICS dose $p=0.135$, $R^2=0.25$).

Leptin levels were associated with neutrophils levels ($r=0.326$, $p=0.002$), however the association between BMI, asthma and neutrophils remained significant after adjusting for the effects of leptin suggesting that it is unlikely to be the only factor responsible for the observed neutrophil trend (BMI $p=0.001$, asthma= 0.001 , leptin= 0.174 , $R^2=0.253$).

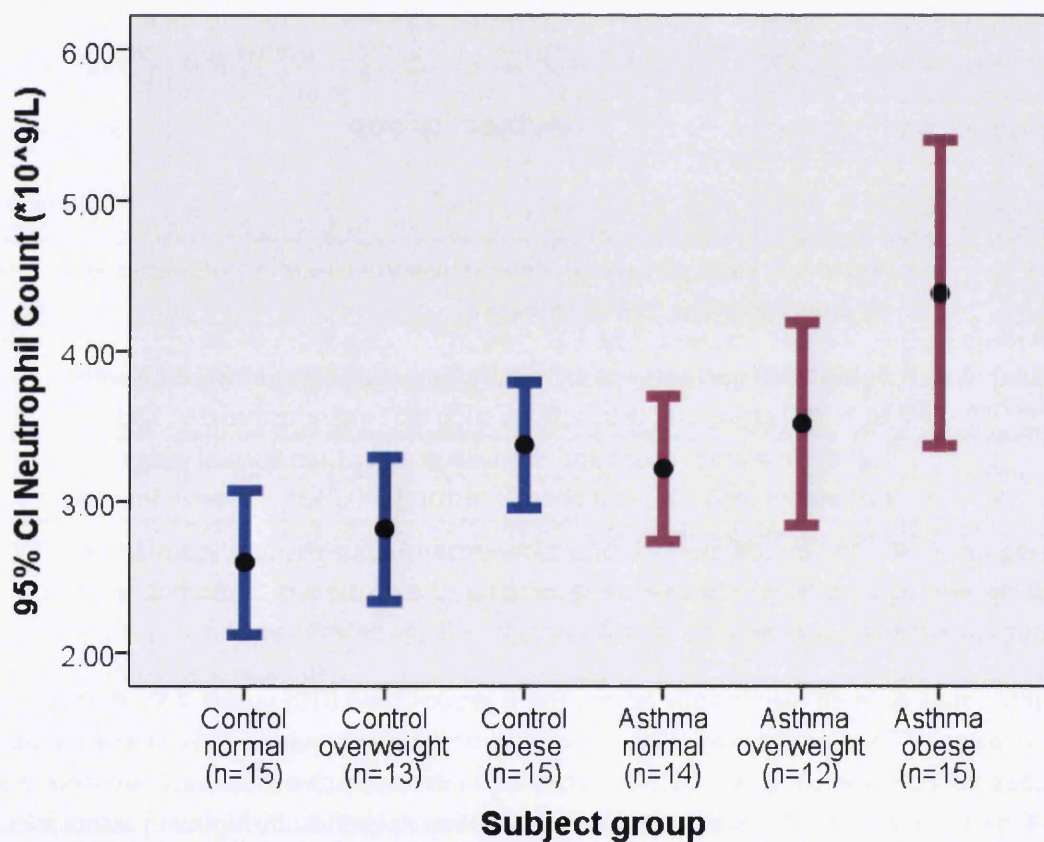


Figure 4.3: Neutrophil count in asthmatics and controls according to BMI category.

Data shown as mean and error bars. Neutrophil count increased with each BMI group and with asthma such that obese asthmatics had the highest neutrophil count which was significantly higher than normal weight asthmatics ($p=0.020$) and obese controls ($p=0.050$).

Eosinophil count differed significantly across the 6 groups (Table 4.8) as across the entire study population asthmatics had significantly higher counts than control subjects ($p < 0.001$). However when each BMI category was compared individually NW asthmatics had significantly higher levels than NW controls ($p < 0.001$) but OW and OB asthmatics did not differ significantly from their control counterparts ($p = 0.073$ and $p = 0.478$ respectively). Across the entire study group BMI category was not associated with a change in eosinophil count ($p = 0.569$). However a significant interaction was seen between asthma status and BMI category ($p = 0.003$), such that there was a trend towards decreased eosinophil count with increasing BMI category in the asthmatics ($p = 0.052$) but not the controls ($p = 0.314$) which was significant when BMI was examined as a continuous variable (controls: BMI $r = 0.182$, $p = 0.242$, asthmatics: BMI $r = -0.316$, $p = 0.044$). Percentage body fat also significantly negatively correlated with eosinophils in the asthmatics ($r = -0.388$, $p = 0.031$) but not in the controls, but no correlation was seen with WHR in either group. Across the entire study population age did not correlate with eosinophil count. Within the asthmatic group asthma control score, asthma severity, asthma duration and current ICS dose were not significantly associated with eosinophil count. Spirometric measurements did not correlate with eosinophil count either.

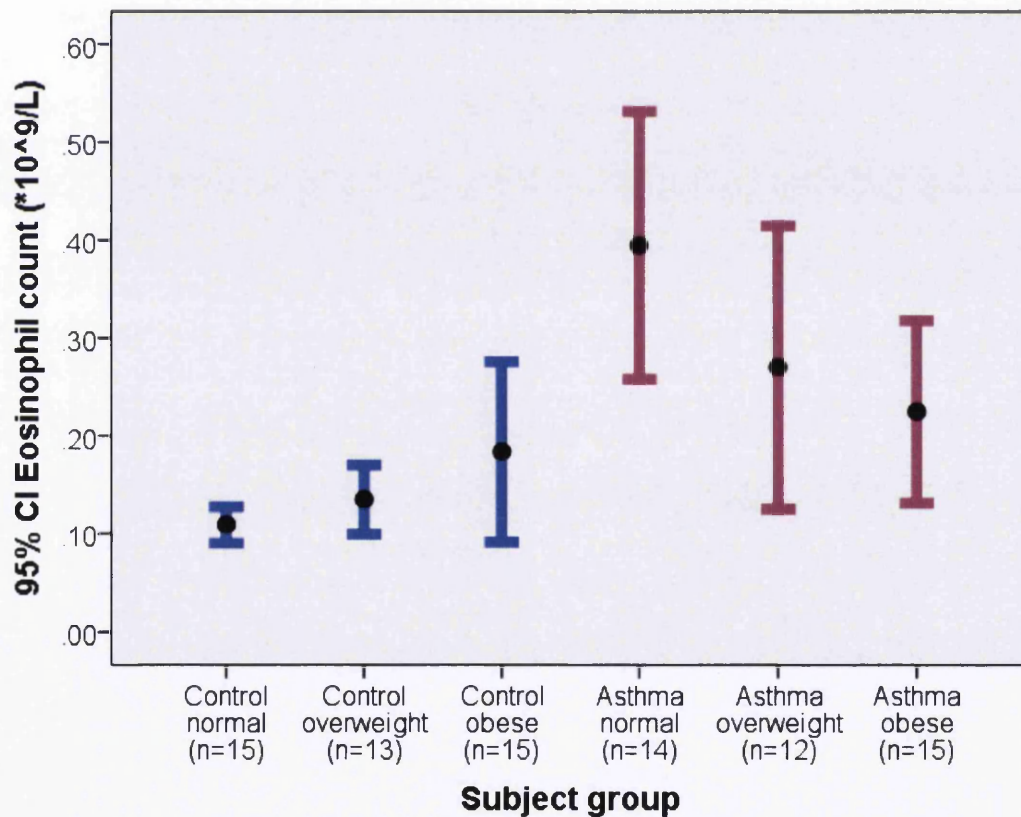


Figure 4.4: Eosinophil count in asthmatics and controls according to BMI category.

Data shown as mean and error bars. Across the study group, mean eosinophil count was significantly higher in asthmatics than controls ($p < 0.001$). Within the asthmatic subgroup increasing BMI category was associated with a trend towards a decrease in eosinophil count ($p = 0.052$).

4.3.6 Insulin resistance

Fasting glucose, insulin levels and their derivatives (β -cell function and IR) are shown in Table 4.9. These did not pass the KS test ($p < 0.001$, $p = 0.047$, $p = 0.066$, $p = 0.05$ respectively) so were logarithmically transformed for analysis. Fasting glucose levels did not differ significantly between the 6 groups ($p = 0.356$). Insulin levels did differ significantly between groups ($p < 0.001$) and this was due to a significant increase in levels with rising BMI category ($p < 0.001$) and BMI as a continuous variable ($r = 0.515$, $p < 0.001$). Percentage body fat ($r = 0.591$, $p < 0.001$) and to a lesser extent WHR ($r = 0.276$, $p = 0.012$) also correlated positively with this variable. Levels in asthmatics were not significantly different to those in controls ($p = 0.157$) across the entire study or when each individual BMI category was compared. No obvious confounders were identified.

β -cell function did differ significantly across the 6 groups studied due to an increase with each BMI category (Table 4.9) and BMI as a continuous variable ($r = 0.399$, $P < 0.001$). β -cell function also increased with body fat composition ($r = 0.473$, $p < 0.001$) but not WHR ($r = 0.162$, $p = 0.145$). β -cell function was not significantly higher in asthmatics vs. control subjects ($p = 0.088$) across the study group. When each BMI group in the asthmatics was compared to their control group no significant differences in β -cell function were observed. (asthma NW vs. control NW $p = 0.16$, asthma OW- vs. control OW, $p = 0.799$, asthma OB vs. control OB $p = 0.36$). No obvious confounders in this association were identified.

IR significantly increased with each BMI category ($p < 0.001$), also with BMI when examined as a continuous variable ($r = 0.506$, $p < 0.001$), percentage body fat ($r = 0.572$, $p < 0.001$) and WHR ($r = 0.286$, $p = 0.009$). Asthmatics did not have higher degrees of IR than controls across the entire study or when each individual BMI category within the asthmatic group was compared with its control counterpart.

4.3.7 Free fatty acid levels

Fasting free fatty acid (FFA) levels did not pass the KS test ($p = 0.037$) and were logarithmically transformed for analysis. Mean FFA levels were similar across the 6 groups (Table 4.8) ($p = 0.736$) and were not associated with asthma status ($p = 0.577$), BMI category ($p = 0.449$), BMI ($r = -0.155$, $p = 0.296$) body fat composition ($r = -0.082$, $p = 0.456$), or waist to hip ratio ($r = -0.106$, $p = 0.344$). Given recent interest in the role of FFA in TLR signalling and neutrophil inflammation, an association between FFA and neutrophil count was examined for, but no correlation was seen ($r = -0.139$, $p = 0.207$).

	Controls			Asthmatics			p value
	NW n=15	OW n=13	OB n=15	NW n=14	OW n=12	OB n=15	
Fasting glucose (mmol/l) Geometric mean (SD)	4.62 (0.53)	4.58 (0.38)	4.83 (0.56)	4.51 (0.57)	4.51 (0.45)	4.64 (0.52)	All 6 groups p=0.356 ¹
Insulin (pmol/l) Geometric mean (SD)	42.09 (20.94)	56.61 (34.57)	85.00 (39.14)	52.15 (63.2)	63.18 (30.70)	108.05 (223.95)	All 6 groups p<0.001 ¹ A vs. C p= 0.157 BMI category p<0.001
Beta cell function (%) Geometric mean (SD)	99.14 (34.07)	117.7 (50.69)	139.46 (59.26)	116.12 (32.36)	131.19 (48.23)	161.95 (70.84)	All 6 groups p=0.011 ¹ A vs. C p=0.088 BMI category p=0.03
Insulin resistance Geometric mean (SD)	0.83 (0.35)	1.02 (0.62)	1.55 (0.70)	0.96 (0.81)	1.14 (0.55)	1.86 (1.52)	All 6 groups p<0.001 ¹ A vs. C p=0.209 BMI category P<0.001
FFA (mmol/l) Geometric mean (SD)	0.36 (0.32)	0.46 (0.14)	0.48 (0.25)	0.40 (0.21)	0.40 (0.24)	0.42 (0.32)	All 6 groups p=0.736 ¹

Table 4.9: Insulin resistance and free fatty acids levels in asthmatics and controls according to BMI category.

All the parameters were not normally distributed and are expressed as geometric mean and standard deviation. Data were logarithmically transformed for analysis. Differences between all 6 groups were analysed using a one-way ANOVA. Differences between asthmatics (A) and controls (C) were analysed using an unpaired t-test and variation between BMI categories using one-way ANOVA.

- (1) One-way ANOVA revealed that insulin levels, beta cell function and insulin resistance did differ across the 6 groups. This was due to a significant increase in these parameters with BMI category with no difference between asthmatics and controls within each BMI category.

4.3.8 Adipokines

Serum levels of leptin, adiponectin and resistin, did not pass the K-S test ($p=0.002$, $p=0.001$, $p=0.099$ respectively) and so were logarithmically transformed for analysis. Serum visfatin and ghrelin were only detectable in some samples processed and therefore not normally distributed. The variable was therefore dichotomised into detectable versus non detectable and analysed using a chi-squared test.

Serum leptin levels differed significantly across the 6 groups (Table 3.10). Across the entire study, leptin levels increased significantly with BMI category ($p<0.001$) and BMI as a continuous variable ($r=0.734$, $p<0.001$). Similarly, correlations were seen between leptin and WHR ($r=0.359$, $p=0.001$) and body fat (%) ($r=0.52$, $p<0.001$). Age of participant did not correlate with leptin levels. Across the entire study group, asthmatics (geometric mean = 39.1ug/l) had significantly higher levels than controls (geometric mean = 20.67ug/l) ($p=0.006$). When each of the BMI categories was analysed individually normal weight and obese asthmatics had significantly higher levels of leptin than their control counterparts (NW asthma vs. NW control $p=0.020$, OW asthma vs. OW control $p=0.177$, OB asthma vs. OB control, $p=0.023$). Using a general linear model it was observed that leptin levels were independently associated with BMI and asthma (BMI: $p<0.001$, Asthma: $p=0.001$, $R^2=0.557$). This meant that obese asthmatics had the highest levels of leptin (Figure 3.5), significantly higher than normal weight asthmatics ($p<0.001$) and obese controls ($p=0.023$). Within the asthmatics studied, age of onset, asthma duration, asthma control, ICS dose and spirometric values did not correlate with leptin levels.

Adiponectin levels did not significantly vary across the 6 categories and were not significantly different in asthmatics and controls (Table 4.10). However levels did significantly decrease with BMI category and when BMI was examined as a continuous variable ($r=-0.324$, $p=0.003$). Furthermore adiponectin was also significantly negatively correlated with body fat (%) and WHR ($r=-0.340$, $p=0.003$ and $r=-0.347$, $p=0.001$ respectively). Age did not correlate with adiponectin levels. Furthermore, within the asthmatics, levels did not correlate with age of onset, asthma duration, asthma control, ICS use or spirometric values.

Resistin levels did not significantly differ across the 6 groups (Table 4.10). Levels did not vary significantly with BMI category ($p=0.158$) and when BMI was examined as a continuous variable ($r=0.186$, $p=0.090$). Across the population studied resistin levels were significantly higher in asthmatics than controls $p=0.024$ (Table 4.10). Age and date of menstrual cycle were not associated with resistin levels. When each of the BMI categories was examined individually, the asthmatics in each category had higher levels than the controls but none of these were significant (NW asthma vs. NW control $p=0.104$, OW asthma vs. OW control $p=0.445$, OB asthma vs. OB control $p=0.150$). Resistin levels remained higher in asthmatics than controls after adjusting for BMI (asthma status: $p=0.033$, BMI: $p=0.122$, $R^2=0.087$). Within the asthmatics, levels did not correlate with age of onset, disease duration, ICS use or spirometric markers.

The proportion of patients with detectable ghrelin and visfatin levels did not vary across the 6 groups (Table 4.10). Furthermore, neither of these parameters significantly differed with BMI category or between asthmatics and controls.

	Controls			Asthmatics			p value
	NW n=15	OW n=13	OB n=15	NW n=14	OW n=12	OB n=15	
Leptin (µg/l) Geometric mean (SD)	7.65 (11.19)	25.88 (18.20)	45.98 (41.61)	17.45 (12.65)	38.02 (35.64)	85.19 (73.03)	All 6 groups: p=<0.001 A-C: p=0.006 BMI: p<0.001
Adiponectin (mg/l) Geometric mean (SD)	5.94 (3.50)	5.31 (3.76)	3.37 (2.77)	5.41 (5.75)	4.47 (3.22)	4.03 (1.91)	All 6 groups: p=0.072 A-C:p=0.206 BMI: p=0.013
Resistin (µg/l) Geometric mean (SD)	5.54 (1.84)	5.29 (2.37)	6.28 (2.98)	7.02 (3.14)	6.03 (2.15)	7.66 (3.18)	All 6 groups: p=0.114 A-C: p=0.024 BMI: p=0.158
Visfatin No. of cases with detectable levels	11/15	7/13	7/15	9/14	7/12	13/15	All 6 groups: p=0.249 A-C: p=0.229 BMI: p=0.557
Ghrelin No. of cases with detectable levels	9/15	4/13	7/15	7/14	2/12	5/15	All 6 groups: p=0.249 A-C: p=0.248 BMI: p=0.062

Table 4.10: Adipokine levels in asthmatics and controls according to BMI category.

Data with regards to leptin, adiponectin and resistin levels were positively skewed and therefore logarithmically transformed for analysis. Data are shown as geometric mean and SD. Differences between all 6 groups and between the 3 BMI categories across the entire study population were analysed using a one way ANOVA. Differences between asthmatics (A) and controls (C) were analysed using an unpaired t-test.

Visfatin and ghrelin levels expressed as the proportion of individuals in each group with detectable levels. Data was analysed using a chi-squared test.

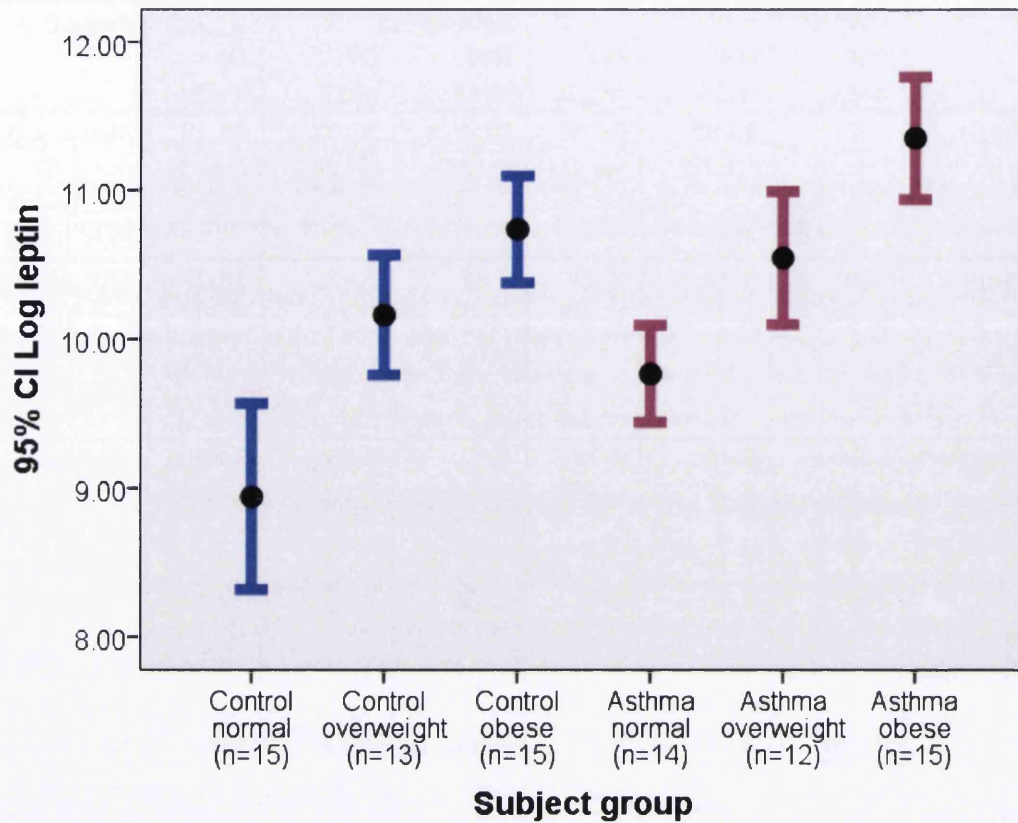


Figure 4.5: Leptin levels ($\mu\text{g/l}$) in asthmatics and controls according to BMI category.

Data shown as mean of logarithmically transformed values and error bars. Obese asthmatics had the highest levels which were significantly higher than normal weight asthmatics ($p < 0.001$) and obese controls ($p = 0.023$).

4.3.9 IgE levels

Total IgE levels did not pass the KS test ($p < 0.001$) and therefore were logarithmically transformed for analysis. Total serum IgE levels for the 6 groups are shown in Figure 4.6. Total IgE levels did differ significantly across the 6 groups ($p = 0.002$) with mean levels significantly higher in the asthmatics than control subjects ($p < 0.001$ (unpaired t-test)). BMI category had no significant association with IgE levels across the entire cohort ($p = 0.528$) neither did BMI as a continuous variable ($r = -0.088$, $p = 0.433$). Although the normal weight asthmatics had the highest mean IgE levels (geometric mean = 191.69 kU/L), and there was a trend towards a reduction in mean levels with increasing BMI within the asthmatics this was not significant ($r = -0.185$, $p = 0.246$). There was no significant association

between IgE and body fat composition or WHR across the entire cohort or when asthmatics and controls were analysed separately.

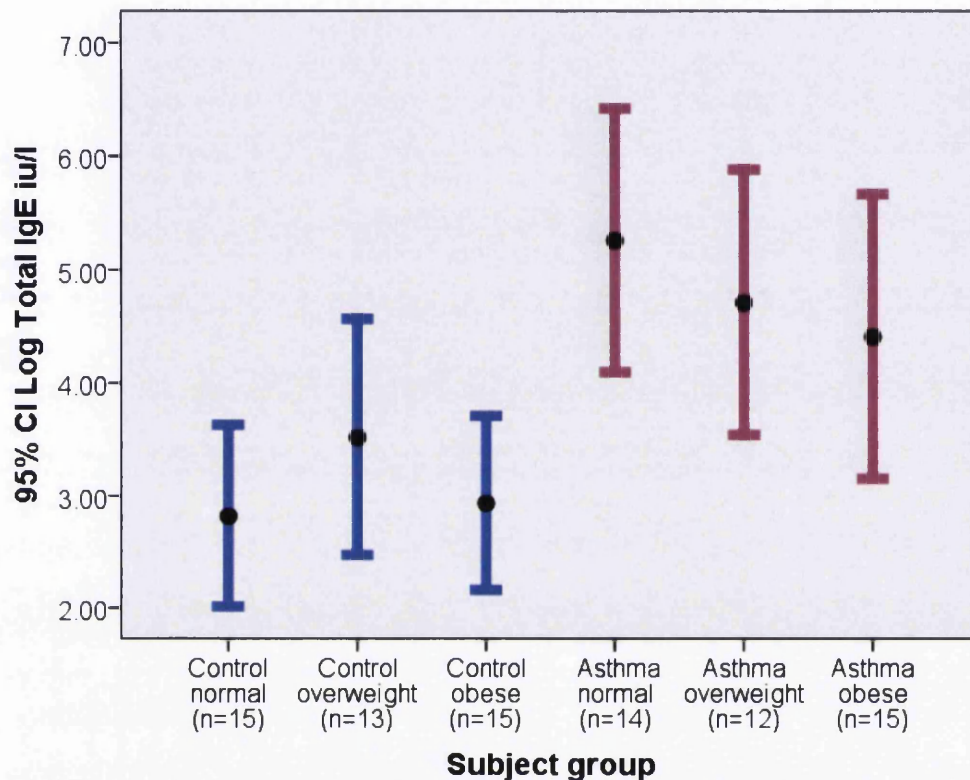


Figure 4.6: IgE in asthmatics and controls according to BMI category.

Data shown as mean 95% of logarithmically transformed values and error bars. Across the study group, IgE count was higher in asthmatics than controls ($p < 0.001$). Within the asthmatics group there was a trend towards reducing levels with increasing BMI but this was not significant ($r = -0.185$, $p = 0.246$).

4.4 Discussion

This study recruited premenopausal women with well defined asthma from a tertiary asthma clinic and surrounding secondary clinics as well as some primary care patients and compared clinical, haematological and metabolic parameters with a well matched group of premenopausal women with little comorbidity. As patients were predominantly recruited from a tertiary asthma service there was a high proportion with moderate or severe persistent disease (75.6%) with very few with intermittent or mild disease, however the BMI were relatively well matched with regards to severity grading. In this study, the patients had relatively early onset disease (mean age of onset 12.8 years) and all were on ICS treatment. Sixty eight percent had a clinical history of atopy and 80% had a positive specific IgE antibodies to a panel of 6 allergens with no significant difference between BMI groups. The high prevalence of atopy may be due to the pool that the patients were recruited from (predominantly a tertiary unit with an interest in allergy), or due to the focus on premenopausal and therefore younger subjects. Although the classical obese phenotype from cluster analyses of asthma is of a late onset obese female predominant phenotype, more recent work suggest that obese asthma encompasses two phenotypes distinguished by age of onset: an early onset disease with a higher incidence of atopy and second group with late onset disease and less atopy [437, 471]. In the study by Holguin et al, early onset disease had more airway obstruction, BHR and a higher incidence of ITU admissions in the previous year [471]. Similarly a cluster analysis identified 2 obese asthma clusters differing by age of onset, with the earlier onset phenotype having worse asthma control, higher FeNO and greater BHR [472]. The response to treatment in these two obese subgroups may also differ with a recent study showing weight loss following bariatric surgery improved BHR in obese asthmatics with no atopy and normal IgE levels, who tended to have late onset disease and marked comorbidity but not in those with atopy and high IgE levels who tended to have early onset disease [229]. Through the recruitment process we have yielded a group of obese asthmatics with early onset disease and high prevalence of atopy and this should be born in mind when interpreting the results.

We used 3 measures of adiposity throughout this works, BMI, body fat composition by biometric impedance and WHR. In our study BMI correlated strongly with both of these parameters, particularly percentage body fat. This is in keeping with large population data which suggests that whilst BMI is poorly sensitive and specific in diagnosing excess body fat in men and the elderly it performs well in women [213]. The correlation with body fat composition may have been higher than expected due to the selection of women in a narrow age range and by controlling for hydration status as all patients were asked to abstain from food and drink from midnight prior to the measurements being taken. Given the tight correlations between the various markers of adiposity it is perhaps not surprising that where various parameters were associated with BMI, they were also significantly associated with percentage body fat and to a lesser extent WHR.

4.4.1 Obesity increases circulating neutrophil count in asthma

These results show that increasing BMI and asthma diagnosis are independently associated with increased number of total leukocytes due to an increased neutrophil count in peripheral blood. Previous studies in non-asthmatics indicate that increasing BMI may be associated with these

changes. A study of Saudi Arabian female university students also found BMI and WHR to be positively associated with increasing total leukocyte count, neutrophils and CD4 count [473]. Similarly a study of 477 bariatric patients (BMI >35kg/m²) undergoing laparoscopic band surgery showed obesity to be associated with higher circulating neutrophil counts, with weight loss causing a marked reduction in neutrophils (11.7%) at 2 years [30]. Similar findings have been reported in the paediatric obese population [474]. This thesis has shown that less dramatic adiposity has the same associations with neutrophils. The observation that asthma diagnosis is also associated with increased leukocytosis and neutrophilia is consistent with previously published work [442, 444]. However the findings from this current study suggest that these effects are additive such that obese asthmatics have the highest neutrophil counts, higher than both normal weight asthmatics and obese controls.

Neutrophils are highly relevant in airways diseases contributing towards localised inflammation through the generation of reactive oxygen species and release of proteases. Such processes are postulated to be important in the development of adult respiratory distress syndrome [164] and chronic obstructive pulmonary disease (COPD), a less steroid responsive smoking related pathology [165], as well as asthma. Studies also show higher levels of sputum neutrophils in refractory asthma patients with irreversible airflow obstruction compared to those with reversible airways disease who tend to have sputum eosinophilia [167]. Sputum neutrophil levels correlate positively with asthma severity [168] and negatively with lung function and markers of airflow obstruction [169]. Similarly, studies on bronchial tissue taken from patients with severe oral glucocorticoid dependent disease, found a 2-fold higher concentration of tissue neutrophils in those with severe disease compared to mild-moderate asthmatics and controls [475].

Recently published studies have suggested that obesity in asthma may be associated with neutrophilic inflammation in the airways, explaining the refractory nature of the disease phenotype to steroid treatment. Two showed a trend towards an increase in sputum neutrophil counts however neither reached statistical significance [25, 362]. Since undertaking this present work, a case-control study of obese and non-obese asthmatics, versus obese and non-obese controls found a significant positive association between obesity and asthma with regards to sputum neutrophils, with obese asthmatics have the highest percentage [429]. When stratified by sex, this observation was only seen in women. If obesity does mediate its effects on asthma through neutrophilic inflammation uniquely in women, this would explain the epidemiological findings of a stronger association in females. Bariatric surgery studies also support the concept of obesity promoting neutrophilic inflammation with trends, although non-significant, towards lower levels of airway neutrophils following surgery [437].

The findings herein of increased blood neutrophils in obese asthmatic women is in keeping with a recently published study which examined 276 well characterised asthmatics, 63 of whom were obese (mean age 45 years) [476]. Telenga et al, found that obese female but not male asthmatics, had significantly higher blood and sputum neutrophils than their lean counterparts. At follow up it was noted that the obese women had a blunted response to corticosteroid treatment which correlated with sputum neutrophil count. However this study lacked a control arm of non-asthmatic obese individuals and therefore it is not certain whether the observations were unique to asthma or just an effect of obesity. Unfortunately the authors did not give information on age of onset of

disease. Our findings confirm in a slightly younger population that in the context of blood neutrophilia, obese asthmatics have higher neutrophils than obese control subjects.

The mechanism linking neutrophilic inflammation in sputum/airways and obesity needs clarification. Given the increase in peripheral blood neutrophils observed here this would appear to be due to a systemic, rather than local effect which seems logical in the context of a systemic condition such as obesity. Furthermore systemic changes in neutrophils, such as increased expression of genes associated with motility and survival [477], have been described in neutrophilic asthma suggesting there is a systemic component to the disease. There are a number of pro-inflammatory cytokines such as IL-17 [91], growth factors including G-CSF [92] and chemokines (e.g. IL-8 [478]), important in mediating neutrophilic responses which warrant further investigation in the context of obesity (see chapter 5).

Some investigators suggest that metabolic factors may mediate neutrophilic inflammation in asthma. FFA can stimulate the innate immune system through ligation of TLR2 and TLR4 [278, 279] activating NF κ B [448] and causing the release of pro-inflammatory cytokines [280]. In view of this fasting FFAs were measured in the study participants. There was no significant association between fasting FFA levels and BMI, body fat composition or WHR, neither was there an association between fasting FFA and neutrophils suggesting that FFA are not promoting the neutrophilia seen. In a case control study examining sputum neutrophils, levels correlated with total fasting plasma saturated fatty acid levels and negatively with mono-unsaturated levels [429]. However this relationship was only seen in men and not women suggesting, together with the current presented findings, that the mechanism behind neutrophilic inflammation in women may not be mediated through fatty acid levels. Post-prandial deposition of fatty acids differs between the sexes with females depositing in the femoral gluteal region [329] and males in visceral fatty tissue [330]. Upon lipolysis and mobilisation from visceral fatty tissue, fatty acids will pass through the portal venous system and into the liver, potentially stimulating TLR receptors on Kupfer cells (hepatic macrophages) resulting in the release of pro-inflammatory cytokines such as IL-6 [331]. Mobilisation of fatty acids from peripheral sites would not have the same effect, explaining why men would be more susceptible to the inflammatory effects of free fatty acids than women.

It may be that peak FFA rather than fasting levels are more relevant. Wood and colleagues examined the acute effects of high fat feeding on male and female non-obese/obese asthmatics versus control subjects [281]. After a single high fat meal they noted that sputum neutrophils and TLR4 mRNA increased in all of the study patients. These changes mirrored levels of total plasma fatty acids and correlated negatively with lung function. The asthmatics also underwent a bronchoprovocation challenge with hypertonic saline followed by administration of the bronchodilator salbutamol. The authors noted that the degree and duration of recovery following this was significantly less in the asthmatics in the high fat feeding group and even worse in those who were obese. In terms of systemic inflammation, a small but significant increase in IL-6 and CRP was seen in the obese asthmatics who underwent high fat feeding but not in non-obese asthmatics or the control group. Furthermore all asthmatics undergoing this diet showed an increase in plasma TNF α . It was noted that the changes in systemic mediators were small compared to those in the airways, suggesting that the airway inflammation seen was not a simple overspill from systemic responses. With regards to the type of fat the investigators noted that trans-saturated fatty acid ingestion provoked a much greater neutrophil response than non-trans-unsaturated fatty acids. This is consistent with previous

work which has shown trans-unsaturated fatty acids to have systemic pro-inflammatory effects [479]. Therefore it is possible that if peak fatty acid levels or the subtypes of FFA had been measured in this study an association with neutrophil levels might have been seen. This warrants further investigation.

Leptin is known to play a role in promoting neutrophil survival and chemotaxis. However, although a significant association between leptin and neutrophils was seen, the relationship between BMI, asthma status and neutrophils remained significant after adjusting for leptin levels suggesting that they are not the sole mediator responsible for the neutrophil trend observed.

Hormonal factors may also play a role. Women have higher neutrophil counts than men, and peripheral blood levels fluctuate throughout the menstrual cycle [388]. We tried to control for this variation by timing the blood sampling to a two hour window between 07.00-09.00am during the first 7 days of the menstrual cycle determined by clinical history. A recent cross-sectional study examined the peripheral white blood cell count in 36 women with polycystic ovary syndrome (PCOS) versus 77 control patients, and noted it was the presence of PCOS not the associated adiposity which predicted increased neutrophils [480]. We excluded patients with known PCOS from our study and due to the difficulties of predicting menstrual dates accurately patients with erratic cycles (a known feature of PCOS), were also excluded.

It is often argued that co-morbidities confound the obesity asthma relationship. Obstructive sleep apnoea is a common pathology in obese individuals and may be associated with worse asthma control in these individuals [258]. Patients with known OSA were excluded and an Epworth score was also determined on all potential subjects in an attempt to exclude undiagnosed individuals. Furthermore recent studies on patients with confirmed OSA have shown that it does not appear to affect peripheral blood counts [481]. GORD is also a comorbidity which increases with BMI [256] and is more common in asthmatics [253]. Three obese and one overweight asthmatic were on treatment for reflux and the resultant mucosal injury could propagate a systemic inflammatory response. However a study examining the effect of varying degrees of mucosal injury from reflux on the peripheral leukocyte count showed a positive association between the degree of reflux and eosinophil count but did not show any correlation with other components of the blood count including neutrophils [482].

Corticosteroids inhibit neutrophil apoptosis and therefore increase survival [483]. All patients involved in this study were free of oral corticosteroids for at least 6 weeks prior to enrolment. There was also no difference in ICS dose taken by the asthmatics across the BMI groups and therefore the association between BMI and neutrophils within the asthmatics persisted after adjusting for ICS dose at the time of sampling. However, the asthmatics were all on ICS treatment whilst the controls were clearly not. It is therefore not possible to exclude the possibility that the higher neutrophil count seen in the asthmatics as a group may be in part due to ICS use. Studies in healthy volunteers suggest that administration of inhaled beclomethasone can increase blood neutrophil count, peaking at 6 hours and returning to baseline by 24 hours, although interestingly this was not seen with inhaled budesonide [484]. It is interesting to note that only two of the asthmatics taking part were on beclomethasone, the remainder on either budesonide or fluticasone. The transient effects of ICS on blood counts were controlled for by asking with all patients to withhold their ICS treatment for 24 hours prior to sampling. Furthermore, the finding that asthmatics had higher neutrophil counts than

controls is in keeping with previously published work. The only way to fully exclude the possibility that ICS use was increasing blood neutrophils between asthmatics and controls would be to administer ICS to the control group, which we did not have the ethical approval to do, or to wean all asthmatics off their inhaled therapy which is a limitation of this work and has its own shortcomings (see chapter 8).

4.4.2 Eosinophils decline with increasing BMI in asthma

The data from this study also show that the eosinophil count was elevated in the lean asthmatics but normal in the overweight and obese individuals. Eosinophilic inflammation is associated with atopic asthma and is more steroid responsive [485]. This study suggests that any inflammation in obese asthmatics is not eosinophil related. This is interesting given animal models show that obesity enhances eosinophil trafficking from the bone marrow to the lung tissues [486]. In addition, a study looking at 26 obese patients undergoing gastric bypass and 10 controls, found obesity to be associated with higher levels of eosinophils [31].

Most human studies have focussed on eosinophilic inflammation locally within the airways measured directly with sputum cell counts or indirectly measuring fractional exhaled nitric oxide (FeNO), a surrogate marker of eosinophilic airway inflammation. Work to date has suggested either no difference or a reduction in eosinophils with increasing BMI in asthma. In a study of 80 women with and without asthma and of varying BMI, the asthmatic patients had a significantly higher mean percentage of sputum eosinophils than the control groups, however there were no significant differences between the obese versus non-obese groups [25]. A cluster analysis of a single primary care cohort and two secondary care cohorts suggested that obese asthma is a female predominant disease characterised by the absence of eosinophilic airway inflammation [8]. In a study by Van Veen of 136 patients with persistent asthma despite high dose corticosteroid treatment, obesity was associated with a significantly lower sputum eosinophil count than lean patients [28]. More recently, the cluster analysis by Sutherland et al also found both obese clusters to be characterised by an absence of sputum eosinophilia [472].

Studies of FeNO in obese individuals have yielded conflicting results but some have shown a reduction in this surrogate marker of eosinophilic airway inflammation with rising BMI. In a study of 67 individuals with moderate to severe asthma, rising BMI and leptin /adiponectin ratio were associated with declining FeNO [27]. In a Dutch study of patients with persistent asthma symptoms despite high dose treatment, BMI negatively correlated with FeNO [28]. More recently a study of Swedish adults with or without symptoms of wheeze found that in non-obese individuals the presence of wheeze was associated with higher rates of atopy and FeNO but that obese patients who reported wheeze had lower levels of FeNO than obese individuals without wheezing irrespective of atopic status [29]. Within the wheezing obese group BMI, percentage body fat, and WHR all correlated negatively with FeNO.

ICS therapy has been shown to affect peripheral blood eosinophil counts [487]. However there was no significant difference in ICS dose between groups and furthermore ICS dose did not correlate with eosinophil count in the asthmatics. In summary our results are consistent with previous studies

showing no evidence of eosinophilic inflammation at a systemic level in obese asthma and complement those revealing similar findings in the airways.

4.4.3 Obesity is associated with insulin resistance

As expected, insulin resistance increased significantly with BMI. However there was no association between insulin resistance and asthma diagnosis in this study and obese asthmatics did not have higher levels than their control groups. There are mechanistic data that hyperinsulinaemia might promote airway hyper-responsiveness [447] and some population studies have revealed an association between insulin resistance and asthma diagnosis [277], although data are conflicting [225]. Patients with confirmed diabetes were excluded from this presented work and study numbers may not have adequately powered for the detection of a difference in this parameter. Given that our study cohort included patients from a difficult asthma service who were on relatively high doses of inhaled steroids and may have used oral steroids previously, a higher degree of insulin resistance in the asthmatic group might have been expected at least due to treatment effects but this was not observed

4.4.4 Leptin levels increase with obesity and asthma

Leptin is an adipokine with multiple effects on the immune system (Table 4.3) and murine models have suggested that it may augment airways inflammation and hyper-responsiveness [23]. In this present study, leptin levels correlated positively with BMI, percentage body fat and WHR with higher levels in asthmatics, such that the highest levels were found in the obese asthmatics, suggesting a possible role for this immunomodulatory adipokine in the obesity-asthma association. A single paediatric study also found leptin levels to be higher in overweight physician diagnosed asthmatics compared to normal weight asthmatics and controls [302], although others have not replicated this finding (Table 4.4). Large population studies have been contradictory: a large cross-sectional study found an association between leptin and self reported asthma diagnosis, independent of BMI [24] whereas another longitudinal study did not note any association with asthma [309]. However these studies whilst large lacked an objective asthma diagnosis, relying on self reporting. A recently published study examining airway inflammation found a trend towards increased leptin levels in obese asthmatics compared to the other groups but this was not significant [429].

The current study differed from previous work as the focus was exclusively on pre-menopausal women. In the large British study mentioned above the association between leptin and asthma diagnosis was stronger in women, especially if pre-menopausal [24]. Mechanistically, leptin secretion is 2-3 fold higher from subcutaneous than visceral adiposity, thus correlating more strongly with adiposity in women [327, 328]. If leptin does play a role in obese asthmatics this could explain why this association is stronger in females. Leptin increases in situations of acute inflammation including sepsis, and in the context of asthma levels increase during exacerbations. A study by Sutherland also focussed on premenopausal females and leptin levels in the obese asthmatics compared to the groups did not significantly differ [25]. However, these investigators attempted to

wear the 40 asthmatics off ICS treatment, resulting in 28 patients being sampled during an exacerbation which may have masked subtle differences in leptin levels between groups. Acute changes in leptin levels were controlled for in this study by recruiting patients who were free of recent infection or exacerbation (>6 weeks) and of note, markers of current asthma control were not different between groups, suggesting that the differential leptin levels seen were not due to acute changes in their disease activity. Hormones may also impact on leptin, with levels rising in the luteal phase of the menstrual cycle or with administration of exogenous oestrogen with progesterone [488]. Sampling all volunteers during the first 7 days of their menstrual cycle limited these effects, and this may also explain why the current results differ from those previously published.

Leptin has a number of effects on neutrophils (Table 4.3) and in mouse pneumococcal pneumonia, administration of leptin increased neutrophils and IL-6 in BAL fluid [455]. In the study herein, serum leptin was strongly positively correlated with neutrophil count however the association between BMI, asthma and neutrophil count remained significant after adjusting for leptin suggesting that it is unlikely to be the sole mediator in this relationship. Given the multitude of immunomodulatory effects of leptin and its association with obesity and asthma in this study group, its correlation with various cell types and cytokines will be examined throughout the remaining results chapters.

Adiponectin has a number of anti-inflammatory effects, so it was of interest to see if serum concentrations were significantly reduced in the obese asthmatics. Although as expected, increasing BMI was associated with a reduction in adiponectin, asthmatics and specifically obese asthmatics did not have significantly different levels to their respective controls. Interestingly resistin, an adipokine with pro-inflammatory effects, was significantly elevated in the asthmatics compared to controls although there was no difference with BMI category. Only a few studies have examined this adipokine in relationship to asthma with contradictory results. In paediatrics Kim et al reported lower levels of resistin in atopic asthmatics than controls [307], whilst Arshi et al found no difference between children with asthma and healthy controls [489]. The results presented here support those of Larochelle et al who found significantly increased levels of resistin in the severe adult asthmatics studied compared to healthy controls [312]. Resistin is a natural TLR4 agonist which results in the activation of NF κ B and induction of a number of pro-inflammatory cytokines [490] which would be of relevance in asthma. Recently a study of 35 steroid naive female adult asthmatics versus controls, found pre-treatment resistin levels to be predictive of a fall in pro-inflammatory blood markers (eosinophil cationic protein, eosinophil protein X and myeloperoxidase) following 8 weeks of inhaled steroids [308]. Steroids exhibit many of their effects via suppression on NF κ B and therefore the authors hypothesised that patients with high resistin levels had high NF κ B activation and therefore would be sensitive to steroid effects. Certainly if resistin is involved in asthma pathogenesis, our study and that of others suggests this is independent of BMI.

4.4.5 Increasing BMI is associated with a trend towards reduction in IgE levels in asthmatics

In this study, markers of adiposity were not associated with IgE levels in non-asthmatic or asthmatic individuals, although there was a non-significant trend towards a reduction in IgE levels in the asthmatics with increasing BMI. These findings are consistent with a recently published study of 666

patients with severe asthma on the National UK registry dedicated to difficult asthma services [491]. In this study serum IgE levels and specific IgE levels to aspergillus decreased with increasing BMI in the severe asthmatics studied. In contrast to this, a study of inner city adults attending an asthma and immunology clinic showed a positive association between obesity and serum IgE levels [445], however only atopic individuals were included in the study. It is increasingly recognised that there might be two sub-phenotypes of obese asthma differentiated by age of onset but also IgE levels. A study of asthmatics undergoing bariatric surgery suggested that early onset disease with higher IgE levels did not experience improvements in BHR unlike those with later onset non-atopic disease with normal IgE levels [229]. Although the obese patients in the study herein did not have as high levels of IgE (geometric mean =82.02 kU/L) as in the atopic group of the bariatric surgery study (mean = 305.3 kU/L).

4.5 Summary

The findings described indicate a switch from systemic eosinophilia to neutrophilia with increasing BMI in asthma. This is in keeping with evidence in the literature showing that obese asthmatics lack significant eosinophilic inflammation but have evidence of neutrophilic inflammation, including in the airways. Despite some good mechanistic and other data in the literature suggesting a role of free fatty acids in mediating neutrophilic airway inflammation in males, this current study suggests fasting free fatty acid levels in women are not associated with obese asthma. Similarly, despite documenting evidence of insulin resistance with increasing BMI there was no association with the obese asthma phenotype. However, leptin, an adipokine associated with numerous immunological effects, was highest in the obese asthmatics, suggesting that it could be playing a role in this disease phenotype. In chapter 5 other potential mechanisms will be explored in more depth, including more detailed investigation of the functional consequences of elevated neutrophil counts and whether there is evidence of neutrophil activation. In addition given the multitude of immunological effects the relationship between leptin and other immunological parameters will be examined.

Chapter 5

Neutrophils and oxidative stress in obesity and asthma

5.1 Introduction

Neutrophils may have an important role in severe asthma phenotypes. In the cluster analysis performed as part of the Severe Asthma Research Programme (SARP), one of the phenotypes described included non-atopic individuals with late onset disease, severely impaired lung function with poor reversibility and higher levels of sputum neutrophilia [16]. Additionally, sputum neutrophil levels are significantly higher in those with severe disease [168] and correlate negatively with lung function and markers of airflow obstruction [169]. In acute severe asthma requiring intubation, the predominant leukocyte in tracheal aspirates is the neutrophil which can be up to ten fold higher than normal levels [492].

Blood neutrophil counts in females have been shown to increase with BMI and work presented in chapter 4 suggests this also occurs in asthmatics, resulting in obese asthmatics having higher levels than normal weight patients and obese controls. This has been noted by one other group [476] and could be due to either increased production of cells, reduced marginalisation and/or delayed apoptosis. In this chapter some of the potential cytokines important in neutrophil regulation will be explored. Furthermore, the association between increased neutrophil count and neutrophil activation and function will be considered.

5.1.1 Cytokines regulating neutrophil levels

5.1.1 (i) Overview of blood neutrophil regulation

Neutrophils are one of the most abundant cell types of the innate immune response and have strong phagocytic and antimicrobial properties, arriving at sites of inflammation within a matter of hours [88]. Part of their activity is based upon an ability to generate reactive oxygen species (ROS), which damage proteins, lipoproteins and DNA. They develop from common myeloid progenitors ($CD34^+CD38^+CD123^{med}CD135^+CD45^-$) which in turn are derived from $CD34^+$ multipotent progenitor cells [493]. Neutrophil turnover is rapid, with their life span short at 7-10 days; the bone marrow contains approximately 2.3×10^9 neutrophils with 1×10^9 cells per kg body weight leaving the bone marrow every day [494, 495]. The blood compartment of neutrophils is only 1/3 the size of the bone marrow and levels are tightly controlled with a mean neutrophil count for Caucasian females of $4.3 \times 10^9/L$ [496].

The blood neutrophil count is regulated predominantly by granulocyte colony stimulating factor (G-CSF), interleukin 17 (IL-17) and IL-23. Briefly, IL-23 is produced by macrophages and dendritic cells on encountering an inflammatory stimulus via activation of the pro-inflammatory transcription factor NF κ B. IL-23 in turn is a potent inducer of IL-17 expression by $CD4^+$ T helper cells termed Th17 cells [90] (see section 2.2.3 (i)). IL-17 then induces IL-1, IL-6, IL-8, G-CSF and GM-CSF production by epithelial, endothelial and other stromal cells which leads to the production, recruitment and activation of neutrophils [91, 497]. Following migration of neutrophils into tissue, apoptotic cells are engulfed by phagocytes and IL-23 is down-regulated therefore forming a negative feedback loop [498]. There are some published data examining these key cytokines in the context of obesity and asthma which will be summarised in the following section.

5.1.1 (ii) Colony stimulating factors (G-CSF & GM-CSF)

The colony stimulating factors are a group of cytokines essential for haematopoiesis. They include G-CSF and macrophage colony stimulating factor (M-CSF), which are lineage specific and important in the differentiation and proliferation of neutrophils and macrophages respectively. They also include GM-CSF and multi-colony stimulating factor (multi-CSF/IL-3) which along with IL-6 and stem cell factor (SCF) act earlier in promoting differentiation of common myeloid progenitors (CD34⁺CD38⁺CD123^{med}CD135⁺CD45⁻) into granulocyte macrophage progenitors (CD34⁺CD38⁺CD123^{med}CD135⁺CD45⁺) [493, 499].

GM-CSF is therefore important in the differentiation and proliferation of macrophage, eosinophil and neutrophil progenitors in the bone marrow and is produced by a vast number of cell types including structural cells (endothelium, osteoblasts, fibroblasts), innate cells (neutrophils, macrophages), and both B and T lymphocytes [499]. Elevated circulating levels of GM-CSF have been observed in the morbidly obese undergoing bariatric surgery compared to healthy controls [500]. GM-CSF is produced locally within the lung and levels within the sputum are increased in patients with asthma, correlating with disease severity [501]. The presence of GM-CSF in the context of asthma correlates with sputum eosinophilia rather than neutrophilia, and animal studies have shown that neutralisation of this factor results in reduced eosinophilic airway inflammation [502]. However GM-CSF is also increased locally in COPD, a more neutrophilic airway disease, where it may be important in promoting neutrophil chemotaxis and inhibiting apoptosis [503].

The major cytokine in neutrophil proliferation and survival is G-CSF: humans deficient in this develop profound neutropenia [92]. Although many cells are capable of its expression, macrophages and monocytes are the primary producers [499]. Although IL-3, SCF and GM-CSF can support the early growth of neutrophil progenitors, G-CSF is required for their terminal differentiation [499]. In addition, upon differentiation these cells remain tethered to the bone marrow through the interaction of C-X-C chemokine receptor 4 (CXCR4) and its ligand, C-X-C chemokine ligand 12 (CXCL12). G-CSF down regulates expression of CXCR4 resulting in release of neutrophils into the blood [504]. Old neutrophils circulating in the blood are thought to start re-expressing CXCR4 and home back to the bone marrow [505]. There is little published work on G-CSF in the wider context of obesity or in studies examining obesity in asthma.

5.1.1 (iii) IL-17

The IL-17 family consists of 6 members designated A-F. IL-17A is produced by Th17 cells [90] and its main function is to promote neutrophilic inflammation through the induction of IL-8 and G-CSF by other structural cells [91]. There is evidence to suggest that IL-17A may be important in neutrophilic asthma and elevated in the obese state. Airway inflammation traditionally has been thought to be a Th-2 mediated process, however, murine models have shown the importance of Th17 cells. Unlike Th2 cells, Th17 cells do not show steroid responsiveness *in vitro* and passive transfer of OVA specific Th17 cells to mice with severe combined immune deficiency has been shown to cause increased G-CSF expression leading to neutrophil influx of the airways and promotion of steroid resistant BHR to methacholine [353]. Interestingly, in human studies, serum levels of IL-17 are elevated in severe

asthma compared to mild-moderate disease with levels >20pg/ml shown to be an independent risk factor for severe disease after controlling for more traditional covariates [354]. Furthermore, sputum IL17A mRNA levels have been shown to correlate with IL-8 and sputum neutrophil counts in asthmatics [171] and correlate clinically with the presence of BHR [506].

Animal models have suggested that obesity results in Th17 bias through an IL-6 dependent process [351]. Circulating IL-17 levels are elevated in obese compared to normal weight women [352]. Despite the weight of evidence suggesting an important role for IL-17A in asthma and the potential for obesity to favour a Th17 bias, to date there has been little work in this area in relation to the obesity asthma association. A Th17 bias in the obese state might be responsible for the enhanced number of neutrophils seen and in the context of asthma, could skew the disease towards a more neutrophilic, treatment refractory phenotype.

5.1.1 (iv) IL-23

IL-23 is a pro-inflammatory cytokine, produced by antigen activated macrophages and DCs. It is important in promoting Th17 differentiation (see chapter 7) and therefore IL-17 mediated neutrophilic inflammation [115]. Evidence suggests that obesity may be associated with Th17 bias. In keeping with this, circulating IL-23 levels have been shown to be elevated in obese women [352] but little work has been done on this cytokine in the context of obesity and asthma.

5.1.1 (v) IL-6

IL-6, first discovered in the 1980s, is produced by a broad range of cells including structural cells (epithelial, endothelial cells and fibroblasts), and cells of the innate (macrophages, dendritic cells and mast cells) and adaptive (B lymphocytes and some CD4 effector cells) immune systems [507]. It acts on the IL-6 receptor (IL-6R) which is expressed on leukocytes and hepatocytes in a process known as "classic signalling". The IL-6 receptor has a short cytoplasmic domain and therefore is associated with another large trans-membrane protein, glycoprotein 130 (gp130), to enable signal transduction. Unlike IL-6R, gp130 is expressed ubiquitously and is not a specific partner to IL-6R. The existence of soluble IL-6 receptor (sIL6R) enables cells which express gp130 but not IL-6R to become responsive, a process known as "trans-signalling"(see Figure 5.1) [508]. Soluble IL6R is produced by neutrophils, macrophages and CD4⁺ T cells. A soluble form of gp130 also exists (sgp130) and this can bind to the IL-6/sIL6R complex preventing it from interacting with surface bound sgp130 and thereby specifically blocking IL-6 trans-signalling (Figure 5.1)[508].

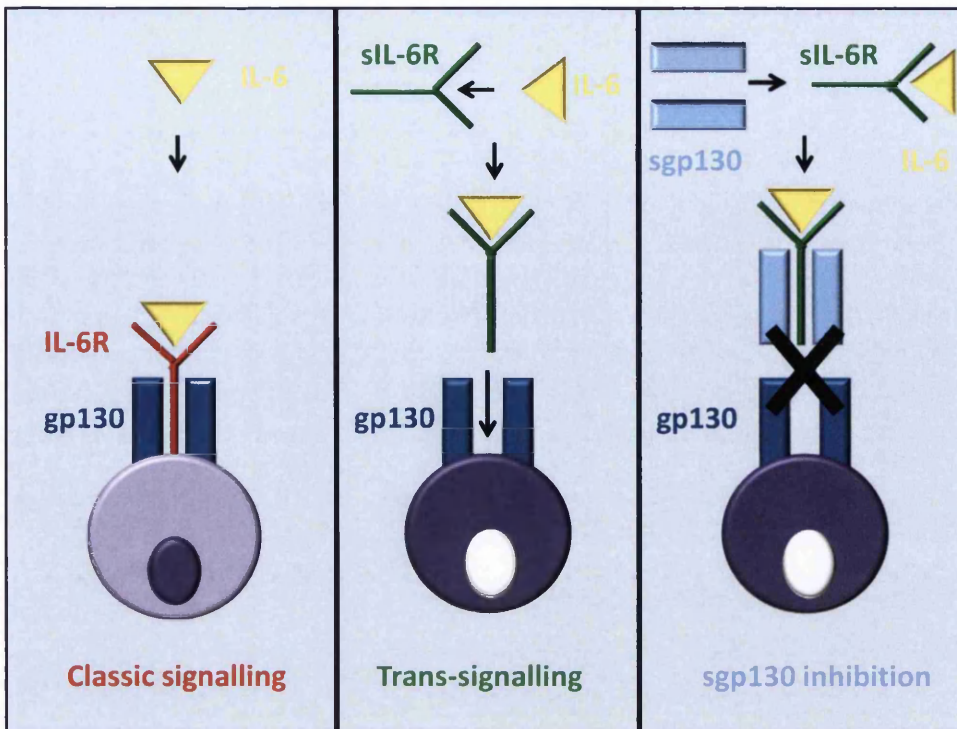


Figure 5.1: Schematic diagram of IL-6 signalling.

Classic signalling: IL-6 binds to surface IL-6 (IL-6R) receptor coupled with membrane gp130.

Trans-signalling: IL-6 binds to soluble IL-6 receptor (sIL-6R). This associates with membrane gp130 on cells which do not express IL-6 receptor enabling them to respond to IL-6.

Soluble gp130 (sgp130) inhibition: IL-6 binds to sIL-6R. This associates with soluble gp130 and prevents it interacting with membrane gp130.

IL-6 has a range of effects on the immune system (Figure 5.2). Mechanistically IL-6 is important in regulating neutrophil trafficking during the acute inflammatory response [509], as well as inhibiting apoptosis [510]. In the context of the lung, murine models show that it enhances neutrophil dependent killing of pulmonary pathogens [511]. IL-6 can also indirectly promote neutrophilic inflammation through its ability to promote Th17 differentiation whilst inhibiting Treg differentiation (see section 7.1.2 (iii)). Recent therapeutic studies of anti-IL-6 therapy in rheumatoid arthritis have shown a transient drop in neutrophil count with blockade of IL-6 again emphasising its importance in mediating neutrophilic inflammation [512].

In the setting of allergic asthma, plasma IL-6 levels have been shown to be elevated compared to control subjects [176] and locally, sputum levels inversely correlate with FEV1 [513]. Furthermore, sIL6R receptor levels are elevated after allergen challenge [514]. A single nucleotide polymorphism (SNP) in the IL-6R has been identified which promotes receptor shedding, increasing sILR-6 levels and thereby promoting trans-signalling. This SNP is more prevalent in patients with severe asthma and in this group serum sIL6R levels inversely correlate with FEV1 and FVC [515].

Circulating IL-6 levels consistently have been shown to be elevated in the obese [516] and it is thought that as much as 25% of circulating IL-6 is derived from adipose tissue [517]. Soluble forms of

IL-6R and gp130 have not been studied extensively in obesity but a single study measuring serum levels in lean and obese pre-menopausal women with and without polycystic ovary syndrome (PCOS) found that serum sIL-6R levels did not differ between BMI groups but that levels were lower in the women with PCOS [518]. However, serum sgp130 levels were significantly higher in the obese subjects and in those with PCOS.

Despite the compelling evidence that IL-6 is increased in both obesity and asthma, there has been little work on this cytokine with regards to obesity in association with asthma. A single study to date showed no significant difference in plasma levels in obese compared to normal weight asthmatics. In a study examining 80 premenopausal women aged 18-50 years with and without asthma, IL-6 levels were higher in the obese and highest in the obese asthmatics, however this did not reach statistical significance [465]. Furthermore, there is no published data on the levels of sIL-6R or sgp130 within this asthmatic phenotype.

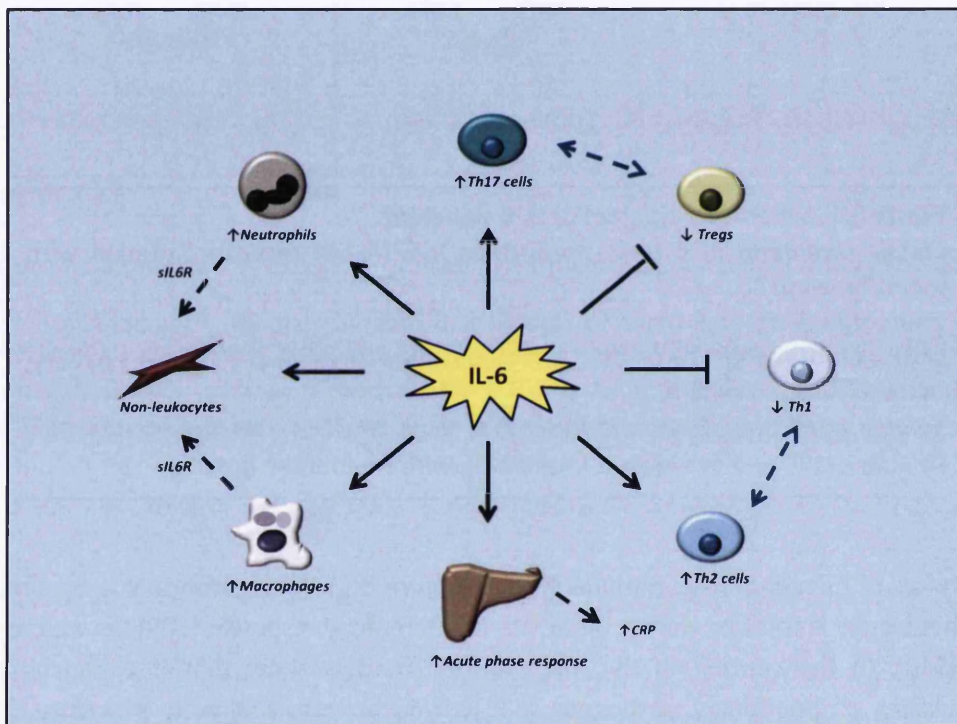


Figure 5.2: Summary of the effects of IL-6 on the innate and adaptive immune response.

5.1.1 (vi) IL-8/CXCL8

IL-8 was first isolated from the supernatant of LPS stimulated mononuclear cells [519]. It is produced by a number of leukocyte types including neutrophils, monocytes, NK cells and T cells as well as structural cells including fibroblasts, endothelial and epithelial cells [520]. Production is induced following ligation of pattern recognition receptors (PRRs) by microbial products or via exposure to other pro-inflammatory cytokines including TNF α and IL-1 [521]. The cytokine mediates its effects through two receptors - chemokine C-X-C receptor 1 (CXCR1) and CXCR2 - and is important

in neutrophil migration into tissues including the pulmonary epithelium. These migratory effects of IL-8 are mediated by downregulating CD62L (L-selectin) and upregulating the integrins CD11b and CD11c. IL-8 also induces neutrophil degranulation and respiratory burst leading to ROS production as well as leukotriene and platelet activation factor synthesis [520].

In severe asthma, sputum IL-8 levels correlate with airway neutrophilia [522] and bronchial smooth muscle cells have been shown to upregulate expression of this cytokine [523]. In individuals with acute severe exacerbations requiring intubation, tracheal aspirates yield high levels of IL-8 up to 19 times normal [492]. Therapeutic advances have also suggested an important role for IL-8 in the pathogenesis of chronic asthma. Macrolide antibiotics reduce neutrophil accumulation in the airways in refractory asthma and this could be due to reduced IL-8 production [524].

In the context of obesity, studies have shown elevated serum levels of IL-8 in the blood of obese children [525] and adults [526] compared to their normal weight counterparts, with a reduction in serum levels seen following weight loss [527]. Only a single study has examined plasma IL-8 levels in obese and non-obese females with and without asthma, finding no association with either obesity or asthma [25]. However when the same group examined sputum, they observed higher levels in asthmatics but no effect with obesity.

In summary a number of cytokines are important in the regulation of neutrophil numbers, activation and trafficking. Studies in asthma and other airway diseases have suggested an importance of some of these in promoting neutrophilic inflammation, especially IL-6, IL-8 and IL-17. Studies to date in obesity have suggested that these same cytokines may be elevated at a systemic level. Therefore it was hypothesised that in the context of asthma, obesity causes changes in these circulating cytokines at a systemic level and these may be responsible for the changes in neutrophil counts seen in the study cohort. If this was found to be the case then this may identify novel therapeutic targets for this specific asthma phenotype

5.1.2 Surface markers of neutrophil activation

Margination of neutrophils to sites of inflammation results from the altered expression of adhesion molecules. CD62L (L-selectin) recognises carbohydrate structures on vascular adhesion molecules and initiates tethering and rolling along the vessel wall surface, whilst CD11b and CD18 facilitate final adhesion to the endothelial surface so that diapedesis can begin [528]. Chemokines such as IL-8 cause shedding of CD62L and upregulation of CD11b, encouraging neutrophil migration into tissue [520]. Therefore, in states of chronic inflammation such as malignancy or chronic renal failure, CD62L is shed whilst CD11b levels increase [529, 530].

Obesity alters expression of neutrophil adhesion molecules. A study of 26 obese patients undergoing gastric bypass and 10 controls examined CD11b and CD62L expression at the cell surface. Consistent with the view that obesity represents a state of chronic inflammation, CD62L was downregulated in the obese patients compared to controls and levels increased following weight loss to supra-normal levels; CD11b levels were unchanged [31]. Using another surface marker of activation, CD66b, a glycoprotein which is translocated from secondary granules to the cell membrane upon activation, Nihjuis et al [337] showed that levels were elevated in obese bariatric patients at baseline compared

to healthy controls. Myeloperoxidase (MPO), the enzyme responsible for ROS generation, was also elevated in the obese but did not change with surgery. However calprotectin, a cytoplasmic bacteriostatic protein which is released on neutrophil activation was found to be elevated in obesity and reduced at 2 years with subsequent weight loss.

In the context of asthma neutrophil activation, has also been shown to be increased at a systemic level. In a study of 30 patients of varying asthma severity and 10 controls, patients with severe disease had higher mean fluorescence intensity of CD11b on circulating neutrophils compared to those with mild-moderate disease and control subjects, although no difference in CD62L expression [531].

In summary there are data that obesity and asthma can both lead to systemic activation of neutrophils. To date there are no published data on these activation markers in obese asthmatics. It postulated that obesity and asthma may interact in an additive manner resulting in not only increased neutrophil numbers but also enhanced cell activation systemically.

5.1.3 Reactive oxygen species production and oxidative stress

5.1.3 (i) Reactive oxygen species production

ROS are molecules which contain unpaired electrons, reacting vigorously with other chemical compounds including proteins, lipoproteins and DNA, altering their structure and function [93]. During activation, neutrophils generate large amounts of ROS via the NADPH oxidase system. ROS are released into phagosomes containing ingested pathogens or into the extracellular space, damaging surrounding tissue and perpetuating inflammation.

In the context of asthma, ROS production by circulating neutrophils in response to an inflammatory stimulus is increased. In a study of asthmatics of varying severity, neutrophil ROS production (measured by flow cytometry using dihydrorhodamine in response to PMA stimulation) was noted to be higher in the asthmatics than controls. This suggested again that changes in the function of circulating neutrophils were seen at a systemic level in these individuals. Interestingly there was no difference in production between those with severe versus mild disease [531]. Studies in obese individuals examining neutrophil PMA-induced ROS activity using a flow cytometry based dihydrofluorescein oxidation assay and chemiluminescence, show that circulating neutrophils derived from obese individuals have a higher ROS production than their lean counterparts, again suggesting systemic neutrophil activation [532].

Despite these findings there has been little work examining the ROS response of circulating neutrophils in obese asthmatics. It was hypothesised that the effect may be additive with neutrophils derived from these individuals exhibiting greater ROS activity than lean asthmatics and obese controls at a systemic level which may then contribute towards local airway inflammation.

5.1.3 (ii) Oxidative stress

Oxidative stress occurs when there is an imbalance between ROS generation and antioxidant defences. A number of mechanisms may promote oxidative stress in obesity [533] and some of these will be summarised here (Figure 5.5). Obesity is associated with chronic inflammation with activation of the innate immune system, resulting in leukocyte activation and resultant ROS generation (see previous section). Hyperleptinaemia is associated with a number of pro-inflammatory effects (Table 4.3) indirectly promoting the generation of ROS. Furthermore, leptin can directly induce ROS production by endothelial cells [534] and monocytes [456]. Obesity is also associated with hyperglycaemia which can promote oxidative stress through several pathways [533]. Chronic hyperglycaemia leads to glycosylation of proteins, lipids and nucleic acids (advanced glycosylation end products (AGE)) which bind to surface receptors (RAGE) promoting NFκB activation and ROS production [535]. Hyperglycaemia also results in activation of NADPH oxidase, resulting in increased NADPH production and therefore ROS generation especially by endothelial cells. Finally glucose can auto-oxidise producing free radicals. Elevated lipid levels may also play a role in oxidative stress through a variety of mechanisms [533]: increased intracellular triglycerides can interfere with the mitochondrial electron transport chain causing enhanced superoxide production and increased FFA can also activate the respiratory burst in leukocytes. The obese state is also associated with reduction in the levels of dietary antioxidants and in the action of antioxidant enzymes including SOD. Finally, increased muscle activity due to higher load carrying can lead to more ROS generation through heightened electron transport chain activity and greater production of the purine derivative hypoxanthine which is converted to urate forming superoxide radicals as a bi-product.

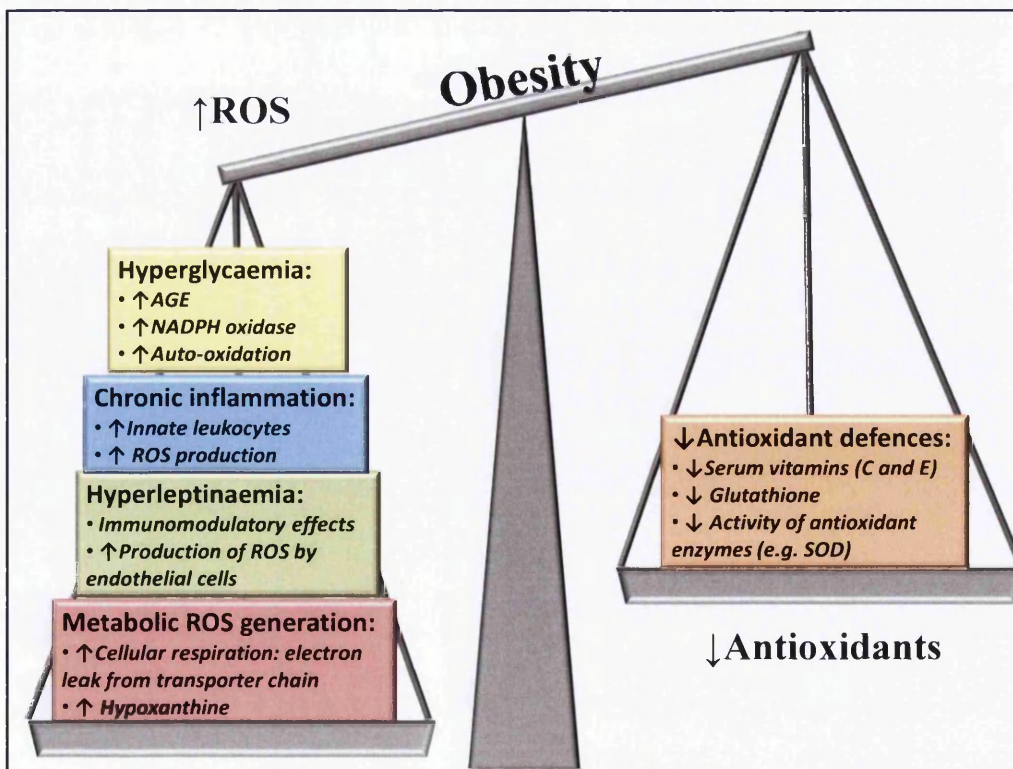


Figure 5.3: Summary of the mechanisms in obesity which promote oxidative stress by increasing ROS production and decreasing antioxidant defences.

A large number of cross-sectional studies have shown obesity to be associated with markers of oxidative stress systemically [94]. Four of these studies have focussed on obese otherwise healthy women using a variety of different measures have all shown evidence of increased oxidative stress in obesity (Table 5.1), particularly with visceral adiposity[536].

Study design	Number of participants	Age range	Outcome	Reference
Obese: mean BMI = 36.7 kg/m ² Non-obese: mean BMI = 21.9kg/m ²	39	Pre-menopausal	↑ plasma TBARS in obese vs. non obese women.	[537]
Obese: mean BMI=39kg/m ² Non-obese: mean BMI 22.5kg/m ²	73	38-45 years	↑ urinary 8 Isoprostanes in obese vs. non-obese, higher levels in android vs. gynoid obesity.	[536]
Obese: mean BMI = 37.1kg/m ² Non-obese: mean BMI = 19.1kg/m ²	73	33.8-36.9 years	↑ serum MDA in obese vs. non-obese.	[538]
Obese: mean BMI = 45.3kg/m ² Non-obese: mean BMI = 20.1kg/m ²	43	31.5-38.2 years	↑ lipid hydroperoxides in isolated HDL and LDL in obese vs. non obese.	[539]

Table 5.1: Summary of cross-sectional studies examining the association between obesity and oxidative stress in women.

The studies are listed in chronological order. A number of different measures were used including the lipid peroxidation product MDA directly in serum, indirectly using TBARS (see section) or another lipid peroxidation production 8 isoprostane in urine or lipid hydroperoxides in HDL and LDL.

Asthma is also associated with increased oxidative stress systemically as evidenced by enhanced ROS production by stimulated blood leukocytes [177], including neutrophils [531], and higher levels of blood markers of lipid peroxidation (plasma TBARS [177] and plasma isoprostanes [540]), and increased protein carbonyls [177], suggesting that novel asthma therapies could be developed focussing on antioxidant defences. During exacerbations systemic oxidative stress increases further, evidenced by higher levels of plasma lipid peroxidation products (measured by TBARS) and a reduction in plasma total oxidant status (TAOS) [179].

Enhanced oxidative stress may modify disease behaviour in asthma. Increased levels of biomarkers of oxidative stress in lavage fluid of patients with severe disease have been associated with a decrease in reduced glutathione and an increase in the oxidised form [541]. Furthermore, in animal models, increasing the ratio of reduced:oxidised glutathione skews T helper responses towards a Th1 bias through IL-12 production. It is therefore conceivable that changes in the redox balance of glutathione within the airways may potentiate a Th1 response and enhanced airway inflammation.

There is also some evidence that enhanced ROS activity may result in glucocorticoid resistance through inactivation of histone deacetylase 2 (HDAC2)[542] and therefore any process which promoted oxidative stress could enhance glucocorticoid resistance.

Over time, chronic oxidative stress in obesity can lead to end organ damage and this is thought to be important in the development of atherosclerosis [343] and non-alcoholic steatohepatitis [543]. Little work has been done on whether this cumulative effect may also impact on the airways, explaining the obesity-asthma association. A study by Sood examined 2,865 individuals taking part in the Coronary Artery Risk Development in Young Adults (CARDIA) study [360]. Of these, 8.1% of had a physician diagnosis of asthma. BMI in women but not men was associated with increased plasma 8-isoprostanes. Asthma diagnosis was also associated with higher levels, however this did not persist after adjusting for gender and BMI differences. Although increasing BMI in women was associated with asthma diagnosis this association was unaffected by adjusting for isoprostane levels, leading the authors to conclude that the BMI-asthma association was not statistically explained by oxidative stress. This study had a number of limitations, including a self reported "physician diagnosis" of asthma rather than objective diagnostic markers and also the authors had no information on asthma control or severity. A further study of plasma 8 isoprostane in 67 non-smoking asthmatics and 33 controls found that whilst plasma isoprostane levels increased with asthma, they did not significantly change across BMI categories [361]. The same group found that increasing BMI was associated with increased exhaled 8 isoprostanes and declining exhaled FeNO, although levels did not differ between asthmatics and controls [27]. However, the study populations used in both these publications had high levels of co-morbidities in the asthmatics (18% diabetic, 12% OSA, 45% hypertensive) which may have confounded results.

In the current study population of exclusively female asthmatics and controls with very little comorbidity it was decided to re-address this question by examining the oxidative stress markers thiobarbituric acid reactive substances (TBARS) and TAOS.

5.2 Methods

A detailed description of the recruitment process is described in section 3.1 but will be summarised here.

5.2.1 Study population

Pre-menopausal women with and without asthma were recruited. Subjects and controls were divided into 3 groups on the basis of body mass index (BMI), giving 6 groups in total (Figure 3.1). Normal weight was defined as BMI 18.5-25kg/m², overweight as a BMI \geq 25kg/m² and < 30kg/m² and obesity as a BMI \geq 30kg/m².

Asthmatic patients were recruited from a local tertiary clinic, 3 surrounding secondary care clinics and a participating GP practice.

After case note review, all asthmatic subjects fulfilling the inclusion/exclusion criteria (Figure 3.1) attended an appointment at a tertiary asthma service where asthma diagnosis was confirmed. The diagnosis required consistent symptoms and demonstrable significant reversible airways obstruction to a β_2 agonist (12%) or if this was not present, significant PEFV variation or a positive bronchial provocation test. Disease severity was graded according to GINA criteria (Appendix VII) and in those with very good disease control, therapy was stepped down to the lowest level to maintain this. Asthmatics were considered stable if they had no exacerbations, oral steroid therapy or respiratory tract infection in the preceding 6 weeks.

Asthmatics were asked to complete a modified European Respiratory Health Survey [391] (Appendix V). Healthy control subjects of varying BMI were recruited from the local university, hospital staff and a Slimming World weight loss club.

5.2.2 Clinical measures and blood collection

Participants were asked to attend during the first 7 days of their menstrual cycle, determined using the date of onset of menstruation, in the fasted state between the hours of 07.00-09.00am. Acute disease control at the time of recruitment in the asthmatics was assessed using the Juniper Asthma Control Questionnaire (Appendix VI) [392].

Spirometry was performed using a portable dry spirometer (Vitalograph) calibrated on the day of use. All asthmatics were asked to withhold medication for the preceding 24 hours. The best of 3 measurements was taken according to a standardised protocol [394]. Following this measures of WHR and percentage body fat composition were taken.

Fasting blood was collected into lithium heparin tubes and gel and clot activator tubes for serum.

5.2.3 Plasma cytokines

Plasma was separated from whole blood by density gradient centrifugation (see section 2.6 for details). Blood (10ml) was gently layered onto Histopaque 1077 (10ml) (Sigma, USA) in a 50ml Falcon tube (Greiner Bio-one, Germany) and centrifuged at 805 x g for 20 minutes (no brake). The plasma was then removed, filtered (0.2 µm polyethersulfone filter, low protein binding; Sigma, USA) and stored at -20°C prior to analysis. Circulating IL-6, IL-8, IL-17A, IL-23 G-CSF and GM-CSF were measured using enzyme linked immunosorbant assay (ELISA). The principles and methods of this technique are outlined in section 3.11. Table 5.2 outlines the commercially available kits used and their respective sensitivities. Note that in this case the sensitivity is set as the least concentrated of the standards included in each assay.

Cytokine	Sample	Sensitivity (pg/ml)	Source
GCSF	Plasma	39.063	Quantikine, R&D systems, Europe.
GMCSF	Plasma	7.813	Quantikine, R&D systems, Europe.
IL-6 (HS)	Plasma	0.156	Quantikine, R&D systems, Europe.
IL-8	Plasma	1.0	Quantikine, R&D systems, Europe.
IL-17A	Plasma	1.563	Platinum, eBiosciences, UK
IL-23	Plasma	39.063	Quantikine, R&D systems, Europe.

Table 5.2: ELISA cytokine kits and their respective sensitivities.

HS: A high sensitivity IL-6 kit was used.

5.2.4 PMA-mediated reactive oxygen species production in whole blood

The principles behind reactive oxygen species production by phagocytes and the use of chemiluminescence in their detection is summarised in section 3.8.1. Whole blood was diluted in PBS (1:10 dilution) and 25µl added to each well. Blood was stimulated using 25µl phorbol myristate acetate (PMA; 1uM Sigma), which was added to each well. Luminol (5-amino-2,3,-dihydro-1,4-phthalazinediane 2mmol/l; Sigma) 25µl/well was added to each well to amplify the reaction. The samples were measured on a plate reader within 5 minutes and the peak light emitted measured.

5.2.5 CD11b and CD62L expression on neutrophils

Neutrophil expression of CD11b and CD62L was measured using flow cytometry. Measurement of monocyte activation markers was done on the same samples (see section 6.2.3). The principles behind this technique are outlined in section 3.7. The antibodies, conjugated fluorochromes and their appropriate isotype controls used are listed in Table 5.3.

Heparinised whole blood (100µl) was added to 4 tubes. Tube 1 contained no additional antibodies. Tube 2 contained anti-CD15:e450 to identify neutrophils, anti-CD14:PerCPCy5.5 to identify monocytes, and isotype controls for the activation makers present in subsequent tubes (mouse IgG1:APC and mouse IgG2a:PE)(see section 5.2.5). Tube 3 contained anti-CD15:e450 and anti-CD62L:PE along with antibodies for the monocyte activation experiments (anti-CD14:PerCP-Cy5. 5 and anti-HLA-DR:FITC. Tube 4 contained anti-CD15:e450 and anti-CD11b:APC along with antibodies for the monocyte experiments (anti-CD14:PerCP-Cy5.5 and anti-CD16:FITC). The samples were vortexed before incubation on ice for 30 minutes. Samples were then treated with 3ml of red blood cell lysis solution (FACS lysing solution; BD Biosciences, USA) and incubated in the dark at room temperature for 10 minutes. Cells were collected by centrifugation (4°C, 515 x g for 7 minutes) and the supernatant discarded before washing in 3ml of FACS buffer (PBS with 0.2% BSA and 0.05% sodium azide). The tubes were centrifuged, supernatant removed and then the samples were fixed using 200µl FACS fix (BD Biosciences, USA).

The stained samples were refrigerated and acquired within 24 hours on a BD FACSAria I flow cytometer: 10,000 events were recorded for each sample. The gating strategy used to measure CD11b and CD62L expression is shown in Figures 5.4 and 5.5, respectively. Neutrophils were identified as a population of cells with high side scatter and by CD15 expression. After gating on this population, CD62L and CD11b expression were measured by median fluorescence intensity. To control for non-specific antibody binding, median fluorescence intensities were also measured in the neutrophils stained with the respective isotype control antibodies. The signal index was calculated from the ratio between median fluorescence intensity in the sample containing the specific antibody (anti-CD11b or anti-CD62L) and the isotype control.

Antigen	Fluorochrome	Cell expression	Clone	Source
CD11b (MAC1-α)	APC	Activation epitope of neutrophils and monocytes	CRBM1/5	eBioscience, UK
CD62L (L-selectin)	PE	Low expression on chronically activated neutrophils and monocytes	DREG-56	eBioscience, UK
CD15	e450	High expression on neutrophils	H198	eBioscience, UK
Mouse IgG1 Isotype control	APC		P3.6.2.8 .1	eBioscience, UK
Mouse IgG2a Isotype control	PE		20102	R&D systems
Rat IgG2a Isotype Control	APC		eBR2a	eBioscience, UK

Table 5.3: List of antibodies and fluorochromes used to measure surface markers of neutrophil activation.

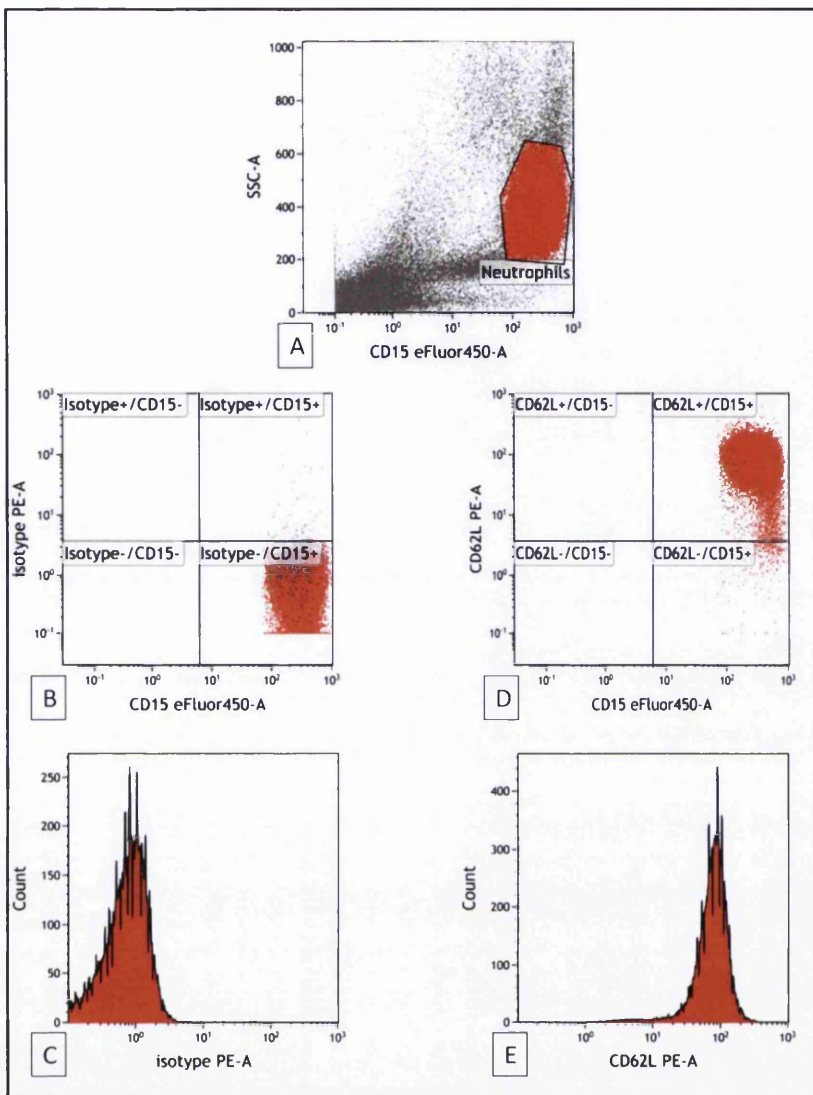


Figure 5.4: Gating strategy for measuring neutrophil CD62L expression.

A: Neutrophil population was identified by side scatter (SSC) and CD15:eFluor450 expression.

B: Neutrophils gated. Mouse IgG2a:PE isotype control used to set gating and control for non-specific binding.

C: Median fluorescence intensity measured using histogram for isotype control.

D: Neutrophils gated. CD62L positive neutrophils identified using gate set by isotype control.

E: Median fluorescence intensity measured using histogram for CD15⁺CD62L⁺ neutrophils.

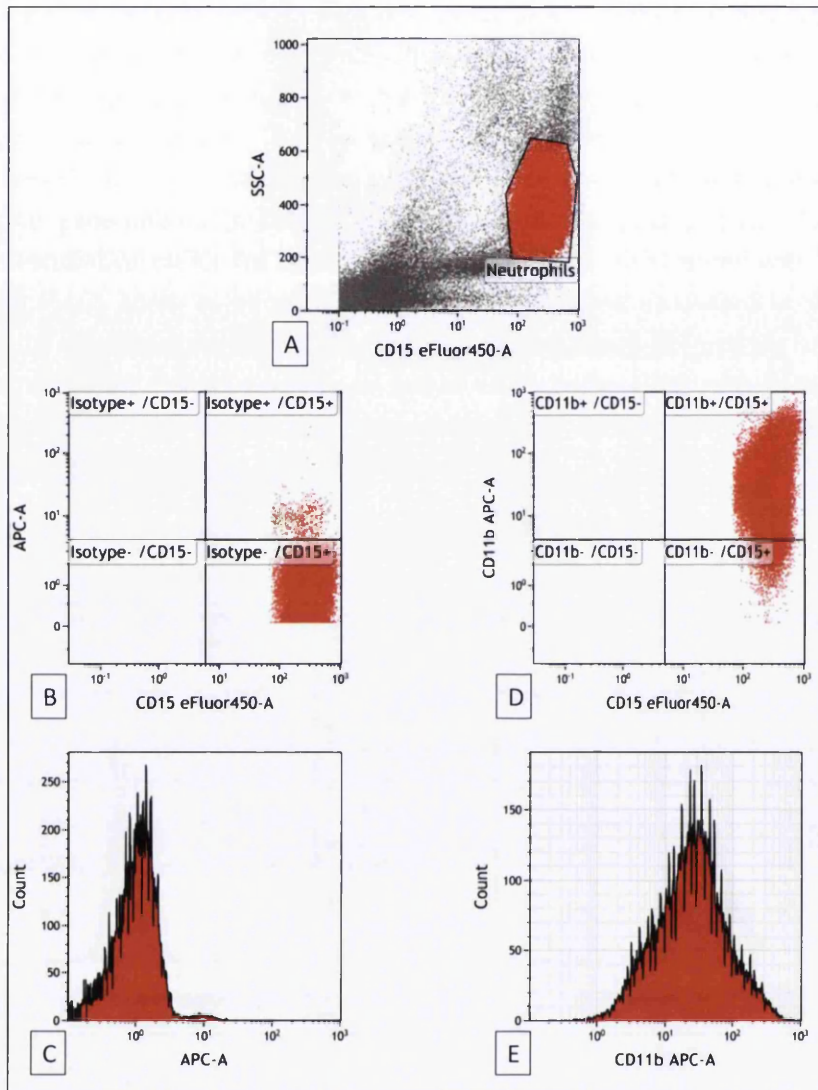


Figure 5.5: Gating strategy for measuring neutrophil CD11b expression.

A: Neutrophil population was identified by side scatter (SSC) and CD15:eFluor450 expression.

B: Neutrophils gated. Mouse IgG1:APC isotype control used to set gating and control for non-specific binding

C: Median fluorescence intensity measured using histogram for isotype control

D: Neutrophils gated. CD11b:APC positive neutrophils identified using gate set by isotype control

E: Median fluorescence intensity measured using histogram for CD15⁺CD11b⁺ neutrophils

5.2.6 Statistical Analysis

Each of the parameters measured was examined visually using a histogram and a KS test for normality was performed. Positively skewed data were logarithmically transformed before analysis. Differences between normally distributed groups were analysed using an unpaired t-test (between 2 categories) or 1 way analysis of variance (ANOVA) (between 3 or more). Associations between normally distributed continuous variables were examined using a Pearson's correlation coefficient. More complex relationships were explored through general linear models. Proportional data were analysed using a chi-squared test of association.

5.3 Results

5.3.1 Circulating plasma cytokines

Plasma cytokine levels were measured in all of the 84 participants in the study. Data regarding IL-6, IL-8 and G-CSF levels did not pass the KS test ($p=0.001$, $p<0.001$ and $p=0.01$) as they were positively skewed so values were logarithmically transformed for analysis. Levels of GM-CSF, IL-23 and IL-17 were only above the sensitivity of the ELISA used in a small number of cases and therefore data were dichotomised into detectable vs. non-detectable.

5.3.1 (i) GM-CSF

Only 38 individuals had detectable levels of GM-CSF in the plasma so data were categorised into whether levels were detectable or not. When analysing GM-CSF as a categorical variable there was no significant difference in the proportion of individuals with detectable levels across the 6 groups (Table 5.4.). Analysing the cohort by BMI category ($p=0.793$) or by asthma diagnosis ($p=0.843$) did not reveal any associations. No differences in the proportion of participants with detectable levels according to BMI category was seen when asthmatics ($p=0.605$) and controls ($p=0.276$) were analysed separately.

5.3.1 (ii) G-CSF

G-CSF levels did differ significantly between the 6 groups (Table 5.4) but were not significantly different in asthmatics compared to controls across the entire study population ($p=0.334$) or when each BMI category was compared individually. However, they did increase significantly with BMI category ($p=0.005$) and continuous BMI ($r=0.278$, $p=0.010$) across the entire population. G-CSF levels also significantly positively correlated with percentage body fat ($r=0.341$, $p=0.002$) but not with WHR ($r=0.203$, $p=0.067$). Levels were not associated with participant age. When asthmatics and controls were analysed separately G-CSF levels correlated significantly with BMI and percentage body fat in the asthmatics ($r=0.395$, $p=0.011$; $r=0.500$, $p=0.001$, respectively) but not the controls ($r=0.147$, $p=0.345$; $r=0.184$, $p=0.236$). However when an interaction between BMI and asthma status with

regards to BMI was formally tested for this was not seen ($p=0.291$). Within the asthmatics studied, GCSF did not correlate with various markers of asthma control, medication usage, FEV1 (expressed as percentage predicted) ($r= -0.298$, $p=0.062$), or PEFr percentage predicted ($r=-0.280$, $p=0.080$).

As G-CSF levels are associated with neutrophil differentiation and release from the bone marrow the association between neutrophil levels and GCSF was explored. There was no association ($r=0.114$, $p=0.301$) and using a general linear model the association between blood neutrophil count, asthma and BMI was not significantly affected by adjusting for GCSF (Asthma $p=0.003$, BMI $p=0.001$ respectively, $p=0.901$ respectively R^2 value =0.222).

	Controls			Asthmatics			p value
	NW n=15	OW n=13	OB n=15	NW n=14	OW n=12	OB n=15	
GM-CSF <i>Number of cases where GMCSF detectable</i>	6/15	4/13	9/15	6/14	7/12	6/15	All 6 groups $p=0.606$ A vs. C $p=0.843$ BMI category $p=0.793$
G-CSF (pg/ml) Geometric mean (SD)	30.45 (12.68)	36.86 (19.60)	34.67 (5.61)	31.15 (5.50)	36.41 (14.14)	39.96 (8.05)	All 6 groups $p=0.041$ A vs. C: $p=0.334$ BMI category: $p=0.005$
IL-17 <i>Number of cases where IL-17 detectable</i>	2/15	2/13	5/15	7/14	7/12	6/15	All 6 groups $p=0.081$ A vs. C $p=0.007$ BMI category $p=0.886$
IL-23 <i>Number of cases where IL-17 detectable</i>	7/15	7/13	8/15	7/14	4/12	7/15	All 6 groups $p=0.922$ A vs. C $p=0.552$ BMI $p=0.903$
IL-6 (pg/ml) Geometric mean (SD)	0.57 (1.57)	0.82 (0.22)	1.13 (0.95)	0.93 (1.71)	1.26 (0.78)	1.79 (0.89)	All 6 groups $p<0.001$ A vs. C: $p=0.001$ BMI category $p<0.001$
sIL6R (ng/ml) Mean (SD)	216.63 (78.22)	274.06 (143.80)	254.98 (84.58)	189.89 (101.71)	216.22 (90.15)	227.26 (84.98)	All 6 groups $p=0.285$ A vs. C $p=0.098$ BMI category $p=0.219$
sgp130 (ng/ml) Mean (SD)	183.09 (40.72)	162.83 (29.58)	183.87 (27.89)	168.81 (26.45)	177.09 (44.73)	182.75 (30.26)	All 6 groups $p=0.462$ A vs. C $p=0.903$ BMI category $p=0.306$
IL-8 (pg/ml) Geometric mean (SD)	4.18 (1.62)	3.59 (0.99)	4.30 (1.14)	4.00 (0.89)	5.76 (15.1)	4.45 (2.62)	All 6 groups $p=0.134$ A vs. C $p=0.144$ BMI category $p=0.702$

Table 5.4: Plasma cytokine levels in asthmatics and controls according to BMI category.

Normally distributed data are expressed as mean and standard deviation (SD). Parameters not normally distributed are expressed as geometric mean and standard deviation and were logarithmically transformed before analysis. IL-17, IL-23 and GM-CSF data are represented as the number of patients who had detectable levels. Differences between all 6 groups and between BMI categories were analysed using a one-way ANOVA. Differences between asthmatics (A) and controls (C) were analysed using an unpaired t-test. Differences between proportional data were analysed using a chi-squared analysis.

Plasma IL-17 levels were only detectable in 29 individuals. Therefore IL-17 was analysed as a binary variable (detectable vs. non-detectable). There was a significant difference in the proportion of patients who had detectable levels across the 6 groups (Table 5.4). This was due to a significantly higher proportion of asthmatics (20/41) having detectable levels than controls (9/43) ($p=0.007$). No differences were seen with BMI category across the entire study group ($p=0.886$) or when asthmatics ($p=0.635$) and controls ($p=0.340$) were analysed separately.

5.3.1 (iv) IL-23

40 individuals within the cohort had detectable levels of IL-23 and therefore it was converted to a binary variable (detectable vs. non-detectable). The proportion of individuals with detectable levels did not significantly vary across the 6 study groups (Table 5.4) or with asthma status ($p=0.552$) or by BMI category across the entire study group ($p=0.903$) or when asthmatics ($p=0.670$) and controls ($p=0.991$) were analysed separately.

5.3.1 (v) IL-6, sIL-6R and sgp130

Plasma IL-6 levels varied significantly across the 6 study groups (Table 5.4). Levels were significantly higher in asthmatics than controls ($p=0.001$) and increased significantly with each BMI category and continuous BMI ($r=0.424$, $p<0.001$) across the entire study population. Using a general linear model, IL-6 levels were independently associated with asthma status and BMI ($p=0.001$ and $p<0.001$ respectively, $R^2=0.279$). The associations appeared to be additive such that obese asthmatics had the highest level (1.79pg/ml) which was significantly higher than normal weight asthmatics (0.93pg/ml, $p=0.012$) and obese controls (1.13pg/ml, $p=0.02$). IL-6 also correlated positively with percentage body fat ($r=0.370$, $p=0.001$) but not significantly with WHR ($r=0.184$, $p=0.098$) across the entire study group. IL-6 levels did not significantly correlate with age. When asthmatics and controls were examined separately IL-6 increased significantly with BMI category ($p=0.025$ and $p=0.007$ respectively) and continuous BMI ($r=0.992$, $p=0.002$ and $r=0.358$, $p=0.022$ respectively). In the asthmatics studied IL-6 levels were not associated with asthma duration, any of the spirometric measures, markers of asthma control or medication usage.

Given that leptin increases with BMI and was higher in asthmatics than controls the relationship between leptin and IL-6 was explored. Leptin did correlate with IL-6 ($r=0.430$, $p<0.001$). However using a general linear model, the relationship between BMI, asthma and IL-6 levels was not significantly affected by retaining leptin levels within the model (BMI $p=0.029$, subject $p=0.005$, leptin $p=0.46$, R squared value =0.284). As FFA can activate TLR and high dietary fat intake has been shown to enhance systemic IL-6 levels, correlation between FFA levels and IL-6 was considered but none was seen ($r=-0.04$, $p=0.972$).

As IL-6 can affect neutrophil survival; possible associations between levels and neutrophil count were explored. There was a significant association between IL-6 and neutrophil count ($r=0.460$, $p<0.001$). Using a general linear model it was possible to see that the association between BMI,

asthma and neutrophil count was not significantly affected by retaining plasma-IL-6 levels in the model (BMI $p=0.013$, asthma $p=0.046$, IL-6 $p=0.008$ $R^2 = 0.314$).

Soluble IL-6 receptor did not differ significantly across the 6 groups (Table 5.4). Levels were lower in the asthmatics than controls although this was not significant ($p=0.098$). When asthmatics and controls were compared for each BMI category no significant differences were seen in sIL-6R levels. Across the whole study group sIL-6R was not associated with BMI category ($p=0.219$) or continuous BMI ($r=0.171$, $p=0.119$). Similarly, levels were not significantly associated with WHR ($r=0.169$, $p=0.129$) or percentage body fat ($r=0.176$, $p=0.109$). When asthmatics and controls were analysed separately no significant associations were seen with sIL-6R and any of the adiposity measures.

Soluble gp130 did not differ significantly across the 6 groups and was not significantly associated with asthma status ($p=0.903$), BMI category ($p=0.306$), continuous BMI ($r=0.097$, $p=0.379$), WHR or body fat composition. No associations were seen with adiposity measures when asthmatics and controls were analysed separately.

5.3.1 (vi) IL-8

IL-8 levels did not differ significantly across the 6 groups (Table 5.4). Plasma IL-8 levels did not significantly vary with asthmatic status, BMI category, or BMI as continuous variable ($r=0.069$, $p=0.534$). Levels were not associated with percentage body fat ($r=0.047$, $p=0.673$) or WHR ($r=0.134$, $p=0.230$) across the entire study group or when asthmatics and controls were analysed in isolation.

5.3.2 CD11b and CD62L expression by neutrophils

CD62L expression on neutrophils (measured as a signal index) was recorded in 36 individuals (20 controls and 16 asthmatics) and data passed the KS test ($p=0.62$). No significant difference in signal index was seen across the 6 groups ($p=0.880$) (Figure 5.6). Asthma status was not associated with CD62L expression ($p=0.661$) and neither was BMI when examined categorically ($p=0.491$) or as a continuous variable across the entire study group ($r=0.074$, $p=0.394$). Other markers of adiposity including body fat composition and WHR were not associated with CD62L expression ($r=0.198$, $p=0.246$ and $r=0.290$, $p=0.096$ respectively). When asthmatics and controls were analysed separately, no correlation between CD62L expression and markers of adiposity were seen in either group.

Neutrophil expression of CD11b (measured as signal index) was examined in 31 individuals (15 controls and 16 asthmatics) and was not normally distributed so was logarithmically transformed for analysis ($p=0.096$). CD11b expression did not vary significantly across the 6 groups ($p=0.368$). Asthma status ($p=0.644$), BMI category ($p=0.925$) and BMI as a continuous variable ($r=0.124$, $p=0.506$) were not associated with CD11b expression, neither were other markers of adiposity including body fat composition and WHR ($r=0.055$, $p=0.768$; $r=0.162$, $p=0.403$ respectively). When

asthmatics and controls were analysed separately no associations between CD11b expression and adiposity measures were seen.

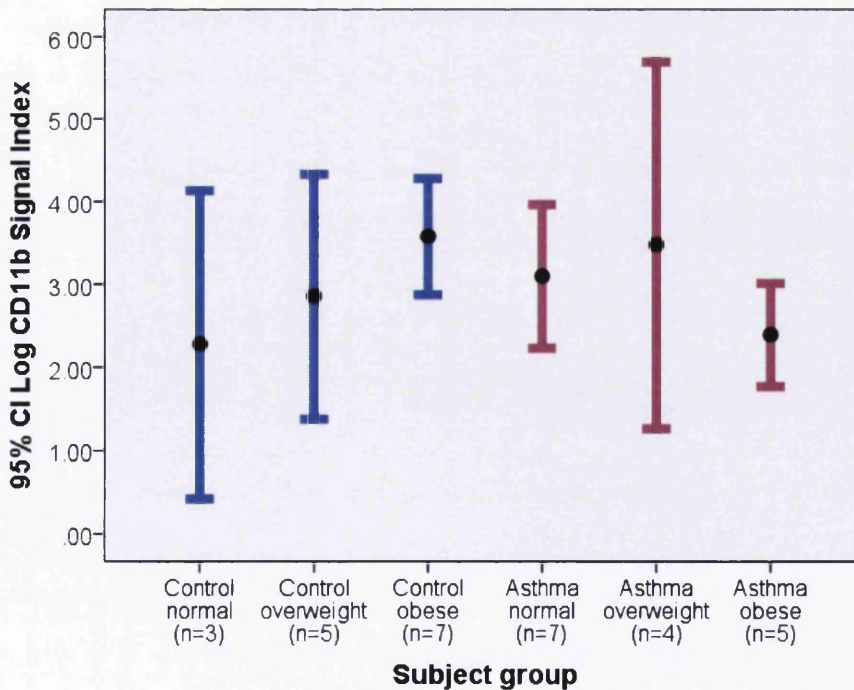
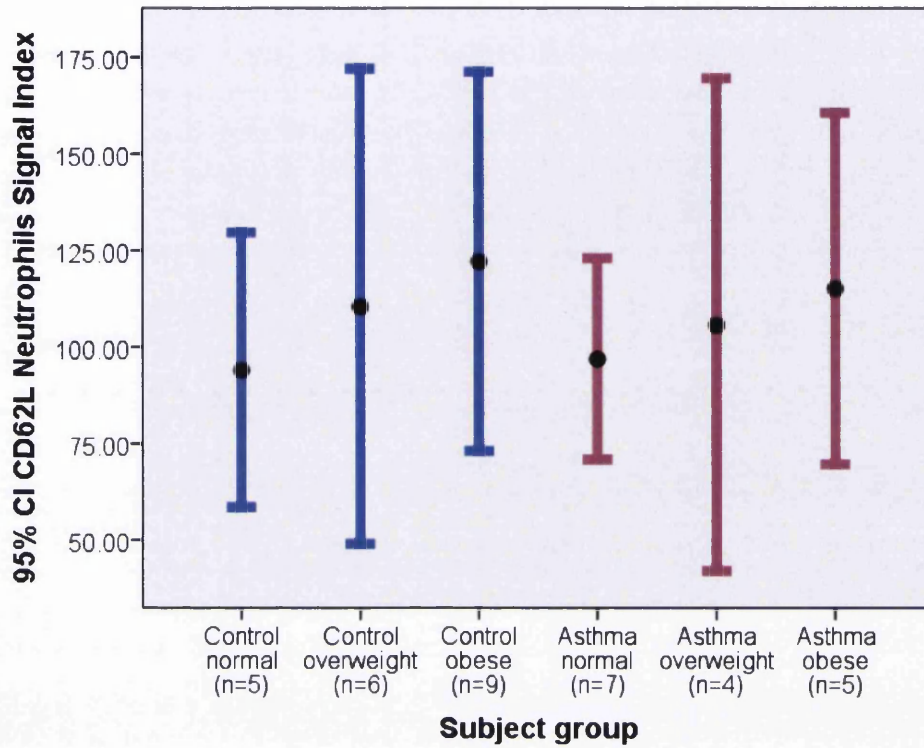


Figure 5.6: CD62L and CD11b expression on neutrophils in asthmatics and controls according to BMI category.
Data displayed as mean and error bars. Neutrophil CD11b expression was not normally distributed and therefore was logarithmically transformed before analysis.

5.3.3 Oxidative stress

5.3.3 (i) PMA-mediated reactive oxygen species production

PMA-induced ROS production was examined in 80/84 participants; due to equipment failure it was not measured in 1 NW control, 2 OW controls and 1 OB asthmatic. Values, expressed as maximal light units produced did not pass the KS test ($p=0.012$) and were logarithmically transformed for analysis. Whole blood PMA-induced ROS production varied significantly across the 6 groups (Figure 4.7). Asthmatics had significantly higher levels than controls and across the entire cohort levels increased with each BMI category ($p=0.012$) and when BMI was analysed as a continuous variable ($r=0.347$ $p=0.002$). Body fat composition also significantly correlated with ROS production ($r=0.388$, $p<0.001$) however, WHR did not ($r=0.155$, $p=0.174$). Age was not associated with a significant change in ROS production.

When asthmatics and controls were analysed separately, the association between BMI category and ROS response was only significant in the controls ($p=0.015$; $r=0.491$, $p=0.001$) and not in the asthmatics ($p=0.140$; $r=0.244$, $p=0.129$), however when a formal interaction between asthma status and BMI with regards to ROS generation was tested for no significant interaction was seen ($p=0.120$, $R^2=0.243$). Within the asthmatics studied, asthma duration, indicators of disease control, ICS usage and spirometric values did not correlate with ROS production. Using a general linear model it was noted that ROS production was independently associated with BMI and asthma status ($p=0.009$ and $p=0.002$ respectively, $R^2=0.196$). Obese asthmatics had the highest ROS activity, higher than non-obese asthmatics ($p=0.054$) and obese controls ($p=0.106$) although this was not significant.

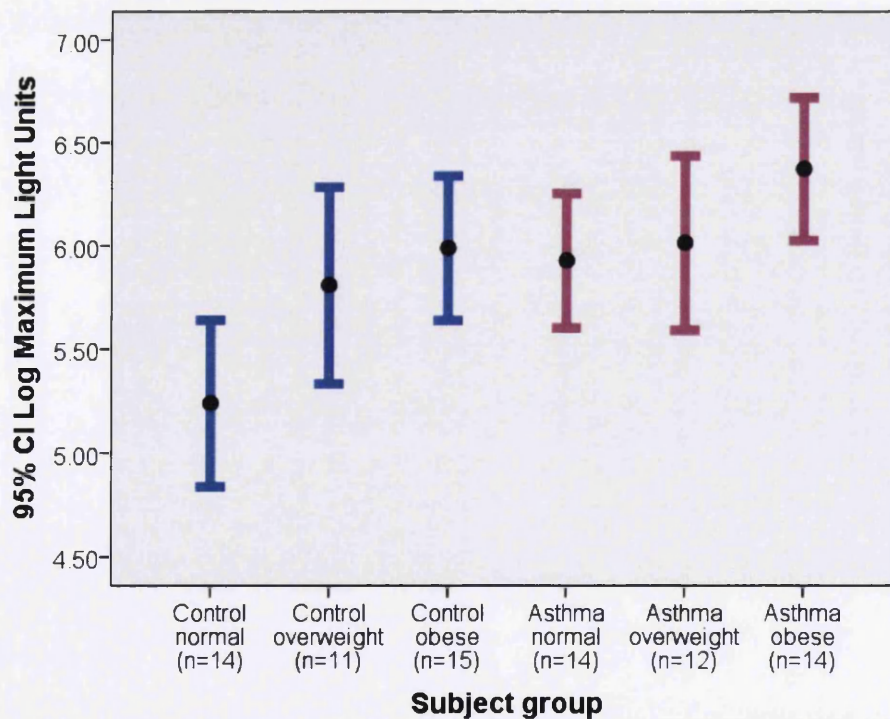


Figure 5.7: Whole blood reactive oxygen species production following PMA stimulation in asthmatics and controls according to BMI category.

Data shown as mean and error bars. ROS production significantly differed across the 6 groups ($p=0.002$), with ROS production increasing with BMI category ($p=0.012$) and higher levels being observed in the asthmatics ($p=0.006$). Obese asthmatics had the highest ROS production in response to PMA stimulation but this was not significant.

Neutrophil count had a very significant association with ROS production ($p < 0.001$) and the association between ROS production, BMI and asthma was less significant when neutrophil count was retained in a general linear model (BMI $p = 0.047$, asthma $p = 0.120$, neutrophils $p < 0.001$, $R^2 = 0.303$) suggesting that the increased neutrophil count is at least partly responsible for the increased ROS activity seen.

5.3.3. (ii) TBARS

Plasma lipid peroxide levels (expressed in terms of concentration of MDA ($\mu\text{Mol/l}$)) and plasma TAOS (expressed as a percentage inhibition of the formation of the peroxidase-mediated reaction of the 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS⁺) radical compared to control (PBS)), were measured in the 84 participants. Both parameters passed the KS test. The mean levels in each of the 6 categories are summarised in Table 5.5. Plasma MDA levels and TAOS did not vary significantly across the 6 categories (Table 5.5), and levels did not differ between the asthmatics and controls ($p = 0.734$ and $p = 0.390$ respectively) across the entire population or when each BMI category was examined individually. Levels did not vary with BMI category (MDA levels: $p = 0.488$, TAOS: $p = 0.205$) or BMI as a continuous variable across the entire population (MDA: levels $r = 0.220$, $p = 0.191$, TAOS: $r = 0.162$, $p = 0.143$) or in the asthmatics and controls when analysed separately. Furthermore, levels did not correlate with other measures of adiposity (WHR and body fat percentage). When asthmatics and controls were analysed separately no associations were seen with any of the adiposity measures. Age did not correlate with this variable and within asthmatics, age of onset, asthma duration, ICS use, control score, and spirometric measures were not associated with this variable.

	Controls			Asthmatics			p value
	NW n=15	OW n=13	OB n=15	NW n=14	OW n=12	OB n=15	
Plasma MDA levels ($\mu\text{Mol/l}$) Mean (SD)	14.52 (6.83)	13.13 (4.61)	14.14 (4.02)	12.38 (2.50)	14.57 (5.01)	15.82 (4.90)	All 6 groups: $p = 0.521$ A-C: $p = 0.734$ BMI category: $p = 0.488$
Plasma TAOS (%) Mean (SD)	48.57 (8.20)	52.66 (9.85)	52.02 (7.74)	47.23 (6.58)	49.77 (7.79)	51.09 (9.98)	All 6 groups: $p = 0.534$ A-C: $p = 0.309$ BMI category: $p = 0.205$

Table 5.5: Plasma MDA levels and TAOS in asthmatics and controls according to BMI category.

Data was normally distributed data and therefore expressed as mean and standard deviation (SD). Differences between all 6 groups were analysed using a one-way ANOVA. Differences between asthmatics (A) and controls (C) were analysed using an unpaired t-test. Differences between BMI categories were analysed using a one-way ANOVA

5.4 Discussion

5.4.1 Circulating cytokines

5.4.1 (i) IL-6 increases significantly with obesity and asthma

Data presented in chapter 4 show that the peripheral blood neutrophil count is higher in asthmatics, and increases with BMI, resulting in obese asthmatics having higher neutrophil counts than the other study groups. By measuring circulating cytokines important in neutrophil regulation, investigation of some of the mechanisms which may be responsible for this was initiated. IL-6 has direct effects on neutrophil survival [510], function [511] and trafficking [509] as well as indirectly promoting neutrophilia through the induction of Th17 cells (see section 7.1.2 (ii)). A high proportion of circulating IL-6 is derived from adipose tissue and in keeping with this, studies have shown elevated levels in the obese [517]. Data herein confirms that IL-6 significantly increases with BMI and the findings suggest that IL-6 may be important in obese asthma as these subjects had the highest circulating levels.

A previous study has shown elevated IL-6 levels in obese compared to non-obese asthmatics, however with no control group, it was not possible to determine if these levels differed from obese non-asthmatics [544]. In a study by Sutherland et al, circulating IL-6 levels were also highest in the obese female asthmatics compared to non-obese asthmatics and obese controls, however this was not significant [25]. The current study was similar in size and design, however in the Sutherland study an attempt was made to wean subjects off ICS, resulting in loss of asthma control in 28/40 patients. This resulted in recruitment of these patients during an exacerbation which may have partially masked differences in circulating inflammatory markers. It also prevented the investigators from timing their sampling around the menstrual cycle. The observation of highest circulating IL-6 levels in the obese asthmatics has recently been confirmed by a study examining airway and systemic inflammation in obese men and women with and without asthma [429]. In that study circulating IL-6 and C reactive protein (CRP) levels increased with obesity and asthma such that obese asthmatics had significantly higher levels than non-obese asthmatics and obese control subjects.

The obese asthma phenotype is characterised by female predominance [8] and in a recently published study by Scott et al, the relationship between circulating IL-6, obesity and asthma was only significant in women [429]. The differential sex effect may be due to hormonal factors. Some circulating cytokines are significantly affected by cyclical hormone changes; plasma IL-8 levels are 4 fold higher in the follicular vs. luteal phase [545] making it imperative to control for this fluctuation. However data suggest that plasma IL-6 levels do not fluctuate with the menstrual cycle [546]. Another explanation may be differences in body fat composition. Body fat deposition tends to occur in the subcutaneous compartment in women which leads to higher levels of leptin release than in men [327]. Leptin was associated with increasing BMI and asthma such that obese asthmatics had significantly higher levels than the other groups (section 4.3.8) and leptin strongly correlated positively with IL-6 levels. This led to the hypothesis that leptin may be important in mediating the observed changes in IL-6. However, the association between BMI, asthma and IL-6 was not

significantly affected by including leptin as a covariate in a general linear model, suggesting that leptin is unlikely to be the sole mediator for the increased IL-6 levels seen.

The mechanism behind the changes in IL-6 levels needs further exploration. A major source of circulating IL-6 is adipose tissue, with as much as 25% derived from this [517]. In obesity adipose tissue contains a much higher proportion of M1 macrophages which produce pro-inflammatory cytokines such as IL-6 [332]. The increased IL-6 production may simply be due to increase in the cell type. The precise mechanism which leads to M1 macrophage accumulation within adipose tissue has yet to be elucidated but mechanisms proposed include changes in local numbers due to response to adipocyte death, activation of common genes important in metabolism and macrophage activation, and local changes in T cell populations [21]. Another potential source of adipose tissue macrophages is increased numbers of circulating activated CD16⁺ monocytes [31], which are programmed to differentiate down a pro-inflammatory M1 phenotype within tissue. This potential mechanism is explored in chapter 6.

IL-6 production may be increased due to changes in PRR signalling, rather than relative cell numbers. FFA can activate TLRs resulting in enhanced IL-6 production acutely after a high fat meal [280]. However FFA levels did not differ significantly between study groups (see section 4.3.7) and there was no significant association between fasting FFA and IL-6. Other TLR ligands including LPS have been shown to be elevated in the obese state [338]; it would be of interest to measure these in this cohort. The response of circulating cells to PRR stimulation may also be altered and this mechanism is explored in chapter 6.

The consequences of increased IL-6 levels may include neutrophilic inflammation through direct and indirect effects via Th17 cells. Neutrophil count did follow a similar pattern to IL-6; levels increased with each BMI category with highest levels in the obese asthmatics (see section 4.3.5). Furthermore IL-6 levels were strongly associated with neutrophil count, however, the association between neutrophil count, BMI and asthma was not significantly altered by retaining IL-6 levels in the model suggesting that IL-6 is unlikely the only mediator in this relationship.

5.4.1 (ii) sIL-6R and sgp130 were not associated with BMI or asthma status

The existence of soluble IL-6 receptor (sIL-6R) enables cells which do not ordinarily express the receptor to respond to IL-6 through trans-signalling (Figure 5.2). The little published work on this receptor in the context of obesity to date has shown no association between serum levels and BMI [518] but a single nucleotide polymorphism (SNP) which promotes IL-6R shedding, increasing sIL-6R levels, has been associated with severe asthma [515]. This presented work is the first to examine sIL-6R in the context of asthma and obesity. Although levels correlated positively with BMI across the entire cohort and were also lower in asthmatics than controls, this was not significant, suggesting that sIL6R is not important in the obesity-asthma association. Trans-signalling with sIL-6R is inhibited by sgp130 (Figure 5.1) and a single study has suggested that sgp130 levels are increased in the obese [518]. The current study showed no association between BMI or other measures of adiposity and sgp130 and no association with asthma diagnosis. These results differ from those of Nikolajuk et al, who examined serum sgp130 in women with and without PCOS categorised according to BMI into lean (BMI<25kg/m²) and overweight/obese (BMI>25kg/m²), showing the latter group to have

significantly higher levels [518]. Even on combining overweight and obese categories, or when examining percentage body fat or WHR, a significant difference in sgp130 levels was not found in the work presented herein. Both studies were similar in design including the sampling window within the menstrual cycle, however, there were significant differences in methodology namely serum versus plasma and different upper BMI cut offs, which might explain the disparity in these results.

5.4.1 (iii) IL-8 levels are not associated with obesity or asthma

Interleukin 8 levels (IL-8) locally within the sputum and airways have been associated with severe neutrophilic asthma [522] and are also increased in acute severe exacerbations of the disease [152]. Circulating levels also increase with BMI [526]. Despite a clear association between obesity, asthma and blood neutrophil count there was no association between plasma IL-8 levels and these parameters. This result is consistent with a previous study examining the obesity-asthma association [25]. The lack of association between BMI and circulating plasma IL-8 levels differs from another previous study [526], however the current study restricted the analysis to pre-menopausal women and controlled for the effects of cyclical effects of hormone changes which have been shown to cause 4 fold changes in the levels of plasma IL-8 [545]. Measurement of levels locally within the airways might have yielded differences but in the single study in which sputum levels was examined, no association was seen [25]. Furthermore whilst basal levels do not differ between groups it may be that levels released on TLR stimulation differ in obese asthmatics; a hypothesis explored in chapter 6.

5.4.1 (iv) IL-17 is more frequently detectable in asthmatics but not in obesity

IL-17A is important in neutrophilic processes and levels are elevated in severe asthma [354] and obesity [352]. Therefore the association between obesity, asthma and levels of this cytokines was explored. The levels of circulating IL-17A in the study participants were generally lower than the detection limits of the ELISA kit used and were only detected in 29/84 participants. However the proportion of patients with detectable levels was significantly higher in the asthmatics than the controls. This is consistent with current literature suggesting higher levels in severe asthma [354]. However we did not observe any association with markers of adiposity, and IL-23 important in Th17 regulation, was not associated with BMI category either. This differs from a previous study which showed elevated IL-17 levels in obese women compared to normal-weight controls [352]. The levels of IL-17 detected in the current study (range 0-7.54pg/ml) are much lower than those in the other cited works (0-22.5pg/ml) [352]. The BMI and age of women within this cohort was similar to that of the previous study, which also took fasting morning samples. This study did differ in the timing of sample in terms of the menstrual cycle with all women recruited early in the follicular phase. There is no literature on whether IL-17 and Th17 levels fluctuate within the menstrual cycle however related cell types - including regulatory T cells which have a reciprocal relationship with Th17 cells in terms of their differentiation - peak in the follicular phase [390]. It seems likely that Th17 cells may follow a reciprocal pattern and therefore may explain the low levels we observed during the early

follicular phase of the menstrual cycle in the women in this study. As for IL-8, whilst basal levels of IL-17 did not differ between BMI groups it will be of interest to see whether levels differ upon stimulation of T cells and this is reported in chapter 7.

5.4.1 (v) G-CSF levels increase with obesity

G-CSF is fundamental to terminal neutrophil differentiation and release from the bone marrow [499]. Despite its importance in neutrophil biology there has been little work on this cytokine in relation to obesity or asthma. Data presented here suggests that G-CSF levels increase with BMI in women, with stronger positive correlations with percentage body fat than BMI or WHR suggesting that the total amount of body fat is important rather than its distribution; an observation not described previously. However this seems unlikely to be the mechanism for the increased neutrophil count observed since G-CSF levels did not correlate with this parameter and the association between asthma, BMI and neutrophil count was not significantly affected by retaining G-CSF in a general linear model.

5.4.2 Neutrophil activation

5.4.2 (i) CD62L and CD11b are unaffected by obesity and asthma

Whilst blood neutrophil levels increase with obesity and asthma, surface markers of neutrophil activation appear unaffected. CD62L (L-Selectin) expression, down regulated in chronic inflammation, was unaffected by obesity or asthma status in the study population. This contrasts with previous reports in the literature which have shown reduced neutrophil expression of this marker in obese patients undergoing bariatric surgery versus lean individuals [31]. However, the mean BMI of the obese patients studied herein was significantly lower (obese group mean BMI= 38kg/m² versus 52kg/ m², respectively); it may be that such changes are only observed at very high BMIs. CD62L signal index was only measured on 16 asthmatics and 20 controls (12NW, 10 OW, 14 OB) as this was not part of the initial study plan, only added following an interim analysis suggesting that neutrophils may be important in obese asthma. The confidence intervals were therefore very wide and it might be that a more adequately powered study is required to detect a difference. There was also no association between BMI and CD11b expression which is consistent with previous work [31]. Asthmatics had no difference in neutrophil CD62L surface expression than controls, an observation also made by another group [531]. However there was also no association between CD11b expression and asthma. Previous work has shown that steroid dependent severe asthmatics have increased levels of this marker on neutrophils compared to mild-moderate asthmatics and healthy volunteers. Whilst this study had similar patient numbers, their patients differed those here in that they were on long-term oral steroids [531], although in the study administration of oral Prednisolone for 1 week did not affect neutrophil CD11b surface expression in the mild-moderate asthmatics. As for CD62L the work herein may have been underpowered to detect a difference in this parameter, only measuring CD11b expression in 15 controls and 16 asthmatics (10NW, 9 OW, 12 OB).

5.4.3 Oxidative stress

5.4.3 (i) Reactive oxygen species generation increases with BMI and asthma

Reactive oxygen species generation from whole blood increased significantly with BMI and asthmatics had higher levels than controls. Obese asthmatics had the highest ROS activity upon PMA stimulation although this was not statistically significant. This is of interest as it suggests systemically that obese asthmatics may be exposed to enhanced oxidative stress when encountering an inflammatory stimulus. This may be of particular relevance to asthma exacerbations, although it should be noted that the association between BMI and ROS generation was only significant in the controls. Neutrophil count was strongly associated with ROS activity suggesting that these were potentially the source of this increased activity seen. It would be of interest to examine ROS activity by flow cytometry as this would enable identification of the specific source of increased ROS activity seen. These data are consistent with previous studies that have shown that PMA-induced oxidative burst in whole blood from asthmatics is higher compared to control subjects [531]. In this current work, the association did not remain significant after adjusting for neutrophil count, suggesting that the enhanced ROS activity seen may be simple due to increased neutrophil numbers rather than enhanced activity at a cellular level.

Similarly our data showed a strong association between markers of adiposity and ROS activity, an observation also noted by others [532]. Adjusting for neutrophil count reduced the significance of this association, however it was still of borderline significance. Dietary intake may be of relevance to the increased ROS activity seen with *in vitro* work showing that certain food groups may inhibit ROS activity [532] and *in vivo* work showing high glucose intake [547] and high fat meals are both associated with enhanced ROS activity in mononuclear cells.

5.4.3 (ii) Plasma markers of oxidative stress were not associated with asthma or obesity

Asthmatics in the study presented herein did not have significantly different markers of oxidative stress compared to control subjects as quantified using plasma TBARS or TAOS. This was an unexpected find as previous studies have shown asthma diagnosis to be associated with increased lipid peroxidation products [177, 540]. There are several possible explanations for the lack of any association. Firstly asthmatic patients were recruited when stable and exacerbation free withholding their regular treatment for only 24 hours prior to sampling; loss of asthma controls is associated with higher plasma levels of oxidative stress [179]. However other studies have also sampled patients when exacerbation free whilst still on inhaled treatment and still found a positive association [177]. It may be that oxidative stress plays more of a role in male asthmatics although from other literature this would also appear unlikely as a study of predominantly women (77-79%) versus controls of varying BMI, found higher plasma 8-isoprostanes in the asthmatics than controls [27]. Sampling at a specific point in the menstrual cycle may have diluted differences between groups, although in otherwise healthy women oxidative stress does not vary across the cycle [548, 549]. It was also not anticipated that BMI and other markers of adiposity would not be associated with markers of oxidative stress. Interestingly a study on obese, predominantly female (77-79%) asthmatics and

controls versus lean individuals (asthmatics = 33kg/m²) also did not find a detectable difference when quantifying oxidative stress using plasma 8-isoprostanes. It may be that this current work was not adequately powered to difference, however from the data presented here there is no supportive evidence that the obesity asthma association is due to changes in systemic oxidative stress.

5.4 Summary

In this chapter, circulating levels of some key cytokines important in neutrophil regulation have been examined. Strong associations were seen between IL-6, obesity and asthma with obese asthmatics having higher levels than the other groups, an observation seen by another group [429]. Furthermore a higher proportion of asthmatics had detectable levels of plasma IL-17 than controls. Interestingly increasing G-CSF levels were seen with obesity, an observation not previously reported. However the association between obesity, asthma and neutrophil count remained significant after adjusting for these variables suggesting other factors must be at play in this complex relationship. Whilst neutrophil levels are increased in obese asthmatics, resulting in enhanced ROS activity to a non-specific stimulus, the markers of activation appear not to change, although the study might be underpowered to detect this. These results suggest that changes in circulating cytokine levels are seen in obesity and asthma. In the next sections differences in dynamic innate and adaptive immune function on stimulation with pattern recognition receptors (chapter 5) and T cell mitogens (chapter 6) will be explored.

Chapter 6

Monocytes, LPS response and dendritic cells in obesity and asthma

6.1 Introduction

The innate immune system provides a non-specific response to potential pathogens through the activation of a limited number of pattern recognition receptors (PRR). These receptors recognise generic molecules present in various types of microorganisms (pathogen associated molecular patterns (PAMPs))[61] (see section 2.2.1). In contrast to the adaptive immune system, this evolutionarily ancient system does not require prior antigen exposure. During this process the inflammatory cytokine profile released shapes the immune response and there is an influx of myeloid derived leukocytes each of which has important functions in the initial response to insult (see section 2.4.1). Beyond the response to the acute stressor the innate immune system coordinates resolution of inflammation and healing. In addition, tissue resident innate immune cells such as macrophages and dendritic cells (DCs) survey the microenvironment, sampling antigen and presenting it to specific T cells to activate the adaptive immune response (see chapter 7). These interactions and the cytokine environment generated, dictate the type of adaptive immune response.

Traditionally, allergic asthma is considered a disease of the adaptive immune response characterised by Th2 biased activity (see chapter 7.1.1). It is now understood that skewing of the adaptive immune response is due to changes in innate immune system priming and behaviour. The hygiene hypothesis for allergy suggests that a lack of early childhood exposure or response to PAMPs primes the innate immune system to promote a Th2 biased adaptive immune response [3]. The innate immune response may also differ in asthmatics with enhanced response to PAMPs such as LPS observed locally and systemically. Finally, the chief coordinators of the adaptive immune response, dendritic cells, have the ability to promote sensitisation or tolerance to environmental exposures depending on the relative abundance of their subtypes (see section 6.1.3).

There is accumulating evidence that obesity is associated with activation of the innate immune system with increased circulating levels of pro-inflammatory cytokines including IL-1 β , IL-6, TNF- α and CRP in the obese compared to normal weight individuals [334]. The pro-inflammatory cytokine profile seen in the obese is similar to that of critically unwell patients in the intensive care unit [550]. The predominant source of these pro-inflammatory mediators is thought to be adipose tissue; as much as 25% of circulating IL-6, is derived from visceral adipose tissue [517]. Obesity is associated with changes in the populations of innate immune cells within adipose tissue but also systemic changes in numbers including neutrophils (see section 4.1.2) and monocytes/macrophages (see section 6.1.1). There is some evidence that on encountering PAMPs the response in obese individuals is also augmented.

In this chapter the effect of obesity and asthma on the response to LPS will be explored. This will be complemented by analysis of the phenotype of circulating monocytes, especially their expression of activation markers. Finally, the relative abundance of circulating dendritic cell subtypes will be determined, an area not well studied to date in the obesity setting.

6.1.1 Monocytes and macrophages in obesity and asthma

6.1.1 (i) Monocytes and macrophages

Monocytes and macrophages play a critical role in innate immunity including phagocytosis, secretion of pro-inflammatory cytokines, and the production of ROS, nitric oxide and myeloperoxidase (see section 2.2.1). Monocytes are classically divided into 2 subsets according to CD16 expression; CD14⁺CD16⁺ and CD14⁺CD16⁻ populations [551]. The CD16⁺ subset comprise 5-8% of circulating monocytes and has a macrophage-like phenotype with augmented phagocytic ability and endothelial affinity promoting tissue migration. They are also potent producers of pro-inflammatory cytokines such as TNF α [552]. As such this subset is increased in pro-inflammatory states including, sepsis, rheumatoid arthritis [552], and ischaemic heart disease [553].

Resident tissue macrophages derived from circulating bone marrow derived monocytes are found in most tissues. These phagocytes are the most abundant haematopoietic cell in the lungs and upon activation of PRRs, serve to eradicate the lung of noxious substances, pathogens and debris. Similarly adipose tissue hosts a population of resident macrophages and the numbers are increased in obesity [332, 554]. As with the Th1/Th 2 paradigm (see section 7.1.1), a model has been proposed whereby two broad populations of macrophages develop depending on exposure to prototypic Th1 and Th2 cytokines [555]. Pro-inflammatory macrophages are activated by IFN γ either alone or in combination with PAMPS, such as LPS leading to high IL-12 and IL-23 production resulting in polarisation towards a Th1 adaptive immune response; hence these pro-inflammatory cells are referred to as M1 macrophages. In contrast exposure to IL-4 and IL-13, results in the development of anti-inflammatory macrophages (M2, or AAMs alternatively activated macrophages).

6.1.1 (ii) Monocytes and macrophages in obesity

Obesity is characterised by an increase in M1 macrophages within adipose tissue [556]. The mechanism behind this remains unclear, however the obese state is characterised by areas of adipose tissue necrosis and M1 macrophages accumulate around these foci in obese mice and humans suggesting that this as a driving factor [554]. Additionally M1 macrophages can also be derived *de novo* from circulating activated CD16⁺ monocytes [76]. Interestingly levels of CD16⁺ monocytes are increased in patients undergoing bariatric surgery, suggesting that obesity is also characterised by systemic activation of the macrophage/monocyte compartment and that these circulating cells may contribute to the changes seen within adipose tissue [31].

6.1.1 (iii) Monocytes and macrophages in asthma

Similarly to in adipose tissue, M1 and M2 macrophages reside in the lung and develop according to the cytokine environment they are exposed to. M1 macrophages in this environment are important in the response to intracellular bacteria including *Mycobacterium tuberculosis* [78], whilst M2 macrophages may also have a role in host defence against parasites [79].

Studies of circulating monocytes in asthmatics indicate that, as with obesity, there is systemic activation of this compartment. A study of 11 untreated asthmatics versus 9 controls, showed increased percentage of CD14⁺ CD16⁺ monocytes derived from peripheral blood mononuclear cells in the asthmatics [173] and functionally this resulted in increased PMA induced superoxide anion release. In concordance, a study of 10 stable asthmatics showed increased MFI of CD16⁺ expression on peripheral blood mononuclear cells compared to controls [557]. Locally within the airways asthma is characterised by marked infiltration of macrophages which share many phenotypic characteristics of blood monocytes [172]. As local macrophage proliferation is not a major contributor to the increased numbers seen [174], recruitment of systemically activated monocytes is the likely source.

Despite the abundance of macrophages in asthmatic airways, the role of different macrophage subtypes in asthma has not been studied widely [558]. Theoretically the typical Th2 environment in atopic asthma should promote the development of M2 macrophages. A study examining ovalbumin induced airway inflammation in a murine model noted increased M2 macrophages in the lung tissue of ovalbumin sensitised mice after exposure compared to controls; adoptive transfer increased the severity of allergen induced disease [559]. In adults with atopic asthma, an increased percentage of M2 macrophages are seen, correlating with PEFV variation [560]. However, whilst M2 macrophages might be involved in the development of allergic airway disease, M1 macrophages could be important in severe or corticosteroid resistant phenotype. A study of 18 asthmatics (divided into steroid responsive and resistant depending on their response to a 1 week course of Prednisolone) and 10 healthy controls examined gene expression of cells from BAL samples. They showed that steroid sensitive asthmatics had up-regulation of genes associated with an M2 macrophage phenotype whilst resistant asthmatics had increased expression of genes associated with an M1 phenotype [561].

In summary, obesity and asthma are associated with systemic activation of the monocyte compartment. Obesity is associated with increased of M1 macrophages within adipose tissue and in asthma accumulation of these cells lungs could be associated with severe disease. The presence of increased numbers of macrophages in the lungs and adipose tissue may be in part due to recruitment from the circulating activated monocyte pools. Therefore markers of systemic activation of monocytes were examined to see if the effects of the two disease states were additive.

6.1.2 LPS response and soluble CD14 in obesity and asthma

6.1.2. (i) LPS signalling

LPS or endotoxin, a constituent of the cell wall of Gram negative cell bacteria, is the most studied of PAMPs. Early detection of this molecule through stimulation of PRRs on innate cells, causes pro-inflammatory cytokine release alerting the host to a bacterial infection (see section 2.2.1). However, an exaggerated response can trigger an overwhelming inflammatory cascade, causing marked tissue damage, organ failure and even death. There is accumulating evidence to suggest that PRR signalling

and response to PAMPs such as LPS may be modified in obesity and asthma yet there has been little work examining this area in the context of obese asthmatics.

CD14 is a pattern recognition receptor and recognises LPS as well as other PAMPs including, lipoteichoic acid, peptidoglycan and phospholipids. Membrane bound CD14 (mCD14) functions as a co-receptor along with TLR4 and MD2 for the detection of LPS [562]. It is expressed in high levels on monocytes, macrophages, DCs and neutrophils. CD14 is also produced by the liver and monocytes in a soluble form, soluble CD14 (sCD14) which can have pro-inflammatory and anti-inflammatory effects depending on its concentration and location [563]. At low concentrations sCD14 can augment LPS responses by transferring monomeric LPS to mCD14 [564] or by interacting directly with MD2/TLR4 complex on cells that do not express mCD14 [565]. In keeping with this, murine models have shown that low levels of sCD14 in cerebrospinal fluid enhance the inflammatory cytokine response in experimental meningitis [566]. Systemically, high levels of sCD14 appear to have an anti-inflammatory role by preventing or limiting LPS interaction with mCD14 [567] and *in vivo* studies have shown that at administration of human recombinant sCD14 can protect mice from the lethal effects of LPS [568].

6.1.2 (ii) LPS response and sCD14 in asthma

Evidence suggests that asthmatics have an enhanced response to LPS at a systemic and a local level. LPS stimulation of blood mononuclear cells from asthmatics results in increased production of IL-1 β and GM-CSF compared to controls [341]. Furthermore challenging asthmatics with inhaled LPS causes a measurable increase in systemic inflammatory cells (neutrophils) and mediators; CRP and TNF α [569]. Functionally there is also evidence of increased BHR in asthmatics following inhalation of LPS which is not seen in controls [570]. Alveolar macrophages isolated from asthmatics and stimulated with LPS have increased production of TNF α and GM-CSF compared to controls [341].

Altered systemic LPS responsiveness in asthmatics may in part be due to increased numbers of circulating pro-inflammatory monocytes as discussed above, however changes in levels of sCD14 may also play a role. Induced sputum from atopic asthmatics has higher levels of sCD14 than controls and levels correlate with neutrophil count after challenge with inhaled LPS [571]. Therefore it has been hypothesised that the enhanced response to LPS seen is as a result of increased CD14. A number of mechanisms have been suggested to account for this including genetic factors, increased vascular permeability and allergen exposure. Not only is the LPS response increased in asthmatics but levels of LPS exposure during childhood and systemic levels of sCD14 may actually modify disease risk. Population studies have examined LPS exposure and a single nucleotide polymorphism (SNP) in the gene encoding for CD14 which modify resultant sCD14 levels and the relationship between these parameters and asthma risk [572]. SNPs exist at two sites on the CD14 gene both resulting in a C to T transition and are associated with changes in sCD14 levels with levels being highest in the TT and lowest in the CC homozygotes respectively. Half of the population are heterozygous for this SNP whilst the remaining 50% are evenly divided between CC and TT homozygotes. Studies of endotoxin exposure and asthma risk have been contradictory with some suggesting a protective effect [573] and others not [574]. Similarly, studies examining SNP in the

CD14 gene have also suggested that CC homozygotes have enhanced risk of atopy and BHR [575] in childhood whilst others have not shown this [576]. The contradiction in these population studies is now thought to be due to an interaction between LPS exposure and CD14 polymorphisms, with increased LPS exposure having a protective effect on atopy risk but only in the CC homozygotes (group with the lower sCD14 expression) [572].

6.1.2 (iii) LPS response and sCD14 in obesity

Animal models of sepsis have shown an increased inflammatory response in obese vs. normal weight subjects [577]. Adipose tissue itself is capable of reacting to PAMPs including LPS [578], but obesity might also modify systemic reactivity to the same triggers. A study of obese African American women showed that those with a BMI > 40 kg/m² had a higher TNF α release in response to LPS stimulation of whole blood compared to normal weight individuals [32]. The authors postulated that this was due to systemic activation of monocytes in the obese state (see section 6.1.1) [31]. Similarly a Japanese study of 34 obese adults (mean BMI = 38.4 kg/m²) versus 50 healthy controls also noted an increased LPS stimulated TNF α response in peripheral blood mononuclear cells from the obese group [579]. Mononuclear cells derived from peripheral blood of obese patients have increased activation of the pro-inflammatory transcription factor NF κ B, reduced levels of its inhibitor I κ B- β and increased mRNA expression of pro-inflammatory cytokines [580]. If obesity is characterised by enhanced responsiveness to PAMPs, especially LPS, then this may be compounded further by higher background levels of circulating LPS which has been demonstrated in obese pregnant women [581] and LPS binding protein in obese but otherwise healthy Chinese adults [582]. The increased LPS level is postulated to be due to changes in the gut; increased chylomicrons favouring LPS transport, changes in intestinal permeability and gut microbiota [583]. Interestingly sCD14 levels are also associated with BMI and levels are significantly reduced on weight loss and sCD14 has been shown to correlate with insulin sensitivity in the obese [584].

6.1.2 (iv) Cytokine response to LPS stimulation

Stimulation of many cell types with LPS results in the release of a number of cytokines including IL-1 β , IL-6, IL-8, IL-10 and TNF α . LPS stimulated whole blood cultures therefore serve as an ideal model to compare LPS responsiveness. Circulating levels of many these same cytokines are altered in obesity and asthma and have biological effects of relevance in the latter, they are summarised in Table 6.1 and will be discussed below.

IL-1 β is produced by many cells including monocytes/macrophages, mast cells, basophils, structural cells (endothelium and smooth muscle), and lymphocytes [585]. IL-1 β production is a two step process involving the synthesis of a pro-molecule in response to a first signal which upon further signalling is then cleaved, producing the active cytokine via a complex containing the enzyme

caspase 1, referred to as the inflammasome [75]. The cytokine has a multitude of effects of relevance to asthma. Instillation of the cytokine into the trachea of ovalbumin sensitised rats given inhaled acetylcholine and bradykinin resulted in enhanced neutrophilic inflammation and BHR [586]. In addition to this levels of IL-1 β are increased in the BAL fluid of patients with symptomatic asthma compared to control subjects [587].

IL-6 has a multitude of effects on innate and adaptive immunity which are summarised in Figure 5.2. Plasma levels are increased in obesity [516] and higher in asthmatics than controls [176]. Evidence presented in this thesis suggests that the effects of obesity and asthma are additive; plasma IL-6 levels increasing with BMI and higher levels in asthmatics than controls with obese asthmatics having the highest levels (see section 5.3.1 (v)). This might be of relevance to neutrophilic disease, and this finding has been confirmed by another group [429]. However the source of this increased IL-6 needs clarification. Potential sources include adipose tissue [588] and the airways [513]. Release from stimulated circulating leukocytes remains another potential source and this was explored in this study.

IL-8 is a pro-inflammatory chemokine important in neutrophilic inflammation (see section 5.1.1 (iv)) and some groups have shown plasma levels to be increased in obesity [526]. IL-8 is increased in the sputum of patients with severe asthma [522]. Total plasma levels were measured in this thesis and no detectable difference were seen across the 6 groups (see section 5.3.1 (vi)). Therefore it would be of interest to see whether stimulation of whole blood with LPS resulted in a differential response in IL-8 release.

IL-10 is a cytokine with anti-inflammatory properties produced by monocytes [589] and regulatory T cells (Tregs) [126]. It has a number of effects including reducing the production of pro-inflammatory cytokines [589] and promoting Treg development [126] (see section 7.1.2 (i)). The adipokine, adiponectin (see section 4.1.3 (iii)), reduced in the obese state [297], promotes IL-10 production and obesity is associated with reduced plasma levels [590]. IL-10 is important in maintaining immune homeostasis at environmental interfaces [191] and may play a role in atopic asthma, with reduced levels seen in the BAL fluid of such individuals and upon stimulation of peripheral blood mononuclear cells with LPS [591].

TNF α is produced by a number of different cell types including monocytes/macrophages, eosinophils, mast cells and epithelial cells [592]. Circulating levels are increased in obesity [593]. TNF α is important in promoting neutrophilic inflammation within the airways with inhalation of the cytokine promoting BHR and airway neutrophilia in rodents [594] and humans [592] and levels are increased in the BAL fluid of those with severe corticosteroid dependent disease [175]. In the same study, treatment with anti-TNF α antibody improved lung function and markers of asthma control suggesting an important role for this cytokine, although unfortunately latter studies did not replicate this [595]. One study has examined circulating TNF α levels in obese asthmatic women and they were not significantly different to normal weight asthmatics or controls [25]. In a study of African women, obese individuals had enhanced TNF α production following LPS stimulation of whole blood [32] and in asthma an augmented response in alveolar macrophages derived from asthmatics has been observed [341].

Cytokine	Source	Principal effects	Findings in obesity	Findings in asthma
IL-1β	Monocytes/macrophages, mast cells basophils, structural cells [585].	Up-regulation of adhesion molecules. Production of other inflammatory cytokines e.g. IL-8 and GM-CSF. Stimulation of myeloid precursors: neutrophilia. Co-stimulator of T cells.	↑ serum levels in the obesity [596].	↑ levels in BAL fluid of asthmatics versus controls [587].
IL-6	Structural cells, monocytes/macrophages, DCs, mast cells [507].	Neutrophilic inflammation. Macrophage activation. Th17 proliferation. Down-regulation of Tregs (Figure 5.2).	↑ plasma levels in obesity [516].	↑ plasma levels in atopic asthma [176].
IL-8	Structural cells, neutrophils, monocytes, NK cells and T cells [520].	Neutrophil migration and activation [520].	↑ plasma levels in obesity [526].	↑ sputum levels in severe asthma [522].
IL-10	Monocytes [589], Tregs [126]	↓ Pro-inflammatory cytokine production. ↑ IL-1RA. ↓ T cell effector responses. ↑ Treg development [126].	↓ plasma levels in obese [590].	↓ levels in BAL fluid of atopic asthmatics. ↓ production on LPS stimulation by mononuclear cells of atopic asthmatics [591].
TNFα	Monocytes/macrophages, structural cells [597]	Promotes airway neutrophilic inflammation.	↑ plasma/serum levels in obesity [579, 593]. ↑ Response in vitro to LPS [32, 579].	↑ levels in BALF of patients with severe disease [175].
IL-12p70	Monocytes/macrophages, DCs [424].	Promotes Th1 differentiation [424].	↑ serum levels with obesity [593].	↑ plasma levels in atopic asthma [176].
IL-23	Monocytes/macrophages, DCs [115].	Promotes Th17 differentiation.	↑ circulating levels in obesity [352].	↑ Serum IL-17 levels in asthma. Correlates with disease severity [354].

Table 6.1: Summary of cytokines related to the innate immune system and related findings in studies on asthma and obesity.

The principal cellular sources of these cytokines are shown together with their main effects.

IL-12 is produced by phagocytes, particularly monocytes/macrophages, dendritic cells and neutrophils. It is a potent inducer of a Th1 response (see section 7.1.1.), resulting in IFN γ production as well as promoting cytotoxicity by NK cells and cytotoxic T cells [424]. Biologically active IL-12p70 is a heterodimer of two covalently bonded molecules, IL-12-p35 and IL-12-p40. The former is expressed ubiquitously at low levels whereas the latter is inducible and only manufactured by IL-12p70 producing cells. Maximum IL-12p70 release by monocytes and DCs require dual stimulation with ligation of pattern recognition receptors with a PAMP such as LPS as well as stimulation from a T cell derived cytokine such as IFN γ [424]. Allergic asthma is characterised by Th2 predominance [176] whereas there is some evidence of Th1 skewing in obesity [288] (see section 7.1.1). Very little is known about systemic levels of IL-12p70 in obesity however a single Mexican study has suggested that serum levels are increased [593]. Although atopic asthma is associated with a Th2 skewed response and IL-12 is important in Th1 priming increased plasma levels have been reported in atopic asthmatics [176].

IL-23 is produced by activated monocytes/macrophages and DCs and is important in the promotion of Th17 differentiation [115]. Activation of the Th17 axis may be important in severe asthma and studies have suggested increased activity of the IL-23/IL-17 axis in obese women (see section 5.1.1 & 7.1.2). Plasma IL-23 did not differ significantly between the groups in this thesis (see section 5.4.1). Therefore it is of interest to see if there is enhanced expression of this cytokine in response to an innate stimulus (LPS).

Given that asthma and obesity are associated with changes in LPS response the possibility that there are additive effects is of interest. To date there has been very little work on this area. It was hypothesised that obese asthmatics may have activation of their monocyte compartment and that this could result in a greatly exaggerated response systemically which may perpetuate the inflammatory response on LPS exposure. The cytokines of particular interest were those with roles in promoting neutrophilic inflammation (IL-1, IL-6, IL-8 and TNF α), and Th1 (IL-12p70) or Th17 (IL-23) skewing.

6.1.3 Dendritic cells

DCs are perfectly placed functionally and anatomically to link the innate and adaptive responses. Functionally they express the innate PRRs and are activated upon encountering microbial ligands, yet at the same time they are able to take up antigen, migrate to lymph nodes and present this on major histocompatibility class I and II molecules enabling recognition by T cell receptors (TCR). DCs produce almost all of the Th polarising cytokines (IL-12, IL-10, TGF β , IL-23 and IL-6) with the exception of IL-4 [100]. Anatomically they are located at epithelial surfaces where they can sample the micro environment. DCs are divided into two main groups: myeloid (mDCs) or conventional (cDCs) dendritic cells share the same lineage as monocytes/macrophages which are further divided into type I mDCs and type II mDCs, whilst plasmacytoid (pDCs) express lymphoid development markers [97]. Identification of DC subtypes in humans has been made easier by the identification of specific surface markers blood dendritic cell antigens (BDCA) (which are summarised in Table 6.2) and studies of lung digests have shown all 3 to be present in the human lung [96].

Antigen	Expression
BDCA-1 (CD1c)	Type I myeloid dendritic cells and B lymphocytes (low level)
BDCA-2 (CD303)	Plasmacytoid dendritic cells
BDCA-3 (CD141)	Type II myeloid dendritic cells and monocytes (low level)
BDCA-4 (CD123)	Plasmacytoid dendritic cells

Table 6.2: Summary of antigens expressed on human dendritic cells.

6.1.3 (i) Plasmacytoid dendritic cells

The development of pDCs is not well understood but they are constantly produced by the bone marrow throughout life. The cytokine, Fms-like tyrosine kinase receptor-3 ligand (Flt3L), is the only one known to be fundamental to their development [598]. At rest pDCs express low levels of MHC class I and II molecules and the co-stimulatory molecules CD80/CD86 [599]. Unlike mDCs, they are able to prime naive CD4⁺ cells to differentiate into IL-10 producing Tregs in their resting state [600]. Upon stimulation, depending on the environment and type of antigen encountered, pDCs may be important in fuelling the immune response to viral and parasitic pathogens. Plasmacytoid DCs express the PRRs, TLR7 and TLR9 within intracellular endosomal compartments which are capable of recognising single stranded RNA and double stranded DNA, respectively. Stimulation of the TLRs causes production of large amounts of type I interferons, particularly interferon- α (IFN α) and TNF α . IFN α is important in fuelling the innate response against viral infection. IFN α and TNF α , production in an autocrine fashion also causes these cells to differentiate into mature DCs which upregulate expression of the MHC class I and II molecules as well co-stimulatory molecules CD80 and CD86. These cells then prime the adaptive response promoting the differentiation of CD4⁺ T cells to produce IFN γ and IL-10. In parasitic infection, pDCs mature and upregulate MHC class II and CD40L expression and prime a Th2 adaptive response in a IL-3 dependent fashion [599].

6.1.3 (ii) Myeloid dendritic cells

Myeloid DCs express TLR2, -4, -5 and -6 which are important in recognising bacterial constituents. Whilst pDCs are thought to be important in innate viral and parasitic immunity as well as promoting immunological tolerance, mDCs are professional antigen presenting cells; they reside in tissues, respond to microbial products and present them to T lymphocytes within regional lymph nodes. They are capable of producing large amounts of IL-12p70, thereby promoting Th1 responses [601]. Two types of mDCs are now known to exist which can be differentiated by surface markers [602].

6.1.3 (iii) Dendritic cells in asthma

In the lung mDCs, are present beneath the epithelium in a prime position to sample the micro-environment and are also found within the lung parenchyma itself [4]. In the context of atopic

asthma, mDCs are thought to play an important role, directing the immune response to allergen and determining the development and perpetuation of allergic inflammation [4, 184]. Work from patients with allergic asthma has shown that within 4-5 hours of allergen challenge there is an influx of mDCs into the bronchial mucosa with a corresponding reduction in blood levels, suggesting that these cells are recruited from the blood to the airways following allergen challenge [603]. In a similar study, an increase in both populations of DCs were observed in the BAL fluid of patients with allergic asthma after antigen challenge, this persisted for 24 hours and was associated with a reduction in blood levels [604]. More recently it has been suggested that type II mDCs have a role in allergic asthma with higher levels seen in BAL fluid [605] and blood of atopic asthmatics [606].

The recruitment of DCs to the airways occurs due to interactions with the airway epithelium. Stimulation of PRRs on airway epithelial cells results in the release of chemokines, including CCL2 [607] and CCL20 [601] which act on CCR2 and CCR6 respectively, expressed on dendritic cells, facilitating their recruitment to the airway epithelium to sample antigen. Production of matrix metalloproteases (MMP) such as MMP9 promotes the migration of these cells through the basement membrane [608]. Upon exposure to antigen and ligation of PRRs, mDCs increase their phagocytic and migratory capacity as well as upregulating surface expression of MHC molecules and co-stimulatory molecules. The epithelium also releases other cytokines which affect DC function including TSLP which causes DCs to release Th2 chemokines (CCL17 and CCL22) [609], as well as upregulating DC expression of the TNF-superfamily protein, OX40L. Upon migration to the local lymph node down a chemokine gradient, DCs present antigen/allergen on MHC class II molecule to CD4⁺ T cells. The interaction of MHC-TCR and of co-stimulatory molecules CD80/CD86 with their ligand, CD28 on T cells, promotes activation and differentiation of CD4⁺ T cells. In the context of allergy up-regulated OX40L interacts with its receptor OX40 on naive T cells promoting Th2 differentiation [181].

Although pDCs can promote Th2 responses in the context of parasitic infection [599], there is evidence to suggest that in the airways they may also play a role in promoting immunological tolerance. Systemic depletion of these cells results in sensitisation to the normally inert antigen, ovalbumin and the development of classical airways changes of asthma [185]. In the same study adoptive transfer of pDCs to pDC-depleted mice prevented sensitisation. Flt3L abolishes the cardinal features of asthma on allergen challenge in sensitised mice and its mode of action might be by altering the balance of mDCs and pDCs in the lung, favouring accumulation of pDCs [610]. Furthermore, depletion of pDCs abolished the protective effects of Flt3L administration in these mice. Mechanisms for the inhibitory effect of pDCs on allergic airway inflammation remain to be fully elucidated but the production of IFN α may be important. On stimulation of TLR7 and TLR9 during viral infection, pDCs release large amounts of IFN α [599], which inhibit development of Th2 cells through suppression of the transcription factor GATA 3 as well as stopping production of IL-4 and IL-5 by Th2 committed cells [611]. IFN α / β have also been shown to inhibit Th17 development [612]. It may be that the IFN α producing ability of pDCs leads to their ability to promote immune tolerance and loss of this may be important in asthma. A study using pDCs derived from atopic asthmatics have shown a reduction in IFN α production in response to influenza exposure compared to controls [613]. The same paper showed that serum IgE levels negatively correlated with *in vitro* IFN α production and that cross-linking of the IgE on pDCs (IgE bound to Fc ϵ receptors at the surface of pDC bind specific antigen via the F $_{ab}$ region inducing cross-linking) inhibits IFN α production.

6.1.3 (iv) Dendritic cells in obesity

Obesity is associated with increased incidence of sepsis [614], pneumonia and wound infections [615]. Such observations have led to some limited works examining changes in DC number and function with obesity. Murine studies have shown that leptin deficient obese mice at steady state have increased epidermal dendritic cells [616]. Interestingly the same group showed that administration of intradermal leptin restored DC numbers to those of controls, suggesting a role for this adipokine in DC regulation (see paragraph below). They also found that mature DCs from the obese mice were less able to stimulate allogeneic T lymphocyte proliferation, despite no differences in expression of surface markers (MHC class II molecules, CD40, CD80 and CD86). Culture supernatants from the DCs derived from obese mice showed increased levels of the immunosuppressive cytokine TGF- β with no differences in IL-12p70, and the authors hypothesised that the enhanced production of this cytokine was the mechanism behind the reduced ability to stimulate T cell responses. A study of obese/normal weight post-menopausal women with and without diabetes found that obesity was associated with increased type I mDCs whilst obese type II diabetics had an increase in circulating type I and II mDCs with no change in the percentage of circulating pDCs [33]. There are very few data on this area and the above findings need clarification, however there are mechanistic data arising suggesting leptin may affect dendritic cell functioning.

Myeloid DCs express leptin receptor, and incubation of blood derived DCs has been shown to protect DCs from spontaneous apoptosis associated with activation of NF κ B [295]. Physiological levels of leptin increased DC production of IL-1 β , IL-6, IL-12, TNF α and MIP-1 α , whilst down regulating IL-10 expression. Leptin also primed DCs to polarise a Th1 response from naive T cells. Leptin may also facilitate DC migration through the up-regulation of the chemokine receptor CCR7 [617]; DCs from leptin receptor deficient mice express low levels of CCR7 [618].

In summary, the balance of DC subtypes appears important in the airways with pDCs promoting immune tolerance perhaps through IFN α production. Very little work has been done on the balance of DCs in obesity but mechanistic data suggest that adipokines may modify DC function and some observational data suggest increased mDCs in the obese. Therefore DC subsets and their possible association with the obese asthma phenotype was investigated.

6.2 Methods

6.2.1 Study population

Pre-menopausal women with and without asthma were recruited. Subjects and controls were divided into 3 groups on the basis of body mass index (BMI), giving 6 groups in total (Figure 3.1). Normal weight is defined as BMI 18.5-25kg/m², overweight as a BMI \geq 25kg/m² and <30kg/m² and obesity as a BMI \geq 30kg/m².

Asthmatic patients were recruited from a local tertiary clinic, three surrounding secondary care clinics and a participating GP practice.

After case note review, all asthmatic subjects fulfilling the inclusion/exclusion criteria (see Figure 3.1) attended an appointment at a tertiary asthma service where asthma diagnosis was confirmed. The diagnosis required consistent symptoms and demonstrable significant reversible airways obstruction to a β_2 agonist (12%) or if this was not present, significant PEFV variation or a positive bronchial provocation test. Disease severity was graded according to GINA criteria (Appendix VII). Asthmatics were considered stable if they had no exacerbations, oral steroid therapy or respiratory tract infection in the preceding 6 weeks.

Asthmatics were asked to complete a modified European Respiratory Health Survey [391] (Appendix V). Healthy control subjects of varying BMI were recruited from the local university, hospital staff and a Slimming World weight loss club.

6.2.2 Clinical measures and blood collection

Participants were asked to attend during the first 7 days of their menstrual cycle, determined using the date of onset of menstruation, in the fasted state between the hours of 07.00-09.00am. Acute asthma control at the time of recruitment was assessed using the Juniper Asthma Control Questionnaire (Appendix VI) [392].

Spirometry was performed using a portable dry spirometer (Vitalograph) calibrated on the day of use. All asthmatics were asked to withhold medication for the preceding 24 hours. The best of 3 measurements was taken according to a standardised protocol [394]. Following this measures of WHR and percentage body fat composition were taken.

Fasting blood was collected into lithium heparin tubes and gel and clot activator tubes for serum.

6.2.3 Expression of CD16 and HLA-DR on peripheral blood monocytes by flow cytometry

Pro-inflammatory CD14⁺CD16⁺ cells account for 5% of circulating monocytes [552]. In contrast surface expression HLA-DR, vital for antigen presentation by monocytes, is down regulated in pro-

inflammatory states [619]. Expression of CD16 and HLADR on CD14⁺ monocytes was measured using flow cytometry. The principles behind this technique are outlined in section 3.7. The antibodies, conjugated fluorochromes and their appropriate isotype controls used are listed in Table 6.3.

Antigen	Fluorochrome	Cell expression	Clone	Source
CD14	PerCP-Cy5.5	High expression on monocytes	61D3	eBioscience, UK
CD16	FITC	High expression NK cells and activated monocytes	3G8	BD Bioscience USA
HLADR	FITC	High expression on activated monocytes	G46-6	BD Biosciences USA
Mouse IgG2b Isotype control	FITC		eBMG2b	eBioscience, UK

Table 6.3: Summary of antibodies used in monocyte activation marker experiment, their respective fluorochromes and sources.

Heparinised whole blood (100µl) was added to each of 4 tubes prepared with antibodies. Tube 1 contained no antibody, tube 2 contained anti-CD14-PerCP Cy5-5 and isotype control (mouse IgG2b-FITC) along with antibodies for the neutrophil activation markers experiment (anti-CD15-e450 and mouse IgG2a-PE (see section 4.2.5). Tube 3 contained anti-CD14 PerCP-Cy5-5 and anti-CD16 FITC along with the neutrophil antibodies (anti-CD15-e450 and anti-CD62L-PE). Tube 4 contained anti-CD14-PerCP-Cy5-5 and anti-HLA-DR-FITC along with the neutrophil antibodies (anti-CD15-e450 and anti-CD11b-APC). The samples were vortexed before incubation in the dark on ice for 30 minutes. Samples were then treated with 3ml of red blood cell lysis solution (FACS lysing solution; BD Biosciences, USA) and incubated in the dark at room temperature for 10 minutes. Cells were collected by centrifugation (4°C, 515 x g for 7 minutes) and the supernatant discarded before washing in 3ml of FACS buffer (PBS with 0.2% BSA and 0.05% sodium azide). The tubes were centrifuged, supernatant removed and then the samples were fixed using 200µl FACSFIX (BD Biosciences, USA).

The samples were refrigerated and acquired within 24 hours on a FACSAria I (BD Biosciences, USA) flow cytometer: 10,000 events were recorded from each sample. The gating strategies used to analyse CD16 and HLA-DR expression are shown in Figures 5.1 and 5.2 respectively. Monocytes were identified as a population of cells with low side scatter and by CD14 expression. After gating on this population the percentage of CD16 positive cells was measured in tube 3 and HLA-DR expression was measured by median fluorescence intensity (MFI) using tube 4. To control for non-specific antibody binding the same measurements were made using the isotype control antibody. The signal index (for HLA-DR expression) was calculated from the ratio between median fluorescence intensity in the sample containing the specific antibody (anti-HLA-DR) and the isotype control.

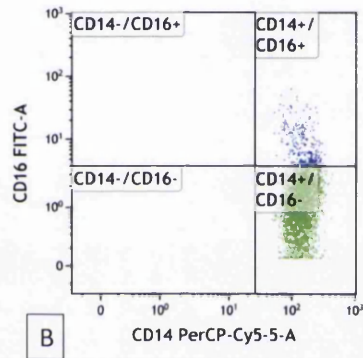
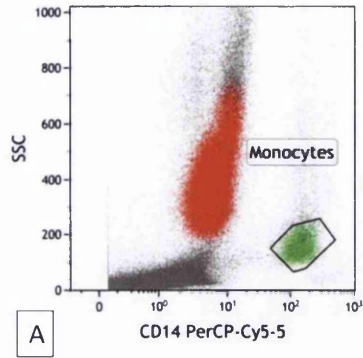


Figure 6.1: Gating strategy used to quantify CD14⁺CD16⁺ monocytes by flow cytometry.

A: CD14⁺ monocytes were identified as a population of cells with low side scatter (SSC) and high CD14 expression.

B: Gating on CD14⁺ monocytes CD16 expression examined and the percentage of CD14⁺CD16⁺ cells expressed as a percentage.

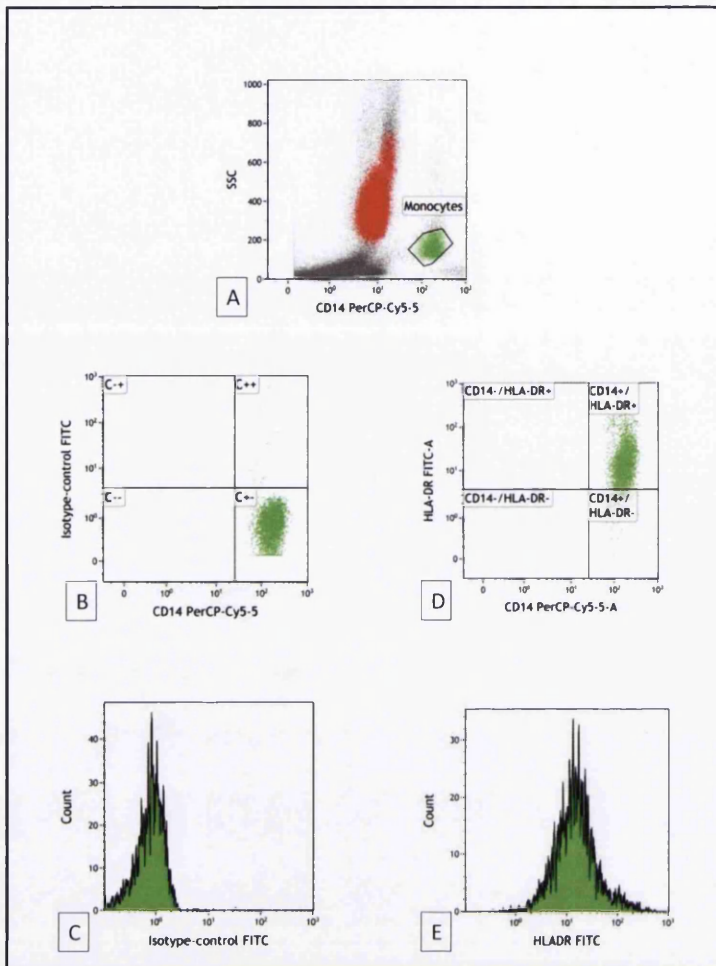


Figure 6.2: Gating strategy for quantifying CD14⁺HLADR⁺ monocytes by flow cytometry.

A: Identification of CD14⁺ monocytes as a population of cells with low side scatter and high CD14 expression.

B: Gating on this population, non-specific binding controlled for using an isotype control.

C: Median fluorescence intensity measured in the isotype control tube.

D: Gating on CD14⁺ monocytes percentage of cells expressing HLADR quantified.

E: Median fluorescence intensity of HLADR-FITC measured.

6.2.4 Measurement of plasma sCD14

Plasma sCD14 was measured using a specific ELISA. The methodology of this technique is detailed previously (section 3.11) and the kit used summarised in Table 6.3. Plasma was separated by density gradient centrifugation: 10ml of heparinised blood was gently layered onto an equal volume of Histopaque (Sigma, USA) in a 50ml Falcon tube (Greiner Bio-one, Germany) and centrifuged at 805 x g for 20 minutes (no brake). The plasma was removed carefully, filtered (0.2 µm polyethersulfone filter; Sigma, USA) and stored at -20°C prior to analysis.

6.2.5 Whole blood culture response to LPS stimulation

The process of whole blood culture is discussed in more detail in section 2.5. The media used for culture preparation was RPMI 1640 + glutamax (Invitrogen UK), supplemented with 50mM of 2-mercaptoethanol (ME; Invitrogen, UK). Culture media (600µl) was added to 8 tubes (4 tests in duplicate) and mixed gently with 200µl of heparinised blood. All culture work was undertaken in a class II tissue culture cabinet. Maximal IL-12p70 is only produced after stimulation of PRRs supplemented by stimuli from activated T cells such as IFN γ [424], therefore IFN γ (10ng/ml; Miltenyi Biotec) was added to 4 tubes (2 to act as controls and 2 to be subsequently stimulated with LPS) (Figure 5.3) to measure IL-12p70 response. The cultures were incubated for 90 minutes. Following this LPS (10ng/ml; Ultrapure, Invivogen) was added to the appropriate tubes and all 8 tubes were incubated at 37°C in 5% CO $_2$ -in-air for 24 hours. After incubation the tubes were centrifuged for 7 minutes at 4°C, 515 x g and cell free supernatants removed for storage at -20°C until analysis.

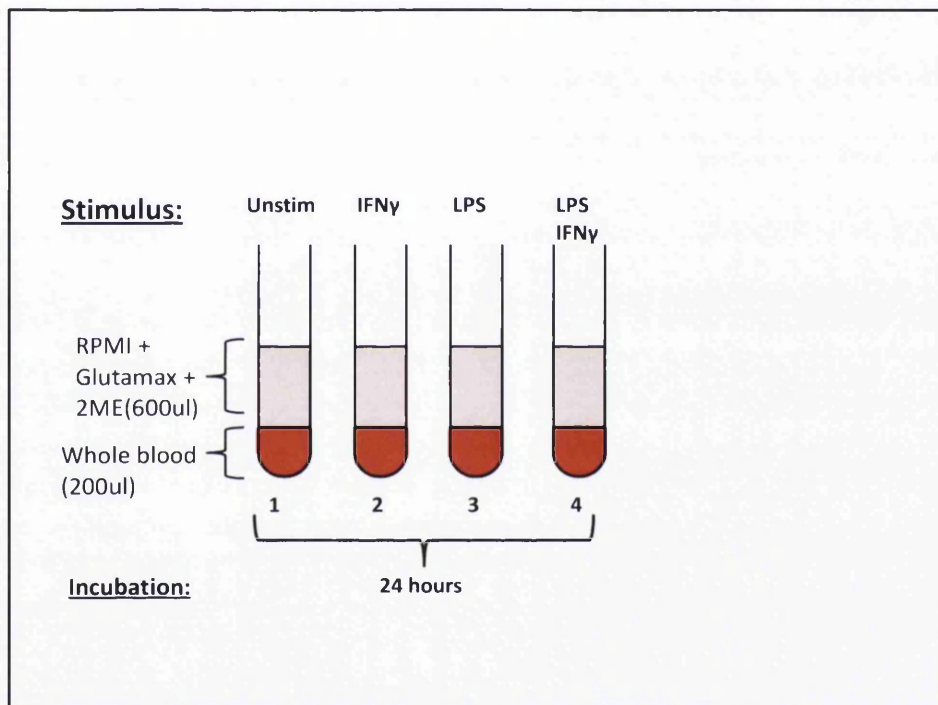


Figure 6.3: Schematic diagram of whole blood cultures examining LPS +/- IFN γ response.

4 tests were set up in duplicate. Control tubes containing no stimulus and IFN γ only were used to look at background levels of the cytokines of

Cytokine responses to culture with the LPS +/- IFN γ were quantified using ELISA. The principles behind this method and the basic protocol are detailed in section 3.11. The commercially available cytokine ELISA kits used, their sources and sensitivities are summarised in Table 6.4.

Cytokine	Sample	Sensitivity (pg/ml)	Source
IL-1 β	Culture supernatants	3.906	Duo Set, R&D Systems, Europe.
IL-6	Culture supernatants	9.375	Duo Set, R&D Systems, Europe.
IL-8	Culture supernatants	15.625	Duo Set, R&D Systems, Europe.
IL-10	Culture supernatants	7.813	Opt-EIA, BD Biosciences, USA
IL-12p70	Culture supernatants	7.813	Ready-SET-Go, eBioscience, UK
IL-23	Culture supernatants	125	Duo Set, R&D Systems, Europe.
IFN γ	Culture supernatants	7.813	Ready-SET-Go, eBioscience, UK
sCD14	Plasma	62.5	Duo Set, R&D Systems, Europe.
TNF α	Culture supernatants	7.813	Opt-EIA, BD Biosciences, USA

Table 6.4: Summary of cytokines measured following LPS stimulation of whole blood, the commercially available kits used and their respective sensitivities.

6.2.6 Dendritic cell subtypes identified by flow cytometry

The major dendritic cell subtypes were quantified using flow cytometry, the principles of this technique are outlined in section 3.3. A commercially available human dendritic cell kit was used (Miltenyi Biotec UK). Two tubes were set up each containing 300 μ l of whole blood. Anti-BDCA cocktail was added to tube one (20 μ l) (containing antibodies to BDCA-1, BDCA-2, BDCA-3, CD14 and CD19) (Table 6.5) and control cocktail (20 μ l) was added to the other tube (containing the respective 3 isotype controls and antibodies to CD14 and CD19). Dead cell discriminator (10 μ l) was added and the samples were incubated on ice under a 60W light (3-5cm away from sample) for 10 minutes. On exposure to light, the dead cell discriminator binds covalently and irreversibly to nucleic acids of dead cells. Following this, 4ml of red blood cell lysing solution was added to each sample and they were incubated in the dark for 10 minutes. Cells were collected by centrifugation (4 $^{\circ}$ C, 515 x g for 7 minutes) and the supernatant discarded before washing in 3mls of FACS buffer (PBS with 0.2% BSA and 0.05% sodium azide). The tubes were centrifuged and the supernatant removed before dead cell discriminator stop reagent was added to each sample. The samples were fixed using 200 μ l FACSFIX (BD Biosciences, USA). The gating strategy used to identify the dendritic cell subsets is shown in Figure 6.4.

Antigen	Fluorochrome	Cell expression
BDCA-1 (CD1c)	PE	Myeloid dendritic cells
BDCA-2 (CD303)	FITC	Plasmacytoid dendritic cells
BDCA3 (CD141)	APC	Type II myeloid dendritic cells
CD14	PE-Cy5	High expression on monocytes
CD19	PE-Cy5	B lymphocytes
Mouse IgG1 Isotype control	APC	
Mouse IgG1 Isotype control	FITC	
Mouse IgG2a Isotype control	PE	

Table 6.5: Antibodies and their conjugated fluorochromes contained within the human dendritic cocktail set (Miltenyi Biotec UK).

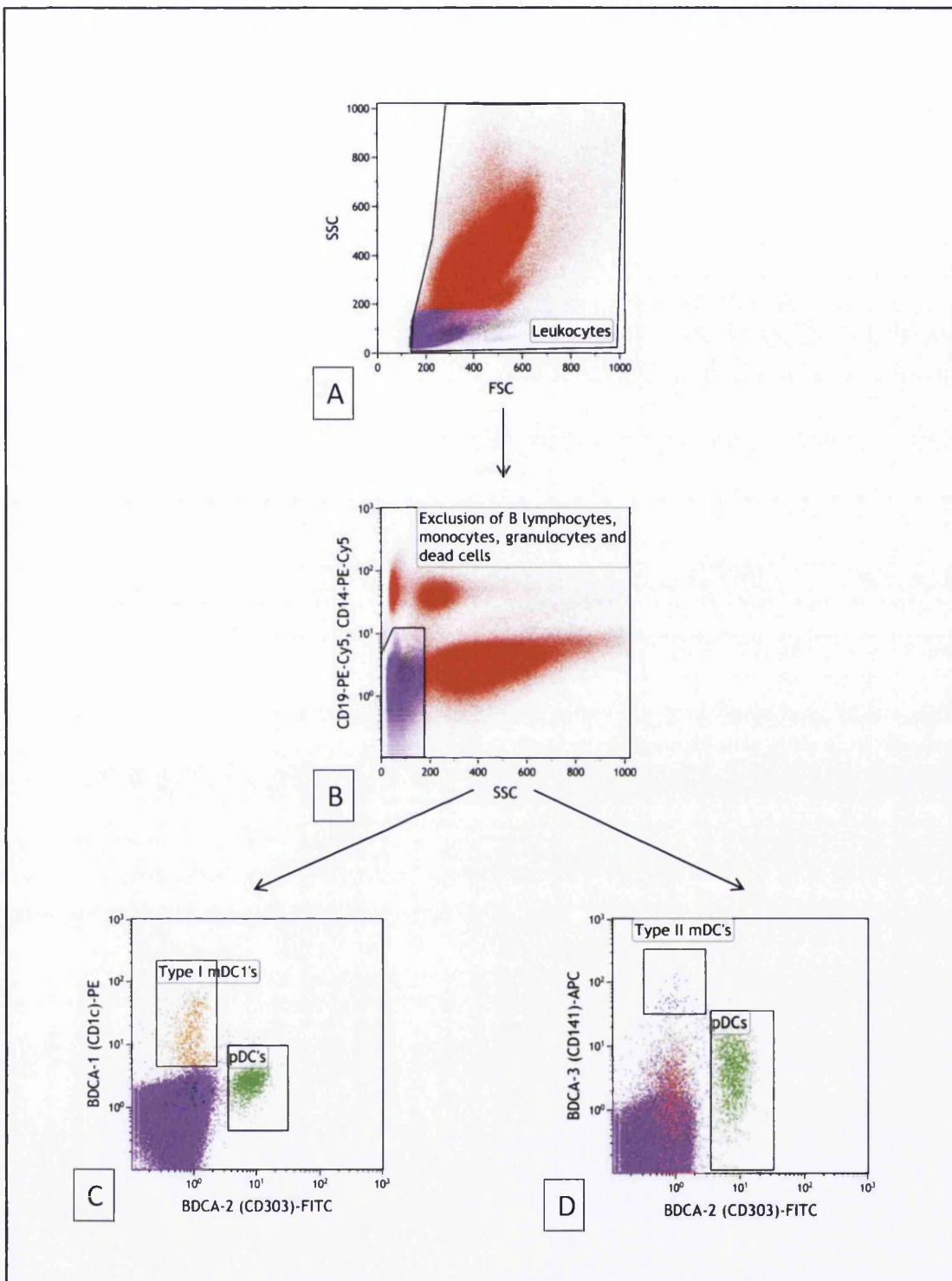


Figure 6.4: Gating strategy for identifying dendritic cell subsets by flow cytometry.

- A:** Identification of leukocytes using forward (FSC) and side scatter (SSC).
- B:** Gating on leukocytes, exclusion of granulocytes (high SSC), B lymphocytes (CD19+), monocytes (CD14+), which also express low levels of BDCA1 and dead cells.
- C:** Identification of Type I mDCs (BDCA-1⁺) and pDC's (BDCA-2⁺).
- D:** Identification of Type II mDCs (BDCA 3⁺).

6.3 Results

6.3.1 Monocyte activation markers

6.3.1 (i) HLA-DR expression

Analysis of monocyte activation using flow cytometry was not included in the range of tests implemented at the outset of this study. It was added, along with evaluation of neutrophil activation status, when interim analysis highlighted the relationship between neutrophils and obesity/asthma. The decision was made to investigate neutrophil phenotype in more detail and as the available literature highlights potential effects on monocyte phenotype this was also added to the revised range of tests. This does mean that only a subset of the cohort was analysed.

HLA-DR expression as signal index on CD14⁺ monocytes was measured in 33 individuals and data gathered passed the KS test ($p=0.847$). There was no significant difference in HLA-DR expression on CD14⁺ monocytes between the 6 groups ($p=0.453$). Across the entire study group BMI category and BMI as a continuous variable were not associated with HLA-DR expression ($p=0.818$; $r=-0.72$, $p=0.694$), neither was percentage body fat or WHR. Participant age did not correlate with this parameter. When the asthmatics and controls were analysed individually HLA-DR expression was not associated with BMI in either group (asthmatics: $r=0.095$, $p=0.717$; controls: $r=-0.267$, $p=0.335$) and neither were the other markers of adiposity.

HLA-DR expression did not differ between asthmatics and controls across the entire study group ($p=0.251$) or when each BMI category was compared separately. Within the asthmatics, asthma duration, age of onset, control score, ICS use and spirometric markers did not correlate with this measure.

6.3.1 (ii) CD16 expression

CD14⁺CD16⁺ monocytes expressed as a percentage of circulating CD14⁺ monocytes were measured in 29 individuals and data passed the KS test ($p=0.524$). There was no significant difference in the percentage of CD14⁺CD16⁺ monocytes across the 6 groups (Figure 6.5) ($p=0.719$). This parameter was not associated with participant age. The percentage of CD14⁺CD16⁺ monocytes was not associated with BMI category across the entire cohort ($p=0.803$) or when BMI was examined as a continuous variable ($r=-0.148$, $p=0.480$). Similarly no associations were seen with percentage body fat or WHR.

When asthmatics and controls were examined separately no correlations were seen between CD14⁺CD16⁺ monocytes and BMI in either group (asthma: $r=-0.264$, $p=0.361$, controls $r=0.057$, $p=0.868$) and no associations were seen with the other markers of adiposity.

Overall levels were higher in asthmatics (mean=4.81%) than controls (mean=3.88%) but this was not significant ($p=0.293$). When each BMI category was compared individually no statistically significant

differences were found although the numbers in each category were very small, as reflected by the wide error bars. CD14⁺CD16⁺ monocyte levels (%) did not correlate with age. Within the asthmatics the percentage of CD14⁺CD16⁺ monocytes did not correlate with asthma control score, ICS use, or spirometric markers.

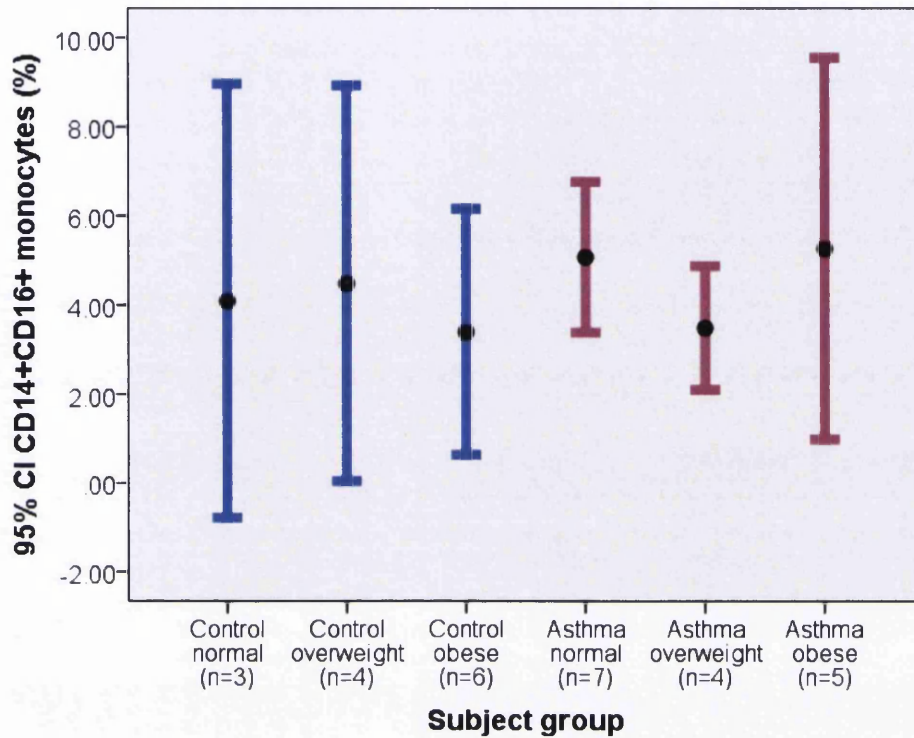


Figure 6.5: Percentage of CD14⁺CD16⁺ monocytes in the asthmatics and controls according to BMI category. Data shown as mean and error bars.

6.3.2 Plasma levels of soluble CD14

Plasma soluble CD14 levels were measured in all 84 individuals and data passed the KS test ($p=0.948$). There was no significant difference in sCD14 levels across the 6 groups and levels were not significantly higher in asthmatics than controls across the entire study group (Table 6.6). However when each group BMI category was compared, normal weight (NW) asthmatics had higher levels of sCD14 than NW controls although this was not statistically significant ($p=0.069$). Overweight and obese asthmatics did not have significantly higher levels compared to their respective control groups ($p=0.261$ and $p=0.711$, respectively).

When BMI category was examined across the entire study group no significant difference was seen in sCD14 levels. However when BMI was examined as a continuous variable a significant positive correlation was seen with sCD14 levels ($r=0.224$, $p=0.041$). No significant association was seen with patient age. The correlation between BMI and sCD14 was only seen in the controls ($r=0.37$, $p=0.027$). There was no correlation between BMI and sCD14 was seen in the asthmatics ($r=0.089$, $p=0.580$). Therefore, the interaction between the BMI and asthma status with regards to sCD14 was

formally tested. No significant interaction effect was seen ($p=0.134$). Within the asthmatics no significant associations were seen between sCD14 levels and asthma duration, asthma control, ICS use, or spirometric markers. Given previous interest in sCD14 levels and insulin sensitivity in obese patients the relationship between these two variables was explored but no significant association was found ($r=-0.166$, $p=0.289$).

	Controls			Asthmatics			p value
	NW n=14	OW n=13	OB n=15	NW n=14	OW n=12	OB n=15	
sCD14 (ng/ml) Mean (SD)	1170.45 (404.01)	1328.74 (257.59)	1434.32 (416.80)	1409.85 (261.72)	1193.26 (321.58)	1386.50 (264.07)	All 6 groups: $p=0.156$ BMI category: $p=0.210$ A vs. C: $p=0.709$

Table 6.6: Plasma sCD14 levels in asthmatics and controls according to BMI category.

Data are expressed as mean and standard deviation (SD). Differences between all 6 groups were analysed using a one-way ANOVA Differences between asthmatics (A) and controls (C) were analysed using an unpaired t-test Differences between BMI categories were analysed using a one-way ANOVA.

6.3.3 Cytokine response to LPS stimulation on whole blood cultures

LPS stimulation of whole blood cultures was used to model and compare the responses by the different study groups. Cytokine responses (IL-1 β , IL-6, IL-8, IL-10, and TNF α) to LPS were measured in 83 individuals and are summarised in Table 6.7. LPS-induced IL-1 β , IL-6, IL-8 and TNF α production in whole blood did not pass the KS test ($p=0.202$, $p=0.076$, $p=0.032$, $P<0.001$, respectively) so therefore data were logarithmically transformed for analysis. The LPS-induced response for all of the cytokines of interest did not vary with BMI category across the entire study group for any of the cytokines studied (Table 6.7). There were also no significant differences when BMI was examined as a continuous variable across the entire study group (IL-1 β : $r=-0.11$, $p=0.306$; IL-6: $r=0.068$, $p=0.583$; IL-8: $r=0.051$, $p=0.649$; IL-10: $r=-0.005$, $p=0.996$; TNF α : $r=-0.672$, $p=0.579$). No correlations were seen with body fat composition or WHR. Age of the participants was not associated with any of the LPS induced cytokine levels.

When the asthmatics and controls were examined separately, none of the LPS-induced cytokine responses were correlated with BMI ((Controls: IL-1 β : $r=-0.161$, $p=0.310$, IL-6: $r=0.204$, $p=0.196$, IL-8: $r=0.002$, $p=0.990$, IL-10: $r=-0.28$, $p=0.862$, TNF α : $r=-0.082$, $p=0.062$) (Asthmatics IL-1 β : $r=-0.097$, $p=0.545$, IL-6: $r=-0.054$, $p=0.737$, IL-8: $r=0.094$, $p=0.558$, IL-10: $r=0.006$, $p=0.968$, TNF α : $r=-0.073$, $p=0.652$)). Similarly none of the cytokine responses correlated with body fat composition or WHR in the asthmatics or controls.

Across the entire cohort, asthmatics had greater LPS induced IL-1 β response than control subjects (geometric mean = 1.63ng/ml versus 1.29ng/ml) but this was not significant ($p=0.089$). LPS induced TNF α response was also higher in asthmatics than controls however this was not significant (geometric mean =139.46pg/ml versus 108.28pg/ml, $p=0.090$). When comparing the individual BMI categories between asthmatics and controls no significant differences were seen for any of the cytokines studied. Within the asthmatics none of the LPS induced cytokine levels correlated with asthma duration, asthma control, ICS use or any of the spirometric markers.

	Controls			Asthmatics			p value
	NW n=14	OW n=13	OB n=15	NW n=14	OW n=12	OB n=15	
IL-1β (ng/ml) Geometric Mean (SD)	1.30 (1.90)	1.53 (0.98)	1.09 (1.30)	1.77 (0.80)	1.49 (0.83)	1.63 (0.57)	All 6 groups: $p=0.387$ BMI category: $p=0.678$ A vs. C: $p=0.089$
IL-6 (ng/ml) Geometric Mean (SD)	9.21 (3.528)	10.65 (3.998)	9.66 (7.350)	10.82 (4.283)	11.95 (7.310)	10.14 (4.672)	All 6 groups: $p=0.701$ BMI category: $p=0.479$ A vs. C: $p=0.265$
IL-8 (ng/ml) Geometric Mean (SD)	2.87 (2.78)	2.57 (3.08)	2.63 (3.49)	2.58 (2.61)	3.85 (2.11)	3.40 (2.81)	All 6 groups: $p=0.625$ BMI category: $p=0.778$ A vs. C: $p=0.264$
IL-10 (pg/ml) Mean (SD)	91.44 (63.05)	114.32 (39.62)	101.21 (54.09)	107.07 (62.20)	118.08 (71.51)	100.02 (89.14)	All 6 groups: $p=0.870$ BMI category: $p=0.511$ A vs. C: $p=0.641$
TNFα (pg/ml) Geometric Mean (SD)	123.30 (313.12)	113.03 (200.02)	186.45 (93.37)	135.67 (390.17)	136.75 (124.60)	145.79 (420.46)	All 6 groups: $p=0.666$ BMI category: $p=0.907$ A vs. C: $p=0.090$

Table 6.7: Plasma cytokine levels from whole blood cultures in response to LPS stimulation in asthmatics and controls according to BMI category.

Normally distributed data are expressed as mean and standard deviation (SD). Parameters not normally distributed were expressed as geometric mean and standard deviation and were logarithmically transformed before analysis. Differences between all 6 groups and the 3 BMI categories were analysed using a one-way ANOVA. Differences between asthmatics (A) and controls (C) were analysed using an unpaired t-test

Production of IL-12p70 requires stimulation with IFN γ prior to PAMP exposure [424], therefore whole blood cultures were also exposed to IFN γ (10ng/ml) 90 minutes prior to the addition of LPS. LPS/IFN γ -stimulated IL-12p70 and IL-23 production did not pass the KS test ($p=0.018$ and $p=0.040$, respectively), therefore values were logarithmically transformed for analysis.

There were no significant differences in values across the 6 groups (Table 6.8) or with BMI category across the entire cohort for either cytokine response. When BMI was analysed as a continuous variable no correlation was seen (IL-12p70: $r=-0.103$, $p=0.356$; IL-23: $r=-0.050$, $p=0.662$) and no correlation was seen with percentage body fat or WHR. Cytokine production did not correlate with age.

When asthmatics and controls were examined individually, IL-12p70 (asthmatics: $r=-0.138$, $p=0.391$; controls: $r=-0.91$, $p=0.571$) and IL-23 (asthmatics: $r=0.101$, $p=0.536$; controls $r=-0.219$, $p=0.174$) did not vary significantly BMI, body fat composition or WHR. LPS/IFN γ -stimulated levels of these cytokines did not significantly differ between asthmatics and controls across the entire cohort (Table 6.8) or when each individual BMI category was compared. No correlations were seen between production of these two cytokines and asthma duration, control, ICS usage or any of the spirometric measurements taken.

	Controls			Asthmatics			p value
	NW n=14	OW n=13	OB n=15	NW n=14	OW n=12	OB n=15	
IL12p70 (pg/ml) Geometric Mean (SD)	204.72 (529.20)	158.37 (393.42)	162.05 (323.29)	248.12 (207.36)	207.36 (319.36)	186.95 (272.85)	All 6 groups: $p=0.847$ BMI category: $p=0.578$ A vs, C: $p=0.348$
IL-23 (ng/ml) Geometric Mean (SD)	1.59 (1.29)	1.52 (1.43)	1.05 (0.85)	1.11 (1.09)	1.10 (0.76)	1.26 (1.30)	All 6 groups: $p=0.333$ BMI category: $p=0.744$ A vs.C: $p=0.333$

Table 6.8: Plasma cytokine levels from whole blood cultures in response to LPS and IFN γ stimulation in asthmatics and controls according to BMI category.

Normally distributed data are expressed as mean and standard deviation (SD). Parameters not normally distributed are expressed as geometric mean and standard deviation and were logarithmically transformed before analysis. Differences between all 6 groups and BMI categories were analysed using a one-way ANOVA. Differences between asthmatics (A) and controls (C) were analysed using an unpaired t-test.

6.3.5 Circulating levels of dendritic cell subtypes

The percentage of circulating dendritic cell subtypes was measured in 39 controls (NW: n=13, OW: n=13, OB: n=13) and 34 asthmatics (NW: n=12, OW: n=10, OB: n=12).

6.3.5 (i) Type I myeloid dendritic cells

Data for the percentage of circulating type I myeloid dendritic cells (mDCs) were normally distributed ($p=0.456$). There was a significant difference in the mean percentage of circulating type I mDCs across the 6 groups (Figure 6.6) ($p=0.002$). This was due to NW asthmatics having significantly higher levels of type I mDCs than the overweight (OW) asthmatics ($p=0.013$) and obese (OB) asthmatics ($p=0.002$) and controls (NW asthma versus NW controls $p=0.002$, NW asthma versus OW control $p=0.005$, NW asthma versus OB control $p=0.002$).

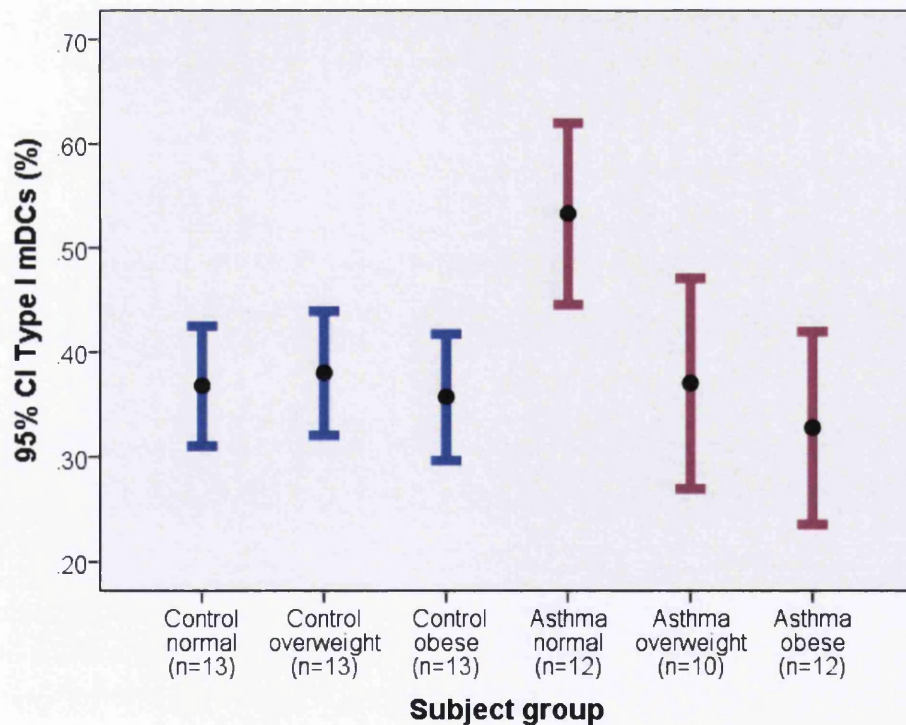


Figure 6.6: Percentage of type I mDCs in the asthmatics and controls according to BMI category. Data shown as mean and error bars.

Examining the asthmatics and controls separately, increasing BMI category and continuous BMI was associated with a reduction in type I mDCs in the asthmatics ($p=0.003$; $r=-0.568$, $p<0.001$) which was not seen in the control group ($p=0.837$; $r=-0.077$, $p=0.647$). This was also seen with percentage body fat (asthmatics $r=-0.529$, $p=0.001$; controls: $r=-0.145$, $p=0.384$) but not WHR. When we tested for this formally a significant interaction was seen between BMI and asthma status with regards to type I mDCs ($p=0.005$). Within the asthmatics, age of onset, duration, asthma control score, ICS use and the spirometric markers were not associated with type I mDCs.

In view of the expression of leptin receptor on DCs, the correlation between type I mDCs and leptin levels was explored and revealed a non-significant negative correlation ($r=-0.244$, $p=0.070$). On examining asthmatics and controls separately neither correlation was significant (asthmatics: $r=-0.329$, $p=0.058$; controls: $r=-0.228$, $p=0.169$). In addition the association between mDCs and within the asthmatics remained significant after retaining leptin the model suggesting that leptin levels are unlikely to be the only explanation for the association between BMI and type I mDCs (BMI $p=0.002$, leptin $p=0.201$, $R^2=0.358$).

6.3.5 (ii) Type II myeloid dendritic cells

Data for the percentage of circulating type II mDCs did not pass the KS test ($p<0.001$) so was logarithmically transformed for analysis. There was no significant difference in the geometric mean levels of type II mDCs (%) across the 6 groups ($p=0.518$) (Figure 6.7). Levels were not associated with BMI category across the entire study group ($p=0.723$) or when BMI was examined as a continuous variable ($r=-0.131$, $p=0.271$). Similarly levels were not associated with percentage body fat or WHR.

Levels were not significantly different between asthmatics and controls across the entire study group ($p=0.697$), or when each BMI category was compared. Although normal weight asthmatics appeared to have higher levels of type II mDCs than normal weight controls and obese asthmatics, this was not significant ($p=0.160$ and $p=0.083$ respectively). Interestingly in the asthmatics, BMI and percentage body fat negatively correlated with type II mDC levels (%), although the latter was not significant ($r=-0.371$, $p=0.031$ and $r=-0.311$, $p=0.074$). Despite this finding, no correlation between leptin levels and Type II mDCs was seen in the asthmatics.

Type II mDCs may play an important role in the polarisation of Th2 responses and higher circulating levels have been described in atopic versus non-atopic individuals with levels rising during acute asthma exacerbations and falling during convalescence in adolescent atopic asthmatics [606]. Within the asthmatics no correlation was seen with asthma duration, asthma control, ICS use or any of the spirometric markers. Given recent findings suggesting that type II mDCs may be important in Th2 response and atopy we looked for correlations between type II mDCs and eosinophils, IgE, PHA-induced IL-13 response and PHA-induced IFN γ response. A negative correlation was seen PHA-induced IFN γ response ($r=-0.345$, $p=0.046$) but with none of the other parameters.

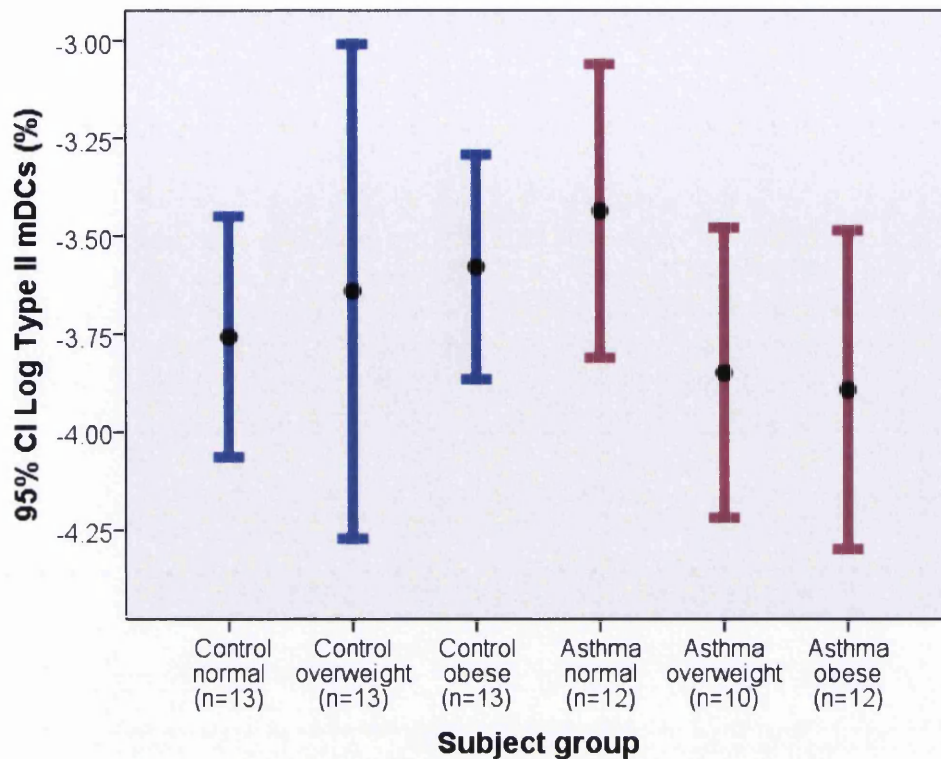


Figure 6.7: Percentage of type II mDCs in the asthmatics and controls according to BMI category. Data shown as logarithmic values, mean and error bars.

6.3.5 (iii) Plasmacytoid dendritic cells

Data for the percentage of circulating pDCs did pass the KS test ($p=0.479$). There was no significant difference in the mean levels of pDCs (%) across the 6 groups ($p=0.141$) (Figure 6.8). Across the entire study group increasing pDCs (%) varied significantly between BMI categories ($p=0.026$) (Figure 6.9), with overweight individuals and obese having lower levels of pDCs than controls ($p=0.011$ and $p=0.064$ respectively), although the latter was not statistically significant. When BMI was examined as a continuous variable there was a significant negative correlation between this and percentage pDCs ($r=-0.233$, $p=0.049$). Negative correlations were also seen with percentage body fat and WHR but the latter was not significant ($r=-0.212$, $p=0.049$ and $r=-0.202$, $p=0.093$ respectively). There was no significant difference in circulating pDCs (%) between the asthmatics and controls ($p=0.526$).

When the asthmatics and controls were examined separately a negative correlation between BMI and pDCs was seen in both groups but neither was significant (asthmatics: $r=-0.254$, $p=0.148$; controls: $r=-0.231$, $p=0.164$). Similar findings were noted for percentage body fat and WHR.

Within the asthmatic group there were no significant associations between pDCs and age of onset, asthma duration, control score, ICS dose or any of the spirometric markers. Plasmacytoid DCs (%) were not associated with age, across the entire cohort; nor were they associated with IR and leptin levels.

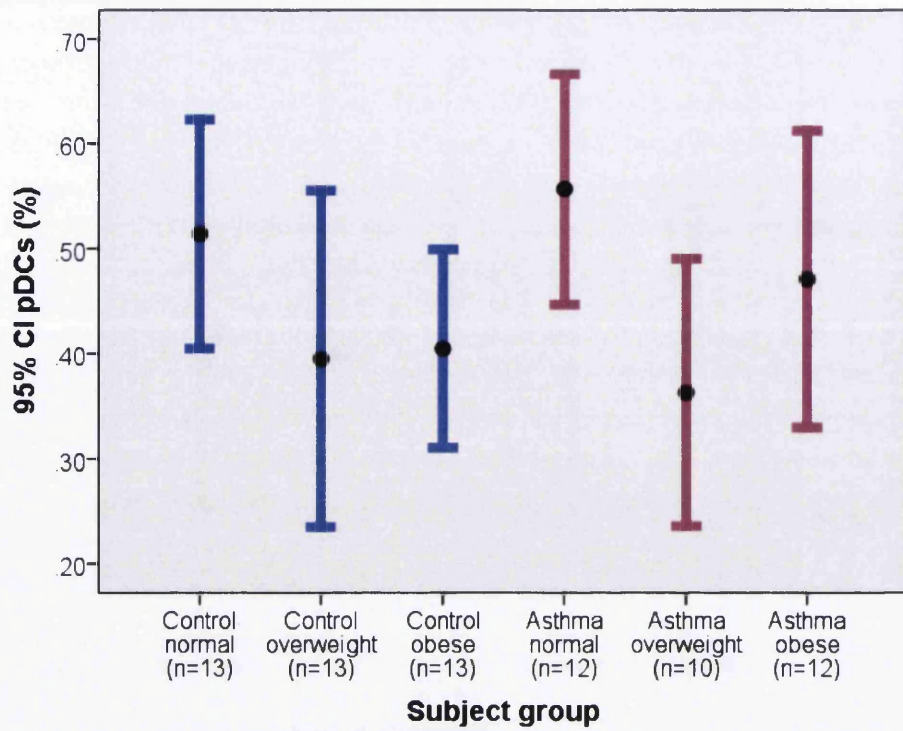


Figure 6.8: Percentage of pDCs in the asthmatics and controls according to BMI category. Data shown as mean and error bars.

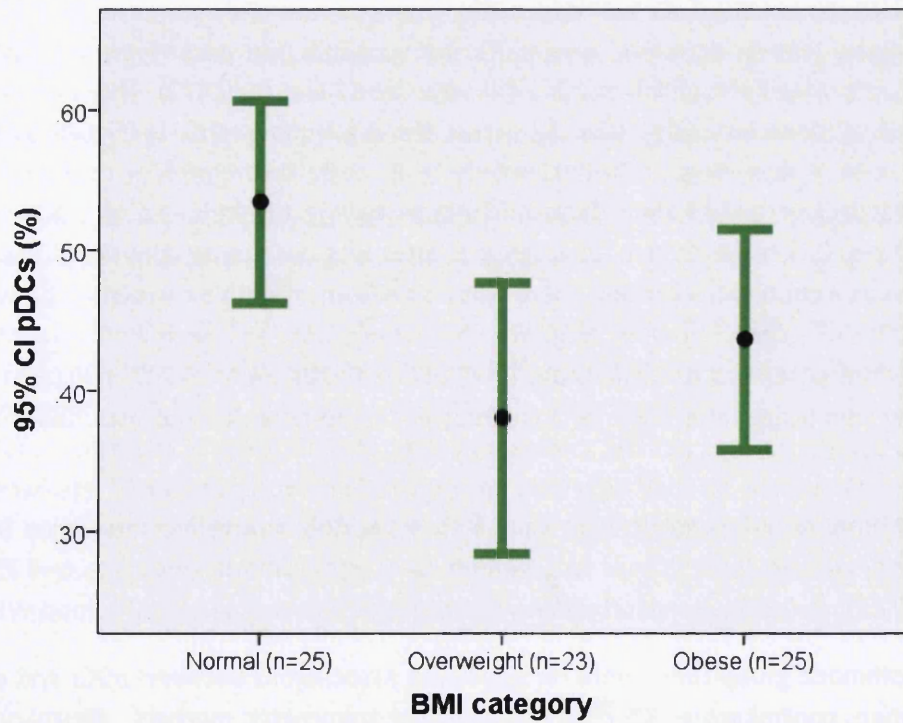


Figure 6.9: Percentage of pDCs across the 3 BMI categories. Data shown as mean and error bars.

6.4 Discussion

6.4.1 Obesity and asthma were not associated with increased expression of monocyte activation markers

Obesity is associated with an increase in pro-inflammatory M1 macrophages within the visceral adipose tissue compartment reflecting at least in part a systemic increase in pro-inflammatory bone marrow derived CD14⁺CD16⁺ monocytes [31]. These M1 macrophages are thought to contribute to the chronic systemic inflammation seen in obesity. There is also some evidence that they might have a role locally in steroid resistant severe asthma with the increased macrophage population seen in asthmatics also derived from increased levels of mature circulating CD16⁺ monocytes [173]. Given that some studies suggest that obesity and asthma are both associated with systemic monocyte activation the percentage of circulating pro-inflammatory monocytes in normal weight, overweight and obese individuals with and without asthma was measured in this study to see if there were discernible differences between the groups. There were no differences in the percentage of circulating CD14⁺ CD16⁺ cells or HLA-DR expression with increasing BMI or between asthmatics and controls suggesting that obesity in asthma is not associated with systemic changes in this marker of monocyte activation. Our results differ from those of Cottam et al, who showed that obese patients undergoing bariatric surgery had significantly higher percentages of circulating CD14⁺/CD16⁺ monocytes than control subjects [31]. Although their study was on almost exclusively females (25/26 in the obese group), the mean BMI of the obese group was significantly higher than ours (mean=52kg/m² vs. 37kg/m²) with no participants with a BMI of <40kg/m². Detectable differences in CD16 expression by monocytes might only be seen at the extremes of obesity. Furthermore they did not control for the effects of fluctuating hormone levels which have been shown to affect other features of mononuclear cell activation such as the response to LPS [620].

These results also differ from those of Rivier et al, who showed significantly increased CD16 expression on blood monocytes in terms of percentage circulating and mean fluorescence intensity in asthmatics than controls [173]. This thesis involved predominantly patients who had moderate to severe disease on ICS treatment, which when given long term may affect surface marker expression on monocytes. Thirty months of ICS treatment increased CD14 expression on peripheral blood monocytes compared to asthmatics not on treatment and controls [621], although patients on ICS treatment did not have detectable differences in CD16 expression compared to untreated asthmatics or controls. In another study, which also showed a higher expression of CD16 on monocytes of asthmatics compared to control subjects, ICS was withheld for 48 hours prior to sample collection unlike the study herein where it was withheld for 24 hours [557]. Tomita et al also withheld theophylline preparations and β 2 agonists for 48 hours as these can potentiate monocyte differentiation [622].

Another major limitation of the study herein is that monocyte activation markers were only determined for 33 (HLA-DR) and 29 (CD16) individuals, so might not be adequately powered. Measurement of monocyte activation was not included in the original study design but following an interim analysis showing measurable differences in circulating neutrophils and monocytes between patient groups it was decided to investigate this area further. Cryopreserved mononuclear cells are

available for the entire study cohort and these samples could be used to measure activation markers in all our individuals retrospectively. It would also be of great interest to look at markers of macrophage activation within the airways themselves by examining BAL fluid samples.

6.4.2 Soluble CD14 levels correlated with BMI in the control group

Soluble CD14 levels significantly positively correlated with BMI in the control group: the obese subjects amongst the controls therefore had the highest mean levels but this was not significantly higher than the normal weight controls. This is similar to findings in another study which also observed that weight loss was associated with a significant reduction in circulating sCD14 [584]. Levels also correlated with insulin sensitivity but this was not seen in the study herein [584]. The biological significance of the positive correlation between BMI and sCD14 levels is not certain. Soluble CD14 potentiates or inhibits LPS signalling depending on its levels and location: it can enable LPS responses by cells that lack mCD14 but can cause efflux of LPS already bound to monocytes and therefore limit the LPS response [567]. Despite the positive correlation between sCD14 and BMI in the controls, we did not see any differential effect on LPS response with BMI (see below). However one key difference between experiments with whole blood versus PBMCs (typically cultured with foetal bovine serum) is the presence of autologous plasma and the inter-subject variation in accessory molecules naturally present in this substrate. Although we measured sCD14 we did not take into account other soluble factors such as lipopolysaccharide binding protein (LBP) [563] and it would be of interest to see if this also changes with BMI.

Given that gene polymorphisms in the CD14 gene and resultant levels modify asthma risk in association with LPS exposure [572] and that asthmatics patients have a higher sCD14 levels in the airways [570], predicting neutrophil influx in response to LPS challenge [571], we measured systemic levels of sCD14 in the asthmatics. Although normal weight asthmatics had higher levels than their control counterparts the same was not seen for overweight or obese asthmatics. In fact in contrast to the observations in the control group, BMI had no correlation with sCD14 levels in the asthmatics.

6.4.3 Obesity and asthma were not associated with detectable differences in cytokine response to LPS stimulation of whole blood

Previous studies suggest that asthmatics have an increased response to LPS systemically [341] and locally in the airways [570]. Some studies suggest that obesity is also associated with increased inflammatory response to LPS systemically [32]. Therefore the response to LPS was measured using a whole blood culture model particularly to determine the response in obese asthmatics, an area which has not been explored previously. However, cytokine responses (IL-1 β , IL-6, IL-8, IL-10, TNF α , IL-12p70 and IL-23) from whole blood exposed to LPS +/- IFN γ did not vary with BMI or asthma status suggesting that at a systemic level, obese asthmatics do not have an increased cytokine

response to endotoxin exposure, at least for the cytokines measured in this study. There was a trend towards an increase in IL-1 β and TNF α production with asthma, however this was not significant. This would be of relevance to severe asthma as IL-1 β increases GM-CSF production by bronchial epithelial cells [623] as well as IL-8 production [624], both of which are important mediators in neutrophilic inflammation and TNF α is increased in BAL of patients with severe corticosteroid dependent disease [175]. In a study by Hallsworth and colleagues, there was increased IL-1 β production following LPS stimulation of peripheral blood mononuclear cells in asthmatics versus controls with no differences in TNF- α or IL-8 production [341]. Our study differed methodologically in that whole blood was used and the patients were on ICS treatment. Glucocorticoids have been shown to reduce IL-1 β production within the airways; it is therefore possible that they may also inhibit production systemically [625]. In contrast to previous study of atopic asthmatics [591], IL-10 production following LPS stimulation was not reduced in the asthmatics studied herein. This may be again due to the effect of ICS treatment; although in the study by Borish et al, *in vitro* use of methylprednisolone reduced LPS induced IL-10 production further. It may also be due to differences in methodology with Borish et al examining LPS response in isolated mononuclear cells rather than whole blood as used here. An advantage of whole blood cultures is that it contains circulating levels of proteins such as sCD14 and LPB, which modify the interaction between LPS and cell surface receptors [563]. Therefore this current work examined LPS responses in a more physiologically accurate environment taking into account these factors.

The lack of any association between BMI and LPS response is in keeping with a recently published *in vivo* study examining the systemic cytokine response to endotoxin infusion in otherwise healthy volunteers of varying BMI [626]. Healthy male volunteers (n=112) were given intravenous LPS and the cytokine responses (TNF α , IL-6, IL-10 and IL-1RA) were measured at time intervals up to 8 hours post administration. Patients who were overweight or obese (BMI>25kg/m²) did not have significantly different cytokine responses compared to normal weight individuals. Our study and that of van Eijk [626] contradicts the findings of Kueht et al [32] and Tanka et al [579] who found an increased TNF α response to LPS stimulation of whole blood and mononuclear cell cultures respectively in obese individuals. However, several key methodological differences exist between their work and ours. Firstly these two studies examined African-American and Japanese adults respectively, whereas our women were exclusively Caucasian. Secondly they did not control for the menstrual cycle. The *in vitro* response of human monocytes to endotoxin changes with the menstrual cycle, peaking in the luteal phase [620]. Furthermore other TLR responses vary with the menstrual cycle [627]. Failure to control for fluctuations in hormone levels may have confounded results. Thirdly it was interesting to note in the study by Kueht et al that whilst overweight and obese category III (BMI>40kg/m²) had an increased response to TNF α , obese category I and II patients did not [32]. The mean BMI of the obese women in our study was lower at 37kg/m² and in the recent *in vivo* study van Eijk, all volunteers had a BMI of <35kg/m² [626]. Differences in the systemic response to LPS might only become apparent when BMI exceeds 40kg/m². Finally, in the Japanese study [579] *in vitro* responses to LPS were measured in mononuclear cell cultures rather than whole blood and therefore will lack the inter-subject variation in natural modulators of the response.

6.4.4 Increasing BMI is associated with a reduction in the percentage of circulating type I and type II myeloid dendritic cells in asthmatics

Within the asthmatics but not controls increasing BMI category was associated with a reduction in the percentage of circulating type I mDCs; this reflected significantly higher levels of type I mDCs in normal weight asthmatics than in the other groups. Obese asthmatics had comparable levels of type I mDCs to the controls. Given that the levels of pDCs did not differ between normal weight asthmatics and controls, this suggests a change in the balance of mDCs versus pDCs. Myeloid DCs have been implicated to have a pathophysiological role in asthma. Airway allergen challenge of atopic asthmatics leads to an increase in mDCs within bronchial biopsies within 4-5 hours [603] and BAL fluid within 24 hours [604] with a concurrent reduction in blood levels [603, 604]. In animal models, upsetting the balance between pDCs and mDCs in favour of mDCs promotes allergen mediated airway inflammation [185] whereas tipping the balance towards pDCs abolishes the cardinal features of asthma in allergen sensitised mice [610]. Examining mDC subsets, type II mDCs are also found in the lung and they outnumber type I mDCs in lung digests [96] and BAL samples [628]. Type II mDCs may play an important role in the polarisation of Th2 responses and higher circulating levels have been described in atopic versus non-atopic individuals with levels rising during acute asthma exacerbations and falling during convalescence in adolescent atopic asthmatics [606]. Within the asthmatics, type II mDCs were highest in the normal weight individuals although this was not statistically significant. Levels did significantly negatively correlate with BMI with obese asthmatics having comparable type II mDC levels to the controls. This is in keeping with the observations in chapter 4 that eosinophils (typically associated with Th2 mediated disease), declined with increasing BMI in the asthmatics and not in the controls. However, in the asthmatics, no correlation between type II mDCs and eosinophils was seen. It will be of interest in chapter 7 to explore if this decline in type II mDCs with BMI in the asthmatics is associated with a T cell cytokine response favouring Th1 skewing.

Differential effects with regards to BMI and DCs dependent on the underlying disease state have been seen in diabetes. Obese diabetics had significantly more circulating type I and type II mDCs than normal weight control patients in one study, however obese control patients did not have significantly increased levels compared to normal weight controls [33]. Leptin receptors are expressed on DCs and leptin has anti-apoptotic effects [295]. Therefore the relationship between leptin levels and type I mDCs was investigated. Perhaps unexpectedly a negative correlation was seen. A potential explanation for this is that a reduction in circulating type I mDCs in obese asthmatics is not due to a reduction in whole body percentages of these cells but actually represents increased migration into tissues, so their loss from peripheral blood. Given the ability of leptin to enhance the migratory capacity of dendritic cells this is a possibility [295]. This might not occur in the controls because the levels of leptin are lower (see chapter 4) or possibly due to leptin resistance, a phenomena associated with obesity, differing between asthmatics and controls. This would be particularly important in a disorder such as asthma that involves mucosal inflammation and, as discussed above, there is already evidence of migration of mDCs from blood to airways [603, 604]. In a murine obesity model induced by leptin deficiency, obesity was associated with increased numbers of dendritic cells locally within the epidermis with restoration in numbers following leptin injection [616]. It would be worthwhile examining local numbers of DCs within the airways as well as

investigating whether leptin sensitivity differs between asthmatics and controls. Interestingly a general linear model incorporating BMI and leptin within the asthmatics showed that the association between BMI and type I mDCs (%) in the asthmatics remained significant whereas the association between leptin and type I mDCs did not, suggesting that whilst leptin may play a role in this relationship it is unlikely to be the sole mediator.

A study of obese but otherwise healthy adults published recently examined the percentage of circulating mDCs and their function compared to lean individuals [629]. In this study adults with a mean BMI of 51.7kg/m², had significantly lower levels of blood mDCs than their lean counterparts. Although we did not find this in our control subjects, across the entire study group increasing BMI was associated with a reduction in myeloid dendritic cells (%) which was entirely due to the changes in the asthmatics. Our study differed from O'Shea et al in that the mean BMI was significantly lower (37.4kg/m²). It may be that a reduction in mDCs is only observed at extremes of BMI and if a leptin mediated mechanism is behind this then this would explain our data since our asthmatics had a higher leptin level compared to our controls in each BMI category.

6.4.5 Increasing BMI is associated with a reduction in pDCs

Increasing BMI as a continuous variable were associated with a reduction in pDCs across the entire study group with overweight and obese patients having lower levels than normal weight individuals, although the latter was not significant. Asthmatics did not have different levels to controls. Plasmacytoid DCs are important in immune regulation and the differentiation of regulatory T cells [599]. It is noteworthy that whilst overweight participants had significantly lower levels of pDCs than those of normal weight, the obese whilst also having lower percentages of pDCs, this was not significant. This suggests that the relationship between BMI and some of the immune parameters might follow a parabolic distribution; cytokine/adipokine resistance or homeostatic mechanisms start taking effect at higher levels of obesity and could explain such a phenomenon. There is very little published work on pDCs in obesity. Others have only examined mDCs [629] although in a study of obese patients with and without diabetes, the obese non-diabetics had lower levels of pDCs than normal weight controls, however as with the present study this did not reach significance [33]. Obesity is associated with a poorer outcome from viral infections [630] and poorer response to vaccination [614] and it has been hypothesised that this is due to changes in DC numbers and function. A reduction in pDCs shown herein might be a potential explanation.

6.5 Summary

In summary, the interaction between obesity and asthma with innate immunity was the theme of this chapter with a specific focus on circulating monocytes, LPS responsiveness, sCD14, and DCs. Obesity and asthma were not associated with significant monocyte activation and in keeping with this, endotoxin responses were not affected. DCs serve as a bridge between the innate and adaptive immune response and the findings reveal elevated type I and type II mDCs in normal weight asthmatics with a decline with increasing BMI in the asthmatics only. Furthermore, increasing BMI,

especially being overweight was associated with a reduction in pDCs which are a cell type associated with immune tolerance and the promotion of regulatory T cells. In the next chapter, changes in adaptive immunity in the study population will be explored with a focus on regulatory T cells. Particular consideration will be given to the possibility that changes in the DC compartment are mirrored by changes in regulatory T cells.

Chapter 7

Adaptive immunity in obesity and asthma

7.1 Introduction

The adaptive immune system provides a temporally delayed response, which is highly specific; recognising peptides unique to a particular pathogen by the use of a seemingly infinite number of randomly generated, clonally expressed receptors [62]. Upon stimulation, the clonal expansion of these cells results in immunological memory, enabling a much faster specific response on subsequent encounter of the same antigen (section 2.2.3).

Asthma, particularly atopic disease, has been traditionally thought of as a disease of the adaptive immune system, a T helper 2 driven inflammatory process (section 2.3.3). However it is increasingly recognised that the inflammation seen in asthma is a result of cross-communication between the innate and adaptive arms of the immune system. With the identification of new T helper subsets (section 2.2.3 (i)), it is now appreciated that the clinical heterogeneity of asthma may partly reflect the varied involvement of different components of both systems. Although studies suggest that obesity may also disturb the Th1/Th2 balance, there is good mechanistic data showing it may modulate other T helper subsets including regulatory T cells (Tregs). To date there is little work on this area in the context of obesity in humans or the obesity-asthma association. In this chapter obesity, asthma and systemic changes in the adaptive immune response will be examined to see whether they provide further insight into the obesity asthma association.

7.1.1 Th1/Th2 balance and cytotoxic T cells

7.1.1 (i) Th1/Th2 balance and cytotoxic T cells in asthma

T cells are produced by the bone marrow but mature in the thymus and express a T-cell receptor (TCR). T cells can be divided into two main groups according to the differential expression of surface glycoproteins; cluster of differentiation 4 (CD4) T cells or T helper (Th) cells and CD8 expressing T cells or cytotoxic (Tc) T cells [104].

CD4⁺ or Th cells recognise antigen bound to MHC class II molecules expressed on professional antigen presenting cells [107]. Their name is derived from their function of “helping” other components of the immune system. They are further subcategorised according to their cytokine expression (Figure 2.2). Initially, two main subsets were identified in animal models [108] and humans [109]. T helper 1 cells (Th1) secrete IFN γ which acts on macrophages increasing their phagocytic capacity, important in responding to bacterial infections. They differentiate in response to IL-12p70 produced by dendritic cells [100] which causes up-regulation of the transcription factor T-bet [110]. Th2 cells produce IL-4, IL-5, IL-6, IL-9 and IL-13 [110]. IL-4 stimulates Th2 differentiation through the activation of transcription factors STAT6 and GATA3 [110]. The source of IL-4 is still under debate but there are two proposed mechanisms. The first suggests that upon antigen stimulation in the absence of Th1 or Th17 polarising cytokines CD4⁺ T cells default to producing IL-4 [631]. Alternatively IL-4 may arise from supporting cells including mast cells and basophils [632].

Other novel subsets have since been identified including regulatory T cells, Th-17 and Th-9 cells (see section below).

Atopic asthma has been characterised by the presence of increased numbers of Th2 cells within the airways [188] and the number of Th2 cells present correlates with disease severity [189]. These changes also occur systemically with atopic asthmatics having lower percentages of Th1 cells than controls with similar levels of Th2 cells suggesting Th2 bias [176]. The signature cytokines released by these cells have a number of effects relevant to disease pathogenesis. IL-4 is involved in immunoglobulin class switching in B cells leading to IgE expression important in the process of allergen sensitisation. IL-5 is fundamental to eosinophil differentiation and survival IL-9 promotes mast cell survival and IL-13 results in BHR [190].

CD8⁺, or Tc cells recognise antigen presented in the cleft of MHC class I molecules (HLA-ABC) [104]. Through the production of cytokines such as IFN γ , perforin and granzyme, they destroy virally infected [105] and cancerous cells [106]. CD8⁺ T cells may also play a role in asthma with increased numbers seen within the airways of affected individuals [199, 200] particularly in those who die from acute asthma [205]. Furthermore the annual decline in FEV1 can be predicted by the number of CD8⁺ cells in the bronchial infiltrate [633]. Animal models have been contradictory with some showing enhanced airway inflammation and remodelling on depletion of CD8 α ⁺ cells, suggesting that their presence may be protective [201, 202], however such studies are limited by the lack of specificity of CD8 α as a marker of cytotoxic T cells as this is also expressed on macrophages, NK cells and dendritic cell subsets. Contrary to these findings, more specific studies in which there has been transfer of CD8 $\alpha\beta$ T cells (see section 2.2.3 (i)) to sensitised animals, show worsening of eosinophilic inflammation and BHR [203, 204]. These cells may be especially important in mediating the inflammatory changes seen during viral exacerbations of asthma with depletion in mice preventing BHR and eosinophilic inflammation in response to respiratory syncytial virus infection [634]. In humans rhinovirus infection is associated with accumulation of these cells within the airway [635]. Consequently there is ongoing debate as to whether their presence is beneficial, incidental or detrimental.

7.1.1 (ii) Th1/Th2 balance and cytotoxic T cells in obesity

Previous studies suggest that obesity may impact on the circulating levels of CD4⁺ and CD8⁺ T cells, however findings have been contradictory. A paper looking at 34 obese Japanese adults and 50 obese controls showed a significant reduction in the absolute count of CD3⁺ T cells as well as CD3⁺CD4⁺ and CD3⁺CD8⁺ subsets in obese compared to normal weight healthy adults [579]. No difference in NK cell or CD19⁺ B lymphocyte percentages was seen. Conversely a retrospective study of 322 women enrolled as a control group in a HIV study found that being overweight, obese or morbidly obese was associated with increased total lymphocyte count and CD4⁺ T cell count, whilst being morbidly obese was associated with a higher CD8⁺ T cell count [636]. In terms of Th1 and Th2 subsets, obesity is associated with increased leptin levels and in murine models leptin has been shown to augment Th1 responses to allogeneic mononuclear cells [288]. This Th1 skewing is abolished in leptin receptor deficient mice suggesting that obesity may promote a Th1 bias through a leptin mediated mechanism.

To date, very little work has been done on Th1 and Th2 immunity in obese asthma. A paediatric study used intracellular flow cytometry to measure Th1 (IFN γ) and Th2 cell (IL-4) responses in peripheral blood, following stimulation with PMA and tetanus toxoid in 120 children: 60 asthmatics (30 obese and 30 non obese) and 60 controls (30 obese and 30 non-obese) [359]. Th1 responses were significantly higher in the obese asthmatics than non-obese asthmatics but did not differ from obese controls. The non-obese asthmatics had higher Th2 responses. The Th1/Th2 ratio correlated positively with serum leptin levels. Contrary to this an adult study by Sutherland and colleagues examined circulating levels of cytokines associated with Th1 (IFN- γ) and Th2 (IL-4, IL-5, IL-13) in obese and non obese premenopausal women with and without asthma and did not find levels of these cytokines to be significantly higher in obese asthmatics vs. normal weight asthmatics and the control group[25]. However, there are new CD4⁺ T cell subtypes which have been discovered and these might contribute to asthma but have not been examined in the obese asthma phenotype.

7.1.2 Regulatory T cells and T helper 17 cells

7.1.2 (i) Regulatory T cells

Regulatory T cells (Tregs), constitute 5-10% of circulating CD4⁺ T cells and are regarded as the principle mediators of immunological tolerance to foreign and self antigen [118]. Tregs were first identified in the mid 1990's when a population of CD4⁺ T cells which co-expressed CD25 (the alpha chain of the IL-2 receptor; IL-2R α) were shown to be important in promoting immunological self tolerance in mice [637].

Although Tregs have traditionally been identified by the presence of CD25, the majority of T lymphocytes will express this surface marker upon activation [120]. As such, early studies using this alone for Treg identification may have been misleading. Recently a more specific marker, the transcription factor forkhead box P3 (FoxP3) has been discovered [638]. This is thought to have a critical role in the development of naturally occurring Tregs; mice lacking a functional FoxP3 gene develop a fatal autoimmune condition characterised by CD4⁺ T cell hyper-responsiveness [121]. The alpha chain of the IL7 receptor (CD127) is also down regulated on Tregs, and has been used to increase the specificity of identification [122]. Tregs can develop in the thymus (natural (nTregs)) or can be induced in the periphery (iTregs) during the course of the immune response. The latter include Tr1 and Th3 cells. Tr1 lymphocytes are induced by IL-10 and can be identified by their high expression of this cytokine [123], however unlike their naturally occurring counterparts murine models suggest they do not express FoxP3 [124]. Th3 cells are another antigen specific subset of CD4⁺ T lymphocytes, induced by TGF- β . They also produce this cytokine in abundance and like nTregs these iTregs express FoxP3 [125].

Tregs exert their effects via contact dependent and independent mechanisms (Figure 7.1). Immunoregulatory cytokines produced by these cells include IL-10, a suppressor of effector T cell responses [126] and an inducer of peripheral Treg cell development [127]. In asthma and allergy IL-

10 is thought to maintain immune homeostasis at environmental interfaces including the lung [191]. TGF- β is also produced by Tregs but takes a more complex role as it has both anti-inflammatory and pro-fibrotic actions [192] with mice deficient in TGF- β having enhanced airway inflammation compared to wild-types [193]. Expression of high levels of the IL-2R α (CD25), is a feature of Tregs and is required for T lymphocyte proliferation. Whilst Tregs do not produce IL-2, they do require it for survival [128] and it has been argued that Tregs partly mediate their effects by competing for IL-2, inhibiting the proliferation of effector T cells [129, 130]. Other mechanisms used by Tregs include: cytotoxicity of pro-inflammatory cells through the expression of granzyme and perforin [131]; expression of CTLA-4 which provides an inhibitory signal to co-stimulatory molecules CD80 and CD86 on dendritic cells, for example by up regulation of the enzyme indoleamine 2,3 dioxygenase (IDO), raising the threshold for dendritic cell activation of T cells [132, 133].

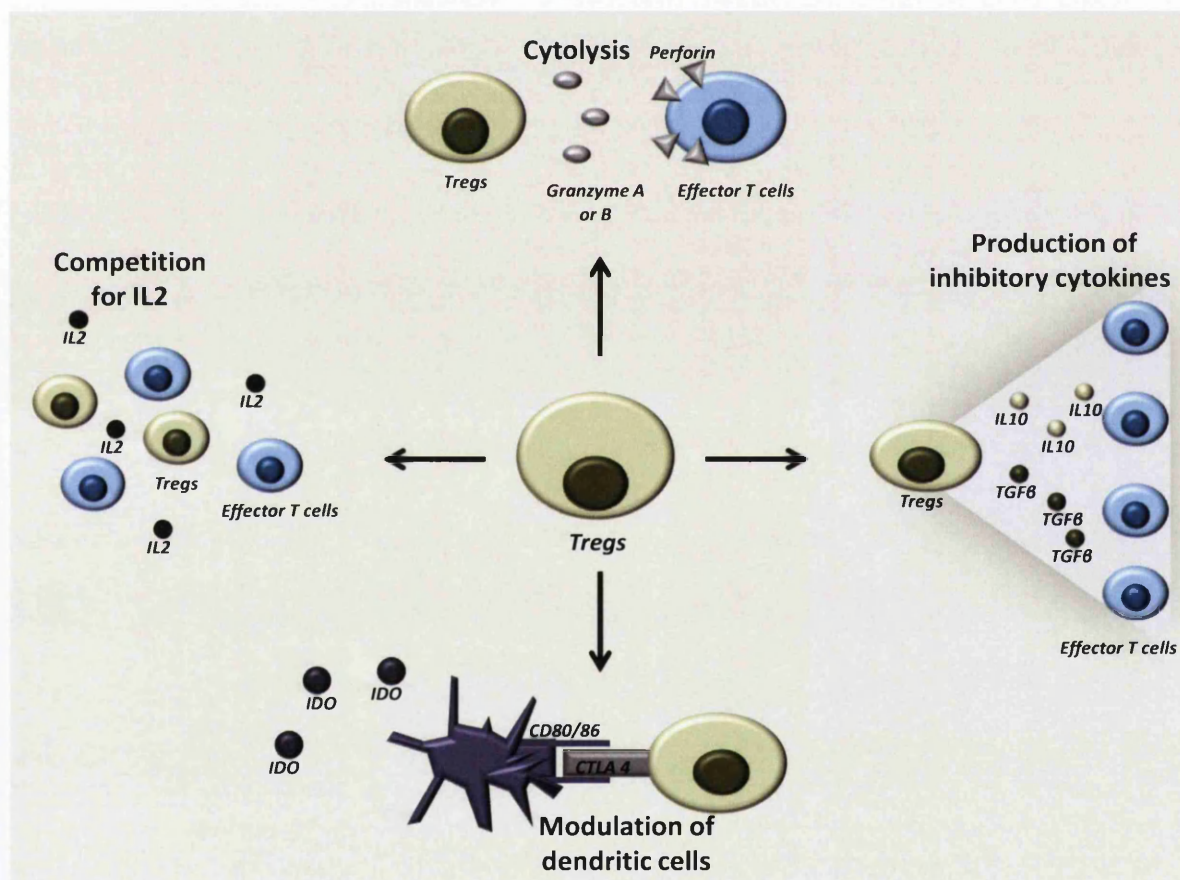


Figure 7.1: Mechanisms of regulatory T cell action.

1. Cytotoxicity of effector T cells via granzyme and perforin mediated mechanisms.
2. Production of inhibitory cytokines.
3. Targeting dendritic cells. Down-regulation of CD80/86 via cytotoxic T-lymphocyte antigen-4 (CTLA-4). CTLA-4 induced production of indoleamine 2,3-dioxygenase (IDO), an immunosuppressive enzyme expressed by dendritic cells.
4. Competition with effector T cells for IL-2 using alpha chain (CD25) receptors.

Although the existence of IL-17 as product of CD4⁺ T cells has been known since the mid 1990's [113], recognition of a distinct subset of Th17 cells responsible for its production has been far more recent [112]. As a major source of IL-17A (see section 5.1.1 (iii)), these cells are important mediators of neutrophilic inflammation [91] and are therefore thought to be important in the defence against extracellular pathogens [116]. They are identified by the expression of the transcription factor retinoic acid related-orphan receptor gamma T (ROR γ T) and the surface expression of the IL-23 receptor, the chemokine receptor CCR6 and the type II transmembrane glycoprotein CD161 [639].

7.1.2 (iii) Development of Tregs and Th17 cells

Treg induction *in vitro* can occur after exposure to IL-2 and TGF- β [640, 641]. Interestingly there appears to be a reciprocal relationship between Treg and Th17 induction. TGF- β is essential for both the induction of Tregs and Th17 cells via the induction of the transcription factor FoxP3, in the case of Tregs and ROR γ T in Th17 cells. In the absence of an inflammatory milieu FoxP3 suppresses the gene for ROR γ T, retinoic acid related orphan receptor C (RORC), promoting the formation of Tregs. However in the presence of pro-inflammatory cytokines including IL-6/IL-21, RORC is relieved from the suppressive effect of FoxP3 resulting in ROR γ T expression. The combination of TGF- β and IL-6/IL-21 also leads to the surface expression of IL-23R on Th17 cells, enabling IL-23 responsiveness. IL-23 subsequently synergises with IL-6 to promote Th17 differentiation, stabilisation, and function [117].

7.1.2 (iv) Regulatory T cells and Th17 cells in asthma

Animal models have shown an important role for Tregs in the development of airways diseases. In murine models, depletion of CD4⁺CD25⁺ Tregs promoted experimentally induced airway inflammation [642]. Furthermore the transfer of ovalbumin specific Tregs to sensitised mice prevents allergen induced airway hyper-responsiveness and inflammation [643]. The principle studies examining Tregs in asthmatic patients are summarised in Table 7.1.

Study	Population	Tissue	Treg findings	Ref
Children (mean age= 5.9-6.8 years)	Severe allergic rhinitis or asthma: n=51 Controls: n=47	Blood	↓ CD4+CD25+ T cells (%) in those with atopic disease vs. controls. ↑CD4+CD25+ T cells (%) in those with severe vs. mild disease. ↑FoxP3mRNA in those with severe vs. mild disease.	[644]
Children	Asthma: n=18 Chronic cough: n=10 Controls: n=13	Blood & BAL fluid	↓ CD4+CD25+ T cells (%) in both compartments (more marked in BAL fluid) in those with untreated asthma. ↓FoxP3 mRNA in both compartments (more marked in BAL fluid) in those with untreated asthma. 4 weeks of ICS treatment: ↑CD4+CD25 T cells (%) and ↑FoxP3 mRNA in both compartments. ↓ Treg function BALF in untreated asthma. Restoration of function with ICS treatment.	[194]
Children (mean age =11.2 years)	Atopic asthma: n=23 Controls: n=16	Blood	↓FoxP3 expression by CD4+CD25+ T cells in asthmatics vs. controls. ↓ Treg function in asthmatics vs. controls. Restoration of function with allergen specific immunotherapy.	[645]
Children (age = 6-13 years)	Controls: n=22 Asthmatics: n=38	Blood BAL fluid	↓CD4 ⁺ CD25 ⁺ FoxP3 ⁺ Tregs (%) in blood and BAL fluid of asthmatics vs. controls.	[195]
Children (age = 7-8 years)	Intermittent asthma: n=13 Mild persistent: n=15 Moderate to severe persistent n=20 Controls: n=93	Blood	↑CD4+CD25+CD127-FoxP3+ Tregs (%) in asthmatics vs. controls. ICS steroid use whether intermittent or chronic was more strongly associated with Tregs than asthma severity.	[646]
Adults	Asthmatics: n=52 Controls: n=20	Blood	↑CD4+CD25+ Tregs (%) in mild asthma vs. controls. ↓↓CD4+CD25+ Tregs (%) in severe asthma compared to mild asthma and controls.	[647]
Adults	Controls: n=6 Mild asthmatics: n=15 Moderate to severe: n=13	BAL fluid	↑CD4+CD25+CD127- Tregs (%) in moderate to severe asthma compared with mild asthma and controls. ↑CD4+FoxP3 Tregs (%) in moderate to severe asthma compared with mild asthma and controls.	[648]
Adults	Controls: n = 20 Mild asthma: n =22 Moderate to severe asthma: n = 17	Blood	↓ CD4 ⁺ CD25 ⁺ FoxP3 ⁺ Tregs (%) in moderate to severe asthmatics. FEV1 positively correlated with CD4 ⁺ CD25 ⁺ FoxP3 ⁺ T cells.	[197]
Adults	Asthmatics: n=71 Controls: n=15	Blood	↓Suppressive function of CD4 ⁺ CD25 ⁺ FoxP3 ⁺ Tregs in the remission group compared to controls.	[649]

Table 7.1: Summary of studies examining Tregs in the blood and BAL fluid of paediatric and adult asthmatics. Studies are in chronological order.

\AZStudies examining the number of Tregs in the peripheral blood of asthmatics have been contradictory. This is likely to be due to the use of CD25⁺ as the principle Treg marker, which is expressed on all effector T cell populations. Nevertheless, paediatric studies using this marker have shown reduced percentages of CD4⁺CD25⁺ Tregs in children with asthma and other atopic diseases [194, 644] compared to control subjects. In one of the studies examining levels in the blood and BAL fluid, the percentage of CD4⁺CD25⁺ T cells in the control group were significantly higher in the BAL fluid than peripheral blood [194]. Treated asthmatics had Treg levels in both compartments which were comparable to the control group. Compared to controls and treated asthmatics, untreated patients had a mild but significant reduction in CD4⁺CD25⁺ T cells in the blood and a marked reduction in the BAL fluid; these were restored with 4 weeks of ICS treatment. When they measured FoxP3 mRNA, the same observations were noted. Not only were the untreated asthmatics deficient in Tregs themselves but the cells present had impaired function and ICS treatment restored both Tregs number and function [194]. Another paediatric paper showed that whilst patients with atopic asthma and rhinitis had reduced blood levels of CD4⁺CD25⁺ T cells compared to control subjects, patients with severe disease had higher levels than those with mild [644]. In contrast a more recent study has shown increased levels of CD4⁺CD25⁺CD127⁺FoxP3⁺ Tregs in the blood of asthmatics. However the authors noted that ICS dose was more strongly associated with Tregs than disease severity suggesting that such discrepancies between studies could be due to the confounding effect of ICS treatment [646].

Studies in adults have also revealed impaired function of blood Tregs isolated in asthmatics [649]. Work suggests a reduction in blood CD4⁺CD25⁺ Tregs [647] and CD4⁺CD25⁺FoxP3⁺ Tregs [197] in patients with severe disease. However unlike the paediatric study a single adult paper found increased levels in the BAL fluid of patients with moderate to severe disease compared to mild asthmatics and controls [648]. It should be noted that unlike the mild asthmatics, patients with moderate to severe disease in this study were all on ICS treatment, which given the observations in the paediatric work (see above) may be responsible for the observation seen.

Th17 cells may be important in neutrophilic asthma with transfer of OVA specific Th17 cells to mice with severe combined immunodeficiency promoting a neutrophilic phenotype which is steroid resistant [353]. This is supported by studies which have shown evidence of enhanced Th17 response systemically and locally within the airways of asthmatics. Serum IL-17 levels are elevated in asthma and correlate with disease severity [354]. Sputum levels have also been shown to be increased in asthmatics compared to controls [650] and IL-17 levels in asthmatic patients correlate with BHR [506].

7.1.2 (v) Regulatory T cells and Th17 cells in obesity

Obesity has been linked with the autoimmune conditions characterised by impaired Treg responses [22, 344]. Leptin deficient mice have higher numbers of circulating Tregs and enhanced FoxP3 expression with improved function of Tregs [296]; leptin receptor deficiency produces a similar profile [346]. Leptin neutralisation with antibody also results in enhanced Treg proliferation in an IL-2 dependent fashion [345]. Furthermore it has been shown that IL-6, which is significantly increased in obesity and asthma, inhibits the suppressive effect of Tregs [348] and promotes Th17 over Treg

differentiation [117]. Regulatory T cells play a role in other obesity related pathologies including non-alcoholic steatohepatitis and high fat feeding of mice has been associated with increased risk of endotoxin induced liver injury and a reduced number of Tregs [350]. This has been suggested by one group to be related to increased oxidative stress, whereas others have highlighted increased leptin levels as a potential mechanism [651].

Normal adipose tissue can be a site for accumulation of Tregs and the percentage CD4⁺CD25⁺FoxP3⁺ cells are reduced in lean vs. obese mice [34, 333]. In humans, obesity is associated with a depletion in Treg numbers within visceral adipose tissue, with a corresponding increase in pro-inflammatory macrophages and Th1 bias [34, 333]. High levels of leptin associated with obesity may be mediating these effects [349]. Obesity is characterised by leptin resistance and it may be argued that Tregs would also be resistant to its effects. However murine models have shown that obesity related leptin resistance may be selective. For example there might be leptin resistance for eating behaviour but no resistance for renal sympathetic activity [347]. There has been little work on circulating Treg levels in obesity but it would be of interest to determine whether these mirror the changes seen in fatty tissue. If obesity does result in reduced numbers of Tregs this would be of relevance to asthma and could explain the obesity-asthma association. This area has not been explored to date.

Murine models indicate that obesity results in a Th17 bias. In diet induced obese mice there were significantly higher levels of Th17 cells than in lean animals; obese IL-6 null mice did not develop this expansion of Th17 cells suggesting an IL-6 dependent process [351]. In obese women, circulating IL-17 levels have been shown to be elevated compared to normal weight women [352] so Th17 bias seen in obese animal models might extend to humans.

7.1.2 (vi) Tregs/Th17 cells: the new paradigm?

It is conceivable that there may be imbalance in the differentiation of these two cell types in severe asthma including in obese patients. A Th17/Treg imbalance in asthma is supported by a paediatric study of Th17 and Tregs in child asthmatics (on ICS) vs. controls. In both BAL fluid and peripheral blood of the asthmatics there was a significant reduction in circulating Tregs but an increase in Th17 cells [195]. This finding is supported by a Chinese adult study of patients with moderate to severe asthma versus mild asthma and healthy controls. Increased blood levels of Th17 and plasma IL-17 were found in the moderate to severe asthmatics compared with the other groups and these same patients also had a reduction in CD4⁺CD25⁺FoxP3⁺ Tregs associated with a reduction in plasma IL-10, suggesting a Th17/Treg imbalance [197]. To date there has been no published literature looking at whether such an imbalance may exist in obesity and asthma.

7.1.3 Th9 cells

Even more recently a subset of IL-9 producing CD4⁺T cells, Th9 cells, has been described [134]. These differentiate in response to TGF- β and IL-4, which firstly cause up regulation of the transcription factors PU-1 and STAT6, respectively, and then GATA3 and IRF4; expression of FoxP3 is suppressed

[135]. Through IL-9 production, enhanced by IL-25 exposure [136], Th9 cells stimulate proliferation of mast cells and may have a role in autoimmune and allergic diseases [137]. Their role in asthma remains to be clarified but PU-1 deficient mice exposed to allergen have reduced airway inflammation [652]. There has been little work looking at IL-9 producing cells in the context of obesity or obesity and asthma.

7.1.4 T cell subsets

7.1.4 (i) Naive, memory and effector subsets

The fundamental immunological benefit of the adaptive immune system is to provide a specific response to antigens, which whilst initially slow, confers long lived immunological memory. Antigen inexperienced (naïve) versus antigen experienced (memory) CD4⁺ T helper cells can be identified by expression of various surface markers using flow cytometry (Figure 7.2) [653]. T cells leaving the thymus, referred to as naive T cells, express the long splice variant of CD45 (CD45RA) and the chemokine receptor 7 (CCR7). Naive T cells produce large amounts of IL-2, a cytokine that encourages T cell survival and growth [653]. After encountering a specific antigen these cells undergo proliferation and differentiation, losing CD45RA surface expression. Depending on the strength of stimulation these cells acquire the ability to respond to homeostatic molecules, anti-apoptotic molecules, homing signals and to perform effector functions [654].

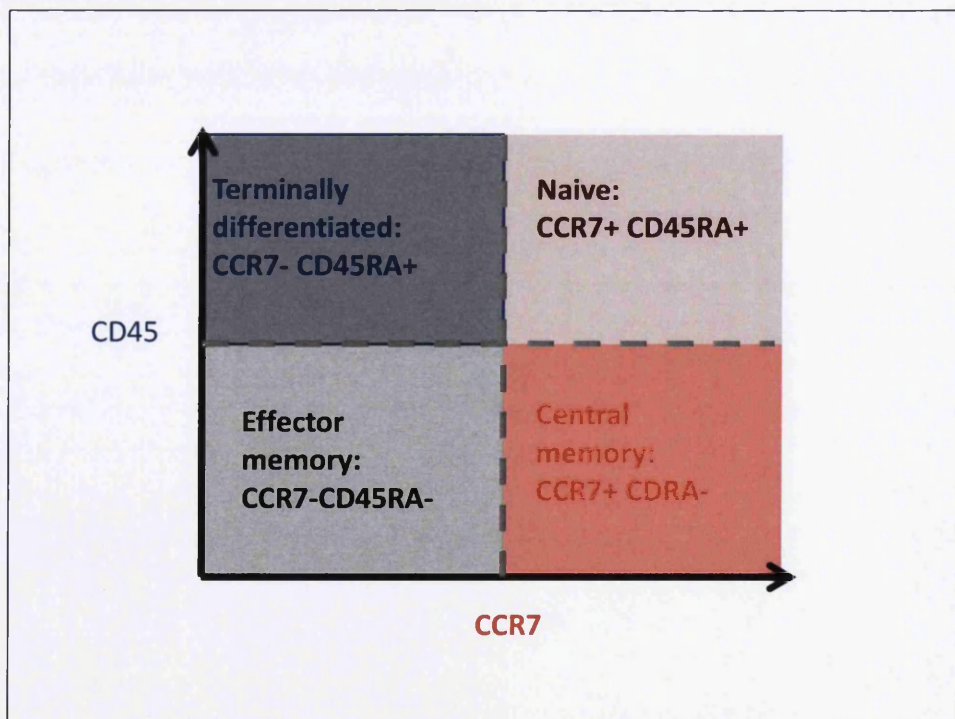


Figure 7.2: Classification of T helper (CD4⁺) subsets according to surface expression of CCR7 & CD45RA. With the use of these two surface markers, naive, central memory, effect memory and terminally differentiated cells can be quantified.

Cells that develop into terminally differentiated effector cells (CCR7⁺CD45RA⁺) perform effector functions, producing cytokines such as IFN γ in the case of Th1 responses, to immediately clear antigen and then die. Those which are less well differentiated exist as central memory (CM; CCR7⁺CD45RA⁻) and effector memory (EM; CCR7⁻CD45RA⁺) cells. Ongoing expression of CCR7 (CD45RA⁻ CCR7⁺) enables CM cells to migrate in response to chemokines. CM cells also retain the ability to produce IL-2, enabling rapid proliferation. They are important in secondary immune responses and confer long term protection but their effector function is limited so on exposure to a second insult they will not provide immediate clearance. Upon a second encounter with their specific antigen, CM cells differentiate into EM cells. EM cells (CD45RA⁺CCR7⁻) lose their homing ability and produce much less IL-2, lacking the proliferative capacity of CM cells. Their ability to rapidly produce effector cytokines enables them to confer immediate protection on second encounter to a specific antigen which is short lived as they terminally differentiate [654].

A large pool of naive T cells with a diverse T cell receptor (TCR) repertoire is paramount to conferring protection against novel antigens. Throughout one's lifetime, continual exposure to different antigens and the generation of memory subsets erodes this pool. Additionally age related thymic involution occurs and so this diminished pool of naive T cells is not replenished [655]. Ageing is hence associated with a reduction in naive CD4⁺ and CD8⁺ T cells and therefore a reduced TCR repertoire with an impaired ability to respond to new antigen. Ageing is also associated with an increase in terminally differentiated and a slight increase in CM cells in the CD4 compartment but and does not appear to affect the proportion of EM cells in either compartment [655].

7.1.4 (ii) Asthma, obesity and T cell subsets

In asthma, the continual stimulation from environmental allergens might sustain effector populations and deplete naive cells. A single study has suggested that asthma may also be associated with a reduction in naive T cells as seen in the ageing process [656]. Obesity may also be associated with changes in T cell subsets typically associated with ageing. Diet induced obesity in a murine model has been associated with a reduction in lymphoid progenitors and accelerated thymic involution [657]. This resulted in a reduction in thymic output and T cell receptor repertoire. The same paper showed that obesity in humans was also associated with a reduction in thymic output.

Given that obesity and asthma both seem to have some association with changes in the T cell population reminiscent of ageing, it would be of particular interest to see whether obesity in asthma accelerates these. Such a finding would suggest that obese asthmatics may be vulnerable to infection and therefore exacerbations of their disease or may have an overactive effector component of their T cell compartment. To date this area has not been explored in the context of obesity and asthma.

7.2 Methods

7.2.1 Identification of lymphocyte subsets, CD4⁺ T cell subsets and regulatory T cells by flow cytometry

Lymphocyte subsets, CD4⁺ T cell subsets and Tregs were identified using flow cytometry. The principles and methods of this procedure are detailed in section 3.11. The identification and quantification of basic lymphocyte subsets, namely CD3⁺ T lymphocytes, CD3⁺CD4⁺ T lymphocytes, CD3⁺CD8⁺ cytotoxic T cells, CD16⁺CD56⁺ NK cells and CD19⁺ B lymphocytes was performed using antibody-fluorochrome conjugates against their respective surface markers. Antibodies against CCR7 and CD45RA were used to measure the major CD4 subsets (naive, CM, EM and terminally differentiated cells). Tregs were identified using surface staining against CD4, CD25 and CD127 as well as intracellular staining against FoxP3. The antibody-fluorochrome conjugates used are listed in Table 7.2

Antigen	Fluorochrome	Cell expression	Clone	Source
CD3	e450	T lymphocytes	UCHT1	eBioscience, UK
CD4	e450	CD4 positive T lymphocytes (Th1, Th2, Th17, Th9 Tregs)	OKT4	eBioscience, UK
	FITC		S3.5	Life Technologies(Caltag), UK
	Alexa Fluor 488		OKT4	eBioscience, UK
CD8	PE	Cytotoxic T cells	OKT8	eBioscience, UK
CD16	APC	High expression NK cells and activated monocytes	CB16	eBioscience, UK
	FITC		3G8	BD Biosciences (Pharmingen), UK
CD19	Alexa Fluor 700	B lymphocytes	HIB19	eBioscience, UK
CD25	APC	Tregs, activated T lymphocytes	BC96	eBioscience
CD28	PE		10F3	Life Technologies(Caltag), UK
CD45RA	FITC	High expression on naive T cells and Terminally differentiated T cells	H1100	eBioscience, UK
CD56	APC	High expression on NK cells	MEM188	eBioscience, UK
CD127	PerCP-Cy55	Low expression in Tregs	RDR5	eBioscience, UK
CD197 (CCR7)	APC	High expression on central and effector memory T cells.	3D12	eBioscience, UK
FOXP3	PE	Tregs	PCH101	eBioscience, UK
Mouse IgG1 Isotype control	PercP-Cy5.5		P3.6.2.8 .1	eBioscience, UK
Mouse IgG2a Isotype control	PE		20102	R&D Systems
Mouse IgG2b Isotype control	FITC		eBMG2b	eBioscience, UK
Rat IgG2a Isotype Control	APC		eBR2a	eBioscience, UK
Rat IgG2a Isotype control	PE		eBR2a	eBioscience, UK

Table 7.2: List of the monoclonal antibodies and fluorochromes used in lymphocyte subsets, T cell subsets and Tregs identification. The commercially available antibodies, respective fluorochromes and sources are listed.

7.2.1 (i) Surface staining

Predetermined volumes of antibodies were added to 100µl of heparinised blood on the day of collection. Isotype controls were also used to control for non-specific antibody binding. The antibodies used to identify the respective cell types are listed in Table 6.3.

Following addition of antibodies to each of the respective tubes, the samples were incubated on ice for 30 minutes. Red blood cell lysis solution (3ml) was then added (FACS lysing solution, BD Biosciences) before incubation in the dark at room temperature for 10 minutes. Cells were collected by centrifugation (4°C, 515 x g for 7 minutes) and the supernatant discarded before washing in 3ml of FACS buffer (PBS with 0.2% BSA and 0.05% sodium azide) by repeat centrifugation. The supernatant was removed and the cells fixed using 200µl FACS fix (BD Biosciences), except for those tubes used for the identification of Tregs which required permeabilisation and intracellular staining to FoxP3.

Experiment	Tube number	Antibody added
Lymphocyte subsets	1	No antibody
	2	CD3 eFluor 450, CD4 FITC, CD8PE, CD16 APC, CD56 APC, CD19 AlexaFluor 700
CD4 subsets	1	No antibody
	2	CD4 eFluor 450.
	3	CD4 eFluor 450, mouse IgG2b FITC, rat IgG2a APC.
	4	CD4 eFluor 450, CD45RA FITC, CCR7 APC.
Regulatory T cells	1	No antibody
	2	CD4 AlexaFluor 488, rat IgG2a APC, mouse IgG1 PerCP-Cy5.5.
	3	CD4 AlexaFluor 488, CD25APC, CD127 PerCP-Cy5.5, rate IgG2a PE
	4	CD4 AlexaFluor 488, CD25APC, CD127 PerCP-Cy5.5, FoxP3 PE

Table 7.3: Flow cytometry panel used to identify and/or phenotype lymphocyte subsets, CD4+ T cell subsets and Tregs. A sample was also used with no antibody added to control for autofluorescence. Samples were also stained with isotype controls to control for non-specific binding.

7.2.1 (ii) Intracellular staining

To identify circulating Tregs, cells were surface stained for CD4, CD25 and CD127 by the methods described above and, to increase the specificity of Treg identification, were also stained with intracellular FoxP3. After surface staining, 1ml of freshly prepared fixation/permeabilisation buffer (eBiosciences) was added to relevant samples followed by incubation on ice for 1 hour. The fixed cells were collected by centrifugation (4°C, 515 x g for 7 minutes) and the supernatant discarded, the samples were then washed twice with 2mls of permeabilisation buffer (eBiosciences) by centrifugation. Anti-FoxP3 antibody and the respective isotype control were added to the

appropriate samples suspended in 100µl permeabilisation buffer for incubation at room temperature in the dark for 30 minutes. The samples were then washed twice with 2ml of permeabilisation buffer as before then fixed with 200µl FACS fix (BD Biosciences, USA). All samples were analysed within 24 hours of processing. The gating strategy used to quantify the percentage of basic lymphocyte subtypes, CD4⁺ T cell subsets and regulatory T cells are shown in Figures 7.3, 7.4 and 7.5 respectively.

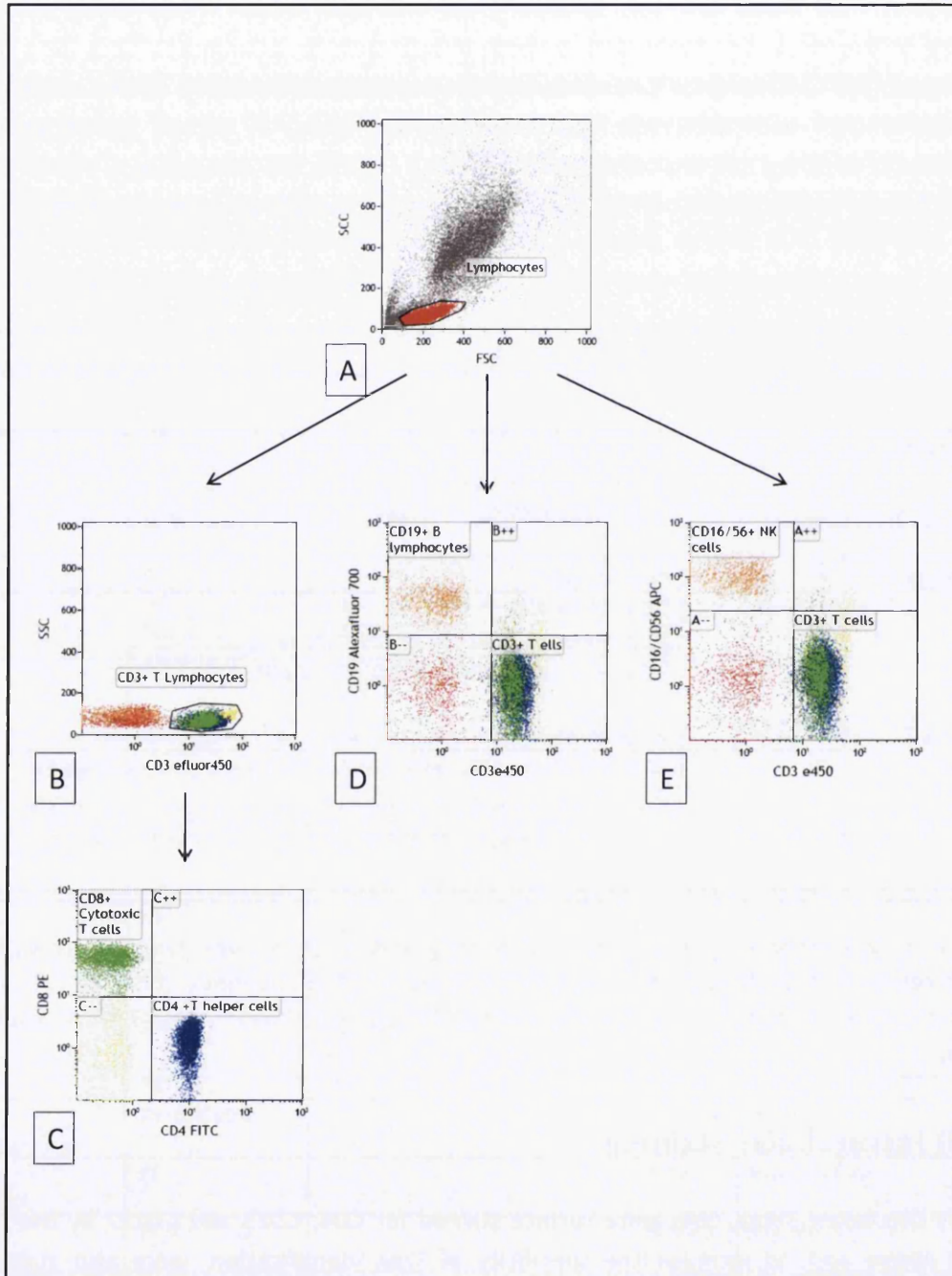


Figure 7.3: Gating strategy to identify lymphocyte subsets.

A: Using forward scatter (FSC) and side scatter (SSC), lymphocyte population identified.

B: Gating on lymphocytes, CD3⁺ T lymphocytes identified.

C: Gating on CD3⁺ T lymphocytes, CD4⁺ and CD8⁺ populations identified.

D: Gating on lymphocytes, CD19⁺ B lymphocytes identified.

E: Gating on lymphocytes CD16⁺CD56⁺ positive NK cells identified.

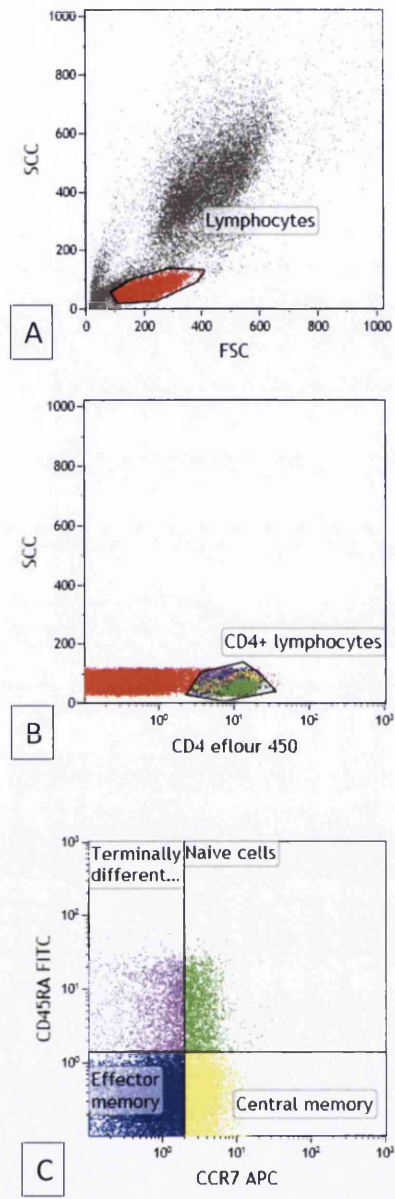


Figure 7.4: Gating strategy to identify CD4⁺ T cell subsets.

A: Using forward scatter (FSC) and side scatter (SCC), lymphocyte population identified.

B: Gating on lymphocytes, CD4⁺ lymphocytes identified.

C: Gating on CD4⁺ lymphocytes, CCR7⁺ CD45RA⁺ (naive), CCR7⁺ CD45RA⁻ (central memory), CCR7⁻ CD45RA⁻ (effector memory) and CCR7⁻ CD45RA⁺ (terminally differentiated) cells identified.

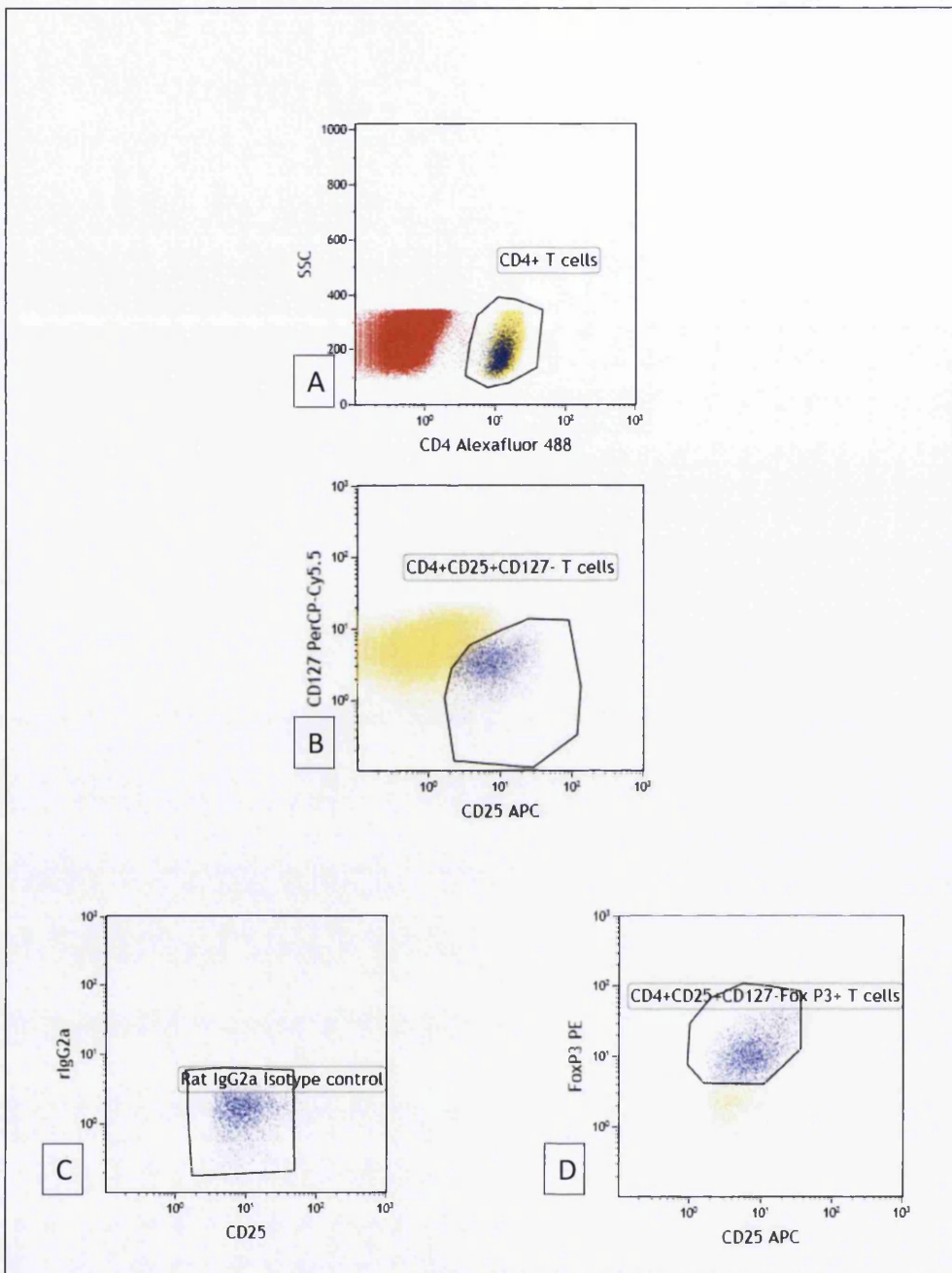


Figure 7.5: Gating strategy to identify regulatory T cells.

A: CD4⁺ lymphocytes identified.

B: Gating on CD4⁺ lymphocytes, population of CD25⁺CD127⁻ lymphocytes identified.

C: Gating on CD4⁺CD25⁺CD127⁻ lymphocytes, using isotype control gate placement determined for FoxP3⁺ cells.

D: Gating on CD4⁺CD25⁺CD127⁻ lymphocytes, CD4⁺CD25⁺CD127⁻FoxP3⁺ cell identified.

7.2.2 Whole blood phytohaemagglutinin stimulated cytokine responses

All culture work was undertaken in a class II tissue culture cabinet. The process of whole blood culture is discussed in more detail in section 2.9. The media used for culture preparation was RPMI 1640+ glutamax (Life Technologies, UK), supplemented with 50mM of 2-mercaptoethanol (ME; Life Technologies, UK). Culture media (600µl) was added to 4 tubes (2 tests in duplicate) and mixed gently with blood (200µl). Phytohaemagglutinin-L (PHA 5µg/ml; Sigma) is a lymphocyte mitogen [426] and was added to two of the 4 tubes prior to incubation of all tubes at 37°C in 5% CO₂-in-air for 48 hours. The other two tubes were left unstimulated to control for background levels of the cytokines. After incubation the tubes were centrifuged for 7 minutes at 4°C, 515 x g and cell free supernatants were removed for storage at -20°C until subsequent cytokine analysis.

The cytokines measured in response to PHA stimulation, the respective kits used, and their sensitivities are summarised in Table 7.4.

Cytokine	Principle lymphocyte source	Sensitivity (pg/ml)	Kit source
IFNγ	Th1	7.813	Ready-SET-Go, eBioscience, UK
IL-13	Th2	7.813	Ready-SET-Go, eBioscience, UK
IL-9	Th9	1.563	Ready-SET-Go, eBioscience, UK
IL-10	Tregs	7.813	Opt EIA, BD Biosciences, USA
IL-17A	Th17	7.813	Ready-SET-Go, eBioscience, UK

Table 7.4: Cytokines measured in response to PHA stimulation. Cytokines representing each T helper subset were assayed. The commercially available kits used and their respective sensitivities are shown.

7.3 Results

7.3.1 Lymphocyte subsets

The percentage of circulating major lymphocyte subsets was measured in 79 individuals (41 controls and 38 asthmatics) and the results are summarised in Table 7.5. The percentage of CD16⁺CD56⁺NK cells did not pass the KS test ($p=0.002$) and therefore data were logarithmically transformed before analysis.

There was no significant difference in the percentage of CD3⁺ T lymphocytes (expressed as a percentage of total lymphocytes) across the 6 groups (Table 7.5). Across the entire study population levels did not differ significantly with BMI category ($p=0.4456$) or when BMI was examined as a continuous variable ($r=-0.44$, $p=0.703$) and no correlation was seen with WHR or percentage body fat. Levels did not correlate with participant age. When asthma and controls were analysed separately no association was seen between CD3⁺ T lymphocytes (%) and BMI category, continuous BMI, percentage body fat, or WHR in either group. Levels were not higher in asthmatics versus controls across the entire study group ($p=0.812$) or when each BMI category was compared individually. Within the asthmatics there were no correlations between this variable and age of onset, asthma duration, control, ICS use or any of the spirometric markers.

CD4⁺ T lymphocytes and CD8⁺ T lymphocytes expressed as a percentage of CD3⁺ T lymphocytes did not differ significantly across the 6 groups or across BMI categories ($p=0.283$ and $p=0.368$ respectively) within the entire cohort (Table 7.5). When BMI was examined as a continuous variable no association was seen with either parameter ($r=0.030$, $p=0.794$ and $r=0.56$, $p=0.625$ respectively) and no correlations observed with WHR or percentage body fat. Similarly no associations were seen with CD4:CD8 ratio. When asthmatics and controls were analysed separately no correlations with either CD4⁺ (%) or CD8⁺ (%) T cells and any markers of adiposity were seen. Percentages of either cell type were not significantly different in asthmatics versus controls across the entire study group (CD4⁺ T cells: $p=0.275$, CD8⁺ T cells: $p=0.197$) or when each BMI category was compared individually. Within the asthmatics neither cell type correlated with age of onset, asthma duration, asthma control, ICS use or any of the spirometric measures.

There were no significant differences in the percentage of CD16⁺CD56⁺ NK cells across the 6 groups (Table 7.5) or across the 3 BMI categories ($p=0.118$). When BMI was examined as a continuous variable, no correlation was seen with NK cells ($r=0.52$, $p=0.652$) across the whole study group or when asthmatics and control were examined separately. Similarly no correlations were seen between CD16⁺CD56⁺ NK cells and WHR or body fat across the entire study group or in the asthmatics and controls when analysed separately. Levels were not higher in asthmatics versus controls ($p=0.684$) across the entire group or when each BMI category was compared individually. Within the asthmatics the percentage of CD16⁺CD56⁺NK cells was not associated with age of onset, asthma duration, asthma control, ICS use or spirometric measures taken.

The percentage of B lymphocytes (expressed as a percentage of total lymphocytes) significantly differed across the 6 groups (Table 7.5). B lymphocytes were significantly higher in asthmatics than controls ($p=0.005$). When the individual BMI categories were compared normal weight (NW)

asthmatics (mean =11.77%) had significantly higher levels than NW weight controls (mean=8.89%) ($p=0.029$), although there was a trend towards higher levels in the obese asthmatics (OB) (mean=11.31%) than OB controls (mean=9.77%), this was not significant ($p=0.162$). No association with BMI category was seen across the entire cohort ($p=0.722$), continuous BMI ($r=0.155$, $p=0.179$), percentage body fat or WHR. Across the entire cohort, age did not correlate with this variable. Within the asthmatics there was no association between B lymphocytes (%) and BMI category ($p=0.720$), BMI as a continuous variable ($r=0.155$, $p=0.179$), WHR or percentage body fat. Similarly in the controls no adiposity markers were associated with this variable. Furthermore there was no association between B lymphocytes and age of onset, disease duration, asthma control score, ICS use or any of the spirometric measures.

	Controls			Asthmatics			p value
	NW n=15	OW n=11	OB n=15	NW n=13	OW n=11	OB n=14	
CD3⁺ T lymphocytes (%) Mean (SD)	71.11 (5.58)	68.66 (6.26)	69.91 (11.28)	69.95 (6.63)	74.48 (6.12)	67.71 (7.71)	All 6 groups: $p=0.381$ BMI category: $p=0.456$ A vs. C: $p=0.812$
CD4⁺ T lymphocytes (%) Mean (SD)	64.40 (7.08)	60.95 (8.54)	63.50 (9.11)	60.68 (8.08)	58.39 (9.63)	63.35 (9.63)	All 6 groups: $p=0.515$ BMI category: $p=0.283$ A vs. C: $p=0.275$
CD8⁺ T lymphocytes (%) Mean (SD)	26.13 (5.31)	30.44 (8.49)	29.09 (10.3)	30.27 (6.39)	32.27 (8.16)	29.98 (9.42)	All 6 groups: $p=0.534$ BMI category: $p=0.368$ A vs. C: $p=0.197$
CD4:CD8 ratio Mean (SD)	2.58 (0.66)	2.26 (1.15)	2.49 (1.03)	2.12 (0.64)	1.95 (0.68)	2.39 (0.97)	All 6 groups $p=0.477$ BMI category: $p=0.389$ A vs. C: $p=0.139$
CD16⁺CD56⁺ NK cells (%) Geometric mean (SD)	11.00 (3.74)	10.41 (3.20)	12.12 (10.6)	11.17 (5.34)	8.65 (3.45)	11.21 (7.57)	All 6 groups: $p=0.448$ BMI category: $p=0.118$ A vs. C: $p=0.684$
CD19⁺ B lymphocytes (%) Mean (SD)	8.89 (2.58)	9.22 (2.36)	9.77 (2.45)	11.77 (3.37)	10.61 (3.61)	11.31 (3.21)	All 6 groups $p=0.092$ BMI category: $p=0.722$ A vs. C: $p=0.005$

Table 7.5: Percentage of circulating lymphocyte subsets in asthmatics and controls according to BMI category.

Normally distributed data are expressed as mean and standard deviation (SD). CD16⁺CD56⁺ NK cells (%) data were positively skewed and therefore expressed as a geometric mean and standard deviation and were logarithmically transformed before analysis. Differences between all 6 groups were analysed using a one-way ANOVA. Differences between asthmatics (A) and controls (C) were analysed using an unpaired t-test. Differences between BMI categories were analysed using a one-way ANOVA. CD3⁺ T cells, CD16⁺CD56⁺ NK cells and CD19⁺ B cells are expressed as a percentage of total lymphocytes. CD4⁺ and CD8⁺ cells were expressed as a percentage CD3⁺ T cells.

Given the importance of B lymphocyte production of IgE and associations reported between B lymphocyte and eosinophils, correlations between B lymphocyte (%) and these variables was examined for but none significant were seen were seen across the entire cohort (IgE: $r=0.174$, $p=0.130$; eosinophils: $r=0.103$, $p=0.371$). Similarly no correlations were observed when asthmatics and controls were analysed separately.

7.3.2 Regulatory T cells

Data regarding the percentages of $CD4^+CD25^+CD127^-$ T cells and $CD4^+CD25^+CD127^-FoxP3^+$ T cells were collected on 79 patients (40 controls, 39 asthmatics). Using flow cytometry, cells were gated as shown in Figure 7.5 to give the percentage of $CD4^+$ T cells which were $CD4^+CD25^+CD127^-$ and $CD4^+CD25^+CD127^-FoxP3^+$. Both parameters passed the KS test ($p=0.675$ and $p=0.950$ respectively) and the data are shown in Table 7.6.

With regards to $CD4^+CD25^+CD127^-$ T cells (expressed as a percentage of $CD4^+$ cells), there was no significant difference in percentages across the 6 groups ($p=0.063$). There was an apparent reduction in the percentage of $CD4^+CD25^+CD127^-$ T cells in the asthmatics (mean =5.89%) versus controls (mean= 6.36%) ($p=0.040$). Over the entire cohort, increase in BMI category from NW to OW and obese was associated with a reduction in $CD4^+CD25^+CD127^-$ T cells, although this was not significant (NW mean=6.55%, OW mean=5.86, OB mean=5.90% $p=0.068$). The overweight and obese individuals had significantly lower levels of $CD4^+CD25^+CD127^-$ T cells than the normal weight group ($p=0.034$ and $p=0.032$, respectively). When BMI was analysed as a continuous variable there was a trend towards a reduction in $CD4^+CD25^+CD127^-$ T cells with increasing BMI but this was not significant ($r=-0.181$, $p=0.110$). Although percentage body fat was negatively correlated with $CD4^+CD25^+CD127^-$ T cells across the entire group this was not significant ($r=-0.126$, $p=0.267$). No correlation was seen with WHR. Across the entire population age did not correlate with this parameter.

The trend towards a negative correlation between $CD4^+CD25^+CD127^-$ T cells and BMI was more apparent in asthmatics ($r=-0.247$, $p=0.130$) than controls ($r=-0.520$, $p=0.748$) although neither was significant. A similar trend was seen with percentage body fat in the asthmatics only but not WHR. Obese asthmatics had the lowest level of $CD4^+CD25^+CD127^-$ T cells, which was lower than obese controls ($p=0.059$) and normal weight asthmatics ($p=0.084$) although this did not reach statistical significance. Across the cohort, age was not associated with $CD4^+CD25^+CD127^-$ T cells. Within the asthmatic group $CD4^+CD25^+CD127^-$ T percentage did not correlate with age of onset, disease duration, asthma control, ICS use or any of the spirometric measures.

In terms of the percentage of circulating $CD4^+CD25^+CD127^-FoxP3^+$ T cells there was no significant difference across the 6 groups ($p=0.203$) (Table 7.6). Levels decreased with increasing BMI category across the entire cohort, however this was not statistically significant ($p=0.087$). Obese participants across the whole cohort had the lowest percentage of $CD4^+CD25^+CD127^-FoxP3^+$ T cells (Figure 7.6), which was significantly lower than normal weight individuals (NW=5.29%, OW= 4.78%, OB=4.73%), ($p=0.038$). Even being overweight appeared to be associated with a reduction in this cell type (Figure 7.6), however this was not significant ($p=0.113$). When BMI was examined as a continuous variable it

was negatively associated with CD4⁺CD25⁺CD127⁻ FoxP3⁺ T cells but this was not significant (r= -0.183 p=0.107). Percentage body fat also negatively correlated with CD4⁺CD25⁺CD127⁻ FoxP3⁺ T cells but this was also not significant (r=-0.126, p=0.267). Across the entire cohort age did not correlate with CD4⁺CD25⁺CD127⁻ FoxP3⁺ T cells.

When asthmatics and controls were analysed separately, a non-significant correlation between CD4⁺CD25⁺CD127⁻ FoxP3⁺ T cells and BMI was only seen in the asthmatics (r=-0.227, p=0.165) and not the controls (r=-0.105, p=0.553). Similar trends were seen with percentage body fat but not WHR. Asthmatics had lower levels of Tregs than controls (mean=4.785% versus mean=5.114%) however this was not significant (p=0.161). Similarly when each BMI category was compared individually no significant differences were seen between asthmatics and controls. Obese asthmatics had the lowest levels of circulating CD4⁺CD25⁺CD127⁻ FoxP3⁺ T cells of all the six groups but this was not significantly lower than obese controls (p=0.094) or normal weight asthmatics (p=0.124). Within the asthmatics, age of onset asthma duration, control, ICS, and spirometric measures did not correlate with CD4⁺CD25⁺CD127⁻ FoxP3⁺ T cells.

As leptin and IL-6 can negatively modulate Tregs, association between CD4⁺CD25⁺CD127⁻ FoxP3⁺ Tregs and these parameters was explored across the entire cohort. No significant correlation was seen with leptin (r=-0.038 p=0.305) or IL-6 (r= -0.109 p=0.399) and no significant correlation was seen when asthmatics and controls were analysed separately. In chapter 6, plasmacytoid dendritic cells (pDCs) were noted to negatively correlate with BMI and in their resting state can promote differentiation of Tregs, however no correlation was seen between pDCs (%) and CD25⁺CD127⁻ FoxP3⁺ T cells (r=0.038, p=0.756). Furthermore as there has been recent interest in the role of these cells in insulin resistance, correlations with fasting glucose and insulin resistance were considered, however none were seen (r=-0.007, p=0.953 and r=-0.56, p=0.626 respectively).

	Controls			Asthmatics			p value
	NW n=15	OW n=12	OB n=13	NW n=14	OW n=12	OB n=13	
CD4⁺CD25⁺CD127⁻ lymphocytes Mean (SD)	6.77 (0.91)	5.95 (0.74)	6.28 (1.04)	6.33 (1.38)	5.76 (1.51)	5.54 (0.87)	All 6 groups: p=0.063 BMI category: p=0.040 A vs. C: p=0.068
CD4⁺CD25⁺CD127⁻ FoxP3⁺ lymphocytes (%) Mean (SD)	5.48 (1.06)	4.82 (0.84)	4.97 (0.91)	5.09 (1.31)	4.74 (1.33)	4.49 (0.35)	All 6 groups: p=0.203 BMI category: p=0.087 A vs. C: p=0.161

Table 7.6: Percentage of circulating CD4⁺CD25⁺CD127⁻ and CD4⁺CD25⁺CD127⁻ FoxP3⁺ Tregs across the 6 study groups. Normally distributed data are expressed as mean and standard deviation (SD). Differences between all 6 groups and BMI categories were analysed using a one-way ANOVA. Differences between asthmatics (A) and controls (C) were analysed using an unpaired t-test. CD4⁺CD25⁺CD127⁻ and CD4⁺CD25⁺CD127⁻ FoxP3⁺ Tregs are expressed as percentage of CD4⁺ cells.

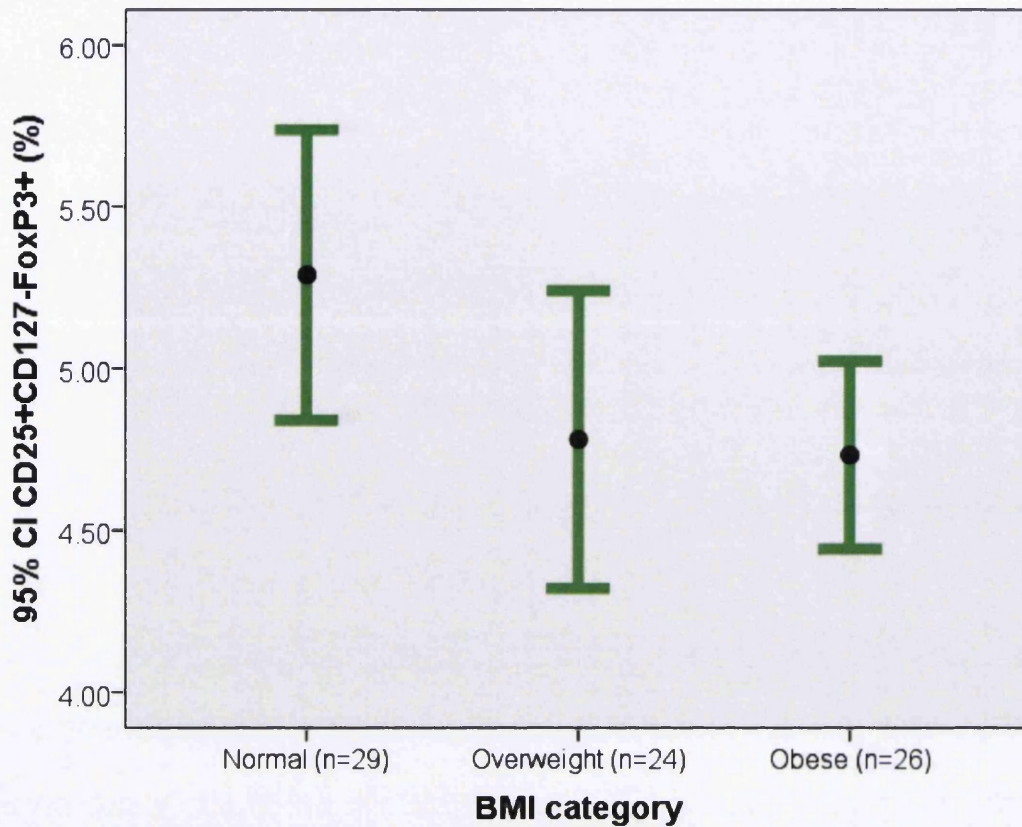


Figure 7.6: Percentage of circulating CD4⁺CD25⁺CD127⁻ FoxP3⁺ Tregs in the 3 BMI categories across the entire study group.

Data displayed as mean and error bars. Obese patients had the lowest levels of CD4⁺CD25⁺CD127⁻FoxP3⁺ Tregs which was significantly lower than normal weight individuals ($p=0.038$). CD4⁺CD25⁺CD127⁻FoxP3⁺ are expressed as a percentage of CD4⁺ cells.

7.3.3 CD4⁺ T cell differentiation

Data was collected on the percentage of circulating CD4⁺ T cells at different stages of differentiation in 77 subjects (38 controls and 39 asthmatics) and these are displayed in Table 7.7. Data on the percentage of circulating naive CD4⁺ T cells passed the KS test ($p=0.765$). Data for the percentage of central memory (CM), effector memory (EM), and terminally differentiated (TERMA) CD4⁺ T cells did not pass the KS test ($p=0.185$, $p=0.222$, and $p=0.154$), so was logarithmically transformed for analysis.

Circulating naive (CCR7⁺CD45RA⁺) CD4⁺ T cells expressed as a percentage of CD4⁺ cells significantly differed across the 6 groups (Table 7.7) with the percentage of these cells decreasing with increasing BMI category across the entire study population ($p=0.013$) (Figure 7.6). Obese participants had the lowest percentage of circulating naive CD4⁺ T cells (NW= 44.7%, OW 37.0%, obese 35.3%) and this was significantly lower than normal weight individuals ($p=0.007$). When BMI was analysed as a continuous variable this negative association was also significant ($r=-0.307$, $p=0.007$); negative correlations were also seen with percentage body fat ($r=-0.295$, $p=0.009$) but not WHR. Age correlated negatively with the percentage of circulating naive CD4⁺ T cells ($r= -0.355$, $p=0.002$), however the association between BMI and circulating naive CD4⁺ T cells persisted after retaining age

in a general linear model (BMI $p=0.025$, age $p=0.006$, R^2 value =0.183). When asthmatics and controls were analysed separately a negative association between BMI category and percentage of naive $CD4^+$ cells was more apparent in the asthmatics than the controls ($p=0.052$ and $p=0.062$ respectively). Similar observations were noted with other adiposity markers

Asthma status had no association with the percentage of circulating naive $CD4^+$ T cells across the entire cohort ($p=0.982$) or when each of the BMI categories was compared individually. The percentage of circulating naive $CD4^+$ T cells correlated negatively with asthma duration ($r=-0.380$, $p=0.017$) however this did not remain significant after adjusting for patient age (asthma duration $p=0.153$, age $p=0.153$, $R^2=0.192$). Asthma control score, medication use including ICS, and spirometric measures were not associated with the percentage of naive $CD4^+$ T cells.

Central memory (CM; $CCR7^+CD45RA^-$) $CD4^+$ T cells expressed as a percentage of $CD4^+$ T cells significantly varied across the 6 groups (see Table 7.7) ($p=0.022$). This was due to an apparent increase in this cell type with each BMI category across the entire cohort (Figure 7.6; NW = 19.5%, OW = 24.0%, Obese=24.8% ($p=0.006$)). Obese women had significantly higher levels than normal weight individuals ($p=0.004$). When BMI was examined as a continuous variable this positive association remained significant ($r= 0.379$, $p=0.001$). Percentage body fat but not WHR also correlated with this variable ($r=0.369$, $p=0.021$). Age was positively associated with the percentage of CM $CD4^+$ T cells ($r=0.275$, $p=0.015$), however the association between BMI and CM $CD4^+$ T cells remained significant after including this covariate in a general linear model (BMI $p=0.002$, age $p=0.057$, $R^2= 0.185$). When asthmatics and controls were analysed separately the positive association between this variable and BMI category was less convincing and only significant in the asthmatics ($p=0.012$, and $p=0.065$ respectively). Similar observations were made when examining the other adiposity markers,

Asthma status across the entire cohort was not associated with the percentage of CM $CD4^+$ T cells or when each individual BMI category was compared. Asthma duration, asthma control score, medication usage and spirometric measures were not associated with this variable.

Circulating effector memory (EM; $CD45RA^+CCR7^-$) cells expressed as a percentage of $CD4^+$ T cells, differed significantly across the 6 groups (Table 7.7). This was due to a positive association between this variable and BMI category across the entire study population (Figure 7.6; NW=22.8%, OW=27.3%, OB=28.9% ($p=0.055$)). Obese had significantly higher levels of this cell type than normal weight individuals ($p=0.025$). When BMI was analysed as a continuous variable there was also a significant positive association with EM $CD4^+$ T cells ($r=0.256$, $p=0.025$), as with percentage body fat ($r=0.342$, $p=0.033$) but not WHR. Age was positively correlated with this variable ($r=0.288$, $p=0.011$) and the association between BMI and the percentage of EM $CD4^+$ T cells became less significant after retaining patient age in the model (BMI $p=0.068$, age $p=0.031$, $R^2=0.124$).

When asthmatics and controls were analysed separately the positive association between BMI category and EM cells was less convincing and not statistically significant in either group ($p=0.094$ and $p=0.060$ respectively). Asthma status was not associated with the percentage of EM $CD4^+$ T cells across the entire population ($p=0.554$) or when each BMI category was compared individually. Within the asthma patients EM $CD4^+$ T cells correlated positively with asthma duration ($r=0.363$, $p=0.023$), however this did not remain significant after adjusting for age (asthma duration $p=0.125$, age $p=0.445$, $R^2=0.146$). Interestingly within the asthmatics FEV1 (% predicted) ($r=-0.4$, $p=0.013$) and

FEF25-75 (% predicted) ($r=-0.407$ $p=0.011$) were both negatively associated with the percentage of EM CD4⁺ T cells.

Terminally differentiated (TERMA; CCR7⁻CD45RA⁺) CD4⁺ T cells, expressed as a percentage of CD4⁺ T cells varied significantly across the 6 groups (Table 7.7) ($p=0.018$). This was due to trend towards a reduction in this cell type with each BMI category (NW = 9.45%, OW= 8.05%, OB=6.86%; $p=0.097$) (Figure 7.7); obese individuals having lower levels than normal weight individuals ($p=0.029$). BMI as a continuous variable ($r=-0.361$, $p=0.001$) and percentage fat ($r=-0.342$, $p=0.02$) also correlated negatively with CCR7⁻CD45RA⁺CD4⁺ T cells. Age was not associated with the percentage of terminally differentiated cells. When analysed separately the association between increasing BMI category and CCR7⁻CD45RA⁺CD4⁺ T cells was only seen in the controls ($p=0.016$) and not the asthmatics ($p=0.206$). Similar findings were noted when examining correlations between the other adiposity measures and CCR7⁻CD45RA⁺CD4⁺ T cells in the asthmatics and controls separately.

	Controls			Asthmatics			p value
	NW n=13	OW n=10	OB n=15	NW n=13	OW n=12	OB n=14	
CD45⁺CCR7⁺ CD4 (naive) lymphocytes (%) Mean (SD)	43.9 (9.06)	33.6 (8.77)	38.1 (11.4)	45.5 (13.6)	39.7 (11.5)	32.3 (15.0)	All 6 groups: $p=0.038$ BMI category: $p=0.013$ A vs.C: $p= 0.982$
CD45⁻CCR7⁺ (CM) lymphocytes (%) Geometric mean (SD)	19.4 (7.43)	22.1 (6.97)	26.6 (8.15)	19.6 (4.6)	25.6 (9.63)	23.0 (5.12)	All 6 groups: $p= 0.022$ BMI category: $p=0.006$ A vs. C: $p=0.899$
CD45⁻CCR7⁻ (EM) lymphocytes Geometric mean (SD)	22.5 (5.21)	30.72 (9.01)	25.5 (9.97)	23.1 (11.0)	25.5 (9.52)	32.8 (14.9)	All 6 groups: $p= 0.060$ BMI category: $p=0.055$ A vs. C: $p=0.554$
CD45⁺CCR7⁻ (TERMA) lymphocytes (%) Geometric mean (SD)	10.4 (7.49)	10.9 (4.58)	5.93 (3.91)	8.61 (3.27)	6.26 (3.87)	8.0 (4.54)	All 6 groups: $p=0.018$ BMI category: $p=0.097$ A vs. C: $p=0.417$

Table 7.7: Percentage of CD4⁺ lymphocyte subsets in asthmatics and controls according to BMI category.

Normally distributed data are expressed as mean and standard deviation (SD). Positively skewed data are expressed as a geometric mean and standard deviation and were logarithmically transformed before analysis. Differences between all 6 groups and BMI categories were analysed using a one-way ANOVA. Differences between asthmatics (A) and controls (C) were analysed using an unpaired t-test. Data are expressed as a percentage of CD4⁺ cells with the respective surface markers detailed.

Asthma status was not associated with the percentage of circulating terminally differentiated CD4⁺ T cells (p=0.417) across the entire study population or when each BMI category was compared individually. Asthma duration, age of onset, control score, ICS use and spirometric measures were not associated with this variable.

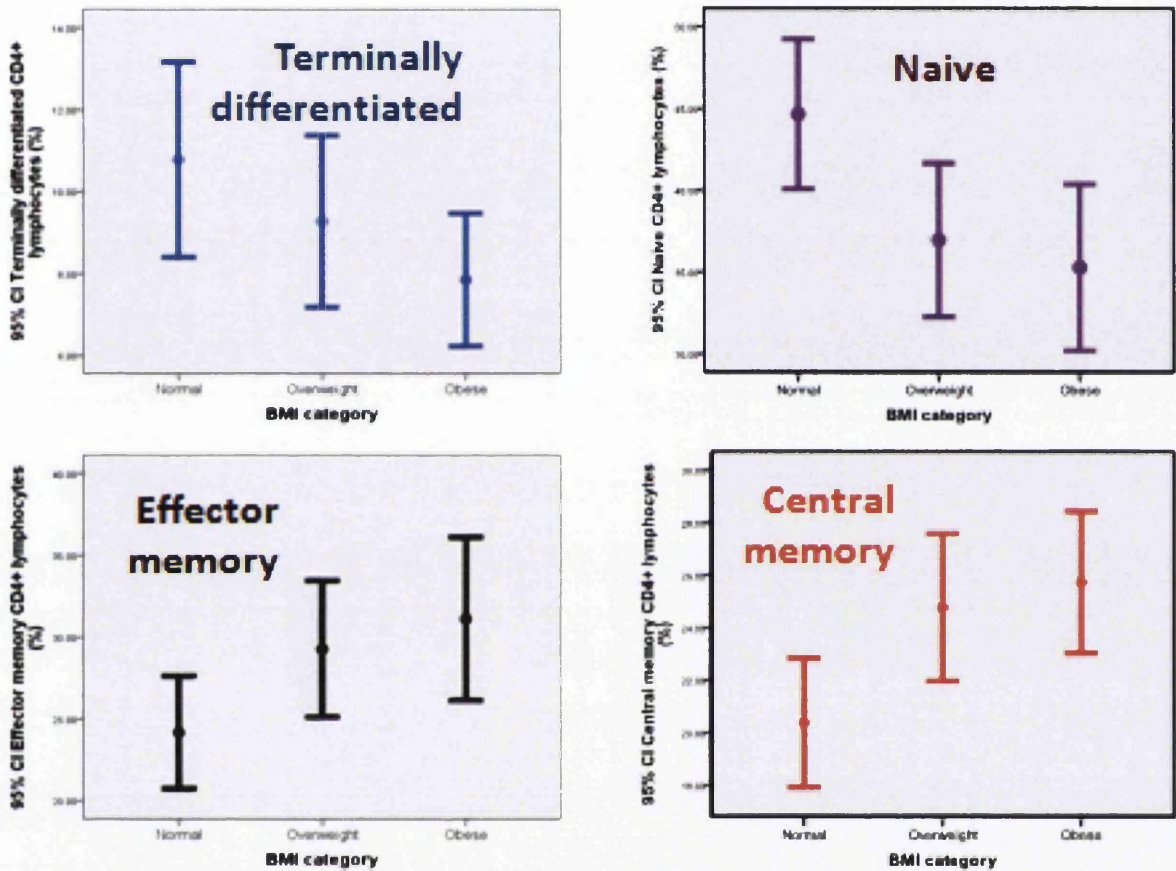


Figure 7.7: Percentage of naive, central memory, effector memory and terminally differentiated CD4⁺ lymphocytes in the three BMI categories across the entire study group. Flow cytometry was used to identify CD4⁺ T cells expressing various combinations of CD45RA and CCR7, giving the 4 cell populations (see Figure 6.2). Across the entire study group, increasing BMI category was associated with decreasing naive CD4⁺ cells (p=0.013) and increasing central memory (p=0.006) and effector memory cells (p=0.055).

7.3.4 Cytokine responses to (PHA) stimulation

Cytokine responses to PHA from whole blood cultures were analysed in 83 individuals (controls n =43, asthmatics n=40) and are summarised in Table 7.8. PHA-induced IFN γ (p=0.001), IL-13 (p<0.001), IL-9 (p<0.001), IL-17 (p=0.001) and IL-10 (p=0.001) were positively skewed and therefore data was logarithmically transformed for analysis.

	Controls			Asthmatics			p value
	NW n=15	OW n=13	OB n=15	NW n=14	OW n=12	OB n=14	
IFNγ (Th1) (ng/ml) Geometric mean (SD)	10.47 (14.83)	10.37 (35.05)	11.02 (15.02)	6.44 (4.28)	18.6 (26.00)	17.73 (14.69)	All 6 groups: p=0.025 BMI category: p=0.052 A-C: p=0.398
IL-13 (Th2) (pg/ml) Geometric mean (SD)	393.77 (482.62)	179.24 (190.07)	382.89 (910.30)	219.26 (169.51)	265.50 (558.89)	305.12 (393.82)	All 6 groups: p=0.259 BMI category: p=0.532 A-C: p=0.164
IL-9 (Th9) (pg/ml) Geometric mean (SD)	63.81 (43.70)	86.26 (152.39)	61.53 (146.22)	60.87 (51.81)	145.37 (158.31)	103.08 (181.23)	All 6 groups: p=0.189 BMI category: p=0.129 A-C: p=0.164
IL-17 (Th17) (pg/ml) Geometric mean (SD)	349.90 (336.87)	816.27 (784.79)	646.74 (709.43)	397.20 (250.84)	497.57 (664.83)	647.09 (645.59)	All 6 groups: p=0.011 BMI category: p=0.003 A-C: p=0.510
IL-10 (Treg) (pg/ml) Geometric Mean (SD)	251.31 (220.14)	384.13 (281.47)	256.20 (164.45)	289.20 (147.61)	274.51 (209.86)	288.39 (167.88)	All 6 groups: p=0.429 BMI category: p=0.374 A-C: p=0.634

Table 7.8: Cytokine responses to PHA stimulation of whole blood in asthmatics and controls according to BMI category.

Data were positively skewed, expressed as a geometric mean and standard deviation (SD) and was logarithmically transformed before analysis. Differences between all 6 groups and the 3 BMI categories were analysed using a one-way ANOVA. Differences between asthmatics (A) and controls (C) were analysed using an unpaired t-test.

PHA-stimulated IFN γ response significantly differed between the 6 groups (Table 7.8). This was due to normal weight asthmatics having a significantly lower geometric mean PHA-stimulated IFN γ response than the overweight and obese asthmatics (OW asthma $p=0.002$ and OB asthma $p=0.001$) and the control groups (NW control $p=0.01$, OW control $p=0.07$, OB control $p=0.005$) (Figure 7.7). When BMI was examined as a continuous variable an asthma-BMI interaction was observed ($p=0.095$, $R^2=0.231$) such that BMI was not associated with PHA-stimulated IFN γ response in the control individuals ($r=0.005$, $p=0.976$) but in the asthmatics BMI was positively correlated with this parameter ($r=0.405$, $p=0.009$). A similar trend was seen with body fat composition but not WHR. Obese asthmatics had the highest PHA-stimulated IFN γ response (mean=17.73ng/ml) which was significantly higher than normal weight asthmatics (mean=6.44ng/ml) ($p=0.001$) and higher than obese controls (mean=11.02ng/ml) although this was not significant ($p=0.148$). Similarly overweight asthmatics also had significantly higher levels than normal weight asthmatics ($p=0.002$) but not overweight controls ($p=0.142$). Age was associated with increasing PHA-stimulated IFN γ response ($r=0.194$ $p=0.078$). However the association between IFN γ PHA response and BMI persisted in the asthmatic group after retaining age in a general linear model (BMI $p=0.017$, age $p=0.488$, $R^2=0.178$). The PHA-stimulated IFN γ response was not associated with asthma duration, age of onset, asthma control score, ICS use or any of the spirometric measures.

As leptin has been associated with Th1 skewing a correlation between leptin and PHA-stimulated IFN γ was considered. Across the whole study population no association was seen ($r=0.138$, $p=0.213$) but there was an asthma-leptin interaction. Leptin had different associations with the PHA-stimulated IFN γ response in asthmatics versus controls ($p=0.011$, $R^2=0.101$). Leptin was not associated with PHA-stimulated IFN γ response in the control group ($r=-0.121$, $p=0.439$), however there was a significant association between BMI and PHA-stimulated IFN γ in the asthmatics ($r=0.413$, $p=0.008$) and this persisted when adjusting for the effects of age (leptin $p=0.004$, age $p=0.195$, $R^2=0.238$). Interestingly, in a general linear model, the association between BMI and PHA-stimulated IFN γ in the asthmatics did not remain significant when leptin levels were incorporated (BMI $p=0.970$, leptin $p=0.110$ R^2 value=0.171). This could suggest that leptin levels may be involved in the association between BMI and PHA-stimulated IFN γ response.

PHA-stimulated IL-13 levels did not differ significantly across the 6 groups (Table 7.8). There was no association with BMI category across the entire cohort ($p=0.532$) or when BMI was examined as a continuous variable ($r=-0.060$, $p=0.586$). Levels were not significantly different in asthmatics vs. controls across the entire cohort ($p=0.164$) or when each of the BMI categories was compared. Across the entire study group, age did not correlate with this variable and within the asthmatics no correlation was seen with asthma duration, control score, ICS use or any of the spirometric measures.

PHA-stimulated IL-9 response did not differ significantly across the 6 groups ($p=0.189$). Levels did not vary significantly across the entire cohort with BMI category, continuous BMI ($r=0.164$, $p=0.139$), or the other adiposity measures. No associations were seen with adiposity marker when asthmatics and controls were analysed separately. Levels were not different for asthmatics vs. controls ($p=0.164$) across the entire cohort or when each BMI category was compared. No correlations were

seen with age across the entire study group or with asthma duration, control score, ICS use or spirometric measures within the asthmatics.

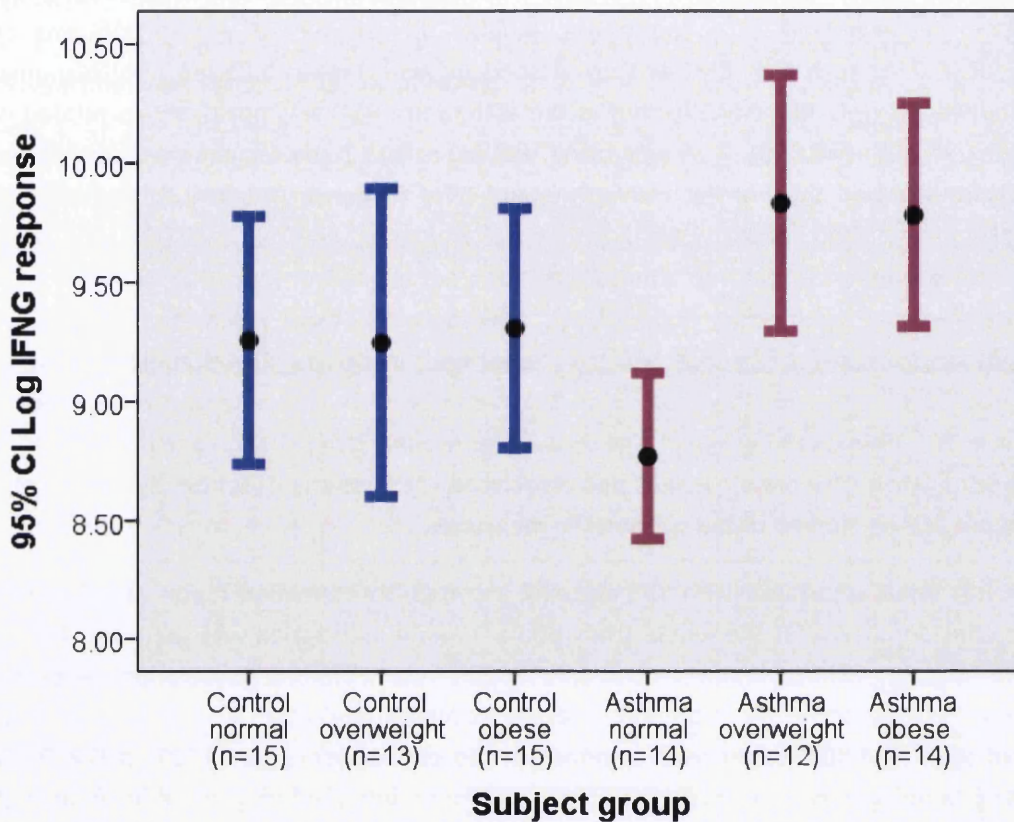


Figure 7.8: IFN γ (IFNG) PHA response in asthmatics and controls according to BMI category. Data was logarithmically transformed and shown as mean and error bars. Levels significantly differed between the 6 groups ($p=0.025$) as normal weight asthmatics had significantly lower levels than the other groups.

PHA stimulated IL-17 response was not normally distributed ($p=0.001$) and did significantly differ across the 6 groups ($p=0.011$) (Table 7.7). This was due to a significant increase with BMI category which was seen across the entire cohort ($p=0.003$). Obese individuals had the highest IL-17 PHA response (geometric mean = 647.00pg/ml) which was higher than NW (geometric mean = 371.00pg/ml) ($p=0.002$) (Figure 6.9). Similarly OW individuals also had higher responses (geometric mean=497.57pg/ml) than NW ($p=0.006$). When examined as a continuous variable, BMI was also associated with the IL-17 PHA response ($r= 0.032$, $p=0.092$) although this was not statistically significant. Similarly percentage body fat ($r=0.193$, $p=0.080$) and WHR ($r=0.202$, $p=0.071$) were correlated with this variable but neither was significant. Participant age was not associated with IL-17 levels. When levels were analysed in the asthmatics and controls separately the association between IL-17 and BMI category was only significant in the controls ($p=0.002$) and not the asthmatics ($p=0.206$), similar trends were seen when BMI was examined as a continuous variable (controls: $r=0.255$, $p=0.099$; asthmatics: $r=0.139$, $p=0.391$). However when an interaction was looked

for between asthmatic status and BMI with regards to PHA induced IL-17 response, this was not significant ($p=0.569$, $R^2=0.047$).

Levels were not significantly higher in asthmatics than controls and no differences were seen when each BMI subgroup was compared. As IL-6 is thought to be important in the development of Th17 cells a correlation between plasma IL-6 levels and IL-17 PHA response was considered, but no significant association was seen ($r=0.159$, $p=0.150$). Similarly no correlation was observed between leptin levels and IL-17 PHA response ($r=0.093$, $p=0.389$).

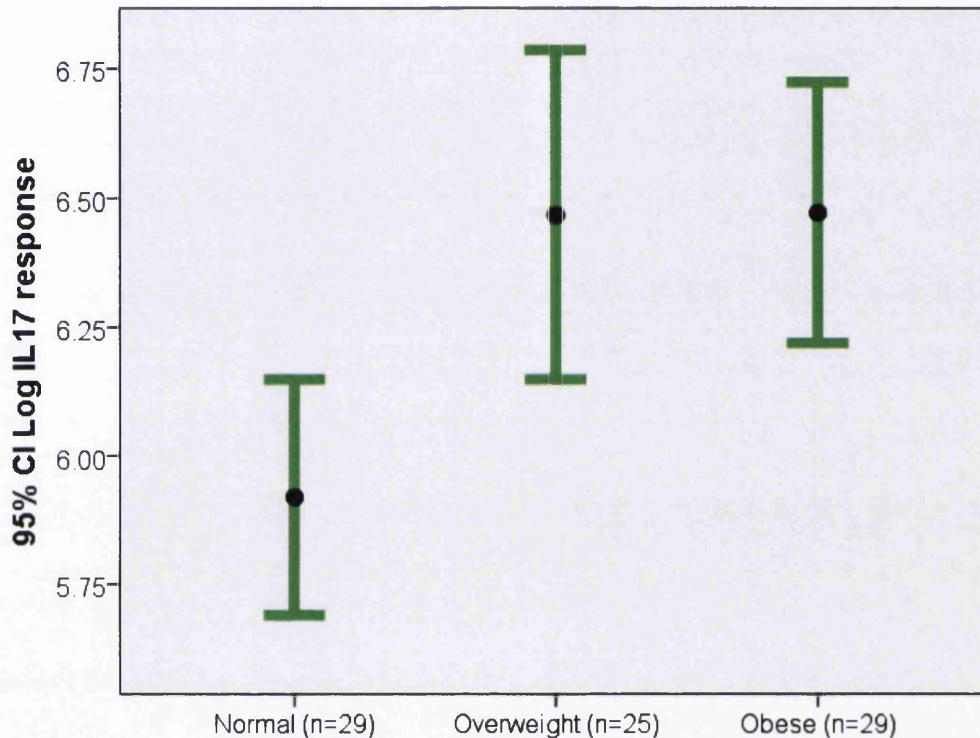


Figure 7.9: PHA-induced IL-17 response according to BMI category. Data was logarithmically transformed and shown as mean and error bars. Cytokine response was significantly increased in the overweight ($p=0.006$) and obese compared to normal weight participants ($p=0.002$).

PHA-induced IL-10 response did not differ across the 6 study groups. Levels did not vary significantly with BMI category across the study population and did not correlate with BMI ($r=0.012$, $p=0.912$) or the other adiposity markers. No associations were seen between IL-10 response and adiposity markers when asthmatics and controls were analysed separately. Responses were not significantly different between asthmatics and controls as a whole or when each BMI category was compared. Age was not associated with this variable and within the asthmatics: asthma duration, asthma control, ICS use and spirometric measures did not correlate with this variable.

7.4 Discussion

7.4.1 Obesity was not associated with systemic changes in major lymphocyte subsets

The percentage of CD3⁺ T cells, CD3⁺CD4⁺ T cells, CD3⁺CD8⁺ cytotoxic T cells, NK cells and B lymphocytes were explored in 79 premenopausal women with and without asthma categorised according to BMI. No significant different differences were seen in the percentage of these cells with BMI category. Asthmatics had a higher percentage of B lymphocytes, but no other differences were seen. Increase in the number of B lymphocytes has been noted locally in the sputum of asthmatics compared to control subjects and correlated with eosinophilia [658]. Normal weight asthmatics had the highest B lymphocyte percentage, IgE level and eosinophil count however no correlations between B lymphocyte (%) and these variables were found.

Previous studies have been contradictory in terms of the effects of obesity on T lymphocytes and their main subtypes. A Japanese group showed a reduction in the absolute count of T cells as well as CD4⁺ and CD8⁺ subsets in obese compared to normal weight healthy adults [579]. In contrast a study in women found that being overweight, obese or morbidly obese was associated with increased total lymphocyte count and CD4⁺ T cell count, whilst being morbidly obese was associated with a higher CD8⁺ T cell count [636]. A recently published paper from the Netherlands found an increase in the circulating absolute count of CD4⁺ T cells in obese individuals with no change in the CD8⁺ T cell count [659].

Our study differed from previous work in several aspects. Firstly, only Caucasian women were included unlike two of the previous in this area [579, 636]; racial differences in lymphocyte subsets have been documented in the literature [660, 661]. Secondly, sample collection was limited to the first 7 days of the menstrual cycle; levels of CD3⁺, CD3⁺CD4⁺ T cells and NK cells can fluctuate throughout the menstrual cycle [389]. Thirdly, the mean BMI was lower in our obese group than in some of the previous work [659] and it may be that only participants with a higher BMI have detectable differences. Finally, although major concomitant diseases were excluded by clinical history some of the other studies were more rigorous in examining all patients as well as performing chest radiographs, electrocardiograms, urinalysis and liver function tests to rule out major end organ disease [579, 659]. It is possible that occult conditions were not fully excluded in our control patients, and this may be a limitation of this work potentially masking subtle differences in the lymphocyte subtypes.

7.4.2 Obesity individuals had reduced numbers of CD4⁺CD25⁺CD127⁻FoxP3⁺ Tregs

Obese individuals had significantly lower percentages of regulatory T cells compared to normal weight individuals and there was a trend towards a reduction in this cell group in the overweight category. When BMI was examined as a continuous variable a negative correlation although non

significant was seen with CD4⁺CD25⁺CD127⁻FoxP⁺ Tregs. The suggestion that obesity may be associated with reduced Tregs has also been noted recently by a German study of 30 obese (defined as BMI >27kg/m²) and 13 non-obese adults; Wagner et al found reduced percentages of circulating CD4⁺CD25⁺FoxP⁺ Tregs in the obese group but a significant proportion of the obese patients had coexisting diabetes mellitus (23% of the obese vs. 0% of the controls) [662]. Changes in Tregs have been noted in diabetes [663]. Our results suggest that the obesity might be associated reduction in Tregs which cannot be explained by the confounding effects of diabetes.

In contrast to our findings, a single paediatric study looking at FoxP3 positive CD4 populations has found that levels were not different in obese vs. normal individuals [664]. In this study by Svec et al, 12 obese children and 10 control subjects were enrolled and the percentage of CD4⁺CD25⁺FoxP⁺ T cells measured within the mononuclear cell compartment derived from whole blood. This thesis differed methodologically in that flow cytometry was performed on whole blood directly and that using the additional marker CD127, increased the specificity of Treg identification. The work presented herein recruited a greater number of participants and the authors of the former study acknowledged that they might have been underpowered to detect a difference.

A recently published adult study from the Netherlands also contradicts our findings. They examined CD4⁺CD25⁺FoxP3⁺ Tregs in previously cryopreserved mononuclear cells from morbidly obese individuals with no other comorbidity and lean individuals [659]. Morbid obesity was associated with an increase in the absolute count of regulatory T cells. The authors hypothesised that in otherwise healthy obese individuals this was to counteract the systemic activation of the macrophage/monocyte compartment seen in obesity. They also postulated that in disease states associated with obesity, such as diabetes and heart disease, this compensatory increase in regulatory T cells is lost leading to a Th1/Th17 biased system.

There are several explanations for the difference in findings between this thesis and that of van der Weerd [659]. Firstly, their methodology differed, as they used cryopreserved mononuclear cells rather than whole blood. Secondly the mean BMI of their obese group was higher (42.4kg/m² vs. 37.4kg/m²). It was interesting to note that in the study herein, the overweight group (BMI 25-30kg/m²) had a reduced percentage of Tregs, although not statistically significant. It is possible that Tregs may follow a parabolic distribution where levels fall with increasing BMI moving from normal weight to overweight and obese category I, whilst at higher levels of obesity they increase. Leptin resistance could explain such a phenomenon. Tregs express leptin receptors, and in animal models receptor deficiency/neutralisation has been associated with increased Tregs [344, 346]. Given that obesity is associated with relative leptin resistance in terms of its effects on satiety, it is conceivable that with increasing BMI leptin reduces the proportion of circulating Tregs but at extreme BMI leptin resistance may again play a role. Given the interest in leptin and its immunomodulatory effects the relationship between leptin levels and Tregs was examined. There was no significant association, suggesting leptin levels are not the main mechanism for the reduction in this cohort. It would be of great interest to examine leptin resistance in regulatory T cells and other T cell subsets (see section 8.3).

Another reason for the difference between our findings and those of previous studies could be that this study better controlled for the confounding effects of cyclical hormone changes. The menstrual cycle dramatically affects numbers of circulating Tregs, with levels peaking in the follicular phase

(6.14%) and dropping dramatically (3.77%) within the luteal phase [665]. A strong correlation between FoxP3⁺ Tregs and oestrogen levels was noted in this study and the authors hypothesised that the peaking of Tregs during the late follicular phase of the cycle may be important in promoting immune tolerance to implantation. Therefore failure to control for stage of cycle might confound results significantly.

It would be of interest to investigate which subtype of circulating Tregs were reduced in our obese individuals. Naturally or thymus derived Tregs (nTregs) can be differentiated from induced Tregs (iTregs) by the expression of Helios, an Ikaros-family transcription factor [666]. In the setting of diabetes, FoxP3⁺ mRNA was reduced in the fat of obese insulin sensitive patients and not in those with insulin resistance. When the authors looked for Helios⁺ mRNA, this was reduced in the visceral adipose tissue of all obese individuals suggesting that obesity leads to reduced nTregs in visceral adipose tissues and that iTregs accumulate in those with insulin resistance [667]. In view of these findings it would be of interest to look at whether the reduction in Tregs observed in our study were nTregs or iTregs and whether asthma is associated with a change in the relative abundance of these subtypes.

IL-6 has been shown to negatively modulate Tregs and given that levels increased significantly with BMI, it was hypothesised that this would be associated with a reduction in Tregs. Whilst a negative association was seen, this was not significant. These findings were also noted in a recent publication [662].

7.4.3 Asthma is not associated with a significant difference in CD4⁺CD25⁺CD127⁻FoxP3⁺ Tregs

Regulatory T cells (Tregs) have been of great interest in the asthma field with some studies showing a reduction in the percentage of such cells in the blood or lungs of asthmatics individuals (Table 7.1). Although a trend towards lower levels of circulating Tregs was observed in asthmatic participants, this did not reach significance. There are several explanations for this. Firstly our study may not have been adequately powered to detect a significant difference. At the time the study was designed, it was not known whether obesity or asthma in adults would be associated with Tregs and there were few data on which to base a formal power calculation. Sample size was therefore based on a pragmatic approach to the number of patients that realistically could be recruited in the time available and on previous work. The final sample size of 79 patients compares favourably to previous studies in this area [194, 644, 647, 648].

Another potential reason is the effect of ICS as patients in this study were not weaned off this medication. Again, this was a pragmatic decision based on adverse findings from other studies. For example, in a study of similar design, patients were weaned off ICS before sampling but 28/33 on treatment exacerbated and were therefore sampled during an exacerbation [25]. As it was likely that a similar problem would occur if patients were weaned off ICS and any exacerbations might bias results, the decision was made to not undertake weaning off ICS. Furthermore markers of Tregs can be increased transiently on activated T cells [668] as occurs during exacerbations and so sampling

patients during exacerbation would not best represent baseline levels of this cell type. However, levels of blood Tregs might be affected by ICS use. Reduced blood levels of Tregs in untreated paediatric asthmatics were restored to normal levels following 4 weeks of ICS treatment [194], and ICS dose was associated more strongly with numbers of Tregs than asthma severity in a study of 66 asthmatic children [646]. A study of adults with moderate asthma not on ICS and moderate and severe asthmatics on ICS +/- oral glucocorticoids, noted that the treated patients had higher levels of FoxP3 mRNA expression within the CD4⁺ T cell compartment of freshly isolated mononuclear cells [669]. In the same study, *in vitro* addition of dexamethasone resulted in increased FoxP3 mRNA expression by CD4⁺ T cells isolated from healthy non-atopic donors [669]. Another study that used CD4⁺CD25⁺CTLA4⁺ to identify Tregs found that low dose fluticasone propionate increased the percentage of cells in induced sputum at 14 days [670].

Despite these observations, ICS dose was not associated with Treg levels in the asthmatics studied in this thesis. If steroids do restore numbers of Tregs in asthmatics, it is worth noting that in our study, the obese asthmatics had a lower mean level of Tregs compared to normal weight asthmatics, although this was not statistically significant ($p=0.084$), despite being on the same level of ICS treatment. There are several possible explanations for this. It could be that the obese patients were all under treated, but this seems unlikely given that there was no statistically significant difference in asthma control score, spirometric markers on the day of sampling, or other markers of asthma control. Secondly, the restorative ability of ICS might be less efficacious in obese asthmatics. This would fit clinically as obese patients are less responsive to ICS treatment [17, 18]. It would be of great interest to perform a longitudinal study looking at whether the changes in the numbers of Tregs described with the initiation of ICS treatment are seen to the same extent in asthmatics of differing BMI.

Our attempts to control for the fluctuations in Treg levels with menstrual cycle may also explain the lack of difference observed between asthmatics and controls. Sampling our individuals just once within their menstrual cycle assumes that the behaviour of Tregs across the menstrual cycle is the same in asthmatics and controls. A study examining the percentage of CD4⁺CD25⁺CD127⁻FoxP3⁺ across the menstrual cycle in asthmatic and non-asthmatic women found differences between the two groups. Tregs increased across the menstrual cycle (by 3%/day) within the asthmatics but not in the control group and levels correlated more strongly with oestrogen in the asthmatics than in the non-asthmatics. [390]. This study was very small (13 women) and their findings need clarification with a longitudinal study in greater numbers.

An additional explanation of the contradictions between published studies is the use of different markers to identify the Treg population. To date, FoxP3 is widely regarded as the most specific marker of regulatory T cells. However it is not a unique marker of Tregs, as levels can be increased transiently during CD4⁺ and CD8⁺ T cell activation [671]. A combination of surface markers and the transcription factor Foxp3 was used to be as specific as possible with regards to identification of Tregs, however without functional assays we cannot be sure whether the observed reduction in percentage of CD4⁺CD25⁺FoxP3⁺ Tregs translates to a reduction in suppressor activity. After sample collection mononuclear cells were collected and cryopreserved from the entire cohort. Tregs isolated from this banked material could be used to verify whether increasing BMI is associated with reduced cell function.

7.4.4 Obesity is associated with reduction in naive CD4⁺ T cells and increase in memory CD4⁺ subsets

Obesity was associated with a significant reduction in the percentage of circulating naive T cells, an association which persisted after adjusting for the effects of age. The ability to provide an effective T cell response to new pathogens depends on having a broad TCR repertoire established by having a large pool of thymic generated naive T cells. Ageing is associated with thymic involution and a reduction in the percentage of naive CD4⁺ T cells, restricting TCR repertoire [672]. A reduction in the circulating pool of naive T cells could explain why, as with ageing, obesity is associated with increased risk of certain infections [615]. Obesity was associated with an increase in the memory CD4⁺ T cell compartments (CM and EM) again a change traditionally associated with ageing. This is similar to observations in diet induced obesity in mice [657]. In the same paper Yang et al, examined T cell receptor excision circles (TRECs), which are extrachromosomal DNA generated during rearrangement of DNA encoding for TCRs and are non-replicable so dilute during T cell proliferation. They are therefore most abundant in truly naive T cells leaving the thymus. Associated with the reduction in naive T cells, the percentage of TRECs was reduced in obese mice and humans again suggesting a reduction in thymic output of naive T cells.

The findings in this thesis are contrary to those recently published by van der Weerd et al who found that morbidly obese individuals had higher levels of circulating CD4⁺ T cells with increased numbers of naive, and central and effector memory cells [659]. Van der Weerd et al also found TREC content to be reduced in $\alpha\beta$ T cells and their subsets from obese individuals and the authors concluded that the changes seen in these cell compartments were due to increased proliferation rather than thymic output. The methodological differences between this and our study have already been highlighted. It remains unclear whether the phenotypic changes observed in the current study translate to functional outcome but the availability of cryopreserved mononuclear cells from this cohort will enable this to be investigated.

7.4.5 Increasing BMI is associated with increased PHA stimulated IFN γ response in asthmatics but not controls

The amount of IFN γ produced into whole blood culture supernatants in response to the T cell mitogen PHA was significantly lower in normal weight asthmatics than all of the other groups. This is consistent with published data where IFN γ producing Th1 cells are reduced in the blood of allergic asthmatics compared to control subjects, in keeping with it being a Th2 mediated disease [176]. Conversely the data presented here suggests that overweight and obese asthmatics have a Th1 skewed response to PHA as they had the highest PHA-stimulated IFN γ response which was significantly higher than normal weight asthmatics. This finding is supported by a previous study of children, where the percentage of IFN γ producing CD4⁺ cells identified by flow cytometry was significantly higher in the obese than normal weight asthmatics and the authors concluded that obesity skewed asthma to a Th1 disease [359]. Although as with this current work the Th1 response was not significantly higher in the obese asthmatics than the obese controls [359]. It would be useful

to clarify whether the changes in PHA stimulated IFN γ response seen in the asthmatics with increasing BMI are due to increased abundance of Th1 cells in the blood and cryopreserved mononuclear cells are available for this.

Higher leptin levels may promote a Th1 response in obese asthma. A murine model had increased Th1 responses (in a mixed lymphocyte reaction) to allogeneic mononuclear cells which was abolished in leptin receptor deficient mice [288]. Additionally, in a paediatric paper where stimulation of whole blood with PMA resulted in an enhanced Th1 response in the obese children studied, the degree of Th1/Th2 skewing correlated with leptin levels [359]. It was of interest to note that leptin levels in the asthmatics sampled herein also correlated with the IFN γ PHA response, yet no association was seen in the control subjects. There may be a differential effect in terms of leptin responsiveness between obese asthmatics and controls: increasing BMI might be associated with leptin resistance in the control group but not in the asthmatics. It would be of great interest to examine leptin responsiveness of the T cell compartment in these two groups. PHA-stimulated IFN γ also varies with the menstrual cycle, dipping in the peri-menstrual period compared to mid-cycle but the potential effects of the menstrual cycle were taken into account [673].

Although normal weight asthmatics had the lowest PHA-induced IFN γ levels of all groups, IL-13 PHA response was no higher than controls or obese asthmatics. This is in keeping with previous observations noting that allergic asthmatics have lower percentages of circulating Th1 cells but similar percentages of Th2 [176]. ICS can also down-regulate IL-13 expression, at least in the airways, so ICS use might explain the lack of differences in PHA-induced IL-13 response across the study groups [674]. There is evidence that atopic asthma is associated with enhanced IL-9 production in the lungs but we did not find such changes systemically in the blood on PHA stimulation. Increasing BMI was not associated with changes in PHA-stimulated IL-9 suggesting at a systemic level that obesity might not impact on Th9 activity.

7.4.6 PHA-stimulated IL-17 response increases with obesity but not asthma

The PHA-stimulated IL-17 response was highest in obese patients across the entire study, significantly higher than normal weight participants and correlated with BMI as a continuous variable, although this was not statistically significant. This is consistent with murine models of diet induced obesity, where expansion of Th17 pools and resultant IL-17 production are described [351]. Circulating plasma levels of IL-17 were not more likely to be detectable in the obese in this current work (see section 5.3.1 (iii)), suggesting that only upon stimulation of the T cell compartment is an augmented IL-17 response seen in obese individuals. Mechanistically an increase in the numbers of circulating Th17 cells with escalating BMI would explain this. Th17 cells and Tregs develop in an antagonistic manner [117] and we found that the latter were reduced in the obese group and a Th17/Tregs imbalance may exist. Although we can speculate that the enhanced PHA-stimulated IL-17 response might represent an increase in the percentage of circulating Th17 cells this needs confirmation which could be achieved using flow cytometry on the cryopreserved samples.

The expansion of Th17 pools with diet induced obesity is abated in IL-6 null mice suggesting this cytokine is important in the differentiation of Th17 cells in this context [351]. Given this association, a correlation between IL-6 and the PHA-stimulated IL-17 response was considered but no significant association was found. This is in keeping with a murine study of zymosan induced peritonitis where peritoneal levels of IL-17A were significantly higher in the obese mice than their lean counterparts but neutralising IL-6 had no effect on IL-17 levels suggesting IL-6 may not be as crucial as previously thought [675]. However in this particular model the principal source of IL-17A was neutrophils so the lack of effect of IL-6 on IL-17A production needs to be interpreted with caution with regards to Th17 cells.

Recently published work suggests that Th17 cells express leptin receptor and that leptin promotes their differentiation *in vitro* and *in vivo* in murine experimental arthritis [459]. Also obese mice genetically deficient in leptin have reduced numbers of Th17 cells with levels restored upon administration of exogenous leptin [676]. Such observations prompted consideration of an association between leptin levels and PHA-stimulated IL-17 but no significant correlation was seen. This suggests that raised leptin levels in isolation are not responsible for the association between BMI and the increased IL-17 response.

PHA-stimulated IL-17 was not increased in the asthmatics in our study but plasma levels of IL-17 were detectable in a significantly higher percentage of asthmatics than controls, a finding confirmed by others [354]. Plasma IL-17 could be derived from multiple tissue sites and one could hypothesise the airways as a potential source. IL-17 levels are higher in the sputum and bronchoalveolar lavage fluid of asthmatics [506] and some have suggested that the predominant source of IL-17 in the airways may be neutrophils [650]. Few studies have looked at the percentage of circulating Th17 cells in the blood of asthmatics but a single paediatric study did show an increase in Th17 cells as identified by flow cytometry compared to the control group. Although our findings do not suggest a systemic increase in Th17 cells within asthma it must be emphasised that this needs clarification by measuring the proportion of these cells directly.

7.4.7 PHA stimulated IL-10 was unaffected by BMI or asthma

PHA-stimulated IL-10 responses did not differ significantly between BMI categories or in asthmatics vs. controls. This was an unexpected finding given the observed reduction in Tregs with increasing BMI. IL-10 production is an important aspect of Tregs function [126] and has a role in mediating immune homeostasis at environmental interfaces [191]. However within the broad classification of Tregs, there are different subtypes. Naturally occurring (nTregs) and some inducible Tregs (iTregs) are both characterised by FoxP3 expression and the former by Helios expression, exert their main effects by cell-to-cell contact. Other types of suppressor T cells also exist including regulatory T cells type 1 (Tr1) which predominantly function by producing immunomodulatory cytokines including IL-10. The lack of specific surface markers for these makes them difficult to fully characterise [677]. Whilst a reduction in FoxP3 positive Tregs was shown, these other regulatory T cells have not been quantified. Furthermore, as noted above, Tregs function was not examined and this would be of great interest.

7.5 Summary

Obesity was associated with a reduction in the percentage of circulating regulatory T cells. Although obese asthmatics had the lowest mean level of these cells this was not significantly lower than obese controls. IL-17 PHA response increased with BMI suggesting that obesity might also be associated with increased Th17 cells. The idea that obesity promotes a Tregs/Th17 imbalance is appealing but further work is needed to clarify that the PHA-stimulated IL-17 response observed is related to changes in Th17 numbers. For normal weight asthmatics, the PHA response was skewed away from a Th1 type response, evidenced by a lower IFN γ output but this was not seen in the obese asthmatics who had the highest response. Leptin may play a role in this observation with levels correlating with PHA-induced IFN γ response in the asthmatics but not the controls. Finally obesity not only appears to skew the adaptive immune system away from regulation and towards a pro-inflammatory phenotype, but might also cause changes typically associated with ageing. Further work is needed and planned to clarify whether these changes in the relative abundance of cell types are associated with functional outcomes.

Chapter 8

Conclusions and future work

8.1 Summary of study aims

The overarching aim of this study was to examine pre-menopausal women with and without asthma of varying BMI to see whether detectable changes in systemic immunity in obese asthmatics might explain the association between obesity and asthma and the obese asthma phenotype observed. The main aims of this study were:

- To examine whether a distinct immunopathological phenotype was linked to the clinical phenotype observed.
- To study metabolic parameters which could impact on immunity including: adipokines, insulin resistance and free fatty acids.
- To investigate the innate immune system including: leukocyte cell counts, markers of neutrophil and monocyte activation and the cytokine response to an inflammatory stimulus in the form of LPS.
- To look at systemic markers of oxidative stress (TBARS and TAOS) as well as acute ROS response to a non-specific inflammatory stimulus.
- To measure changes in dendritic cell populations.
- To explore changes in adaptive immunity including the percentage of circulating regulatory T cells and markers of T cell ageing.

8.2 Summary of main findings

8.2.1 A phenotype within a phenotype

Premenopausal asthmatic women of varying BMI with minimal co-morbidity were recruited for this study predominantly from a local asthma service with a special interest in allergy. Various immune parameters were compared to that of a well-matched group of women with little comorbidity without asthma. Due to the inclusion criteria used and the pool of patients available, asthmatics with relatively early onset disease were recruited; these had a high degree of atopy across all of the BMI groups. Cluster analyses have suggested obese asthma to be a late onset female predominant disease, however it is increasingly recognised that two phenotypes may exist within the umbrella term of obese asthma; the aforementioned late onset phenotype but also a second characterised by earlier onset disease with higher incidence of atopy [437]. The clinical features of these two sub-phenotypes differ with the early onset disease exhibiting worse disease control, more airflow obstruction and BHR [471, 472]. The response of these two sub-phenotypes to weight loss, differs with a recent study of asthmatics undergoing bariatric surgery showing that those with late onset disease who tended to have more comorbidities less atopy and normal IgE levels, experienced improvements in BHR with weight loss, whereas those with early onset disease who were more atopic and had less comorbidity did not experience an improvement [229]. The pathogenesis of these two phenotypes is likely to differ, so any study must differentiate between them. The profile of obese asthmatics in this study and thus any conclusions drawn reflect an early onset more atopic obese phenotype.

8.2.2 Leptin was independently associated with BMI and asthma, with obese asthmatics having the highest fasting levels

Adipocytes produce a number of hormones, so-called adipokines, which have immunomodulatory effects (section 4.1.3 (iii)). Leptin has a wide range of impacts on innate and adaptive immunity which are of relevance to asthma (Table 4.2). Murine models have shown that infusion of this adipokine augments BHR and inflammation [23, 461]. Large population studies, limited by the lack of robust definitions of asthma, have yielded contradictory results on the role of leptin in asthma. Leptin levels can be elevated as part of the acute inflammatory response in conditions such as sepsis [453] and acute asthma exacerbations [306]. Levels correlate more specifically with adiposity in women [327] are also affected by cyclical hormonal changes [488] making it a challenging adipokine to measure in a controlled manner, especially in women. However in the current study it was found that in fasted, pre-menopausal, stable female asthmatics sampled during the first 7 days of their menstrual cycle, leptin levels increased with BMI with higher levels in asthmatics such that obese asthmatics had higher levels than obese controls and normal weight asthmatics suggesting that in premenopausal obese women with early onset asthma, this adipokine may play a role. Methodological differences including controlling for exacerbations, cyclical hormonal influences and recruiting a specific female obese asthma phenotype sets this study apart from previous and may explain why a positive association has been found when other studies have not observed this (see section 4.4.4). Throughout this current work, given the diverse effects of leptin, the association of this adipokine with other changes in immunity observed was examined and will be discussed in the relevant sections.

Adiponectin has anti-inflammatory properties and is reduced in the obese. In this study, asthmatics did not have significantly different levels of this cytokine compared to the control group. Several other adipokines not well studied in the context of obesity and asthma were also measured. Resistin levels were significantly higher in asthmatics than in controls, however this appeared independent of BMI, a finding supported by another study [312]. These findings support a role for this pro-inflammatory cytokine in asthma, however it seems unlikely that it contributes to the obese asthma phenotype.

8.2.3 Obese asthmatics have a peripheral blood profile characterised by higher levels of circulating neutrophils and IL-6, with low eosinophil counts

Neutrophils are important mediators of airways diseases, playing a role in adult respiratory distress syndrome [164], COPD [678] and asthma. Their presence in asthma is associated with less reversible disease [167], poorer lung function [169] and as a result is associated with greater disease severity [522]. In keeping with previous studies, increasing BMI was associated with higher blood neutrophil counts in the controls. However asthmatics had higher levels than controls resulting in obese asthmatics having the highest neutrophil counts (see section 4.3.5). Although this finding is in peripheral blood, since commencing this study another group has reported similar findings in the airways of obese women [429] and a further group has shown increased neutrophilic inflammation

in both compartments [476]. Functionally in this current work, this trend was mirrored by an increase in ROS production measured in peripheral blood in response to a non-specific stimulus (PMA) which was not seen when adjusted for neutrophil count suggesting that neutrophils were the source of this (see section 5.3.4 (ii)), although this warrants clarification. Despite the obese asthmatics having the highest levels of neutrophils, these cells did not express higher levels of surface markers associated with activation, however as this was only performed on a subset of the population, it is possible that the study was not adequately powered to detect this. Although neutrophil count increased with BMI within the asthmatics, eosinophil conversely declined suggesting that eosinophilic inflammation may not play a role in this disease phenotype an observation noted by others when measuring counts locally in the airway [8, 472].

The mechanism behind the increase in neutrophils with BMI and asthma was examined and several key cytokines and adipokines which promote neutrophilic inflammation were measured. IL-6 has several direct effect on neutrophils including inhibiting apoptosis [510] and promoting migration (see section 5.1.1 (v)). When measuring plasma levels a trend that mirrored that of the neutrophil count was observed: IL-6 levels increased with BMI category with higher concentrations in asthma, resulting in obese asthmatics having the highest circulating levels (see section 5.3.1 (v)). In fact there was a very strong association between plasma IL-6 and neutrophil count in keeping with mechanistic data. However, the association between BMI, asthma and neutrophil counts remained significant after retaining IL-6 as a covariate in a general linear model, suggesting that it may not be the sole explanation for the change in neutrophil count. IL-6 is produced by many cells of the innate immune system upon stimulation of PRRs. In chapter 6, the response of whole blood upon stimulation with the well-studied TLR ligand LPS resulted in an elevated IL6 (and other cytokines) response, but an enhanced IL-6 response was not seen with increasing BMI and/or asthma (see section 6.3.3). The source of the increased IL-6 therefore may not be circulating innate cells and other likely sources include adipose tissue, which is thought to contribute up to 25% of IL-6 in the obese [517], or the airways as levels have been shown to be increased in sputum and correlate with markers of airflow obstruction [513]. It would be of interest to examine IL-6 expression locally in these tissues (see further works). The possibility that this increase in total IL-6 was associated with changes in sIL-6R, a molecule capable of enabling cells which do not express the receptor to respond to IL-6 (trans-signalling) and/or sgp130, a molecule capable of blocking trans-signalling was also examined. Little work has been published on this area in the wider field of obesity or in asthma; there were no significant associations with either of these molecules and BMI and/or asthma (see section 5.3.1 (v)).

IL-6 can also indirectly promote neutrophilic inflammation by promoting Th17 development [117]. On measuring plasma IL-17, levels were more frequently detectable in asthmatics than controls which may be in part responsible for the increased neutrophil levels seen with asthma, although there was no association with BMI (see section 5.3.1 (iii)). However when whole blood was stimulated with the T cell mitogen PHA, increasing BMI category was associated with an increase in IL-17 levels in cell culture supernatants. The overweight and obese categories had significantly higher IL17-responses compared to normal weight categories suggesting that obesity may be associated with increased Th17 cells (see section 7.3.4), although this warrants clarification by measuring the percentage of circulating Th17 cells across the 6 groups (see future works). If this is the case, it is therefore conceivable that the increased plasma IL-6 seen in obesity favours Th17 development promoting neutrophilic inflammation, although there was no correlation between IL-6 and PHA-stimulated IL-17 response. Interestingly, IL-23, another cytokine important in Th17

development, was not detectable in a higher proportion of obese vs. normal weight individuals although the results may have been limited by sensitivity of the assay. Asthmatics did not have a higher IL-17 response to PHA stimulation than controls (section 7.3.4), suggesting that the increased plasma IL-17 observed with asthma (section 5.3.1 (iii)) may not be derived from blood Th17 cells. The source of the increased plasma IL-17 could be inflammatory cells within the airways. Doe et al, found increased levels of IL-17 in bronchial biopsies of moderate asthmatics compared to controls but it was noted that the predominant source was neutrophils rather than T cells [650].

Other cytokines associated with neutrophilic inflammation include G-CSF, which is fundamental for terminal differentiation in the bone marrow [499]. Levels of G-CSF were increased with increasing BMI category, an observation not previously reported to the author's knowledge, suggesting that the higher number of blood neutrophils seen with obesity are likely due to a multitude of effects.

Free fatty acids (FFA) through their action on TLRs can activate the innate immune system and promote neutrophilic inflammation. A high fat meal in asthmatics is associated with increased sputum neutrophils and impaired bronchodilator induced recovery after a bronchoprovocation test [281]. Therefore a correlation between neutrophils and FFA levels (section 4.3.7) was considered, however none was seen. A recently published study did find an association between FFA and sputum neutrophils in obese asthmatics but only in men [429], supporting the findings herein that FFA may not contribute to the obese female asthma phenotype.

8.2.4 Obesity in asthma was associated with skewing towards a Th1 response

Allergic asthma is characterised by a shift in the Th1/Th2 balance towards a Th2 bias. Studies of the peripheral blood of allergic asthmatics have demonstrated a reduction in the percentage of Th1 cells compared to control subjects with no change in the percentage of Th2 cells resulting in a reduction in the Th1/Th2 ratio, favouring Th2 immunity [176]. Prototypical Th1 (IFN γ) and Th2 (IL-13) responses to the T cell mitogen PHA in whole blood were examined and it was noted that normal weight asthmatics had a reduced PHA-stimulated IFN γ response in keeping with a reduction in Th1 immunity. The PHA-stimulated IL-13 response did not differ from that of controls meaning the ratio of PHA-stimulated Th1/Th2 response favoured Th2 skewing in the normal weight asthmatics compared to controls as expected [176]. This was not seen in the overweight and obese asthmatics who had a higher IFN γ response than normal weight asthmatics but a similar IL-13 response, in keeping with Th1 bias as seen in the control group. In fact the obese asthmatics had the highest IFN γ response although this was not significantly higher than the control groups. Whilst these results suggest skewing to Th1 predominance in obese asthmatics, this needs clarification by measuring the percentage of circulating Th1 vs. Th2 cells in these patients (see future works). The findings are supported by a similar paediatric study [359] suggesting that obese asthma is not characterised by Th2 bias which may explain the lack of eosinophilic inflammation. This is particularly interesting given that the phenotype of the obese asthmatics in this study was early onset disease with high prevalence of atopy.

Leptin levels correlated positively with PHA-induced IFN γ response in the asthmatics, a finding also noted in two paediatric studies [302, 359], although no correlation was seen in the control group. Leptin resistance is an obesity related phenomena which has effects on appetite [285]. Murine studies have shown that whilst obese mice may be resistant to the effects of leptin on appetite suppression and body weight, they are not resistant to the impact of hyperleptinaemia on other physiological effects such as sympathetic excitatory actions with regards to blood pressure, suggesting that leptin resistance in obesity may be selective [347]. It is possible that the differential associations between leptin and the PHA-stimulated IFN γ response in asthmatics versus controls may be due to differences in leptin resistance and this warrants further study (see future works).

8.2.5 Obesity is associated with a reduction in cells promoting immunotolerance: regulatory T cells and plasmacytoid dendritic cells

The development of Tregs and Th17 cells are closely linked. Tregs differentiate on exposure to TGF- β [640], whereas in the presence of pro-inflammatory cytokines such as IL-6 and IL-21, Th17 cells develop [117]. Obesity is associated with a reduction in regulatory T cells in adipose tissue in both murine models and humans [34, 333]. Similarly several studies have suggested that obesity is associated with increased levels of IL-17, a product of Th17 cells [352]. Therefore the percentage of circulating CD4⁺CD25⁺FoxP3⁺ Tregs (as a percentage of CD4⁺ cells) was determined in the study groups with obese participants being found to have significantly lower levels than normal weight participants. When BMI was examined as a continuous variable there was a trend towards a negative correlation although it was not significant. Although asthmatics had lower levels of CD4⁺CD25⁺FoxP3⁺ Tregs than controls this was not significant; similarly obese asthmatics had the lowest levels but again this was not significantly lower than obese controls. As already mentioned above, obese women had a higher PHA-stimulated IL-17 response than normal weight women, suggestive of increased numbers or responsiveness of Th17 cells. Given the mechanistic data suggesting the importance of IL-6 in mediating Treg vs. Th17 development, the possible correlation between plasma IL-6, Treg percentages, or IL-17 PHA response was examined but no significant association was found.

Leptin deficient mice have higher numbers of circulating Tregs, enhanced FoxP3 expression with improved Treg cell function [296]; leptin receptor deficiency produces similar effects [345]. Very recent work suggests that leptin receptors are expressed on Th17 cells, that the adipokine promotes their differentiation in murine models [459] and that leptin deficiency results in reduced Th17 cells [676]. This prompted consideration of the relationship between leptin levels, FoxP3⁺ Tregs (%), and PHA-stimulated IL-17 but no correlations were seen.

Dendritic cells are professional antigen presenting cells and secrete most of the Th polarising cytokines, with the exception of IL-4. Myeloid dendritic cells (mDCs) are important in sensitisation [184] and are recruited to the lung rapidly following allergen challenge [603], whilst plasmacytoid DCs (pDCs) are able to prime regulatory T cells and promote tolerance (see section 6.1.3). Type II mDCs may play an important role in Th2 polarisation and higher circulating levels have been noted in

atopic individuals with levels rising during acute atopic asthma exacerbations [606]. Normal weight asthmatics were found to have higher levels of type I mDCs than overweight or obese asthmatics and the control group, again emphasising a different endotype. These cells may play a role in promoting the inflammatory response seen in normal weight asthmatics but not in obese individuals. Furthermore, in the asthmatics type II mDCs were significantly negatively correlated with BMI. Given that these cells are important in promoting Th2 responses, associations between type II mDCs and PHA IFN γ and IL-13 responses, as well as blood eosinophils, were examined for. A significant negative correlation was seen with PHA IFN γ response suggesting that the lack of Th2 response seen in obese asthmatics may be driven by changes in dendritic cell profile. Interestingly, in keeping with the FoxP3⁺ Treg findings, pDCs were negatively correlated with BMI across the entire study group, but no significant association between pDCs and Tregs was seen. It is also noteworthy that the greatest difference in pDC percentages was between overweight and not obese participants compared to controls. Similarly with PHA induced IL-17 PHA response across the entire cohort and IFN γ response in the asthmatics, significant detectable differences were noted in the overweight individuals compared to the normal weight category with no further increases in these cytokine responses in the obese. It is likely that the assumption that BMI correlates with such markers in a linear fashion is an oversimplification and that some immune parameters follow a parabolic distribution with homeostatic mechanisms of cytokine/adipokine resistance coming into play at higher degrees of adiposity.

8.2.6 Obesity is associated with a reduction in naive CD4⁺ T cells

As with ageing, epidemiological data suggest that obesity is associated with risk of infections including post-operative and other nosocomial infections [615]. Using flow cytometry obese women across the study groups were found to have reduced numbers of CCR7⁺CD45RA⁺CD4⁺ naive T cells with a corresponding increase in memory T cells (both central and effector memory). Such changes could limit T cell repertoire diversity reducing the number of novel antigens the obese immune system can respond to. Atopic asthma is characterised by repeated immune response to specific environmental allergens and therefore it was speculated that this might be associated with an increase in memory subsets, however this was not seen. The data are consistent with murine models which have also shown a reduction in naive T cells with diet induced obesity [657]; this was associated with a reduction in T cell receptor excision circles (TRECs) which are a measure of thymic output. It would be of interest to explore this further in the current cohort but it must be stressed that whilst obesity may be associated with a reduction in the expression of surface markers associated with naive T cells we cannot be certain as to whether this has any functional consequences.

8.3 Study strengths and limitations

Studies examining the mechanisms underpinning the association between obesity and asthma are fraught with difficulty. Asthma is a disorder characterised by reversible airways obstruction, therefore at times between exacerbations objective clinical and spirometric signs may be absent

leading to difficulties in confirming or refuting the diagnosis. This can lead to over diagnosis of the condition which is as high as 30% in some studies [20]. Many large population studies on the obesity-asthma association have lacked robust definitions of asthma, a potential problem given the high levels of over diagnosis, although this appears to be no more of a problem in the obese than normal weight patients [20]. This issue was addressed by recruiting patients with definite evidence of airways obstruction with significant reversibility or in two case clear variable airflow obstruction on peak flow recording and in one evidence of BHR to methacholine. It is also becoming increasingly accepted that obese asthma may contain two distinct phenotypes [437]; by recording data with regards to age of onset and atopy status it could be seen that this study predominantly examined obese asthmatics with earlier onset disease with a high prevalence of clinical atopy and so provides some insight into this sub-phenotype.

This study was mainly a hypothesis generating work. Sample size was based on previous studies in this area and also on what was practically feasible from the source of patients available. The aim was to recruit 90 individuals but this target number was not met due to the challenges in recruitment. Obesity is also associated with a number of co-morbidities which may confound any associations found and it was attempted to control for this by excluding patients with co-existing sleep apnoea, cardio respiratory disease, malignancy, diabetes, or other systemic inflammatory disorders. This presented a particular challenge in the obese category as many potential recruits had co-existing disease, especially diabetes, which made achieving the planned numbers very difficult. In addition all of the volunteers underwent Epworth scoring to try and clinically exclude undiagnosed sleep apnoea and the controls completed a modified bronchial symptom questionnaire in an attempt to exclude occult respiratory disease.

Asthma is a fluctuating chronic condition characterised by exacerbations interspersed with periods of disease control. This was addressed by recruiting patients who were 6 weeks free of exacerbation or infection. Evidence suggests that the obesity asthma association may be stronger in women [15] and cluster analyses have identified an obese phenotype which is female predominant [8]. Furthermore, body fat distribution and resultant release of adipokines differs between the sexes [328]. Therefore exclusively premenopausal women were recruited. A number of innate immune cells studied are known to be affected by the menstrual cycle including neutrophils, monocytes, eosinophils, basophils [388] and NK cells [389]. The responses of these cells to stimulation with PAMPs also fluctuate across the cycle [620, 627]. In terms of adaptive immunity, levels of CD3⁺ T cells, CD3⁺CD4⁺ T cells [389] and Tregs [665] more specifically have been shown to significantly vary throughout the cycle, making it important to control for this. Venepuncture of all the participants was undertaken when they were in the fasted state during the first 7 days of the menstrual cycle. Further to control for diurnal variation in the level of cells and asthma control, blood was taken within a very narrow two hour time window (0700-0900). Recruiting patients who were exacerbation/infection free and not taking oral steroids within the correct time within their menstrual cycle was a real challenge and in some cases required many months of regular phone contact to achieve the optimal sample timing.

In terms of the laboratory methodology, with the exception of the metabolic parameters (adipokines, insulin, glucose and FFA), IgE, and TAOS/TBARS all of the laboratory work was performed exclusively by the candidate so there was no inter-person variation in the methods applied or interpretation of data.

Despite these efforts the study does have a number of limitations. As a cross-sectional study, although associations can be observed between obesity, asthma and various immunological parameters and hypotheses generated, no causal link can be inferred from the current data. Without performing a longitudinal study - which was outside the resources and time constraints of this study - any direction of causality cannot be determined.

In terms of recruitment, whilst all of the asthmatics had documented reversible airways disease to a β_2 agonist, or significant PEF variability or positive methacholine challenge test if no evidence of airway obstruction, the controls were recruited on the basis of no clinical history of asthma or atopy, no symptoms on a modified bronchial symptom questionnaire and no airflow obstruction on spirometry. However due to resource limitations they were not subjected to clinical tests for atopy (IgE or measurement of specific IgE) and they did not undergo bronchoprovocation challenges which might have detected undiagnosed BHR. However such omissions may lead to the recruitment of undiagnosed asthmatics or atopics in the control group and are likely to increase the chances of a false negative rather than a false positive result. Questionnaires were used to exclude significant co-morbidities in the asthmatics and controls and an Epworth score to look for undiagnosed OSA. However in the obese group in particular this may not have been sufficient. Due to measurements of fasting glucose we can be confident that all diabetics were excluded, however we cannot be certain with regards to occult heart disease, obstructive sleep apnoea, or malignancy. Furthermore, given that the majority of patients were recruited from secondary care or tertiary care and local practices, some were on treatment for possible coexisting gastro-oesophageal reflux (GORD) which may have confounded results (see chapter 4). Ideally all recruited participants should be subjected to a more intensive medical assessment including ECG, echocardiography, plain film radiology, limited channel sleep studies and oesophageal pH monitoring to ensure that potential confounding co-morbidities were further addressed, but with the resources available this was not feasible. Similarly whilst every effort was made to control for hormonal fluctuations by sampling patients within the first 7 days of their menstrual cycle this was based on the onset of menses and not on measured hormone levels which would have clarified that all patients were sampled at the same time within the cycle.

Asthma is an inflammatory disease and especially in the context of a tertiary asthma clinic, all patients were on ICS and a high number of maintenance oral steroids or steroid sparing immunosuppression. Given the significant systemic immunological effects of oral steroids and other immunosuppressants such patients were excluded from the study which severely limited potential participants and those who did need intermittent steroids courses were required to be at least 6 weeks clear prior to recruitment. At the outset of the study it was planned to wean all subjects off ICS treatment prior to sampling. However it immediately became clear that this limited recruitment severely as many patients were understandably reluctant to do this. Review of previous studies in this area revealed that a high number of patients would end up being sampled during an exacerbation, and, in fact, the first two asthmatic recruits both exacerbated on weaning off their treatment. A study by Sutherland and colleagues also tried to examine the obesity asthma association whilst controlling for confounders including hormonal fluctuation [25]. They weaned patients off therapy and noted that 28/33 patients on ICS lost control of their disease within 30 days meaning that they were sampled during exacerbation. Therefore it was decided to wean patients down to the lowest dose able to achieve disease control, which should be the aim of any asthma service, and once stable recruit them into the study. Whilst there were no differences in the daily dose of ICS between BMI categories and whilst all subjects were 24 hours off therapy at the timing

of venesection, this means that all patients were on ICS treatment at sampling which limits the results. ICS therapy has an impact on a number of cell types; they have been shown to inhibit neutrophil apoptosis and increase their survival [483] and in healthy volunteers beclomethasone (but not budesonide) has been shown to increase neutrophil counts at 6 hours, returning to normal at 24 hours [484]. ICS therapy has been shown to restore peripheral blood Treg numbers in asthmatic children [194] and in adults increased FoxP3 mRNA expression has been seen in freshly isolated CD4⁺ T cells from the blood of moderate to severe asthmatics treated with ICS compared to those not on therapy [669]. Such a phenomenon may explain why the study asthmatics did not have significantly lower levels of Tregs than the control group. The acute effects of ICS treatment were limited by withholding therapy for 24 hours prior to blood sampling, however it is possible that this still may have affected the results, although adjustments for ICS dose were made when potential confounding could have been an issue.

Our laboratory techniques also have some potential limitations. Firstly, whilst the majority of techniques were performed by the candidate ensuring consistency in the methods applied this meant that the candidate was not blinded to study subject and this could have biased results. Initially it was planned for other blinded individuals to analyse the data, especially the flow cytometry data where gating is open to some subjectivity, however due to lack of local resources this was not possible. Whilst the data underwent an interim analysis in bulk and then final analysis, prior knowledge of the patient identifiers could have introduced bias. Looking in more detail at specific techniques, a multiplex assay was used to be measure the levels of serum adipokines. This requires all the proteins of interest to be measured at the same dilution. Adiponectin, which is known to be abundant, is not included in the multiplex assay for this reason. It became clear after running the study samples for the first time that the leptin and resistin levels obtained in the dilution recommended by the manufacturer fell above the optimal range of the assay, whilst the visfatin and ghrelin levels obtained fell below the optimal range. Due to resource limitation the assay could only be repeated once and the decision was made to focus on optimising the leptin and resistin results. This means that the visfatin and ghrelin results could only be expressed as a binary variable (detectable vs. non detectable) which may limit interpretation. It was also demonstrated that reactive oxygen species production in response to a non-specific stimulus (PMA) increased with BMI, however as chemiluminescence of whole blood was the chosen technique, it is not possible to clarify the cellular source of this. One can speculate that as this trend was abolished on correcting for neutrophil count that the neutrophils were the likely source. However, this needs confirmation (see future works) and in response to the findings of this study a flow cytometry based approach which enables cellular production of ROS to be determined has been optimised in the laboratory.

Asthma is predominantly a disease of the airways yet the local inflammatory response is associated with measurable changes systemically, with many of the cells involved recruited from the circulation. Obesity is clearly a systemic disorder and this and other work has demonstrated that it is associated with detectable changes in systemic immunity. Therefore the possibility that detectable changes in innate and adaptive immunity could be observed at a circulatory level in obese asthmatics was investigated. Clearly this does not provide information about the airways of such individuals and ideally simultaneous investigation of cellular changes in the airways by measuring cell counts within sputum or BAL samples would be pursued but local resources did not allow this.

8.4 Future work

The study was a hypothesis generating work and has yielded a number of results which require further exploration. As mononuclear cells were cryopreserved from all participants at the time of recruitment the research group will continue some of this investigation in the very near future. Also, ethical approval has been obtained to examine other areas such as the effects of weight loss on the immune changes observed and collecting sputum and BAL samples from the participants for analysis. Detailed below are the areas which the research group will be taking forward.

8.4.1 Quantification of other CD4⁺ T helper cell subsets, common myeloid and common lymphoid progenitors using cryopreserved material

The current work showed that increasing BMI is associated with increased PHA-stimulated IFN γ in asthmatics and IL-17 in obesity. This may be due to increased circulating Th1 and/or Th17 cells in these individuals however this warrants clarification. The use of intracellular flow cytometry for IFN γ , IL-4, and IL-17, and more detailed analysis of surface phenotypes on cryopreserved CD4⁺ cells will enable evaluation of a genuine shift in the relative abundance of Th1, Th2, or Th17 cells with asthma and/or obesity.

It was also found that circulating neutrophil counts increased with BMI. A number of important cytokines have been shown to vary with BMI, however after incorporating these in a general linear model the association between BMI and neutrophils persisted suggesting that other factors may play a role. Neutrophils develop from common myeloid progenitors and more specifically granulocyte/macrophage progenitors (see section 5.1.1). Using antibodies against cell surface markers human common myeloid (CD34⁺CD38⁺CD123^{med}CD135⁺CD45⁻) progenitors, more specifically granulocyte macrophage progenitors (CD34⁺CD38⁺CD123^{med}CD135⁺CD45⁺) as well as common lymphoid progenitors (CD34⁺CD10⁺CD7⁺) can be identified [493]. Studies in diet induced obese mice have shown that obesity was associated with an increase in common myeloid progenitors and a reduction in lymphoid progenitors [657]. Such changes could explain an expansion in neutrophil numbers in the obese whilst noting a reduction in the percentage of naive CD4⁺ T lymphocytes. Cryopreserved MNCs will be used to explore the relative abundance of common myeloid and lymphoid progenitors by flow cytometry.

8.4.2 Tregs subtype and function

Findings suggest that obesity in women across the BMI range measured is associated with a reduction in percentage of circulating regulatory T cells. Studies of Treg percentages in the adipose tissue of obese patients have suggested that obesity is associated with a reduction in nTregs whilst insulin resistant individuals have an increase in the number of iTregs [667]. Again, the cryopreserved mononuclear cells could be used for flow cytometry using antibodies against the Ikaros-family transcription factor Helios, enabling differentiation of the relative abundance of nTregs and iTregs.

The obese asthmatics had the lowest levels of Tregs of the 6 groups however this was not statistically significant. It may be that the study was not sufficiently powered to detect a difference (see limitations), or that differences are only seen in the morbidly obese. Given the known effects of corticosteroids as discussed above, it would be of interest to isolate CD4⁺ T cells from the cryopreserved mononuclear cells and determine the effect of *in vitro* steroid exposure on FoxP3 expression and whether this differs with BMI. Some studies have shown asthma to be associated with changes in Treg function. A paediatric study has shown a reduction in the ability of BAL Tregs to suppress *in vitro* proliferation and cytokine response of autologous T cells in untreated asthmatics compared to controls [194]. A study in adults showed that Tregs isolated from peripheral blood of asthmatics also had impaired ability to suppress *in vitro* effector T cell proliferation [649]. The cryopreserved mononuclear cells could also be used to examine Treg suppressor function.

8.4.3 T cell ageing

In this current work obese individuals were found to have a reduced percentage of naive (CD45RA⁺CCR7⁺) CD4⁺ T cells and an increase in memory (CM and EM) subsets, changes classically observed with ageing. Obesity is associated with a number of conditions classically connected with ageing including cardiovascular disease. Telomeres are non-coding, repeat sequences of DNA at the end of chromosomes which protect the coding sequences of DNA from enzymatic degradation. Telomere length is lost with each mitosis due to the inability of DNA polymerase to completely replicate terminal sequences of DNA and so decreases with ageing and eventually results in cellular apoptosis (the Haylick effect) [679]. Within the literature, leukocyte telomere length has been shown to reflect systemic telomere length and therefore ageing [680]. The association between obesity and telomere length has not been clarified. A study of 309 Caucasian men and women aged 8-80 years showed a negative association between BMI and telomere length which was more marked in the younger ages [681] whilst a longitudinal study of 435 obese post menopausal women showed no association between BMI and telomere length and no effect with weight loss [682]. Other inflammatory conditions such as systemic lupus erythematosus are also associated with telomere shortening, however little work has been done on this area in asthma. Real time PCR can be used to measure telomere length in the cryopreserved mononuclear cells to see if the changes in surface markers on CD4⁺ T cells, reflect a more general ageing of the immune system in obesity and also explore whether telomere shortening is seen in asthma. Similarly, analysis of T receptor excision circles (TRECS) can be considered to determine if the observed reduction in naive CD4⁺ T cells is due to a reduction in thymic output, which has been suggested in animal models [657]. The functional consequences of changes in the relative abundance of naive and memory CD4⁺ T cell populations could also be explored using intra-cellular flow cytometry of cytokines such as IL-2, IFN γ , and TNF α .

8.4.4 Leptin resistance

Obesity is characterised by a state of leptin resistance with regards to its effects on satiety. Leptin levels increased with BMI category in both asthmatics and controls with highest levels in the obese

asthmatics (section 4.3.8). Given the multitude of immunomodulatory effects, possible relationships between leptin and other immune parameters were considered throughout this work. Correlations were seen with some parameters which differed between asthmatics and controls. Examples of these included PHA-stimulated IFN γ which correlated with leptin in the asthmatics and not the controls and also correlation between leptin and mDCs which was more marked in the asthmatics than controls. In addition, in the context of PHA-stimulated IFN γ within the asthmatics the transition from normal weight to overweight category was associated with an increased response but no further escalation was seen in the obese asthmatics. Differences in leptin sensitivity may explain such observations and this should be explored in more detail. Leptin has a number of effects on the phenotype and function of mononuclear cells and these could be explored to identify differences in the leptin response within each of the groups. *In vitro* leptin has been shown to promote Th1 polarisation, increasing the production of Th1 cytokines (IL-2 and IFN γ) when monocyte depleted lymphocytes are cultured with leptin and the T cell mitogen PHA [458]. It would be of interest to repeat this experiment with lymphocytes isolated from the 6 study groups see if there are differential effects with regards to Th1 polarisation ability of leptin.

8.4.5 Weight loss

A number of weight loss studies of obese patients, by either bariatric surgery or diet, have shown benefits in asthma control (see Tables 2.6 and 2.7), however the mechanisms remain to be elucidated. One well designed study of bariatric patients with asthma (defined as demonstrable BHR or significant reversibility to β 2 agonist) showed a clear improvement in asthma control, quality of life and BHR [229]. Interestingly the improvement in BHR was only seen in those with a normal IgE. A rise in lymphocytes in the BAL fluid with weight loss was noted but there were no other changes in the cellular constituents. An increase in cytokine outputs (IL-5, IFN γ and IL-6) on T lymphocyte stimulation was also noted but this may have been confounded by treatment effects, with the patients on significantly less ICS post surgery. As of October 2013 another MD student will explore the effects of bariatric surgery on asthma and immunity. It would be of great interest to look at whether weight loss leads to an increase in Tregs and whether this was responsible for the increased lymphocytes seen in the BAL fluid in the study by Dixon and colleagues. Furthermore a number of controls in the study described herein were recruited from weight loss groups (Slimming World) and therefore it would be feasible and of interest to resample these women to examine whether weight loss by dietary modifications has any immunomodulatory effect.

8.4.6 Examining airway inflammation

A limitation of the work as previously discussed is the lack of simultaneous examination of the airways. This was due to the unavailability of suitable resources. Given the systemic changes in a number of immune cells in obese asthmatics it would be of great importance to determine whether these are mirrored in the airways; ethical approval has been obtained to gather sputum samples from these individuals and BAL samples from the asthmatics. Two groups have recently shown neutrophils to be increased within sputum samples of obese asthmatics [429, 476] suggesting that the systemic changes seen herein are also apparent within the airways. However, it would still be of

value to verify this finding in this very well matched study group. It would also be interesting to determine whether the systemic changes in IL-6 observed were mirrored in the airways. Flow cytometry on BAL samples would enable investigation of whether changes in Th1, Th17, and Tregs as observed systemically occur locally within the airways. As the presence of detectable plasma IL-17 did not correlate with PHA-stimulated IL-17 analysis for IL-17 expression within the airways would be worthwhile. Finally as there were no differences in terms of LPS responsiveness with obesity or asthma and specifically no augmented response in the obese asthmatics and that, at least in terms of asthma such changes have been seen locally within the airways [341], BAL derived macrophages could be used to examine LPS responsiveness locally.

8.5 Final summary

This case control study examined systemic immunity in obesity and asthma and more specifically whether obese asthmatics had detectable differences which may explain their different clinical phenotype. By choosing a very specific population of premenopausal women and controlling stringently for comorbidity and cyclical hormonal changes, an area not addressed well in previous studies, confounders which might impair the interpretation of results were limited. Furthermore this study focuses on a specific phenotype of obese asthma; an earlier onset atopic phenotype.

Several novel areas were the focus of investigation including the role of innate immunity, changes in dendritic cells populations and regulatory T cells. Results suggest that obesity in asthma is associated with systemic changes in innate immunity with increased numbers of neutrophils observed, and higher levels of circulating pro-inflammatory mediators including IL-6 and leptin. Within asthmatics obesity was associated with changes in the percentage of mDCs and a PHA-stimulated cytokine response more in keeping with Th1 skewing. Such observations support the notion that systemic immunity may play a role in this increasingly common disease phenotype.

Finally obesity itself was observed to be associated with a number of changes in innate immune function including increased neutrophilia, and increased levels of neutrophil related cytokines including IL-6 and G-CSF, as well as sCD14. In addition obesity is associated with changes in pDCs and adaptive immunity including a reduction in regulatory T cells, increased PHA-induced IL-17 and increased surface expression of CD4+ T cell markers associated with immune ageing. Such changes may explain why this disease state promotes the development of inflammatory conditions such as asthma and provides a gateway for further research to target these areas with the aim of modifying the detrimental effects of this endemic disease.

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APPENDIX I



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Abertawe Bro Morgannwg
University Health Board

Patient Identification Number for this trial: _____

CONSENT FORM –Study Participants

10/WMW02/4 Version 3 14.09.2011

Title of Project: Asthma and Immunity – effect of adipokines on immune function

Name of Researchers: Dr M Pynn, Dr GA Davies, Dr CA Thornton, Prof JM Hopkin

Please initial box

I confirm that I have read and understand the information sheet dated 13.09.2011 (version 4) for the above study. I have had the opportunity to consider the information; ask questions and have had these answered satisfactorily.

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

I understand that I will have blood **and sputum samples** which may be stored for future testing, with data being anonymised.

I understand that all samples will be fully anonymised and I will not be identifiable.

I understand that if it is felt to be useful in the future, I may be contacted again to give a further sample (blood/**sputum test**).

I agree to take part in the above study. I understand that the results of the research may appear in a medical journal in an anonymised fashion.

Name of Patient/Volunteer Date Signature

Name of Person taking consent Date Signature
(If different from researcher)

Researcher Date Signature



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Abertawe Bro Morgannwg
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Patient Information Sheet

10/WMW02/4 Version 3 26.11.2010

Asthma and Immunity – effect of adipokines on immune function

You are invited to take part in a research study. Before you decide it is important for you to understand why the research is being undertaken and what it will involve. Please take time to read the following information carefully. Discuss it with your friends, family and GP if you wish. Ask us if there is anything you do not understand or if you would like further information. Take time to decide whether or not you wish to take part.

Part 1 tells you about the purpose of this study and what will happen if you decide to take part.

Part 2 gives you more detailed information about the conduct of the study.

Part 1

What is the purpose of this study?

As you are aware, you have asthma, which is an inflammatory condition affecting the lungs. We know that certain types of allergic immune cells are involved in the inflammation we see in asthma. Recently, there is evidence that there may be reduced numbers of protective or regulatory immune cells in asthma, so that there is less control over the allergic cells.

In recent years there has been a worldwide increase in the number of asthma sufferers and there is some evidence to suggest that this may be linked directly to a corresponding increase in occurrence of obesity although why this should be so is still unclear. This study sets out to explore this further by comparing test results from a group of asthmatics with those obtained from a group of healthy females with similar physical characteristics. In particular the study will look at the role played by a specific class of immune cells (regulatory T cells) and whether immune function is linked to measures of weight such as body mass index.

Why have I been chosen?

Because you have been diagnosed as having asthma and fulfil the criteria for entry to the study.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

You will initially be assessed in Chest Clinic by a specialist doctor as you have been previously. The diagnosis of asthma will be confirmed by performing a breathing test (as you have done on previous clinic visits). We will record whether you are on any steroid treatment and if your asthma is controlled. If it is stable we will decide whether it is appropriate to consider step-down of this treatment (as would be done as part of your usual asthma care) and treatment. An investigator will ask some simple questions to make sure you don't have sleep apnoea (a disorder of abnormal breathing at night). Measurements will be taken of your height, weight, waist circumference and body fat (on digital scales). You will be asked to give a blood sample in the morning after fasting overnight. As we want to control for the effects of hormones on the immune system we would like to take the blood sample during the first 7 days of your menstrual cycle.

In addition to your usual clinical assessments, taking part in the study will mean one further visit for blood and body mass measurements. Other visits and tests would be part of your usual asthma management. As further information becomes available, it may be helpful to ask patients to return in the future to give a further sample and we would seek your consent to have the option to contact you again if needed.

What samples are we measuring in the study?

We are taking blood samples to measure cells relating to asthma and immune regulation. We are also measuring the protein products of fat cells (adipokines) in the blood to see whether these are linked to immune regulation.

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

Blood test may cause minor discomfort.

What are the possible benefits of taking part?

There will be no direct benefit to you from taking part in this study. However, the results may give us a better understanding of your condition which in turn may help patients in the future.

What happens if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the disease being studied. However, we do not feel that this is likely with this study as it does not involve any new or experimental drugs, nor does it involve any change in the standard of your care.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2. The telephone number to contact if you wish to lodge a complaint is 01792 703410.

Will my taking part in the study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. Any information which leaves the hospital will have your name and address removed so that you cannot be recognised from it. The details are included in Part 2.

Contact Details:

Dr M Pynn

Chest Physician

Department of Respiratory Medicine

Singleton Hospital

Swansea SA2 8PP

Tel: 

If this information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

This completes Part 1 of the Information Sheet.

Part 2

What will happen to any samples I give?

Samples will be processed in the laboratory for analysis. Appropriate samples will be stored so that further tests may be carried out in the future as more information about asthma becomes known. Cell samples and biopsy samples will be frozen and stored in a tissue bank. There are strict rules regarding storage of samples and tissue banks are certified to show they are compliant with these. Research samples will be anonymised and researchers will not be able to identify you from these samples.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be identified from it.

Procedures for handling, processing, storage and destruction of your data are compliant with the Data Protection Act 1998.

You will have the right of access to your results at any time.

What will happen to the results of the research study?

The results may be published as a conference presentation to other medical personnel involved with the management of asthma patients. You will not be identified in any publication arising from your participation in this study. You will have ready access to the results from the study if you wish by contacting the principal study investigator.

What if there is a problem?

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

The test procedures are routine and pose negligible risk to those taking part. In the very unlikely event that something does go wrong and you suffer harm during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for legal action for compensation against Abertawe Bro Morgannwg University Health Board but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

Who is organising and funding the research?

The study is being organised by Dr GA Davies in the Asthma Clinic at Singleton Hospital, Swansea. The doctors conducting the research are not receiving any payment for the project.

Who has reviewed the study?

The South West Wales Research Ethics Committee.

Will I receive a copy of this Information Sheet and the consent form?

Yes, a copy of both the information sheet and a signed consent form will be given to you to keep.

Thank you for taking the time to read this information sheet and considering taking part in this study.



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University Health Board

Healthy volunteer - Information Sheet

10/WMW02/4 Version 5 14.09.2011

Asthma and Immunity – effect of adipokines on immune function

You are invited to take part in a research study. Before you decide it is important for you to understand why the research is being undertaken and what it will involve. Please take time to read the following information carefully. Discuss it with your friends, family and GP if you wish. Ask us if there is anything you do not understand or if you would like further information. Take time to decide whether or not you wish to take part.

Part 1 tells you about the purpose of this study and what will happen if you decide to take part.

Part 2 gives you more detailed information about the conduct of the study.

Part 1

What is the purpose of this study?

In recent years there has been a worldwide increase in the number of asthma sufferers and there is some evidence to suggest that this may be linked directly to a corresponding increase in weight gain although why this should be so is still unclear. This study sets out to explore this further by comparing test results from a group of asthmatics with those obtained from a group of healthy females with similar physical characteristics. In particular the study will look at the role played by a specific class of immune cells (regulatory T cells) and whether immune function is linked to measures of weight such as body mass index.

Why have I been chosen?

Because you are a healthy person of a similar age to the patients with asthma that we are studying.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time without giving a reason.

What will happen to me if I take part?

An investigator will ask some simple questions to make sure you don't have asthma, significant allergy or sleep apnoea (a disorder of abnormal breathing at night). Measurements will be taken of your height, weight, waist circumference and body fat (on digital scales). Your lung function will be assessed by a simple blowing test (Spirometry). You will then be asked to give a blood sample (in the morning) after fasting overnight. As we want to control for the effects of hormones on the immune system we would like to take the blood sample during the first 7 days of your menstrual cycle.

What samples are we measuring in the study?

We are taking blood samples to measure cells relating to asthma and immune regulation. We are also measuring the protein products of fat cells (adipokines) in the blood to see whether these are linked to immune regulation.

What are the other possible disadvantages and risks of taking part

Blood tests may cause minor discomfort.

What are the possible benefits of taking part?

There will be no direct benefit to you from taking part in this study. However, the results may give us a better understanding of asthma which may help patients in the future.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2. The telephone number to contact if you wish to lodge a complaint is 01792 703410.

Will my taking part in the study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

Contact Details:

Dr M Pynn

Respiratory Registrar and Clinical Lecturer

Immunity and allergy

Institute of Life Science

Swansea University

Email: m.c.pynn@swansea.ac.uk

Tel: 

If this information in Part 1 has interested you and you are considering participating, please continue to read the additional information in Part 2 before making any decision.

This completes Part 1 of the Information Sheet.

Part 2

What will happen to any samples I give?

Samples will be processed in the laboratory for analysis. Appropriate samples will be stored so that further tests may be carried out in the future as more information about asthma becomes known. Cell samples and biopsy samples will be frozen and stored in a tissue bank. There are strict rules regarding storage of samples and tissue banks are certified to show they are compliant with these. Research samples will be anonymised and researchers will not be able to identify you from these samples.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be identified from it.

Procedures for handling, processing, storage and destruction of your data are compliant with the Data Protection Act 1998.

You will have the right of access to your results at any time.

What will happen to the results of the research study?

The results may be published as a conference presentation to other medical personnel involved with the management of asthma patients. You will not be identified in any publication arising from your participation in this study. You will have ready access to the results from the study if you wish by contacting the principal study investigator.

What if there is a problem?

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

The test procedures are routine and pose negligible risk to those taking part. In the very unlikely event that something does go wrong and you suffer harm during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for legal action for compensation against Abertawe Bro Morgannwg University Health Board but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

Who is organising and funding the research?

The study is being organised by Dr GA Davies in the Asthma Clinic at Singleton Hospital, Swansea. The doctors conducting the research are not receiving any payment for the project.

Who has reviewed the study?

The South West Wales Research Ethics Committee.

Will I receive a copy of this Information Sheet and the consent form?

Yes, a copy of both the information sheet and a signed consent form will be given to you to keep.

Thank you for taking the time to read this information sheet and considering taking part in this study.

APPENDIX IV

MODIFIED IUATLD BRONCHIAL SYMPTOMS QUESTIONNAIRE

Questionnaire-Volunteers without Asthma

To answer the questions, please choose the appropriate box; IF YOU ARE UNSURE OF THE ANSWER, PLEASE CHOOSE 'NO'

All answers are strictly confidential

Asthma

1. Have you ever had asthma? No Yes
[] []

Hayfever and eczema

2. Have you ever had any nasal allergies including hayfever? No Yes
[] []

If yes to 2a/b. If no to 3

2a) Do you still have it? No Yes
[] []

b) Are you currently on any medications
Including tablets, nasal sprays [] []

3. Have you ever had eczema? No Yes
[] []

If yes to 3a/b

. If no to 4

3a) Do you still have it? No Yes
[] []

3b) Are you currently on any medication for it
Including tablets or topical treatments [] []

Smoking

4. Have you ever smoked for as long as one year? No Yes
[] []

If yes to 4a:

If no to 5

4a. Do (did) you usually smoke: cigarettes? []
pipe? []

cigars? []
Other (precise please) _____

4b. How many cigarettes do (did) you smoke each day, on average? _____

4c. Have you:

continued to smoke? []
given up smoking altogether, but less than 4 weeks ago? []
given up smoking altogether, at least 4 weeks ago? []

4d. For how many years have you smoked (did you smoke) _____

Other conditions

5. Do you have any other medical conditions including diabetes or reflux disease?

No Yes
[] []

If yes to 5a:. If no 6

5a) Please list medical conditions

Sleep apnoea

6. Do you have a history of Obstructive sleep apnoea?

No Yes
[] []

7. In the following situations please grade from 0-3 your chances of falling asleep.

(0=would never doze, 1= slight chance of dozing, 2= moderate chance of dozing,
3= high chance of dozing)

Sitting reading []
Watching TV []
Lying down to rest in the afternoon when circumstances permit []
Sitting inactive in a public place []
Sitting and talking to someone []
Sitting after lunch without alcohol []
As a passenger in a car for an hour without a break []
In a car whilst stopped for a few minutes in the traffic []

TOTAL SCORE []

8. Are you on any medication?

No Yes
[] []

If yes to 8a:

If no to 9

8a) Please list medications

Wheeze and tightness in the chest

9. Have you, at any time in the last 12 months, had wheezing or whistling in your chest?

No Yes
[] []

If yes to 9a/9b/9c. If no, go to question 10.

9a. Have you been at all breathless when the wheezing noise was present?

No Yes
[] []

9b. Have you had this wheezing or whistling when you did *not* have a cold?

No Yes
[] []

9c. Have you, at any time in the last 12 months, woken up with a feeling of tightness in your chest first thing in the morning?

No Yes
[] []

Shortness of breath

10. Have you, at any time in the last 12 months, had an attack of shortness of breath that came on during the day when you were not doing anything strenuous?

No Yes
[] []

11. Have you, at any time in the last 12 months, had an attack of shortness of breath that came on after you stopped exercising?

No Yes
[] []

12. Have you, at any time in the last 12 months, been woken at night by an attack of shortness of breath?

No Yes
[] []

Cough and Phlegm from the chest

13. Have you, at any time in the last 12 months, been woken at night by an attack of coughing?

No Yes
[] []

14. Do you usually cough first thing in the morning?

No Yes
[] []

If yes to 14a/14b. If no to 15.

14a. Do you have a cough like this most mornings for as much as 3 months per year?

No Yes
[] []

14b. How many years have you had this cough? _____ YEARS

15. Do you usually bring up phlegm from your chest first thing in the morning?

No Yes
[] []

If yes to 15a. If no to 16.

15a. Do you have phlegm like this most mornings for as much as 3 months per year?

No Yes
[] []

15b. How many years have you had this phlegm? _____ YEARS

Breathing

16. Which of the following statements best describes your breathing? check only one:

I never or only rarely get trouble with my breathing []

I get repeated trouble with my breathing but it always gets completely better []

My breathing is never quite right []

More about yourself

17. When were you born

--	--	--	--	--	--

18. What was the date of your last period

day		month		year	

19. How long is your menstrual cycle

--

 days

20. What is the anticipated date of your next period

day		month		year	

21. What is today's date?

day		month		year	

22. Are you a student (undergraduate/postgraduate)?

No	Yes
[]	[]

23. What is your height and weight (approximately)?

24. What is your ethnic group?

- a) White []
- b) Black African []
- c) Black Caribbean []
- d) Black other []
- e) Indian []
- f) Pakistani []
- g) Bangladeshi []
- h) Chinese []
- i) Arab []
- j) Turkish []
- k) Other ethnic group []
- l) If other, please state [e.g. a) and b)] _____

MODIFIED ECRHS II QUESTIONNAIRE

Questionnaire-Volunteers with asthma

Asthma

1. Have you ever had asthma? No Yes
[] []

1.1 Do you still have it? No Yes
[] []

1.2 Was it confirmed by a doctor? No
Yes
[] []

1.3 At what age did it start? _____ Age in years

1.4 If you no longer have it, at what age did it stop? _____ Age in years

2. Have you had an attack of asthma at any time in the last 12 months? No Yes
[] []

If yes:

2.1 How many attacks of asthma have you had in the last **12 months**? _____
Attacks

2.2 How many attacks of asthma have you had in the last **3 months**? _____
Attacks

1.3 How old were you when you had your most recent attack of asthma? Years
[] []

3. How many times have you woken up because of your asthma in the last **3 months**?

Tick one box only

every night or almost every night	1	
more than once a week, but not most nights	2	
at least twice a month, but not more than once a week	3	
less than twice a month	4	
not at all	5	

4. How often have you had trouble with your breathing because of your asthma in the last **3 months**?

	Tick one box only
continuously	1 <input type="checkbox"/>
about once a day	2 <input type="checkbox"/>
at least once a week, but less than once a day	3 <input type="checkbox"/>
less than once a week	4 <input type="checkbox"/>
not at all	5 <input type="checkbox"/>

Asthma treatment

5. Are you currently taking any medicines (including inhalers, aerosols or tablets) for asthma?

No	Yes
[]	[]

6. Have you used any **inhaled** medicines to help your breathing at any time in the last **12 months**? No Yes

IF 'YES':

Which of the following have you used in the last **12 months**?

6.1 short acting beta-2-agonist inhalers No Yes

(Please include combinations that include beta 2 and steroids in section 6.5)

6.1.1 If used, which one?

6.1.2 What type of inhaler do you use?

6.1.3. What is the dose per puff (in micrograms)?

6.1.4. In the last 3 months, how have you used them:

	TICK ONE BOX ONLY
a) when needed	1 <input type="checkbox"/>
b) in short courses	2 <input type="checkbox"/>
c) continuously	3 <input type="checkbox"/>
d) not at all	4 <input type="checkbox"/>

If answer to 6.1.4 is when needed:

6.1.5 Number of puffs per month

If answer to 6.1.4 is in short courses

6.1.6 number of courses

6.1.7 number of puffs per day

6.1.8 average number of days per month

If answer to 6.1.4 is continuously

6.1.9 number of puffs per day

6.2 long acting beta-2-agonist inhalers

No	Yes
<input type="checkbox"/>	<input type="checkbox"/>

(Please include combinations that include beta 2 and steroids in section 76.5)

6.2.1 If used, which one? _____

<input type="checkbox"/>
<input type="checkbox"/>

6.2.2 What type of inhaler do you use?

6.2.3. What is the dose per puff (in micrograms)?

<input type="text"/>	<input type="text"/>
----------------------	----------------------

6.2.4. In the last 3 months, how have you used them:

- a) when needed
- b) in short courses
- c) continuously
- d) not at all

Tick one box only

1	<input type="checkbox"/>
2	<input type="checkbox"/>
3	<input type="checkbox"/>
4	<input type="checkbox"/>

If answer to 6.2.4 is when needed:

6.2.5 Number of puffs per month

<input type="text"/>	<input type="text"/>
----------------------	----------------------

If answer to 6.2.4 is in short courses

6.2.6 number of courses

<input type="text"/>	<input type="text"/>
<input type="text"/>	<input type="text"/>
<input type="text"/>	<input type="text"/>

6.2.7 number of puffs per day

6.2.8 average number of days per month

If answer to 6.2.4 is continuously

6.2.9 number of puffs per day

<input type="text"/>	<input type="text"/>
----------------------	----------------------

6.3 non-specific adrenoreceptor agonist inhalers

No	Yes
<input type="checkbox"/>	<input type="checkbox"/>

6.3.1 If used, which one? _____

<input type="text"/>	<input type="text"/>
----------------------	----------------------

6.4 anti-muscarinic inhalers

No	Yes
<input type="checkbox"/>	<input type="checkbox"/>

6.4.1 If used, which one? _____

<input type="checkbox"/>	<input type="checkbox"/>
	<input type="checkbox"/>

6.4.2 What type of inhaler do you use?

6.4.3. What is the dose per puff (in micrograms)?

<input type="text"/>	<input type="text"/>	<input type="text"/>
----------------------	----------------------	----------------------

6.4.4. In the last 3 months, how have you used them:

- a) when needed
- b) in short courses
- c) continuously
- d) not at all

Tick one box only

1	<input type="checkbox"/>
2	<input type="checkbox"/>
3	<input type="checkbox"/>
4	<input type="checkbox"/>

If answer to 6.4.4 is when needed:

6.4.5 Number of puffs per month

<input type="text"/>	<input type="text"/>
----------------------	----------------------

If answer to 6.4.4 is in short courses

6.4.6 number of courses

<input type="text"/>	<input type="text"/>
<input type="text"/>	<input type="text"/>
<input type="text"/>	<input type="text"/>

6.4.7 number of puffs per day

6.4.8 average number of days per month

If answer to 6.4.4 is continuously

6.4.9 number of puffs per day

<input type="text"/>	<input type="text"/>
----------------------	----------------------

6.5 inhaled steroids

(if combined B2 and steroid please insert inhaled steroid dose)

No Yes

<input type="checkbox"/>	<input type="checkbox"/>
--------------------------	--------------------------

6.5.1 If used, which one? _____

<input type="text"/>	<input type="text"/>
<input type="text"/>	<input type="text"/>

6.5.2 What type of inhaler do you use?

6.5.3. What is the dose per puff (in micrograms)?

<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
----------------------	----------------------	----------------------	----------------------

6.5.4. In the last 3 months, how have you used them:

- a) when needed
- b) in short courses
- c) continuously
- d) not at all

Tick one box only

1	<input type="checkbox"/>
2	<input type="checkbox"/>
3	<input type="checkbox"/>
4	<input type="checkbox"/>

If answer to 6.5.4 is when needed:

6.5.5 Number of puffs per month

<input type="text"/>	<input type="text"/>
----------------------	----------------------

If answer to 6.5.4 is in short courses

6.5.6 number of courses

<input type="text"/>	<input type="text"/>
<input type="text"/>	<input type="text"/>
<input type="text"/>	<input type="text"/>

6.5.7 number of puffs per day

6.5.8 average number of days per month

If answer to 6.5.4 is continuously

6.5.9 number of puffs per day

--	--

6.6 inhaled cromoglycate/nedocromil

No	Yes
<input type="checkbox"/>	<input type="checkbox"/>

6.6.1 If used, which one? _____

--	--

6.6.2. What is the dose per puff (in micrograms)?

--	--

6.6.3. In the last 3 months, how have you used them

- a) when needed
- b) in short courses
- c) continuously
- d) not at all

Tick one box only

1	<input type="checkbox"/>
2	<input type="checkbox"/>
3	<input type="checkbox"/>
4	<input type="checkbox"/>

If answer to 6.6.3 is when needed:

6.6.4 Number of puffs per month

--	--

If answer to 6.6.3 is in short courses

6.6.5 number of courses

6.6.6 number of puffs per day

6.6.7 average number of days per month

If answer to 6.6.3 is continuously

6.6.8 number of puffs per day

--	--

6.7 inhaled compounds

No	Yes
<input type="checkbox"/>	<input type="checkbox"/>

6.7.1 If used, which one?

6.7.2 What type of inhaler do you use?

6.7.3. What is the dose per puff (in milligrams)?

--	--	--	--

7. Have you used any **pills, capsules, tablets** or **medicines**, other than inhaled medicines, to help your breathing at any time in the last **12 months**?

No	Yes
<input type="checkbox"/>	<input type="checkbox"/>

IF 'NO' GO TO QUESTION 8, IF 'YES':

Which of the following have you used in the last **12 months**?

7.1 oral beta-2-agonists

7.1.1 If used, which one? _____

--	--

7.1.2. What is the dose of tablet?

--	--

7.1.3. In the last 3 months, how have you used them

- a) when needed
- b) in short courses
- c) continuously
- d) not at all

Tick one box only

1	
2	
3	
4	

If answer to 7.1.3 is when needed:

7.1.4 Number of tablets per month

--	--

If answer to 7.1.3 is in short courses

7.1.5 number of courses

7.1.6 tablets per day

7.1.7 average number of days per month

If answer to 7.1.3 is continuously

7.1.8 tablets per day

--	--

7.2 oral methylxanthines

	NC	YES

7.2.1 If used, which one?

7.2.2. What is the dose of tablet?

7.2.3. In the last 3 months, how have you used them

- a) when needed
- b) in short courses
- c) continuously
- d) not at all

TICK ONE BOX ONLY

1	
2	
3	
4	

If answer to 7.2.3 is when needed:

7.2.4 Number of tablets per month

--	--

If answer to 7.2.3 is in short courses

7.2.5 number of courses

7.2.6 tablets per day

7.2.7 average number of days per month

If answer to 7.2.3 is continuously

7.2.8 tablets per day

--	--

7.3 oral steroids

NC YES

--	--

7.3.1 If used, which one?

7.3.2. What dose of tablet?

7.3.3. In the last 3 months, how have you used them

TICK ONE BOX ONLY

- a) when needed
- b) in short courses
- c) continuously
- d) not at all

1	
2	
3	
4	

If answer to 7.3.3 is when needed:

7.3.4 Number of tablets per month

--	--

If answer to 7.3.3 is in short courses

7.3.5 number of courses

7.3.6 tablets per day

7.3.7 average number of days per month

If answer to 7.3.3 is continuously

7.3.8 tablets per day

--	--

7.3.9. Have you used them in the last **3 months**?

NO YES

--	--

7.4 oral anti-leukotrienes

NO YES

--	--

7.4.1 If used, which one?

7.4.2. What is the dose of tablet?

7.4.3. In the last 3 months, how have you used them

- a) when needed
- b) in short courses
- c) continuously
- d) not at all

Tick one box only

1	<input type="checkbox"/>
2	<input type="checkbox"/>
3	<input type="checkbox"/>
4	<input type="checkbox"/>

If answer to 7.4.3 is when needed:

7.4.4 Number of tablets per month

<input type="text"/>	<input type="text"/>
----------------------	----------------------

If answer to 7.4.3 is in short courses

7.4.5 number of courses

<input type="text"/>	<input type="text"/>
<input type="text"/>	<input type="text"/>
<input type="text"/>	<input type="text"/>

7.4.6 tablets per day

7.4.7 average number of days per month

If answer to 7.4.3 is continuously

7.4.8 tablets per day

<input type="text"/>	<input type="text"/>
----------------------	----------------------

7.5 ketotifen

No	Yes
<input type="checkbox"/>	<input type="checkbox"/>

7.5.1 If used, which one?

<input type="text"/>	<input type="text"/>
	<input type="text"/>

7.5.2. What dose of tablet?

7.5.3. In the last 3 months, how have you used them

- a) when needed
- b) in short courses
- c) continuously
- d) not at all

Tick one box only

1	<input type="checkbox"/>
2	<input type="checkbox"/>
3	<input type="checkbox"/>
4	<input type="checkbox"/>

If answer to 7.5.3 is when needed:

7.5.4 Number of tablets per month

<input type="text"/>	<input type="text"/>
----------------------	----------------------

If answer to 7.5.3 is in short courses

7.5.5 number of courses

<input type="text"/>	<input type="text"/>
<input type="text"/>	<input type="text"/>
<input type="text"/>	<input type="text"/>

7.5.6 tablets per day

7.5.7 average number of days per month

If answer to 7.5.3 is continuously

7.5.8 tablets per day

8. Have you ever used inhaled steroids (show list)?

No	Yes
<input type="checkbox"/>	<input type="checkbox"/>

IF NO GO TO QUESTION 9, IF YES

8.1. How old were you when you first started to use inhaled steroids?

Years

--	--

8.2. Have you now stopped using inhaled steroids?

No	Yes
<input type="checkbox"/>	<input type="checkbox"/>

IF NO, GO TO Q8.3, IF YES

8.2.1 How old were you when you stopped using inhaled steroids?

Years

--	--

8.3 Have (did) you used inhaled steroids *every year* since you started using them?

No	Yes
<input type="checkbox"/>	<input type="checkbox"/>

IF NO GO TO QUESTION 8.4, IF YES

8.3.1. On average how many months each year have you taken them (or did you take them)?

Months

--	--

NOW GO TO Q9

8.4 How many of the years since you started using them have you taken inhaled steroids?

Years

--	--

8.5 On average how many months of each of these years have you taken them?

Months

--	--

Asthma Severity

9. Have you visited a hospital casualty department or emergency room because of asthma, shortness of breath or wheezing in the *last 12 months*?

No	Yes
<input type="checkbox"/>	<input type="checkbox"/>

IF YES

9.1 How many times *in the last 12 months*?

Times

--	--

10. Have you spent a night in hospital because of asthma, shortness of breath or wheezing in the *last 12 months*?

No	Yes
<input type="checkbox"/>	<input type="checkbox"/>

IF YES

10.1 How many nights have you spent in hospital because of asthma, shortness of breath or wheezing in the *last 12 months*?

Nights

--	--

10.1.1 Have you spent a night in ITU because of asthma, shortness of breath or wheezing in the *last 12 months*?

No	Yes
<input type="checkbox"/>	<input type="checkbox"/>

IF YES

10.1.2 How many nights have you spent in ITU because of asthma, shortness of breath or wheezing in the **last 12 months**?

11. Have you been seen by a general practitioner because of asthma, shortness of breath or wheezing in the **last 12 months**?

No	Yes
<input type="checkbox"/>	<input type="checkbox"/>

IF YES

11.1 How many times have you been seen by your general practitioner because of asthma, shortness of breath or wheezing in the **last 12 months**?

Times	
<input type="checkbox"/>	<input type="checkbox"/>

12. Have there been days when you have had to give up work or other activities because of asthma, shortness of breath or wheezing in the last **12 months**?

No	Yes
<input type="checkbox"/>	<input type="checkbox"/>

IF YES

12.1 How many days on average each month?

Days	
<input type="checkbox"/>	<input type="checkbox"/>

Hayfever and eczema

13. Do you have any nasal allergies including hayfever?

No	Yes
[]	[]

If yes to 13a/b. If no to 14.

13 a) Do you still have it?

No	Yes
[]	[]

b) Are you currently on any medications
Including tablets, nasal sprays

[]	[]
-----	-----

14. Have you ever had eczema? _____

No	Yes
[]	[]

If yes to 14a. If no to 15.

14a) Do you still have it?

No	Yes
[]	[]

14b) Are you currently on any medication for it
Including tablets or topical treatments

[]	[]
-----	-----

Other conditions

15. Do you have any medical conditions including diabetes or reflux disease

No	Yes
[]	[]

If yes to 15a. If no to 16.

15.a Please list medical conditions

Sleep apnoea

18. Do you have a history of Obstructive sleep apnoea? No Yes
[] []

19. In the following situations please grade from 0-3 your chances of falling asleep.

(0=would never doze, 1= slight chance of dozing, 2= moderate chance of dozing,
3= high chance of dozing)

Sitting reading []

Watching TV []

Lying down to rest in the afternoon when circumstances permit []

Sitting inactive in a public place []

Sitting and talking to someone []

Sitting after lunch without alcohol []

As a passenger in a car for an hour without a break []

In a car whilst stopped for a few minutes in the traffic []

TOTAL SCORE []

20. Are you on any medication?

No Yes
[] []

If yes to 20a. If no to 21

16.1 Please list medications including any hormonal or contraceptive medications

Smoking

21. Have you ever smoked for as long as one year?

No Yes
[] []

If yes to 21a. If no to 22

21a). Do (did) you usually smoke:

cigarettes? []

pipe? []

cigars? []

Other (precise please) _____

21b). How many cigarettes do (did) you smoke each day, on average? _____

21c). Have you:

continued to smoke?

given up smoking altogether, but less than 4 weeks ago?

given up smoking altogether, at least 4 weeks ago? []

Check one:

[]

[]

21d). For how many years have you smoked (did you smoke) _____

More about yourself

22. When were you born

day month year

23. What was the date of your last period

day month year

24. How long is your menstrual cycle

days

26. What is the anticipated date of your next period

day month year

27. What is today's date?

day month year

28. What is your ethnic group?

- a) White []
- b) Black African []
- c) Black Caribbean []
- d) Black other []
- e) Indian []
- f) Pakistani []
- g) Bangladeshi []
- h) Chinese []
- i) Arab []
- j) Turkish []
- k) Other ethnic group []
- l) If other, please state [e.g. a) and b)] _____

APPENDIX VI Juniper asthma control questionnaire [392]

Please answer questions 1-6

Circle the number of the response that best describes how you have been during the past week

1. On average, during the last week, how often were you **woken by your asthma** during the night?
 0. Never
 1. Hardly ever
 2. A few minutes
 3. Several times
 4. Many times
 5. A great many times
 6. Unable to sleep because of asthma

2. On average, during the last week, how **bad were your asthma symptoms when you woke up** in the morning?
 0. No symptoms
 1. Very mild symptoms
 2. Mild symptoms
 3. Moderate symptoms
 4. Quite severe symptoms
 5. Severe symptoms
 6. Very severe symptoms

3. In general, during the past week, how **limited were you in your activities** because of your asthma?
 0. Not limited at all
 1. Very slightly limited
 2. Slightly limited
 3. Moderately limited
 4. Very limited
 5. Extremely limited
 6. Totally limited

4. In general, during the past week, how much **shortness of breath** did you experience because of your asthma?
 0. None
 1. A very little
 2. A little
 3. A moderate amount
 4. Quite a lot
 5. A great deal
 6. A very great deal

5. In general, during the past week, how much of the time did you **wheeze**?
 0. Not at all
 1. Hardly any of the time
 2. A little of the time
 3. A moderate amount of the time
 4. A lot of the time
 5. Most of the time
 6. All of the time

6. On average, during the past week, how many **puffs of short acting bronchodilator** (e.g. Ventolin) have you used?
 0. None
 1. 1-2 puffs most days
 2. 3-4 puffs most days
 3. 5-8 puffs most days
 4. 9-12 puffs most days
 5. 13-16 puffs most days
 6. More than 16 puffs most days

7. To be completed by a member of the clinic staff

FEV1 pre-bronchodilator:

FEV1 predicted

FEV1 %

(Record the actual values on the dotted lines and score the FEV1 % predicted in the next column)

0. >95% predicted
1. 95-90%
2. 89-80%
3. 79-70%
4. 69-60%
5. 59-50%
6. <50% predicted

APPENDIX VII

Global Initiative for Asthma (GINA) programme classification of asthma severity [393].

GINA grading	Symptoms in the day	Symptoms at night	PEFR or FEV1	PEFR variability
Step I Intermittent	<1 time a week. Asymptomatic and normal PEFR between attacks.	< 2 times a month.	≥80%	<20%
Step II Mild persistent	>1 time a week but < 1 time a day. Attacks may affect activity	>2 times a month	≥80%	20-30%
Step III Moderate persistent	Daily Attacks affect activity	>1 time a week	60-80%	>30%
Step IV Severe persistent	Continuous Limited physical activity	Frequent	<60%	>30%

The presence of any one of the listed criteria is enough to place the asthmatics in the respective severity grade.

APPENDIX VIII

Krebs-Ringer bicarbonate buffer made up in distilled water:

- Na_2HPO_4 (12.7 mM),
- $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (3.06 mM),
- NaCl_2 (120 mM),
- KCl (4.8 mM),
- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1.2 mM),
- Dextrose (11 mM)
- CaCl_2 (0.71 mM)(Fisher Biosciences).