Swansea University
Prifysgol Abertawe

Cronfa
Setting Research Free

Swansea University E-Theses

Asthma and Th2 immunity: The impact of genetic factors and obesity.

Davies, Gwyneth Ann

How to cite:

Davies, Gwyneth Ann (2007) *Asthma and Th2 immunity: The impact of genetic factors and obesity..* thesis, Swansea University.
http://cronfa.swan.ac.uk/Record/cronfa42321

Use policy:

This item is brought to you by Swansea University. Any person downloading material is agreeing to abide by the terms of the repository licence: copies of full text items may be used or reproduced in any format or medium, without prior permission for personal research or study, educational or non-commercial purposes only. The copyright for any work remains with the original author unless otherwise specified. The full-text must not be sold in any format or medium without the formal permission of the copyright holder. Permission for multiple reproductions should be obtained from the original author.

Authors are personally responsible for adhering to copyright and publisher restrictions when uploading content to the repository.

Please link to the metadata record in the Swansea University repository, Cronfa (link given in the citation reference above.)

http://www.swansea.ac.uk/library/researchsupport/ris-support/

Asthma and Th2 immunity – the Impact of Genetic Factors and Obesity

A thesis submitted for the degree of
Doctor of Medicine
to Swansea University

by

Gwyneth Ann Davies, MB BCh, MRCP (UK)

School of Medicine
University of Wales Swansea

May 2007

UNIVERSITY OF WALES SWANSEA LIBRARY

ProQuest Number: 10798029

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 10798029

Published by ProQuest LLC (2018). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Thinking is more interesting than knowing,
but less interesting than looking.

GOETHE

Cyflwynir y gwaith yma i fy nheulu
am eu cefnogaeth bob amser

DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed ..... (candidate)

Date 28/07/08

STATEMENT 1

This thesis is the result of my own investigations, except where otherwise stated. Where correction services have been used, the extent and nature of the correction is clearly marked in a footnote(s).

Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

Signed ..... (candidate)

Date 28/07/08

STATEMENT 2

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed ..... (candidate)

Date 28/07/08

Abstract

Introduction: Th2 immune signalling is key to the pathological substrate of asthma. The primary objective of this thesis was to identify the predictive effects of genetic variants of Th2 signalling at the population level, and to examine interactions between different loci, with relation to asthma and associated phenotypes. This genetic epidemiological study provided an opportunity to explore also the predictive effects of different measures of obesity on these phenotypes.

Methods: An unselected population of 1614 young adults was recruited. Responses to validated questionnaires and spirometry provided clinical and physiological asthma phenotypes. Anthropometry provided the measures of obesity. Assays on venous blood provided immune phenotypes (IgE, eosinophils, eotaxin). Genotyping encompassed 22 polymorphisms in the *IL13*, *IL4RA* and *STAT6* genes, representing the Th2 immune signalling pathway. Data analysis used linear and logistic regression models, and a modified regression to address haplotypes.

Results: Single polymorphisms and haplotypes of *IL13*, *IL4RA* and *STAT6* loci significantly predicted asthma, eczema and hayfever at the population level. Several novel associations were shown for serum IgE levels and airflow obstruction. Significant interaction (epistasis) was identified between variants of *IL13* and *STAT6* for total IgE levels. Adiposity indices (BMI, waist circumference, body fat) showed consistent associations with asthma and airflow obstruction, but also with Th2 inflammatory markers (IgE, eotaxin and eosinophils) with modulation by sex/smoking status.

Conclusions: Genetic variants of Th2 immune signalling are one important source of risk for asthma and allergy in the general population, with variants operating both singly and in combination. Obesity and its causes are also likely to be significant contributors to the occurrence of asthma in the population, and may operate through pro-inflammatory mechanisms. These findings exemplify the polygenic and multifactorial determination of asthma and allergy, through genetic and environmental effects.

Acknowledgements

Diolch yn bennaf i Julian am ei holl gefnogaeth. Mae ei ddoethineb a'i frwdfrydedd dros ymchwil wedi bod yn ysbrydoliaeth. Ni allwn wedi gobeithio am well arweiniad gyda'r gwaith.

A heartfelt thank you to Mike Gravenor for his considerable contribution to this thesis. His encouragement and patience was very much appreciated.

I would like to thank the following for their help during the preparation of this thesis:

Maria Moller, for her support, and for supervising and assisting with the genotyping, and with data collection.
Cathy Thornton, for invaluable advice and for supervising and assisting with the eotaxin assays.

Divya Gopalakrishnan and Priyanka Bikhchandani for all the hours they spent helping with the data collection and laboratory work, and to Keiko Tanaka, Silas Benjamin, Mike Sansbury and Mark Roberts for their help also. Thanks to Mary Cratchley for administrative support.

Dr Ann Benton, for undertaking the eosinophil count measurements.
Professor Gareth Morgan for helping to facilitate the total IgE assays and to Graham Bishell for overseeing the measurement of total IgE.

Professor Rhys Williams, for his guidance on the obesity and epidemiology aspects.

A warm thanks to all the volunteers who took part in this study.

This thesis was supported by funding from the Stone-Mallabar foundation and Swansea Asthma Research Fund, with contributions from Llanelli Asthma Link, and my thanks for the generous donations received.

Table of contents

List of Figures

List of Tables

Abbreviations

AD	Atopic dermatitis
ADAM33	A disintegrin and metalloproteinase
AHR	Airway hyperresponsiveness
AR	Allergic rhinitis
Bp	Base pairs
BF	Body fat
BMI	Body mass index
BSA	Bovine serum albumin
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
ECRHS	European community respiratory health survey
ELISA	Enzyme-linked immunosorbent assay
EM	Expectation maximisation
EtBr	Ethidium bromide
FEF	Forced expiratory flow
FEV1	Forced expiratory volume in 1 second
FRC	Functional residual capacity
FVC	Forced vital capacity
GINA	Global initiative for asthma
HDM	House dust mite
HZ	Heterozygosity
IgE	Immunoglobulin E
IGF	Insulin-like growth factor
IL	Interleukin
ISAAC	International study of asthma and allergies in childhood
IUATLD	International union against tuberculosis and lung disease
L	Litres
LD	Linkage disequilibrium
LPS	Lipopolysaccharide

LREC	Local research and ethics committee
mRNA	Messenger ribonucleic acid
NCBI	National centre for biotechnology information
NHANES	National health and nutrition examination survey
OD	Optical density
OR	Odds ratio
PAPA	*Poblogaeth Asthma Prifysgol Abertawe* (Swansea University Asthma Population)
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEF	Peak expiratory flow
RAST	Radioallergosorbent test
Rpm	Revolutions per minute
RSV	Respiratory syncytial virus
SD	Standard deviation
SM	Smooth muscle
SNP	Single nucleotide polymorphism
SNS	Sympathetic nervous system
SPT	Skin prick test
STAT	Signal transducer and activator of transcription
TARC	Thymus and activation regulated chemokine
TGF	Transforming growth factor
Th	T helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
UCP	Uncoupling protein
UV	Ultra-violet
WC	Waist circumference
WHR	Waist/hip ratio

Introduction

Asthma is a common disease affecting 1 in 10 children and 1 in 13 adults in the UK and represents the commonest chronic disease of childhood [1]. Asthma is a chronic inflammatory disorder of the airways, characterised by variable airflow obstruction and an increase in airway responsiveness to a number of stimuli. Airflow obstruction is usually reversible, either spontaneously or with treatment. Patients present with episodic wheeze, breathlessness or cough. Subjects vary in the severity of disease, with some requiring only occasional inhaler use whilst others need regular medication to achieve disease control. The worldwide prevalence of asthma has been rising over recent decades, pointing to potent environmental effects, and has been mirrored by a similar rise in the prevalence of obesity [2-5]. The aetiology of asthma remains far from being fully understood. The origins of asthma lie early in life and include a vital genetic component. Environmental factors interact with underlying genetic susceptibility to initiate development of this complex trait. Atopy is the main predictor of asthma between the ages of 5 and 25 years, and is a T helper cell type 2 (Th2) driven hypersensitivity to innocuous antigens (allergens), clinically manifested by epithelial inflammation [6].

More than 60 studies have delineated chromosomal regions/candidate genes associated with asthma and its related allergic disorder of atopy, with heritability for asthma ranging around 50-60%. In a recent review, Cookson reported that at least 11 genome scans have between them shown ten chromosomal regions to be associated with asthma [7]. The evidence for a genetic contribution is strong, not only for asthma, but also for atopy, described in terms of elevated IgE levels.

Prospects of modifying the progress of asthma, through intervention, would be substantially advanced by early diagnosis or pre-diagnosis. Because of the substantial contribution of genetic variation to risk, useful prediction of disease might be achieved by genetic testing. Linkage studies and candidate-gene association studies indicate that asthma is a polygenic disorder. Meta-analysis of genetic association studies supports a contribution of common variants to the susceptibility to common disease and it is likely

that several genes contribute interactively to produce the asthmatic phenotype [8]. There is now overwhelming evidence that signalling by the Th2 cytokine interleukin (IL)-13 is a key molecular pathway driving bronchial inflammation in asthma [9]. This Th2 pathway involves IL-13, IL-4 receptor alpha (IL-4RA) and the transcription factor STAT6, signal transducer and activator of transcription.

Case-control studies have demonstrated an association between variants of *IL13*, *IL4RA* and *STAT6* and asthma and associated Th2 phenotypes [10, 11]. Recent work has demonstrated the importance of examining variants in a common pathway in conjunction with one another, when individual small changes can lead to a large combined effect [12-14].

Case-control studies have not allowed the predictive effects of variants to be fully estimated at the population level. The primary objective of this thesis was to determine which *IL13*, *IL4RA* and *STAT6* loci – acting singly and in combination - predict asthma phenotypes in a general population. The strength of these effects, and the interactions between different loci, will be clarified. A comprehensive genetic analysis will include all potential significant variants spanning each entire gene and examine gene-gene and gene-environment interactions. Novel epistatic interactions will be examined. Identifying the most important loci predicting asthma will advance the development of a pre-diagnostic genetic test for asthma and further our understanding of the molecular mechanisms underlying this disease.

The population study also provides an excellent opportunity to explore the relationship between different measures of obesity and the state of asthma, in addition to atopy and Th2 immune phenotypes. Therefore a further aim of the thesis is to examine the association between obesity, overweight and asthma and associated Th2 phenotypes. Alongside the worldwide increase in asthma prevalence has been a parallel obesity epidemic, leading to speculation on a causal link between these two complex traits. Obesity has important cultural and environmental causes – including the availability and character of foods – but genetic factors are also believed to contribute. There are few

well-characterised genetic causes for obesity as it occurs in the substantial minority (35%) of the general population. This contrasts with well-characterised loci such as leptin (LEP) and leptin receptor (Lep-R) which have been implicated in individuals with extreme, familial obesity [15, 16].

Cross-sectional and prospective studies have largely supported the association between asthma and obesity but not all studies have been consistent [5, 17, 18]. Investigators have predominantly used body mass index (BMI, weight/height^2) as a measure of obesity, with few using measures of central obesity (waist circumference, waist-hip ratio). In this thesis, appropriate measures of body mass and body composition are used, including percentage body fat and measures of central obesity in addition to BMI, to clarify the risk of asthma conferred by obesity in a young adult population.

The potential mechanistic basis for the apparent association between obesity and asthma has yet to be elucidated. Although emerging evidence points to the role of leptin (secreted by adipose tissue) as an inflammatory cytokine, recent work suggests that leptin does not explain the link between obesity and asthma [19, 20]. The Th2 chemokine eotaxin is also secreted by adipose tissue and a recent study found elevated eotaxin levels in a small group of obese humans with weight loss leading to reduced eotaxin levels [21]. This thesis examines whether adiposity associates with asthma and focuses on the effects of adiposity on associated Th2 immune phenotypes, namely IgE, eotaxin and eosinophil counts, in a large unselected population. The role of adipose tissue in these phenotypes will be further elucidated and the question of whether Th2 signalling provides a potential mechanistic basis for the relationship between obesity and asthma will be explored.

This thesis specifically addresses the relationships of both genetics and of obesity on Th2 phenotypes including clinical asthma and atopy, IgE, eotaxin and eosinophil counts in an unselected population.

Hypotheses

1) Genetic variants of Th2 signalling, acting singly and in combination, are important predictors of asthma and associated Th2 and physiological phenotypes in an unselected population.

2) Adiposity promotes asthma and its associated physiological and immune phenotypes.

The study of the association of Th2 signalling single nucleotide polymorphisms (SNPs) with clinical, Th2 immune phenotypes and physiological phenotypes is reported in Chapters 4-6. Chapter 5 reports epistatic interactions and haplotype associations between Th2 SNPs and clinical, immune and physiological phenotypes. The association of obesity with asthma, Th2 phenotypes and physiological phenotypes is described in Chapter 7.

CHAPTER 1

Background

1.1. Overview of the aetiology of asthma

1.1.1. Introduction

The origins of asthma are multifactorial, arising from a complex interaction of genetic and environmental factors. Genetic factors underlie the population *susceptibility* to asthma which is then induced by particular environmental stimuli. Genetic variation may influence asthma at a number of levels – such as resistance of the bronchial epithelial barrier to insult (e.g. allergen, virus); Th2 immunity (upregulated in asthma and atopy); and response of the whole airway to injury (relevant to airway remodelling). Atopy is an inherited tendency to develop IgE mediated allergic disease of the airways and skin, namely asthma, rhinitis, and eczema. Atopy mediates about 80% of childhood asthma and approximately 40–50% of adult asthma [22]. However, atopy is not synonymous with asthma, which occurs without atopy in up to 30% of cases.

The reasons for the recent impressive rise in asthma and atopy in Westernised countries are as yet unclear. It is likely that relevant environmental factors are exerting an influence early in life, in those with genetic predisposition. Theories include changes in microbial exposure in childhood, house dust mite sensitisation, diet and air pollution.

1.1.2 Genetic susceptibility

Human chromosomes

Asthma clusters in families and first-degree relatives of asthmatics have a significantly higher prevalence of asthma than relatives of non-asthmatics. Twin studies estimate the proportion of asthma due to genetic variation to be 50-60% [23]. Genome-wide screens have shown repeatable linkages of chromosomal regions with asthma and related traits of airway hyperresponsiveness (AHR) and atopy (total IgE levels, IgE to specific allergens, skin prick tests) (Figure 1.1). The most replicated linkages are to the following regions: 2p; 4q; 5q23-31; 6p24-21; 11q13-21; 12q21-24; 13q12-14; 16p12;

16q21-23; and 19q [10, 11]. Most of these chromosomal regions contain hundreds of genes, many of them potential asthma-susceptibility genes. For instance, the 5q region - implicated in phenotypes ranging from asthma and AHR to total IgE levels - contains the cytokine gene cluster and the gene coding for the B2-adrenergic receptor (Figure 1.2).

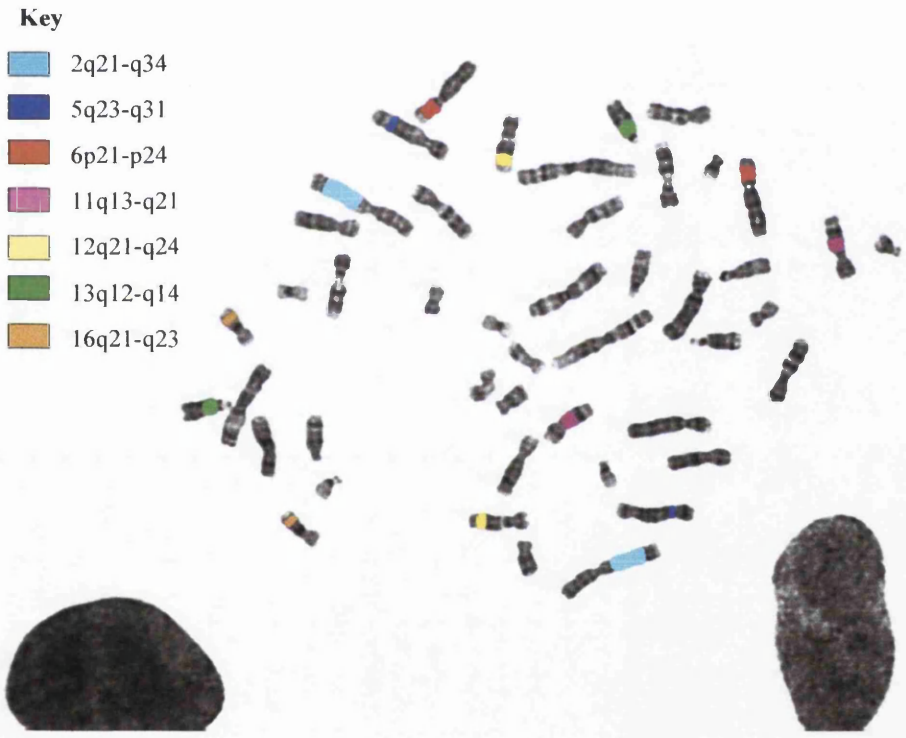


Figure 1.1 A diploid set of human chromosomes, with highlighted regions showing the most widely replicated linkage regions with asthma and related traits. Image by Dr Shareen H Doak, School of Medicine, University of Wales Swansea.

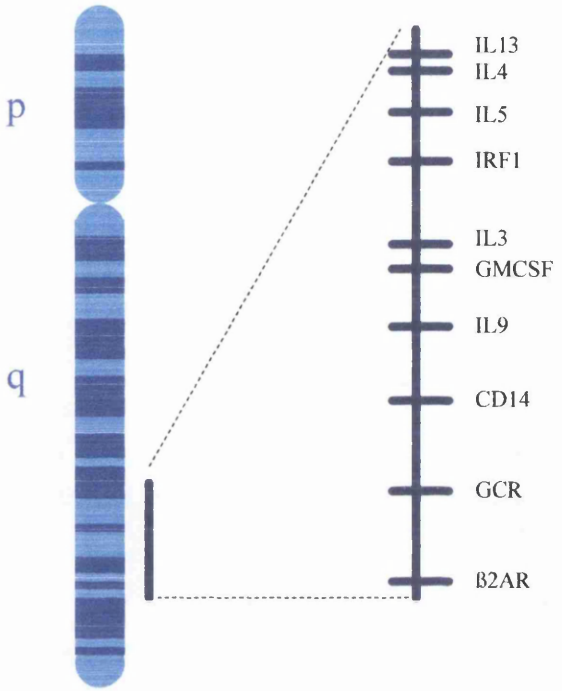


Figure 1.2 The cytokine gene cluster on chromosome 5q31-q33. This region contains several genes with well-replicated associations with asthma.

Major genes in asthma, AHR, IgE/atopy

Asthma is a polygenic disease. Variants in 80 genes have been associated with asthma or related traits, but it is likely that only a few genes are important, with several common variants each exerting a modest functional effect (Figure 1.3).

Genes associated with asthma and related traits include the cytokine cluster on chromosome 5 (*IL4*, *IL13*, *IL9*, *IL5*, *CD14* and *B2-adrenergic receptor*); the HLA and TNF genes on chromosome 6; the B chain of the high affinity IgE receptor and the Clara cell secretory protein on chromosome 11; and the alpha chain of the IL4 receptor on chromosome 16 [11, 24]. Associations are often not replicated in other populations, implying that different populations may have different asthma characteristics associated

with particular genetic markers.

Good examples of widely replicated associations are the genes coding for the IL-4/IL-13 pathway. This makes biological sense since IL-4 and IL-13 are important Th2 cytokines, central to the bronchial inflammation seen in asthma, with actions including isotype switching to IgE. Asthma/atopy phenotypes have been associated not only with variants in the *IL4/IL13* genes themselves, but also in their common receptor (*IL4RA*) and transcription factor *STAT6*. Interactions between these variants increase the risk of asthma and atopy further [13]. The *IL13* Gln110 variant reduces binding to a decoy receptor, with resultant increased IL-13 levels seen in asthmatics, demonstrating its functional effect [25]. The IL-13 signalling pathway will be discussed in further detail in the next part of this chapter.

Five novel candidate genes have been identified by positional cloning strategies over the last 3 years [26-30]. These fine-mapping techniques allow progress from broad chromosomal linkage regions to gene identification. These genes (*ADAM33*, *DPP110*, *PHF11*, *GPRA*, *HLAG*) are not involved in known asthma pathways. These genetic studies offer new insights into asthma pathogenesis, suggesting that pathways relating to tissue growth and remodelling may be important.

The genetic contribution to asthma is complex and involves polygenic inheritance and genetic heterogeneity (different combinations of genes causing asthmatic traits in different individuals). Gene-gene and gene-environment interactions underpin the aetiology of asthma, the complexity of which is just beginning to be unravelled.

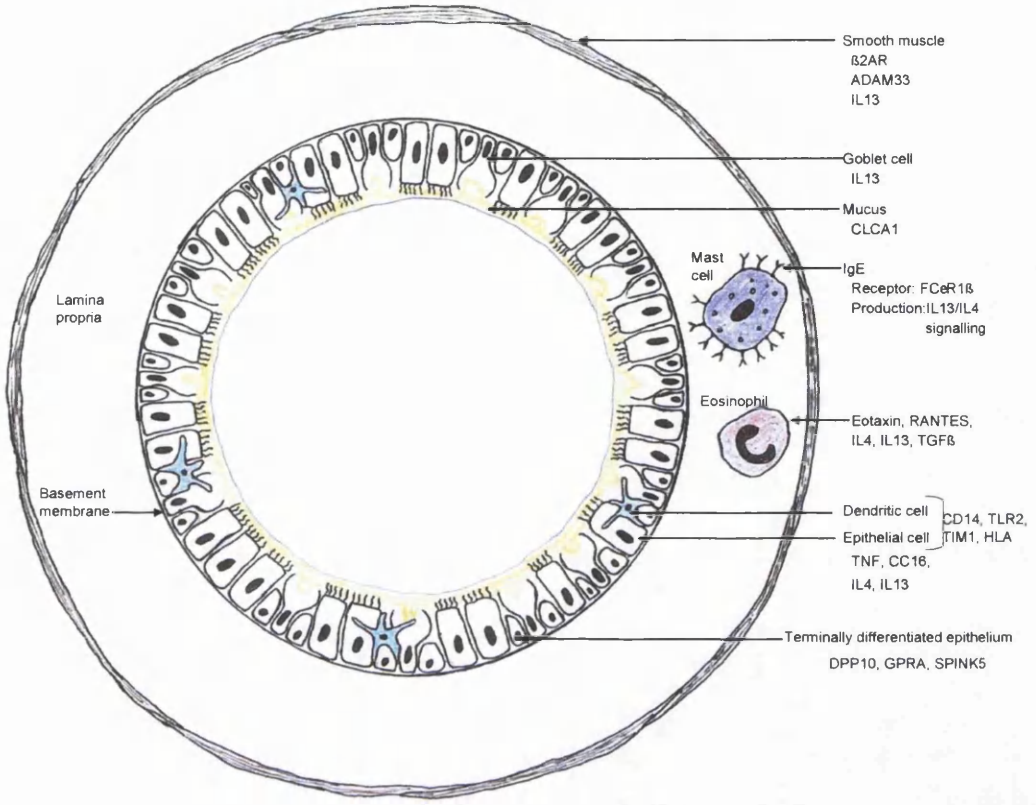


Figure 1.3 An airway diagram illustrating the proteins encoded by asthma-susceptibility genes. ADAM, a disintegrin and metalloproteinase; CLCA, calcium-dependent chloride channel; RANTES, regulated upon activation, normally T-expressed and secreted; TGF, transforming growth factor; TLR, toll-like receptor; TIM, T-cell immunoglobulin and mucin containing molecules; CC, clara cell; TNF, tumour necrosis factor; DPP, dipeptidyl peptidase; GPRA, G-protein-coupled receptor for asthma susceptibility; SPINK5, serine protease inhibitor, kazal type 5.

1.1.3 Environmental factors

Migrants moving to Westernised countries have an increased risk of acquiring asthma, emphasising the importance of environment in disease development [31]. Maternal asthma confers a greater risk of asthma than paternal asthma [32], and increased IL-13

production in cord blood has been shown to predict subsequent atopic disease, suggesting that the *in-utero* environment may account for later asthma [33]. Since the recent rise in asthma and atopy in the developed world has occurred over a matter of decades, environmental factors must be responsible – but which environmental factors have driven the rising prevalence in asthma?

Common allergens

Allergens are defined as antigens that promote IgE sensitisation. They are diverse in origin and character and unified by their resistance to heat and protein digestion. They can act as proteases (house dust mite antigens); non-specific lipid transfer proteins (certain food allergens); or gelsolins that interfere with the actin cytoskeleton of cells. Allergens important in asthma are common antigens which are efficiently delivered into the airways within respired particles. Allergen proteases can trigger epithelial cells directly or via protein activating receptors (especially PAR-2), releasing Th2 cytokines and chemokines.

House dust mite (HDM) accounts for the highest sensitisation rates in the UK, followed by cat and grass pollen. Other common aeroallergens include tree pollens, dog, and the moulds *Aspergillus fumigatus* and *Alternaria alternata*. In the humid south-eastern American states, cockroach sensitisation is more common.

Role of allergen sensitisation

Allergens penetrate the mucosal epithelial lining where they induce the allergic response. Sensitisation is defined by positive skin prick tests or specific IgE directed against common allergens. Early allergic sensitisation, particularly to indoor allergens,

is a major risk factor for the development of asthma in genetically susceptible individuals. HDM sensitisation is a significant risk factor for the development of asthma, hayfever and eczema [34]. Furthermore, sensitisation to common aeroallergens is associated with increased AHR [34, 35].

The highest risk of childhood asthma has been seen in children sensitised very early in life [34]. However this was only true for children with a family history of asthma/atopy leading to speculation about whether the relationship between allergic sensitisation and asthma is actually causal or represents parallel pathways arising from common genetic origins.

Role of allergen exposure

The development of allergen sensitisation requires exposure to that allergen. Once sensitisation has occurred, repeated exposure to that allergen is likely to trigger symptoms. Though HDM levels are related to mite sensitisation and wheezy illness [36], rising HDM levels are not felt to account for the general increased prevalence of atopic disease. Asthma prevalence has also risen in hot dry areas where mite levels are low. It seems unlikely that increased allergen exposure accounts for the rise in atopy, since sensitisation to all allergens has increased simultaneously, suggesting a generally heightened allergic response [37]. Higher allergen exposure may mean more sensitisation to that allergen but does not necessarily translate into increased childhood asthma [38].

Having a cat or dog at home may actually be protective against the development of atopy and wheeze [39-41]. Hence the relationship between allergen exposure and asthma is far from being completely resolved. Whilst cats have been shown to be

protective against wheeze in children *without* a maternal history of asthma, risk of wheeze was *increased* in those *with* a maternal history, indicating the importance of gene-environment interactions [41].

Finally, primary prevention studies involving reduction of HDM exposure have been disappointing [42]. Allergen exposure is not a proven risk factor for childhood asthma. Allergen exposure may increase the risk of atopic disease, but outcome is highly dependent on dose, type of allergen, age at exposure and host susceptibility [43].

1.1.4 Occupational Asthma

Occupational irritants can worsen pre-existing asthma. Occupational asthma per se is asthma caused by exposure to a particular occupational agent. These agents can be divided into reactive chemicals (e.g. isocyanates) or organic antigens (e.g. wheat flour). For both, the pathological and physiological changes in the airway are similar, but the mechanisms underlying the development can be different. Organic antigens promote IgE response. Reactive chemicals act in various ways - the acid anhydrides produce haptens with native proteins, resulting in IgE production to these complexes. The mechanism for the highly reactive isocyanates remains unclear. High exposures to occupational agents increase the risk of asthma. Other risk factors interact e.g. smoking increases the risk of isocyanate occupational asthma. Pre-existent atopy predisposes to occupational asthma from organic antigens (e.g. flour). HLA Class 2 variation influences risk of response to reactive chemicals (e.g. DQB1 for isocyantes; DRB1 for Western Red Cedar).

1.1.5 Role of air pollution

Air pollutants such as ozone can exacerbate asthma. It is less clear whether air

pollutants - including nitrogen dioxide, ozone, sulphur dioxide and particulate carbons - contribute to the development of asthma [44-48]. Declining air pollution in Western industrialised countries has occurred concurrently with increasing asthma prevalence which argues against a strong effect on asthma causation. Asthma rates were lower in former East Germany (with greater air pollution) compared with West Germany, with similar findings from large surveys elsewhere [49, 50].

Pollution is unlikely to be a primary cause of asthma but epidemiological and experimental evidence suggests that diesel exhaust particulates and ozone promote allergen sensitisation. Individuals with particular genotypes related to antioxidant defences (e.g. glutathione S-transferase variants) may be more susceptible to the effects of pollution [44, 45, 51].

1.1.6 Role of smoking

Exposure to tobacco smoke *in-utero* is associated with increased risk of allergic sensitisation and asthma. Passive smoking - especially maternal smoking - confers an increased risk of wheezing and/or asthma at least until a child reaches 7 years and active smoking increases the risk of adult-onset wheeze [52-54]. If the father also smokes, the risk of childhood wheeze increases but maternal smoking confers the greatest risk [54]. A recent study has shown that parental smoking impairs vaccine responses in children with atopic genotypes, highlighting the importance of gene-environment interactions which underpin the aetiology of asthma and atopy [55].

1.1.7 Role of diet

Change in the Western diet, with reduced intake of antioxidants, has led to speculation

on the role of nutrients (including antioxidant vitamins, omega-3 fatty acids, selenium, magnesium, sodium and zinc). Some cross-sectional studies support an association but there is no conclusive evidence (Table 1.1) [56-59].

Early life studies show breast-feeding to reduce eczema but remain inconclusive regarding protection against asthma [60]. Diet in pregnancy may affect the developing immune system and omega-3 fatty acid supplementation has shown beneficial effects [61-63].

An "obesity epidemic" has paralleled the rise in asthma in the Western world. An increased prevalence of asthma has been observed in overweight individuals, especially females [5, 17]. Weight loss trials have shown a reduction in asthma signs and symptoms but may be confounded by related factors of diet and physical activity [64-66]. The effects of obesity on asthma, lung function and Th2 immunity are examined in this thesis in Chapter 7. The potential mechanisms underlying any association between obesity and asthma are discussed in part 1.3 of this chapter.

Table 1.1 A summary of the evidence on the role of dietary nutrients in asthma [56-59]

Nutrient	Cross-sectional	Case-control	Longitudinal	Interventional
Vitamin C	↓ risk of asthma Mixed results	Protective Mixed results	No effect on asthma incidence	Limited evidence of protection, given with other antioxidants
Vitamin E	No effect	Protective Mixed results	Limited evidence of protection	Limited evidence of protection, given with other antioxidants
Vitamin A or beta-carotene	Inconclusive	Protective Mixed results	No effect on asthma incidence	Limited evidence of protection, given with other antioxidants
Omega-3 fatty acids	N/A	Limited evidence of protection	No effect on asthma incidence	Protective Mixed results
Selenium	No effect	Protective	N/A[1]	No effect
Magnesium	Protective	Protective	N/A	No effect
Sodium	Mostly no effect	Limited evidence of ↑ airway reactivity	N/A	Some evidence of ↑ risk

[1] N/A, Not applicable

1.1.8 Role of infection

The "Hygiene Hypothesis"

The pattern of microbial exposure in childhood has changed considerably in Westernised countries, as a result of a cleaner environment, widespread antibiotic use and immunisations. The "hygiene hypothesis" suggests that lack of exposure to childhood infection, endotoxin and microbial products causes persistence of Th2 responses, thus increasing the likelihood of atopic disease [67-69].

Observations supporting the hygiene hypothesis are that asthma and atopy are:

1 less common in children with more siblings or in those attending day-care, presumably due to increased exposure to childhood infections [70-73],

2 strikingly less common in children living on livestock farms, who have a higher exposure to endotoxin [74-76],

3 more common in Westernised countries.

Further refinements to the hygiene hypothesis stem from the suggestion that specific infections occurring at critical times in immune development are most important:

1 *Mycobacterial infection.* Some epidemiological studies have suggested an inverse relationship between mycobacterial exposure and asthma/atopy [77, 78].

2 *Intestinal flora.* The gut is a critical regulator of the immune response and an adequate intestinal flora may be important in guarding against atopy [79]. This is supported by the association between enteric infection (Hepatitis A), family size and absence of atopy in Italian recruits [80]. Probiotics studies are underway, with one showing reduced risk of eczema by age 2, but no change in allergic sensitisation nor asthma [81].

3 *Helminth infection.* The Th2 response forms the basis of the natural immune response against parasites, with increased IgE production and eosinophilia. Interestingly, worm infestation appears to be protective against asthma despite the marked Th2 response common to both [82-84]. The STAT6 variant G2964A and IL-13 variant -1024C/T – both associated with asthma - have been shown to be protective against ascaris and schistosoma infestation respectively and the survival advantage conferred may explain the persistence of these alleles in the population [84-86]. Moreover, anti-helminth treatment appears to increase allergen sensitisation [87]. One explanation is that helminths stimulate exuberant IL-10 production which reduces

specific IgE to aeroallergens, thus causing deviation away from an atopic phenotype [88].

Toll-like receptors (TLRs) are recognised to be key molecules in innate and adaptive immunity [89]. These are pathogen associated molecular pattern receptors that recognise extracellular microbes and trigger anti-pathogen signalling cascades. TLR activation has been shown to induce antigen-presenting cells to produce cytokines that favour Th1-type immune responses. TLRs, such as TLR9, TLR7, and TLR8, are considered to be good potential target candidates to modulate immune responses. TLR ligands, such as CpG DNA, are effective adjuvants, and induce downregulation of both IL-5 and eosinophilia, and their relation to allergen epitopes could offer opportunities for the development of allergen-specific immunotherapy [90]. There is evidence to suggest that reduced microbial stimulation of TLRs in early life, may lead to a weaker Th1 response and a stronger Th2 response to allergens. The individual immunological response is shaped by the interplay between the dose and timing of endotoxin exposure, other environmental factors and genetic predisposition.

1.1.9 Role of viruses

The sensitive method of reverse transcriptase-DNA amplification, detecting viruses in respiratory materials, emphasises the role of viral infections in exacerbations of asthma (85% in children, 70% in adults) [91, 92]. Asthmatic respiratory epithelial cells appear more susceptible to viruses, though it is unclear whether this is a primary epithelial defect or the result of Th2 induced inflammatory damage. Viral infection promotes further inflammation in the airway - with prominent actions from NF Kappa B and AP1, and specific enhancement of Th2 induced inflammation by CD8+ T-lymphocytes and chemokines CCL3 and CCL5.

It has been suggested that viral infections, in particular respiratory syncytial virus

(RSV), act as an independent risk factor for asthma and atopy [93-95]. Recent work suggests a common genetic background in children with severe RSV infection and bronchial asthma, with an association found between IL13 variant -1024C/T and severe RSV infection, with stronger associations seen for certain haplotypes [96]. Bronchiolitis associated with viruses apart from RSV may be associated with an even greater risk of childhood asthma [97].

This would appear to contradict the hygiene hypothesis which suggests childhood infections to be protective against the development of asthma. However, since RSV infection is almost universal by the age of 2 years, host susceptibility must play a part in which children go on to develop asthma. Fascinating interactions between genes and environment may explain these apparent paradoxes e.g. differential responses of specific genotypes to viral infections and day-care [98]. Another explanation is that viral infection with wheeze acts as a marker for asthma and reflects impaired viral handling by the asthmatic lung [99-101]. Studies are ongoing to answer this intriguing question.

1.1.10 Role of the epithelial mesenchymal trophic unit

Whilst the central role of Th2 inflammation is undisputed, bronchial remodelling represents a crucial component of bronchial hyper-reactivity in established asthma, and is an important cause of declining lung function with time [102]. This remodelling includes epithelial goblet cell hyperplasia, thickening of the basement membrane by collagen deposition and smooth muscle hypertrophy. Is it simply the result of chronic Th2 inflammation or has it a more primary causation? The interaction between epithelium and mesenchyme is exemplified by transforming growth factor (TGF) beta 2 release from injured or stimulated epithelial cells, which acts on fibroblasts to cause myofibroblast development and smooth muscle hypertrophy. Variants of ADAM33 (a zinc dependent metalloprotease) are strongly linked with bronchial reactivity, and this

protein is specifically expressed in smooth muscle and myofibroblasts - therefore innate susceptibility within the mesenchyme may be a primary disposer towards remodelling [103]. Hence the epithelium and the mesenchyme together play important mechanistic roles in asthma, complementing Th2 immune mechanisms and IgE sensitisation.

1.2 Genetic epidemiology of the IL-13 signalling pathway in asthma and atopy – an overview of the evidence

1.2.1. Introduction

An overview of the genetic basis of asthma has already been provided in part 1.1. Genetic factors at a number of loci have been associated with the disease. Some of the most replicated loci are genetic variants in the IL-13 signalling pathway. Here there will follow a more detailed summary of the current state of knowledge on this Th2 signalling pathway in asthma and atopy, in terms of evidence from immunological and genetic studies.

1.2.2. The role of the IL-13 signalling pathway in asthma and atopy

There is now overwhelming evidence that signalling by the Th2 cytokine IL-13 is a key molecular pathway driving bronchial inflammation in asthma. IL-13 promotes IgE isotype switching, CD23 induction, epithelial cell damage, stimulation of eosinophils, goblet cell hyperplasia with mucus production and bronchial smooth muscle activity [9]. IL-13 also stimulates airway fibrosis, mainly by the induction of matrix metalloproteinases 9 and 12 which activate pro-fibrotic transforming growth factor-β1 (TGF-β1) [104]. Although exerting many similar biological activities to IL-4, IL-13 has several unique activities. Unlike IL-4 deficient mice, IL-13 null mice fail to generate goblet cells, fail to recover basic IgE levels after IL-4 stimulation and fail to clear helminth [105]. Recent murine studies on dendritic cells suggest that IL-4 promotes the secretion of Th2 cytokines, whereas IL-13 acts by suppressing T cell secretion of IFN-gamma, an inhibitory cytokine for the Th2 immune pathway [106].

The *IL13* gene is located only 25 kilobases away from the *IL4* gene which is in the same orientation, leading to the suggestion that these genes may have arisen from a duplication event during evolution. IL-13 and IL-4 share a common receptor chain, IL-4 receptor alpha (IL-4RA), giving rise to the overlapping functions of these two

cytokines. IL-13 acts via a heterodimer receptor composed of IL-4RA and IL-13RA1 and intracellular signalling is initiated through the Janus tyrosine kinase mediated phosphorylation of signal transducer and activator of transcription (STAT)6 (Figure 1.4). STAT6 plays a crucial role in this pathway, by transducing the signal to the nucleus: once phosphorylated, STAT6 molecules form homodimers, which penetrate the nucleus, activating the transcription of target genes involved in the multiple functions of IL-13 [9]. STAT6 is not only involved in the regulation of its own pathway but is also a key regulator of Th2 immune responses, activating several Th2 specific gene promoters. STAT6 deficient mice lack IgE production, have an impaired proliferative response to IL-4 and cannot differentiate naïve T cells into Th2 cells [107].

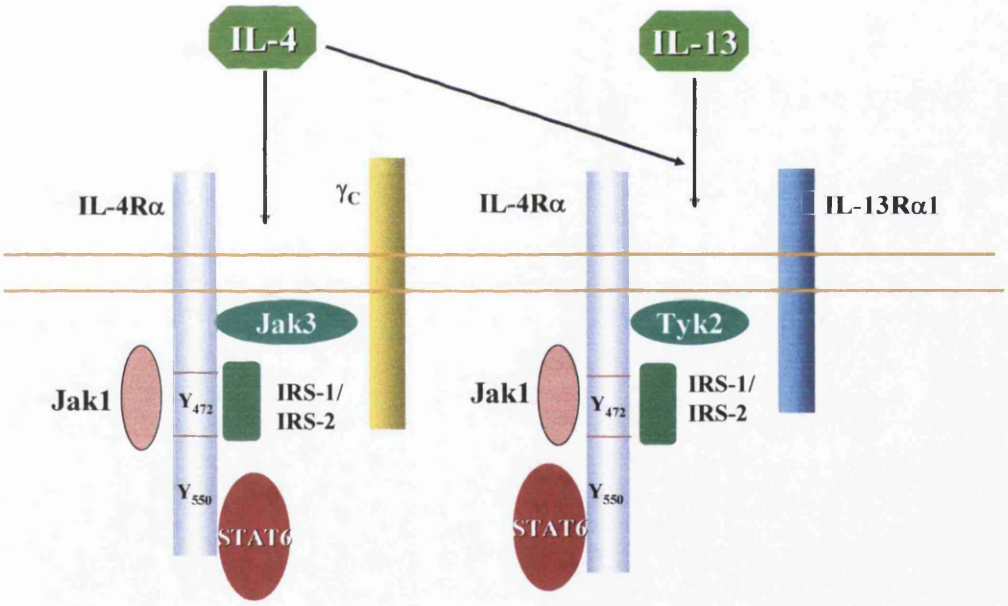


Figure 1.4 Diagram of the IL-13 signalling pathway. IL-4 and IL-13 share a common receptor, the alpha chain of the IL4 receptor (IL4RA). Phosphorylation of the kinases (Jak, Janus kinase; Tyk, tyrosine kinase) and IRS (insulin receptor substrate) results in activation of STAT6, signal transducer and activator of transcription which transduces the signal to the nucleus, leading to the transcription of multiple genes involved in asthma pathogenesis.

1.2.4. Genetic studies of the IL-13 signalling pathway in asthma and atopy

Genome wide screens have revealed repeated linkages to the chromosomal regions housing genes along the IL-13 signalling pathway. Linkages have been found with the regions 5q23-31 (including the Th2 cytokine gene cluster); 12q21-24 (including signal transducer and activator of transcription-6: *STAT6*); and 16p12 (including IL4 receptor alpha: *IL4RA*) [10, 11].

Candidate gene association studies have demonstrated an association between variants of *IL13*, *IL4Rα*, *STAT6* and asthma phenotypes and these studies are summarised in Tables 1.2-1.4. However, many case-control studies have been conducted in small samples and findings have not been replicated in further studies [108-110]. Publication bias is likely to lead to the reporting of initial striking positive findings in small case-control studies, which fail to be replicated in larger populations, emphasising the importance of replication in an unselected population study to confirm true positive associations with disease [111, 112].

IL13

The human *IL13* gene on chromosome 5q31 encompasses 2938 bp and includes four exons, 56 bp of 5'UTR and 828 bp of 3'UTR. Arg130Gln (+2044G/A) is the only coding SNP in a block of common SNPs in almost complete linkage disequilibrium (LD), which spans the third intron, the fourth exon, and the 3′ UTR of the gene. Two SNPs in the promoter (–1512A/C and –1024C/T) are also in strong, but not complete, LD with the distal polymorphisms [113].

Association studies examining the *IL13* SNPs considered in this thesis are summarised in Table 1.2. Van der Pouw Kraan et al first described a C/T nucleotide exchange at position -1055 in the *IL13* promoter region and found that the T/T genotype associated with allergic asthma, altered regulation of IL-13 production and increased binding of nuclear proteins [99]. This variant - located in a region containing a nuclear factor of activated T cells transcription factor binding site - was subsequently described at

position -1111 (or -1112) and reported in association with asthma, bronchial hyper-responsiveness and skin test positivity [114]. Hummelshoj et al then reported the variant to be located at position -1024 relative to ATG translation initiation codon, with an association between the T/T genotype and inhalation allergy [115]. The -1512 A/C promoter polymorphisms has been described in association with IgE levels in various populations (see Table 1.2).

In vivo, *in vitro*, and *in silico* analyses have shown that the *IL13*-1024T allele (found to be the ancestral allele) enhances *IL13* promoter activity in primary human and murine CD4(+) Th2 lymphocytes, and attenuates STAT6-mediated repression of *IL13* transcription [116]. IL-13 secretion was increased in *IL13*-1024T/T homozygotes and it is interesting to note strong associations between *IL13*-1024T and protection from Schistosoma hematobium in Africa [85], lending support to the hypothesis that asthma susceptibility variants arise in part due to an evolutionary protection against worm infestation (discussed further in section 1.1.8).

To date, the only coding variant in *IL13* results in an amino acid change from arginine to glutamine at position 110 (Figure 1.5). Several studies have reported positive associations between Arg110Gln (R110Q) and asthma and various atopic markers, but other studies show conflicting results. Heinzmann et al found Arg110Gln to be associated with asthma rather than IgE levels in case-control populations from Britain and Japan and the variant predicted higher serum IL-13 levels in a general Japanese paediatric population [117].

Molecular modelling studies have found the Arg110Gln variant to be crucial to ligand receptor interactions and functional studies have demonstrated Arg110Gln to be significantly more active than wild-type IL-13 in inducing STAT6 phosphorylation and CD23 expression in monocytes and hydrocortisone-dependent IgE switching in B cells [117]. Furthermore, mutant IL-13 was neutralized less effectively than wild-type IL-13 by an IL-13R2 decoy, which could contribute to its enhanced in vivo activity [118]. This variant has also been shown to upregulate IL-13 concentration *in vivo* [25].

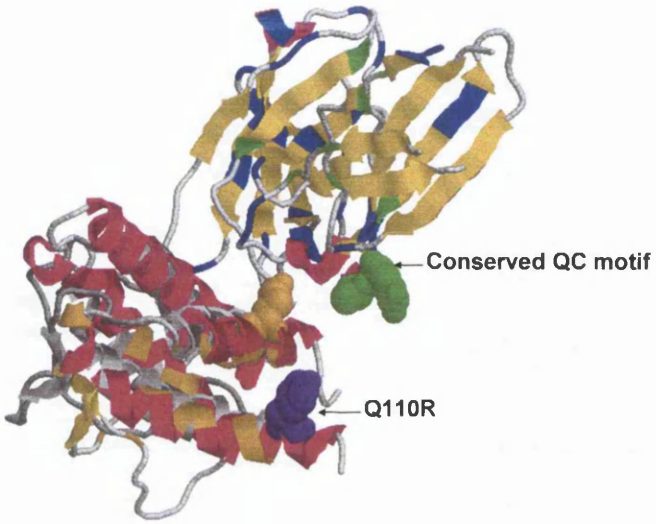


Figure 1.5 Molecular model of the IL-13 cytokine-receptor complex. This illustrates the proximity of the Arg110Gln (R110Q) polymorphism to the binding regions of the IL-13 receptor (pink). On the receptor (yellow), a conserved QC motif (at positions 181-182 in IL-4RA, positions 80-81 in IL-13RA1, and positions 86-87 in IL-13RA2), is likely to be involved in cytokine binding. The variable residues surrounding this motif provide the basis for the different affinities observed between different receptor chains and IL-13 variants. Molecular modelling by Dr Jonathan Mullins, School of Medicine, University of Wales Swansea.

IL4RA

The human *IL4RA* gene on chromosome 16p12.2 encompasses 50374 bp and is highly polymorphic, including 12 exons, with 13 polymorphisms that result in amino acid substitutions. Three of these SNPs are associated with functional alterations of the receptor. Association studies examining the *IL4RA* SNPs considered in this thesis are summarised in Table 1.3.

Mitsuyasu et al found that the Ile50 variant (of an amino acid in the extracellular part of the receptor subunit) associated with atopic asthma and IgE in a Japanese case-control population. Furthermore, they demonstrated that Ile50 augmented STAT6 activation and increased IgE production [119]. However, other case-control studies have shown inconsistent results (Table 1.3). The Arg551 variant has been associated with increased CD23 expression in peripheral blood mononuclear cells after IL-4 challenge [120]. However Kruse et al showed that the Pro478 and Arg551 variants – in the intracellular part of the receptor – associated with *lower* IgE levels and *downregulated* signal transduction [121]. Studies in other populations have shown the commoner Ser478 allele to be associated with higher IgE, asthma and atopy [13, 122]. Despite some conflicting results, there is now a robust body of evidence that the *IL4RA* locus has a role in IgE regulation, and specific alleles are likely to have different effects in different ethnic populations, depending in part on allele frequency.

STAT6

Human *STAT6* (12q13.3-14.1) spans 19 kb of genomic DNA containing 23 exons. The first 2 exons of the *STAT6* gene are non-coding regions. The AUG translation initiation codon, encoding the first amino acid, is located within exon 3 [123]. During the progress of this thesis, four non-synonymous substitutions have been identified but none are common (http://www.ncbi.nlm.nih.gov/projects/SNP).

In comparison with *IL13* and *IL4RA*, there have been relatively few case-control association studies on *STAT6* variants, with several of these studies having negative findings. Association studies examining the *STAT6* SNPs considered in this thesis are summarised in Table 1.4. Gao et al demonstrated the association of the 3'UTR G2964A variant with atopic asthma in a Japanese population [124]. This association was not replicated among the British patients, nor in later studies on Japanese populations and German/Swedish sibpairs [125-127]. Another 3'UTR variant (rs1059513) has been associated with IgE but not examined in relation to asthma [128]. Intronic variants C2892T and C1570T have been associated with IgE levels rather than asthma [127-

129]. A GT repeat polymorphism in exon 1 was associated with eosinophil count in the German/Swedish study and with allergic diseases and childhood asthma in Japanese studies [126, 127, 130, 131].

Genetic studies using unselected populations

There have been few unselected population studies to ascertain which loci may be of clinical use in predicting asthma, in comparison to case-control studies. Case-control studies provide an indication of some of the loci contributing to asthma susceptibility but only unselected population studies can estimate the actual risk conferred by a single variant, or combinations of variants, at the population level.

A recent analysis of the 1958 British Birth Cohort has robustly confirmed several *IL13* variants - including promoter polymorphisms and Arg110Gln – to be associated with IgE levels [132]. A large German cross-sectional paediatric study showed the *IL13* -1024C/T promoter variant and *STAT6* variant C2892T to be associated with IgE levels with the *IL13* variant also associated with asthma, with synergistic effects from combinations of loci in this signalling pathway [12]. A German adult study also showed C2892T was associated with IgE levels [129].

Epistatic effects

Recent work has demonstrated the importance of examining loci in the same pathway in combination, since small individual effects on phenotype may combine to produce much larger synergistic effects. Kabesch et al found that by combining polymorphisms in 4 major IL-4/IL-13 signalling pathway genes, the risk for high serum IgE levels increased 10.8-fold and the risk for the development of asthma increased by a factor of 16.8-fold compared with the maximum effect of any single polymorphism [12]. Chen et al demonstrated functional synergistic effects of *IL13* and *IL4RA* polymorphisms on IL-13-dependent gene induction, when both variants occurred in combination [133]. Thus variants may only be relevant to population risk when occurring in combination. This

may be true not only for gene-gene (epistatic) interactions but also for haplotype combinations on the same gene. Risma et al found that the association of two common *IL4RA* variants (Val50/Arg551) with atopic asthma was greater than for either allele alone, the association for Arg551 being dependent on the presence of Val50 [14]. Epistatic and haplotype effects will be discussed further in Chapter 6.

Table 1.2 Summary of known heterozygosity and associations for selected *IL13* SNPs.

	Location	*HZ*[1]	*Population*	*Associations*
SNP 10 rs1881457	5'UTR - 1512A/C	.25	British	IgE [132]
			Japanese	Eosinophils Negative for AD[2], IgE [134]
			German & American	IgE (only in SPT[3] negative group) [113]
SNP 1 rs1800925	5'UTR - 1024C/T	.39	British	IgE [132]
			Dutch	Allergic asthma [135] Asthma, AHR[4], SPT Non-significant for IgE [114]
			Icelandic	Negative for atopic asthma [136]
			Danish	Inhalation allergy, AD [115] (IgE,RAST[5],SPT)
			German	Asthma [12] Total IgE [12, 137], RAST [138]
			German & American	IgE (only in SPT[3] negative group) [113]
			European American	Negative: IgE [139]
			White Canadian	Negative: atopy, AD [140]
			African Americans	Asthma/ atopy [141]
			Chinese	RAST (house dust mite) Negative for total IgE, AR[6] [142], AD [143]
			Japanese	Negative for AD, eosinophils, IgE [134]
SNP 7 rs2066960	Intron 1	.22	British	IgE [132]
SNP 2 rs1295686	Intron 3	.45	British	IgE [132]
			Icelandic	Negative for atopic asthma [136]
			European American	Negative: IgE [139]

	Location	*HZ*[1]	*Population*	*Associations*
SNP 5 rs20541	Exon 4 R110Q	.32	British	IgE [132]
			British	Asthma, negative for IgE [117]
			Japanese	
			Hutterites	Negative for asthma/atopy (SPT) [144]
			Icelandic	Negative for atopic asthma [136]
			Dutch	Negative for asthma, AHR, SPT, IgE [114]
			German	AD , IgE [145]
			German & American	IgE (only in SPT[3] negative group) [113]
			American	Atopy (grouped: eosinophils, IgE, SPT), eosinophils [110], IgE (aged 1 year) [139]
				Negative for asthma, lung function, AHR [110]
			White Canadian	Atopy, AD [140]
			Costa Rican	Negative for asthma, IgE, SPT [146]
			Chinese	Total IgE [142, 143, 147]
				Negative for asthma, AD [143], RAST, AR[6] [142]
			Japanese	AD
				Negative for eosinophils, IgE [134]
			Korean	Asthma [148]
SNP 13 rs1295685	3'UTR	.26	Dutch	Asthma, SPT Negative for AHR, IgE [114]
SNP 6 rs848	3'UTR	.45	Icelandic	Negative for atopic asthma [136]
SNP 9 rs847	3'UTR	.33		

[1] HZ, Heterozygosity on SNP database http://www.ncbi.nlm.nih.gov/projects/SNP
[2] AD, atopic dermatitis
[3] SPT, skin prick tests
[4] AHR, airway hyperresponsiveness
[5] RAST, radioallergosorbent test
[6] AR, allergic rhinitis

Table 1.3 Summary of known heterozygosity and associations for selected *IL4RA* SNPs

	Location	*HZ*[1]	*Population*	*Associations*
SNP 3 rs2283563	5'UTR	.44		
SNP 5 rs1805010	Exon 5 I50V	.50	British	Negative: IgE [132]
			German	IgE: in haplotype with SNP 12 & 13 [149] Negative: RAST[2] [138], IgE [12, 137], asthma [12]
			Danish	Negative: RAST [150]
			Italian	Negative: atopic asthma, AHR[3], IgE, SPT [109]
			European American	Negative: IgE (aged 1 year) [139]
			Mexican	Negative: atopic asthma [151]
			Asian (mixed)	IgE (Malay; negative in Indian/Chinese) [152]
			Chinese	Negative: asthma, IgE [153]
			Singapore (mixed)	Negative: IgE, RAST, SPT[4,5] [154]
			Japanese	Atopic asthma [119, 155], IgE [119], cedar pollinosis [156], Negative: atopic asthma [108], Negative: AD[6] [157, 158]
			Korean	Negative: atopic asthma, AHR, IgE, eosinophil (%) [159]
SNP 7 rs3024622	Intron 7	.47		
SNP 8 rs2891058	Intron 8	.46		
SNP 12 rs1805015	Exon 12 S478P	.30	British	Negative: IgE [132]
			Dutch	Asthma, AHR, IgE, SPT [13]
			German	IgE [121] IgE: in haplotype with SNP 12 & 13 [149] Negative: RAST [138]
			Italian	Negative: atopic asthma, AHR, IgE, SPT [109]
			European American	Negative: IgE (aged 1 year) [139]
			Korean	Negative: atopic asthma, AHR, IgE, eosinophil (%)[159]

	Location	*HZ*[1]	*Population*	*Associations*
SNP 13 rs1801275	Exon 12 R551Q	.47	British German	Atopic asthma [[160], IgE [132] Asthma [161], IgE [121] IgE: in haplotype with SNP 5 & 12 [149] RAST (to cat, if maternal smoking; negative for other RAST) [138]
			Danish	Negative: RAST [150]
			Italian	Negative: atopic asthma [109, 162], AHR, IgE, SPT [109]
			Spanish	Negative IgE (except in subgroup with family history) [163]
			Canada	FEV1 [164]
			American	Asthma (and severity) [165] AD, Hyper-IgE syndrome, IgE/RAST [120]
			European American	Negative: IgE (aged 1 year) [139]
			Chinese	Atopic asthma [166], asthma [167] Negative: IgE [167], AD [143]
			Japanese	AD [157] Negative: Asthma, IgE/RAST [108], Negative: Atopic asthma [155], AD [158]
			Asian (mixed)	Negative : asthma, IgE [167]
			Korean	Atopic asthma, AHR, eosinophil (%), negative for IgE [159]
SNP 16 rs1049631	3'UTR	.49		

[1] HZ, Heterozygosity on SNP database http://www.ncbi.nlm.nih.gov/projects/SNP
[2] RAST, radioallergosorbent test
[3] AHR, airway hyperresponsiveness
[4] SPT, skin prick tests
[5] Except in Indian subgroup where Val50 associated with atopy
[6] AD, atopic dermatitis

Table 1.4 Summary of known heterozygosity and associations for selected *STAT6* SNPs

	Location	*HZ*[1]	*Population*	*Associations*
SNP 8 rs324011	Intron 2 C2892T	.37	German Swedish	IgE [127-129] Negative: asthma [12, 127] Negative: asthma, IgE [168]
SNP 12 rs841718	Intron 16	.47	German	IgE [127] Negative: asthma [127]
SNP 11 rs3024974	Intron 17 C1570T	.19	German	IgE [127] Negative: IgE (positive in haplotype) [128, 129]
SNP 1 rs324015	3'UTR G2964A	.45	British German Swedish Japanese	Negative: atopic asthma [124] Nut allergy [169] Negative: asthma, IgE [127] Negative: asthma, IgE [168] Atopic asthma [124] Negative: allergic disease [125]
SNP 2 rs703817	3'UTR	.49	Swedish	Negative: asthma, IgE [168]
SNP 3 rs1059513	3'UTR	.15	German	IgE [128] Weak AHR[1], negative IgE [127]
SNP 4 rs4559	3'UTR	.42	German Swedish	Weak: IgE [127] Negative: IgE (positive in haplotype) [129] Negative: asthma, IgE [168]

[1] HZ, Heterozygosity on SNP database http://www.ncbi.nlm.nih.gov/projects/SNP
[2] AHR, airway hyperresponsiveness

1.3 Obesity and asthma: potential underlying mechanisms linking two common complex traits

Evidence for the association between obesity and asthma will be summarised in Chapter 7. In this section I will provide a summary of the potential mechanisms for an association between obesity and asthma, and including the possibility that both conditions are linked by an upregulation in Th2 immunity. This thesis will present data addressing this possibility, in addition to exploring more generally the relationship between different measures of obesity and the asthma syndrome.

The majority of cross-sectional studies show a consistent association between obesity and asthma with prospective studies lending support to that association being causal. Further support comes from the observation of a dose-response effect and the association of obesity with intermediate asthma phenotypes [5, 17] (discussed in detail in Chapter 7).

There are potential confounders in the association between obesity and asthma. These include the effects of diet, exercise, gastro-oesophageal reflux and sleep-disordered breathing. However it is possible that some of these factors, rather than being confounders, may be part of a causal pathway between obesity and asthma. Notwithstanding these potential confounders, there is increasing evidence that obesity is a pro-inflammatory state and therefore predisposes to asthma. Evidence for adipocytes being pro-inflammatory cells will be outlined in the following section and other mechanisms explaining obesity in asthma causation will be discussed. A diagrammatic schema illustrating potential interactions between these two complex traits is shown in Figure 1.6.

1.3.1 Mechanical effects

It has long been accepted that obesity causes a restrictive defect in lung function, with a

generalised reduction in lung volumes including tidal volume, functional residual capacity (FRC) and expiratory reserve volume [170]. Accumulation of fat around the ribs, diaphragm and abdomen leads to an approximately 25% reduction in chest wall compliance in simple obesity [171]. Central obesity has been found to further reduce lung volumes [172]. The European Prospective Investigation into Cancer and Nutrition (EPIC) examined a general population of older adults and found abdominal obesity to be inversely related to FEV1 and FVC, but not to airflow obstruction. Waist/hip ratio was a better predictor of lung function than body mass index (BMI), although body fat was not measured [173]. Increased visceral fat mass causes increased intra-abdominal pressure so that the diaphragm is pumped upwards, compressing the lung parenchyma. Additionally, over-stretching of the diaphragmatic muscle leads to reduced diaphragmatic contractility [174]. Another explanation is that compression of the abdominal viscera by fat causes increased blood volume to be redistributed to the thorax, thus reducing vital capacity which explains the fall in vital capacity that occurs in the supine position. Moreover, increased pulmonary blood volume may lead to congestion of airway submucosal vessels, thickening of the airway wall, and smaller airway size [175]. Increased epicardial fat may also reduce vital capacity.

In contrast to earlier studies showing a purely restrictive defect, more recent studies have shown chronic obesity to be associated with peripheral airways obstruction, independent of smoking [176, 177]. Since lung volume is an important determinant of airway diameter, a decrease in FRC is likely to unload airway smooth muscle resulting in an excessive shortening on activation [178].

The "Latch-bridge Hypothesis" has been formulated to explain the association between obesity and asthma (characterised by airflow obstruction) [17]. This suggests that smaller lung volumes lead to reduction in smooth muscle stretch or "latching", giving rise to smaller tidal breaths during exercise and thus altering smooth muscle contraction. This vicious cycle leads to further deconditioning of the smooth muscle. The smooth muscle effects are mediated by the conversion of normal rapidly cycling actin-myosin cross-bridges to slowly cycling latch-bridges [179]. Reduced tidal expansion impairs

force fluctuations acting on the airways, which exert potent bronchodilatory effects. Reduced tidal volumes in the obese are thus associated with a loss of bronchodilation which may lead to increased aiway hyperresponsiveness (AHR) [178]. Furthermore, it has been shown that obese subjects lose the protective deep inhalation prior to a methacholine challenge, leading to greater bronchoconstriction compared with non-obese subjects [180]. Moreover, morbid obesity can cause extra-thoracic obstruction.

Mechanical factors are likely to play a role in the relationship between obesity and asthma. However, obese mice have persistent AHR even when mechanical load is removed (by opening the chest wall and fixing tidal volume by artificial ventilation) suggesting that alternative mechanisms exist to explain this association [181].

Obesity may exert an effect on airway bronchoconstriction via gastro-oesophageal reflux. Obesity leads to relaxation of the oesophageal sphincter resulting in acid reflux. This can cause bronchoconstriction either by microaspiration into the airways or by vagally mediated reflexes [182]. Since obesity, asthma and gastro-oesophageal reflux are all conditions of high prevalence in the population, it is difficult to disentangle the relationships between them and much work remains to be done in this field.

1.3.2 Obesity as a pro-inflammatory state

Evidence is mounting for the role of the adipocyte as an inflammatory cell, releasing pro-inflammatory cytokines. The adipocyte releases tumour necrosis factor (TNF), IL-6 and monocyte chemoattractant protein 1. Obesity is associated with a low-grade systemic inflammation, with increased levels of C reactive protein and markers of lipid peroxidation [183-185]. Obesity and asthma are both associated with upregulation of the TNF pathway. TNF is associated with a more severe relatively steroid-resistant asthma phenotype and is upregulated by allergen exposure. It is possible that obesity-related TNF contributes to the development of an asthmatic phenotype.

It has been suggested that obesity-related hormones such as leptin, adiponectin, insulin

and neuropeptide may exert important immune effects in asthma. Leptin is produced by adipocytes, acting as a satiety signal on the hypothalamus to increase basal metabolic rate and is elevated in obesity [186]. Leptin also has pro-inflammatory effects and whereas it generally promotes T helper type 1 cytokines via IL-12, leptin also promotes IL-6 release which in turn stimulates Th2 cytokines [187].

Leptin levels may reflect sex differences in the natural history of asthma, with higher levels in asthmatic boys than girls and higher levels in adult women, both of which groups have a higher prevalence of asthma [188-190]. Moreover, leptin levels are higher in obese asthmatic subjects compared with obese non-asthmatic subjects [191]. Correlations have been found between leptin and IgE levels in allergic subjects, with positive findings only for boys in one study [188, 192].

Obese mice have been shown to have innate AHR and increased AHR and airway inflammation in response to ozone, in comparison to their lean wild-type counterparts [193]. Leptin administration increases Th2 cells' proliferative responses to T-cell mitogens and increases AHR and Th2 cytokine production in ovalbumin-sensitized mice but its potential molecular mechanisms in asthma development remain unclear [194]. Conversely, adiponectin is *lower* in obese subjects, who may develop AHR and airway inflammation partly as a result of the loss of adiponectin's anti-inflammatory properties [178]. Adiponectin is reduced by allergic pulmonary responses and attenuates allergic airway inflammmation in mice [195]. Adipocytes also release plasminogen activator inhibitor (PAI-1) which is a potent inhibitor of fibrinolysis and plasmin activation and is increased in the obese and reduced by weight loss [196]. Experimental data from murine asthma models suggest that PAI-1 is required for airway hyperresponsiveness and remodelling in response to lipopolysaccharide (LPS) sensitisation and chronic challenge [178].

An intriguing link between fatty acid metabolism and allergic inflammation was suggested by Shum et al [197]. They report that the adipocyte fatty acid-binding protein aP2, which regulates systemic glucose and lipid metabolism, was markedly upregulated

on human airway epithelial cells following stimulation with IL-4 and IL-13. Moreover, regulation of aP2 mRNA expression by Th2 cytokines was highly dependent on STAT6, which has a major regulatory role in allergic inflammation. Eosinophilic airway infiltration was highly dependent on aP2 function. Bronchial epithelial cells were implicated as the likely site of action of aP2 in allergic airway inflammation, thus providing a potential link between adipocyte metabolic pathways and asthma.

1.3.3 Endocrine factors

There has been increasing interest in the idea of asthma as an endocrine condition [198]. The stronger association of obesity with asthma in women has led to the hypothesis that the effects may be related to female sex hormones. Pre-puberty, asthma is commoner in boys but post-puberty and into adulthood, females are at an increased risk of developing asthma [199, 200]. Aromatase, the enzyme which converts androgens to oestrogen, is found in adipose tissue. Obesity tends to increase oestrogen levels, and is therefore associated with early menarche in females and delayed puberty in males [201, 202]. Data from the Nurses Health Study suggested exogenous oestrogen to be an independent risk factor for the development of asthma in adult women [203, 204]. Hormonal influences have also been implicated in asthma severity. Varraso and colleagues found severity to increase with BMI in women only, and the association was stronger in women with early menarche [205]. Another study suggested hormone replacement therapy (HRT) to be associated with asthma in lean women only, whilst it appeared to confer protection in obese women, suggesting that the interactions between obesity and hormonal influences with asthma are unlikely to be straightforward [206]. In clinical practice, severe refractory steroid-resistant asthma is seen more often in obese females than in other groups but the question of whether this is oestrogen-mediated and if so, by what mechanism, is as yet unanswered. However, animal models have shown oestrogen to increase AHR; effect a shift from Th1 to Th2 immune responses; and increase eosinophil recruitment, all changes typically found in asthma [207-209]. Oestrogen has also been shown to increase production of IL-4 and IL-13 from human blood monocytes [210]. Effect modulation by sex may indicate different

causative mechanisms in the association between obesity and asthma in men and women.

Svanes et al found a strong association between asthma, atopy and menstrual irregularity in a study of 8000 women [211]. They proposed an intriguing explanation whereby these conditions are linked by insulin resistance, with asthma as another manifestation of metabolic syndrome, which would explain the previous documented association between insulin resistance and lung function [212].

Other hormones may also play a role. Leptin's role as a pro-inflammatory cytokine has already been discussed. In addition, leptin activates the sympathetic nervous system (SNS), which controls airway tone and diameter; factors of central importance in the airflow obstruction seen in asthma. If leptin is administered to normal weight mice, stimulation of the SNS ensues but obese mice already have high circulating leptin levels and are resistant to its effects [213]. This is similar to the insulin resistance seen in the metabolic syndrome associated with obesity and the possible overlap between these phenomena and asthma warrants further research.

1.3.4 Common genetic predisposition and overlapping metabolic pathways in asthma & obesity?

A common genetic pathway to obesity and asthma may underlie their association in the population. Genes are usually pleiotropic (having multiple effects) and so upregulation in specific genes could lead to increased susceptibility to both obesity and asthma development. The authors of a large twin study recently concluded that the covariation between obesity and asthma is predominantly caused by shared genetic risk factors for both conditions [214]. Genes which may contribute to the control of both traits include those encoding for the beta2-adrenergic receptor, tumour necrosis factor-alpha (TNF-alpha) and the insulin-like growth factor 1 (IGF-1). Genome wide linkage studies have identified several consensus linkage peaks: the chromosomal regions 5q, 6p, 11q, 12q and 17q might be important in both these complex traits.

The 5q region contains the gene for the adrenergic B2 receptor, involved in SNS activation with effects including control of airway tone and metabolic rate. The Glu 27 variant of this receptor has been associated with an increased prevalence of obesity [215] as well as increased serum IgE and protection against methacholine challenge [216, 217]. The 5q region also contains the glucocorticoid receptor gene (modulating inflammation seen in both conditions); and the genes for the Th2 cytokines IL-4 and IL-13. The 6p region contains the *HLA* gene cluster and *TNF*, related to immune and inflammatory responses in both conditions. *TNF p*olymorphisms are associated with asthma, and bronchial hyper-responsiveness [218, 219] and a linkage has also been found between obesity and a marker near the *TNF-α* locus [220]. Chromosome 11q contains genes encoding the uncoupling proteins (UCP2 and UCP3) which influence metabolic rate (with as yet no known role in asthma), in addition to the low affinity immunoglobulin E receptor (*FCεRB*) which is associated with allergic inflammation and not yet examined in obesity. The 12q region contains the inflammatory cytokine genes *STAT6*, *IGF1*, *IL1A* and *LTA4H* with potential roles in inflammatory pathways in both conditions [11, 221]. An *IGF1* variant is associated with body mass index [222] and IGF-1 secreted by bronchial epithelial cells has been shown to stimulate myofibroblast proliferation after injury [223].

Intriguingly, a recent study has found significant evidence of linkage to BMI on chromosome 17q23-q24 amongst female Costa Ricans (with modest linkage for males on chromosome 6p) [224]. Both these chromosomal regions have previously been shown to demonstrate linkage with asthma and investigators are currently exploring which genes may be responsible for these linkage peaks.

Genetic differences may underlie common immune and endocrine mechanisms already discussed. For instance, a polymorphism of oestrogen receptor 1 has recently been associated with airway hyperresponsiveness and lung function decline, particularly in female asthmatic subjects [225].

1.3.5 Diet, physical activity and the "foetal origins" hypothesis

An imbalance between dietary intake and physical activity leads to obesity. There is a postulated link between asthma and inadequate antioxidant intake (e.g. vitamin C and E) which has been discussed in detail in section 1.1.7. It is feasible that individuals eating an unhealthy diet are less likely to have an adequate intake of potentially protective nutrients in addition to an excessive energy intake leading to obesity.

Likely to be of more interest is the effect of maternal diet and physical activity on prenatal programming. Diet in pregnancy may influence the developing immune system and omega-3 fatty acid supplementation has shown modifying effects on allergen specific immune responses and clinical outcomes [61-63]. Maternal diet and physical activity may affect the "programming" of foetal genes, thus influencing foetal growth, birth weight and immune development. These factors may be responsible for the later development of asthma and obesity: the so-called "foetal origins" hypothesis where foetal programming is suggested to account for chronic disease in later life [226]. Low birth weight is associated with higher body fat later in life [227]. The association between asthma and birth weight (independent of gestational age) appears to be U-shaped with increased asthma risk at both extremes of birth weight [228, 229] and modest positive associations between birth weight and lung function on meta-analysis [230].

Maternal diet and physical activity may influence foetal sympathetic nervous system (SNS) development [231]. As previously discussed, the SNS is relevant to both asthma and obesity because of its roles in regulating smooth muscle tone and basal metabolic rate. Physical activity activates brown fat, regulated by the SNS, resulting in increased basal metabolism via activation of uncoupling proteins [232]. B adrenergic receptors are important in SNS signalling (and modulate airway tone) and are expressed by adipose tissue. It is plausible that prenatal defects in SNS development - modulated by maternal diet and physical activity - may contribute to both asthma and obesity in later life.

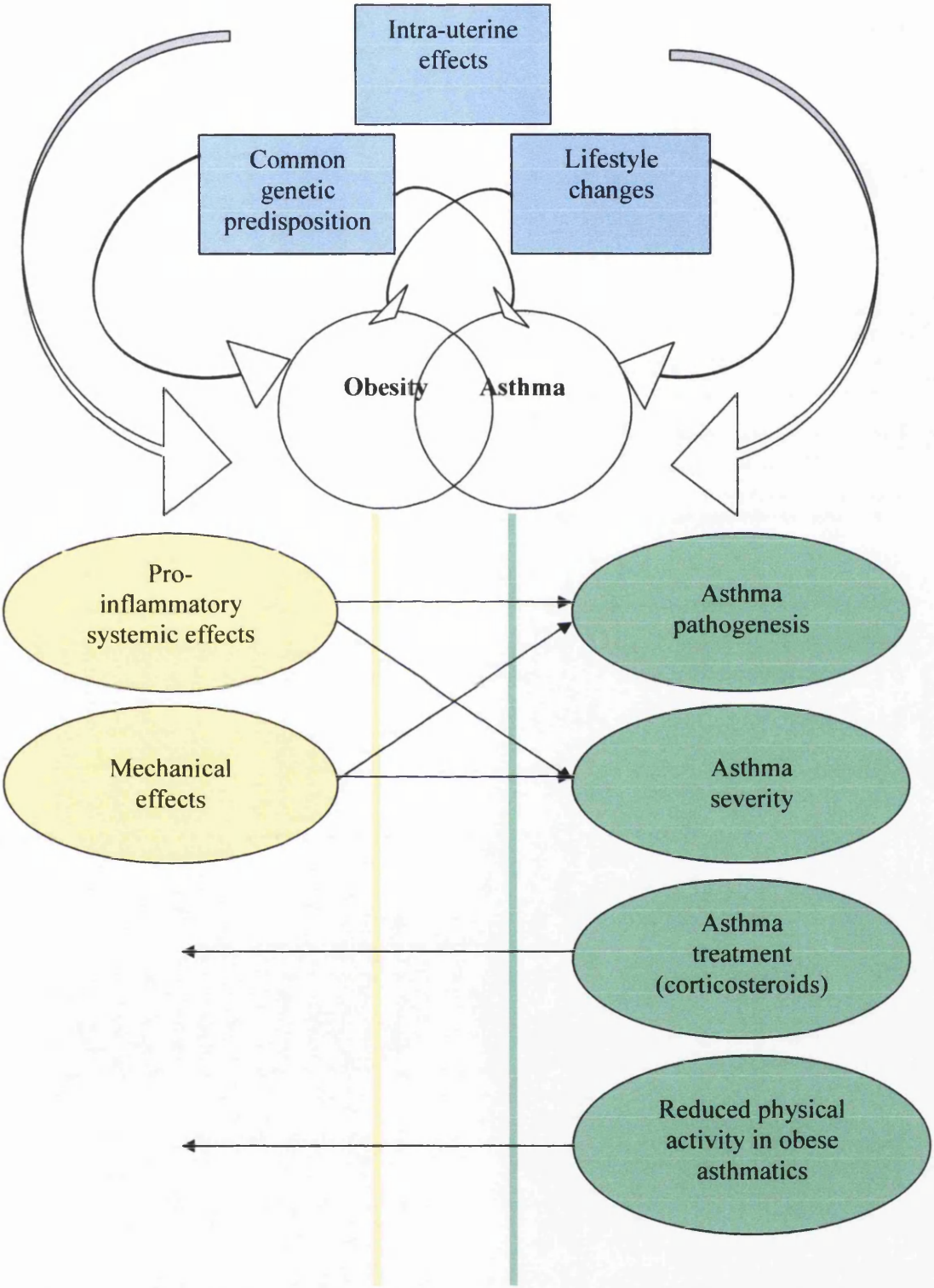


Figure 1.6 Diagram illustrating the complex inter-relationships between obesity and asthma. The pro-inflammatory and mechanical effects of obesity are likely to influence both asthma pathogenesis and severity of disease. Conversely, factors such as corticosteroid use and reduced physical activity may contribute to increased overweight and obesity.

CHAPTER 2

Methodology

2.1 Study design

This is a cross-sectional population study designed to test which genetic variants of *IL13*, *IL4RA* and *STAT6*, acting singly and in combination, predict asthma phenotypes in an unselected population of young adults. Our subjects were recruited from a population of university students and staff, many of whom had varying degrees of mild asthma while some 10-15% would be expected to have clear asthma requiring treatment periodically or regularly. The objective of the population recruitment was to assemble as far as possible an unselected population of young adults who would be of dominantly Caucasian ethnic type.

Researchers have previously performed case-control studies on smaller groups of subjects confined to marked asthma and controls, which do not allow for the predictive effects of the variants to be fully estimated at a population level. Here asthma was treated as a *spectrum* of disease rather than a single disease entity. Comprehensive phenotyping encompassed clinical, physiological and immune asthma phenotypes. Clinical phenotype included measures of disease severity, such as symptom frequency and volume of medication use.

Asthma is a complex trait, consisting of overlapping phenotypes giving rise to the triad of airway inflammation, bronchial obstruction and airway hyperresponsiveness. Given the heterogeneity of asthma phenotypes, asthma is difficult to define for the purposes of clinical research. A large number of studies support our use of a self-report of "physician diagnosed asthma" as a robust definition for epidemiological research [233-235]. In this thesis the aim was to provide a comprehensive view both of asthma and Th2 immune phenotypes and of a range of Th2 genotypes spanning entire loci. To this end, phenotypes encompassed clinical, immune and physiological parameters to build a comprehensive picture of the role of genetic variants of Th2 immunity in predicting asthma and associated phenotypes.

This association study matched genetic variants to fully quantitative assays of *lung*

function and *immune manifestations* of asthma. Multiple genetic variants were examined - through molecular analysis of specific immune regulatory genes in one key pathway – and risk was defined against quantitative traits including asthma severity, lung function and immune allergic disorder, utilising logistic and linear regression models.

IL-13 and STAT6 variants previously associated with asthma in case-control studies were validated by examining their effects in a large population thus giving a true estimation of the population risk conferred by these genetic factors. In addition, common polymorphisms in these genes were examined where an association with asthma had not yet been documented and epistatic relationships between these genes in asthma were investigated. Thus a population risk at each of these loci, acting singly and in combination, was provided, building a unique population picture of the genetic epidemiology of asthma in relation to the IL-13 signalling pathway.

This population study provided an opportunity to clarify genetic factors that significantly predict asthma in the general population. This might allow the identification of at-risk individuals at a young age where early interventions would be important and could prevent asthma from continuing into later life. New targets for therapeutic interventions in asthma are needed and in the future it may be possible to identify particular subgroups of patients who would benefit from particular therapies from their genotypes. We know that the IL-13 signalling pathway is vital in asthma. The interaction between different variants in this pathway (i.e. IL-13 and STAT6) in promoting asthma has not been properly clarified. Improved understanding of the interplay between such genetic variants may clarify potential targets for therapeutic intervention as well as population risk. This population-based approach offers valuable opportunities to identify significant genetic predictors of allergic asthma.

2.2 Ethical approval

Ethical approval was obtained from the local research and ethics committee, LREC for

Bridgend, Neath Port Talbot & Swansea (REC reference number 04/WMW02/29). Informed consent was obtained from all volunteers (Appendix II).

2.3 Subjects

Subjects comprised 1614 unselected volunteers (aged 18-30) from students and staff at Swansea University, Swansea Institute and Singleton Hospital, Swansea. The population was named the PAPA population (*Poblogaeth Asthma Prifysgol Abertawe*, Swansea University Asthma Population). Exclusion criteria were significant lung disease other than asthma (e.g. single lung) and pregnancy since these states would act as a potential confounders for the associations studied. Subjects were studied when free of acute lower respiratory infection which would be likely to result in transient reduced lung function.

2.4 Subject recruitment

Subjects were recruited by the following methods:

1. A manned stall at the students' *Freshers' Fair*
2. A week spent recruiting/collecting data at Swansea Institute
3. Announcements and advertisements in undergraduate lectures
4. Posters around the University and at the University Health Centre (Appendix IA)
5. Sandwich board outside the University reception area
6. Advertisement by email to all students and staff (Appendix IB)
7. Use of flier advertisements

2.5 Anthropometric measurements

The following measurements were recorded on a data sheet (Appendix III).

1. **Height** measured by staediometer to 0.1cm (Leicester; Chasmors, UK)
2. **Waist and hip circumference** to 0.1cm (D loop non-stretch fibreglass tape; Chasmors, UK).
3. **Weight** to 0.1kg (Seca 873 digital scales; Cranlea, Birmingham, UK).
4. **Percentage body fat** to 0.1% (Tanita digital scales, Tanita UK Ltd, Yiewsley, UK)

The following were then calculated:

1. **Waist/hip ratio**
2. **Body mass index** (BMI) = weight (kg)/ [height (m)]^2

2.6 Phenotyping

2.6.1 Clinical phenotype

Clinical phenotype was defined by validated questionnaire. Demographic data were collected and subjects completed a validated bronchial symptoms questionnaire which included questions on symptoms (e.g. wheeze, chest tightness) and presence of asthma, hayfever and eczema (Appendix IV) [236]. Smoking status (pack-years) was recorded. Those who answered positively to "doctor-diagnosed asthma" completed a further validated asthma and allergy symptom questionnaire, based on the European Community Respiratory Health Survey (Appendix V) [237]. Quantitative traits were defined e.g. asthma severity was graded as mild intermittent, moderate persistent or severe persistent according to GINA criteria [50]. Further quantification of severity included recording symptom frequency, volume of medication and number of doctor visits over the previous 12 months.

2.6.2 Physiological phenotype

Given recent evidence that airway remodelling is an early phenomenon [238], a spirometric measure was included in the phenotyping. Morning spirometry (before

noon) capitalised on the diurnal variation in asthma and maximised the discriminatory value of this test in subjects with airflow obstruction. Forced Expiratory Volume in 1 second (FEV1), Forced Expiratory Flow at 25-75% of expired vital capacity (FEF 25-75), Forced Vital Capacity (FVC), FEV1/FVC ratio and Peak Expiratory Flow (PEF) were measured by standardised protocol [239], using a dry spirometer (Vitalograph, UK). The best value of three manoeuvres was expressed as an absolute and percentage of the age-gender-stature predicted value. If on inhaler treatment for asthma, volunteers were asked to omit their morning dose until after the spirometry measurements were taken.

2.6.3 Immune phenotype

Since there is an established association between atopy and asthma, serum total IgE levels were measured, providing a prediction of immune phenotype in addition to clinical and physiological phenotype. Immunophenotype also included eosinophil count and plasma eotaxin (a chemokine effector molecule of IL-13).

Total IgE

Whole blood was collected in vacutainers (serum separator clotted tube) and centrifuged for serum separation (2500 rpm, 4°C for 10 minutes). Serum total IgE (kIU/L) was measured using an ELISA-type sandwich assay (Roche Diagnostics, Lewes, UK) at Morriston Hospital Chemical Pathology. A "sandwich type" assay utilising electrochemiluminescent technology was performed using the Roche Elecsys Modular E analyser. The method has a similar principle to the ELISA (enzyme-linked immunosorbent assay) method: rather than the antibody being attached to a microplate well, it is attached to magnetic particles, and separated by magnet to allow quantitation. Further details are as follows:

a. First incubation: IgE from 10 µl serum sample, a biotinylated monoclonal IgE-specific antibody and a monoclonal IgE-specific antibody labelled with a ruthenium

complex form a sandwich complex.

b. Second incubation: After addition of streptavidin labeled microparticles, 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 microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

d. 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.

Eotaxin

Materials (BD OptEIA™, BD Biosciences)

Capture Antibody: Anti-human eotaxin monoclonal antibody.

Detection Antibody: Biotinylated anti-human eotaxin monoclonal antibody.

Enzyme Reagent: Streptavidin-horseradish peroxidase conjugate (SAv-HRP).

Standards: Recombinant human eotaxin, lyophilised

Buffers and solutions (BD OptEIA™, BD Biosciences)

Coating Buffer: 0.1 M Sodium Carbonate, pH 9.5, 8.4 g NaHCO3, 3.56 g Na2CO3; q.s. to 1.0 L; (prepared from carbonate-bicarbonate buffer capsules; Sigma).

Block Buffer: 1% Bovine serum albumin (BSA) in phosphate-buffered saline (PBS).

Wash Buffer: PBS with 0.05% Tween-20.

Assay Buffer: 1% BSA in PBS/0.05% Tween-20.

Substrate Solution: Tetramethylbenzidine (TMB) and Hydrogen Peroxide (TMB substrate set; BD Biosciences).

Stop Solution: 2 N H_2SO_4.

Whole blood was collected in EDTA vacutainers and centrifuged for plasma separation (2500 rpm, 4°C, 10 minutes). Plasma eotaxin (pg/ml) was measured using ELISA (BD

OptEIA™, BD Biosciences), under the supervision of Dr Cathy Thornton, Senior Lecturer in Newborn Immunity. Assays were performed in duplicate and assay procedure was as follows.

a. Dilution of capture antibody (1:250) into coating buffer and 50 µl added to each well (Costar ½ area 96 well plate) and incubated overnight at 4°C.

b. Coating antibody tipped off and 150 µl/well blocking buffer added. Incubated for 1 hour at room temperature.

c. Blocking buffer tipped off and plate washed 3 times (Wash Buffer).

d. Standard curve: Stock eotaxin at 20ng/ml. Standard curve of 400, 200, 100, 50, 25, 12.5, 6.25 and 0 pg/ml in duplicate for each plate, at 50 µl/well.

e. Samples added at 50 µl/well, following optimal dilution in assay buffer (initially 1:2 and further dilutions in repeat assays if needed). Incubated for 2 hours at room temperature.

f. Standards/samples tipped off and plate washed 4 times (Wash Buffer).

g. Detection complex diluted in assay buffer: (1:250 detection antibody and 1:250 enzyme reagent), 50 µl/well added and incubated for 1 hour at room temperature.

h. Detection complex tipped off and plate washed 6 times (Wash Buffer).

i. Addition of 50 µl/well substrate solution and blue colour allowed to develop (Figure 2.1a).

j. Reaction stopped with 50 µl/well 2N H_2SO_4, with colour changing to yellow (Figure 2.1b).

k. Absorbance read at 450 nm (Multiskan Ascent; Thermo Labsystems).

l. Calculation of results: mean absorbance calculated for each set of duplicate standards and standard curve plotted for each plate. An example of plotted standard curve is shown in Figure 2.2. Mean absorbance was calculated for each set of duplicate samples and eotaxin level calculated from the derived equation from the standard curve (Excel 2003).

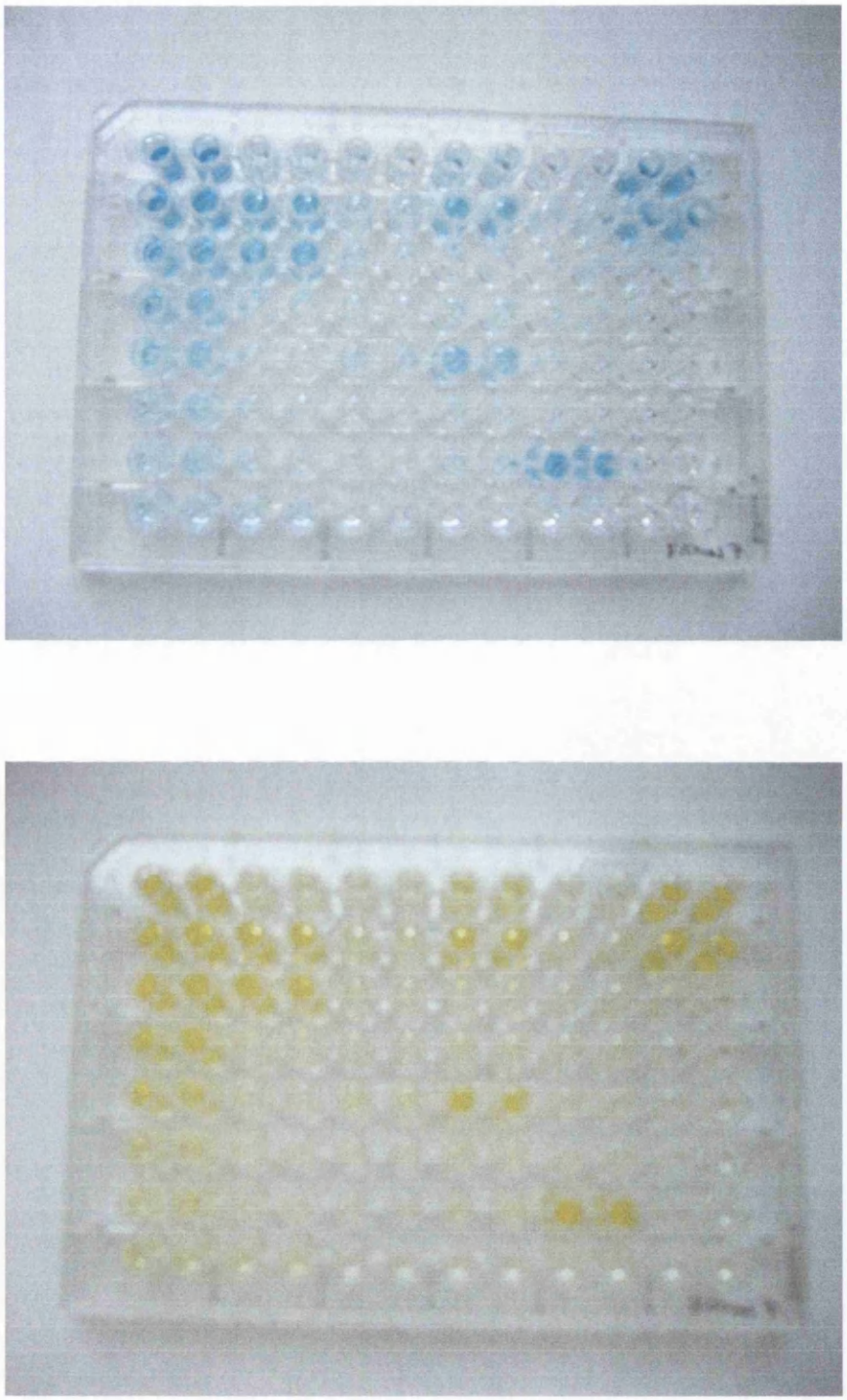


Figure 2.1 Plates showing eotaxin reactions measured by ELISA. Eotaxin standard curves were derived from the first two columns containing stock solution of 400, 200, 100, 50, 25, 12.5, 6.25 and 0 pg/ml in duplicate for each plate. Upper plate, after addition of TMB substrate; lower plate, after reaction stopped by H_2SO_4.

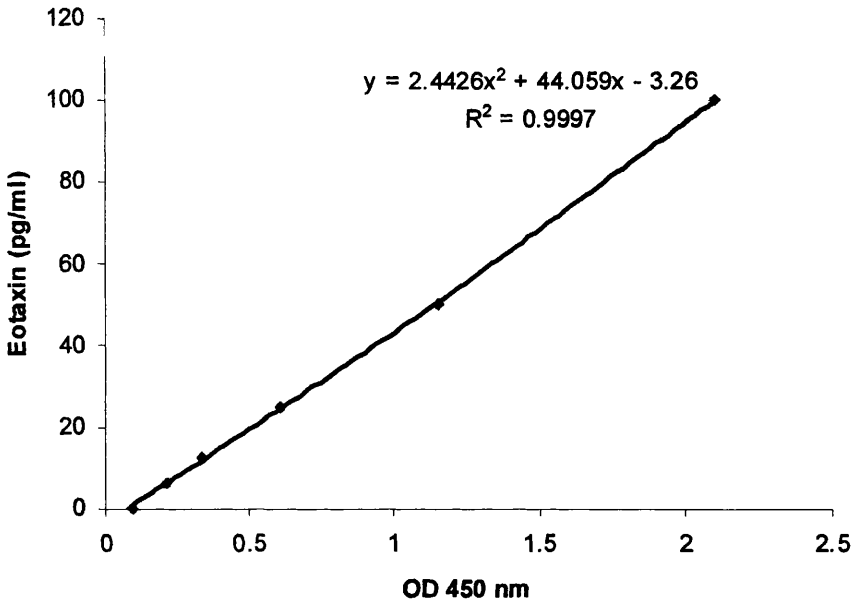


Figure 2.2 Example of a plotted standard curve for eotaxin. Mean absorbance was calculated for each set of duplicate samples and eotaxin level calculated from the derived equation from the standard curve

Eosinophil counts

Whole blood was collected in EDTA vacutainers and eosinophil counts were measured by Haematology staff at Singleton Hospital, Swansea NHS Trust, under the supervision of Dr Ann Benton, Consultant Haematologist. All samples were processed on the same day as collection on a Sysmex XE-2100 automated analyser which provides a full blood count including a five part differential of the white cell count (neutrophils, lymphocytes, monocytes, eosinophils and basophils). Full blood counts were reviewed and validated as per standard protocol and blood films were made on all samples in case a manual differential was needed (if analyser unable to produce results) and in case review was required for abnormalities found on full blood count.

2.7 Genotyping

2.7.1 SNP selection

The approach to single nucleotide polymorphism (SNP) selection was that of ensuring comprehensive coverage of the entire *IL13*, *IL4RA* and *STAT6* loci. Therefore, SNPs were selected in the 5'UTR; intronic and exonic regions; and the 3'UTR. The SNPs with highest heterozygosities were chosen from these regions. Heterozygosities were initially obtained from the NCBI SNP database: http://www.ncbi.nlm.nih.gov/projects/SNP (and further examined in our own population when necessary, see below). In addition, through extensive literature review, SNPs were chosen on the basis of their association with asthma and atopy in previous studies; these were primarily non-synonymous SNPs causing an amino acid change.

In the case of *STAT6*, previous work by our unit and other groups and the choice of high heterozygosity SNPs to attain comprehensive gene coverage, yielded 11 SNPs of interest across the *STAT6* gene. In the case of *IL13*, several of the SNPs were similar in location and heterozygosity and so were examined further in our population in order to refine our selection and choose the SNPs most likely to be important. This was achieved by genotyping a small sample of our population for the *IL13* SNPs of interest by sequencing. The *IL4RA* is highly polymorphic with multiple SNPs causing amino acid change: SNPs in this gene were chosen on the basis of location, heterozygosity and previous literature.

Figure 2.3 outlines the molecular methods of DNA extraction, polymerase chain reaction (PCR) and sequencing which will be described in detail in the subsequent section.

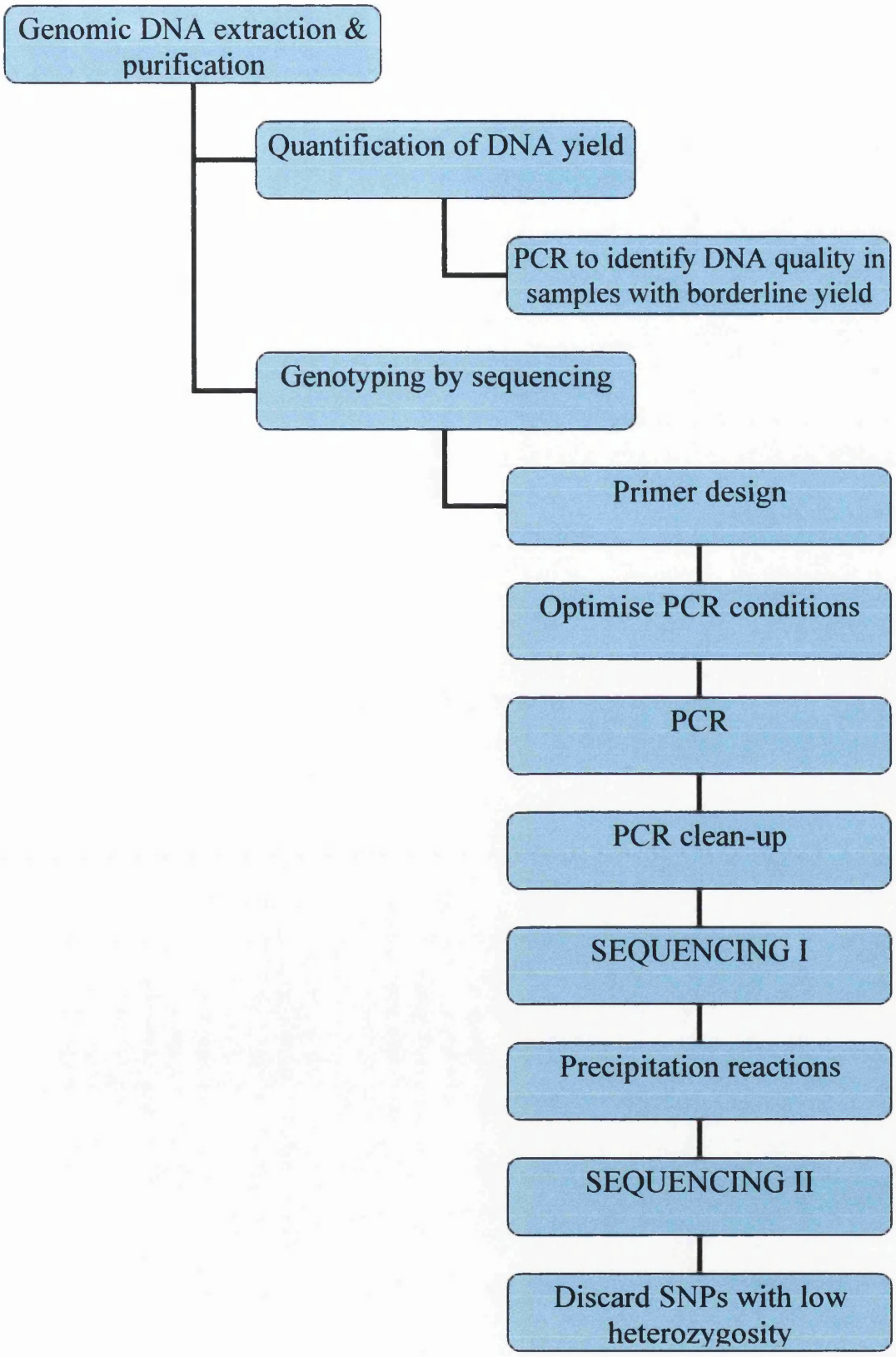


Figure 2.3 Flow chart of molecular methods.

2.7.2 Genomic DNA extraction & purification

Materials (QIAamp 96 DNA Blood Kit, Qiagen, Germany)
QIAGEN Protease.

Buffers and solutions (QIAamp 96 DNA Blood Kit, Qiagen, Germany)
Lysis Buffer AL.
Wash Buffer AW1 & AW2.
Elution Buffer AE (10mM TrisCL, 0.5 mM EDTA, pH 9.0).
See QIAamp 96 DNA Blood Handbook for further details (http://www1.qiagen.com/literature/handbooks/default.asp). Further details regarding buffer composition are proprietary.

Whole blood was collected in EDTA vacutainers and stored at -80°C. Genomic DNA was isolated from whole blood using the QIAamp 96 DNA Blood. The kit utilises the selective binding properties of a silica-gel membrane with a high-throughput 96-well format, allowing us to process 190 samples at a time (with one negative control in each plate for quality control). A 200µl sample of whole blood yields 3-12 µg of DNA (average final concentration of 34 ng/µl). The purified DNA ranges in size up to 50 kb, mainly comprising fragments of 20-30 kb.

a. Lysis was initiated by adding 200µl of whole blood to 20µl of QIAGEN Protease stock solution in a 96 well block.
b. 200µl of lysis Buffer AL was added to each sample.
c. The samples were centrifuged briefly at 3000 rpm and incubated at 70°C for at least 10 minutes.
d. The samples were centrifuged briefly at 3000 rpm to collect any lysate from the caps.
e. Purification on the QIAamp membrane

- 200µl of ethanol (100%) was added to each well to optimise the binding of DNA to the QIAamp membrane

- The wells were sealed before centrifuging briefly at 3000 rpm.
- The lysates were loaded onto the QIAamp plate and the DNA adsorbed onto the QIAamp membrane in a brief centrifugation step (6000 rpm for 4 minutes).
- DNA bound to the membrane was then washed in two centrifugation steps: two wash buffers AW1 and AW2 were used (500µl). Samples were centrifuged at 6000 rpm between these washes and at 6000 rpm after the second wash.

f. DNA was eluted from the membranes using 200µl of elution buffer.

g. Purified DNA was stored at -20 °C.

Quantification of DNA yield

Following DNA extraction, DNA was quantified in ng/µl by measuring and comparing its optical density (OD) at 260nm and 280nm using a NanoDrop 1000 Spectrophotometer. Pure DNA has an A_{260}/A_{280} ratio of 1.7-1.9. An OD of 1 is equivalent to 50µg/ml of dsDNA. DNA extraction and purification were repeated for samples with low DNA concentrations (defined as $\leq$ 20ng/µl) and/or unsatisfactory OD ratios. If the values were borderline, then PCR was done to decide whether repeat DNA extraction was necessary.

PCR to identify DNA quality in samples with borderline yield

Materials

Purified DNA.

Forward and reverse primers for SNP 5 [IL13(5)F and IL13(5)R].

ABgene PCR Master Mix [1.25 units *Taq* DNA polymerase, 75mM Tris-HCl, 20mM $(NH_4)_2SO_4$, 1.5mM $MgCl_2$, 0.01% Tween 20, 0.2mM each of dATP, dCTP, dGTP, dTTP].

- Twelve samples were deemed to show borderline DNA yield on spectrophotometry and PCR was performed on these samples. SNP 5 primers were chosen for PCR because of their consistently good performance.

- In a total volume of 20µl, 1µl (approx. 50ng) DNA was added to 17µl ABgene PCR Master Mix, and 10pmol of each primer.
- PCR conditions were as follows: 1 cycle of 5 min at 95^0C; followed by 35 cycles of 30s at 94^0C, 30s at 60^0C, 40s at 72^0C; and a final extension of 10 min at 72^0C.
- PCR products were run on 1.5% agarose gel at 140V for 15 minutes and visualised with ethidium bromide (EtBr) staining in a UV dark chamber.
- All 12 PCR products showed satisfactory bands (Figure 2.4).

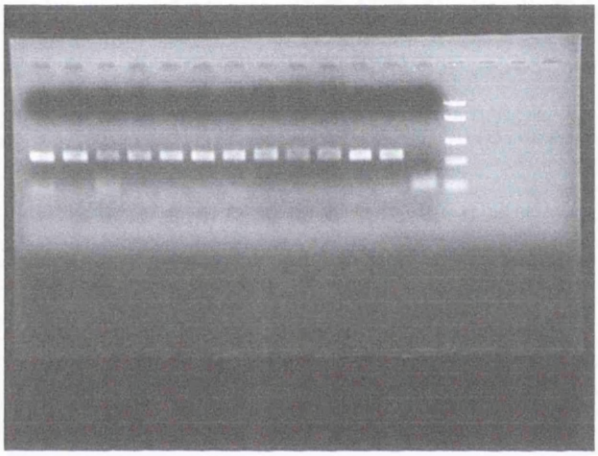


Figure 2.4 PCR products from 12 samples showing borderline DNA yield on spectrophotometry (lane 1-12), negative control (lane 13), size ladder (lane 14). Satisfactory bands are seen on gel electrophoresis.

2.7.3 Genotyping *IL13* SNPs by automated DNA sequencing

Aim: To determine the heterozygosities of *IL13* SNPs in a small sample of our population in order to select the highest heterozygosity SNPs when genotyping the total population.

Up to 30 randomly selected samples were sequenced from our study population in order to ascertain whether any of the *IL13* SNPs 1-6 could be discarded on the basis of low heterozygosity.

Primer design

A website was used to aid primer design (www.wi.mit.edu/genome_software/other/primer3.html). A sequence surrounding the SNP of interest (60 base pairs upstream, 50 base pairs downstream) was cut and pasted into the Primer3 design website and the best sequence match primers (Primer F, forward; Primer R, reverse) were selected and ordered from MWG (Germany, www.mwgbiotech.com) (Table 2.1).

Table 2.1 PCR primers used in sequencing *IL13*.

	Forward primer (5'-3')	**Reverse primer (5'-3')**
IL13 SNP 1 rs1800925	AGGAAGTGGGTAGGGGAGAA	GCAGAATGAGTGCTGTGGAG
IL13 SNP 2 rs1295686	GTCAGGTCCTGTCTCTGCAA	TCCGTGAGGACTGAATGAGA
IL13 SNP 3 rs2069744	TGCACGTGCTTTATGTGTCA	GCCACTGTTGACCAAGACAA
IL13 SNP 4 rs3212145	CAGCTCACATGTCTGAGCACT	CATGGACACTCTGGTGTTGG
IL13 SNP 5 rs20541	CAGCACAGGCTGAGGTCTAA	CGAGACACCAAAATCGAGGT
IL13 SNP 6 rs848	GTTCTGCCCCTCTCCTGAC	AGTGTGTTTGTCACCGTTGG

Optimising PCR conditions

In a total volume of 20μl, 1μl (approx. 50ng) of DNA from each of 6 subjects was added to 17μl ABgene PCR Master Mix (ABgene, as previous), and 12pmol of each primer. This was done for SNP 1- 6.

PCR was then performed at different annealing temperatures (56^0C, 58^0C and 60^0C) (GeneAmp PCR System 9700, Applied Biosystems). PCR conditions were as follows: 1 cycle of 5 min at 95^0C; followed by 35 cycles of 30s at 94^0C, 30s at $56/58/60^0$C, 30s at

72^0C; and a final extension of 10 min at 72^0C.

PCR products (10µl) were run on 1.5% agarose gel at 140V for 15 minutes and visualised with EtBr staining in a UV dark chamber. A 100bp DNA ladder was loaded for comparison.

Temperatures 56-60^0C yielded satisfactory bands, except for SNP 1 where at least one other PCR product was identified at these temperatures (Figure 2.5) and an optimal temperature of 61^0C was selected (Figure 2.6). For SNP 2-6, a temperature of 56^0C was selected.

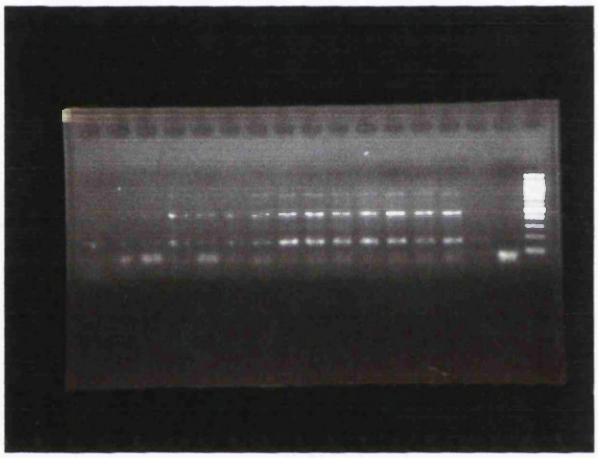


Figure 2.5 PCR bands for *IL13* SNP 1 in 14 samples (lane 1-14), negative control (lane 15), 100bp size ladder (lane 16). At least 2 PCR products were present at an annealing temperature of 58^0C and bands were not demonstrated for some samples. PCR was repeated until an optimal temperature of 61^0C was found (Figure 2.6).

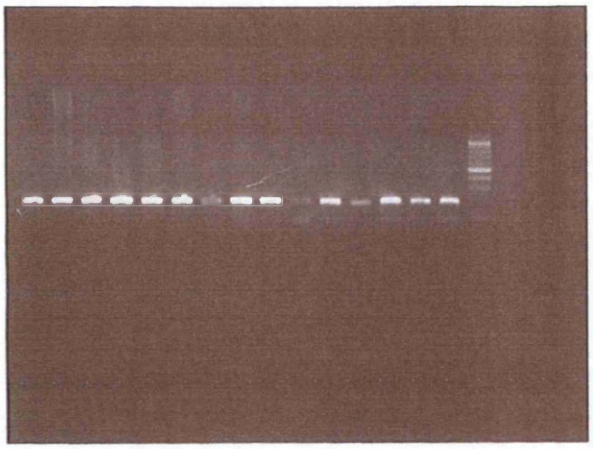


Figure 2.6 PCR bands for *IL13* SNP 1 at annealing temperature of 61^0C in 15 samples (lane 1-15), 100bp size ladder (lane 16). Satisfactory bands are shown.

PCR amplification

In a total volume of 20µl, 1µl (approx. 50ng) of DNA from each subject was added to 17µl ABgene PCR Master Mix (ABgene), and 10 pmol of each primer.

PCR conditions were as follows: 1 cycle of 5 min at 95^0C; followed by 35 cycles of 30s at 94^0C, 30s at 56^0C (61^0C for SNP 1), 30s at 72^0C; and a final extension of 10 min at 72^0C.

PCR products were loaded onto 1.5% agarose gel and run at 140V for 15 minutes, as previously outlined. Representative bands are shown for SNP 3 (Figure 2.7). If bands were unsatisfactory (e.g. for 3 of 20 samples for SNP 2), PCR was repeated for those samples and the bands rechecked before proceeding to PCR clean-up.

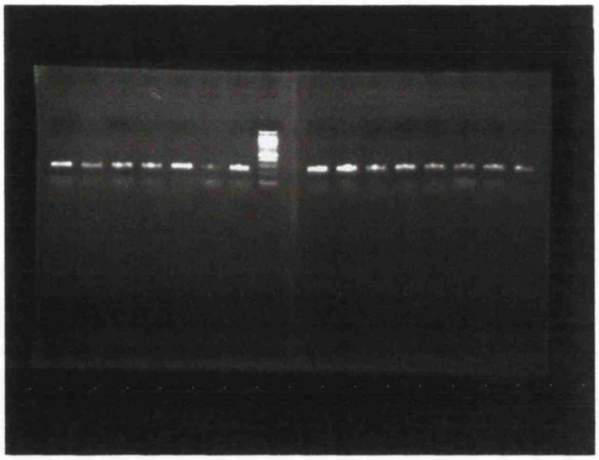


Figure 2.7 PCR products for *IL13* SNP 3 for 15 samples (lane 1-7, 9-16) with 100bp size ladder (lane 8). Satisfactory bands are shown.

Purifying PCR products

Prior to sequencing, the PCR products were purified using the microCLEAN™ DNA clean-up reagent (Microzone) which works on differential precipitation of DNA by size.

a. 15µl microCLEAN added to 15µl of PCR product, and allowed to stand at room temperature for 5 minutes.

b. Spun at high speed (13000 rpm in microfuge) for 7 minutes, supernatant removed, spun again to remove dregs and pellet then resuspended in 20µl distilled water (dH_2O). Allowed to stand for 5 minutes to rehydrate DNA and stored at 4^0C.

The purity of cleaned PCR products was checked by running 5µl of cleaned PCR product on 1.5% agarose gel at 140V for 15 minutes. This was done on 5 samples for each SNP.

Automated DNA sequencing

Aim: To sequence up to 30 samples from our population for SNP 1-6, to ascertain whether any of SNPs could be discarded on the basis of low heterozygosity.

Sequencing I

a. In a total volume of 8µl, the master mix comprised 2µl BIG DYE, 1µl BIG DYE sequence buffer (ABgene), 5pmol of primer (Primer F chosen) and 4µl of H_20. Quantities were multiplied according to number of samples to be sequenced.

b. 2µl cleaned PCR product was added to the master mix in each well

Sequencing reactions were performed under the following conditions: 1 cycle of 1 min at 96^0C; followed by 25 cycles of 10s at 96^0C, 5s at 50^0C, and 4 min at 60^0C.

Precipitation reactions

Aim: To remove residual dye terminators from sequencing reactions

a. After completion of sequencing reaction, the extension products (10µl) were added to 10µl dH_2O.

b. The following reagents were added:

- 2µl of 3M NaOAc, pH 4.6
- 50µl of 95% EtOH at room temperature

c. The tubes were capped, vortexed briefly and allowed to stand at room temperature for 15 minutes to precipitate the extension products.

d. The capped tubes were centrifuged for 20 minutes at maximum speed (13000 rpm).

e. Without disturbing the pellet, the supernatant was carefully aspirated and discarded.

f. The pellet was rinsed with 250µl of 70% EtOH, vortexed briefly and centrifuged

at maximum speed for 5 minutes.

g. The supernatant was carefully aspirated again and discarded.

h. The pellets were dried in a heat block at 90^0C for 1 minute.

i. The samples were resuspended in 10µl template suppressant reagent (TSR) and stored at 4^0C.

Sequencing II

Samples were denatured by incubation at 90^0C for 2 minutes and put on ice for 2 minutes. Samples underwent capillary electrophoresis in an ABI PRISM™ 310 Gene Analyser.

Figure 2.8a-c shows the sequenced genotype at the SNP 1 locus for subjects 1,2 and 3.

Figure 2.8a Sequenced genotype at SNP 1 of *IL13* for subject 1: C/C. Blue peaks, C allele; red peaks, T allele.

Figure 2.8b Sequenced genotype at SNP 1 of *IL13* for subject 2: T/T. Blue peaks, C allele; red peaks, T allele.

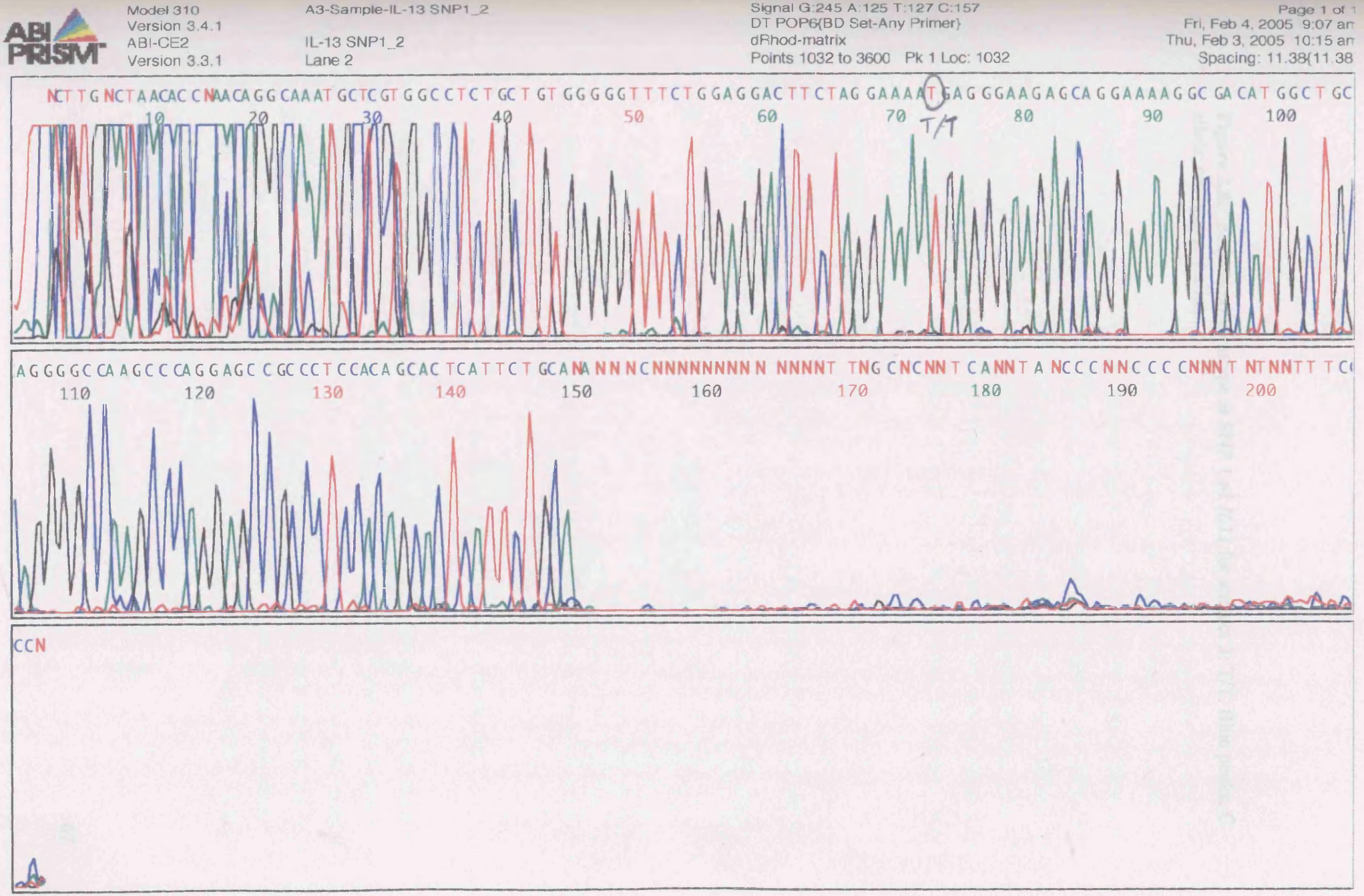

Model 310
Version 3.4.1
ABI-CE2
Version 3.3.1
A3-Sample-IL-13 SNP1_2
IL-13 SNP1_2
Lane 2
Signal G:245 A:125 T:127 C:157
DT POP6{BD Set-Any Primer}
dRhod-matrix
Points 1032 to 3600 Pk 1 Loc: 1032
Page 1 of 1
Fri, Feb 4, 2005 9:07 an
Thu, Feb 3, 2005 10:15 an
Spacing: 11.38{11.38
T/T
CCN

Figure 2.8c Sequenced genotype at SNP 1 of *IL13* for subject 3: T/T. Blue peaks, C allele; red peaks, T allele.

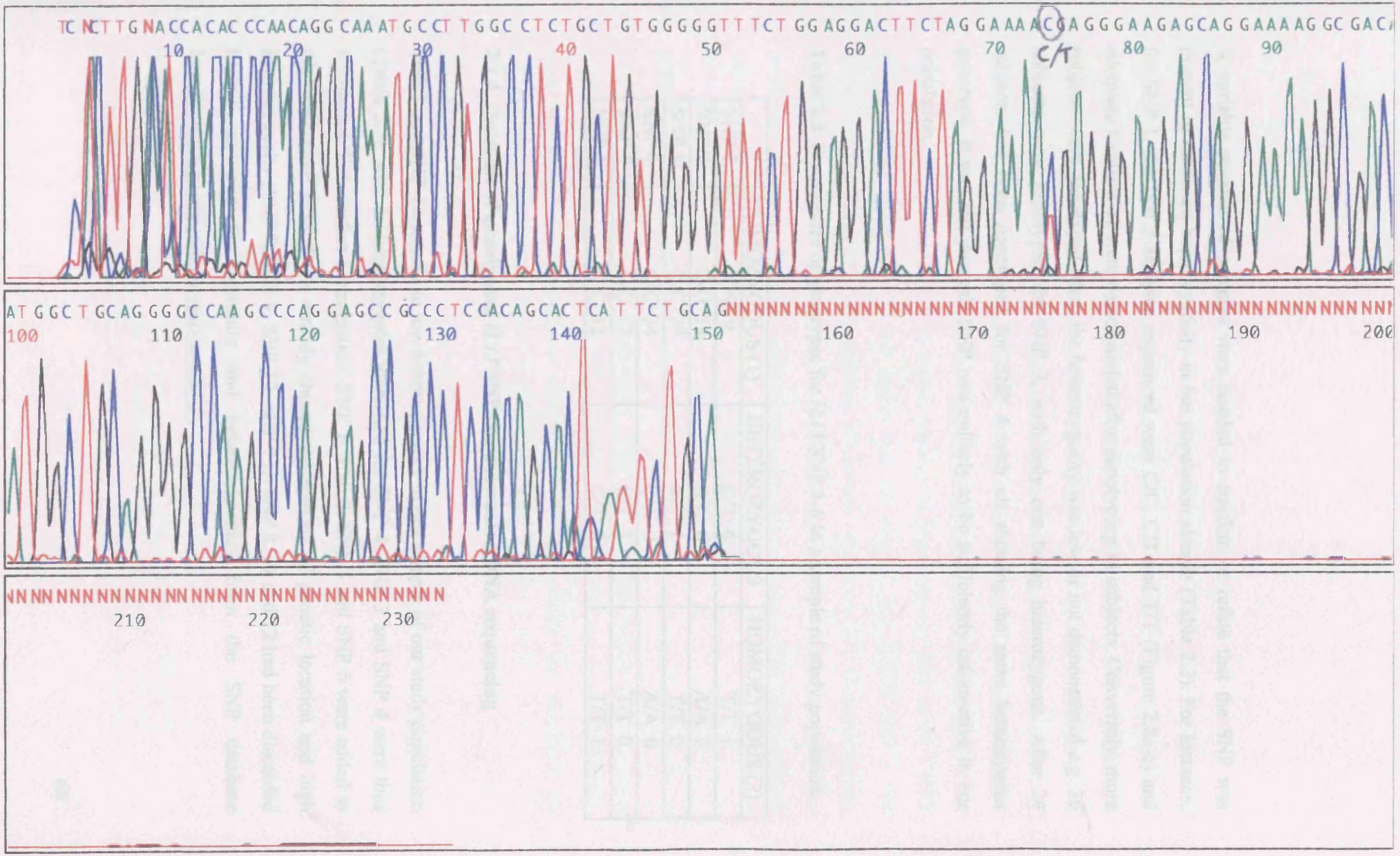
Model 310
Version 3.4.1
ABI-CE2
Version 3.3.1

A5-Sample-IL-13 SNP1_3

IL-13 SNP1_3
Lane 3

Signal G:2359 A:1295 T:985 C:1419
DT POP6{BD Set-Any Primer}
dRhod-matrix
Points 898 to 3600 Pk 1 Loc: 898

Fri, Feb 4, 2005 9:07 am
Thu, Feb 3, 2005 11:02 am
Spacing: 10.45{10.45



A variable number of samples were needed to confirm or refute that the SNP was present in sufficient heterozygosity in our population sample (Table 2.2). For instance, for SNP 1, the first 3 samples sequenced were C/C, C/T and T/T (Figure 2.8a-c) and adequate frequencies were demonstrated after genotyping 14 subjects. Conversely, more subjects were genotyped when the heterozygosity was low or not demonstrated e.g. 30 subjects were genotyped for SNP 3, with only one being heterozygous. After 24 subjects had been genotyped for SNP 4 with all showing the same homozygous genotype, it was felt that this SNP was unlikely to be sufficiently informative in our population.

Table 2.2 Frequencies of genotypes for *IL13* SNP 1-6 in a sample of study population.

	HOMOZYGOUS (1)	HETEROZYGOUS	HOMOZYGOUS (2)
SNP 1	C/C 9	C/T 4	T/T 1
SNP 2	G/G 14	A/G 6	A/A 2
SNP 3	C/C 29	C/T 1	T/T 0
SNP 4	C/C 24	C/A 0	A/A 0
SNP 5	C/C 17	C/T 8	T/T 0
SNP 6	C/C 12	C/T 6	T/T 1

2.7.4 Outcome of genotyping *IL13* SNPs by automated DNA sequencing

SNP 3 and SNP 4 occurred in low heterozygosity in this sample of our study population (Table 2.2), with no heterozygotes identified for SNP 4. SNP 3 and SNP 4 were thus eliminated from further investigation. SNP 1, SNP 2, SNP 5 and SNP 6 were added to the other three *IL13* SNPs already chosen for reasons of genetic location and high heterozygosity (SNP 7, SNP 9, SNP 10, SNP 13). SNP 8, 11 and 12 had been discarded having reviewed the literature and heterozygosities from the SNP database http://www.ncbi.nlm.nih.gov/projects/SNP.

2.7.5 Genotyping of selected SNPs in *IL13*, *IL4RA*, *STAT6* genes

Figure 2.9 shows a schematic representation of the SNPs chosen for genotyping of the population in the *IL13*, *IL4RA* and *STAT6* genes. For *IL4RA*, SNP 1, SNP 4 and SNP 6 were initially selected but the assay validation set-ups for genotyping were unsuccessful and so SNP 2, SNP 3 and SNP 7 were then substituted for reasons of genetic proximity and high heterozygosity. Four exonic SNPs were discarded for reasons of low heterozygosity in the SNP database http://www.ncbi.nlm.nih.gov/projects/SNP. For logistic reasons, it was decided to look at the highest heterozygosity SNP in the 3'UTR and so an alternative SNP in this region was not selected. For *STAT6*, SNP 5 was rejected for reasons of low heterozygosity in a British population [82]. On panel testing, SNP 6, 7 and 9 were monomorphic in a population sample (n=42) and so no further genotyping was done. The assay validation set-ups were unsuccessful for SNP 10 which was therefore not genotyped.

Following SNP selection, genotyping of our population was performed by KBioscience (www.kbioscience.co.uk). A competitive allele specific PCR system (KASPar) was used, utilising fluorescently labelled primers. This proprietary system has been developed from the Ampliflour system (no further details are disclosed by KBioscience).

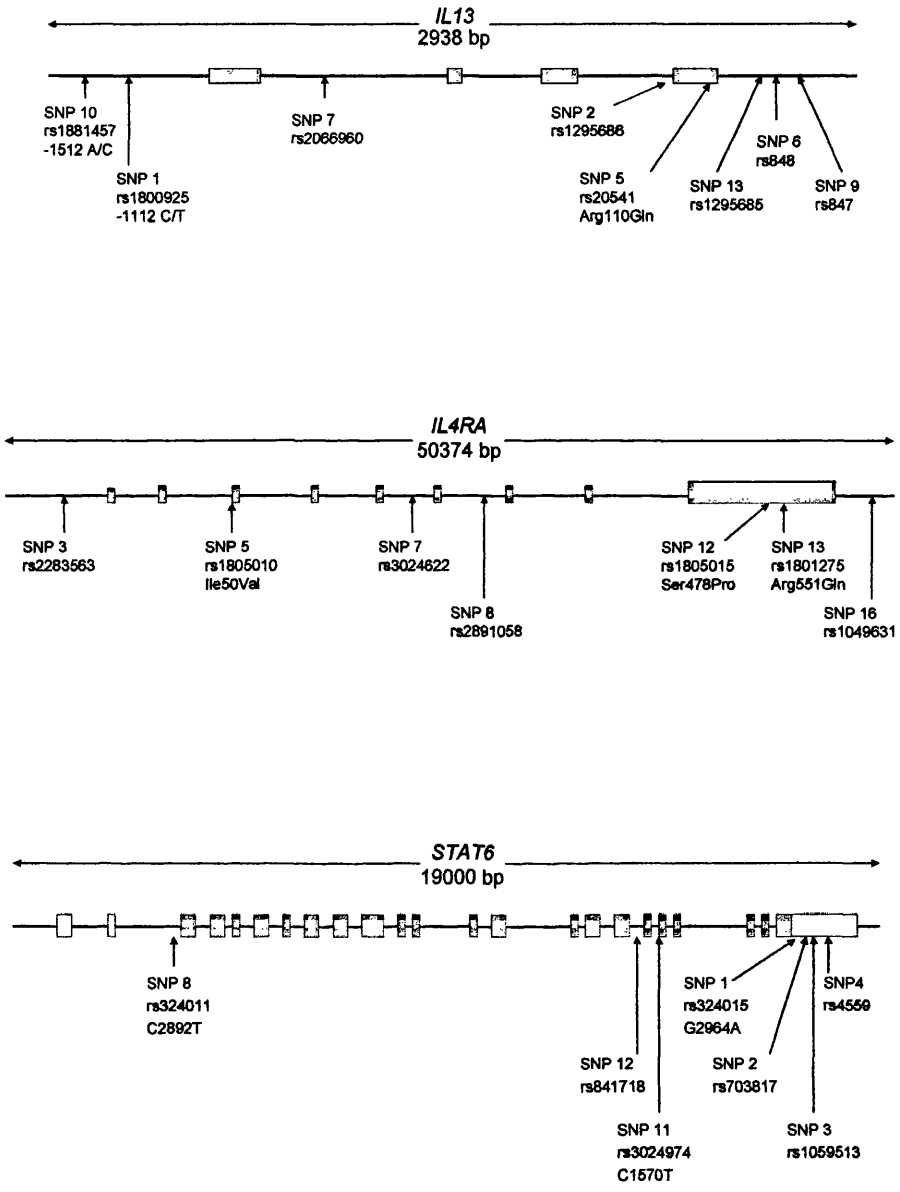


Figure 2.9 Genomic architecture of *IL13*, *IL4RA* and *STAT6* selected SNPs for population genotyping. Reference SNP ID (rs) shown (http://www.ncbi.nlm.nih.gov/projects/SNP) and polymorphism defined for well-described SNPs.

2.8 Data analysis

Published odds ratios (OR) for risk of asthma and variants in the genes considered in this study tend to be in the region of 1.5 to 3.0. Thus in a targeted population of 2000, and assuming an asthma prevalence of 10%, the study has a power of between 70% (OR = 1.5) and <99% (OR = 3.0) to detect a risk factor that is present in approximately 25% of the (control) population. Power is reduced for more rare risk factors, for example between 47% (OR = 1.5) and <99% (OR = 3.0) for variants present in only 10% of the population [240]. However the power of the design is strengthened by the additional information provided by quantitative phenotypes, which typically require much smaller sample sizes. The above calculation therefore represents a conservative assessment of statistical power.

For the realised study (asthma prevalence of 'current asthma' of 11.1% and 'physician diagnosed asthma ever' of 23.9%, and the Caucasian population (n = 1445), estimates of study power are as follows.

- For *current asthma*, and an exposure present at a frequency of 25% (in the normal population), the power is approximately 61% (OR=1.5), 97% (OR=2.0) and >99% (OR=3.0). For an exposure present at a frequency of only 10% (in the normal population), the power is approximately 40% (OR=1.5), 83% (OR=2.0) and >99% (OR=3.0).

- For *'physician diagnosed asthma ever'*, and an exposure present at a frequency of 25% (in the normal population), the power is approximately 85% (OR=1.5), >99% (OR=2.0) and >99% (OR=3.0). For an exposure present at a frequency of only 10% (in the normal population), the power is approximately 60% (OR=1.5), 97% (OR=2.0) and >99% (OR=3.0).

The database was compiled using Access 2003 software. The analysis of the association between genotype/haplotype and risk of asthma took the form of a series of standard

logistic regression models, with adjustment made for relevant covariates (age, sex, smoking, height etc). The analysis was expanded upon to include the quantitative asthma phenotypes, using general linear models. Linear and logistic regression analysis was performed using SPSS 13.0.

Our model building approach was as follows. Initially the relationship between the primary predictors (e.g. genotype) and outcome was explored in a univariate manner. The effect of adjustment for relevant covariates (age, sex, height, smoking) was explored by adding the variables to the model in a stepwise manner and (at each step) retaining the covariates if there was either 1) a significant ($p<.05$) improvement in the likelihood of the fit of the new model, or 2) the covariate caused a substantial alteration of the coefficients representing the effect of the primary predictor. Lastly, when the above process had settled on a 'final model' for individual predictors, all pairwise interactions involving the primary predictors were tested.

Since this was a hypothesis generating study, generating candidate associations, adjusting for multiple comparisons was not applied (e.g. Bonferroni correction) [241]. Using the adjustments results in a large loss of power (for those relationships that are not null), in particular for the testing of the rarer genotypes with the risk that we would overlook potentially important genotypes. Bonferroni is only valid under a strict set of circumstances. By applying the Bonferroni adjustment, the null hypothesis would be universal, that "none of these genotypes have any effect on asthma" and chance underlies the full set of observations. Since the selected loci are part of a functional pathway known to be related to asthma pathogenesis, with known previous associations and physiological effects in case-control studies, we would assume that this global null hypothesis is wrong (i.e. some variants are likely to have true associations with asthma at the population level) but the adoption of the Bonferroni method results in a lack of power to reject the global null hypothesis i.e. an unacceptably high false negative rate. This approach means that some false positive associations may be generated and so identified candidate genotypes in the study need to be interpreted in light of previous studies and should be confirmed in additional populations of sufficient size, a strategy

advocated by reviewers in this field [242, 243].

Haploview software was used to calculate linkage disequilibrium. The haplo.stats 1.3.0 package and the haplo.glm function were used for haplotype analysis. Haplotype frequencies were estimated from genotype data – at the *IL13*, *IL4RA* and *STAT6* loci - using the Expectation Maximisation (EM) algorithm [244, 245] and R statistical environment package [246]. Further details of this more complex statistical analysis are given in Chapter 6.

CHAPTER 3

Genetic association of single nucleotide polymorphisms with asthma and clinical atopy phenotypes

3.1 Introduction

A number of case-control studies have reported associations between polymorphisms in the IL-13 signalling pathway and asthma, allergic rhinitis and atopic dermatitis (Tables 1.2-1.4). In *IL13*, the main polymorphisms examined have been Arg110Gln in exon 4 and the promoter polymorphism 1024C/T. Many positive associations are reported but there are several inconsistent findings, especially for Arg110Gln, with two studies finding a positive association with asthma [117, 247], but a further seven studies reporting no significant association [114, 136, 144, 146, 147, 153, 248]. *IL4RA* is a highly polymorphic locus, containing twelve reported non-synonymous SNPs resulting in amino acid change. Reported associations with asthma have focussed mainly on the Ile50Val variant in exon 5 and Arg551Gln in exon 12, with several conflicting reports regarding association and very few reports on clinical atopic disease other than asthma (Table 1.3). Gao et al found a positive association between a 3'UTR variant of *STAT6* and atopic asthma in a Japanese population [124]. However, this finding has not been replicated in other diverse populations where *STAT6* variants have primarily been reported in association with IgE levels rather than clinical atopic disease (Table 1.4).

Case-control studies provide an indication of some of the loci contributing to asthma susceptibility but only unselected population studies can estimate the actual risk conferred by a single variant, or combinations of variants, at the population level. Moreover, given the degree of inconsistent results in case-control studies, unselected population studies are required in order to estimate the true population risk conferred by a polymorphism, or groups of polymorphisms. There have been few unselected population studies examining asthma and related atopy phenotypes to date. Only one German study has examined asthma in the context of IL-13 signalling polymorphisms; only one polymorphism was examined at each of the *IL13*, *IL4RA* and *STAT6* loci. The *IL13* -1024C/T promoter polymorphism was found to associate with asthma [12]. No unselected populations have been used to examine IL-13 signalling variants relating to the clinical atopic phenotypes of hayfever and atopic eczema.

In this chapter, associations between IL-13 signalling variants and asthma, hayfever and atopic eczema will be examined in an unselected population, in order to estimate the actual population risk conferred by these single nucleotide polymorphisms. Selected polymorphisms span each of the *IL13*, *IL4RA* and *STAT6* genes.

3.2 Methods

An unselected population of 1614 adults aged 18-30 years was recruited according to General Methodology outlined in Chapter 2. Of these, genotyping results are reported on the majority ethnic population, the Caucasian group (n=1445). Clinical phenotyping of "physician diagnosed asthma ever" was obtained by validated questionnaire (Appendix IV) [236] (answering affirmatively to "Have you ever had asthma?" and "Was it confirmed by a doctor?"). Those classified as having "current eczema" had answered affirmatively to "Have you ever had eczema?" and "Do you still have it?" and those with hayfever had answered affirmatively to "Do you have any nasal allergies including hayfever?" Genotyping was performed for a total of 22 polymorphisms; eight spanning the *IL13* locus and seven spanning each of the *IL4RA* and *STAT6* loci, with polymorphisms spanning each entire gene, including non-synonymous SNPs and SNPs in intronic and 3' and 5' untranslated regions (see Chapter 2 for further detail). The analysis of the association between genotype/phenotype took the form of a series of standard logistic regression models, with adjustment made for relevant covariates (age, sex, smoking, height etc). Logistic regression analysis was performed using SPSS 13.0.

3.3 Results

3.3.1 Population characteristics

There were 1614 participants in our cross-sectional sample. Of these, 829 (51.4%) were men, 1445 (90%) were white, 53 (3.3%) were Chinese and 24 (1.5%) were Indian. The mean (standard deviation, SD) age of the participants was 21 (2.89) years. Self-reported subjects with "physician diagnosed asthma ever" represented 23.3% of the study

population (n=374) with "current physician diagnosed asthma" represented by 10.9% (n=176). Bronchodilators were taken by 10.8% (n=175) and 3.5% (n=56) were taking regular inhaled corticosteroids. Subjects reporting "smoking for as long as a year" represented 26.8% of the population (n=433) with 16.9% reporting current smoking (n=271). A summary of the characteristics of the Caucasian group is shown in Table 3.1.

As expected, there was a high correlation between asthma and other clinical manifestations of atopy, namely hayfever (or allergic rhinitis) and current eczema (or atopic dermatitis) (Table 3.2). Odds ratios measuring the association of asthma with hayfever and eczema were 5.0 (95% CI 3.6-7.0) and 2.7 (95% CI 1.7-4.2) respectively.

Table 3.1 Clinical characteristics of Caucasian participants.

	Male		Female		All	
Current asthma[1]	79	(10.6)	82	(11.7)	161	(11.2)
"Asthma ever"	187	(25.2)	159	(22.8)	346	(24.0)
Hayfever	246	(33.1)	258	(37.1)	504	(35.0)
Current eczema	60	(8.1)	87	(12.6)	147	(10.2)

Data are number (percentage) of subjects.
[1]"Asthma" refers to a subjective report of physician diagnosed asthma.

Table 3.2 Clinical atopy in asthma[1] versus non-asthma.

	Current asthma		Never asthma	
Hayfever	106	(65.8)	300	(27.9)
No hayfever	55	(34.2)	772	(71.8)[2]
Eczema	31	(19.3)	88	(8.2)
No eczema	130	(80.7)	980	(91.2)[2]

Data are number (percentage according to asthma status) of subjects.
[1]"Asthma" refers to a subjective report of physician diagnosed asthma.
[2] Total less than 100% due to missing data.

3.3.2 Genotyping results

All genotype frequencies were concordant with Hardy-Weinberg equilibrium. Allele frequencies and call rates for each SNP are shown in Tables 3.3-3.5. Call rates were over 97% for all SNPs. For *IL13*, SNPs 10, 2, 5, 13, 6 and 9 were in strong linkage disequilibrium (LD) (Figure 3.1). The *IL4RA* SNPs showed considerably less linkage disequilibrium, with SNP 3, 7 and 13 showing strong LD (Figure 3.2). *STAT6* showed the highest LD with all SNPs in strong LD except SNP 12 and SNP 3 (Figure 3.3). Figures 3.1-3.3 show LD for the Caucasian population but patterns were almost identical when considering all ethnicities together (with only a very slight reduction in LD of D' ~.01 for the majority of SNPs).

Table 3.3 Description of *IL13* SNPs with allele frequencies and call rates

	Location	Allele frequency	Call rate
SNP 10 rs1881457	5'UTR -1512A/C	C 0.18 A 0.82	0.991
SNP 1 rs1800925	5'UTR -1024C/T	T 0.17 C 0.83	0.991
SNP 7 rs2066960	Intron 1	A 0.11 C 0.89	0.996
SNP 2 rs1295686	Intron 3	A 0.20 G 0.80	0.994
SNP 5 rs20541	Exon 4 Arg110Gln	A 0.18 G 0.82	0.989
SNP 13 rs1295685	3'UTR	T 0.17 C 0.83	0.992
SNP 6 rs848	3'UTR	T 0.19 G 0.81	0.994
SNP 9 rs847	3'UTR	A 0.18 G 0.82	0.973

Table 3.4 Description of *IL4RA* SNPs with allele frequencies and call rates

	Location	Allele frequeny	Call rate
SNP 3	5'UTR	A 0.33	0.986
rs2283563		G 0.67	
SNP 5	Exon 5	G 0.44	0.993
rs1805010	Ile50Val	A 0.56	
SNP 7	Intron 7	G 0.36	0.978
rs3024622		C 0.64	
SNP 8	Intron 8	G 0.15	0.991
rs2891058		A 0.85	
SNP 12	Exon 12	G 0.40	0.993
rs1805015	Ser478Pro	A 0.60	
SNP 13	Exon 12	G 0.23	0.985
rs1801275	Arg551Gln	A 0.77	
SNP 16	3'UTR	T 0.41	0.991
rs1049631		C 0.59	

Table 3.5 Description of *STAT6* SNPs with allele frequencies and call rates

	Location	Allele frequeny	Call rate
SNP 8	Intron 2	T 0.40	0.993
rs324011		C 0.60	
SNP 12	Intron 16	C 0.41	0.991
rs841718		T 0.59	
SNP 11	Intron 17	T 0.09	0.996
rs3024974		C 0.91	
SNP 1	3'UTR	A 0.24	0.997
rs324015		G 0.76	
SNP 2	3'UTR	A 0.49	0.995
rs703817		G 0.51	
SNP 3	3'UTR	G 0.12	0.990
rs1059513		A 0.88	
SNP 4	3'UTR	G 0.35	0.989
rs4559		A 0.65	

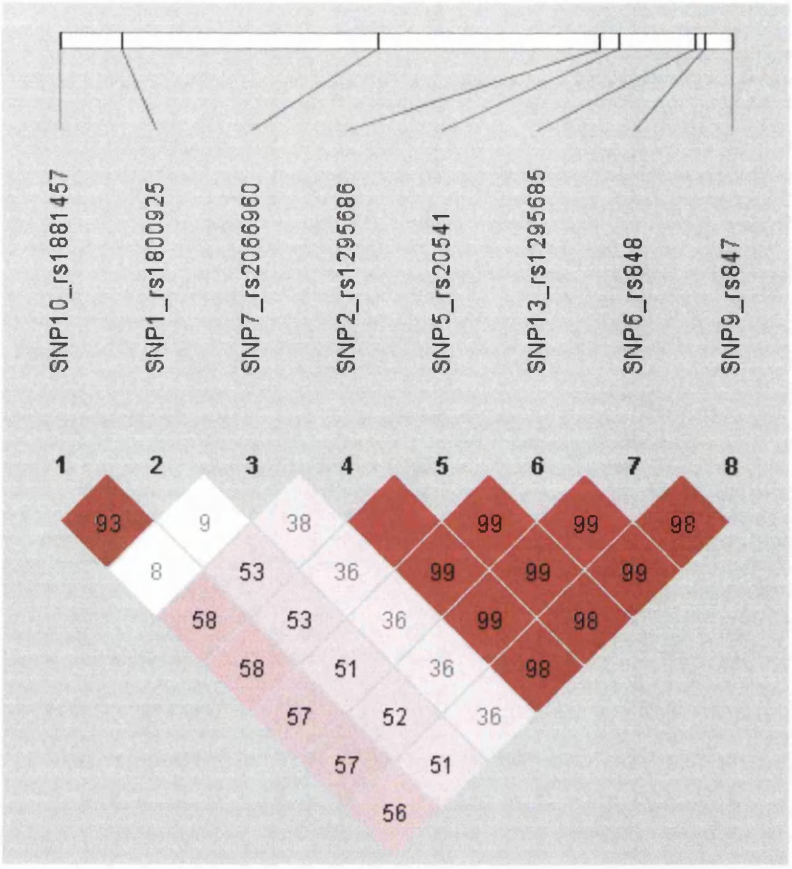


Figure 3.1 Linkage disequilibrium (LD) map for *IL13*. D' values are shown with higher LD shown in darker red. Strong LD is demonstrated between variants in an extended haploblock spanning intron 3, exon 4 and the 3'UTR.

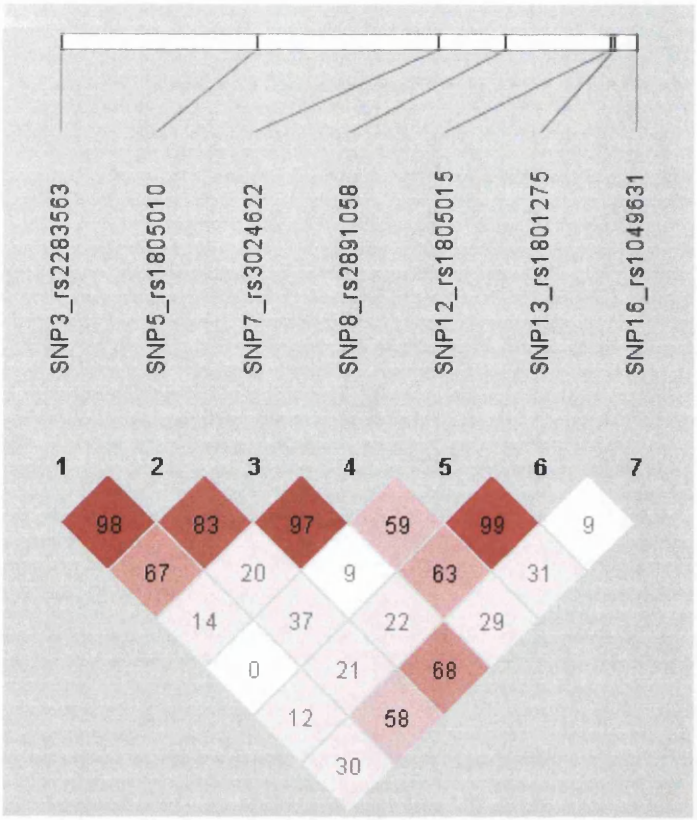


Figure 3.2 Linkage disequilibrium (LD) map for *IL4RA*. D' values are shown with higher LD shown in darker red. *IL4RA* is a highly polymorphic gene with relatively low LD between the majority of variants examined.

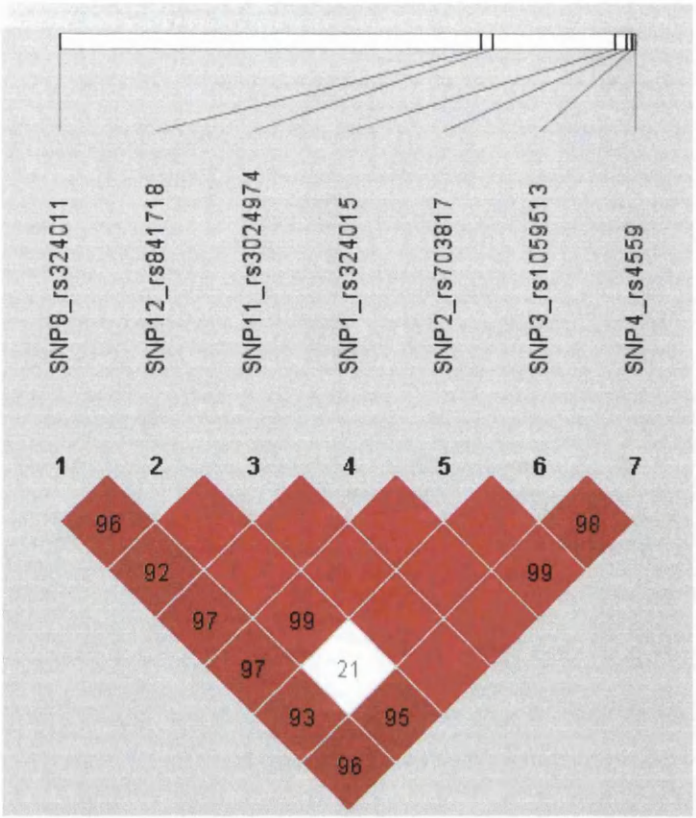


Figure 3.3 Linkage disequilibrium (LD) map for *STAT6*. D' values are shown with higher LD shown in darker red. *STAT6* is a highly conserved gene with strong LD between polymorphisms.

3.3.3 Logistic and linear regression analysis

For all genotype analysis, genotype-phenotype associations were analysed for the Caucasian group initially (n=1445) since numbers in other ethnic subgroups were small, the largest being the Chinese (n=53) and Indian subgroups (n=24). Sex, age and height are unlikely confounders as they are expected to be distributed randomly with respect to genotype. This was confirmed and these variables did not alter the relationship between asthma and genotype. Logistic regression also confirmed no associations between these variables and asthma. Both BMI and current smoking status were associated with a physician diagnosis of "asthma ever" and these variables were therefore adjusted for in subsequent logistic analysis although they were again unlikely to confound the genotype

effects: for association of BMI with "asthma ever", p=.004 (see Chapter 7 for full details); for current smoking and "asthma ever", OR 1.50 (95% CI 1.11-2.02, p=.008). "Asthma" refers to a subject reported physician-diagnosis of asthma. Asthma severity data are presented with the lung function data in Chapter 5, where Table 5.11 summarises the main findings for genotype associations with clinical, immune and physiological phenotypes from Chapters 3-5.

There was an association between age and "eczema ever" (OR 1.05, 95% CI 1.00-1.09, p=.03) and between sex and "current eczema" (OR 1.63, 95% CI 1.15-2.31, p=.006 for females compared to males). Therefore age and sex were considered and interactions examined. There was an association between short stature and current eczema (for height, p.006, beta =-.026) and a trend towards an association with hayfever. Height was therefore included in the model and is likely to be a marker for sex.

Crosstabulations were performed and odds ratios plotted. In cases where the genetic association appeared clearly additive, the locus was modelled as a continuous variable whereas if there was a clear dominant effect, the locus was considered as two categories. Unless otherwise stated, p values are given for associations after adjusting for relevant covariates, which are summarised in the relevant Tables.

Logistic regression

Asthma

IL13

Table 3.8 summarises the significant associations seen. The Arg110Gln variant (R110Q) associated with asthma with an OR of 1.38 for genotypes Q/Q and R/Q compared with R/R (p=.015, 95% CI 1.06-1.79) (Figure 3.4). The heterozygotes had the highest odds: OR 1.80 (95% CI 1.00-2.60) for Arg/Gln; OR 1.25 (95% CI 0.47-2.03)

for Arg/Arg versus Gln/Gln respectively (p=.018). Genotype data are presented in two rather than three categories due to small numbers in the Gln/Gln group (n=43).

The 3'UTR SNPs 6, 9 and 13 associated with asthma. As previously noted, this is expected since these 3'UTR loci are in strong linkage disequilibrium with Arg110Gln (D'>0.98). Crosstabulations for SNP 5 and 6 are shown in Tables 3.6 and 3.7. Table 3.7 for SNP 6 is almost identical to Table 3.6 for SNP 5, illustrating the effects of strong linkage disequilibrium. There were no significant interactions with sex/BMI/smoking. There were no other significant associations in the Caucasian group. The overall association between the intronic SNP 2 and asthma approached significance (p=.056) with OR 1.65 (95% CI 0.89-2.41) for G/G and G/A versus A/A group.

Table 3.6 Crosstabulation for *IL13* R110Q (SNP 5) and asthma.

	Q/Q		Q/R[1]		R/R	
Asthma[2]	216	(22.2)	116	(29.1)	8	(18.6)
No asthma	756	(77.8)	282	(70.9)	35	(81.4)
Total	972	(100)	398	(100)	43	(100)

Data are number (percentage, %) of subjects, with % having asthma in each genotype group highlighted in red.
[1] R, Arginine; Q, Glutamine.
[2] "Asthma" refers to a subjective report of physician diagnosed asthma ever.

Table 3.7 Crosstabulation for *IL13* SNP 6 and asthma.

	G/G		T/G		T/T	
Asthma[1]	218	(22.4)	117	(28.7)	8	(18.2)
No asthma	754	(77.6)	290	(71.3)	36	(81.8)
Total	972	(100)	407	(100)	44	(100)

Data are number (percentage, %) of subjects, with % having asthma in each genotype group highlighted in red.
[1] "Asthma" refers to a subjective report of physician diagnosed asthma ever.

Table 3.8 Significant associations of *IL13* SNPs with asthma[1].

	Location	Odds ratio	95% CI	P value
SNP 5 rs20541	Exon 4 R110Q	1.38 for A/A+A/G vs G/G	1.06-1.79	.015
SNP 13 rs1295685	3'UTR	1.38 for T/T+C/T vs C/C	1.07-1.79	.014
SNP 6 rs848	3'UTR	1.33 for T/T+G/T vs G/G	1.03-1.72	.029
SNP 9 rs847	3'UTR	1.34 for A/A+A/G vs G/G	1.03-1.73	.029

[1] After adjusting for relevant covariates, see Table 3.14.

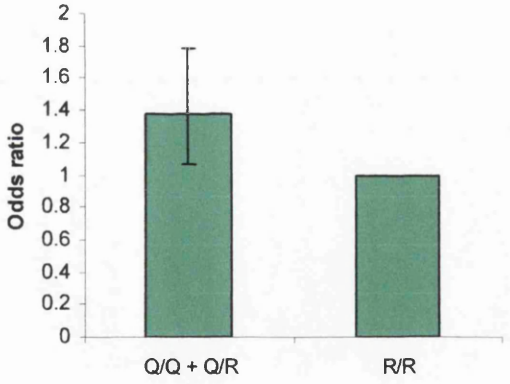


Figure 3.4 Odds ratio of asthma at *IL13* R110Q (SNP 5). Very similar results were seen for the three 3'UTR variants in strong linkage disequilibrium with R110Q.

IL4RA

SNP 5 (Ile50Val) associated with asthma with an additive effect demonstrated shown in Table 3.9 and Figure 3.5: OR 1.25 for G/G vs G/A and A/G vs A/A, where the A allele codes for Isoleucine (I) and the G allele for Valine (V) (p=.013, 95% CI 1.05-1.49). There were no significant interactions with sex/BMI/smoking.

Table 3.9 Crosstabulation for *IL4RA* I50V (SNP 5) and asthma.

	V/V		V/I		I/I	
Asthma[1]	77	(28.3)	173	(24.7)	90	(20.1)
No asthma	195	(71.7)	526	(75.3)	357	(79.9)
Total	272	(100)	699	(100)	447	(100)

Data are number (percentage, %) of subjects, with % having asthma in each genotype group highlighted in red.
[1] "Asthma" refers to a subjective report of physician diagnosed asthma ever.

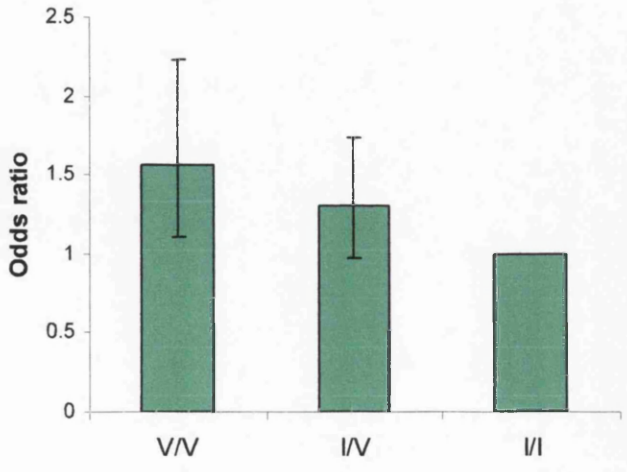


Figure 3.5 Odds ratio of asthma at *IL4RA* I50V (SNP 5). An additive effect is shown for the Valine variant.

STAT6

When examining the association with current asthma (which is likely to provide a more robust diagnosis of asthma than that made during childhood which has now remitted), dominant effects were seen for 3'UTR SNP 1 (Figure 3.10) and intronic SNP 11. These variants were associated with the highest odds ratios of current asthma: SNP 1, OR 2.21 (1.17-4.17, p=.014) for A/A vs A/G and G/G, after adjusting for relevant covariates (BMI); SNP 11, OR 3.60 (1.10-11.84, p=.035) for T/T vs C/T and C/C. However, the association at SNP 11 did not quite reach significance after adjusting for BMI with OR 3.24 (95% CI 2.01-4.47, p=.061). Crosstabulation tables 3.10 and 3.11 are shown: although heterozygote A/A numbers are small for SNP 1 (n=62), the association with current asthma remained significant after adjusting for relevant covariates. There were no significant interactions with sex/BMI/smoking.

Table 3.10 Crosstabulation for *STAT6* SNP 1 and current asthma.

	A/A		A/G		G/G	
Current asthma[1]	13	(21.0)	49	(9.5)	97	(11.4)
No asthma	49	(79.0)	467	(90.5)	751	(88.6)
Total	62	(100)	516	(100)	848	(100)

Data are number (percentage, %) of subjects, with % having asthma in each genotype group highlighted in red.
[1] "Asthma" refers to a subjective report of physician diagnosed asthma.

Table 3.11 Crosstabulation for *STAT6* SNP 11 and current asthma.

	T/T		C/T		C/C	
Current asthma[1]	4	(30.8)	19	(8.4)	136	(11.5)
No asthma	9	(69.2)	206	(91.6)	1051	(88.5)
Total	13	(100)	225	(100)	1187	(100)

Data are number (percentage, %) of subjects, with % having asthma in each genotype group highlighted in red.
[1] "Asthma" refers to a subjective report of physician diagnosed asthma.

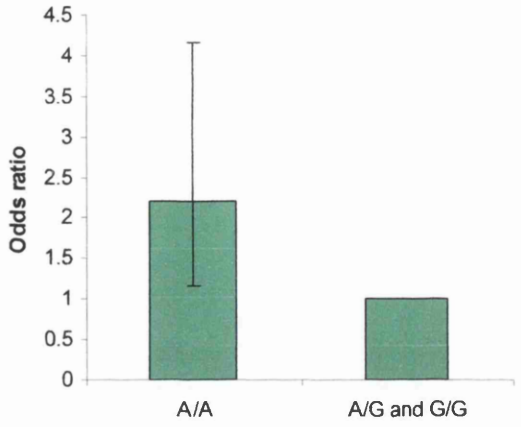


Figure 3.6 Odds ratio of current asthma at *STAT6* G2964A (SNP 1).

Clinical atopy

STAT6

An association between the 3'UTR SNP 1 and "eczema ever" was of borderline significance after adjusting for age (p=.050). When including only those with current eczema (which, like asthma, is likely to exclude some cases where the diagnosis is uncertain e.g. neonatal eczema), the association with SNP 1 became more significant (p=.010 overall): OR 2.62 (1.40-4.89, p=.003) for A/A vs. A/G and G/G, after adjusting for relevant covariates (Figure 3.7). SNP 1 also associated with hayfever: OR 1.99 (1.19-3.32, p=.008) (Figure 3.8). Crosstabulations are shown (Tables 3.12, 3.13) with a strong recessive effect seen for homozygotes A/A. There were no significant interactions with sex/BMI/smoking.

Table 3.12 Crosstabulation for *STAT6* SNP 1 and current eczema.

	A/A		A/G		G/G	
Current eczema	14	(22.6)	52	(10.1)	80	(9.5)
No asthma	48	(77.4)	462	(89.9)	763	(90.5)
Total	62	(100)	514	(100)	843	(100)

Data are number (percentage) of subjects, with % having current eczema in each genotype group highlighted in red.

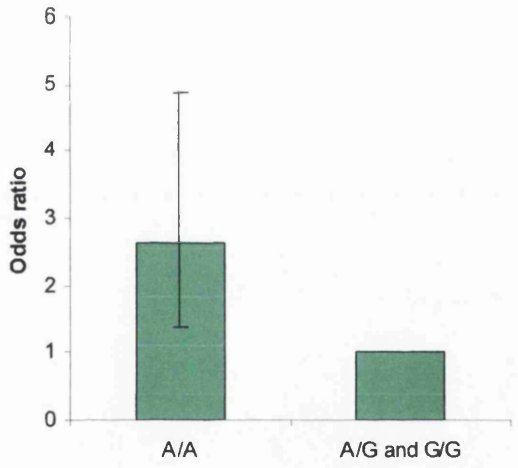


Figure 3.7 Odds ratio of current eczema at *STAT6* 3'UTR G2964A (SNP 1).

Table 3.13 Crosstabulation for *STAT6* 3'UTR SNP 1 and hayfever

	A/A		A/G		G/G	
Hayfever	32	(51.6)	169	(32.8)	300	(35.5)
No hayfever	30	(48.4)	346	(67.2)	545	(64.5)
Total	62	(100)	515	(100)	845	(100)

Data are number (percentage, %) of subjects, with % having hayfever in each genotype group highlighted in red.

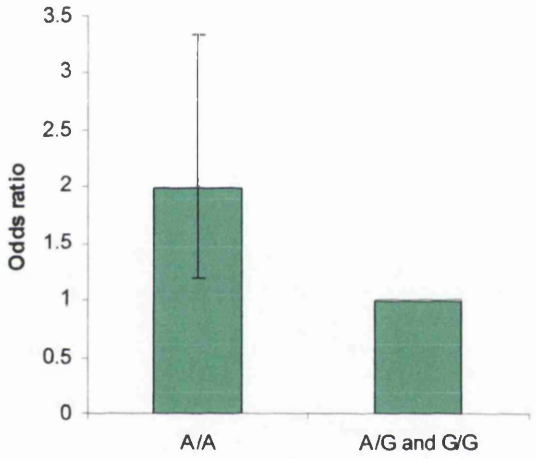


Figure 3.8 Odds ratio of hayfever at *STAT6* 3'UTR SNP 1.

IL13 and ***IL4RA***

IL13 and *IL4RA* SNPs showed no significant associations with hayfever or eczema. A summary of variants where associations approached significance and where $p<.05$ for differences between individual pairs of genotypes (rather than for the overall factor 'genotype') is provided in Appendix VI.

Table 3.14 Final models of genetic associations for dichotomous outcomes including relevant covariates (sex, smoking, BMI, age, height if significant at 5% level)[1]

	Location	Outcome	P value
IL13			
SNP 5	Exon 4 Arg110Gln	Asthma ever	.016[2]
SNP 5 + smoking[3] + BMI			.017
SNP 13	3'UTR		.017
SNP 13 + smoking + BMI			.016
SNP 6	3'UTR		.029
SNP 6 + smoking + BMI			.028
SNP 9	3'UTR		.043
SNP 9 + smoking + BMI			.038
IL4RA			
SNP 5	Exon 5 Ile50Val	Asthma ever	.010
SNP 5 + smoking + BMI			.013
STAT6			
SNP 1	3'UTR	Current asthma	.027
SNP 1 + BMI			.031
SNP 11	Intron 18	Current asthma	.046
SNP 11 + BMI			.071
SNP 1	3'UTR	Current eczema	.007
SNP 1 + sex + height			.010
SNP 1		Hayfever	.015
SNP 1 + sex + height			.018

1. Covariates shown if $p<.05$ when included in final model.
2. Overall p value at this locus before considering relevant covariates.
3. Refers to current smoking status.

3.4 Discussion

Associations were demonstrated for a "physician diagnosis of asthma ever" with all three loci – *IL13*, *IL4RA* and *STAT6*. In *IL13*, the non-synonymous substitution Arg110Gln in exon 4, associated with asthma, consistent with findings from case-

control studies in British, Japanese and German populations previously [117, 247]. This is the first time this variant has been confirmed in association with asthma in an unselected population study and is a relevant finding since several case-control studies have failed to confirm this association. This is likely to be due to the differing frequency of the variant across ethnic groups [249]. Moreover, Gln110 is of particular interest since this variant is found in approximately 33% of our population and previously observed in 25% of a mixed American/German general population [113]. Heterozygotes were observed to have the highest odds of asthma, which is likely to be simply due to low numbers in the Gln homozygote group but the potential functional relevance of this observation, with regard to epistatic interactions, is discussed further in Chapter 6. Structural and functional analyses provide insights into the mechanistic basis for the increased activity of Gln110. The SNP results in the non-conservative replacement of a positively charged arginine with a neutral glutamine at position 130 (this numbering includes the signal peptide; 130 is referred to as position 110 when numbering does not include the signal peptide) [117, 133]. The Arg110Gln substitution occurs in α-helix D, the region of IL-13 that is thought to interact with IL-4RA/IL-13RA1 heterodimers [250]. Thus, Arg110Gln has the potential to affect IL-13–dependent signalling events.

The observed association for Arg110Gln is strengthened by the finding of almost identical quantitative associations with the three 3'UTR SNPs which are in almost complete linkage disequilibrium (LD) with the non-synonymous SNP. This extended LD haploblock spans intron 3, exon 4 and the 3'UTR and poses difficulties in identifying the mechanisms underlying observed associations. While it is not possible to exclude that the association with clinical phenotype may be due in part to the biologic activities of these 3'UTR SNPs, they are not located in consensus sequences known to regulate gene-splicing, gene expression nor IL-13 mRNA stability, and it seems reasonable to conclude that the association with asthma is more likely due to the biological activity of Arg110Gln.

However, in the absence of functional studies investigating all *IL13* SNPs, it is not possible to exclude the presence of other functional SNPs in this region. There is the

possibility of allelic heterogeneity, that is, several disease-associated *IL13* variants may exist. Population theory suggests that for common disease, only a few alleles in a gene are likely to contribute to disease i.e. allelic heterogeneity is low for common conditions [251]. It is possible that the causal variant remains untyped, but in linkage disequilibrium with the examined SNPs and is located elsewhere in this chromosome region, possibly regulating IL-13 expression. This can only be ascertained through analysis of a complete polymorphism and linkage disequilibrium map of the whole region in a large sample set, and thereby excluding regions where a causal variant is unlikely to exist.

Arg110Gln has known functional effects with upregulation of IL-13 activity demonstrated *in vivo* [25, 118]. Functional studies show that this may be due to decreased affinity of the minor variant for the decoy receptor IL-13R2. This receptor is part of a complex feedback loop involved in controlling IL-13–dependent responses, acting as a key negative regulator of IL-13 (by binding the cytokine with high affinity) whilst IL-13 itself enhances decoy receptor expression. The minor variant was more active than wild-type IL-13 in inducing STAT6 phosphorylation and CD23 expression in monocytes and hydrocortisone-dependent IgE switching in B cells [118].

No associations with clinical phenotype were demonstrated for the *IL13* promoter polymorphisms. The -1512A/C SNP has not previously been examined in association with asthma. However, the -1024C/T SNP has been associated with asthma in Dutch, German and African-American case-control populations [12, 114, 135, 141], although not in an Icelandic population [136]. This could be postulated to be due to the strong LD observed previously between this promoter SNP with Arg110Gln in the German population [113], but not in our population. However, LD was not observed between these variants in the Dutch population [114] and the lack of association in our population may be due to the -1024C/T variant not been clinically relevant at the population level, or due to differences in allele frequencies between British and Dutch populations.

This is the first time the Ile50Val variant of *IL4RA* has been found to be associated with asthma at the population level, having been previously reported in Japanese case-control studies [119, 155]. A gene-dosage effect was observed, with increasing odds of asthma for each Valine allele. However in contrast, the Japanese studies found Ile50 to confer asthma risk, whereas Val50 was associated with increased risk in our study, emphasising that susceptibility loci may differ in diverse ethnic populations (and discussed further in Chapter 6). Furthermore, Ile50 differs in frequency between populations, for instance reported at 59% vs 41% in European and Japanese populations respectively [119, 252] and the association between *IL4RA* gene polymorphisms, its haplotypes and asthma have been noted to vary between different Asian populations [152]. Interestingly, Risma et al reported that the Val50Arg551 haplotype was associated with allergic asthma and enhanced IL-4R function [14]. Kabesch et al did not find an association when the Ile50Val variant was examined singly in a German population [12] and several case-control studies have reported negative findings (Table 1.2). This thesis clearly demonstrates that the Val50 allele confers an additive effect for asthma risk at the population level in a British Caucasian population.

This thesis describes a novel association with asthma at the population level for the *STAT6* 3'UTR variant G2964A, which demonstrated the highest odds ratio for asthma (2.21, CI 1.17-4.18). Only one Japanese case-control study has previously reported a positive association for this (or any other) *STAT6* variant with asthma [124], with recent data showing association on haplotype analysis [82], and negative findings in other diverse populations on single polymorphism analysis [124, 127, 168]. In contrast to our population, the G/G genotype (rather than A/A) related to mild atopic asthma in the Japanese population. This may be due to ethnic differences in allele frequency and risk susceptibility and it is worth noting also that 2964G/G was only associated with *mild* atopic (rather than marked atopic) asthma in the Japanese and no association was found in the British population [124]. Kabesch et al reported negative findings in a German unselected population for the C2892T promoter SNP which is in strong LD with G2964A [12]; our study confirmed these negative findings in a British population.

With regard to our findings of increased asthma susceptibility conferred by *IL4RA*-Val50 and *STAT6*-2964A, there are now several examples of established associations with different functional variants within the same gene or with opposite alleles at the same SNP in different populations [11]. Cameron et al found that the *IL13*-1024T allele enhanced *IL13* promoter activity but only in the context of Th2 differentiation [116]. Thus it can be speculated that seemingly contradictory results for specific alleles in different populations may in some cases be the result of complex interactions between genes (their transcription and the actions of their products) and the environmental *milieu.*

It is perhaps surprising that only the *STAT6* 3'UTR variant G2964A associated with asthma, whereas this SNP (and almost all *STAT6* SNPs) are in strong LD with the other *STAT6* SNPs. However, a biological role for this variant is suggested by highly consistent associations for this SNP demonstrated in addition for current eczema and hayfever in this population, and the high odds ratio found. The highest odds ratio was demonstrated between this SNP and current eczema (2.62, CI 1.40-4.89). Associations with eczema and hayfever were only demonstrated at the *STAT6* locus, for the G2964A polymorphism, and these associations are novel. Only one previous Japanese case-control study has examined this variant in association with eczema (a composite of "allergic diseases" was examined), with negative findings [125]. This variant has not previously been examined in relation to hayfever/allergic rhinitis. There are few reports of IL-13 signalling variants being examined in the context of rhinoconjunctivitis: an association has been reported between IL-4RA Ile50Val and Japanese cedar pollinosis and Ser478Pro showed a trend towards a protective effect against allergic rhinitis [149, 156]. Whilst our study did not specifically document an atopic component for hayfever/eczema, previous epidemiological studies show these definitions to be robust markers for atopic rhinitis/atopic dermatitis [253, 254]. We examined total IgE levels separately since this is more robust than using a composite clinical/IgE score where the parameters are likely to be under different genetic influences [255, 256].

Populations of different ethnic origin have been observed to have different allele

frequencies for many genes, which provides one explanation for non-replication of genetic association in different populations. For instance, the observation of differential allele frequency and disease association for *STAT6* G2964A emphasises the genetic heterogeneity of atopic disease, even within one locus in different ethnic groups [124, 257]. Whilst founder effects and genetic drift may account for some of these differences, consistent observations have been made relating to IL-13 signalling pathway genes, suggesting that evolutionary pressures may also be important [86].

Whilst asthma is a genetically heterogenous disease, findings from this study, and across ethnically diverse populations, support a role for IL-13 signalling variants in asthma and atopy susceptibility. When examining *IL13* haploblock structure across ethnic groups, investigators have shown a significant excess of high frequency-derived SNPs in Caucasian and Chinese populations suggesting a positive selection effect [249]. Conserved regions of the gene are likely to confer some evolutionary advantage and there is evidence that Th2 variants conferring asthma/atopy susceptibility may have arisen due to their evolutionary protection against worm infestation [82, 84, 86]. The *STAT6* variant G2964A and *IL13* variant -1024C/T, both associated with asthma, have been shown to be protective against ascaris and schistosoma infestation respectively and the survival advantage conferred may explain the persistence of these alleles in the population. Moller et al recently reported that *STAT6* haplotypes relate to IgE levels, allergy and worm burden, with a cross-population comparison in Chinese and British subjects showing that haplotypes relating to low worm burden/high IgE confer increased risk of asthma and allergic disorder [82]. Since genes exhibit pleiotropy, having multiple roles within living organisms, it is entirely plausible that alleles conferring advantage in one system may confer disadvantage when different environmental pressures apply. Thus Th2 immune variants offering protection against helminth infestation in non-western environments, confer increased susceptibility to asthma and associated Th2 allergic disease in the context of a western environment.

This thesis suggests that genetic variation at the *IL13*, *IL4RA* and *STAT6* loci is implicated in asthma and atopy susceptibility. In particular, identification of the

underlying mechanisms remains difficult for *IL13* due to the extended haploblocks in chromosome 5q31 and requires clarification by functional studies. Moreover this region has been found to show a high level of diversity across different ethnic populations, emphasising the importance of determining allele frequencies and analysing haplotype structure across ethnically and geographically diverse populations. This has important implications for the HapMap initiative, which is based on the premise that it will be possible to identify a common pattern of haplotype structure (and htSNPs) for gene mapping studies across geographic regions [258].

The definition of asthma gives rise to difficulties since the term encompasses a heterogenous spectrum of disorder rather than a single clinical entity. The diagnosis can be difficult to make clinically at a single timepoint and is usually made over a period of observation, based on symptoms, peak flow lability and bronchodilator reversibility. For the purposes of epidemiological study, the definition of 'physician-confirmed asthma' is regarded as robust and the bronchial symptoms IUATLD questionnaire (similarly the ISAAC questionnaire in children) has been validated and widely used in asthma research [233-235]. Given the age range of this study, the majority of volunteers with asthma are likely to have atopic asthma. Oryszczyn et al found in a recent epidemiological study that almost 90% of their asthmatic subjects had a co-existing marker for atopy, and in our younger population, we found co-existent hayfever and eczema in 63.1% and 19.9% of asthma subjects respectively [259]. The data on clinical atopy, in addition to quantitative markers of Th2 immunity and lung function provided comprehensive additional phenotypic information.

There are inherent difficulties in obtaining a truly random sample representative of the general population, which apply to any epidemiological study. An advantage of this study was that volunteers were recruited from one particular city rather than being recruited via hospital clinic or family members with the associated potential confounding effects therein, and the study was inclusive of all volunteers within the stated age range.

It is possible that the nature of the recruitment process may have enriched the population for those with asthma and smokers. However, the prevalence of current asthma and smoking was not higher than expected in a young adult (predominantly student) population, at 10.9% and 16.9% respectively. Furthermore, many fit non-smokers were keen to participate in order to obtain a measure of their health status, and conversely, some students with asthma declined to take part since they felt they did not wish to perform further testing; effects which are likely to counterbalance any potential enrichment of the population described above. Since this was not a study designed to test the prevalence of asthma or smoking, potential enrichment of the sample is not relevant, and smoking was adjusted for in the statistical analysis.

Since the selected 22 SNPs span the three loci, it is unlikely that important associations were missed. It is possible that observed associations were the result of non-genotyped SNPs in LD with selected SNPs, but this seems unlikely given the consistency of results, and gene coverage achieved. Results of haplotype analysis also showed these particular SNPs to be mainly responsible for the haplotype results, as described in Chapter 6. The possibility of extended LD beyond the examined loci cannot be excluded and has been previously reported [260].

This thesis examined non-coding region polymorphisms in addition to the coding variants traditionally thought to be of paramount importance. The observations presented here indicate a contributory role for a non-coding variant of *STAT6* in asthma and associated atopic conditions. Emerging evidence suggests that SNPs in non-coding regions may have important functional activities in gene regulation and expression. A 3'UTR polymorphism in the E-Cadherin Gene has recently been found to be associated with primary open angle glaucoma [261]. It is of note that G2964A is located in a C-rich region of the 3'UTR regulatory elements of *STAT6*, where there is increasing evidence that these elements regulate gene expression in a variety of ways, including the determination of mRNA transcript half-life [262]. The next step will be functional studies to unravel the biological role of this non-coding variant, and determine the underlying basis of the association with asthma and clinical atopy.

CHAPTER 4

Genetic association of single nucleotide polymorphisms with Th2 immune phenotypes

4.1 Introduction

The asthmatic response is characterised by elevated production of IgE, cytokines, chemokines, eosinophilia, mucus hypersecretion, airway obstruction, and airway hyper-responsiveness to allergens. Clinical and functional studies have shown a strong correlation between the presence of CD4+ Th2 cells, eosinophils, and disease severity, suggesting a crucial role for these cells in the pathophysiology of asthma. Although recently it has become apparent that the Th1/Th2 paradigm is insufficient to completely explain the underlying basis of allergic disease, Th2 cells are recognised to be critical to the development of allergic responses [263].

Th2 cells induce asthma through the secretion of specific cytokines, in particular IL-13 and IL-4, which activate inflammatory and residential effector pathways both directly and indirectly. IL-13 has been shown to induce a complex array of genes in resident airway cells (smooth muscle cells, fibroblasts, epithelial cells and monocyte/macrophages) independently of traditional effector cells such as mast cells and eosinophils. Effector cells contribute to disease via their ability to produce IL-13 in response to IL-13-induced mediators, thus perpetuating the allergic response and leading to disease chronicity [9]. The importance of early life immunomodulatory effects on asthma inception is demonstrated by easily detected levels of IL-13 in the placenta and the secretion of IL-13 by neonatal T cells [118].

Experimental work has shown chemokines to have an important role in the allergic inflammation characteristic of asthma and related atopic disease, mediated by their functions as potent leukocyte chemoattractants, cellular activating factors, and histamine-releasing factors. Recent evidence points to the role of the eotaxin subfamily of chemokines and their receptor CC chemokine receptor 3 (CCR3) in promoting IL-13-associated Th2 allergic responses. Epithelial and endothelial cells express eotaxin-1 while its receptor, CCR3 is highly expressed on eosinophils [264]. IL-13 upregulates eotaxin expression in the airway – which is increased in asthmatics - by STAT6 dependent mechanisms [265, 266].

The main role of eotaxin is as a potent attractant for eosinophils, which are the main effector cells in airway inflammation, and an important cellular source of IL-13 [267]. Recent murine studies of gene deletions eliminating eosinophils emphasise their role in asthma [268]. Transgenic mice over-expressing IL-13 have a marked increase in eotaxin-1 along with other characteristics of asthma including eosinophil infiltration [269]. A complex integrated pathway exists whereby IL-13 promotes inflammation partly by induction of eotaxin-1, which provides regulatory feedback on IL-13 production, directly via eosinophils and indirectly via Th2 cells [270]. Eotaxin-1 can be regarded as a downstream effector molecule of the IL-13 pathway, with roles including eosinophil recruitment, activation and degranulation [270]. This thesis considers total IgE, eotaxin-1 (CCL11) levels and eosinophil counts as the primary Th2 immune phenotypes in asthma and related atopic disease.

Events mediated by IgE are integral to allergic reactions and induction of IgE is likely to be critical to the role of IL-13 signalling variants in asthma and atopy. Isotype class switching from IgM to IgE is a result of the activation of the IL-4/IL-13 cytokine pathway and genetic variants within this pathway may influence the regulation of IgE levels. STAT6 activates germline transcription from the epsilon heavy chain gene locus, and induces isotype switching in B cells (in combination with signals from the B cell surface molecule CD40) and activates other genes involved in IgE production [271]. Genetic susceptibility to heightened IgE responses is likely to be caused by a pattern of polymorphisms in several genes involved in Th2 immunity.

Association studies have suggested that IgE levels are under genetic control but case-control studies have shown conflicting results for many common variants of IL-13 signalling [117, 121, 145, 152]. There have been very few studies on unselected populations to test whether these variants confer risk of higher IgE levels in a general population. Studies in British and German populations have found that some of these variants confer susceptibility to higher IgE levels at a population level [12, 129, 132]. This chapter describes the largest unselected study to date, to examine the role of

UNIVERSITY OF WALES SWANSEA LIBRARY

variants of *IL13*, *IL4RA* and *STAT6* in relation to IgE regulation, and the only study to examine these IL-13 signalling variants with relation to the additional Th2 immune phenotypes of eosinophil counts and eotaxin levels.

4.2 Methods

An unselected population of 1614 adults aged 18-30 years was recruited according to the Methodology outlined in Chapter 2. Genotyping results are reported on the Caucasian group (n=1445). Serum total IgE (kIU/L) was measured by ELISA-type sandwich assay and plasma eotaxin (pg/ml) was measured using ELISA (BD OptEIA™, BD Biosciences) as outlined in Chapter 2. Eosinophil counts were measured by automated analyzer, providing a five part differential of the white cell count, as outlined in Chapter 2. Genotyping was performed for a total of 22 polymorphisms at the *IL13*, *IL4RA* and *STAT6* loci (see Chapter 2 for further detail). The analysis of the association between genotype/phenotype took the form of a series of standard linear regression models, with adjustment made for relevant covariates (age, sex, smoking, height etc). Linear regression analysis was performed using SPSS 13.0. Since IgE, eosinophil counts and eotaxin were not normally distributed, statistical analysis was performed on log-transformed values. The phenotype data were analysed as continuous variables on a logarithmic scale, as ln(x+1) to overcome errors when levels were undetectable and thus counted as zero. Analysis of continuous data was chosen, rather than choose a widely used arbitrary cutoff such as 100 kIU/L for IgE, because continuous data may much better resemble the distribution of immune markers in the population.

4.3 Results

4.3.1 Population characteristics

The Caucasian group for this analysis consisted of 1445 subjects. Of the Caucasians, subjects reporting "smoking for as long as a year" represented 27.7% of the population (n=399) with 17.6% reporting current smoking (n=253). Current asthmatics had

significantly higher total IgE and eosinophil counts, after adjusting for relevant covariates (p<.001) but not higher levels of eotaxin (Table 4.1).

Table 4.1 Baseline characteristics of study participants.

	Current asthma[1]	"Never asthma"
IgE (kIU/L)	113 (91-141)[2]	41 (38-45)
Eosinophil count (cells x10^9/L)	0.24 (0.22-0.27)[2]	0.17 (0.16-0.18)
Eotaxin (pg/ml)	11.5 (8.9-13.9)[2]	12.5 (11.6-13.5)

[1] "Asthma" refers to a subjective report of physician diagnosed asthma.
[2] Geometric mean (95% confidence interval, CI).

Males had higher total IgE, eotaxin levels and eosinophil counts (p<.001). Current smokers had higher eosinophil counts (p=.001) but not significantly higher total IgE nor eotaxin (Table 4.2). However smoker pack years were related to eotaxin (p=.036). A small increase in total IgE levels with increasing age was observed (p=.004, beta=.04) per year but not between age and eosinophil count and of borderline significance for eotaxin (p=.052). Height was not associated with total IgE nor eosinophil count but was associated with eotaxin (p<.001) where it is likely to act as a marker for sex. Relevant covariates were included in the linear regression models and interactions examined.

Table 4.2 Mean total IgE, eotaxin and eosinophil count according to sex and smoking status.

	Geometric mean total IgE (kIU/L)	95% CI	P value
Males	55	49-62	<.001
Females	37	33-42	
	Geometric mean eotaxin (pg/ml)	95% CI	P value
Males	15.0	13.7-16.4	<.001
Females	9.9	8.9-11.0	
	Geometric mean eosinophil count (cells x10^9/L)	95% CI	P value
Males	0.20	0.19-0.21	<.001
Females	0.17	0.16-0.18	
Smokers	0.21	0.19-0.22	.001
Non-smokers	0.18	0.17-0.18	

4.3.2 Linear regression analyses

Genetic associations with total IgE

IL13

After adjusting for sex, age and height, the association between the 3'UTR SNP 6 and IgE just reached significance (p=.048), due largely to the difference between the G/T and G/G group (p=.014) (Figure 4.1). The heterozygotes had the highest IgE levels: T/T (42, 95% CI 28-67), G/T (54, 95% CI 47-64), G/G (42, 95% CI 40-48). Data are presented in two categories since the number in the T/T group was small (n=44). Due to associations in other populations, SNP 10, SNP 1 and SNP 5 were examined in more detail. No significant association with IgE was seen for SNPs 1 (-1024C/T) and 5 (Arg110Gln). For SNP 10 (-1512A/C), there was a trend towards a higher IgE level for heterozygotes A/C compared to A/A homozygotes, with p=.048 before adjustment and p=.064 after adjustment for sex, height and age.

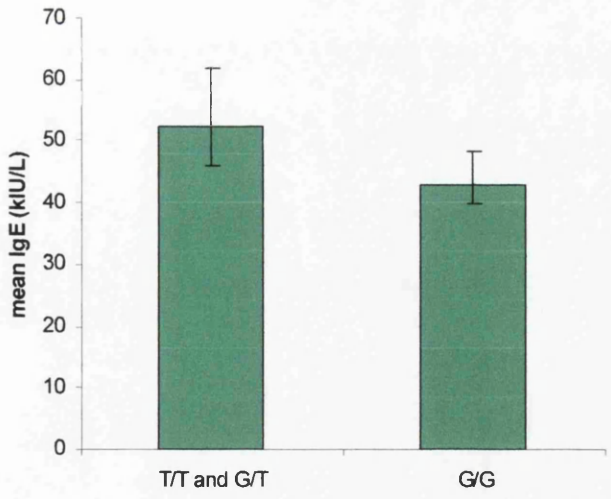


Figure 4.1 Mean IgE at 3'UTR SNP 6 of *IL13*.

IL4RA

Three of the *IL4RA* variants showed strongly significant associations with IgE. These were intronic SNP 8, Arg551Gln (SNP13) (Figure 4.2) and the 3'UTR SNP 16, shown in Table 4.3. A codominant mode of inheritance was observed, with heterozygotes having intermediate levels compared with both homozygote groups.

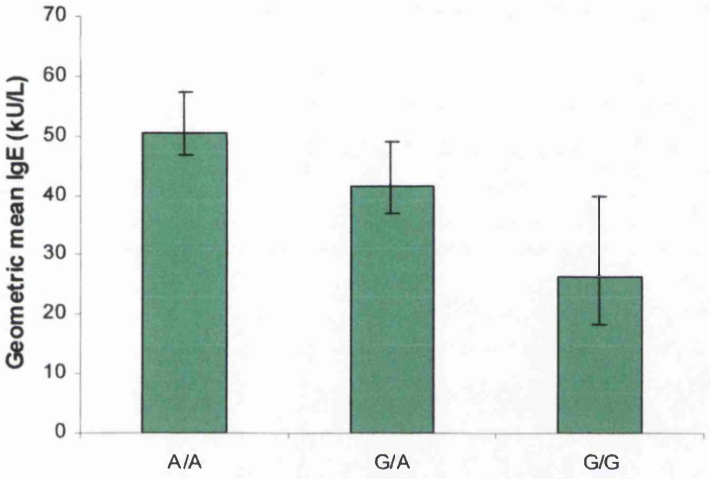


Figure 4.2 Mean IgE at Arg551Gln SNP 13 of *IL4RA*.

STAT6

Significant associations with IgE were seen at the 3'UTR SNP 3 and intronic SNP 8, shown in Figure 4.3 and Table 4.3.

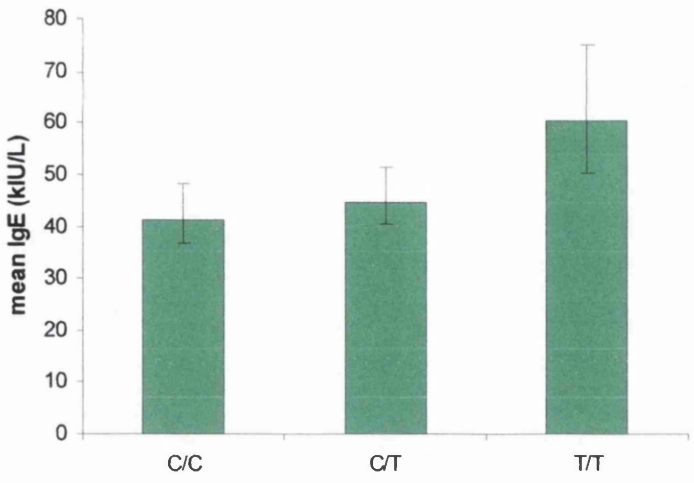


Figure 4.3 Mean IgE at *STAT6* intronic SNP 8 (C2892T).

Table 4.3 Mean IgE level at *IL13*, *IL4RA* and *STAT6* loci.

	Location	Genotype	Mean IgE (kIU/L)	95% CI	P value[1]
IL13					
SNP 6	3'UTR	T/T	42	28-67	.048
rs848		G/T	54	47-64	
		G/G	43	40-48	
IL4RA					
SNP 8	Intron 8	A/A	50	46-55	.011
rs2891058		A/G	37	32-45	
		G/G	33	19-60	
SNP 13	Exon 12	A/A	51	47-57	.001
rs1801275		A/G	42	37-49	
Arg551Gln		G/G	26	18-40	
SNP 16	3'UTR	T/T	50	45-56	.006
rs1049631		C/T	38	34-46	
		C/C	30	17-55	
STAT6					
SNP 8	Intron 2	T/T	61	50-75	.007
rs324011		C/T	45	41-51	
		C/C	41	37-49	
SNP 3	3'UTR	A/A	50	46-56	.001
rs1059513		A/G	34	30-42	
		G/G	38	20-75	

[1] After adjusting for relevant covariates, see Table 4.4.

Table 4.4 Final models of genetic associations for total IgE after considering relevant covariates (sex, smoking, age, height if significant at 5% level)[1]

	Location	Outcome	P value
IL13			
SNP 6	3'UTR	IgE	.056[1]
SNP 6 + sex + age + height			.048[2]
IL4RA			
SNP 8	Intron 8	IgE	.007
SNP 8 + sex + age + height			.011
SNP 13	Exon 12	IgE	.001
SNP 13 + sex + age + height	Arg551Gln		.001
SNP 16	3'UTR	IgE	.005
SNP 16 + sex + age + height			.006
STAT6			
SNP 8	Intron 2	IgE	.009
SNP 8 + sex + age + height			.007
SNP 3	3'UTR	IgE	.001
SNP 3 + sex + age + height			.001

[1]Overall p value at this locus before considering relevant covariates.
[2] p value after adjusting for relevant covariates.

Genetic associations with eosinophil count

Associations with eosinophil count for the *IL4RA* variants are summarised in Tables 4.5-4.6. There were no significant genetic associations with eosinophil count for *IL13* and *STAT6* variants.

Table 4.5 Genetic associations with eosinophil count.

	Location	Outcome	P value
IL4RA			
SNP 3	Promoter	Eosinophil count	.035
SNP 3 + sex + smoking			.030
SNP 5	Exon 5	Eosinophil count	.024
SNP 5 + sex + smoking	Ile50Val		.020
SNP 12	Exon 12	Eosinophil count	.031
SNP 12 + sex + smoking	Ser478Pro		.032

Table 4.6 Geometric mean eosinophil count for *IL4RA* genotypes.

	Location	Genotype	Geometric mean eosinophil count (cells x 10^9/L)	95% CI	P value[1]
IL4RA					
SNP 3	Promoter	G/G	0.19	0.17-0.21	.030
		A/G	0.18	0.16-0.21	
		A/A	0.17	0.15-0.18	
SNP 5	Exon 5	V/V	0.20	0.18-0.21	.020
	Ile50Val	V/I	0.18	0.17-0.20	
		I/I	0.17	0.16-0.19	
SNP 12	Exon 12	A/A	0.17	0.15-0.19	.032
	Ser478Pro	A/G	0.18	0.17-0.20	
		G/G	0.19	0.18-0.21	

[1] P value after adjusting for relevant covariates.

Genetic associations with plasma eotaxin

Genetic associations with eotaxin are illustrated in Table 4.7. For *IL13*, there was an association between intronic SNP 7 and eotaxin ($p<.05$). An association was demonstrated between *STAT6* 3'UTR SNP 1 and eotaxin levels ($p<.05$). These associations were adjusted for sex.

Table 4.7 Geometric mean eotaxin for genotypes of *IL13* and *STAT6* variants.

	Location	Genotype	Geometric mean eotaxin (pg/ml)	95% CI	P value[1]
IL13					
SNP 7	Intron 1	C/C	12.9	11.9-13.9	.048
		A/C	10.7	9.0-12.7	
		A/A	7.0	3.0-15.1	
STAT6					
SNP 1	3'UTR	A/A	14.8	10.8-20.2	.047
		A/G	11.0	9.7-12.3	
		G/G	13.1	11.9-14.4	

[1] P value after adjusting for relevant covariates.

Table 4.8 Summary of significant associations of *IL13* signalling variants with immune phenotypes.

	Location	IgE P value[1]	Eosinophil count P value[1]	Eotaxin P value[1]
IL13				
SNP 7 rs2066960	Intron 1			.048
SNP 6 rs848	3'UTR	.048		
IL4RA				
SNP 3 rs2283563	5'UTR		.030	
SNP 5 rs1805010	Exon 5 Ile50Val		.020	
SNP 8 rs2891058	Intron 8	.007		
SNP 12 rs1805015	Exon 12 Ser478Pro		.032	
SNP 13 rs181275	Exon 12 Arg551Gln	.001		
SNP 16 rs1049631	3'UTR	.005		
STAT6				
SNP 8 rs324011	Intron 2 C2892T	.009		
SNP 1 rs324015	3'UTR			.047
SNP 3 rs1059513	3'UTR	.001		

[1] P value after adjusting for relevant covariates.

4.4 Discussion

Highly significant associations ($p<.01$) with total IgE levels were seen at the *IL4RA* and *STAT6* loci, rather than at *IL13*. A significant association was demonstrated between

Gln551 and higher IgE levels, having been observed previously in a German case-control study [121], but with many case-control reports of negative findings (see Table 1.3). A gene-dosage effect was observed, with increasing IgE levels for each A allele. Our study has replicated findings from the only other unselected population study to examine this association, where Gln551 was associated with higher IgE in the British 1958 Birth Cohort, with stronger associations and higher IgE levels observed in our population [132].

Associations were demonstrated for *IL4RA* intron and 3'UTR variants which do not appear to have been previously examined in genetic association studies and whilst these variants were not in strong LD with the Arg551 variant, it is possible that these associations might be due to LD between these variants and alternative variants which were not examined. On individual SNP analysis, Ile50Val and Ser478Pro variants do not appear to be important in IgE regulation, which confirms findings from large unselected [12, 132] and smaller (partially unselected) studies [137]. Haplotype analysis indicated that associations with IgE focus on Arg551Gln but also suggested that Ile50Val may have a contributory role and these data are presented in Chapter 6.

This thesis provides the largest unselected study to date of the association between IL-13 signalling variants and asthma and atopy phenotypes. Confirmation was found for the association between *STAT6* cis-regulatory element and 3'UTR variants (rs324011 and rs1059513) with IgE levels, previously documented in German populations [127-129]. Although these are non-coding variants, they are likely to be of functional importance. The function of the 3'UTR of eukaryotic genes is not yet fully understood. Human diseases such as myotonic dystrophy arise as a result of 3'UTR variation. Variants in the 3'UTR region have been shown to affect gene expression by influencing translation, coding capacity, mRNA stability and localisation of RNA in the cytoplasm and therefore may have biological relevance [272, 273]. The 3'UTR affects protein binding but may also exert its effects in its binding with short RNA [274].

Schedel et al proposed that carriers of the T allele for the 3'UTR variant would have

larger amounts of STAT6 mRNA available for translation due to increased stability, and thus increased STAT6 availability for intracellular signalling and induction of IgE [128]. Variant rs324011 is located in the cis-regulatory element of intron 2, where it is positioned within the matrix of one of two transcription binding sites which are in close proximity. Thus a SNP here may alter the binding of NF-κB and therefore have important effects on STAT6 transcription. Moreover, although STAT6 is central in controlling the IgE germline gene promoter, NF-κB has synergistic effects, and the presence of two NF-κB sites within intron 2 suggests feedback regulation between these two transcription factors [275]. Schedel et al found that this SNP was much less well-preserved in human DNA compared with primates, suggesting a functional role for this polymorphism [128]. Since both *STAT6* SNPs are in strong LD, it is proposed that it is the cis-regulatory SNP which is of primary functional importance although further expression analysis studies are needed for confirmation.

Given the strong associations of *STAT6* 3'UTR variant G2964A with clinical atopic disease in this study, it was unexpected that an association was not seen with IgE levels, although it is of note that no investigators have found a positive association with IgE for this variant. This suggests that total IgE levels are controlled by different sets of polymorphisms than clinical atopy *per se,* which will be influenced to a greater degree by environmental factors including allergen exposure and early sensitisation. Previous work points to IgE levels being under greater genetic control than clinical manifestations of atopy [255, 256]. Total IgE and eosinophil counts are likely to represent the most useful immunophenotypes in this random population since eotaxin levels may be more dependent on clinical status at the time of testing, being induced early in the allergic response [270].

This thesis does not confirm some previously documented associations between *IL13* variants and IgE levels in British and German populations of adults and children respectively [12, 132]. The only *IL13* variant showing an association with IgE in our study was the 3'UTR variant rs848, which was in strong LD with Arg110Gln. Heterozygotes had the highest IgE levels, mirroring the highest odds of asthma for the

heterozygotes also, and this is discussed further in Chapter 6. For promoter polymorphism -1512A/C, there was a trend towards A/C heterozygotes having higher IgE than A/A homozygotes which may have been significant in an even larger study. The main explanation for the inconsistency with the German data was that the German study only examined very high IgE levels i.e. a more extreme phenotype (IgE >90th centile; 457 kIU/ml), whereas in our study IgE was examined as a continuous variable.

We did not replicate associations between *IL13* SNPs and total IgE levels reported in the 1958 British Birth Cohort [132]. Explanations for the non-replication of genetic association include differential exposure to environmental factors, heterogeneity in allele frequencies, true differences in IgE associations between populations, ascertainment bias, different methodology and classification of outcome and the use of inadequate sample size to detect a true effect. The study population was a similar British Caucasian population to the British Birth Cohort. However environmental factors may differ between the populations, and for the Birth Cohort, IgE was measured at the ages of 45/46 years rather than an average age of 21 years in our study. This is a possible explanation since in our study, a small increase in IgE levels with increasing age was observed, and environmental factors are also likely to be important since twin studies report 40% of the variance in IgE to be due to environmental factors [276]. Whilst genotype is unchanged, differential gene expression will be influenced by environmental factors. It is also possible that our study of 1445 Caucasians was insufficiently large to detect a modest effect of *IL13* on IgE levels. Although no significant associations were observed when *IL13* variants were examined in isolation, highly significant associations were demonstrated for a risk haplotype containing the previously described promoter and coding *IL13* risk variants and these data are presented in Chapter 6.

Our data are in concordance with the findings of Heinzmann et al who found Arg130 to be associated with asthma rather than IgE in British and Japanese case-control populations [117]. Furthermore, Arima et al found that a recombinant Gln130 variant did not alter STAT6 activation, induction of germline transcripts, or IgE synthesis,

although there was a lower affinity with a decoy receptor IL-13RA2, leading to reduced clearance [25]. Moreover, our data are consistent with the emerging paradigm whereby IL-13 has been shown to induce allergic responses via resident airway cells rather than through traditional effector pathways involving eosinophils and IgE-mediated events [9].

Eosinophils have been measured in few studies examining the IL-13 signalling pathway [110, 134, 159]. In this thesis, weak associations were observed for *IL4RA* variants – a promoter variant and two coding variants. The positive associations for Ile50Val and Ser478Pro and lack of association for Arg551Gln are in contrast to findings from a Korean case-control study [159]. The association with the promoter variant rs2283563 may have been due to the strong LD between this SNP and Ile50Val but it is not possible to exclude an individual biological effect for this variant. It should be noted that although the results reported are statistically significant, the magnitude of the difference between mean eosinophil counts by genotype was small. We found no associations for *IL13* variants with eosinophil count. For *IL13*, Japanese and American investigators have reported associations for promoter variants -1512A/C and Arg110Gln, with negative findings for promoter variant -1024C/T and Arg110Gln (in the Japanese) [110, 134]. *STAT6* variants have not previously been examined in relation to eosinophils in an unselected population study. Only one published study has examined *STAT6* in relation to eosinophils, in a sib-pair design, and similarly to our unselected population, found no positive associations with the *STAT6* locus [127].

The Th2 cytokines IL-13 and IL-4 are potent inducers of epithelial cell expression of the chemokine eotaxin. Eotaxin can be regarded as an effector molecule for the IL-13 signalling pathway and this study examined whether genetic variants of IL-13 signalling had effects on plasma eotaxin levels. This is the first time *IL13*, *IL4RA* and *STAT6* variants have been examined with respect to eotaxin. Associations observed for an intronic *IL13* SNP and *STAT6* 3'UTR G2964A variant with eotaxin levels were of borderline significance and must be interpreted with caution, but serve as hypothesis-generating observations. Of particular interest was the association with G2964A in

STAT6 since this SNP showed a significant association with asthma, eczema and hayfever, although not with total IgE levels. Thus it may be speculated that the clinical effects of this *STAT6* variant may be mediated by mechanisms other than IgE induction.

In conclusion, these data indicate that Th2 immune phenotypes (IgE, eosinophils and eotaxin) are under the genetic influence of variants in IL-13 signalling, with different patterns of variants influencing specific phenotypes. The data presented here indicate that genetic variations in *IL4RA* and *STAT6* contribute significantly to the regulation of total IgE levels in British adults, which have been shown to be under strong genetic control in segregation analysis. Of the immune phenotypes examined, total IgE levels appear to be under the strongest genetic influence. Novel associations were demonstrated and robust confirmation of previously documented associations between *STAT6* SNPs and IgE was provided. The study provides new evidence for the effect of Ile50Val on eosinophil count.

CHAPTER 5

Genetic association of single nucleotide polymorphisms with physiological phenotype and asthma severity

5.1 Introduction

Few genetic association studies have attempted to examine lung function, and in particular, there is a paucity of data testing the physiological effects of genetic variants at the population level. A small number of studies have examined the role of IL-13 signalling variants in asthma severity [164, 165, 277] but there are no published studies on the effects of these variants on more detailed measures of lung function, nor on the predictive effects of these variants in an unselected population. Pulmonary function may be used to categorise the severity of asthma, in terms of percentage predicted FEV1 and PEF. Asthma severity may be categorised as "mild", "moderate" or "severe" by FEV1 (or PEF) of $\geq$80%, 60-80% and $\leq$60% respectively.

The first study to examine the association between a genetic marker and asthma severity found that Caucasians with the *IL4* 589T/T genotype had a 4.5% lower predicted FEV1 than C/C individuals [278]. Two studies have demonstrated a correlation between the R576 coding variant of *IL4RA* and asthma severity in terms of baseline FEV1 [165], although numbers of asthmatic subjects were small in one study (n=60) [164]. A Swedish study found *IL4RA* haplotypes to be associated with asthma severity in terms of % predicted PEFR [277].

Smokers with the *IL4RA* 576R/R genotype have been shown to have a more rapid decline in lung function (FEV1) (odds ratio, 2.24; P = 0.043) and this decline was more significant in subjects who also had either the *IL13* 110R/R or -1024T/T genotypes [140].

This thesis examined the effects of IL-13 signalling variants on lung function in an unselected population and a subgroup analysis was undertaken to examine whether these genetic variants had an impact on asthma severity in terms of lung function.

5.2 Methods

Morning spirometry (before noon) was performed by standardised protocol [239], using a dry spirometer (Vitalograph, Buckingham, UK). Spirometry was undertaken by one researcher for the majority of cases and supervised by the same researcher for the remainder (GD) in order to minimise inter-observer variation. Measurements included Forced Expiratory Volume in 1 second (FEV1), Forced Expiratory Flow at 25-75% of expired vital capacity (FEF 25-75), Forced Vital Capacity (FVC), FEV1/FVC ratio and Peak Expiratory Flow (PEF). The best value of three manoeuvres was expressed as an absolute and percentage of the age-gender-stature predicted value. If on inhaler treatment for asthma, measurements were taken before the morning dose. Genotyping was completed as outlined in Chapter 2.

A subgroup analysis was undertaken on subjects with a current physician diagnosis of asthma to examine the effect of genotype on physiological and clinical severity. Asthma severity was quantified by the following measures:

1. Clinically categorised into "intermittent", "mild persistent", "moderate persistent" and "severe persistent" asthma according to GINA criteria, shown in Table 5.10. Current therapy was accounted for e.g. a subject having intermittent symptoms but on "mild persistent" therapy of low-dose inhaled corticosteroids would be classified as having "mild persistent" asthma [50].
2. Lung function: % predicted FEV1; % predicted PEF.

Ordinal regression was applied to identify genotype associations with clinical severity categories. Linear regression was applied for continuous lung function data. SPSS 13.0 was used for statistical analysis. In all cases, adjustments were undertaken for relevant covariates.

5.3 Results

5.3.1 Effect of genotype on lung function in an unselected population

IL13

There was an association between promoter SNP 1 (-1024C/T) and % predicted FEV1: after adjusting for relevant covariates (smoking, BMI), mean %FEV1 was approximately 2% lower in the C/C compared with the C/T and T/T group (p=.002) (Figure 5.1). This is of uncertain clinical relevance, but it is worth noting that there was no overlap in the 95% confidence intervals here. There was also an association between promoter SNP 10 (-1512A/C) and %FEV1 (Figure 5.2). As seen with SNP 1, after adjusting for relevant covariates, mean %FEV1 was lower in the C/C compared with the C/T and T/T group (p=.007). As noted previously, SNP 10 was in strong linkage disequilibrium with SNP 1 in the promoter region (D'=0.93).

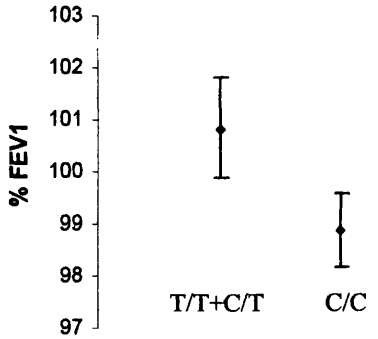


Figure 5.1 Mean % FEV1 by genotype at *IL13* -1024 promoter (SNP 1).

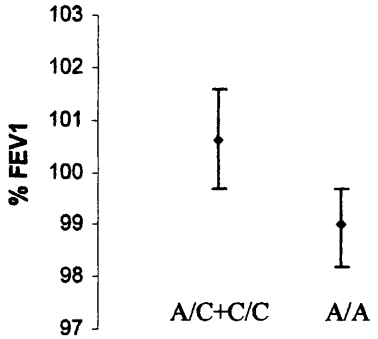


Figure 5.2 Mean % FEV1 by genotype at *IL13* -1512 promoter (SNP 10).

An association was seen between SNP 5 (Arg110Gln) and % predicted FVC such that the mean value was lowest in the R/R (Arg/Arg) group (p=.036) (Table 5.1). SNP 13 was in almost complete LD with SNP 5 (D'=0.99). As expected, a similar association was seen between SNP 13 and %FVC (p=.028) (Table 5.2).

Table 5.1 Mean % FVC by genotype at *IL13* R110Q (SNP 5).

	Mean %FVC	N	95% CI	p value[1]
R/R	100.4	973	99.7-101.1	.036
R/Q	102.1	399	100.9-103.0	
Q/Q	103.0	43	99.7-105.6	
Total	101.0	1415		

[1]After adjusting for relevant covariates.

Table 5.2 Mean % FVC by genotype at *IL13* 3'UTR SNP 13.

	Mean %FVC	N	95% CI	p value[1]
C/C	100.4	981	99.7-101.1	.028
C/T	102.1	396	99.5-104.5	
T/T	102.9	42	100.0-105.9	
Total	101.1	1419		

[1]After adjusting for relevant covariates.

IL4RA

There was an association between SNP 12 (Ser478Pro) and PEF with an additive effect seen with A/A having the lowest mean PEF (p=.033) (Figure 5.2). A similar association was seen for % predicted PEF (p=.026) (Table 5.3).

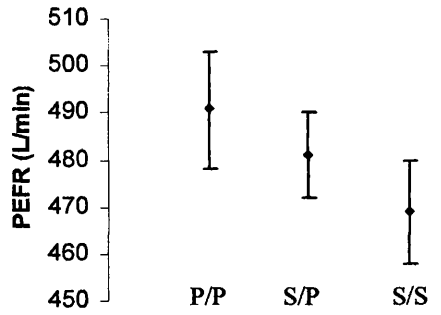


Figure 5.3 Mean PEFR by genotype at *IL4RA* S478P (SNP 12).

Table 5.3 Mean % PEFR by genotype at *IL4RA* S478P (SNP 12).

	Mean % PEFR	N	95% CI	p value[1]
S/S	90.5	413	89.0-92.1	.026
S/P	91.7	687	90.5-92.9	
P/P	93.8	323	92.1-95.5	
Total	91.8	1423		

[1]After adjusting for relevant covariates.

The 3'UTR SNP 16 showed an association with FEF 25-75 such that genotypes C/T and T/T had a significantly lower mean value than the C/C group (Figure 5.4). Although numbers in the homozygote C/C group were small (n=39), this difference remained statistically significant after adjusting for relevant covariates (sex, height, age) (p=.004). A similar association was seen with % predicted FEF 25-75, which remained significant after adjusting for smoking (p=.004) (Figure 5.5).

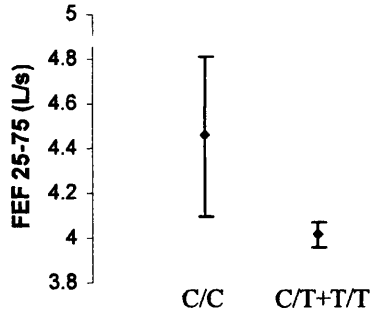


Figure 5.4 Mean FEF 25-75 by genotype at *IL4RA* 3'UTR SNP 16.

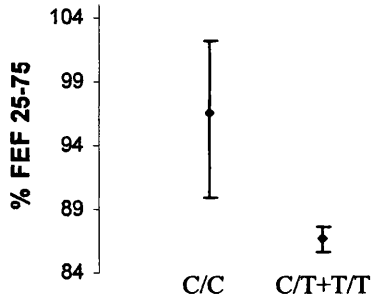


Figure 5.5 Mean % FEF 25-75 by genotype at *IL4RA* 3'UTR SNP 16.

There was an association between the intronic SNP 8 and FVC after adjusting for relevant covariates (p=.024) (Figure 5.6). There was also an association between this locus and FEV1/FVC before adjusting for covariates (p=.036). However, after appropriate adjustment for relevant covariates – which proved to be sex, current smoking, BMI, height, age – the association did not quite reach significance at the 5% level (p=.068). The main reason for this was a difference in the distribution of genotypes between males and females: males represented 53.2% of the A/A group and 46.9% of the G/G group. The effect of sex is described in further detail in the *STAT6* section, where differences were more significant. There was also an association between the 3'UTR SNP 16 and FEV1/FVC (p=.026). However this was of borderline significance after adjusting for relevant covariates (p=.051) – sex, current smoking, age, height and BMI - and is described in Appendix VII.

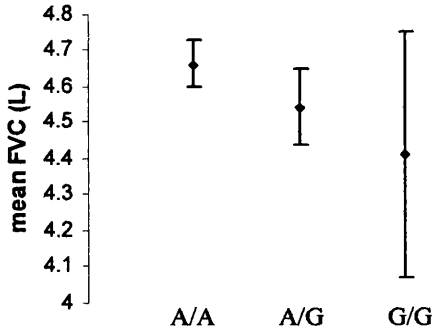


Figure 5.6 Mean FVC by genotype at *IL4RA* intron SNP 8.

STAT6

Intronic SNP 11 showed an association with FVC, FEV1, PEF, FEF 25-75 ($p<.05$ for all) before adjusting for relevant covariates. However, sex showed an unexpected non-random distribution at this locus which meant that the associations between SNP 11 and these lung function parameters were *not* significant after adjusting for sex. Crosstabulation by sex at this locus is shown in Table 5.4. There were approximately equal proportions of males and females in the C/C group but on examining the combined C/T and T/T group, 42.3% were female and 57.7% were male.

Table 5.4 Crosstabulation for *STAT6* SNP 11 and sex.

	C/C		C/T		T/T	
Male	605	(50.9)	135	(59.7)	3	(23.1)
Female	583	(49.1)	91	(40.3)	10	(76.9)
Total	1188	(100)	226	(100)	13	(100)

Data are number (percentage) of subjects.
Percentages for combined C/T+T/T group are given in text since only 13 subjects in T/T group.
Chi^2=.006.

SNP 11 showed an association with % predicted FEF 25-75, after adjusting for smoking (p=.036) (Figure 5.7). Mean % FEF 25-75 was 13.8% lower in the T/T group compared to the C/C group. Although numbers in the T/T group are small (n=13), the individual difference between the T/T and C/C groups remains significant after adjustment for smoking (p=.013) and there is a clear difference between confidence intervals.

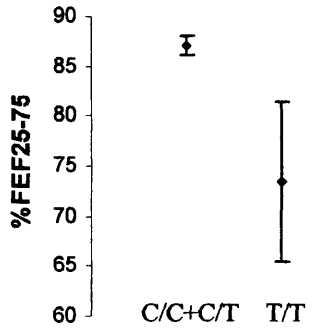


Figure 5.7 Mean % FEF 25-75 by genotype at *STAT6* intron SNP 11.

Table 5.5 Summary of significant physiological phenotype-genotype associations at *IL13*, *IL4RA* and *STAT6* loci in an unselected population.

	Location	Phenotype	p value
IL13			
SNP 10 (-1512A/C)	Promoter	% FEV1	.031[1]
SNP 10 + smoking[3] + BMI			.008[2]
SNP 1 (-1024C/T)	Promoter	% FEV1	.009
SNP 1 + smoking + BMI			.008
SNP 5 (R110Q)	Exon 4	% FVC	.038
SNP 5 + BMI			.036
SNP 13	3'UTR	% FVC	.028
SNP 13 + BMI			.028
IL4RA			
SNP 8	Intron 8	FVC	.023[4]
SNP 8 + sex + height + BMI			.027
SNP 12 (S478P)	Exon 12	PEF	.012[4]
SNP 12 + sex + height + age +BMI			.010
SNP 12		% PEF	.006[4]
SNP 12 + smoking + BMI			.010
SNP 16	3'UTR	FEF 25-75	.013[4]
SNP 16 + sex + height + age			.006
SNP 16		% FEF 25-75	.006[4]
SNP 16 + smoking			.008
STAT6			
SNP 11	Intron 18	% FEF 25-75	.034
SNP 11 + smoking			.036

[1] p value for overall association at this SNP (with genotype as 3 categories).
[2] p value after adjusting for relevant covariates.
[3] Current smoking.
[4] p value for overall association with SNP modelled as a continuous variable (when clear additive effect observed).

5.3.2 Effect of genotype on pulmonary function and clinical severity in asthma

Subjects with current physician diagnosed asthma represented 10.9% of our population (n=176) with a further 12.3% (n=198) having had asthma which had remitted. Subgroup analysis of the "current asthma" group was undertaken to ascertain whether certain genotypes were associated with asthma severity. Firstly, lung function, including physiological severity criteria (%FEV1, %PEF) was considered. Secondly, GINA severity categories were considered [50].

The majority of asthma subjects had mild disease, since this was an unselected population sample. Tables 5.6, 5.7 and 5.10 show asthma subjects categorised by severity, by %FEV1, %PEF and by GINA criteria whereby severity is categorised by symptom frequency and medication usage is taken into account. It can be seen that there were few subjects categorised as "severe" in terms of %FEV1 alone; a greater number when categorised by %PEF; and the greatest number when categorised by GINA criteria. This is because the GINA criteria takes into account medication usage, so that an individual with "mild" disease according to symptoms (or FEV1) would be classified as having more severe disease if taking high dose inhaled steroids to achieve asthma control.

5.3.3 Effect of genotype on lung function and physiological severity in asthma

Baseline characteristics

Tables 5.6-5.7 illustrate asthma severity when classified according to %FEV1 and %PEF: the majority of subjects fall into the "mild" category when using a purely physiological definition of severity (and it should be noted that this does not take into account medication usage, in contrast to the GINA severity criteria).

Table 5.6 Asthma severity by % FEV1 in current asthmatics.

	Number of subjects	*Percentage*
"Mild" FEV1[1] ≥80% predicted	154	(95.7)
"Moderate" FEV1 60-80% predicted	5	(3.1)
"Severe" FEV1 ≤60% predicted	2	(1.2)
Total	161	(100)

[1] Pre-bronchodilator FEV1 taken before morning dose of inhalers, if on inhalers.

Table 5.7 Asthma severity by % PEF in current asthmatics.

	Number of subjects	*Percentage*
"Mild" PEF [1] ≥80% predicted	130	(80.7)
"Moderate" PEF 60-80% predicted	25	(15.5)
"Severe" PEF ≤60% predicted	6	(3.7)
Total	161	(100)

[1] Pre-bronchodilator PEF taken before morning dose of inhalers, if on inhalers.

5.3.4 Genotype associations with severity of airflow obstruction in asthma

IL13 promoter SNP 10 (-1512A/C) associated with percentage FEV1 in subjects with asthma, with an approximately 4% difference between groups, after adjusting for BMI (p=.04) (Figure 5.8). BMI was adjusted for since, although percentage FEV1 is height-adjusted, BMI acted as a significant covariate (p=.011) above the effects of height alone. The same SNP was also associated with % FEF 25-75, after adjusting for relevant covariates (current smoking) (p=.027) (Figure 5.9).

IL4RA intronic SNP 8 was associated with FEF 25-75 and FEV1/FVC after adjusting for relevant covariates (sex, height, smoking) (p=.030, p=.034) (Figures 5.10, 5.12). An association of borderline significance was observed for the same SNP with % FEF 25-75 (p=.045) (Figure 5.11). The relationship between genotype and FEV1/FVC appeared additive, with a decrease in FEV1/FVC of 3% for each A allele when genotype was modelled as a continuous variable (p=.019 after adjusting for relevant covariates). A summary of genotype associations with severity of airflow obstruction is shown in Table 5.8.

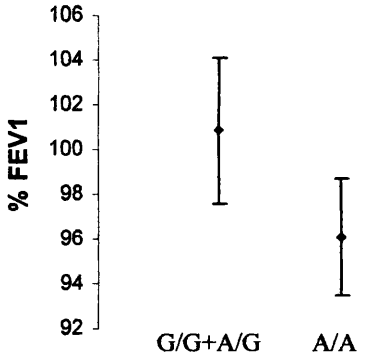


Figure 5.8 Mean % FEV1 by genotype at *IL13* -1512 promoter (SNP 10) in asthma.

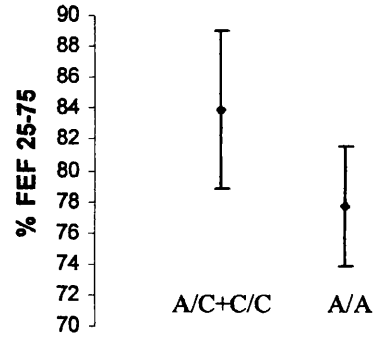


Figure 5.9 Mean % FEF 25-75 by genotype at *IL13* -1512 promoter (SNP 10) in asthma.

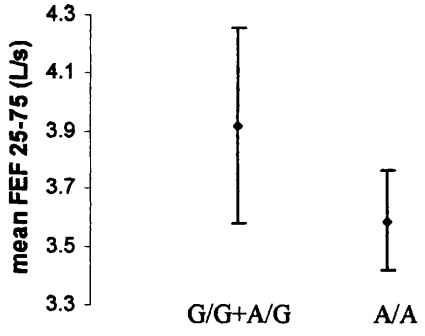


Figure 5.10 Mean FEF 25-75 by genotype at *IL4RA* intron SNP 8 in asthma.

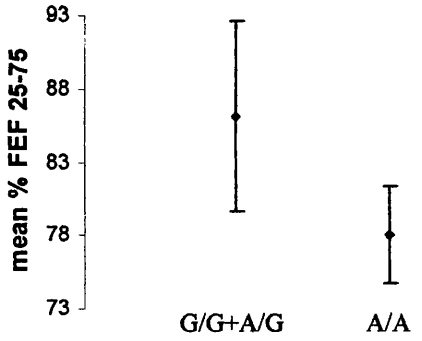


Figure 5.11 Mean % FEF 25-75 by genotype at *IL4RA* intron SNP 8 in asthma.

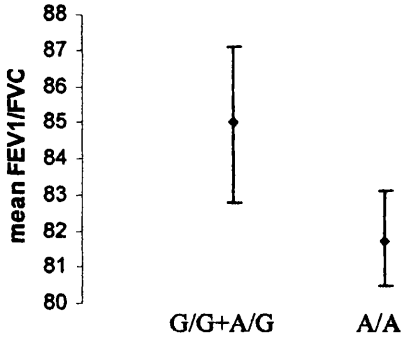


Figure 5.12 Mean FEV1/FVC by genotype at *IL4RA* intron SNP 8 in asthma.

Table 5.8 Summary of significant genotype associations with lung function severity in asthma.

	Location	Phenotype	p value[1]
IL13			
SNP 10	Promoter	% FEV1	.037
-1512A/C		% FEF 25-75	.027
IL4RA			
SNP 8	Intron	FEV1/FVC	.034
		FEF 25-75	.030
		% FEF 25-75	.045

[1]After adjusting for relevant covariates.

5.3.5 Effects of genotypes on clinical asthma severity

Baseline characteristics

Table 5.9 summarises the clinical characteristics of the group and Table 5.10 illustrates the number of subjects with asthma of various clinical severity by GINA criteria. Only one subject was taking inhaled cromoglycate, anti-muscarinic, oral methyl xanthine or an oral leukotriene antagonist respectively. Only one subject had spent nights in hospital over the last 12 months and no subjects had been admitted to ITU in the last 12 months. Forty subjects had had to give up days of work or other activities over the last 12 months: of these, the number of days lost per month was 1.01 ± 1.30.

Table 5.9 Summary of clinical characteristics of current asthmatics.

	Number of subjects	*(Percentage)*
Using inhaled short acting beta-2 agonist in last 12 months	159	(90.3)
Using inhaled long acting beta-2 agonist in last 12 months	29	(16.5)
Using regular inhaled corticosteroid in last 3 months	56	(31.8)
Using oral corticosteroids in last 12 months	8	(4.5)
Visiting hospital casualty department because of asthma, shortness of breath or wheezing in last 12 months	7	(4.0)
Seen by a general practitioner because of asthma, shortness of breath or wheezing in last 12 months	99	(56.3)
Seen by specialist because of asthma, shortness of breath or wheezing in last 12 months	5	(2.8)
Had to give up days of work/other activities because of asthma, shortness of breath or wheezing in last 12 months	40	(22.7)

Table 5.10 GINA classification of asthma severity in current asthmatics [50].

	Number of subjects	*(Percentage)*
Intermittent Symptoms less than once a week Nocturnal symptoms not more than twice a month	74	(46.0)
Mild Persistent Symptoms more than once a week but less than once a day Nocturnal symptoms more than twice a month	38	(23.6)
Moderate Persistent Symptoms daily Nocturnal symptoms more than once a week Daily use of inhaled short-acting beta2-agonist	29	(18.0)
Severe Persistent Symptoms daily Frequent nocturnal asthma symptoms Limitation of physical activities	20	(12.4)

Genotype associations with clinical asthma severity

Associations were seen for two *IL4RA* variants and clinical severity. These were Ile50Val (SNP 5) and promoter SNP 3 (rs2283563) which were in strong linkage disequilibrium. Promoter SNP 3 showed an association with clinical severity, after adjusting for relevant covariates (sex, height) (p=.011). The OR for being in a higher severity group was 3.14 for the G/G and G/A group versus the A/A homozygotes. For Ile50Val, the OR was 2.46 for the Ile/Val heterozygotes versus the Ile/Ile homozygotes (p=.012), suggesting that Val50 (which was associated with asthma) may have an effect on disease severity. However interpretation is limited by the small number in the Val/Val group (n=36).

5.4 Discussion

This is the first study to examine the association of variants of IL13 signalling with lung function in an unselected population. Strongest effects were seen between *IL13* and *IL4RA* variants with markers of airflow obstruction. Interestingly, both promoter SNPs of *IL13* were associated with %FEV1 ($p<.01$) with an approximately 2% difference between genotype groups. Although a relatively small difference, this may reflect a biologically important difference in an epidemiological study of this size, and may be compared to small blood pressure changes demonstrated in large Cardiology trials which are accepted as being clinically important [279]. It is worth noting that recent evidence points to lung function being a more important predictor of mortality than previously thought, with a recent study showing that moderately impaired lung function was associated with higher mortality, after appropriate adjustments [280].

It is of note that the *IL13* promoter variants were solely associated with %FEV1 rather than the clinical or immune phenotypes examined. A summary of phenotype associations for clinical, immune and physiological phenotypes from Chapters 3-5 is included in Table 5.11. The -1512C/T promoter SNP was also associated with asthma severity in terms of %FEV1 with an over 4% difference between genotype groups, and with % FEF 25-75 with an over 6% difference shown. This suggests that it may be the -1512 promoter SNP which is of primary importance, with the associations for -1024 being a result of the strong linkage disequilibrium between these variants. The -1024C/T variant has been more widely studied, with gene expression studies and transcription factor binding analysis by electrophoretic mobility shift assay with this site supporting a functional role of this variant altering *IL13* gene expression [281]. The promoter SNPs are located within the extended matrix of a STAT binding site and phylogenetic shadowing has shown a high degree of conservation in this area, indicating a potential functional role. Given their location, these SNPs may be involved in a STAT6-dependent regulatory loop in the signalling pathway [12].

Taken together, these findings suggest that the promoter region of *IL13* may have a

specific effect on lung physiology, influencing this particular asthma phenotype, and may be particularly important in airway remodelling. A possible underlying mechanism is demonstrated by the effects of IL-13 on remodelling shown in functional studies [282]. Experimental work has show prominent effects of IL-13 on airway smooth muscle, suggesting that gene modulation by IL-13 in these cells may be an essential event leading to the development of allergic asthma [283]. Of special interest is the observation of an association between the -1024 variant with chronic obstructive pulmonary disease (COPD) in comparison to healthy controls and smokers with normal lung function [284]. A recent study has found this variant to enhance the adverse effect of smoking on % FEV1 [285]. Of further note is the observation of an association between the -1024 variant and severe RSV infection in children [96]. Whereas the -1024 promoter variant has been associated with airway hyper-responsiveness (AHR) in one study [114], the -1512A/C variant has been associated with IgE levels [132] rather than AHR, and an association with AHR warrants examination.

The potential biological role of non-coding variants is discussed further in Chapter 3 and 8. Variation in the 5'UTR may have regulatory effects on mRNA translation or RNA processing. Several studies suggest that this type of non-coding variant causes changes in the stability and splicing of the encoded mRNAs and therefore has a biological effect [286, 287]. Cameron et al have shown that the *IL13*-1024T allele enhances IL13 promoter activity in primary human and murine CD4(+) Th2 lymphocytes, and attenuates STAT6-mediated repression of *IL13* transcription [116]. The gain-of-function associated with the *IL13*-1024T allele was evident only in differentiated Th2 cells suggesting that *IL13*-1024C>T is likely to influence risk of asthma and related phenotypes in the context of an established Th2 response. Thus it may be postulated that this polymorphism may contribute to the maintenance and/or exacerbation of airway pathology (such as remodelling) rather than to its inception, in the context of an established Th2 immune response. Functional studies are lacking for the -1512 promoter and further investigation is needed.

In our unselected population, weak associations were demonstrated for *IL13* Arg110Gln

and 3'UTR variants with %FVC. The 3'UTR association is likely to arise as a consequence of the almost complete linkage disequilibrium between these two variants. The small additive effect seen for Arg110Gln – with a 1% decrease with each G allele – is of uncertain clinical significance. It should be noted that no associations were seen for this variant with lung function in the asthmatic population, consistent with findings from other studies [147, 248] and the association observed with asthma (Chapter 3) was in the opposite direction i.e. the A allele (resulting in Arg→Gln) conferred asthma risk. Our study was consistent with other populations with only a low level of LD between the coding variant and -1024 (and -1512) promoter variants [249], although high LD has been observed in some populations [118]. Since these SNPs have been identified as htSNPs belonging to different *haploblocks*, both should be included in disease association studies. It has been postulated that the modest transcriptional enhancement conferred by -1024C/T may act in synergy with that conferred by Arg110Gln to amplify IL-13–dependent events [118].

For *IL4RA*, the strongest association was between a 3'UTR variant and absolute and % FEF 25-75 ($p<.01$) with an almost 10% difference in % FEF 25-75. A weaker association was seen for Ser478 and absolute and %PEF ($p=.01$), with an additive effect seen and a reduction of over 3% for S/S compared to P/P subjects. The data also suggested that Val50 may be associated with clinical severity in addition to the previously demonstrated association with asthma, but these findings are to be interpreted with caution given the small number of Valine homozygotes, and warrant replication in a further population.

In contrast to other studies examining genotype association with asthma severity [164, 165, 277], our study included not only a definition of asthma severity by symptom frequency, but a correction for medication usage. Other studies have tended not to correct for medication usage, which is likely to be a strong confounder for severity since a subject requiring high dose inhaled steroid to maintain a good %FEV1 would be incorrectly classified as having mild disease.

A strength of this study was that lung function was examined as a continuous variable rather than simply a categorical marker of severity. This enabled the use of a quantitative marker for asthma severity. Since this was primarily an unselected population study of young adults, the asthma population within the study largely consisted of milder asthma by lung function criteria, reflecting asthma in the general population rather than asthma in a hospital setting. This contrasts with other studies examining genotype associations with asthma severity, where subjects were recruited from hospital outpatients [164, 165, 277, 278]. Thus our effect sizes may be considerably more marked in a more severe asthma population. The main finding was that the *IL13* promoter variant -1512A/C associated with asthma severity, with >4% difference in %FEV1. This variant was also associated with small airflow obstruction (%FEF 25-75) as discussed above.

The other findings of note for the group with asthma, were the unexpected associations between *IL4RA* intronic SNP 8 and airflow obstruction (FEV1/FVC; absolute and % FEF 25-75). It is possible that this does not represent a true association but that this variant is in linkage disequilibrium with another marker which affects airflow obstruction in asthmatics and this requires further study in a separate population. The previously observed association for *IL4RA* R576 [164, 165] was not replicated in our population and this may be due to the small number of subjects with severe airflow obstruction in our population.

In conclusion, IL-13 signalling variants were found to have identifiable predictive effects on lung function in an unselected population. The most interesting findings were the associations of *IL13* promoter polymorphisms with % FEV1. It is striking that these effects were demonstrable in an unselected population of young adults and adds to emerging evidence supporting a role for these promoter variants in influencing physiological outcomes, perhaps via airway remodelling. Replication is needed in other populations, including examination of airway hyperresponsiveness, and functional work is needed to explore the mechanistic basis of these associations.

Table 5.11 Summary table for associations between *IL13*, *IL4RA* and *STAT6* and clinical, immune and physiological phenotypes.

	Location	**Asthma**[1] OR (95% CI, p value)[2]	**Eczema**[3] OR (95% CI, p value)[2]	**Hayfever** OR (95% CI, p value)[2]	**Total IgE**	**% FEV1**
IL13						
SNP 10 -1512A/C	5'UTR					P=.008
SNP 1 -1024C/T	5'UTR					P=.008
SNP 5 (R110Q)	Exon 4	1.38 (1.06-1.79, p=.015) A/A + G/A vs G/G				
SNP 13	3'UTR	1.38 (1.07-1.79, p=.014) T/T + C/T vs C/C				
SNP 6	3'UTR	1.33 (1.03-1.72, p=.029) T/T + G/T vs G/G G/G			P=.048	
SNP 9	3'UTR	1.34 (1.03-1.73, p=.029) A/A + A/G vs G/G				
IL4RA						
SNP 5 (I50V)	Exon 5	OR 1.25 V/V vs I/V and I/V vs I/I (1.05-1.49, p=.013).				
SNP 8	Intron 8				p=.007	
SNP 13 (Q551R)	Exon 12				p=.001	
SNP 16	3'UTR				p=.005	
STAT6						
SNP 8 C2892T	Intron 2				P=.009	
SNP 1 G2964A	3'UTR	2.21 (1.17-4.18, p=.015 A/A vs A/G + G/G[4]	2.62 (1.40-4.89, p=.003) A/A vs A/G +G/G	1.99 (1.19-3.32, p=.008) A/A vs A/G and G/G		
SNP 3	3'UTR				P=.001	

[1] "Physician diagnosed asthma ever", [2] after adjusting for relevant covariates, [3] Current eczema, [4] "Physician diagnosed current asthma".

CHAPTER 6

Epistatic and haplotype effects of IL-13 signalling variants on asthma and associated Th2 immune phenotypes

6.1 Introduction

Recent studies have demonstrated the importance of examining loci in the same biological pathway in combination, since small individual effects on phenotype may combine to produce much larger synergistic effects. Examining combinations of multiple alleles, in haplotypes or combinations of haplotypes, has proved more fruitful than examining individual SNPs, because the significance of a SNP may only be apparent in the context of an additional SNP in the same or other genes in the same pathway [12, 14, 133]. The body of literature on association studies points to the existence of genetic effects via complex networks involving gene-gene and gene-environment interactions in complex disease, and it is thus important to model epistatic interactions simultaneously with single polymorphism associations.

Howard et al examined the interaction between *IL13* and *IL4RA* genes. In their population, the *IL13* locus was most significantly associated with bronchial hyper-responsiveness and the *IL4RA* locus with IgE but the combined effects of the two susceptibility loci conferred an increased asthma risk [13]. Risma et al. found that two variants of *IL4RA*, when occurring together, were more strongly associated with asthma than either allele alone [14]. In a recent study, Hytonen et al demonstrated that haplotypes of *IL4RA* (but not individual SNPs) were associated with asthma susceptibility and to severity of disease [277]. Thus the combination of *IL4RA* polymorphisms - and the coexistence of polymorphisms in *IL4RA* and *IL13* - are more strongly associated with asthma [12-14, 133, 277].

Kabesch et al combined one functional polymorphism in *IL13*, *IL4*, *IL4RA* and *STAT6*, and found that this increased the risk for high serum IgE levels 10.8-fold ($p = .02$) and the risk for the development of asthma by a factor of 16.8 ($p = .005$) compared with the maximum effect of any single polymorphism. They observed significant interactions in a model with additive and dominant effects, for both pair and triplet combinations for asthma (lowest $p = .005$), and for pairs of polymorphisms in IgE regulation (lowest $p = .054$) [12].

The functional impact of SNP/SNP interactions within the same gene could be further amplified by gene/gene interactions along the same pathway [8]. Chen et al demonstrated functional synergistic effects of *IL13* and *IL4RA* polymorphisms on IL-13-dependent gene induction, when both variants occurred in combination. The Gln110 variant of *IL13* and the Val50Arg551 variant of *IL4RA* act in a concerted fashion to enhance IL-13 responsiveness in mouse cell lines [133].

Similarly, it has been suggested that haplotype analyses may be more informative for drawing associations between phenotypes and genetic variation than SNPs, especially when considering common variants which have small individual effects on disease risk [288, 289]. Haplotype analysis may be considered as representing *cis*–interactions, with epistatic interactions representing *trans*-interactions. Recent German and Swedish population studies support the approach of studying the effect of combinations of multiple alleles, in haplotypes or in combinations of haplotypes, on complex phenotypes within a biological pathway [12, 128, 168].

In this study, the combined effects of *IL13*, *IL4RA* and *STAT6* polymorphisms, including epistatic interactions and haplotype effects, were examined in relation to asthma and atopy phenotypes for the first time in a large population based cross sectional cohort of adults.

6.2 Methods

Physician-diagnosed asthma and clinical atopy (eczema, hayfever) were defined by validated bronchial symptoms questionnaire (IUATLD) as outlined in section 2.6.1. Spirometry was measured as previously described (2.6.2). Total IgE, eosinophil counts and eotaxin levels were measured as previously described (2.6.3). Quantitative variables (IgE, eosinophil counts, eotaxin levels) were analysed as continuous data (on a logarithmic scale) rather than choose a widely used arbitrary cutoff such as 100 kIU/L for IgE, to increase the power of the analysis.

To guard against multiple-testing, we examined only those SNPs for which a main effect had been documented in chapters 3-5. Using the basic linear and logistic regression models, we then examined all possible 2-way interactions involving the significant factors, and retained them in the final model if the interaction coefficients were significant at the 5% level.

To assess the effects of haplotypes in our cross-sectional study population of unrelated subjects, haplotypes were estimated from genotype data, at the *IL13*, *IL4RA* and *STAT6* loci, according to recently derived methods using the Expectation Maximisation (EM) algorithm [244, 245] and R statistical environment package [246]. When linkage phase is unknown, for each individual there will be a set of haplotypes that are consistent with the genotype data. In essence, the method assigns probabilities to the potential haplotypes for each individual by using the available information on unambiguous haplotypes and by assuming that there is random mating in the population. The code was written by Dr Mike Gravenor in the R statistical language. The relationship between haplotypes and traits is then assessed by a range of regression models that take into account the estimated haplotype probabilities.

All haplotype analyses were carried out using the haplo.stats 1.3.0 package, and the haplo.glm function. Haplotype analysis was run for the following binary outcomes: asthma, eczema, hayfever, and the following quantitative outcomes: absolute lung function parameters, total IgE, eosinophil count and eotaxin levels. For most of the quantitative traits (IgE, eosinophil counts, eotaxin) a log transformation was applied as necessary. For all haplo.glm analyses, all the key algorithm parameters were explored to check for consistent results. Problems were encountered with algorithm conversion for age-gender-stature adjusted lung function, PEFR and FEV1/FVC ratio, with different starting conditions for the haplo.glm generating different results, and so these were omitted. Significant associations are reported after adjusting for relevant covariates (such as age, sex, smoking).

6.3 Results

6.3.1 Two locus interaction effects on asthma and immune phenotypes

Epistatic interactions for asthma

***IL13* Arg110Gln and *IL4RA* Ile50Val interaction in asthma**

The interaction between *IL13* Arg110Gln (R110Q, SNP 5) and *IL4RA* Ile50Val (I50V, SNP 5) was examined. Individually, these loci associated with physician "asthma ever" as follows:

- *IL13* Arg110Gln (SNP 5): OR 1.37 for Gln/Gln + Gln/Arg vs Arg/Arg (95% CI 1.06-1.77, p=.016). Previous studies have shown that Gln is the risk allele for asthma at this locus. This SNP was considered as a categorical (binary) variable since there was no evidence of a clear additive effect in the initial logistic regression.
- *IL4RA* SNP 5 (Ile50Val): for OR 1.25 for Val/Val vs Ile/Val and Ile/Val vs Ile/Ile (p=.013, 95% CI 1.05-1.49). A clear additive effect was demonstrated and this SNP was considered as a continuous variable in subsequent logistic regression analysis.

Logistic regression analysis of *IL13* SNP 5 (Arg110Gln)* *IL4RA* SNP 5 (Ile50Val) in asthma

Although 5% statistical significance was not quite reached for the interaction term (p=.082), the trends seen were of interest and were as follows. The probability of asthma associated with different genotype combinations is shown in Figure 6.1.

Odds for *IL4RA* Ile50Val genotypes stratified by *IL13* Arg110Gln

1) For Gln/Gln individuals, Ile/Ile is highest risk genotype to have in combination: OR 16.09 compared to Val/Val.
2) For Gln/Arg individuals, Ile/Ile confers protection: OR 0.71 for Ile/Ile compared to

Val/Val. Thus conversely for these individuals, Val/Val has an OR of 1.40 compared to Ile/Ile.

3) For Arg/Arg individuals, as for Gln/Arg, Ile/Ile confers protection: (OR 0.55 for Ile/Ile compared to Val/Val. Conversely, Val/Val has an OR of 1.81 compared to Ile/Ile for Arg/Arg individuals.

4) Therefore Ile may only act as a risk allele in the presence of a Gln/Gln "risk" variant of *IL13*.

Odds for *IL13* genotypes stratified by IL4RA

1) For Ile/Ile individuals, Gln/Gln confers higher risk than Arg/Arg: OR 2.39 compared to Arg/Arg.

2) For Ile/Val individuals, Gln/Gln is *protective:* OR 0.44 compared to Arg/Arg. Conversely, OR 2.26 for Arg/Arg vs Gln/Gln.

3) For Val/Val individuals, Gln/Gln also appears *protective:* OR 0.08 compared to Arg/Arg. Thus OR 12.17 for Arg/Arg vs Gln/Gln.

4) Therefore, Gln may only act as a risk allele in the presence of an Ile/Ile genotype at the *IL4RA* locus.

Table 6.1 shows crosstabulations for the presence or absence of physician-diagnosed asthma according to different combinations of genotypes for these two variants at IL13 and *IL4RA*. Figure 6.1 shows the probability of asthma, according to different genotype combinations derived from Table 6.1. It can be seen that individuals having both Gln/Gln and Ile/Ile have the highest probability of asthma, having an OR of 1.36 compared with Arg/Arg Val/Val individuals. However, it can be seen that the Arg/Arg Val/Val individuals do not have the lowest probability of asthma; individuals having either a Val/Val or Ile/Val genotype in combination with Gln/Gln have the lowest probability of asthma. The OR cannot be calculated for Val/Val Gln/Gln since n=4 only in this group with none having this genotype and asthma. The OR for the highest risk Gln/Gln Ile/Ile individuals compared to Gln/Gln Ile/Val individuals is 3.5.

The interaction between this functional variant Arg110Gln of *IL13* with the functional

variant Ile50Val of *IL4RA* has been presented here. As previously noted, *IL13* 3'UTR SNPs 6, 9 and 13 were in strong linkage disequilibrium with the functional variant Arg110Gln (SNP 5). On examining the interaction between Ile50Val with these three *IL13* SNPs, the interaction terms more closely approximated statistical significance for SNP 9 and 13, with a p value of .054 and .067 respectively. Figure 6.2 shows the probability of asthma for different combinations of genotypes for SNP 9 in *IL13* and Ile50Val in *IL4RA*: the probabilities shown are virtually identical to those for combinations of genotypes for Arg110Gln and Ile50Val (Figure 6.1).

Table 6.1 Crosstabulation for *IL13* Arg110Gln and *IL4RA* Ile50Val by asthma.

			Ile50Val			
			Ile/Ile	Ile/Val	Val/Val	Total
Asthma[1]	Arg110Gln	Arg/Arg	50	115	50	215
		Arg/Gln	34	54	26	114
		Gln/Gln	5	3	0	8
	Total		89	172	76	337
No asthma		Arg/Arg	244	372	136	752
		Arg/Gln	97	130	53	280
		Gln/Gln	10	21	4	35
	Total		351	523	193	1067

[1] "Physican diagnosis of asthma ever".

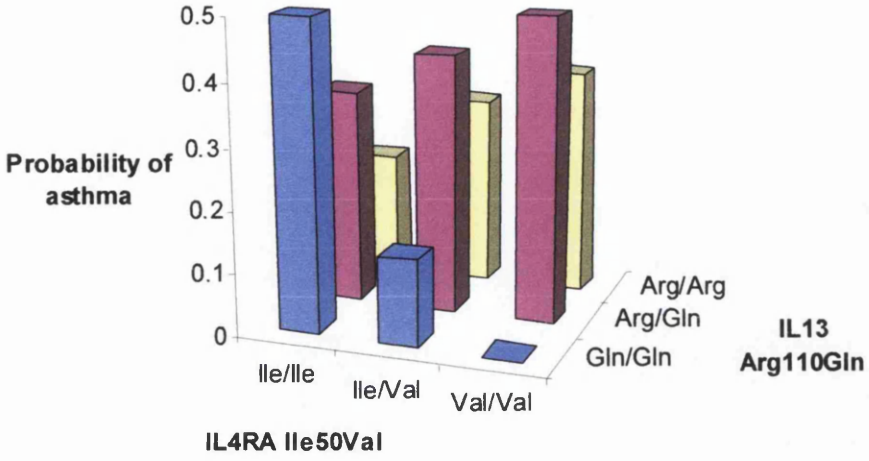


Figure 6.1 Probability of asthma for different combinations of genotypes for *IL13* Arg110Gln and *IL4RA* Ile50Val.

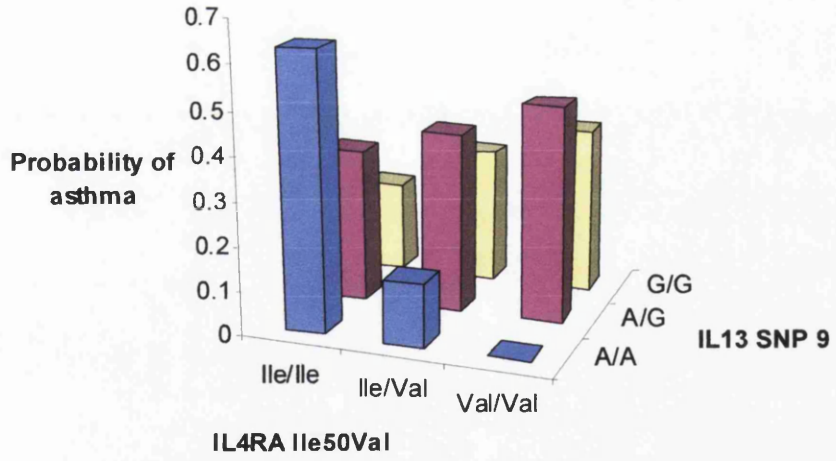


Figure 6.2 Probability of asthma for different combinations of genotypes for *IL13* SNP 9 and *IL4RA* Ile50Val.

Epistatic interactions for total IgE levels

Interaction between *IL13* SNP 6 and *STAT6* SNP 8 with IgE

The individual loci associated with IgE as follows:

- *IL13* 3'UTR SNP 6: T/T+G/T group had a geometric mean IgE of 52 (95% CI 45-61), G/G group had a geometric mean IgE of 43 (95% CI 39-47) (p=.027).
- *STAT6* intronic SNP 8: T/T group had a geometric mean IgE of 61 (95% CI 49-74), C/C+C/T group had a geometric mean IgE of 43 (95% CI 39-47) (p=.003).

When examining these two loci in a general linear model, there was a significant interaction between them in the association with IgE. For individuals with both genotypes associated with higher IgE levels – T/T at *STAT6* locus and either T/T or G/T at IL-13 locus – the geometric mean IgE was raised to 102 kIU/L (interaction effect 59, 95% CI 19-181, p=.003) (Figure 6.3).

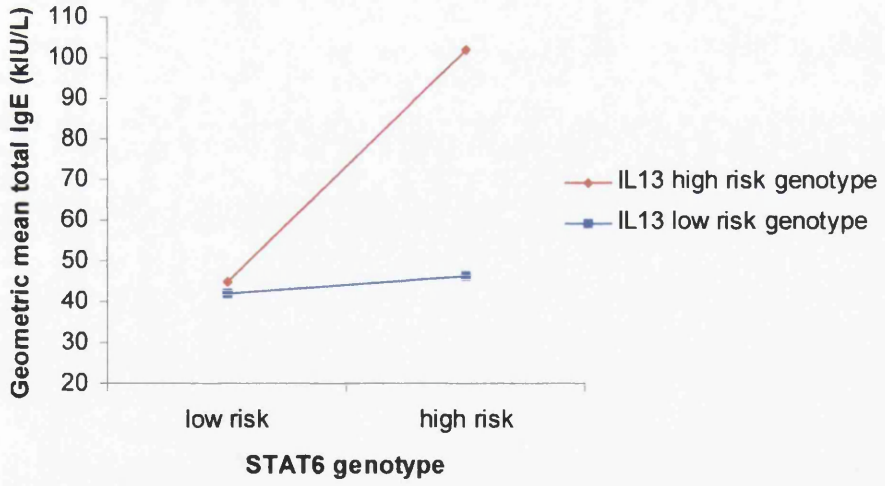


Figure 6.3 Graph showing the interaction effects of high risk genotypes of *IL13* and *STAT6* on total IgE level. A significant effect is only seen when both high risk genotypes are present in combination.

Genetic interactions for eosinophil count

Interaction between Ile50Val and Ser478Pro variants of *IL4RA* and eosinophil count

The individual variants associated with eosinophil count as follows:

- *IL4RA* SNP 5 Ile50Val: Ile/Ile, Ile/Val and Val/Val groups had a geometric mean eosinophil count of 0.20, 0.18 and 0.17 respectively (p=.020).
- *IL4RA* SNP 12 Ser478Pro: Pro/Pro, Pro/Ser and Ser/Ser groups had a geometric mean eosinophil count of 0.19, 0.18 and 0.17 respectively (p=.032).

When examining these two variants in a general linear model, there was a significant interaction between them in the association with eosinophil count (interaction effect 0.10, 95% CI 0.01-0.17, p=.016). Table 6.2 shows the effects of individual genotypes and different genotype combinations for these two variants on eosinophil count. The highest mean eosinophil count was observed for individuals with Val/Val Ser/Ser genotypes. The effects of these genotypes were interdependent in that significant effects were only seen when both high risk genotypes were present (Figure 6.4). In fact, when examined in combination, mean eosinophil count is actually lower (though not significantly) for the Ser/Ser group – which is associated with higher eosinophil count when examined in isolation - when in the presence of the Ile/Ile low risk *IL13* genotype.

Table 6.2 Geometric mean eosinophil count by individual genotype and genotype combinations for *IL4RA* SNP 5 and SNP 12

	Genotype	Geometric mean eosinophil count (cellsx10^9/L)	95% CI	P value
IL4RA				
SNP 5	G/G	0.20	0.18 - 0.21	.020
Ile50Val	A/G	0.18	0.17 - 0.20	
	A/A	0.17	0.16 - 0.19	
SNP 12	G/G	0.19	0.18 - 0.21	.032
Ser478Pro	A/G	0.18	0.17 - 0.20	
	A/A	0.17	0.15 - 0.19	
Genotype combinations				
SNP 5	G/G	0.29		.016
SNP 12	A/A			
SNP 5	G/G	0.19		
SNP12	A/G or G/G			
SNP 5	A/G or A/A	0.18		
SNP12	A/G or G/G			
SNP 5	A/G or A/A	0.17		
SNP12	A/A			

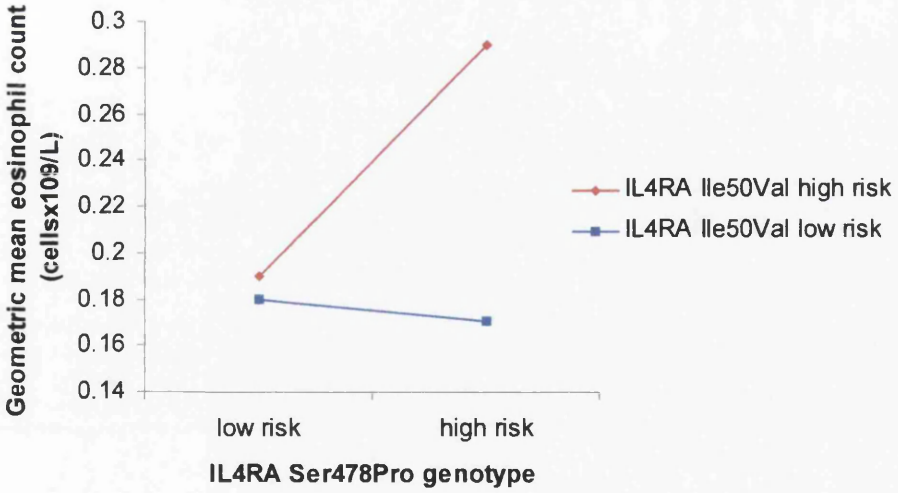


Figure 6.4 Graph showing the interaction effects of Ile50Val and Ser478Pro on eosinophil count. There is no effect by Val/Val (high risk genotype) unless in the presence of Ser/Ser and vice versa i.e. the effects of each genotype on eosinophil count are interdependent.

6.3.2 Haplotype analysis

IL13

When maximum-likelihood haplotype frequencies were estimated using the EM method in the 1445 Caucasian samples for which complete genotyping information was available, seven (out of a possible $2^8 = 256$) exceeded a frequency of 1%, with a cumulative frequency of over 95% (Table 6.3).

The most common or 'baseline' haplotype was estimated to have a frequency of 67.8%, and this was used as the baseline for calculation of odds ratios.

Table 6.3 *IL13* haplotypes based on total genotyped polymorphisms and haplotype frequencies (haplotypes with frequencies<1% were not considered).

Haplotype number	Haplotypes	Frequencies
*IL13*01*	11121121	0.678
*IL13*02*	22211212	0.087
*IL13*03*	21121111	0.055
*IL13*04*	11122121	0.052
*IL13*05*	12211222	0.037
*IL13*06*	12212222	0.028
*IL13*07*	22212212	0.014

Haplotype associations are summarised in Table 6.4. Haplotype analysis revealed the existence of two *IL13* haplotypes associated with asthma: *IL13*03* (OR 1.42, p=.003) and *IL13*06* (OR 1.59, p=.03). Smoking was identified as a potential confounding factor and only *IL13*03* was shown to be a risk haplotype for asthma after adjusting for smoking (TGGGCGCC, from consecutive *IL13* SNP 1, 2, 5, 6, 7, 9, 10, 13). This haplotype contains the A allele for Arg110Gln (which was protective in single analysis), in combination with the T allele of -1024C/T which has been identified as a risk allele for asthma by others [12, 114, 135, 141] but not in SNP analysis in this study. *IL13*06* was found to confer borderline increased risk for eczema (OR 1.36, p=.055). In this case, the risk haplotype contained both risk alleles found to also confer borderline risk

when examined individually (Chapter 3).

Two haplotypes, *IL13*07* and *IL13*02*, comprised -1024T, -1512C and +2044A (Gln110), and these haplotypes were both associated with higher IgE levels and *IL13*07* with additional Th2 phenotypes. *IL13*07* was identified as a risk haplotype for hayfever, with OR 1.15 (95% CI, 1.04-1.29, p=.009). *IL13*07* was also associated with higher IgE levels, with a borderline association shown for *IL13*02.* This was consistent with individual risk effects seen for IgE levels for intron SNP 6 and -1512 promoter SNP 10 (section 4.3). Highly significant associations were also shown for *IL13*07* and eotaxin levels and eosinophil counts, whereas only weak associations had been demonstrated for the individual SNPs, with no individual associations with eosinophil counts. In addition, *IL13*07* was associated with FEV1, in concordance with the individual effects of the promoter alleles contained within this haplotype on % FEV1. *IL13*04* associated with higher FEV1 and FVC levels which was consistent with individual protective effects for Q110R with % FVC within the haplotype, but also contained the -1024 promoter allele associated with lower % FEV1 when examined individually.

Table 6.4 Summary of *IL13* haplotype-phenotype associations.

Haplotype	Phenotype	OR	p value[1]
*IL13*03*	Asthma[2]	1.42	.003
*IL13*07*	Hayfever	1.15	.009
	IgE		<.0001
	Eotaxin		<.0001
	Eosinophil count		<.0001
	FEV1		<.001
*IL13*02*	IgE		.046
*IL13*04*	FVC		.044
	FEV1		.035

[1] After adjusting for relevant covariates.
[2] "Physician diagnosed asthma ever".

IL4RA

When maximum-likelihood haplotype frequencies were estimated using the EM method in the 1445 Caucasian samples for which complete genotyping information was available, fourteen (out of a possible 2^7 =128) exceeded a frequency of 1%, with a cumulative frequency of over 94% (Table 6.5). Three haplotypes covered 56% of all chromosomes, differing in 4 of 7 alleles. *IL4RA*01* was the most common haplotype, accounting for 23% of the haplotypes.

Table 6.5 *IL4RA* haplotypes based on total genotyped polymorphisms and haplotype frequencies (haplotypes with frequencies<1% were not considered).

Haplotype number	Haplotypes	Frequencies
*IL4RA*01*	1121111	0.226
*IL4RA*02*	2111112	0.199
*IL4RA*03*	2111122	0.138
*IL4RA*04*	2121122	0.091
*IL4RA*05*	1122211	0.062
*IL4RA*06*	2212222	0.050
*IL4RA*07*	1222212	0.041
*IL4RA*08*	1211112	0.031
*IL4RA*09*	2112121	0.030
*IL4RA*10*	1111112	0.023
*IL4RA*11*	1111111	0.018
*IL4RA*12*	2121112	0.016
*IL4RA*13*	1121112	0.013
*IL4RA*14*	1112111	0.011

There were no significant associations between *IL4RA* haplotypes and asthma. However, for *IL4RA*06* (AAAGCAC, from consecutive *IL4RA* SNP 5, 8, 16, 13, 12, 3, 7) a borderline protective effect was seen, with OR 0.62 (p=.058). This haplotype contained the Ile50 allele, found to be protective in previous individual SNP analysis (section 3.3). For hayfever, *IL4RA*05* and *IL4RA*11* confer risk (p<.001) and *IL4RA*06* is protective (p=.02). In this case, the risk haplotypes contained the Val50 risk allele and conversely, the protective haplotype contained the Ile50 protective allele

(SNP 5). There were no associations with eczema.

Haplotype associations are summarised in Table 6.6. Three haplotypes showed highly significant associations with IgE levels: *IL4RA*14* and *IL4RA*06* were associated with lower IgE levels and *IL4RA*11* with higher IgE levels. The strongest contribution to the haplotype effects observed was made by Q551R (SNP 13), where the T allele also associated with higher IgE levels when examined individually. *IL4RA*06*, which was protective for IgE levels, contained all protective alleles associated individually with lower IgE: GGA for intron SNP 8, Q551R (SNP 13) and 3'UTR SNP 16 respectively. *IL4RA*14* also exerted a protective effect for eotaxin levels and *IL4RA*06* was identified as a risk haplotype for higher eotaxin levels. When examined individually, no *IL4RA* variants were associated with eotaxin levels but it is worth noting that the protective haplotype for both IgE and eotaxin levels contained the T allele of Q551R SNP 13. *IL4RA*11* was shown to be associated with higher eosinophil counts in addition to IgE levels. *IL4RA*11* comprised both "risk" alleles for the coding variants I50V and Q551R. Consistent effects were observed for haplotypes *IL4RA*06* (protective) and *IL4RA*12* (risk) in their effects on Th2 immune marker levels. In contrast, *IL4RA*14* (protective haplotype for IgE) was associated with higher eosinophil counts but effects were small. Three *IL4RA* haplotypes were associated with risk of higher eosinophil counts and *IL4RA*06* was found to be protective. When haplotype effects for eosinophil counts were compared with individual allele effects, the strongest contributions to the observed haplotype effects on total IgE were made by promoter SNP 3 and I50V SNP 5, rather than S478P SNP 12, with effects for the former two variants largely concordant with the effects seen in single analysis. *IL4RA*05* comprised all three risk alleles that were individually associated with higher eosinophil counts.

*IL4RA*14* was associated with higher FVC but lower FEF 25-75. *IL4RA*11* was associated with lower FVC and higher FEF 25-75. *IL4RA*12* was associated with higher FEV1 and FEF 25-75. This suggests that *IL4RA*11*and *IL4RA*12* may be protective against airflow obstruction, whereas *IL4RA*14* may confer increased risk.

Table 6.6 Summary of *IL4RA* haplotype-phenotype associations.

Haplotype	Phenotype	p value[1]
*IL4RA*14*	IgE	<.0001
	Eotaxin	<.0001
	Eosinophil count	<.0001
	FVC	<.0001
	FEF 25-75	.003
*IL4RA*11*	IgE	<.0001
	Eosinophil count	<.001
	FVC	.003
	FEF 25-75	<.001
*IL4RA*06*	IgE	<.001
	Eosinophil count	.028
*IL4RA*12*	Eotaxin	<.0001
	Eosinophil count	.007
	FEV1	<.001
	FEF 25-75	<.0001
*IL4RA*05*	Eosinophil count	.011

[1] After adjusting for relevant covariates.

STAT6

When maximum-likelihood haplotype frequencies were estimated using the EM method in the 1445 Caucasian samples for which complete genotyping information was available, eight (out of a possible 2^7 =128) exceeded a frequency of 1%, with a cumulative frequency of over 98% (Table 6.7). Two haplotypes covered 63% of all chromosomes, differing in 5 of 7 alleles. *STAT6*01* was the most common haplotype, accounting for 39% of the haplotypes.

Table 6.7 *STAT6* haplotypes based on total genotyped polymorphisms and haplotype frequencies (haplotypes with frequencies<1% were not considered).

Haplotype number	Haplotypes	Frequencies
*STAT6*01*	1222212	0.393
*STAT6*02*	2121111	0.237
*STAT6*03*	1222112	0.096
*STAT6*04*	1121121	0.090
*STAT6*05*	1112112	0.081
*STAT6*06*	1122111	0.041
*STAT6*07*	1112111	0.032
*STAT6*08*	1121112	0.014

Haplotype *STAT6*08* (GGAGCCT, from consecutive STAT6 SNP 1, 2, 3, 4, 8, 11, 12) was observed to be a risk haplotype for asthma, with OR 1.99 (95% CI, 1.06-3.74, p=.03). Smoking was identified as a potential confounding factor since a high proportion of subjects with this haplotype were smokers, and the association therefore became non-significant after adjusting for smoking. The same haplotype was also associated with hayfever, with OR 1.05 (95% CI, 1.01-1.09, p=.01). Haplotype *STAT6*07* was associated with eczema, with OR 1.73 (95% CI, 1.11-2.67, p=.01). Both these haplotypes contain the G2964 allele of SNP 1, whereas it was the A/A homozygotes that had an increased risk of asthma and clinical atopy when this SNP was examined in isolation.

Haplotype analysis revealed the existence of a protective *STAT6* haplotype for total IgE levels. *STAT6*05* was associated with lower IgE levels (p<.0001), with a geometric mean of 34 kIU/L compared to 57 kIU/L for baseline *STAT6*01*. The strongest contribution to the observed haplotype effects on total IgE was made by 3'UTR SNP 3 (rs1059513) and intron SNP 8 (rs324011) variants, which were also significant in the single analysis (section 4.3). *STAT6*08* was associated with lower eosinophil counts although these effects are unlikely to be of clinical relevance with negligible differences between mean absolute counts despite p<.05. No associations were demonstrated with eotaxin levels.

*STAT6*08* paradoxically associated with a small increment in FEV1 and FVC, after adjusting for relevant covariates: differences were small, with FEV1 0.05 litres greater than baseline haplotype *STAT6*01* (p<.0001). *STAT6*02*, *STAT6*06* and *STAT6*08* were associated with higher FEF 25-75 suggesting a protective effect against small airflow obstruction, although the association was of borderline significance for *STAT6*06*. The association demonstrated for *STAT6*02* was concordant with the individual variant association of intron SNP 11 with % FEF 25-75 and it is worthy of note since this haplotype with potential protective effects for airflow obstruction has a relatively high frequency in our population with a prevalence of 23.7%.

Table 6.8 Summary of *STAT6* haplotype-phenotype associations.

Haplotype	Phenotype	OR	p value[1]
*STAT6*08*	Hayfever	1.05	.009
	Eosinophil count		<.0001
	FVC		<.0001
	FEV1		<.0001
	FEF 25-75		<.0001
*STAT6*07*	Eczema	1.73	.015
*STAT6*05*	IgE		<.0001
*STAT6*02*	FEF 25-75		.033
*STAT6*06*	FEF 25-75		.049

[1] After adjusting for relevant covariates.

6.4 Discussion

Interesting trends were noted for *IL13-IL4RA* epistatic interactions for Arg110Gln and Ile50Val with asthma, but differences did not reach statistical significance. These trends were worthy of note since they may suggest one possible explanation for non-replication of findings by previous investigators regarding Ile50Val, whereby in some populations it is the Val50 which confers asthma risk, whilst others have observed the

Ile50 allele to confer risk [119, 155]. In our population, trends were observed for Val50 conferring risk only in those with a low risk *IL13* genotype whereas elevated risk of asthma in Ile50 carriers was in those with a high risk genotype of *IL13*. A case-control study of Chinese children showed similar effects which were significant with an OR of high risk to low risk group of 3.9 for asthma [153]. It is possible that statistically significant differences would be seen in our study if groups were stratified according to environmental exposures. A recent study by Bernstein et al found a significant epistatic interaction between these two variants and diisocyanate asthma, but only in a subgroup stratified by a specific diisocyanate exposure (OR 4.13, p=.01) [290]. Further analysis in groups stratified for example by atopy or smoking exposure may yield interesting results.

As previously discussed (Chapter 3), it was the heterozygote Arg/Gln110 group that had the highest odds of asthma. This may have been due to small numbers in the homozygote group but another explanation is that the presence of both alleles has biological relevance and increases the chance of a functional interaction with Ile50Val (or with another locus). Two different protein copies, given that there is bi-alleleic expression, give greater opportunity for interaction with variants proteins from other loci in promoting asthma. *IL13* heterozygotes for other SNPs (promoter and 3'UTR, Chapter 4) were also noted to have the highest IgE levels.

There are several points to be made regarding Figure 6.1. The most striking trends of interest are that the probability of asthma *increases* in a dose-response manner in the presence of Val50, for the Arg/Gln and Arg/Arg group. However the trend is *reversed* for the Gln homozygotes, with probability of asthma increasing in the presence of Ile50. Secondly, Gln may only act as a risk allele in the presence of the Ile/Ile genotype and Ile may only act as a risk allele in the presence of the Gln/Gln genotype. Lastly, the Arg/Gln heterozygotes would appear to have a relatively high probability of asthma with the two groups having the highest (and almost identical) probability of asthma being Arg/Gln Val/Val and Gln/Gln Ile/Ile at 0.49 and 0.50 respectively. As pointed out, these trends did not reach significance, probably due to the rarity of the joint variant

homozygotes. However, taken together, these epistatic effects could provide an explanation of disease susceptibility in heterozygotes and suggest that the "risk" conferred by a particular allele is likely to be determined by the genetic *milieu* in which it exists. This may contribute to the non-replication of genetic associations in different populations where allele frequencies, and thus the strength of epistatic effects, may differ. Therefore, this may provide one explanation of why the Val50 variant of *IL4RA* and A2964 variant of *STAT6* were found to confer risk of asthma/atopy in our population, whereas these were found to be protective variants in other populations. The observations presented here hint at the potential complexity of genetic interactions and suggest that effects are unlikely to be straightforward and additive.

Epistatic interactions were observed between *IL13* and *STAT6* genes in their effects on total IgE levels. The effects of a variant of one gene were dependent on the presence of a high risk genotype related to an entirely separate gene, that is, a true epistatic effect. In the examination of the isolated effects of IL-13 signalling polymorphisms, these two *IL13* and *STAT6* variants had minor effects on IgE levels. However, in this combined analysis, each polymorphism amplifies the effects of the other polymorphism in the signalling pathway. This is all the more striking since these loci are located on different chromosomes, emphasising the importance of examining the co-effects of genes coding for molecules which signal and interact in a particular biological pathway. This is especially intriguing since both polymorphisms are non-coding variants, suggesting yet again the potential functional role of non-coding variants, which awaits further clarification in functional work, and which is discussed further in section 4.4. Another possibility is that the observed association is due to strong LD with coding variants: in the case of the *IL13* polymorphism, there was strong LD with a coding variant but it should be noted that an interaction was not seen when examining the coding variant itself. Other investigators have found a gene-gene interaction between risk alleles of each *IL13* promoter polymorphism and an *IL13RA1* polymorphism in predicting higher total IgE in children with atopic asthma [148].

Similarly, interactive effects were seen for eosinophil counts, in this case between two

coding variants of *IL4RA*. Both high risk genotypes needed to be present for a significant effect to be seen, with the effect of one again dependent on the combined presence of the other high risk genotype. In the analysis of the isolated effects of *IL4RA* polymorphisms, Ile50Val and Ser478Pro had minor effects on eosinophil counts. However, in combination, each polymorphism amplifies the effects of the other.

Haplotype analysis revealed much stronger associations when alleles were examined in combination rather than in isolation. A high risk *IL13*03* haplotype was identified for asthma, which contained -1024C/T, which has been identified as a risk allele for asthma by others [12, 114, 135, 141] but not in single analysis in this study. Haplotypes containing multiple risk alleles - for instance *IL13* haplotypes comprising -1024T, -1512C and +2044A (Gln110) - showed strong associations with Th2 phenotypes including IgE levels. This is concordant with a Korean study which showed a three locus haplotype for *IL13* (comprising the described high risk alleles) to be associated with higher IgE levels in children with atopic asthma [148]. We have thus replicated these findings at the population level and found an identifiable haplotype effect in an unselected population rather than the findings being limited to subjects with asthma.

A high risk atopy haplotype was identified for *IL13*, comprising three high risk alleles, which associated with risk of hayfever and higher levels of all Th2 immune markers (IgE, eotaxin and eosinophil counts), whereas individually, only weak effects were seen for *IL13* variants and IgE levels. The Val50 allele of *IL4RA* showed only a trend towards risk of hayfever in isolation, but a very significant association was observed when examined in combination with other variants as part of a haplotype ($p<.00001$). Similarly, associations with IgE levels became much stronger when SNPs were examined in haplotype combinations, with *IL4RA* haplotypes associated with p values as low as 1.75×10^{-14}.

This is the first study to examine the influence of *IL13*, *IL4RA* and *STAT6* polymorphisms and haplotypes on IgE regulation in a large population based cross-sectional cohort of adults. The data indicate that *IL13*, *IL4RA* and *STAT6* haplotypes

contribute significantly to the genetic determination of total IgE levels in a British population of adults, although when taking these results together with data from the individual SNP analysis, it would seem that *IL4RA* and *STAT6* have stronger regulatory effects on total IgE than *IL13*. These data are concordant with recent findings by Moller et al, who showed that *STAT6* haplotypes relate to IgE levels, allergy and worm burden, in a comparison of Chinese and British populations [82].

When considering *IL13* risk haplotypes, the promoter variant -1512A/C seemed to be mainly responsible for the detected alterations in total serum IgE regulation, and a non-significant trend towards higher IgE levels had been detected for this variant in isolation. Associations for *IL4RA* with elevated serum IgE clearly focus on the Q551R variant, with consistent protective/risk haplotype effects determined at this locus for each of the significant haplotypes examined. This variant showed significant association in single analysis and these findings are concordant with findings from the British 1958 Birth Cohort [132], with higher total IgE levels found in our population.

For *STAT6*, the effects on IgE were focused on two polymorphisms located in intron 2 and the 3′ UTR region, identical to findings from a large German population of children [128]. Although in strong linkage disequilibrium, alleles at these loci were not invariably inherited in identical haplotype combination. On examining the relative contribution of each variant to the direction of risk/protection for each haplotype, it would appear that intron SNP 8 (rs324011, C2892T) had the strongest effects. A functional role for this intron 2 variant is suggested by sequence analysis showing this variant to alter a NF-κB transcription binding site [128]. However it remains possible that the 3'UTR variant, or another variant in linkage disequilibrium with rs324011, is functionally important in the regulation of IgE production.

Risk haplotypes did not invariably contain individual "risk" alleles. For example, *STAT6*08* which associated with asthma contained the G2964 allele (3'UTR SNP 1) which was found to be protective against asthma when examined individually. This

demonstrates that asthma results from a complex interaction between multiple polymorphisms and that risk is not conferred by a single variant. The epistatic interactions described demonstrate the potential complexity of interactions between individual SNPs: in some cases an allele found to confer risk when examined in isolation, was actually found to be protective when its interaction in combination with another risk allele was examined. Also, these findings may suggest that an asthma susceptibility variant is on *STAT6*08* but is not any of the individual SNPs comprising this haplotype but rather that the true susceptibility SNP(s) are in linkage disequilibrium with one or more of these. It is worth noting that *STAT6* is a highly conserved gene, with strong linkage disequilibrium found between the examined SNPs. Only one *STAT6* haplotype (*STAT6*02*) contained the A2964 "risk" allele: this was the second commonest haplotype (with a trend towards a risk of asthma but not reaching significance) suggesting that alleles conferring asthma risk may confer another survival advantage and are thus conserved. This may be explained by Peisong et al's observation that the *STAT6* 3'UTR variant that conferred asthma risk was protective against ascaris worm infection in a population of Chinese children [84].

The protective effect of *IL13*07* on FEV1 was compatible with the observed individual effects of the promoter alleles contained within this haplotype on % FEV1. Two *IL4RA* haplotypes were found to be protective against airflow obstruction (*IL4RA*11* and *IL4RA*12*) with one haplotype associating with lower FEF 25-75 (*IL4RA*14*). A common *STAT6*02* haplotype was associated with higher FEF 25-75. Associations with lung function, although adjusted for relevant covariates, are difficult to interpret in isolation; unfortunately problems encountered with algorithm conversion for percentage predicted values and FEV1/FVC did not allow further clarification of these associations.

For *IL13*, genetic variability is characterized by a high level of nucleotide diversity and haplotype structure and linkage disequilibrium (LD) has been shown to differ across ethnic and geographical groups, emphasising the importance of determining haplotype structure in a particular population for gene association studies. Since the identification of single causal variants is hampered by the extended LD haploblock spanning intron 3,

exon 4 and the 3'UTR, haplotype analysis may be of greater utility than single nucleotide analysis alone. The HapMap initiative is likely to provide important information which should be applicable to the investigation of common disease aetiology across a range of populations, with its remit of identifying common patterns of haplotype structure (and htSNPs) for use in association studies across geographical regions [258].

In this thesis, we have employed a standard, step-wise, approach to detecting interactions (epistatic effects). This is partly to guard against false positives deriving from multiple testing, and also partly a pragmatic approach given the vast numbers of interactions that could be tested in the limited time frame. Our results, however, have highlighted an interesting potential drawback with this approach. In several instances we have found the effect of two interacting SNPs to be fully dependent on each other. That is, the increased risk allele at one locus is only manifest if a certain other risk allele is present at another locus. The effect of this situation is to dilute the impact of any particular locus, when it is examined individually, and its marginal effect 'averaged' over the alleles at the other locus. Here, the standard approach runs the risk of not selecting out the key loci in the first set of analyses, in which case their epistatic effects would not be tested. Therefore this conservative approach to haplotype and interaction modeling may mean that some true interactions were not identified. To extend our work further, we propose the exhaustive testing of all possible interaction terms (a combinatorially large process if more than 2-way interactions are also considered) and this is outlined in Chapter 8. Although one would have to tackle the issues of multiple testing, it would be very interesting to use this method as a means of identifying combinations of risk loci. Given available flexible software (the R language) and the availability of supercomputing, this task is not as daunting as it once appeared, and is likely to become a standard component of analyses of genome scans for complex diseases.

This thesis shows that analysis of epistatic and haplotype effects, in addition to individual SNP analysis, provides a more complete picture of how variants along the IL-

13 signalling pathway may interact to produce measurable asthma and atopy phenotypes. These findings may have practical clinical utility in the development of a diagnostic or predictive genetic test which could be used in infancy. The identification of an isolated high risk SNP is unlikely to be highly predictive of the development of asthma, whereas a combined analysis of SNPs in the IL-13 pathway may prove useful as a predictor of atopy and asthma in children. Even though the prevalence of high risk haplotypes was low, the highly significant associations demonstrated mean that the identification of these haplotypes in selected children (e.g. with a strong family history of atopy) may predict those with the highest risk. Epistatic and haplotype analysis suggests that the identification of combinations of multiple alleles in haplotypes is likely to advance towards differentiating the child with high genetic risk for the development of asthma and atopy from the child at low risk. Further assessment of these variants is required, with further replication studies in other populations to evaluate their utility as predictive markers for asthma and atopy.

The observations presented here support the approach of studying the effect of combinations of multiple alleles, in haplotypes or in combinations of haplotypes, on complex phenotypes within a biological pathway. Whilst the data are supportive of a biological interaction between polymorphisms along the IL-13 signalling pathway, this requires confirmation in functional assays *in vitro* and *in vivo*. One such study has been undertaken by Faffe et al who have shown that expression of the Val50/Pro478/Arg551 haplotype of *IL4RA* in human airway smooth muscle cells is associated with greater IL-13-stimulated TARC (thymus and activation regulated chemokine) production by these cells. Since TARC has an important role in recruiting Th2 cells, expression of the variant *IL4RA* haplotype results in a positive feedback loop promoting the recruitment of Th2 cells and IL-13 production in the asthmatic airway [291]. Since complex diseases are likely to result from combinations of, and complex interactions between, variants with moderate effects (in contrast with monogenic disorders), interpreting the contribution of these variants to the pathogenesis of allergic inflammation requires advancing beyond the one SNP by one SNP approach taken so far, and interrogating multiple genes within a biological pathway.

CHAPTER 7

The impact of obesity on asthma, lung function and Th2 immune phenotypes

7.1 Introduction

The concomitant rise in the prevalence of both asthma and obesity has led to speculation on a causal link between these conditions. Obesity has reached epidemic proportions in affluent societies with the adult prevalence of obesity in the UK almost trebling since 1980. In 2002, 23% of British men and 25% of women were obese [292]. Worldwide obesity prevalence has risen from 200 million obese adults in 1995 to 300 million in 2000 [293]. There is an inherent difficulty in proving a causal link between obesity and asthma since both are common conditions and may be rising in parallel with no association between them. However, evidence of an association comes from cross-sectional and prospective studies in addition to weight loss studies in asthmatics which show an improvement in lung function and asthma symptoms [5, 17]. The studies used body mass index (BMI), calculated as weight/height^2, as the predominant measure of obesity. Adult obesity is represented by a BMI of at least 30 kg/m^2 and overweight by a BMI of 25-29.9 kg/m^2. In UK centile charts for children, overweight and obesity are defined as a BMI greater or equal to the age and sex specific 91st and 98th percentiles of the population distribution in 1990 [294]. Evidence on the role of obesity in asthma is summarised below, in addition to an account of the available evidence on the relationship between obesity and atopy.

7.1.1 Cross-sectional studies

Cross-sectional studies in different countries have found adult asthmatics to have a higher prevalence of obesity than non-asthmatics, although this is not universally the case [5]. Self-reported asthma was used in the majority, with or without a physician's diagnosis. Several studies have reported obesity to be more strongly associated with wheeze and asthma diagnosis in females [190, 295, 296] but other studies have not shown a significant modification by sex [297, 298] or have shown a stronger association between obesity and airway hyperresponsiveness (AHR) in males [299, 300].

A large Chinese study of families of asthmatics found a U-shaped relationship between

obesity and AHR with both extremes of BMI being associated with symptomatic AHR in men and women [301]. This was also the case for the association between obesity and asthma in women, but in men, asthma was associated only with being underweight rather than overweight. Conversely, a US study demonstrated this U-shaped relationship in men but in women, asthma was associated with overweight and obesity only [298]. The Humbolt study showed both BMI and waist circumference to be significantly associated with asthma in women but not in men and a Swedish study showed these associations without sex-specific effects [302, 303]. These associations were also observed in Hispanic women but not men [304].

Studies in adolescents and children show a less consistent association between obesity and asthma. BMI is the most widely used measure, but other anthropometric measures have also been used. Again, some studies showed an association with females only [305] with other studies showing no sex difference [306].

7.1.2 Prospective studies

Cross-sectional studies have demonstrated an association between obesity and asthma. Prospective studies allow exploration of the causal nature of this association. A recent meta-analysis of cohort studies concluded that children with high body weight were at increased risk of developing future asthma. Combined results from four studies examining the effect of high body weight during middle childhood on the outcome of future asthma showed a 50 percent increase in relative risk (RR 1.5, 95% confidence interval [CI], 1.2-1.8). Combined results from nine studies examining the effect of high birth weight on subsequent asthma had a pooled RR of 1.2 [307]. Seven of eight adult prospective studies have shown an association between baseline BMI and subsequent development of asthma [203, 297, 308-313]. A recent meta-analysis of adult studies concluded that overweight and obesity are associated with a dose-dependent increase in the odds of incident asthma in both men and women [314]. Thus strong evidence for causality is provided, since overweight and obesity antecede the development of asthma, with relative risk ranging from 1.6 to 3, with several studies showing a dose-

response relationship. Prospective studies showing obesity to antecede the development of asthma refute the suggestion that obesity is related either to corticosteroid use or reduced physical activity in asthmatics.

Some (but not all) studies showed sex-specific effects which may reflect differences in the shape of the relationship in men and women, as seen in some of the cross-sectional studies. A study including only men demonstrated a U-shaped relationship between AHR and both extremes of weight [315], whereas a study including only women detected a monophasic relationship between asthma and overweight development [203]. In the Coronary Artery Risk Development in Young Adults study, 4547 adults from the same age range as our own study (18-30 years) were followed for 10 years. An association between asthma and obesity was seen for females but not males. However the definition of asthma was less strict, not requiring a physician diagnosis [311]. A similar association in females has been noted in other studies [310, 313]. A Norwegian study of 135,000 found an association in both sexes: for each unit of increased BMI between 25 and 30, the risk of asthma increased by 10% and 7% for men and women respectively [316]. The European Community Respiratory Health Survey found that the association between asthma symptoms and obesity was similar in both men and women [317].

In children and adolescents, two US studies showed conflicting results on obesity and risk of asthma development according to sex, one showing an association in females (population aged 6-14) and the other in males (population aged 7-18) and it may be that the effects were modified by the slightly different ages in the study populations [318, 319]. A recent birth cohort followed for up to 14 years, showed an association for boys only [320]. In a British cohort of 5-6 year olds followed for 12 years, obese children had an increased risk of asthma development after 4 years. However the investigators concluded that obesity was not causing increased asthma prevalence but that lifestyle changes common to obesity and asthma were responsible for the associations demonstrated [321]. A New Zealand birth cohort study showed an association between BMI and asthma in females only and estimated a population attributable fraction of 28%

(95% confidence interval 7–45) of asthma developing in females after age 9 to be due to overweight [322].

7.1.3 Effects of weight change

In two prospective studies, weight gain was associated with an increased incidence of asthma, with a non-significant trend seen in another study [203, 311, 312]. The National Population Health Surveys did not find an increase in weight or BMI to be associated with increased asthma incidence over a 2 year follow-up [313]. In children, an association was seen between an increase in BMI and asthma incidence or asthma symptoms and bronchial responsiveness in females only [319, 323].

Conversely, weight loss in obese asthmatic patients has been shown to improve symptoms. Bariatric surgery in morbidly obese asthmatics led to resolution of asthma in up to half of patients with an improvement in symptoms, exacerbations, hospitalisations and medication use in up to 90% of cases [324, 325]. A limitation of bariatric studies is the difficulty in generalisability to obese asthmatics in the general population, since bariatric surgery is only offered to the morbidly obese (BMI $\geq$40kg/m^2) or those with comorbidities and BMI $\geq$35kg/m^2. An alternative explanation to weight loss improving asthma per se is that *symptoms* are improved rather than the condition itself and bariatric studies have provided no objective measures of lung function or bronchial hyperreactivity. Other explanations are that the change in diet or improvement in gastro-oesophageal reflux following surgery may be important.

Low calorie diets in obese asthmatics have been found to improve FEV1, FVC, variation in peak expiratory flow, ratio of forced midexpiratory flow rate to FVC; number of exacerbations and use of rescue medications [64, 176]. An uncontrolled study which combined a low calorie diet with an exercise programme demonstrated an improvement in FEV1 and FVC but no significant improvement in bronchial hyperreactivity [65]. The obvious problem with a weight loss intervention is that this either involves dietary change, increased exercise or a combination of these factors,

both of which may act as confounders beyond the effects of weight loss alone. This has led to the suggestion that obesity may be an epiphenomenon related to the effects of diet and/or physical activity on asthma. It is difficult to measure physical activity accurately but three of the prospective studies attempted to adjust for it [203, 308, 309]. At least one study found physical activity to be inversely related to asthma (in men only) [312] but no significant association was seen in two other studies [203, 311] and population studies have not shown that asthmatics are significantly less physically active [308, 313]. The potential role of diet in asthma aetiology has been reviewed in Chapter 1.

7.1.4 Evidence of the effects of obesity on atopy

Whereas several studies have examined the association between obesity and asthma, there is a paucity of data on the relationship between obesity and atopy, and the reported data are conflicting. Positive associations between body mass index (BMI) and atopy have been reported in cross-sectional surveys of children/adolescents [326-328]. Any association with atopy is likely to be age-dependent and influenced by the developmental stage of the immune system with modifying effects by gender. For instance, Schachter et al found no association between BMI and atopy when examining Australian adults, but an association was seen for girls, but not boys, aged 7-12 years [326, 329]. A similar association was seen for girls in the Dunedin cohort study, with a higher frequency of atopy in those who became both asthmatic and obese after puberty, and BMI was positively associated with total IgE [322]. Likewise for Taiwanese girls, obesity was related to atopy, including an association with rhinitis [327]. Obesity was reported in association with atopy in a longitudinal Finnish study from birth to 31 years [330].

In contrast, several studies have also reported negative findings. In a study of 9000 German schoolchildren aged 5–6 years, physician-diagnosed asthma, but not hayfever and eczema, was associated with overweight in girls [331]. In the NHANES-III study, no association was found between BMI and atopy or eosinophilia in over 7500 children aged 4–17 years, although a positive association was seen for asthma [332], with a New

Zealand study reporting similar negative findings for atopy [333]. Moreover, the Children Health Study, found that overweight/obese children had an increased risk of incident asthma during about 4 years after recruitment , but that this effect was limited to non-allergic children [334]. The European Community Health Survey (ECRHS) found that BMI was related to airway hyperresponsiveness but not to atopy in a cross-sectional survey of adults [317].

It is difficult to draw any definitive conclusions from these conflicting data. It is important to clearly define the atopic phenotype to be examined, since recent data show that BMI was associated with skin sensitisation but not with corresponding serum IgE antibodies among South African schoolchildren with exercise-induced bronchospasm, as well as in age-matched controls [335]. Moreover, most studies have defined atopy by objective measures (skin test reactivity, IgE) in addition to self-reported allergic symptoms, including asthma symptoms, and have made no attempt to adjust for asthma. Thus the effect of obesity on atopy, independent of asthma, has been difficult to assess. Clearly more research is needed to explore the association between obesity, asthma and atopy, and in particular to define effects by gender and in different age groups and to clarify other environmental effects which may impact upon this relationship.

7.2 Study design

The majority of studies used BMI as the only measure of obesity, which takes no account of body frame and muscle mass, and BMI may be an unreliable indicator of adiposity in men in particular [336]. Potential misclassification of adiposity might explain why the association with asthma appears weaker in men. Other measures of obesity might clarify the associations, possible modification by sex and provide new insights into the mechanisms behind the relationship. Measuring waist circumference (WC) and waist/hip ratio (WHR) advances our understanding of the role of abdominal obesity specifically. In Europids, central obesity relates to a waist circumference $\geq$ 94cm and 80cm in males and females respectively, whereas in the Chinese, the cut-off for males only is reduced to 90cm [337]. Cut-off points for waist/hip ratios are 0.9 and

0.85 for males and females respectively [338]. It is also important to provide an assessment of body fat to clarify the role of the adipose tissue itself in the development of asthma. In this way, new light may be shed on molecular interactions at the adipocyte level. Here for the first time we examine percentage body fat in addition to BMI, WC and WHR.

In order to explore whether obesity was associated with atopy and an upregulation of Th2 pro-inflammatory markers, multiple Th2 immune phenotypes were included, in additional to clinical data on the presence of asthma, hayfever and eczema. The Th2 immune phenotypes examined included total IgE levels, eosinophil counts and plasma eotaxin, a Th2 chemokine which acts as a chemoattractant for eosinophils.

7.3 Methods

Methods have been outlined in detail in the main Methodology section (Chapter 2). Here I will focus on the particular methods relevant to examining the association between obesity, asthma and atopy and lung function.

Subjects comprised 1614 unselected volunteers (aged 18-30) from students and staff at Swansea University, Swansea Institute and Singleton Hospital, Swansea. Subjects were studied when free of acute lower respiratory infection which would be likely to result in transient reduced lung function.

7.3.1 Anthropometric measurements

The following measurements were recorded on a data sheet (Appendix III)

1. **Height** measured by staediometer to 0.1cm (Leicester; Chasmors, UK)
2. **Waist** and **hip circumference** to 0.1cm (D loop non-stretch fibreglass tape; Chasmors, UK). Waist was measured at the smallest circumference between the ribs and iliac crest, with the participant standing with abdomen relaxed at the end of a normal

expiration. Hip circumference was measured at the point of maximum circumference over the buttocks [339].

3. **Weight** to 0.1kg (Seca 873 digital scales; Cranlea, Birmingham, UK).
4. **Percentage body fat** to 0.1% (Biolelectrical impedance; Tanita digital scales, Tanita UK Ltd, Yiewsley, UK)

The following were then calculated:

1. **Waist/hip ratio**
2. **Body mass index** (BMI) = weight (kg)/ [height (m)]^2

7.3.2 Clinical variables

Demographic data were collected and subjects completed a validated bronchial symptoms questionnaire which included questions on symptoms (e.g. wheeze) and presence of asthma, hayfever and eczema [236]. Smoking status (pack-years) was recorded. Those who answered positively to "doctor-diagnosed asthma" completed a further validated asthma and allergy symptom questionnaire, based on the European Community Respiratory Health Survey [237]. Asthma severity was graded as mild intermittent, moderate persistent or severe persistent according to GINA criteria [50]. Quantification of severity included recording symptom frequency, volume of medication and number of doctor visits over the previous 12 months.

7.3.3 Spirometry

Forced Expiratory Volume in 1 second (FEV1), Forced Expiratory Flow at 25-75% of expired vital capacity (FEF 25-75), Forced Vital Capacity (FVC), FEV1/FVC ratio and Peak Expiratory Flow (PEF) were measured by standardised protocol [239], using a dry spirometer (Vitalograph, UK). The best value of three manoeuvres was expressed as an absolute and percentage of the age-gender-stature predicted value. Spirometry was performed in the morning (before noon) in order to capitalise on the diurnal variation in asthma thus maximising the discriminatory value of this test in subjects with airflow

obstruction. If on inhaler treatment for asthma, measurements were taken before the morning dose.

7.3.4 Severity criteria

A subgroup analysis was undertaken on subjects with a current physician diagnosis of asthma to examine the effect of obesity on physiological and clinical severity. Asthma severity was quantified by the following measures:

1. Clinically categorised into "intermittent", "mild persistent", "moderate persistent" and "severe persistent" asthma according to GINA criteria (shown previously in Table 5.10), ascertained by validated questionnaire (ECRHS, Appendix V). Current therapy was accounted for e.g. a subject having intermittent symptoms but on "mild persistent" therapy of low-dose inhaled glucocorticoids would be classified as having "mild persistent" asthma [50].
2. Number of asthma attacks in last 3 months.
3. Frequency of waking because of asthma in last 3 months.
4. Frequency of problems with breathing because of asthma in last 3 months.
5. Volume of daily inhaled beta-2 agonist therapy in last 3 months.

A daily equivalent dose of Salbutamol was estimated, taking long-acting beta agonist usage into account e.g. if on Salmeterol 50mcg daily, this was considered equivalent to 800mcg Salbutamol.

6. Volume of daily inhaled corticosteroid therapy in last 3 months.
7. Lung function: % predicted FEV1; % predicted PEF.

7.3.5 Th2 immune phenotypes

Total IgE

Whole blood was collected in vacutainers (serum separator clotted tube) and centrifuged for serum separation. Serum total IgE (kIU/L) was measured using an ELISA-type

sandwich assay (Roche Diagnostics, Lewes, UK) at Morriston Hospital Chemical Pathology, as outlined in Methodology section 2.6.3.

Eotaxin

Whole blood was collected in EDTA vacutainers and centrifuged for plasma separation. Plasma eotaxin (pg/ml) was measured using enzyme-linked immunosorbent assays (ELISA) (BD OptEIA^{TM}, BD Biosciences), under the supervision of Dr Cathy Thornton, Senior Lecturer in Newborn Immunity, as outlined in Methodology section 2.6.3.

Eosinophil counts

Whole blood was collected in EDTA vacutainers and full blood count with white cell differentials including eosinophil count was measured on a Sysmex XE-2100 automated analyser, as outlined in Methodology section 2.6.3. Analysis was performed by Haematology staff at Singleton Hospital, Swansea NHS Trust, under the supervision of Dr Ann Benton, Consultant Haematologist.

7.3.6 Data analysis

The analysis of the association between obesity measures – BMI, waist circumference, waist/hip ratio, % body fat - and risk of asthma took the form of a series of standard logistic regression models, with adjustment made for relevant covariates (sex, smoking etc). Since smoking is directly associated with asthma and inversely associated with weight, it acts as a potential confounder for which adjustment must be made [5]. The analysis was expanded upon to include the quantitative outcomes – e.g. lung function, IgE, eosinophil count, eotaxin - using general linear models. A quadratic model was chosen as the best model for the data when the quadratic form of the covariate showed a significant association with the dependent variable at the 5% level.

Th2 immune markers were analysed on a logarithmic scale since they were non-normally distributed. All were analysed as ln(x+1) in order to overcome an error occurring when x was undetectable and counted as zero.

A subgroup analysis was undertaken on subjects with a current physician diagnosis of asthma to examine the effect of obesity on physiological and clinical severity. Ordinal regression was applied to identify associations of obesity with clinical severity categories. Linear regression was applied for continuous lung function data.

Our model building approach has been outlined in section 2.8 and will be briefly reiterated. Initially the relationship between the primary predictor and outcome was explored in a univariate analysis. The effect of adjustment for relevant covariates was explored by adding the variables to the model in a stepwise manner and (at each step) retaining the covariates if there was either 1) a significant ($p<.05$) improvement in the likelihood of the fit of the new model, or 2) the covariate caused a substantial alteration of the coefficients representing the effect of the primary predictor. Lastly, when the above process had settled on a 'final model', all pairwise interactions involving the primary covariate were tested.

7.4 Results

7.4.1 Population characteristics

There were 1614 participants in our cross-sectional sample. Of these, 829 (51.4%) were men, 1445 (90%) were white, 53 (3.3%) were Chinese and 24 (1.5%) were Indian. The mean ± SD age of the participants was 21 ± 2.89 years. Twenty-six percent of the population were either overweight or obese (n=420): 21% were overweight and a further 5% were obese (Figure 7.1). Self-reported subjects with "doctor confirmed diagnosis of asthma ever" represented 23.3% of the study population (n=374) with "current doctor diagnosed asthma" represented by 10.9% (n=176). Bronchodilators were taken by 10.8% (n=175) and 3.5% (n=56) were taking regular inhaled

corticosteroids. Subjects reporting "smoking for as long as a year" represented 26.8% of the population (n=433) with 16.9% reporting current smoking (n=271). Population characteristics are shown in Figure 7.1 and Tables 7.1-7.3 with correlations between the adiposity indices shown in Table 7.4.

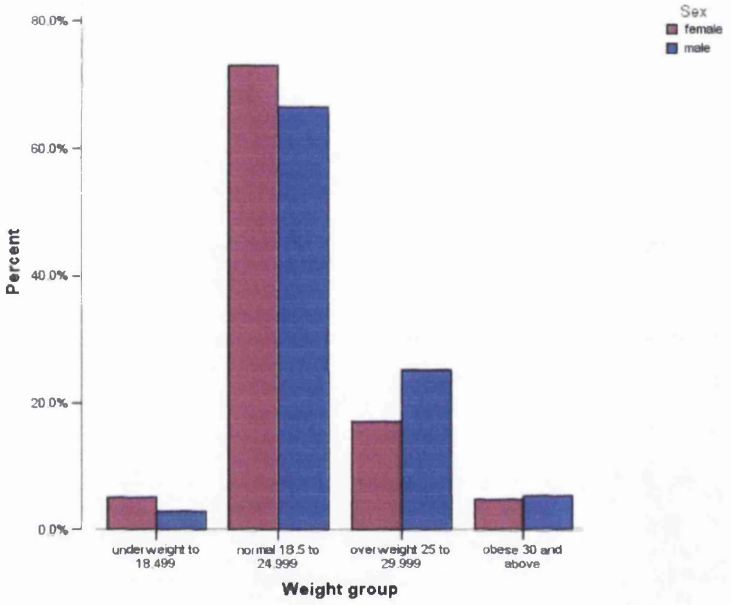


Figure 7.1 Baseline characteristics of the population by weight category, subdivided by gender. Males are slightly over-represented in the overweight group.

Table 7.1 Baseline characteristics of study participants, by adiposity indices.

	Males	Females
BMI	23.8 ± 3.5[1]	23.1 ± 3.6
Waist circumference	82.8 ± 8.6	74.0 ± 8.1
Waist/hip ratio	0.82 ± 0.05	0.75 ± 0.05
Percentage body fat	17 ± 6.0	28 ± 7.0

[1] x ± SD (all such variables).

Table 7.2 Baseline characteristics of study participants in relation to asthma and adiposity indices.

	Current asthma	"Never asthma"
BMI	24.5 ± 4.2[1]	23.3 ± 3.4
Waist circumference	80.4 ± 11.1	78.1 ± 9.1
Waist/hip ratio	0.79 ± 0.07	0.78 ± 0.06
Percentage body fat	24.3 ± 9.3	22.0 ± 8.4

[1]x ± SD (all such variables).

Table 7.3 Baseline characteristics of asthmatics vs. non-asthmatics, according to weight category.

	Current asthma		"Never asthma"	
Underweight	4	(2.3)	52	(4.3)
Normal weight	110	(62.5)	856	(70.7)
Overweight	49	(27.8)	243	(20.1)
Obese	13	(7.4)	59	(4.9)

Data are number (percentage) of subjects.

Table 7.4 Correlations between adiposity indices.

	BMI	*Waist circumference*	*Waist/hip ratio*	*Percentage body fat*
BMI		.823[1]	.462	.876
Waist circumference	.776		.575	.834
Waist/hip ratio	.180	.578		.509
Percentage body fat	.794	.782	.460	

Data are R^2

[1]Model included sex in all cases except analysis of waist/hip ratio dependent on waist circumference where sex interaction was non-significant.

7.4.2 Logistic regression analysis of adiposity and clinical outcomes

Logistic regression analysis was performed for the clinical outcomes of asthma, hayfever and eczema and the results are outlined in this section. Analysis of the association between adiposity and asthma severity then follows: severity was analysed by a combination of logistic and linear regression. Lastly, the linear regression results are presented, examining the association between adiposity and lung function, IgE, eosinophils and eotaxin. The outcomes of "current asthma", "asthma ever" and "asthma attack in last 12 months" all refer to a physician diagnosis of asthma.

BMI was a highly significant predictor of current asthma ($p<.001$); "asthma ever" ($p<.001$); "attack of asthma in last 12 months" ($p<.01$) and "wheeze in last 12 months" ($p<.001$). The odds ratio for the association with current asthma was 1.083 for a unit change in BMI (95% CI, 1.04-1.13). Thus a clinically meaningful change in BMI would be associated with increased risk of asthma as follows:

- a BMI of 29 (overweight range) compared to BMI 24 (normal range) equates to a 41.5% increased risk of asthma
- a BMI of 34 (obese range) compared to BMI 24 (normal range) equates to a 83% increased risk of asthma

When comparing the odds of asthma in the obese versus the non-obese categories, the odds ratio was 1.6 (95% CI 0.98-2.22): this is just non-significant at the 5% level due to the small number of obese subjects, and examining BMI as a continuous variable is more informative. When the overweight and obese are grouped together, the odds ratio is almost identical at 1.61 (95% CI 1.28-1.94), which is a significant difference, with $p=.005$.

The prevalence of asthma according to weight group is shown in Figure 7.2 and the estimated probabilities derived from the logistic regression model are shown in Figure 7.3. It can be seen that prevalence rises across weight groups for the group as a whole,

with a dose-response effect of elevated BMI on asthma, with much more striking effects seen for females. For the males, prevalence drops slightly between the overweight and obese groups but this is not a significant difference, and as stated, numbers of obese subjects were relatively small. For both sexes, there was a significant and positive association between BMI and asthma, with larger effects for females: OR 1.093 (95% CI 1.04-1.14) for females; OR 1.076 (95% CI 1.02-1.13) for males.

There was a sex modifying effect in the association with "asthma ever" such that there was only a significant association for females ($p<.001$; OR 1.094, 95% CI, 1.05-1.14). The association with "attack of asthma in last 12 months" did not remain significant after adjusting for body fat.

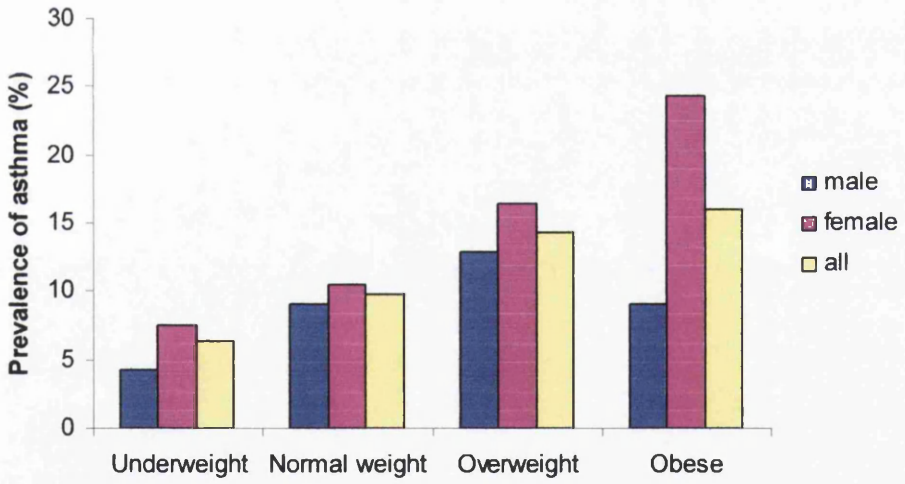


Figure 7.2 Prevalence of asthma according to weight group. The prevalence of asthma increases with weight, with the effects being more marked in the females. A dose-response effect of elevated BMI on asthma is seen across the group as a whole.

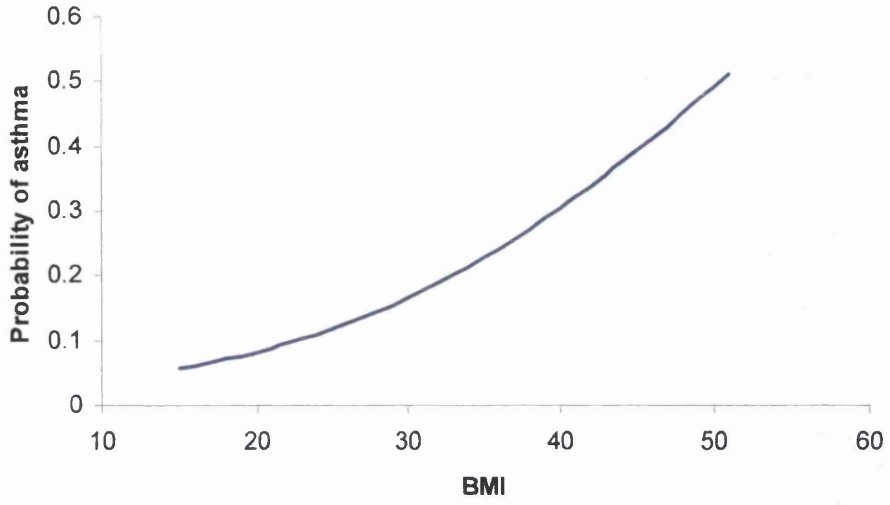


Figure 7.3 Estimated relationship between probability of asthma and BMI, as obtained from fit of logistic regression model.

Waist circumference was significantly associated with current asthma ($p<.01$; OR 1.022, 95% CI, 1.01-1.04; Figure 7.4) and "wheeze in last 12 months" ($p<.001$). Putting this into clinical context, a 10cm increase in girth would equate to a 22% increase in risk of asthma. These associations were not significant after adjusting for BMI. The association with wheeze (but not current asthma) remained significant after adjusting for body fat. Again the association with "physician-diagnosed asthma ever" was only significant in females ($p<.001$; OR 1.039, 95% CI, 1.02-1.06).

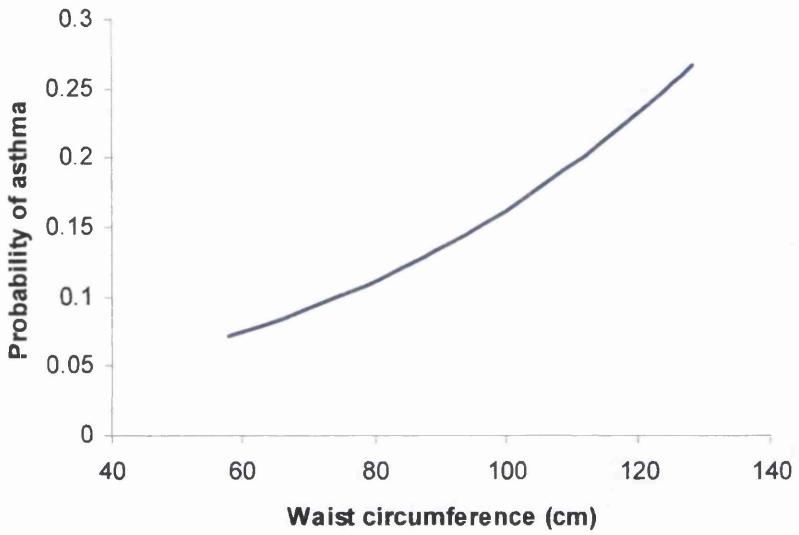


Figure 7.4 Estimated relationship between probability of asthma and waist circumference (WC), as obtained from fit of logistic regression model.

Waist/hip ratio showed no significant associations with any of the binary asthma outcomes described. There was a significant association with "wheeze in last 12 months" with a high OR of 8.25 (p=.02, 95% CI, 1.50-45.39). There was an inverse association with "eczema ever" (p=.03, OR 0.12, 95% CI, 0.05-0.32) but this is of questionable relevance since no significant association was seen for males or females when analysed as separate subgroups, and there was no association with "current eczema".

Percentage body fat was significantly associated with current asthma (p=.001; OR for unit increase of 1.032, 95% CI, 1.01-1.05) (Figure 7.5) after adjusting for waist circumference but not after adjusting for BMI. However the association with "asthma attack in last 12 months" (p=.001; OR 1.04) remained significant after adjusting for BMI and WC indicating an effect over and above these factors. The association with "wheeze in last 12 months" did not quite reach significance. Body fat significantly predicted current eczema (p=.001; OR for unit increase of 1.031, 95% CI, 1.01-1.05). Thus to extrapolate to a clinical context, if we consider a male with low-normal BF of

12% compared to male with BF 22% which is just beyond normal range, this 10% increase in body fat would equate to:

- 32% increase in risk of current asthma
- 31% increase in risk of current eczema

A summary of the associations between adiposity indices and clinical outcomes is shown in Table 7.5. None of the obesity measures showed significant associations with hayfever.

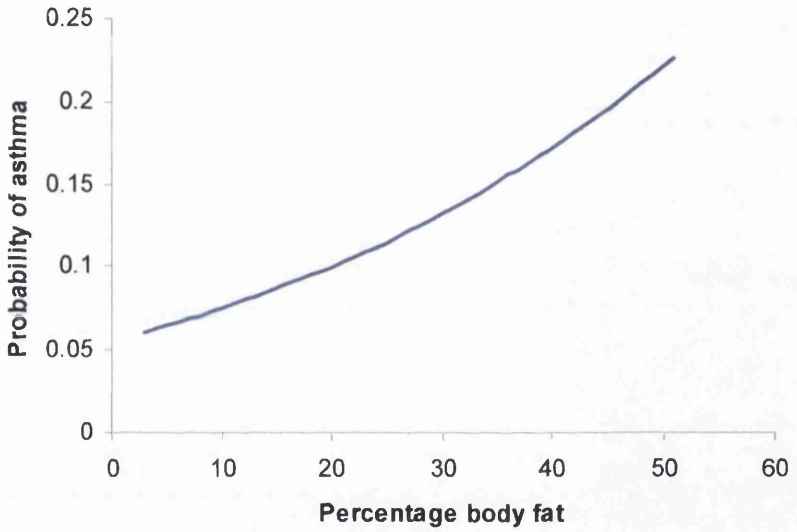


Figure 7.5 Estimated relationship between probability of asthma and body fat (BF), as obtained from fit of logistic regression model.

Table 7.5 Associations between adiposity indices and clinical outcomes.

	Current doctor-diagnosed asthma	*Wheeze last 12 months*	*Current eczema*
BMI	Positive association p < .001 OR 1.08 95% CI 1.04-1.13	Positive association p < .001 OR 1.06 95% CI 1.03 to 1.09	No association
WC	Positive association p < .01 OR 1.02 95% CI 1.01- 1.04	Positive association p < .001 OR 1.02 95% CI 1.01-1.03	No association
WHR	No association	Positive association p < .001 OR 8.25	No association
% BF	Positive association P =. 001 OR 1.03 95% CI 1.01-1.05	Association NS[1] P = .055	Positive association P = .001 OR 1.03 CI 1.01-1.05

[1] NS, non-significant

7.4.3 Asthma severity

Subjects with current asthma represented 10.9% of our population (n=176) with a further 12.3% (n=198) having had asthma which had remitted. Subgroup analysis of the "current asthma" group was undertaken to ascertain whether adiposity indices were associated with asthma severity. The characteristics of the asthma group have previously been shown in Table 5.9. Only one subject was taking inhaled cromoglycate, anti-muscarinic, oral methyl xanthine or an oral leukotriene antagonist respectively. Only one subject had spent nights in hospital over the last 12 months and no subjects

had been admitted to ITU in the last 12 months. Forty subjects had had to give up days of work or other activities over the last 12 months: of these, the number of days lost per month was 1.01 ± 1.30.

A subgroup analysis was undertaken on subjects with a current physician diagnosis of asthma to examine the effect of obesity on physiological and clinical severity. BMI predicted frequency of symptoms over the last 3 months, after adjusting for sex and smoking ($p<.05$, OR 1.086). Therefore for each unit change in BMI, there was an 8.7% increased risk of having a higher frequency of symptoms e.g. "continuous" compared with "daily" symptoms. BMI also predicted daily inhaled bronchodilator volume ($p<.05$). There was a significant sex modifying effect such that the association was only significant in men ($p<.01$) (Figure 7.6). There was also a smoking interaction ($p=.048$) such that a positive linear association was seen for the current smokers or those who had smoked for up to one year ($p<.001$), whereas a quadratic relationship provided the best fit for the data for the non-smokers, where a J-shaped curve shows volume of medication to be increased at both weight extremes, with a rise also for the underweight group (Figure 7.7).

BMI did not predict any other of the severity criteria, including classification by GINA criteria (shown previously in Table 5.10) nor daily volume of inhaled corticosteroid. None of the other adiposity measures predicted asthma severity. None of the adiposity indices were associated with worse lung function severity (% predicted FEV1 and PEF) in the current asthmatics. In fact, BMI was positively associated with % predicted FEV1 and PEF; and waist circumference and body fat were positively associated with % PEF. These data are difficult to interpret and the main reason for the positive association is likely to be that the quadratic variable (BMI^2, WC^2, BF^2) failed to reach significance for this subgroup compared to the population as a whole, probably because of the much smaller number in the current asthma subgroup (n=176). However, waist circumference and waist/hip ratio were inversely associated with FEV1/FVC ratio ($p<.05$, $p<.01$) with the association only significant at the 10% level for body fat, in the asthma group ($p=.09$).

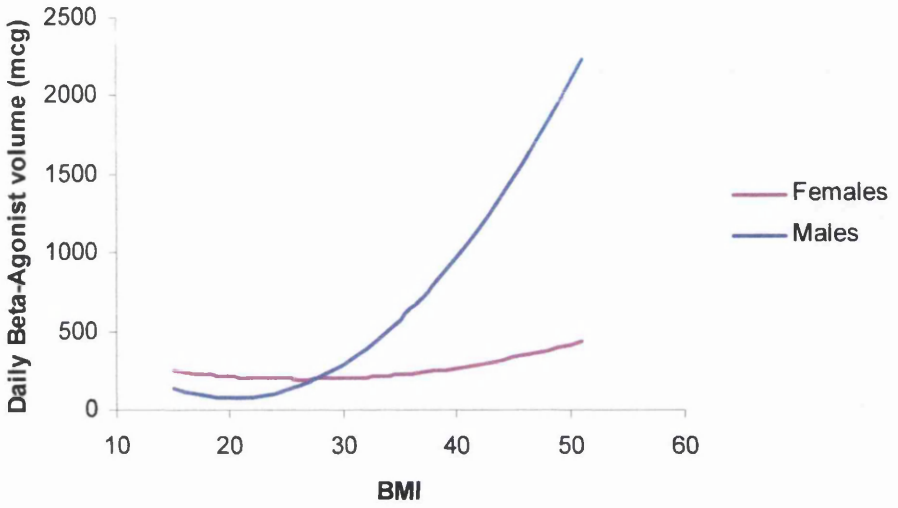


Figure 7.6 Estimated relationship between daily beta-agonist usage and BMI according to gender. Volume of medication rises sharply in males with increasing levels of obesity. No association is seen for females. Plot lines are obtained from linear regression. Data points are omitted for reasons of clarity here, since the large number of data points obscures the intersect.

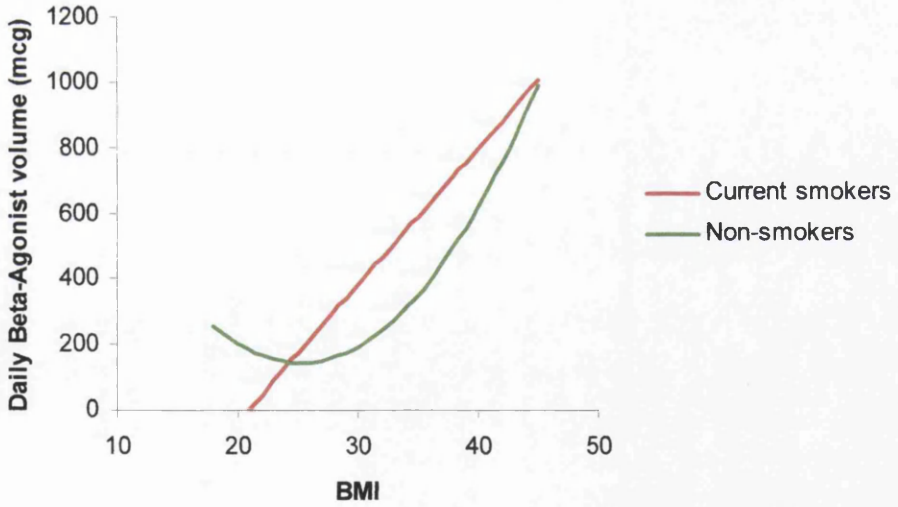


Figure 7.7 Estimated relationship between daily beta-agonist usage and BMI according to smoking status. For smokers, a linear association is seen, with volume of medication rising steadily with increasing levels of obesity. For non-smokers, a J-shaped curve is seen, with volume of medication increased at both weight extremes, with a rise also for the underweight group. Data points are omitted for reasons of clarity here, since the large number of data points obscures the intersect.

7.4.4 Linear regression analysis of adiposity and lung function in an unselected population

Although there is considerable scatter about the regression lines, the large population makes it possible to detect significant relationships. The obesity measures showed non-linear associations with lung function parameters. For the majority of associations, a quadratic curve provided the best fit for the data in regression analysis. That is, lung function increased as anthropometric measurements increased over normal ranges but as higher values were reached, lung function then decreased. A quadratic model was chosen as the best model for the data when the quadratic form of the covariate showed a significant association with the dependent variable at the 5% level.

A summary of the associations between adiposity indices, asthma and lung function is shown in Table 7.6.

BMI

BMI was significantly associated with absolute and percentage of age-gender-stature predicted values of FVC, FEV1 and PEF ($p<.001$) (Figure 7.8 & 7.9). BMI was inversely associated with FEV1/FVC ratio which indicates degree of airflow obstruction ($p<.001$) (Figure 7.10). Smoking (history of "smoking for as long as a year") modified the effects on FVC and FEV1. There was no significant association between BMI and flow rates at 25-75% of expired vital capacity (FEF 25-75). BMI remained a significant predictor of lung function after adjusting for WC and body fat, but was not an independent predictor of FVC, FEV1 and PEF once waist/hip ratio was adjusted for.

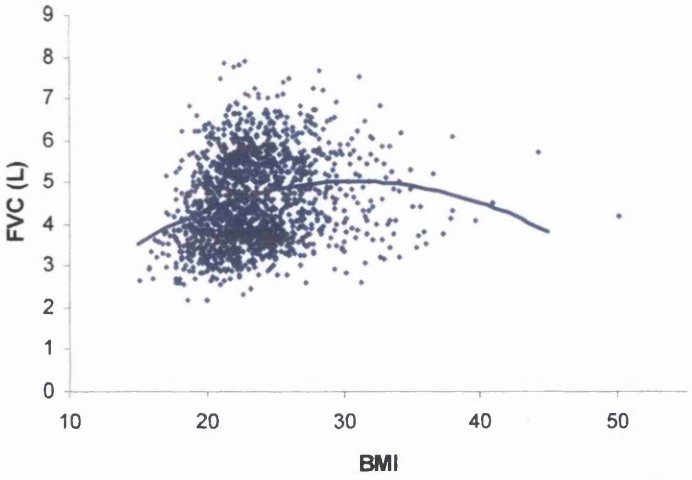


Figure 7.8 The association between FVC and BMI. A quadratic relationship is seen, with FVC increasing across normal anthropometric ranges of BMI, with an inverse association seen as BMI approaches obese levels.

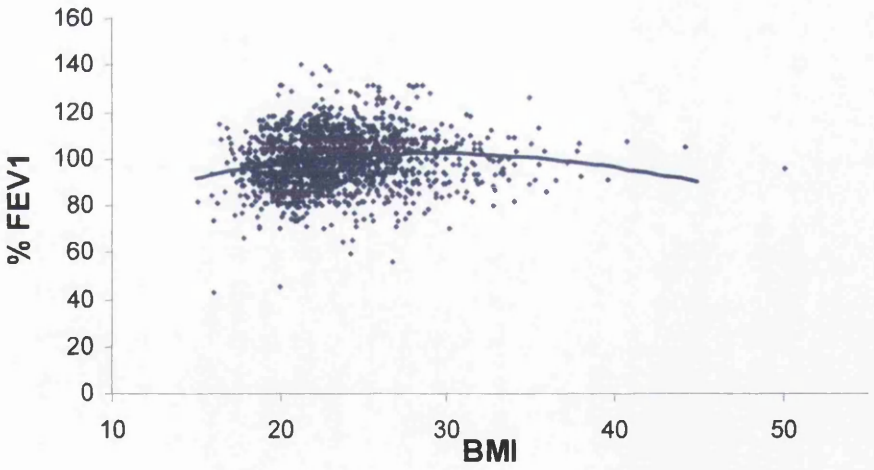


Figure 7.9 The association between % FEV1 and BMI. A quadratic relationship is seen, with % FEV1 increasing across normal anthropometric ranges of BMI, with an inverse association seen as BMI approaches obese levels. An almost identical association is seen as for FVC in Figure 7.8 above: similar quadratic associations were seen for both absolute and age-gender-stature predicted values.

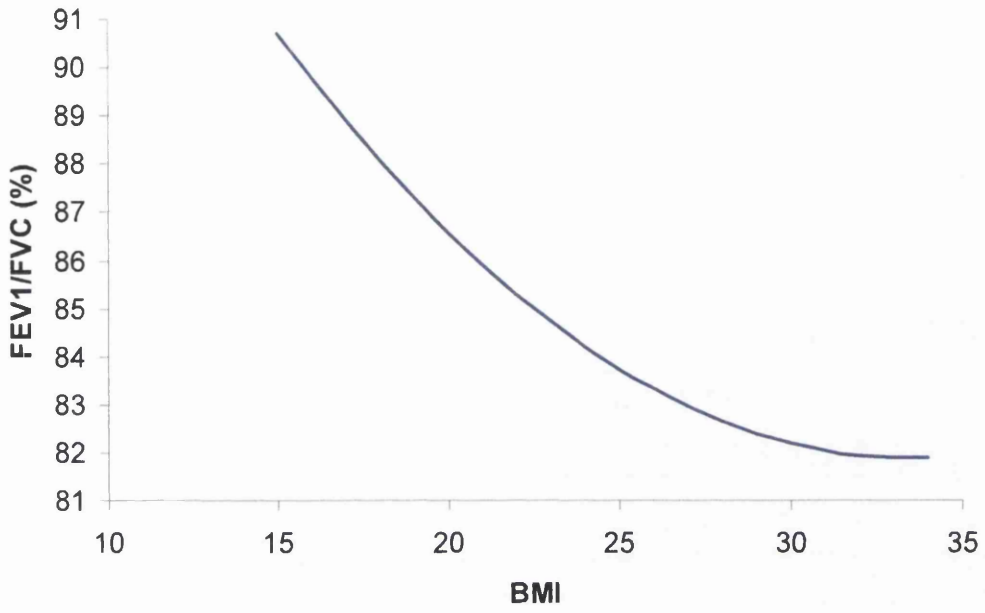


Figure 7.10 Estimated association between FEV1/FVC and BMI. A decline in FEV1/FVC, indicating increased airflow obstruction, is seen with increasing BMI. As for other variables, there is considerable scatter about the curve. Data points are omitted here because they obscure the inflexion point at around BMI=30.

Waist circumference

Waist circumference (WC) was significantly associated with FVC, FEV1, PEF and also FEF 25-75 (unlike BMI) ($p<.001$) (Figures 7.11-7.13). Similarly to BMI, smoking modified the effects of WC on FVC, FEV1 and also showed an interaction with PEF. Sex also modified the effects on PEF such that the relationship was more marked in the males ($p<.05$) (Figure 7.12). Like BMI, WC was inversely associated with FEV1/FVC ratio ($p<.001$) (Figure 7.14). The associations between WC and absolute lung function and FEV1/FVC ratio remained significant after adjusting for BMI but the associations observed for % predicted values for FVC and PEF did not remain significant after adjusting for BMI.

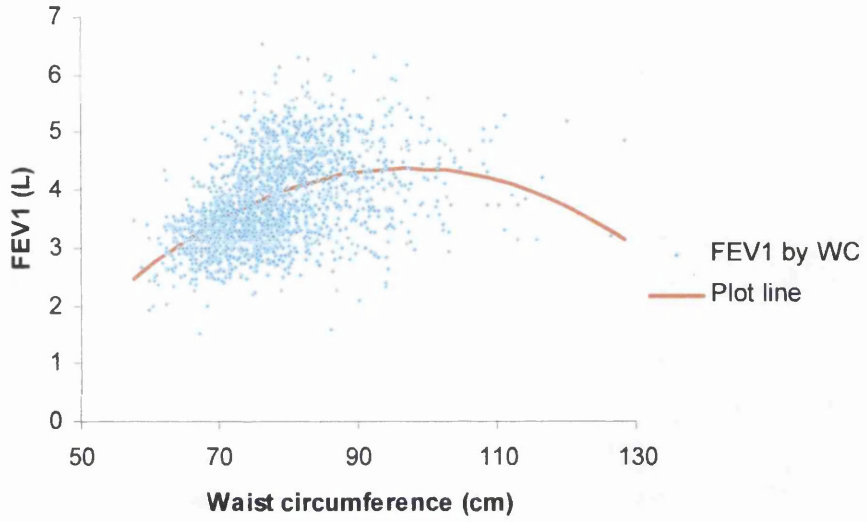


Figure 7.11 The association between FEV1 and waist circumference (WC). A quadratic relationship is seen, with FEV1 increasing across normal anthropometric ranges of WC, with an inverse association seen at higher levels of adiposity.

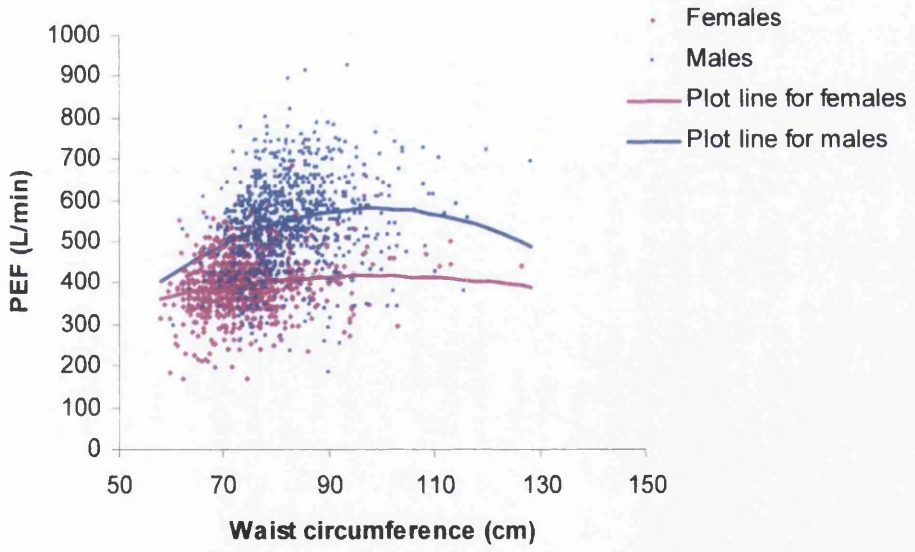


Figure 7.12 The association between PEF and waist circumference (WC), according to gender. A quadratic relationship is seen for both sexes, with PEF increasing across normal anthropometric ranges of WC, with an inverse association seen at higher levels of adiposity. The relationship is more marked in the males.

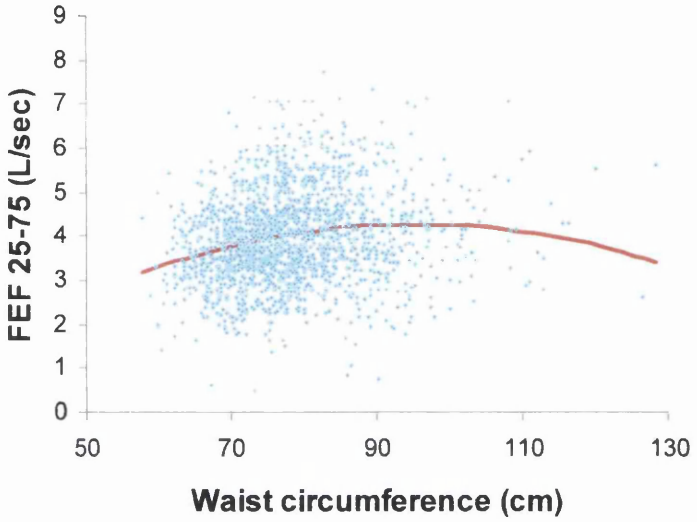


Figure 7.13 The association between FEF 25-75 and waist circumference (WC). A quadratic relationship is seen, with FEF 25-75 increasing across normal anthropometric ranges of WC, with an inverse association seen at higher levels of adiposity.

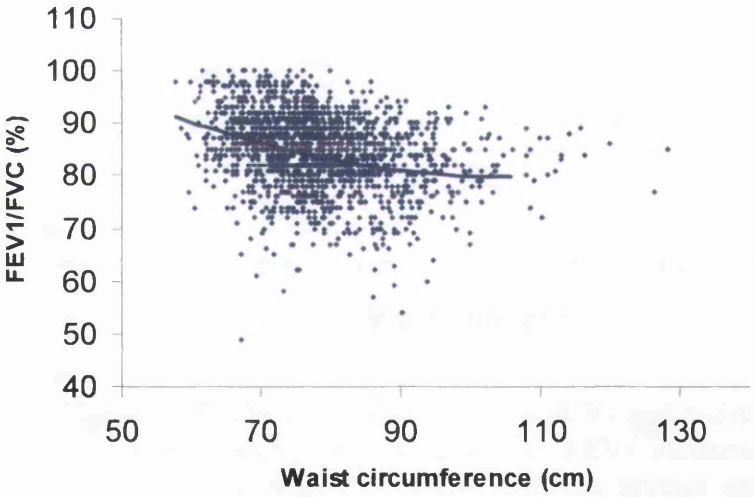


Figure 7.14 The association between FEV1/FVC and waist circumference (WC). A decline in FEV1/FVC, indicating increased airflow obstruction, is seen with increasing WC.

Waist/hip ratio

Waist/hip ratio (WHR) was significantly associated with FVC, FEV1, PEF, FEF 25-75, FEV1/FVC and % predicted FVC (p<.001) (Figure 7.15-7.16). These associations remained significant after adjusting for BMI and body fat, except for the association with % predicted FVC which was not significant after adjusting for BMI . Interestingly WHR was an independent predictor of FVC, FEV1, PEF and FEF 25-75 whereas BMI was not an independent predictor once WHR was adjusted for. An inverse association was again seen for the relationship between WHR and FEV1/FVC ratio (p<.001) (Figure 7.16).

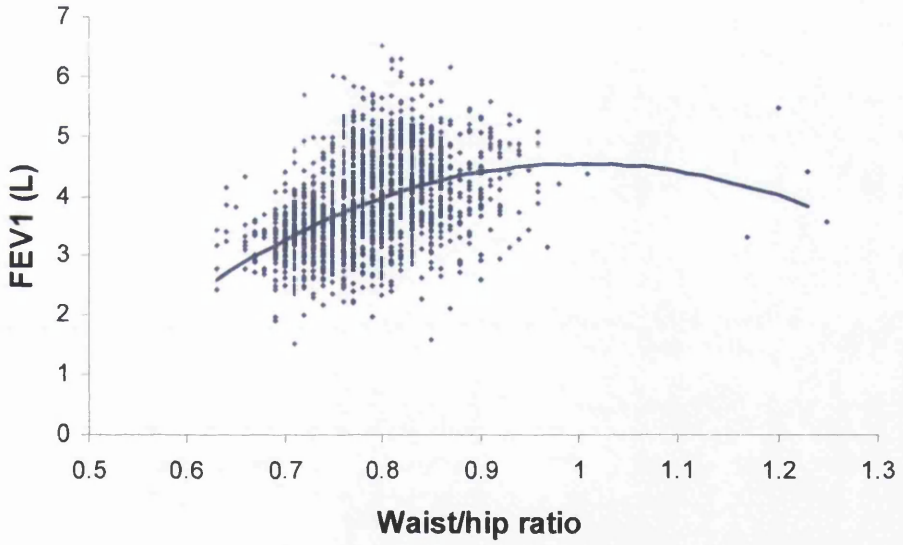


Figure 7.15 The association between FEV1 and waist/hip ratio (WHR). A quadratic relationship is seen, with FEV1 increasing across normal anthropometric ranges of WHR, with an inverse association seen at higher levels of adiposity. Note that the quadratic parameter is not (strongly) determined by the outlying very high WHR individuals.

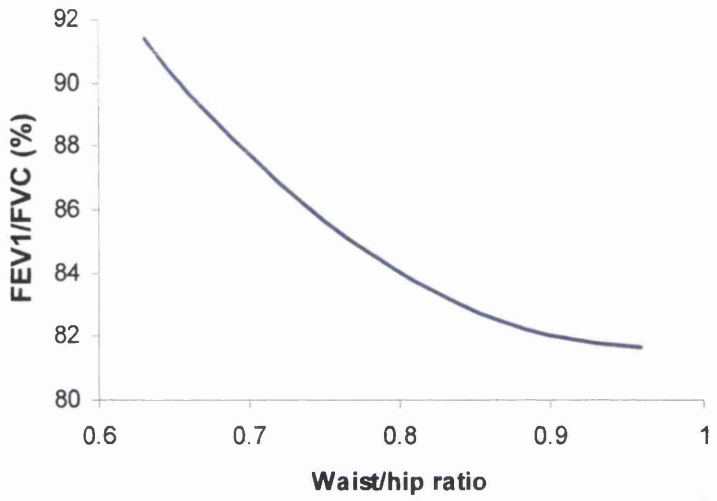


Figure 7.16 Estimated association between FEV1/FVC and waist/hip ratio (WHR). A decline in FEV1/FVC, indicating increased airflow obstruction, is seen with increasing WHR. Data points are omitted here since they obscure the plot line, obtained from linear regression.

Body Fat

Body fat showed significant associations with FVC, FEV1, PEF, FEF 25-75 and % predicted FVC, FEV1 and PEF ($p<.001$ except for % FEV1 where $p<.05$) (Figures 7.17-7.20). Associations remained significant after adjusting for BMI, waist circumference or waist/hip ratio, except for the association with % predicted values which were not significant after adjusting for BMI. There were sex-modifying effects on the associations with absolute lung function, which was most significant for the interaction with FEV1 ($p<.001$). Of interest was the importance of analysing the data for male and female subgroups separately, since both absolute lung function and body fat ranges are different for males and females. Thus when analysing the group as a whole, the associations between body fat and absolute lung function were inverse i.e. lung function declined with increasing body fat across the entire range (Figure 7.17). However, analysis by sex subgroup clarified the association with the same quadratic relationship

demonstrated as with other obesity measures (Figure 7.18). When analysing separate subgroups of males and females, there was an inverse association of body fat with FEV1/FVC, i.e. an increase in airflow obstruction with BF for both sexes ($p<.001$). Exploration of quadratic terms clarified the association, with a linear inverse relationship for females and a quadratic relationship shown for males (Figure 7.21).

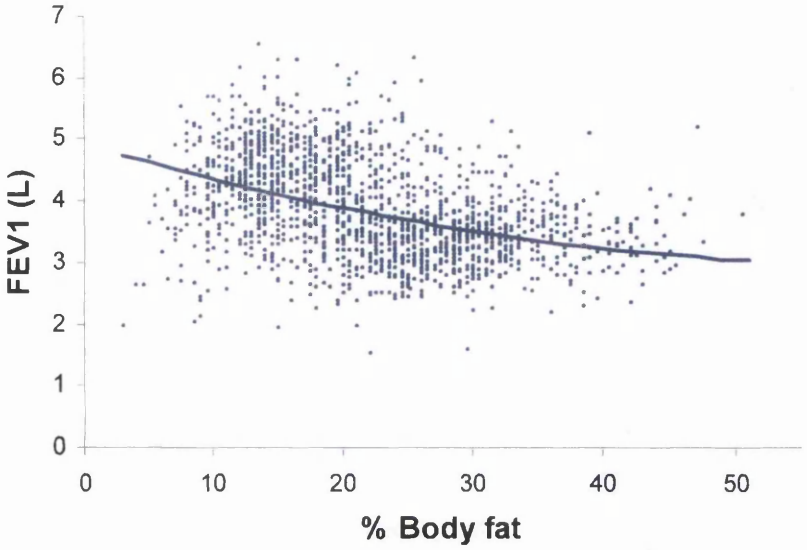


Figure 7.17 The association between FEV1 and body fat (BF). A decline in FEV1 is seen with increasing BF, for the group as a whole.

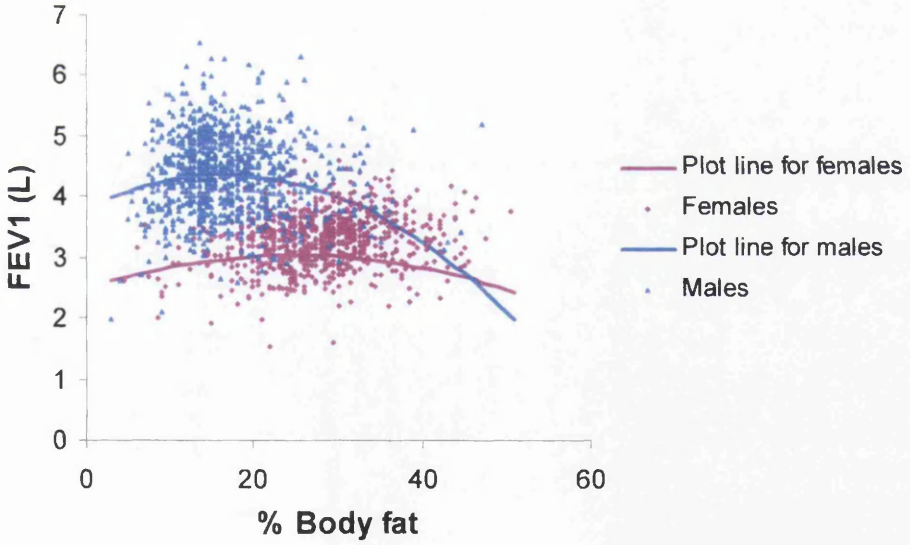


Figure 7.18 The association between FEV1 and body fat (BF), according to gender. A quadratic relationship is seen for both sexes, with FEV1 increasing across normal anthropometric ranges of BF, with an inverse association seen at higher levels of adiposity. The relationship is more marked in the males.

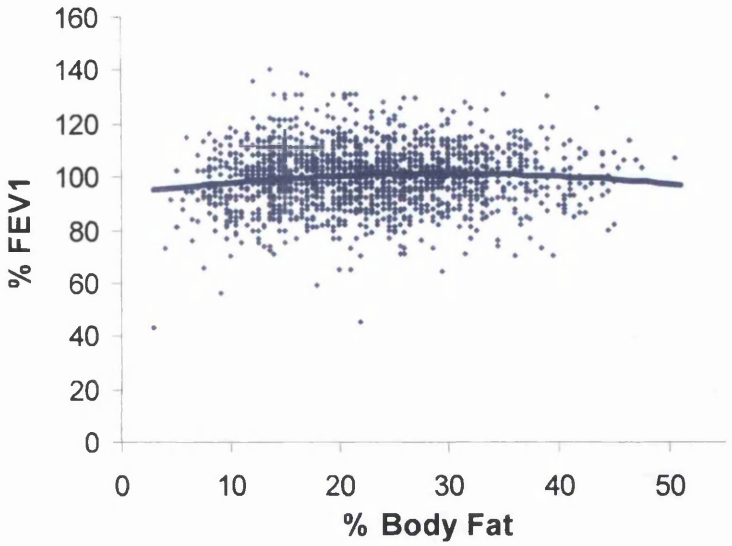


Figure 7.19 The association between % FEV1 and body fat (BF). A weak quadratic relationship is seen, with % FEV1 increasing across normal anthropometric ranges of BF, with an inverse association seen as BF reaches higher levels. Small effects are seen, individual data points are removed in Figure 7.20 to show the association more clearly.

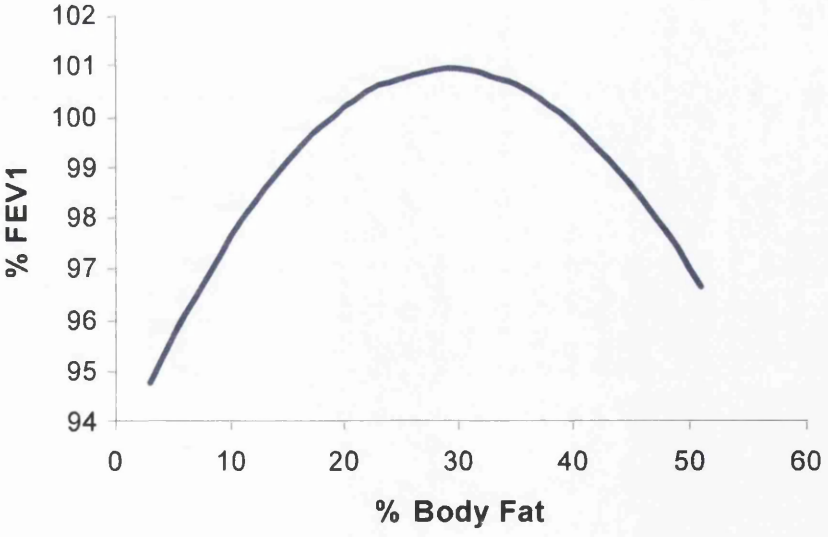


Figure 7.20 Estimate of relationship between % FEV1 and body fat (BF), obtained from regression fit in Figure 7.19. Note the difference in scale presented in the two graphs.

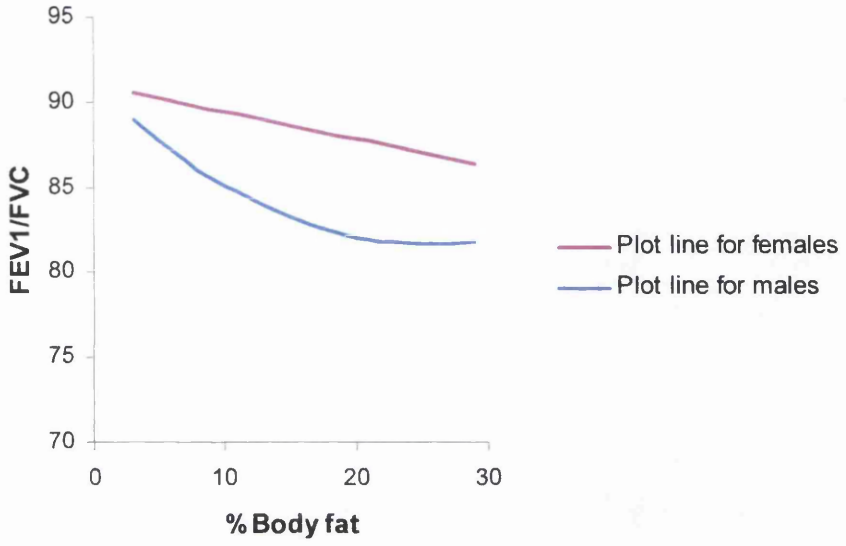


Figure 7.21 Estimated relationship between FEV1/FVC and body fat (BF) according to gender. For females, an inverse linear association is seen. For males, a quadratic relationship is seen. Data points are omitted for reasons of clarity here, since the large number of data points obscures the plot lines.

7.4.5 Linear regression analysis of adiposity and Th2 immune markers in an unselected population

Total IgE

The geometric mean IgE was 49kIU/L (95% CI 40-59). The highest IgE was 18,802kIU/L from a male ex-asthmatic with moderately severe eczema (subsequently referred for investigation of hyper IgE syndrome). Males had significantly higher total IgE ($p<.001$), outlined in detail in Chapter 4.

IgE significantly predicted current asthma, after adjusting for sex (OR, on log scale, 1.51, 95% CI 1.35-1.67, $p<.001$). Thus for a 2.7 fold increase in IgE, there was a 51%

increased risk of having current asthma. For current asthmatics, IgE was positively associated with an attack of asthma in the last 12 months, after adjusting for sex and height (OR, on log scale, 1.53, 95% CI 1.20-1.94, p<.001). Thus a 2.7 fold increase in IgE was associated with a 53% increased risk of having an attack of asthma in the last 12 months. IgE was not associated with other severity indices, including severity score, symptom frequency, % predicted FEV1 and PEF.

BMI

The association between BMI and IgE was non-significant for the group as a whole (p=.09). However there was a significant sex interaction with BMI and IgE (p=.016) such that there was a significant positive association between BMI and IgE in the female subgroup only (Figure 7.22). For the entire range of BMI (15-51), this equates to an increase from IgE 29 to 120kU/L. The positive association for females remained significant after adjusting for current asthma (p=.04).

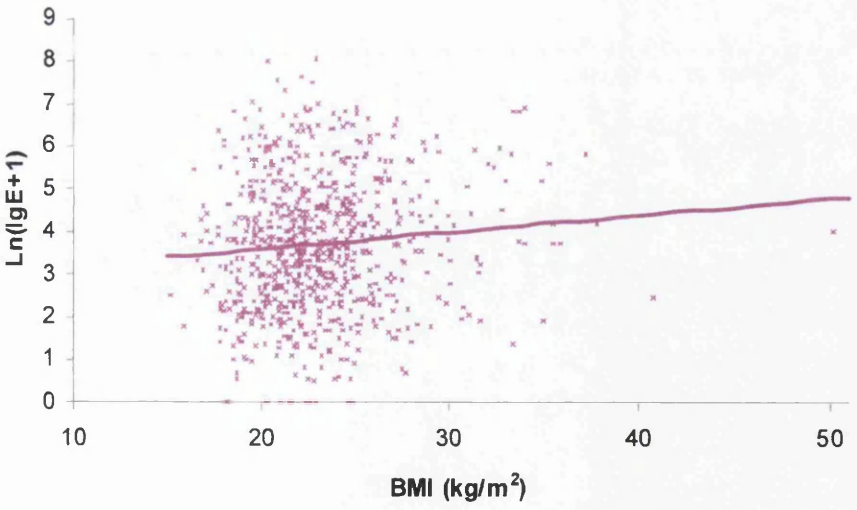


Figure 7.22 The association between total IgE and BMI for the females. A positive association was seen for the females but not the males.

Waist circumference

For the group as a whole, there was an association between waist circumference and IgE, after adjusting for age (p=.012). There was a sex interaction (p=.044) such that there was a positive association for females but no significant association for males, with a small positive effect seen with increasing age (Figure 7.23). The association remained significant after further adjustment for current asthma, in addition to age-adjustment (p=.04).

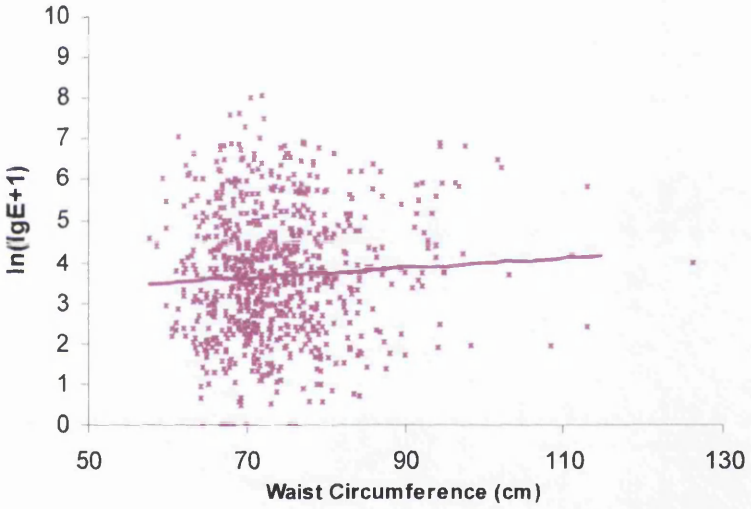


Figure 7.23 The association between total IgE and waist circumference (WC) for the females. A positive association was seen for the females but not the males.

Waist/hip ratio

There was a similar pattern for waist/hip ratio as for waist circumference, with a small positive effect seen with increasing age (p<.001 after adjusting for age). A positive

association was seen in females (Figure 7.24) but the sex interaction did not quite reach significance (p=.053). The association was of borderline significance for females when further adjustment for current asthma in addition to age was applied (p=.052).

When examining the group with current asthma (n=176), the only significant association was between waist/hip ratio and IgE (p=.024).

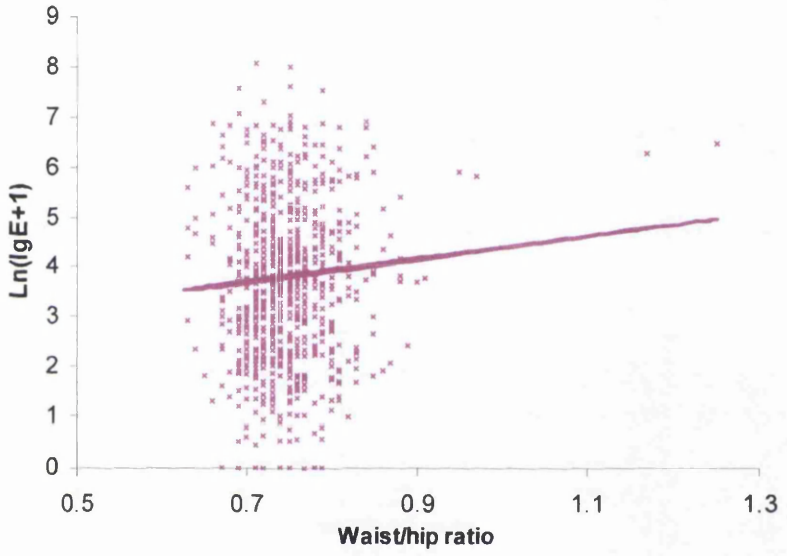


Figure 7.24 The association between between total IgE and and waist/hip ratio (WHR) for the females. A positive association was seen for the females but not the males. Note that the fit obtained from linear regression is not dependent on the outlying very high WHR individuals.

Body fat

There was no relationship between body fat and IgE except for sex being associated with body fat which is expected since normal body fat ranges are different for males and females.

Eotaxin

BMI

BMI showed a significant association with eotaxin, after adjusting for sex, age and number of pack years ($p<.01$). There was a sex-modifying effect such that the association was only significant for males. The relationship was also modified by age and number of pack years such that the relationship was more marked with increasing age and pack years. A quadratic model provided the best fit for the data, with improvement in the fit of the model being significant at the 5% level (likelihood ratio test). Figure 7.25 shows the relationship for males, with a comparison of the different shaped relationship for those aged 30 years versus those aged 18 years. An adjustment for asthma was not undertaken since there was no significant correlation between asthma and eotaxin levels (in contrast to correlations for asthma with IgE and eosinophil count, presented previously in Table 4.1).

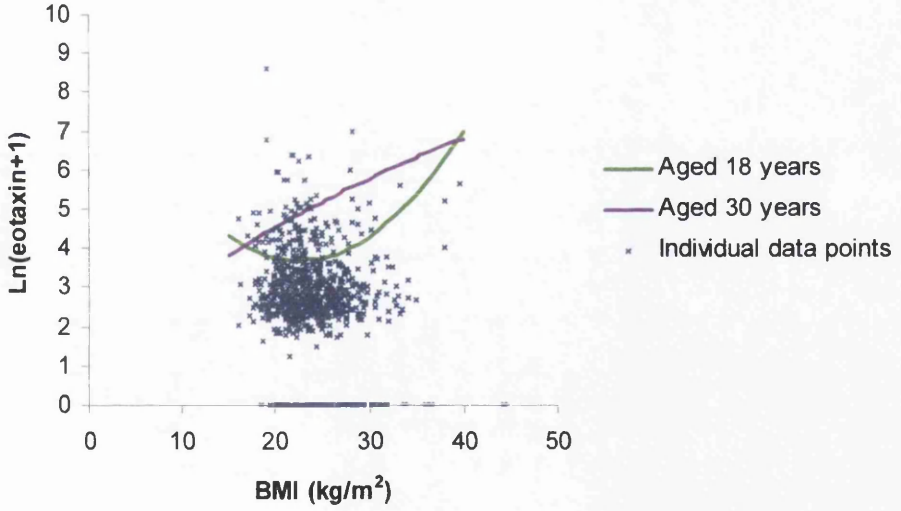


Figure 7.25 The relationship between eotaxin and BMI for males, with plot lines derived from linear regression showing the shape of the relationship at age 30 years in comparison to age 18 years. An essentially U-shaped curve is seen for those aged 18 years, with an increase in eotaxin at both extremes of BMI, whereas the relationship approximates linearity for those aged 30 years. Individual data points are not shown by age since the majority of the population fell between the ages of 18-30 years. No significant associations were seen for females.

Waist circumference

Waist circumference showed a positive linear association with eotaxin, after adjusting for number of pack years ($p<.05$), for both males and females. The relationship was more marked with increasing pack years. However the effects were small: across the range of WC (57.8-128.4cm), geometric mean eotaxin increased from 10.2-17.3 pg/ml. Figure 7.26 shows ln(eotaxin+1) plotted against WC.

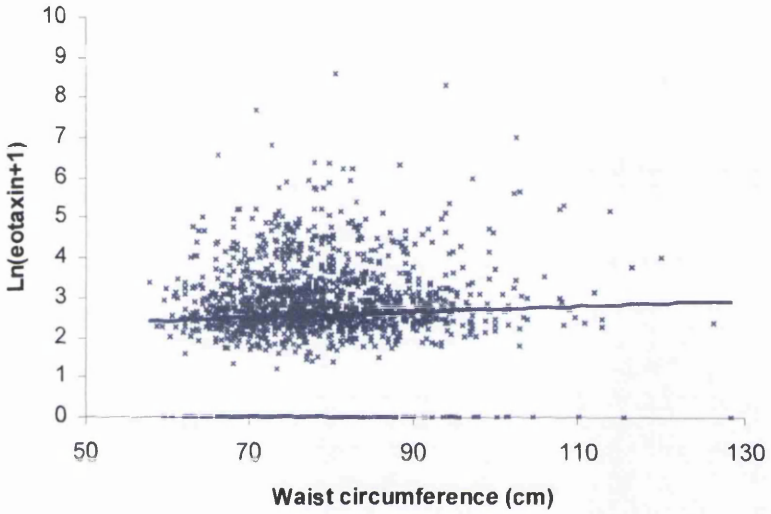


Figure 7.26 The association between between eotaxin and waist circumference (WC). Given the sample size, the undetectable eotaxin levels (counted as zero) did not affect the analysis since ln(eotaxin+1) closely approximated normal distribution. Moreover, since the sample size is large, the regressions are robust to departures from normal assumptions.

Waist/hip ratio

Waist/hip ratio (WHR) showed a similar positive linear association with eotaxin as WC, after adjustment for pack years ($p<.001$), with a more marked relationship with increasing pack years. The effects were larger than for WC: across the range of WHR (0.63-1.25), geometric mean eotaxin increased from 8.7-30.8 pg/ml (Figure 7.27).

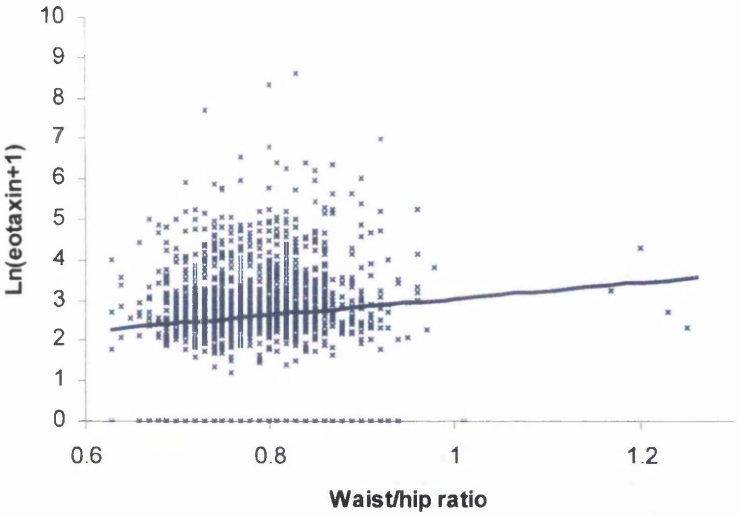


Figure 7.27 The association between between eotaxin and waist/hip ratio (WHR). Given the sample size, the undetectable eotaxin levels (counted as zero) did not affect the analysis since ln(eotaxin+1) closely approximated normal distribution. Moreover, since the sample size is large, the regressions are robust to departures from normal assumptions.

Body fat

Body fat showed a significant association with eotaxin, after adjusting for age and number of pack years ($p<.001$). A quadratic model provided the best fit for the data. There was a sex-modifying effect such that the association was only significant for males. The relationship was also modified by age and number of pack years such that the relationship was more marked with increasing age and pack years (Figure 7.28). For example, when comparing males with a body fat beyond the normal range at 33% with no pack years versus 15 pack years, the geometric mean eotaxin is 14.8 vs 48.3 pg/ml respectively, with greater differences at the extreme upper end of the range.

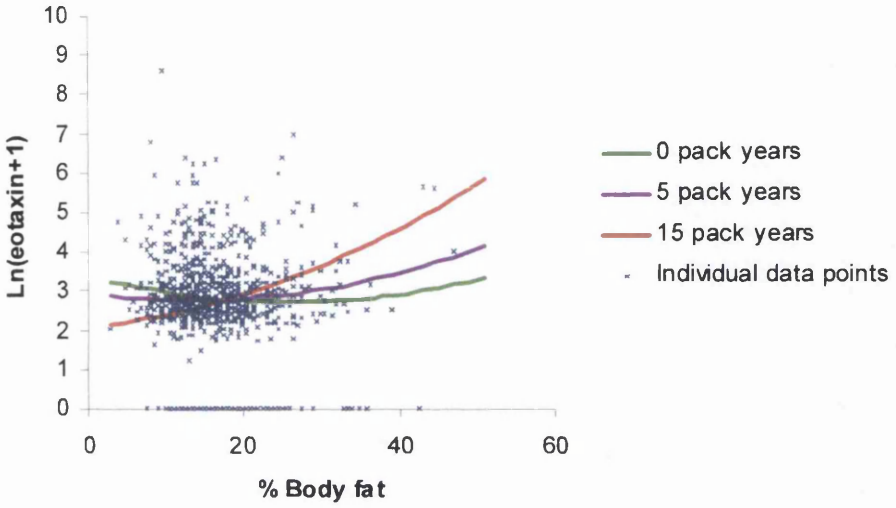


Figure 7.28 The relationship between eotaxin and body fat (BF) for males, with plot lines derived from linear regression. The curves become steeper with increasing smoking history (pack years) indicating a more marked increase in eotaxin with BMI with increased smoking. Associations were not significant for females.

Eosinophil count

Waist circumference showed a significant positive association with eosinophil count ($p<.001$ after adjusting for current smoking) and there was a smoking interaction such that the association was more marked in the current smokers ($p<.05$) (Figure 7.29). Waist/hip ratio showed similar associations ($p<.001$, smoking interaction $p<.05$) (Figure 7.30). The associations for central adiposity measures and eosinophil count remained highly significant after adjustment for current asthma, with adjusted p values of .001 and <.001 for waist circumference and waist/hip ratio respectively. It was noted that smoking pack years predicted eosinophil count such that an increase of 10 pack years equated to a 0.07 increase in eosinophil count ($p<.001$). For BMI, there was a smoking-modifying effect such that a positive association with eosinophil count was only seen in current smokers, although the interaction with current smoking just failed to reach statistical significance ($p=.054$). The associations for smokers remained significant after adjustment for current asthma ($p =.027$). For body fat, the only significant association was between sex and body fat, with no direct relationship with eosinophil count.

A summary of the associations between adiposity indices and immune phenotypes is shown in Figure 7.7.

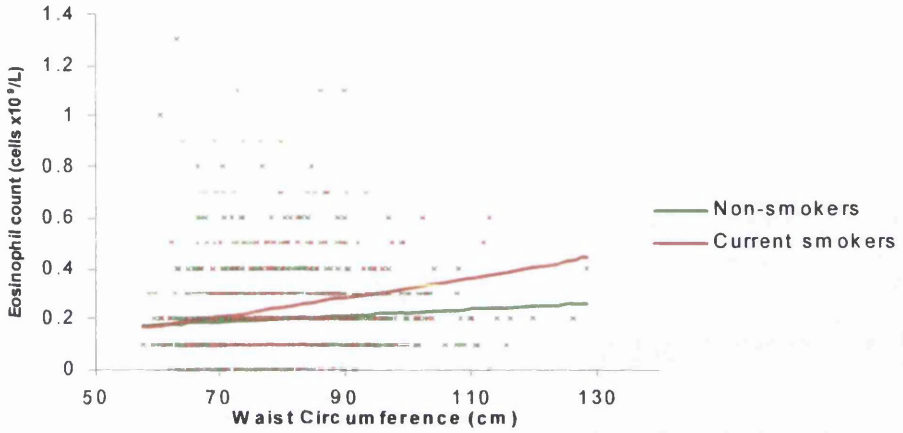


Figure 7.29 The relationship between eosinophil count and waist circumference (WC), with plot lines derived from linear regression. The gradient is steeper for the smokers indicating a more marked increase in eosinophils with WC for smokers.

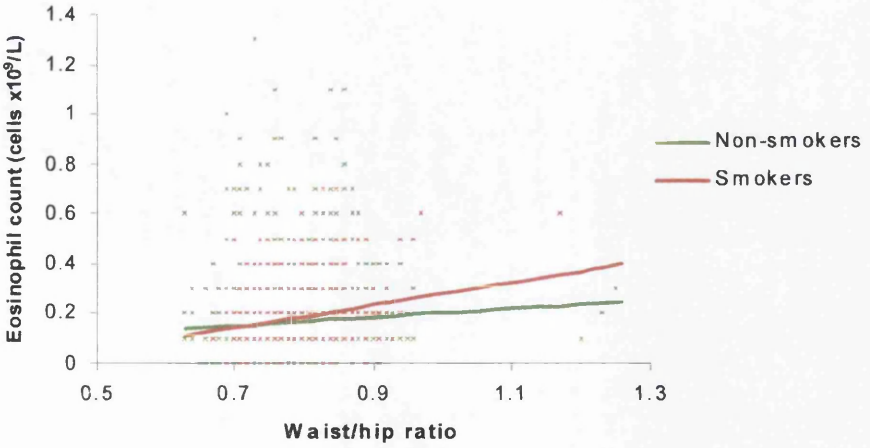


Figure 7.30 The relationship between eosinophil count and waist/hip ratio (WHR), with plot lines derived from linear regression. The gradient is steeper for the smokers indicating a more marked increase in eosinophils with WHR for smokers.

Table 7.6 Summary of associations between adiposity indices and asthma and lung function.

	Asthma[1]	*FVC*	*FEV1*	*PEF*	*FEF 25-75*	*FEV1/FVC*	*% FVC*	*% FEV1*	*% PEF*	*% FEF 25-75*
BMI	Association P <.001 OR 1.08 95% CI 1.04-1.13	Association[2] P <.001	Association P <.001	Association P <.001	No association	Inverse association P<.001	Association P <.001	Association P <.001	Association P <.001	No association
WC	Association P <.01 OR 1.02 95% CI 1.01- 1.04	Association P <.001	Association P <.001	Association P <.001	Association P <.001	Inverse association P<.001	Association P <.001	No association	Association P <.001	No association
WHR	No association	Association P <.001	Association P <.001	Association P <.001	Association P <.001	Inverse association P<.001	Association P <.001	No association	No association	No association
% BF	Association P =.001 OR 1.03 95% CI 1.00-1.05	Association P <.001	Association P <.001	Association P <.001	Association P <.001	Association P <.001 For male and female subgroups	Association P <.001	Association P =.03	Association P <.001	No association

[1] Current physician-diagnosed asthma.

[2] Quadratic relationships were seen: see section 7.4.4.

Table 7.7 Summary of associations between adiposity indices and immune phenotypes.

Adiposity measures	Immune phenotype	P value[1]
BMI, WC, WHR	Total IgE	<.05 for females
BMI, %BF	Eotaxin	<.01 for males
WC, WHR	Eotaxin	<.05 for whole group
WC, WHR	Eosinophil count	<.001 for whole group
BMI	Eosinophil count	<.05 for current smokers

[1] After adjusting for relevant covariates.

7.5 Discussion

General adiposity measures (BMI, BF) were associated with increased odds of asthma. Waist circumference, as a measure of central adiposity, was also associated with increased odds of asthma but WHR was not. Several studies have shown the association between BMI and asthma is exclusively or predominantly seen in females. Our study showed adiposity indices to be associated with increased risk of asthma in both sexes, but with more marked effects in females.

Due to the more marked effects in females, a smaller sample size is likely to result in significant association being seen only in females; this is supported by our analysis of the first 1000 subjects initially, where significant associations were seen only for the female group, with smaller but significant effects seen for males on analysis of the total dataset. This highlights the importance of sample size in such studies. As expected from diseases with complex aetiology, there tends to be large scatter around the average trend lines for individual factors, and adiposity measures are not expected to explain a high percentage of the variance in asthma risk. However, within this large study we were able to demonstrate the significant involvement of adiposity in a range of asthma outcomes.

The age of our population may have had some bearing regarding differential sex effects also since this was a young adult population; sex differences may become apparent in older age groups and this may skew results unless analysis of subgroup according to age is undertaken.

Relatively few studies have examined the association between central adiposity and asthma. Studies in Canadian and Hispanic populations demonstrated an association between waist circumference and asthma, but only in women [302, 304]. Our study demonstrated an association in both sexes, corroborated by a Swedish study [303]. The relationship with WHR has only been examined in one other study, which found no association with asthma, as did our own study [304]. A study of morbidly obese subjects found WHR to be associated with increased odds of asthma [340].

The examination of body fat as a predictor of asthma is relatively novel, having been examined in few other studies. Body fat has the advantage of being the most direct marker of adiposity and significantly predicted current asthma in our study. A recent Dunedin study, which examined a similar age group to our own population, found body fat to associate with asthma and airflow obstruction in females only [341]. However, it is of note that this study used an identical sample size as included in our preliminary analysis (n=1000), with additional effects only apparent in our male population when the total subset was analysed. Body fat was associated with rhinoconjunctivitis but not asthma in a study of 1185 Vietnamese adolescents [342]. Our findings of an association between body fat and asthma further clarifies the role of adiposity in asthma, suggesting that an increase in adipocytes themselves may contribute to asthma – possibly via pro-inflammatory effects - beyond the mechanical effects of increased body mass alone. Ideally, the gold standard of dual-energy X-ray absorptiometry would have been used to measure body fat. However, this was not practical in a field study and for large epidemiological studies, body impedance analysis is regarded as a simple and valid measure of determining body composition [343]. Moreover, differences between fat measurement using these techniques are small [344] and other investigators have used bioelectrical impedance with reproducible results [345, 346].

There are methodological weaknesses to several studies reporting an association between obesity and asthma (e.g. use of self-reported asthma, weight and height) and there is a possibility of bias in that the obese may be more likely to present to the healthcare system and so asthma may be diagnosed more often. Also, it is difficult to assess the extent of publication bias for studies showing a positive association. A strength of our study was that we used exact anthropometric measurements rather than self-reported measurements; the majority of measurements being done by the same operator and the remainder supervised by that same operator (myself), minimising inter-operator variation. Furthermore, the use of the outcome of *current* doctor-diagnosed asthma in addition to "doctor-diagnosed asthma ever", is likely to strengthen our findings. Many studies used only the latter definition which may lack specificity by including uncertain cases diagnosed during childhood (where asthma has now remitted) and of dubious relevance to the relationship between obesity and asthma, in particular when examined in a cross-sectional study in adulthood. A weakness is that we did not confirm the diagnosis of asthma by objective measures (PEF variability, AHR). However only cases of "physician-confirmed asthma" were included which is accepted as a relatively robust measure for epidemiological purposes [234, 235]. Furthermore, intermediate phenotypes were quantified (IgE, eosinophils, eotaxin, lung function).

Our study had the advantage of examining BMI (in addition to other adiposity indices) as a continuous variable rather than simply as weight group categorical variables, which is likely to lead to more informative results. For comparison purposes, odds ratio by weight category was also reported: compared with normal weight, overweight/obesity conferred increased odds of asthma, with an odds ratio of 1.61 (95% CI 1.28-1.94), which is comparable to other studies, but with smaller effect sizes seen than in some [297, 298]. A dose-response effect of elevated BMI on asthma was observed. The effects are especially striking since stricter definitions of physician-diagnosed asthma were used in the study, as outlined above, and also since a narrow age range of young adults were examined, and the effects of obesity may be more marked in older groups. Publication bias is likely to lead to the reporting of striking positive associations in

smaller studies, whereas effects tend to be more modest when studied in larger populations [347]. Since obesity may be a more specific phenomenon to certain subsets - and this was true for females in our study – dilutional effects are likely to be relevant when examining the group as a whole, emphasising the importance of examining effects by gender [328].

Adiposity had few discernible effects on asthma severity in terms of GINA criteria or lung function, on subgroup analysis. This is not surprising since this was an unselected population with few subjects having severe disease, and the majority having mild asthma, as expected in a community sample. However, BMI predicted frequency of symptoms in the previous 3 months and an interesting positive correlation between BMI and daily bronchodilator volume was observed for a subset of male smokers. It may be tentatively hypothesised that BMI may relate to airway hyperresponsiveness (AHR). It was not logistically feasible to measure AHR in this field study, but it is interesting to note that Chinn et al found BMI to associate with AHR only in men [299].

There are inherent difficulties in obtaining a truly random sample representative of the general population, which apply to any epidemiological study. This study may underestimate the effects of obesity on asthma since overweight/obese students may be less likely to volunteer for anthropometric measurements and lung testing. Overweight/obese females in particular may be reluctant for reasons of body image, supported by females being under-represented in the overweight group. However, the prevalence of overweight/obesity overall was comparable to national figures for this age range, at 26% of our population.

With regard to physiological measures, adiposity measures showed a non-linear positive association with lung function over normal ranges and negative association as adiposity measures increased beyond the normal range, suggesting extremes of adiposity to be associated with lower lung function. A similar inverted U-shaped curve was demonstrated from the NHANES-III data (for BMI quintiles) and for children aged >12 years in a recent Mexican study [348, 349]. However previous studies have in general

shown solely an inverse association between BMI and lung function [294]. Our data are consistent with increasing muscle mass leading to improved ventilatory function but as subjects become more adipose, a detrimental effect on lung function is seen.

In addition to BMI, we examined the effects of increasing body fat and central adiposity (WC, WHR) about which there is a relative paucity of data. We also examined the effects on other lung function parameters in addition to FEV1 and FVC, giving further information on expiratory flow rates (PEF, FEF 25-75) and airways obstruction (FEV1/FVC ratio). Additional adiposity measures (BF, WC, WHR) showed similar non-linear association with lung function as BMI. Unlike BMI, this association was also seen for mid-expiratory flow rates (FEF 25-75). Separate studies in children and elderly men have shown body fat to be inversely associated with ventilatory function but have not examined flow rates [345, 350].

In our study, both BMI and central adiposity measures were inversely associated with FEV1/FVC ratio. Inverse effects were also seen for body fat in male and female subgroups. This is interesting since several previous studies have shown adiposity to have no effect on this ratio, with similar reduction in FEV1 and FVC leading to a restrictive pattern of lung function [351-353]. The NHANES-III study found the prevalence of significant airflow obstruction (FEV1/FVC<80%) to be *lower* in the obese, despite an increased prevalence of self-reported asthma [348] and the Normative Ageing Study on 30-79 year olds found a *positive* association between BMI and FEV1/FVC ratio [354]. However data from children and adolescents have shown a tendency for FEV1/FVC to decline with BMI, supporting our findings, and it is possible that an inverse relationship exists in childhood and early adulthood rather than in later adulthood [332, 349].

Decline in FEV1/FVC along with reduced PEF and FEF 25-75 suggest both central and peripheral airways obstruction with increasing adiposity. This is consistent with previous findings of reduced flow rates with increasing BMI [355, 356]. Of interest are the findings of Mansell et al who report an increase of FEV1 with BMI in normal

subjects (comparable with the first part of our quadratic curve), but found opposing patterns in subjects with asthma, with decreased FEF 25-75 with BMI demonstrated [357]. In our study, body fat and central adiposity measures, but not BMI, predicted FEF 25-75 (showing a quadratic association). This suggests that central or visceral adiposity may have a greater effect on small airways function than the effect of weight itself, which is corroborated by a weight loss study which found loss of truncal fat mass to be most strongly related to improvements in FEF 25-75 [358]. A proposed explanation is that obesity may lead to accelerated remodelling of the asthmatic airway with each exacerbation. Supporting evidence comes from the observation that weight loss in obese asthmatics leads to improved lung function but has no effect on airway hyperresponsiveness (AHR) [65], which may be because of increased remodelling, which tends to be irreversible. Other investigators have examined AHR with conflicting results; some studies show no association with BMI [322, 329], and others show an association only for men [299].

Despite a decline in FEV1/FVC ratio with increasing adiposity in our study, it should be noted that clinically relevant reductions in FEV1/FVC ratio (<80%) were only seen at upper extremes of adiposity e.g. WC > 100cm. The data support a relationship between obesity and baseline flow limitation. The well-documented reduction in end-expiratory lung volume in obesity could be a cause of expiratory flow limitation.

The influence of body weight on lung function is complex and is likely to be dependent on a combination of *muscularity* effects (directly proportional to lung function) and *adiposity* effects (inversely associated with lung function). This explains the non-linear relationship demonstrated in our study whereby lung function increases initially with increasing muscle mass but then decreases above a certain threshold of adiposity. Weak inspiratory and expiratory muscles decrease vital capacity [359] whereas stronger muscles tend to improve ventilatory function. The contrasting effects of muscularity and adiposity may well also explain the purely inverse association seen in older age groups (where an increased weight correlates more closely with increased adiposity rather than muscle mass) compared with our population of young adults.

There are some limitations to our study. Firstly, we did not measure pre and post-bronchodilator spirometry or serial peak flow rates and so did not document reversibility to any airways obstruction present. However, given recent evidence that airway remodelling is an early phenomenon in asthma [238], pre-bronchodilator spirometry is likely to provide useful information beyond peak flow variability alone, especially in cases of more fixed airways obstruction associated with remodelling and occurring in a more severe asthma phenotype. Secondly, some of our asthmatic subjects were on bronchodilator and/or inhaled corticosteroid treatment which is likely to improve lung function. Participants on bronchodilators and inhaled corticosteroids represented 10.8% and 3.5% of our population respectively. This potential bias was minimised by asking asthmatics to avoid taking their inhalers prior to spirometry if possible and taking all measurements in the mornings to maximise the effects of diurnal variation in spirometry in asthma, characterised by morning dipping of flow rates. Grading of asthma severity also takes these medications into account. Moreover, this could only lead to a Type 1 error if our asthmatic population were leaner than our non-asthmatic population which was not the case.

Potential underlying mechanisms linking adiposity and asthma are outlined in detail in section 1.3. A body of evidence supports a role for the adipocyte as a pro-inflammatory cell [328]. For instance, microarray data have shown that eotaxin is expressed by adipocytes, with expression upregulated by TNF-alpha [360]. Whilst several studies have examined the association between obesity and asthma, only a minority have examined whether obesity may also associate with atopy, with BMI being almost exclusively the only adiposity measure used, and conflicting findings in different populations. In this study, clinical atopic states (hayfever, eczema) were examined in additional to quantitative Th2 immune markers.

Body fat was found to associate with current eczema. No other associations were demonstrated between adiposity indices and clinical atopy, in particular, no associations for BMI or between adiposity indices and hayfever were demonstrated, consistent with findings from a German study [331] but in contrast to those of a Taiwanese study who

reported an association between BMI and rhinoconjunctivitis in girls [327]. The negative findings described for BMI do not exclude that adiposity may have pro-atopic effects, since BMI is a rather indirect measure of adipose tissue, being influenced to a large extent by body build, and measuring adipose tissue by more direct methods (e.g. body fat, skin fold thickness) may provide the tools to answer questions on the effects of adiposity on allergic inflammation in a more informative way.

In this cross-sectional study of young adults, adiposity measures (BMI, WC) were associated with IgE in females but not in males, suggesting differential pro-inflammatory or endocrine effects by gender. These data are concordant with the finding of higher total IgE in females with higher BMI after puberty [322]. A possible mechanistic basis is provided by Shore and colleagues' murine studies where leptin administration has been shown to increase IgE production, airway hyperresponsiveness and Th2 cytokine production in ovalbumin-sensitized mice [178, 194]. As discussed in Chapter 1, leptin is increased in obesity and is higher in women. Thus females in our study may have higher leptin levels with potential effects on IgE production, although this requires further exploration in functional experiments.

Most studies have examined atopy together with asthma, with no attempt to correct for the effects of asthma. Thus the effects of obesity on atopy, independent of asthma, have tended not be examined. Although asthma and atopy are inextricably linked, we also made adjustments for asthma in our analysis and found that the associations described between adiposity and Th2 markers remained significant after adjusting for asthma. This suggests that adiposity has effects on Th2 inflammatory markers, over and above the effects on asthma alone. Recent evidence suggests that obesity may lead to a pre-allergic state. A study examining the relationship between obesity and serum IgE levels and the appetite-regulating peptides leptin and ghrelin, found that obese children had significantly higher IgE levels and that ghrelin levels inversely correlated with IgE levels [192].

Our study included an exploration of the modifying effects of gender and smoking on

the associations between adiposity indices and markers of Th2 immunity. Novel differential effects were seen, which included the observation of an association between BMI and eosinophil count, which was confined to the subgroup of current smokers, and WC was associated after adjusting for current smoking. The National Health and Nutrition Examination Survey found obesity to be associated with skin test reactivity but not eosinophils but did not look specifically at the smoking subgroup [332]. Elevated levels of eosinophils have previously been observed in a small group of morbidly obese individuals [361].

Eotaxin has not been widely studied in obesity. Vasudevan et al examined 49 obese patients and found higher eotaxin levels, demonstrated in humans and mice, and adipose tissue explants were found to secrete eotaxin [21]. A small Japanese study showed eotaxin levels to correlate with BMI and WHR, in addition to raised eotaxin-2 and monocyte chemoattractant-4 in the obese [362]. In our much larger study, BMI and body fat associated with eotaxin for the males rather than the females, suggesting differential effects by sex according to the specific phenotype examined. Furthermore, smoking was observed to potentiate these effects, in addition to influencing the associations described for eosinophil counts. It can be postulated that smoking enhances the pro-inflammatory effects of adiposity. Murine studies have shown that acute concurrent exposure to allergen and cigarette smoke enhances airway inflammation, with increased eotaxin levels in bronchoalveolar fluid and increased airway responsiveness in sensitized mice [363].

The data suggest that the mechanisms driving the adiposity-asthma association are complex. Differential effects by sex and smoking were seen and it is likely that other environmental factors will also have modifying influences on the underlying mechanisms responsible in different phenotypes. Thus future work should explore the mechanistic basis of the association in different homogenous subsets, e.g. sex, smoking, allergy. Outcomes must be clearly identified, and if examining sex-specific effects, then the age range of subjects is relevant since associations by gender are likely to occur at specific stages of development of the immune and endocrine systems.

This thesis provides an analysis of multiple adiposity phenotypes in relation to asthma, lung function and Th2 immune parameters. A significant association is found between adiposity measures and asthma with adverse effects demonstrated on lung function, including increased airflow obstruction with obesity. The positive associations between adiposity measures with IgE, eosinophils and eotaxin suggest that the association between obesity and asthma may in part be mediated via an upregulation of Th2 immunity, with modifying effects by sex and smoking status.

Since this thesis only examined Th2 immune markers, it may be that obesity has a non-specific pro-inflammatory effect, and that Th1 markers may also be raised in certain subsets. However, the positive associations seen between adiposity and all three Th2 immune markers would suggest that Th2 upregulation is certainly likely to have a contributory role. Analysis of other pro-inflammatory molecules, including Th1 subsets, warrants further examination.

Since this was a cross-sectional study, a causal relationship between obesity and asthma cannot be inferred. However, several prospective studies have shown that an initial raised BMI is associated with an increased odds of developing asthma in the future [203, 297, 308-313]. The increased risk of asthma in the obese has significant public health implications since the prevalence of obesity has risen dramatically and is likely to continue to rise. Obesity has a particularly high prevalence in socially deprived areas where individuals already have an increased risk of asthma [364]. As many as 70% of severe asthmatics are obese [365] and findings from this thesis are likely to be especially pertinent to this severe refractory asthma phenotype which is associated with female gender, obesity and smoking and which poses the greatest challenge in terms of asthma management and resources [366]. Quantifying the impact of obesity on asthma will be crucial in guiding public health strategies and emphasising weight loss as a central part of asthma prevention and treatment. Further work is needed to explore the mechanistic basis of the relationship between obesity and asthma which may identify novel targets for treatment of this susceptible population.

CHAPTER 8

Conclusions

8.1 CONCLUSIONS (I)

8.1.1 Effects of genetic variants of IL-13 signalling molecules on asthma, Th2 immunity and physiological phenotypes

This thesis describes the largest population study to date to examine how common variants of IL-13 signalling genes relate to asthma and atopy. The primary objective was to test genetic variants of Th2 immune signalling at the population level, to clarify the strength of these effects and examine the interactions between different loci. Common variants, encompassing the span of the *IL13*, *IL4RA* and *STAT6* loci, were used and associations with a range of phenotypes (including the validated phenotype of physician diagnosed asthma) were tested.

Use of a large population of young volunteers, representative of the general population from the same ethnic group and geographical background, allowed the estimation of the true population risks conferred by specific genetic variants. However, the population cannot be said to be wholly 'unselected' since there was an element of self-selection inherent in the recruitment process. Since the population is predominantly formed of university students, it cannot be said to be socioeconomically representative of the general population as a whole. However, the assembled population of 1614 young adult volunteers did prove to be representative of a general population of adults of this age group, in terms of asthma, overweight and obesity prevalence.

Genetic effects were clearly seen in our population, with effects evident across a range of continuous variable clinical, immune and physiological phenotypes. Given the polygenic nature of asthma and atopy with gene-environment interactions, it is striking that we see these effects consistently in a population of young volunteers, where only extremes of phenotype will manifest (as actual disease) clinically in what we would label "asthma". Recent reviews of genetic association studies have emphasized the importance of large sample size both to confirm previous findings and to uncover novel associations [242, 243, 367].

Asthma is a heterogenous disorder and is therefore difficult to define for research purposes. For this reason, the genetic basis of complex diseases such as asthma has proved difficult to unravel. The clinical diagnosis of asthma is based on a combination of clinical features (wheeze, breathlessness, cough) and evidence of lability/reversibility (variability in peak flow rate, bronchodilator reversibility). When there is diagnostic doubt, methacholine challenge testing may be employed. In this thesis, asthma was defined as 'physician confirmed asthma' using a validated bronchial symptoms questionnaire. This thesis describes a genetic epidemiology study, and the phenotype of 'physician diagnosed asthma' has been validated and accepted as a robust tool for epidemiological purposes. Ideally, a quantifiable asthma phenotype such as AHR (defined by challenge testing) would have been used in addition. This was not feasible in this initial field study but would be incorporated in future work. Whilst it is likely that AHR would provide useful additional information and allow narrowing of the asthma phenotype, it is an intermediate phenotype and does not provide sufficient diagnosis of asthma when examined in isolation. Another potentially useful measure would have been bronchodilator reversibility of FEV1, although many volunteers with asthma had a normal FEV1 in their stable state, and FEV1 is also influenced by asthma medication, limiting the usefulness in this context.

Another limitation of the study is that although total IgE provided an intermediate phenotype for atopy, no direct quantifiable measures of atopy were used. 'Atopy' describes a tendency to mount specific IgE responses to allergens and sensitisation is defined by positive skin prick tests (SPT) or specific IgE directed against common allergens. Early sensitization is a major risk factor for the development of asthma in genetically susceptible individuals and asthma is associated with atopy in around 80% of childhood asthma and up to 50% of adult asthma. Measurement of SPT or specific IgEs should have been included in the study but given the large sample size was omitted for logistic reasons and limited resources.

A striking distribution of phenotype associations was evident across the three genes

examined. Whereas *IL13* variants associated with asthma and lung function variables, it was variants of *IL4RA* and *STAT6* that predicted higher IgE levels. This is consistent with the lack of association between *IL13* variants and IgE levels demonstrated in previous case-control work [117] but contrasts with recent findings in British and German unselected populations [12, 132]. Our findings are consistent with a large body of functional work which implicates IL-4 (interacting with IL-4RA) in the initiation of Th2 immune responses to allergenic peptides leading to IgE production, whereas IL-13 mediates the main physiological consequences of disease (AHR, mucus hypersecretion, and subepithelial fibrosis leading to airway remodelling) [9]. Figure 8.1 illustrates the various functional roles of IL-13 in the pathophysiology of asthma, with effects on residential effector cells in addition to circulating immune cells.

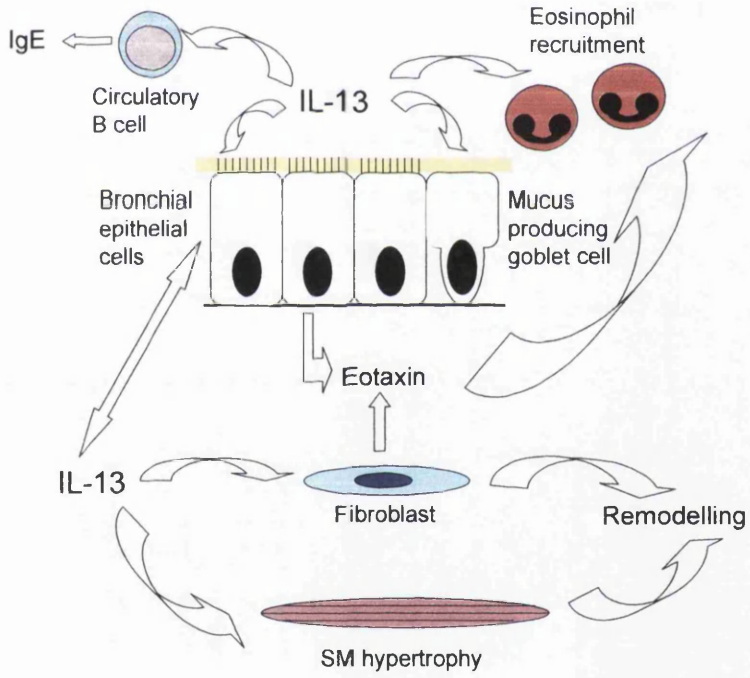


Figure 8.1 A diagrammatic illustration of the complex interplay between IL-13 and residential effector cells and circulating immune cells in asthma. IL-13 acts on B cells in the circulation to cause isotype switching to IgE production. IL-13 recruits eosinophils from the circulation into the airway. The cytokine acts on goblet cells to increase mucus production and causes bronchial epithelial cells and fibroblasts to produce eotaxin, a chemokine which in turn acts as a chemoattractant for further eosinophil recruitment. IL-13 acts on fibroblasts and smooth muscle (SM) within the mesenchyme leading to airway remodelling, with resultant disease chronicity.

The differential genetic associations with specific outcomes emphasise the importance of a well-defined phenotype. This highlights asthma as a heterogenous composite of different phenotypes rather than a single disease entity and illustrates the importance of teasing out the individual phenotypes within this group. This has been exemplified by work on the *ADAM33* susceptibility locus where examination of a phenotype of asthma and airways hyperresponsiveness markedly increased the linkage signal in comparison to the much weaker association with IgE levels [26].

This thesis demonstrated for the first time that the Arg110Gln variant of IL-13 may be a predictor of asthma at the population level. This is of relevance since case-control studies have shown inconsistent results, with some but not all demonstrating this association. *IL13* promoter variants were shown for the first time to predict %FEV1 and asthma severity. Genotype associations with lung function have not been widely studied, especially in unselected populations, and variants across the 3 loci were associated with airflow obstruction, the most marked findings being for *IL13* and *IL4RA* variants. This is a striking and novel finding, since discernible genetic effects on lung function were evident in an healthy young adult population. *IL13* promoter associations with airflow obstruction were also demonstrated in the group with asthma. This is consistent with a role for IL-13 in airway remodelling leading to chronicity of disease, and IL-13 has been shown to induce of a complex array of genes in resident airway cells (smooth muscle cells, fibroblasts, epithelial cells and monocyte/macrophages) independently of traditional effector cells such as mast cells and eosinophils [9].

Another coding variant of interest, Ile50Val of *IL4RA*, was also shown for the first time to predict asthma at a population level. Other findings of note for *IL4RA* and *STAT6* were the strong associations seen with total IgE levels, confirming the role of Arg551Gln in IgE regulation, observed in another unselected population [132]. Other associations for IL4RA variants with immune phenotypes including that of Ile50Val and Ser478Pro with eosinophil counts, which showed significant interaction effects with each high risk genotype potentiating the effects of the other.

The main *STAT6* SNP of interest proved to be the G2964A 3'UTR variant, which showed the strongest associations with asthma, with consistent associations seen for both clinical atopy phenotypes (hayfever, eczema). These data are concordant with emerging evidence of the potential functional importance of non-coding regions. There is evidence that the 3'UTR affects gene expression by influencing translation, coding capacity, mRNA stability and localisation of RNA in the cytoplasm [272]. This region affects protein binding but may also exert its effects in its binding with short RNA [368]. Non-coding regions may play an important role in mRNA expression or mRNA stability. Alternatively, the G2964A variant may be in LD with as yet unidentified but functional variants in regulatory or coding regions, or variants of the immediately adjacent genes.

Epistatic interactions were observed for *IL13* and *STAT6* polymorphisms which in combination predicted higher IgE levels than for each genotype alone. The association of each individual high risk allele with IgE was dependent on the coexistence of the high risk allele for the other gene. The same was true for eosinophil counts for a combination of two *IL4RA* coding genotypes (which were not in strong LD): Ile50Val and Ser478Pro, where each high risk genotype potentiated the effects of the other.

For asthma, whilst the interaction between Arg110Gln and Ile50Val was significant only at the 10% level, interesting trends were observed suggesting that the asthma risk attributable to one locus may be dependent on the genotype held in combination at a distant locus. This may provide an explanation for the genetic heterogeneity of atopic disease, even within one locus in different ethnic groups, which is suggestive that interaction effects are important. Epistatic effects may also explain why heterozygotes for certain variants, rather than having an intermediate phenotype, have the highest risk phenotype, which is discussed in Chapter 6.

Haplotype analysis revealed stronger associations when alleles were examined in combination. Although no significant associations were observed for single *IL13*

variants with total IgE, highly significant associations were demonstrated for a risk haplotype containing the promoter and coding *IL13* risk variants. Haplotype analysis indicated that *IL4RA* and *STAT6* haplotypes played an important role in IgE regulation and supported genetic associations for *IL4RA* Q551R and *STAT6* C2892T identified on individual SNP analysis.

Caution must be exercised in the interpretation of these findings, as emphasised in the General Methodology chapter. Correction for multiple testing was not applied since a candidate gene approach was used, and based on previous genetic and functional studies we would expect to find at least some true positive associations. In this context, and in particular for a large number of variables tested that are clearly not independent, the use of methods such as the Bonferroni correction would result in a large loss of power and inability to reject a global null hypothesis thus resulting in an unacceptable number of false negatives. It should be emphasised that the findings must therefore be interpreted with caution and accepting there are likely to be some false positives. The findings which can be regarded with most confidence are those where there are consistent results and concordance with previous genetic association or functional studies. Clearly the findings require replication in another population. Then further clarification of the biological role of these polymorphisms is required by functional studies to complement this epidemiological work.

This thesis did not examine all components of the IL-13 signalling pathway, but focused on IL-13, IL-4RA and STAT6. Important omissions were IL-4, which is currently being genotyped, and IL-13RA (1 and 2) which we will examine in future work. IL-13RA2 is likely to act as a decoy receptor and work emerging since completion of this thesis suggests the presence of an important feedback mechanism between IL-13 and IL-13RA2. The Arg110Gln variant has been shown to lead to increased eotaxin production by human lung fibroblasts [369], but only from cells expressing low levels of IL-13RA2. This suggests that there may be important interactions with implications for asthma susceptibility here and it would be interesting to examine epistatic interactions initially in this context. Findings from this thesis, and across ethnically diverse

populations, suggest that genetic variation at the *IL13*, *IL4RA* and *STAT6* loci is implicated in asthma and atopy susceptibility. The data suggest that asthma is both a genetically heterogenous and polygenic disease, and individual variants may only cause disease when in combination with other variants, or in gene-environment interactions. This is supported by functional studies which have shown significant but modest differences in activity between variants and wild-type counterparts. IL-13 signalling polymorphisms and haplotype combinations are likely to contribute significantly to asthma and atopy phenotypes, including the regulation of total IgE levels. This thesis supports the approach of studying the effect of combinations of multiple alleles, in haplotypes or in combinations of haplotypes, on complex phenotypes within a biological pathway. The potential clinical utility of these findings includes progress toward the development of diagnostic/predictive genetic testing for asthma in childhood and the development of new asthma therapies targeting specific ligands and receptors in the IL-13 signalling pathway.

8.1.2 Future directions

Storage of DNA, serum and plasma from this study provides an opportunity to use this biobank to explore further genotypes and phenotypes relating to Th2 immunity. The biobank can also be used as a resource to confirm findings of other asthma/atopy studies in a large well-phenotyped unselected population.

If designing this study again, and with no resource limitation, I would include the following measures: challenge testing as a measure of AHR; bronchodilator reversibility; SPT and specific IgE to common allergens as a direct measure of atopy; extended genotyping of the IL-4/IL-13 signalling pathway, to include in particular, variants of IL-4, IL-13RA1 and IL-13RA2; and advanced statistical methods (with supercomputer analysis) of all possible interactions.

Further genotyping is planned:

1. At the *IL13*, *IL4RA* and *STAT6* loci, to examine further variants of interest, including *STAT6* GT dinucleotide repeats (in particular the GT repeat in the first exon of *STAT6*, shown to be associated with elevated eosinophil levels among Caucasians and with allergic phenotypes and childhood asthma in Japanese case-control studies [126, 127, 130, 131] in addition to examining new non-synonymous *STAT6* SNPs.
2. At related loci, such as *IL4*, which is part of the same biological pathway, signalling via the IL-4R alpha chain. Another important locus is IL-13RA2 as outlined previously. Further analysis of epistatic interactions and haplotype associations will be undertaken.
3. At distant loci, such as the eotaxin genes, *ADAM33*. IL-13 signalling, whilst central to bronchial inflammation, is clearly not the only biological pathway relevant in asthma. Variation in other independent pathways is important to asthma pathogenesis e.g. ADAM33 has been shown to have a key role in remodelling, and the phenotype of AHR. Phenotypes can be considered in relation to other loci which shape disease.

Functional studies are needed to unravel the underlying molecular mechanisms and clarify which of the variants confer the greater functional effects. Much of the evidence-base for the role of the IL-13 signalling pathway in asthma comes from murine models rather than studies in humans. Problems with murine models include the absence of naturally occurring asthma-like syndromes in mice and important anatomical differences between the mouse and human lung such as the absence of smooth muscle bundles in most mouse airways [370]. Studies on human cells (including leukocytes, fibroblasts, bronchial epithelial cells) are now needed to better understand the effects of specific genetic variants on human airways and systemically. Immunological studies have been initiated in order to explore and clarify whether the variants found to associate with asthma/atopy phenotypes at the population level have a biological role, or act merely as markers for unidentified variants in linkage disequilibrium with the examined polymorphisms. Initial functional studies will focus on the C2892T intron variant of *STAT6* (rs324011, SNP 8) located in a transcription factor binding site and found to have effects on IgE regulation in single polymorphism and haplotype analysis. Selection by genotype will allow functional comparison of four groups: C/C and T/T

homozygotes, having high/low IgE levels (90^{th} centile). Th2-trophic activity will be examined by measuring outputs such as upregulation of the low affinity IgE receptor (CD23) by flow cytometry and phosphorylation of STAT6 (ELISA based method measuring total and phospho-specific STAT6), using B cells and monocytes stimulated by IL-4/IL-13. For further studies, selection by genotype will allow examination of genetic associations with other Th2 immune phenotypes. Currently, the population is being phenotyped for serum IL-13 levels, and measurement of soluble IL-4RA is planned, thus direct associations between genetic variants and expressed proteins can be examined. Other phenotypes of interest include specific IgE sensitisation to common allergens, since there is evidence that different genetic influences are responsible for the inheritance of basal and allergen specific IgE regulation [138].

Individual genetic variants have modest functional effects in asthma. This thesis suggests that it is the combination of modest effects at a number of loci that confer susceptibility to disease. The very large amount of genetic information offers an opportunity for more detailed statistical analysis based on haplotype analysis and multivariate techniques. Where haplotypes need to be reconstructed and where a huge number of potential genetic interactions are possible, considerable computing power is required to fit models to large data sets, and for testing large numbers of potential models. To extend our work further, we propose to address the exhaustive testing of all possible interaction terms (a combinatorially large process if more than 2-way interactions are also considered), discussed in Chapter 6. This advanced analysis was beyond the scope of this thesis and future analysis will be provided by the Institute of Life Sciences' Blue-C supercomputer (maximum speed 2 teraflops), which can be used for visualization of this complex dataset, and novel means of data mining, in combination with available flexible software (the R language). Future work to encompass a combination of functional genomics utilising microarray technology (allowing the simultaneous monitoring of thousands of genetic or expression data points) coupled with sufficiently powerful supercomputer analysis is likely to begin to tease out the complex networks of gene-gene and gene-environment effects in asthma. This will involve a multidisciplinary approach involving collaboration between the

overlapping fields of epidemiology, clinical medicine, genetics & bioinformatics.

The potential clinical utility of identifying the most important genetic predictors of asthma and associated phenotypes are twofold. Firstly, this will advance towards the development of genetic testing for asthma. Since there is no gold standard diagnostic test for asthma, a simple inexpensive test would be especially useful in childhood, to aid diagnosis or even to simply predict which children are at highest risk, thus identifying those in need of asthma surveillance and avoidance of potential precipitants where appropriate. Secondly, unraveling the molecular basis of asthma would allow drug development and targeting of specific ligands and receptors involved in Th2 signalling, leading to novel asthma therapies. Preliminary studies of antagonists targeting this pathway have shown promising results in animal models [371, 372] and it has recently been shown that inhaled IL-4RA antisense oligonucleotide has anti-inflammatory activity in mice, suggesting the potential utility of a dual IL-4 and IL-13 oligonucleotide inhibitor in targeting asthma and allergy [373].

This biobank provides a well-phenotyped population with stored biological data which may be used as a resource, both in terms of replicating findings from association studies in other population and in investigating asthma pathogenesis in more diverse ways. We plan a collaboration with investigators of the Isle of White cohort (Arshad Hassan, Wilfried Karmaus, Marianne Huebner, Susan Ewart) which will involve investigating whether our findings are replicated in another population, and also using the PAPA population for replication work. Regarding use of the biobank as a resource, future collaborations of interest include using this population to undertake replication studies of genes involved in GPCR signalling pathways identified by SNP based association approaches in two other cohorts (Professor Ian Hall, University Hospital of Nottingham). A current collaboration relates to the investigation of the role of CMV infection in the development of atopy (Professor Paul Moss, University of Birmingham).

8.2 CONCLUSIONS (II)

8.2.1 Effects of adiposity on asthma, Th2 immunity and physiological phenotypes

The parallel rise in prevalence of the complex traits of obesity and asthma over recent decades has led to speculation on a possible causal role of obesity in asthma pathogenesis. Prospective studies have tended to confirm that there is an association between BMI earlier in life and the development of later asthma, with varying differential effects by sex. Obesity, in common with asthma, is a complex trait with multiple phenotypes. For example, central adiposity associates with insulin resistance whereas centrifugal or gluteal adiposity does not. Investigators have focused on the effects of obesity (BMI>30) on asthma but alternative adiposity phenotypes, such as central adiposity and body fat, have not been widely examined. This population study provided an opportunity to test the relationship between different measures of obesity and the state of asthma, in addition to atopy and putatively Th2 signalling. This thesis provides the first investigation of how BMI, central adiposity and body fat relate to asthma and associated phenotypes.

This thesis confirmed the association between BMI and asthma in a cross-sectional population of young adults. In contrast to some studies, showing an effect only in females, effects were seen for both sexes, although effects tended to be stronger for females. Observed effects for both sexes are likely to be due to the large sample size in this study, since when examining the first 1000 volunteers, significant associations were found only for females. Differential effects by sex were seen for specific outcomes such as 'physician diagnosed asthma ever', with the association for BMI seen only in females whereas this was observed in both sexes when considering 'current physician diagnosed asthma'. Similar associations were seen for central adiposity (waist circumference) and body fat.

Interesting effects of adiposity on lung function were observed in this cross-sectional unselected population of young adults. Lung function was observed to *increase* initially

across more normal anthropometric ranges, presumably due to muscularity effects. However, when higher anthropometric ranges indicative of adiposity were reached, lung function declined. A quadratic curve provided the best fit for the data in regression analysis.

Since this was a cross-sectional rather than a prospective study, it is not possible to conclude that there is a causal link between obesity and asthma, merely that there is an association between these two complex traits. To explore the possible mechanistic basis for this association, the association between adiposity and markers of Th2 immunity was examined. This is the first study to examine a comprehensive range of both adiposity and Th2 phenotypes.

Adiposity measures were found to be associated with higher levels of Th2 immune markers, and these effects were significantly modified by both sex and smoking. Differential effects by sex were clearly seen for the association between BMI/central adiposity and total IgE levels, with a positive association for the females but not the males. Conversely, plasma eotaxin showed a positive association with BMI/body fat in the males, associating with central adiposity in both sexes, with a modifying effect by smoking which caused more marked effects. There was a similar smoking-modifying effect for the association between BMI/central adiposity and eosinophil count, with the association between BMI and eosinophil count confined to current smokers. Differential effects by sex and smoking were seen when examining different adiposity phenotypes. This illustrates the importance of exploring separate and well-defined phenotypes for a complex trait such as the obese state.

Since sample size was large, it was possible to detect highly significant associations between adiposity with the phenotypes of asthma, lung function and Th2 immunity, despite some of the effects being relatively small in comparison to some previous epidemiological studies. The reason for relatively small effects in some cases was likely to be the age of our population. A narrow age range of young adults (18-30 years) was examined, in comparison to other studies, where the age range is broader e.g. 18-80

years. The advantage of our study is that using a narrow age range allows us to answer questions relating to adiposity associations in a specific and relatively homogenous young adult population, whereas the examination of broader age ranges is more likely to introduce potential confounders. When analyzing a broad age range of adults, subgroup analysis by age is important, since associations have been found to change with age. Analysis of too heterogenous an age group is likely to lead to masking of effects, for instance, an obstructive defect may be seen with increasing adiposity in the young, but with increasing age, it may be that restrictive effects predominate. We found lung function to increase initially with anthropometric measures due to muscularity effects, until higher levels of adiposity were reached, but it may be hypothesized that the point at which lung function begins to decline becomes lower with age i.e. at lower levels of adiposity than in the young. Larger effect sizes might be expected for the association between adiposity and asthma, if we had also included volunteers over the age of 30 years.

It is difficult to unravel the potential confounding effects of factors such as diet and exercise, which may have effects on both obesity and asthma traits. In order to provide as comprehensive a picture as possible, in addition to the validated phenotype of 'current physician diagnosed asthma', associated/intermediate phenotypes were also quantified including lung function and markers of Th2 immunity (total IgE, eotaxin levels, eosinophil count). In addition to a highly significant association with asthma, adiposity measures also impacted negatively on lung function at higher values. Moreover, positive association were observed between adiposity indices and markers of Th2 immunity, as outlined, with modifying effects by sex and smoking.

The potential mechanistic basis of an association between adiposity and asthma is explored in Chapter 1. One possible explanation is that both complex traits share common biological pathways, and rather than being causally linked, their prevalence may be rising in parallel. The data presented supports the hypothesis that adiposity and asthma may be linked by an upregulation of Th2 immunity, given the positive correlation between adiposity indices and the Th2 immune markers examined. This is

supported by animal models, in particular the work by Shore et al who have demonstrated an *innate* airway hyperresponsiveness in obese (*ob/ob*) mice, in addition to increased Th2 responses on allergen challenge [181]. Moreover, the effects of obesity are not merely mechanical, since experimental murine data has shown that when the mechanical load of obesity is removed (by opening the chest wall and applying artificial ventilation), the effects of adiposity on airways resistance is unchanged [181]. Evidence is mounting for the role of the adipocyte as a pro-inflammatory cell, and leptin, which is elevated in the obese, has been found to act as a cytokine, and has been shown to be associated with higher IgE levels [188, 192].

The biological pathways regulating obesity and asthma may not be mutually exclusive. It is possible that these complex traits have genetic pathways in common, with specific variation promoting both obesity and asthma. The overlap between these two complex traits and potential common pathways linking the two are shown in Figure 8.2. Data described in this thesis suggest that obesity and genetic factors have contributory roles in asthma. Examining common genetic pathways was beyond the scope of this thesis, but will be explored in future work. If obesity and asthma have genetic pathways in common, then care must be taken when including BMI as a covariate in genetic studies of asthma, since it may act a true confounder to any relationships seen. However, in Chapter 3, BMI was only found to act as a confounding variable in one case, so the potential confounding effect of BMI does not seem to be relevant to the genetic pathways studied in this thesis.

In conclusion, adiposity was found to associate with asthma, have a negative impact on lung function over the upper range, and showed a positive association with markers of Th2 immunity. The effect of adiposity on specific immune phenotypes suggests that pro-inflammatory Th2 signalling may provide one potential mechanistic basis for this association. This epidemiological study suggests that obesity contributes to the causation of asthma and associated phenotypes and upregulation of Th2 signalling may provide one common pathway. Much work is needed to address this hypothesis, with replication required in other populations including different age groups, in parallel with

functional work to examine the mechanistic basis for the association between obesity and asthma.

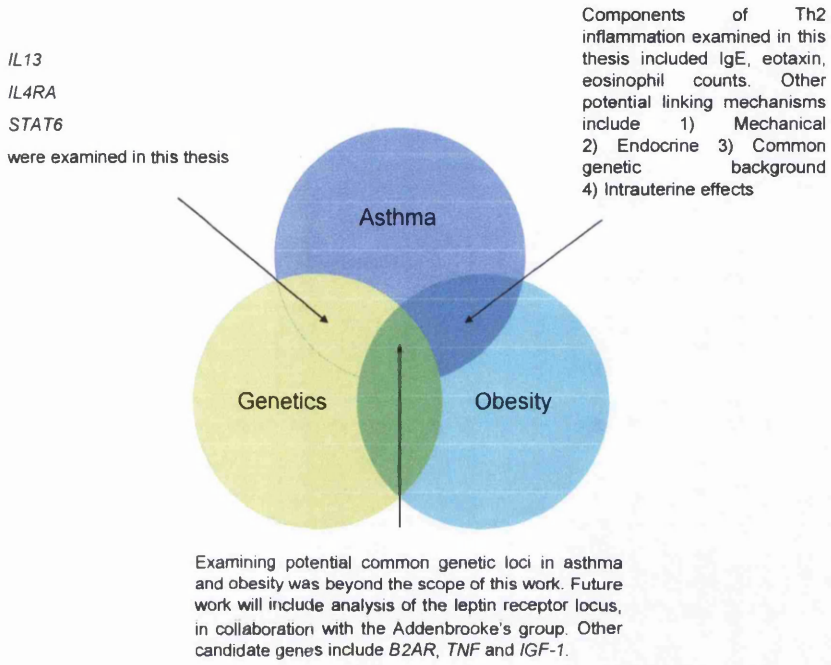


Figure 8.2 A diagrammatic representation of the overlap between the complex traits of asthma and obesity and the potential common pathways linking these conditions. The areas of potential overlap which are addressed in this thesis are highlighted.

8.2.2 Future directions

Exploratory functional studies are needed to investigate the mechanistic basis of the obesity-asthma association and to test whether adiposity *per se* results in upregulation of Th2 pathways. To investigate this, a comparison of leptin levels is being made in 4 subject groups: normal weight/obese with/without asthma. In addition, leptin levels will be compared with levels of Th2 immune markers (total IgE, eotaxin, eosinophil count) and serum IL-13 levels. (in collaboration with Dr Sadaf Farooqi, Professor Steve

O'Rahilly, Addenbrooke's Hospital, University of Cambridge). Further work of similar design will involve the analysis of differential proteome and metabolome profiles in serum/urine in obesity and asthma (Dr Cathy Thornton, Swansea University, Dr Ruta Furmonaviciene, DeMontfort University, Leicester, Dr Egidijus Machtejevas, Johannes Gutenberg University, Mainz, Germany). Further exploration of the role of adiposity in allergic phenotypes will include the measurement of specific IgE levels in our population. Additional work in collaboration with the Cambridge group, will involve further genotyping of our population, to investigate whether obesity and asthma may share common genetic pathways. In particular, the leptin receptor gene will be focused upon.

REFERENCES

1. AsthmaUK, *Where do we stand? Asthma in the UK today.* www.asthma.org. 2004.
2. Whinchup, P., D. Cook, D. Strachan, and O. Papacosta, *Time trends in respiratory symptoms in childhood over a 24 year period.* Arch Dis Child, 1993. **68**: p. 729-34.
3. Magnus, P. and J. Jaakkola, *Secular trend in the occurrence of asthma among children and young adults: critical appraisal of repeat cross-sectional surveys.* BMJ, 1997. **314**: p. 1795-9.
4. Woolcock, A. and J. Peat, *Evidence for the increase in asthma worldwide.* The Rising Trends in Asthma, ed. C. Foundation. Vol. 206. 1997, Chichester: Wiley. 122-4.
5. Ford, E.S., *The epidemiology of obesity and asthma.* Journal of Allergy and Clinical Immunology, 2005. **115**(5): p. 897-909.
6. Larsen, G., *Asthma in children.* New Engl J Med, 1992. **326**(1): p. 540-5.
7. Cookson, W., *The immunogenetics of asthma and eczema: a new focus on the epithelium.* Nat Rev Immunol, 2004. **4**(12): p. 978-988.
8. Lohmueller, K.E., C.L. Pearce, M. Pike, E.S. Lander, and J.N. Hirschhorn, *Meta-analysis of genetic association studies supports a contribution of common variants to the susceptibility to common disease.* Nat Genet, 2003. **33**: p. 177-182.
9. Wills-Karp, M., *Interleukin-13 in asthma pathogenesis.* Immunol Rev, 2004. **202**: p. 175-90.
10. Wills-Karp, M. and S.L. Ewart, *Time to draw breath: asthma-susceptibility genes are identified.* Nat Rev Genet, 2004. **5**(5): p. 376-87.
11. Ober, C. and S. Hoffjan, *Asthma genetics 2006: the long and winding road to gene discovery.* 2006. **7**(2): p. 95-100.
12. Kabesch, M., M. Schedel, D. Carr, B. Woitsch, C. Fritzsch, et al., *IL-4/IL-13 pathway genetics strongly influence serum IgE levels and childhood asthma.* J Allergy Clin Immunol, 2006. **117**(2): p. 269-74.
13. Howard, T.D., G.H. Koppelman, J. Xu, S.L. Zheng, D.S. Postma, et al., *Gene-gene interaction in asthma: IL4RA and IL13 in a Dutch population with asthma.* Am J Hum Genet, 2002. **70**(1): p. 230-6.
14. Risma, K.A., N. Wang, R.P. Andrews, C.M. Cunningham, M.B. Ericksen, et al., *V75R576 IL-4 receptor alpha is associated with allergic asthma and enhanced IL-4 receptor function.* J Immunol, 2002. **169**(3): p. 1604-10.
15. Roth, H., T. Korn, K. Rosenkranz, A. Hinney, A. Ziegler, et al., *Transmission disequilibrium and sequence variants at the leptin receptor gene in extremely obese German children and adolescents.* Hum Genet, 1998. **103**(5): p. 540-6.
16. Jiang, Y., J.B. Wilk, I. Borecki, S. Williamson, A.L. DeStefano, et al., *Common variants in the 5' region of the leptin gene are associated with body mass index in men from the National Heart, Lung, and Blood Institute Family Heart Study.* Am J Hum Genet, 2004. **75**(2): p. 220-30.
17. Weiss, S., *Obesity: insight into the origins of asthma.* Nat Immunol, 2005. **6**(6): p. 537-9.

18. Shore, S.A., *Obesity and asthma: cause for concern.* Curr Opin Pharmacol, 2006. **6**(3): p. 230-6.
19. Otero, M., R. Lago, R. Gomez, C. Dieguez, F. Lago, et al., *Towards a pro-inflammatory and immunomodulatory emerging role of leptin.* Rheumatology, 2006. **45**(8): p. 944-950.
20. Sood, A., E.S. Ford, and C.A. Camargo, Jr, *Association between leptin and asthma in adults.* Thorax, 2006. **61**(4): p. 300-305.
21. Vasudevan, A.R., H. Wu, A.M. Xydakis, P.H. Jones, E.O.B. Smith, et al., *Eotaxin and Obesity.* J Clin Endocrinol Metab, 2006. **91**(1): p. 256-261.
22. Johansson, S.G.O., J.O.B. Hourihane, J. Bousquet, C. Bruijnzeel-Koomen, S. Dreborg, et al., *A revised nomenclature for allergy. An EAACI position statement from the EAACI nomenclature task force.* Allergy: European Journal of Allergy and Clinical Immunology, 2001. **56**(9): p. 813-824.
23. Koppelman, G.H., H. Los, and D.S. Postma, *Genetic and environment in asthma: the answer of twin studies.* Eur Respir J, 1999. **13**(1): p. 2-4.
24. Hoffjan, S., D. Nicolae, and C. Ober, *Association studies for asthma and atopic diseases: a comprehensive review of the literature.* Respir Res, 2003. **4**(1): p. 14.
25. Arima, K., R. Umeshita-Suyama, Y. Sakata, M. Akaiwa, X.Q. Mao, et al., *Upregulation of IL-13 concentration in vivo by the IL13 variant associated with bronchial asthma.* J Allergy Clin Immunol, 2002. **109**(6): p. 980-7.
26. Van Eerdewegh, P., R. Little, J. Dupuis, R. Del Mastro, K. Falls, et al., *Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness.* Nature, 2002. **25**(418): p. 426-30.
27. Zhang, Y., N. Leaves, G. Anderson, C. Ponting, J. Broxholme, et al., *Positional cloning of a quantitative trait locus on chromosome 13q14 that influences immunoglobulin E levels and asthma.* Nat Genet, 2003. **34**: p. 181-86.
28. Allen, M., A. Heinzmann, E. Noguchi, G. Abecasis, J. Broxholme, et al., *Positional cloning of a novel gene influencing asthma from chromosome 2q14.* Nat Genet, 2003. **358**: p. 258-63.
29. Laitinen, T., A. Polvi, P. Rydman, J. Vendelin, V. Pulkkinen, et al., *Characterization of a common susceptibility locus for asthma-related traits.* Science, 2004. **304**: p. 300-304.
30. Nicolae, D., N.J. Cox, L.A. Lester, D. Schneider, Z. Tan, et al., *Fine mapping and positional candidate studies identify HLA-G as an asthma susceptibility gene on chromosome 6p21.* Am J Hum Genet, 2005. **76**(2): p. 349-57.
31. Leung, R., J. Carlin, J. Burdon, and D. Czarny, *Asthma, allergy and atopy in Asian immigrants in Melbourne.* Med J Aust, 1994. **161**(7): p. 418-25.
32. Litonjua, A., V. Carey, H. Burge, S.T. Weiss, and D. Gold, *Parental history and risk of childhood asthma. Does mother confer more risk than father?* Am J Respir Crit Care Med, 1998. **158**(1): p. 176-81.
33. Ohshima, Y., M. Yasutomi, N. Omata, A. Yamada, K. Fujisawa, et al., *Dysregulation of IL-13 production by cord blood CD4(+) T cells is associated with the subsequent development of atopic disease in infants.* Pediatric Research, 2002. **51**(2): p. 195-200.

34. Illi, S., E. von Mutius, S. Lau, R. Nickel, B. Niggermann, et al., *The pattern of atopic sensitization is associated with the development of asthma in childhood.* J Allergy Clin Immunol, 2001. **108**(5): p. 709-14.
35. Ramsdale, E., M. Morris, R. Roberts, and F. Hargreave, *Asymptomatic bronchial hyperresponsiveness in rhinitis.* J Allergy Clin Immunol, 1985. **75**(5): p. 573-77.
36. Sporik, R., S. Holgate, T.A. Platts-Mills, and J. Cogswell, *Exposure to house dust mite allergen (Der P I) and the development of asthma in childhood. A prospective study.* N Engl J Med, 1990. **323**(8): p. 502-7.
37. Gassner, M., *[Imunological-allergological reactions with changed environmental conditions}.* Schweiz Rundsch Med Prac, 1992. **81**(14): p. 426-30.
38. Lau, S., S. Illi, C. Sommerfeld, B. Niggermann, R. Bergmann, et al., *Early exposure to house-dust mite and cat allergens and development of childhood asthma: a cohort study.* Lancet, 2000. **356**: p. 1392-7.
39. Litonjua, A., D. Milton, J.C. Celedon, L. Ryan, S.T. Weiss, et al., *A longitudinal analysis of wheezing in young children: The independent effects oof early life exposure to house dust endotoxin, allergens, and pets.* J Allergy Clin Immunol, 2002. **110**(5): p. 736-742.
40. Remes, S.T., J.A. Castro-Rodriguez, C.J. Holberg, F.D. Martinez, and A.L. Wright, *Dog exposure in infancy decreases the subsequent risk of frequent wheeze but not of atopy.* Journal of Allergy and Clinical Immunology, 2001. **108**(4): p. 509-515.
41. Celedon, J.C., A.A. Litonjua, L. Ryan, T. Platts-Mills, S.T. Weiss, et al., *Exposure to cat allergen, maternal history of asthma, and wheezing in first 5 years of life.* The Lancet, 2002. **360**(9335): p. 781-782.
42. Brunekreef, B., J. Smit, J. de Jongste, H. Neijens, J. Gerritsen, et al., *The prevention and incidence of asthma and mite allergy (PIAMA) birth cohort study: design and first results.* Pediatr Allergy Immunol, 2002. **13**(Suppl 15): p. 55-60.
43. Wright, A.L., *The epidemiology of the atopic child: Who is at risk for what?* Journal of Allergy and Clinical Immunology, 2004. **113**(1, Supplement 1): p. S2-S7.
44. Peden, D.B., *The epidemiology and genetics of asthma risk associated with air pollution.* Journal of Allergy and Clinical Immunology, 2005. **115**(2): p. 213-219.
45. Bernstein, J.A., N. Alexis, C. Barnes, I.L. Bernstein, J.A. Bernstein, et al., *Health effects of air pollution.* Journal of Allergy and Clinical Immunology, 2004. **114**(5): p. 1116-1123.
46. McDonnell, W.F., D.E. Abbey, N. Nishino, and M.D. Lebowitz, *Long-Term Ambient Ozone Concentration and the Incidence of Asthma in Nonsmoking Adults: The AHSMOG Study.* Environmental Research, 1999. **80**(2): p. 110-121.
47. McConnell, R., K. Berhane, F. Gilliland, S.J. London, T. Islam, et al., *Asthma in exercising children exposed to ozone: a cohort study.* Lancet, 2002. **359**(9304): p. 386-391.

48. Nicolai, T., D. Carr, S.K. Weiland, H. Duhme, O. von Ehrenstein, et al., *Urban traffic and pollutant exposure related to respiratory outcomes and atopy in a large sample of children.* Eur Respir J, 2003. **21**(6): p. 956-963.
49. von Mutius, E., F.D. Martinez, C. Fritzsch, T. Nicolai, G. Roell, et al., *Prevalence of asthma and atopy in two areas of West and East Germany.* Am J Respir Crit Care Med, 1994. **149**(2 pt 1): p. 358-64.
50. GINA, *Global Strategy for Asthma Management and Prevention.* 1995, NIH Publication N002-3659. www.ginasthma.org.
51. Romieu, I., J.J. Sienra-Monge, M. Ramirez-Aguilar, H. Moreno-Macias, N.I. Reyes-Ruiz, et al., *Genetic polymorphism of GSTM1 and antioxidant supplementation influence lung function in relation to ozone exposure in asthmatic children in Mexico City.* Thorax, 2004. **59**(1): p. 8-10.
52. von Mutius, E., *Environmental factors influencing the development and progression of pediatric asthma.* J Allergy Clin Immunol, 2002. **109**(6 Suppl): p. S525-32.
53. Martinez, F.D., M. Cline, and B. Burrows, *Increased incidence of asthma in children of smoking mothers.* Pediatrics, 1992. **89**(1): p. 21-6.
54. Cook, D.G., D.P. Strachan, and I.M. Carey, *Health effects of passive smoking.* Thorax, 1999. **54**(5): p. 469.
55. Baynam, G., S.K. Khoo, J. Rowe, G. Zhang, I. Laing, et al., *Parental smoking impairs vaccine responses in children with atopic genotypes.* J Allergy Clin Immunol, 2007. **119**(2): p. 366-74.
56. McKeever, T.M. and J. Britton, *Diet and Asthma.* Am. J. Respir. Crit. Care Med., 2004. **170**(7): p. 725-729.
57. Romieu, I. and C. Trenga, *Diet and obstructive lung diseases.* Epidemiol Rev, 2001. **23**(2): p. 268-87.
58. Smit, H.A., *Chronic obstructive pulmonary disease, asthma and protective effects of food intake: from hypothesis to evidence?* Respir Res, 2001. **2**(5): p. 261-4.
59. Fogarty, A. and J. Britton, *The role of diet in the aetiology of asthma.* Clin Exp Allergy, 2000. **30**(5): p. 615-627.
60. Friedman, N. and R. Zeiger, *The role of breast-feeding in the development of allergies and asthma.* J Allergy Clin Immunol, 2005. **115**(6): p. 1238-48.
61. Spector, S.L. and M.E. Surette, *Diet and asthma: has the role of dietary lipids been overlooked in the management of asthma?* Ann Allergy Asthma Immunol, 2003. **90**(4): p. 371-7.
62. Dunstan, J.A., T.A. Mori, A. Barden, L.J. Beilin, A.L. Taylor, et al., *Fish oil supplementation in pregnancy modifies neonatal allergen-specific immune responses and clinical outcomes in infants at high risk of atopy: A randomized, controlled trial.* Journal of Allergy and Clinical Immunology, 2003. **112**(6): p. 1178-1184.
63. Mihrshahi, S., J.K. Peat, G.B. Marks, C.M. Mellis, E.R. Tovey, et al., *Eighteen-month outcomes of house dust mite avoidance and dietary fatty acid modification in the childhood asthma prevention study (CAPS).* Journal of Allergy and Clinical Immunology, 2003. **111**(1): p. 162-168.
64. Stenius-Aarniala, B., T. Poussa, J. Kvarnstrom, E.-L. Gronlund, M. Ylikahri, et

al., *Immediate and long term effects of weight reduction in obese people with asthma: randomised controlled study.* BMJ, 2000. **320**(7238): p. 827-832.
65. Aaron, S.D., D. Fergusson, R. Dent, Y. Chen, K.L. Vandemheen, et al., *Effect of Weight Reduction on Respiratory Function and Airway Reactivity in Obese Women.* Chest, 2004. **125**(6): p. 2046-2052.
66. Cheng, J., T. Pan, G. Ye, and Q. Liu, *Calorie controlled diet for chronic asthma.* The Cochrane Database of Systematic Reviews, 2003. **Issue 3:CD004674**.
67. Romagnani, S., *The increased prevalence of allergy and the hygiene hypothesis: missing immune deviation, reduced immune suppression, or both?* Immunology, 2004. **112**(3): p. 352-363.
68. Yazdanbakhsh, M., P.G. Kremsner, and R. van Ree, *Allergy, Parasites, and the Hygiene Hypothesis.* Science, 2002. **296**(5567): p. 490-494.
69. Strachan, D.P., *Hay fever, hygiene and household size.* BMJ, 1989. **299**: p. 1259-1260.
70. Ramsay, C. and J. Celedon, *The hygiene hypothesis and asthma.* Curr Opin Pulm Med, 2005. **11**: p. 14-20.
71. Svanes, C., D. Jarvis, S. Chinn, E. Omenaas, A. Gulsvik, et al., *Early exposure to children in family and day care as related to adult asthma and hay fever: results from the European Community Respiratory Health Survey.* Thorax, 2002. **57**(11): p. 945-950.
72. Kramer, U., J. Heinrich, M. Wjst, and H.-E. Wichmann, *Age of entry to day nursery and allergy in later childhood.* The Lancet, 1999. **353**(9151): p. 450-454.
73. Ball, T.M., J.A. Castro-Rodriguez, K.A. Griffith, C.J. Holberg, F.D. Martinez, et al., *Siblings, Day-Care Attendance, and the Risk of Asthma and Wheezing during Childhood.* N Engl J Med, 2000. **343**(8): p. 538-543.
74. von Ehrenstein, O., E. von Mutius, S. Illi, L. Baumann, O. Bohm, et al., *Reduced risk of hay fever and asthma among children of farmers.* Clin Exp Allergy, 2000. **30**(2): p. 187-193.
75. von Mutius, E., C. Braun-Fahrlander, R. Schierl, J. Riedler, S. Ehlermann, et al., *Exposure to endotoxin or other bacterial components might protect against the development of atopy.* Clin Exp Allergy, 2000. **30**(9): p. 1230-1234.
76. Riedler, J., C. Braun-Fahrlander, W. Eder, M. Schreuer, M. Waser, et al., *Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey.* The Lancet, 2001. **358**(9288): p. 1129-1133.
77. Shirakawa, T., T. Enomoto, S.-i. Shimazu, and J.M. Hopkin, *The Inverse Association Between Tuberculin Responses and Atopic Disorder.* Science, 1997. **275**(5296): p. 77-79.
78. Matricardi, P.M. and M. Yazdanbakhsh, *Mycobacteria and atopy, 6 years later: a fascinating, still unfinished, business.* Clin Exp Allergy, 2003. **33**(6): p. 717-720.
79. Rautava, S., O. Ruuskanen, A. Ouwehand, S. Salminen, and E. Isolauri, *The hygiene hypothesis of atopic disease--an extended version.* J Pediatr Gastroenterol Nutr, 2004. **38**: p. 378-388.
80. Matricardi, P.M., F. Rosmini, L. Ferrigno, R. Nisini, M. Rapicetta, et al., *Cross*

sectional retrospective study of prevalence of atopy among Italian military students with antibodies against hepatitis a virus. BMJ, 1997. **314**(7086): p. 999-.
81. Kalliomaki, M., S. Salminen, H. Arvilommi, P. Kero, P. Koskinen, et al., *Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial.* The Lancet, 2001. **357**(9262): p. 1076-1079.
82. Moller, M., M.B. Gravenor, S.E. Roberts, D. Sun, P. Gao, et al., *Genetic haplotypes of Th-2 immune signalling link allergy to enhanced protection to parasitic worms.* Hum Mol Genet, 2007.
83. Scrivener, S., H. Yemaneberhan, M. Zebenigus, D. Tilahun, S. Girma, et al., *Independent effects of intestinal parasite infection and domestic allergen exposure on risk of wheeze in Ethiopia: a nested case-control study.* The Lancet, 2001. **358**(9292): p. 1493-1499.
84. Peisong, G., A. Yamasaki, X.Q. Mao, T. Enomoto, Z. Feng, et al., *An asthma-associated genetic variant of STAT6 predicts low burden of ascaris worm infestation.* Genes Immun, 2004. **5**(1): p. 58-62.
85. Kouriba, B., C. Chevillard, J.H. Bream, L. Argiro, H. Dessein, et al., *Analysis of the 5q31-q33 locus shows an association between IL13-1055C/T IL-13-591A/G polymorphisms and Schistosoma haematobium infections.* J Immunol, 2005. **174**(10): p. 6274-81.
86. Le Souef, P., P. Candelaria, and J. Goldblatt, *Evolution and respiratory genetics.* Eur Respir J, 2006. **28**(6): p. 1258-63.
87. van den Biggelaar, A.H.J., L.C. Rodrigues, R. van Ree, J.S. van der Zee, Y.C.M. Hoeksma-Kruize, et al., *Long-term treatment of intestinal helminths increases mite skin-test reactivity in Gabonese schoolchildren.* J Infect Dis, 2004. **189**: p. 892-900.
88. van den Biggelaar, A.H., R. van Ree, L.C. Rodrigues, B. Lell, A.M. Deelder, et al., *Decreased atopy in children infected with Schistosoma haematobium: a role for parasite-induced interleukin-10.* The Lancet, 2000. **356**(9243): p. 1723-1727.
89. Meylan, E., J. Tschopp, and M. Karin, *Intracellular pattern recognition in the host response.* Nature, 2006. **177**(9): p. 6245-55.
90. Gangloff, S.C. and M. Guenounou, *Toll-like receptors and immune response in allergic disease.* Clin Rev Allergy Immunol, 2004. **26**(2): p. 115-25.
91. Johnston, S.L., P.K. Pattemore, G. Sanderson, S. Smith, F. Lampe, et al., *Community study of role of viral infections in exacerbations of asthma in 9-11 year old children.* BMJ, 1995. **310**(6989): p. 1225-1229.
92. Wark, P.A.B., S.L. Johnston, I. Moric, J. Simpson, M. Hensley, et al., *Neutrophil degranulation and cell lysis is associated with clinical severity in virus-induced asthma.* Eur Respir J, 2002. **19**: p. 68-75.
93. Lemanske, J., Robert F., *Viral infections and asthma inception.* Journal of Allergy and Clinical Immunology, 2004. **114**(5): p. 1023-1026.
94. Openshaw, P.J.M., Y. Yamaguchi, and J.S. Tregoning, *Childhood infections, the developing immune system, and the origins of asthma.* Journal of Allergy and Clinical Immunology, 2004. **114**(6): p. 1275-1277.
95. Peebles, J., R. Stokes, *Viral infections, atopy, and asthma: Is there a causal*

relationship? Journal of Allergy and Clinical Immunology, 2004. **113**(1, Supplement 1): p. S15-S18.
96. Puthothu, B., M. Krueger, J. Forster, and A. Heinzmann, *Association between severe respiratory syncytial virus infection and IL13/IL4 haplotypes.* J Infect Dis, 2006. **193**(3): p. 438-41.
97. Kotaniemi-Syrjanen, A., R. Vainionpaa, T.M. Reijonen, M. Waris, K. Korhonen, et al., *Rhinovirus-induced wheezing in infancy--the first sign of childhood asthma?* Journal of Allergy and Clinical Immunology, 2003. **111**(1): p. 66-71.
98. Hoffjan, S., D. Nicolae, I. Ostrovnaya, K. Roberg, M. Evans, et al., *Gene-environment interaction effects on the development of immune responses in the 1st year of life.* Am J Hum Genet, 2005. **76**(4): p. 696-704.
99. Wark, P.A.B., S.L. Johnston, F. Bucchieri, R. Powell, S. Puddicombe, et al., *Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus.* J. Exp. Med., 2005. **201**(6): p. 937-947.
100. de Kluijver, J., C.E. Evertse, J.K. Sont, J.A. Schrumpf, C.J.G. van Zeijl-van der Ham, et al., *Are Rhinovirus-induced Airway Responses in Asthma Aggravated by Chronic Allergen Exposure?* Am. J. Respir. Crit. Care Med., 2003. **168**(10): p. 1174-1180.
101. Mallia, P. and S.L. Johnston, *Respiratory viruses: do they protect from or induce asthma?* Allergy, 2002. **57**(12): p. 1118-1129.
102. Holgate, S.T., J. Holloway, S. Wilson, P.H. Howarth, H.M. Haitchi, et al., *Understanding the pathophysiology of severe asthma to generate new therapeutic opportunities.* Journal of Allergy and Clinical Immunology, 2006. **117**(3): p. 496-506.
103. Haitchi, H.M., R.M. Powell, T.J. Shaw, P.H. Howarth, S.J. Wilson, et al., *ADAM33 expression in asthmatic airways and human embryonic lungs.* American Journal Of Respiratory And Critical Care Medicine, 2005. **171**(9): p. 958-965.
104. Lee, C.G., R.J. Homer, Z. Zhu, S. Lanone, X. Wang, et al., *Interleukin-13 Induces Tissue Fibrosis by Selectively Stimulating and Activating Transforming Growth Factor beta-1.* J Exp Med, 2001. **194**(6): p. 809-822.
105. McKenzie, G.J., A. Bancroft, R.K. Grencis, and A.N. McKenzie, *A distinct role for interleukin-13 in Th2-cell-mediated immune responses.* Curr Biol, 1998. **8**(6): p. 339-42.
106. Webb, D.C., Y. Cai, K.I. Matthaei, and P.S. Foster, *Comparative roles of IL-4, IL-13, and IL-4Ralpha in dendritic cell maturation and CD4+ Th2 cell function.* J Immunol, 2007. **178**(1): p. 219-27.
107. Shimoda, K., J. van Deursen, M.Y. Sangster, S.R. Sarawar, R.T. Carson, et al., *Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene.* Nature, 1996. **380**(6575): p. 630-3.
108. Noguchi, E., M. Shibasaki, T. Arinami, K. Takeda, Y. Yokouchi, et al., *No association between atopy/asthma and the ILe50Val polymorphism of IL-4 receptor.* Am J Respir Crit Care Med, 1999. **160**(1): p. 342-5.
109. Migliaccio, C., C. Patuzzo, G. Malerba, E. Trabetti, R. Galavotti, et al., *No linkage or association of five polymorphisms in the interleukin-4 receptor*

alpha gene with atopic asthma in Italian families. Eur J Immunogenet, 2003. **30**(5): p. 349-53.
110. DeMeo, D.L., C. Lange, E.K. Silverman, J.M. Senter, J.M. Drazen, et al., *Univariate and multivariate family-based association analysis of the IL-13 ARG130GLN polymorphism in the Childhood Asthma Management Program.* Genet Epidemiol, 2002. **23**(4): p. 335-48.
111. Ioannidis, J., E. Ntzani, T. Trikalinos, and D. Contopoulos-Ioannidis, *Replication validity of genetic association studies.* Nat Genet, 2001. **29**: p. 306-309.
112. Bain, S. and J. Rippin, *Complex disease genetics enters the high street.* Eur J Clin Invest, 2003. **33**(3): p. 186.
113. Graves, P.E., M. Kabesch, M. Halonen, C.J. Holberg, M. Baldini, et al., *A cluster of seven tightly linked polymorphisms in the IL-13 gene is associated with total serum IgE levels in three populations of white children.* Journal of Allergy and Clinical Immunology, 2000. **105**(3): p. 506-513.
114. Howard, T.D., P.A. Whittaker, A.L. Zaiman, G.H. Koppelman, J. Xu, et al., *Identification and association of polymorphisms in the interleukin-13 gene with asthma and atopy in a Dutch population.* Am J Respir Cell Mol Biol, 2001. **25**(3): p. 377-84.
115. Hummelshoj, T., U. Bodtger, P. Datta, H.J. Malling, A. Oturai, et al., *Association between an interleukin-13 promoter polymorphism and atopy.* European Journal of Immunogenetics, 2003. **30**(5): p. 355-359.
116. Cameron, L., R.B. Webster, J.M. Strempel, P. Kiesler, M. Kabesch, et al., *Th2 Cell-Selective Enhancement of Human IL13 Transcription by IL13-1112C>T, a Polymorphism Associated with Allergic Inflammation.* J Immunol, 2006. **177**(12): p. 8633-8642.
117. Heinzmann, A., X.Q. Mao, M. Akaiwa, R.T. Kreomer, P.S. Gao, et al., *Genetic variants of IL-13 signalling and human asthma and atopy.* Hum Mol Genet, 2000. **9**(4): p. 549-59.
118. Vladich, F.D., S.M. Brazille, D. Stern, M.L. Peck, R. Ghittoni, et al., *IL-13 R130Q, a common variant associated with allergy and asthma, enhances effector mechanisms essential for human allergic inflammation.* J Clin Invest, 2005. **115**(3): p. 747-54.
119. Mitsuyasu, H., K. Izuhara, X.Q. Mao, P.S. Gao, Y. Arinobu, et al., *Ile50Val variant of IL4R alpha upregulates IgE synthesis and associates with atopic asthma.* Nat Genet, 1998. **19**(2): p. 119-20.
120. Hershey, G.K., M.F. Friedrich, L.A. Esswein, M.L. Thomas, and T.A. Chatila, *The association of atopy with a gain-of-function mutation in the alpha subunit of the interleukin-4 receptor.* N Engl J Med, 1997. **337**(24): p. 1720-5.
121. Kruse, S., T. Japha, M. Tedner, S.H. Sparholt, J. Forster, et al., *The polymorphisms S503P and Q576R in the interleukin-4 receptor alpha gene are associated with atopy and influence the signal transduction.* Immunology, 1999. **96**(3): p. 365-71.
122. Ober, C., S.A. Leavitt, A. Tsalenko, T.D. Howard, D.M. Hoki, et al., *Variation in the interleukin 4-receptor alpha gene confers susceptibility to asthma and atopy in ethnically diverse populations.* Am J Hum Genet, 2000. **66**(2): p. 517-

26.
123. Patel, B.K.R., C.L. Keck, R.S. O'Leary, N.C. Popescu, and W.J. LaRochelle, *Localization of the Human Stat6 Gene to Chromosome 12q13.3-q14.1, a Region Implicated in Multiple Solid Tumors.* Genomics, 1998. **52**(2): p. 192-200.
124. Gao, P.S., X.Q. Mao, M.H. Roberts, Y. Arinobu, M. Akaiwa, et al., *Variants of STAT6 (signal transducer and activator of transcription 6) in atopic asthma.* J Med Genet, 2000. **37**(5): p. 380-2.
125. Tamura, K., M. Suzuki, H. Arakawa, K. Tokuyama, and A. Morikawa, *Linkage and association studies of STAT6 gene polymorphisms and allergic diseases.* Int Arch Allergy Immunol, 2003. **131**(1): p. 33-8.
126. Shao, C., Y. Suzuki, F. Kamada, K. Kanno, M. Tamari, et al., *Linkage and association of childhood asthma with the chromosome 12 genes.* J Hum Genet, 2004. **49**(3): p. 115-22.
127. Duetsch, G., T. Illig, S. Loesgen, K. Rohde, N. Klopp, et al., *STAT6 as an asthma candidate gene: polymorphism-screening, association and haplotype analysis in a Caucasian sib-pair study.* Hum. Mol. Genet., 2002. **11**(6): p. 613-621.
128. Schedel, M., D. Carr, N. Klopp, B. Woitsch, T. Illig, et al., *A signal transducer and activator of transcription 6 haplotype influences the regulation of serum IgE levels.* J Allergy Clin Immunol, 2004. **114**(5): p. 1100-5.
129. Weidinger, S., N. Klopp, S. Wagenpfeil, L. Rummler, M. Schedel, et al., *Association of a STAT 6 haplotype with elevated serum IgE levels in a population based cohort of white adults.* J Med Genet, 2004. **41**(9): p. 658-63.
130. Tamura, K., H. Arakawa, M. Suzuki, Y. Kobayashi, H. Mochizuki, et al., *Novel dinucleotide repeat polymorphism in the first exon of the STAT-6 gene is associated with allergic diseases.* Clinical & Experimental Allergy, 2001. **31**(10): p. 1509-1514.
131. Gao, P.S., N.M. Heller, W. Walker, C.H. Chen, M. Moller, et al., *Variation in dinucleotide (GT) repeat sequence in the first exon of the STAT6 gene is associated with atopic asthma and differentially regulates the promoter activity in vitro.* J Med Genet, 2004. **41**(7): p. 535-9.
132. Maier, L.M., J.M. Howson, N. Walker, G.P. Spickett, R.W. Jones, et al., *Association of IL13 with total IgE: evidence against an inverse association of atopy and diabetes.* J Allergy Clin Immunol, 2006. **117**(6): p. 1306-13.
133. Chen, W., M.B. Ericksen, L.S. Levin, and G.K. Khurana Hershey, *Functional effect of the R110Q IL13 genetic variant alone and in combination with IL4RA genetic variants.* J Allergy Clin Immunol, 2004. **114**(3): p. 553-60.
134. Tsunemi, Y., H. Saeki, K. Nakamura, T. Sekiya, K. Hirai, et al., *Interleukin-13 gene polymorphism G4257A is associated with atopic dermatitis in Japanese patients.* Journal of Dermatological Science, 2002. **30**(2): p. 100-107.
135. van der Pouw Kraan, T.C., A. van Veen, L.C. Boeije, S.A. van Tuyl, E.R. de Groot, et al., *An IL-13 promoter polymorphism associated with increased risk of allergic asthma.* Genes Immun, 1999. **1**(1): p. 61-5.
136. Hakonarson, H., U.S. Bjorndottir, E. Ostermann, T. Arnason, A.E. Adalsteindottir, et al., *Allelic Frequencies and Patterns of Single-nucleotide*

Polymorphisms in Candidate Genes for Asthma and Atopy in Iceland. Am J Respir Crit Care Med, 2001. **164**(11): p. 2036-2044.
137. Liu, X., T.H. Beaty, P. Deindl, S.K. Huang, S. Lau, et al., *Associations between total serum IgE levels and the 6 potentially functional variants within the genes IL4, IL13, and IL4RA in German children: the German Multicenter Atopy Study.* J Allergy Clin Immunol, 2003. **112**(2): p. 382-8.
138. Liu, X., T.H. Beaty, P. Deindl, S.K. Huang, S. Lau, et al., *Associations between specific serum IgE response and 6 variants within the genes IL4, IL13, and IL4RA in German children: the German Multicenter Atopy Study.* J Allergy Clin Immunol, 2004. **113**(3): p. 489-95.
139. Hoffjan, S., I. Ostrovnaja, D. Nicolae, D.L. Newman, R. Nicolae, et al., *Genetic variation in immunoregulatory pathways and atopic phenotypes in infancy.* J Allergy Clin Immunol, 2004. **113**(3): p. 511-8.
140. He, J.Q., M. Chan-Yeung, A.B. Becker, H. Dimich-Ward, A.C. Ferguson, et al., *Genetic variants of the IL13 and IL4 genes and atopic diseases in at-risk children.* Genes Immun, 2003. **4**(5): p. 385-9.
141. Moissidis, I., B. Chinoy, K. Yanamandra, D. Napper, T. Thurmon, et al., *Association of IL-13, RANTES, and leukotriene C4 synthase gene promoter polymorphisms with asthma and/or atopy in African Americans.* Genet Med, 2005. 7(6): p. 406-10.
142. Wang, M., Z.M. Xing, C. Lu, Y.X. Ma, D.L. Yu, et al., *A common IL-13 Arg130Gln single nucleotide polymorphism among Chinese atopy patients with allergic rhinitis.* Hum Genet, 2003. **113**(5): p. 387-90.
143. Chang, Y.T., W.R. Lee, C.W. Yu, H.N. Liu, M.W. Lin, et al., *No association of cytokine gene polymorphisms in Chinese patients with atopic dermatitis.* Clin Exp Dermatol, 2006. **31**(3): p. 419-23.
144. Ober, C., A. Tsalenko, R. Parry, and N.J. Cox, *A second-generation genomewide screen for asthma-susceptibility alleles in a founder population.* Am J Hum Genet, 2000. **67**(5): p. 1154-62.
145. Liu, X., R. Nickel, K. Beyer, U. Wahn, E. Ehrlich, et al., *An IL13 coding region variant is associated with a high total serum IgE level and atopic dermatitis in the German multicenter atopy study (MAS-90).* J Allergy Clin Immunol, 2000. **106**(1 Pt 1): p. 167-70.
146. Celedon, J.C., M.E. Soto-Quiros, L.J. Palmer, J. Senter, J. Mosley, et al., *Lack of association between a polymorphism in the interleukin-13 gene and total serum immunoglobulin E level among nuclear families in Costa Rica.* Clinical & Experimental Allergy, 2002. **32**(3): p. 387-390.
147. Leung, T.F., N.L. Tang, I.H. Chan, A.M. Li, G. Ha, et al., *A polymorphism in the coding region of interleukin-13 gene is associated with atopy but not asthma in Chinese children.* Clin Exp Allergy, 2001. **31**(10): p. 1515-21.
148. Kim, H.-B., Y.-C. Lee, S.-Y. Lee, J. Jung, H.-S. Jin, et al., *Gene-gene interaction between IL-13 and IL-13RA1 is associated with total IgE in Korean children with atopic asthma.* J Hum Genet, 2006. **51**(12): p. 1055-1062.
149. Woitsch, B., D. Carr, D. Stachel, I. Schmid, S.K. Weiland, et al., *A comprehensive analysis of interleukin-4 receptor polymorphisms and their association with atopy and IgE regulation in childhood.* Int Arch Allergy

Immunol, 2004. **135**(4): p. 319-24.
150. Haagerup, A., T. Bjerke, P.O. Schiotz, R. Dahl, H.G. Binderup, et al., *No linkage and association of atopy to chromosome 16 including the interleukin-4 receptor gene.* Allergy, 2001. **56**(8): p. 775-779.
151. Mujica-Lopez, K.I., S.E. Flores-Martinez, R. Ramos-Zepeda, S.A. Castaneda-Ramos, A. Gazca-Aguilar, et al., *Association analysis of polymorphisms in the interleukin-4 receptor (alpha) gene with atopic asthma in patients from western Mexico.* European Journal of Immunogenetics, 2002. **29**(5): p. 375-378.
152. Zhang, W., X. Zhang, D. Qiu, A. Sandford, and W.C. Tan, *IL-4 receptor genetic polymorphisms and asthma in Asian populations.* Respir Med, 2007. **101**(1): p. 186-90.
153. Chan, I.H., T.F. Leung, N.L. Tang, C.Y. Li, Y.M. Sung, et al., *Gene-gene interactions for asthma and plasma total IgE concentration in Chinese children.* J Allergy Clin Immunol, 2006. **117**(1): p. 127-33.
154. Tan, E., B. Lee, A. Tay, L. Shek, F. Chew, et al., *Interleukin-4 receptor variant Q576R: ethnic differences and association with atopy.* Clinical Genetics, 1999. **56**(4): p. 333-334.
155. Takabayashi, A., K. Ihara, Y. Sasaki, Y. Suzuki, S. Nishima, et al., *Childhood atopic asthma: positive association with a polymorphism of IL-4 receptor alpha gene but not with that of IL-4 promoter or Fc epsilon receptor I beta gene.* Exp Clin Immunogenet, 2000. **17**(2): p. 63-70.
156. Nakamura, H., K. Miyagawa, K. Ogino, T. Endo, T. Imai, et al., *High contribution contrast between the genes of eosinophil peroxidase and IL-4 receptor alpha-chain in Japanese cedar pollinosis.* J Allergy Clin Immunol, 2003. **112**(6): p. 1127-31.
157. Oiso, N., K. Fukai, and M. Ishii, *Interleukin 4 receptor alpha chain polymorphism Gln551Arg is associated with adult atopic dermatitis in Japan.* British Journal of Dermatology, 2000. **142**(5): p. 1003-1006.
158. Tanaka, K., H. Sugiura, M. Uehara, Y. Hashimoto, C. Donnelly, et al., *Lack of association between atopic eczema and the genetic variants of interleukin-4 and the interleukin-4 receptor alpha chain gene: heterogeneity of genetic backgrounds on immunoglobulin E production in atopic eczema patients.* Clinical & Experimental Allergy, 2001. **31**(10): p. 1522-1527.
159. Lee, S.G., B.S. Kim, J.H. Kim, S.Y. Lee, S.O. Choi, et al., *Gene-gene interaction between interleukin-4 and interleukin-4 receptor alpha in Korean children with asthma.* Clin Exp Allergy, 2004. **34**(8): p. 1202-8.
160. Beghe, B., S. Barton, S. Rorke, Q. Peng, I. Sayers, et al., *Polymorphisms in the interleukin-4 and interleukin-4 receptor alpha chain genes confer susceptibility to asthma and atopy in a Caucasian population.* Clin Exp Allergy, 2003. **33**(8): p. 1111-7.
161. Wjst, M., S. Kruse, T. Illig, and K. Deichmann, *Asthma and IL-4 receptor alpha gene variants.* European Journal of Immunogenetics, 2002. **29**(3): p. 263-268.
162. Malerba, G., E. Trabetti, C. Patuzzo, M.C. Lauciello, R. Galavotti, et al., *Candidate genes and a genome-wide search in Italian families with atopic*

asthmatic children. Clin Exp Allergy, 1999. **29 Suppl 4**: p. 27-30.
163. Isidoro-Garcia, M., I. Davila, E. Moreno, E. Laffond, F. Lorente, et al., *[IL4RA gene polymorphism (Q576R) is associated with higher total IgE levels in Spanish patients with family history of atopy].* Med Clin (Barc), 2005. **124**(6): p. 211-2.
164. Sandford, A.J., T. Chagani, S. Zhu, T.D. Weir, T.R. Bai, et al., *Polymorphisms in the IL4, IL4RA, and FCERIB genes and asthma severity.* J Allergy Clin Immunol, 2000. **106**(1 Pt 1): p. 135-40.
165. Rosa-Rosa, L., N. Zimmermann, J.A. Bernstein, M.E. Rothenberg, and G.K. Khurana Hershey, *The R576 IL-4 receptor alpha allele correlates with asthma severity.* J Allergy Clin Immunol, 1999. **104**(5): p. 1008-14.
166. Cui, T., J. Wu, S. Pan, and J. Xie, *Polymorphisms in the IL-4 and IL-4R alpha genes and allergic asthma.* Clin Chem Lab Med, 2003. **41**(7): p. 888-92.
167. Zhang, A.M., H.L. Li, P. Hao, Y.H. Chen, J.H. Li, et al., *[Association of Q576R polymorphism in the interleukin-4 receptor gene with serum IgE levels in children with asthma].* Zhongguo Dang Dai Er Ke Za Zhi, 2006. **8**(2): p. 109-12.
168. Pykalainen, M., R. Kinos, S. Valkonen, P. Rydman, M. Kilpelainen, et al., *Association analysis of common variants of STAT6, GATA3, and STAT4 to asthma and high serum IgE phenotypes.* J Allergy Clin Immunol, 2005. **115**(1): p. 80-7.
169. Amoli, M.M., S. Hand, A.H. Hajeer, K.P. Jones, S. Rolf, et al., *Polymorphism in the STAT6 gene encodes risk for nut allergy.* Genes Immun, 2002. **3**(4): p. 220-4.
170. Luce, J.M., *Respiratory complications of obesity.* Chest, 1980. **78**(4): p. 626-31.
171. Rochester, D., ed. *Obesity and pulmonary function.* The Heart and Lung in Obesity, ed. M. Alpert and J. Alexander. 1998, Futura Publishing: Armonk. 108-131.
172. Collins, L., P. Hoberty, J. Walker, E. Fletcher, and A. Peiris, *The effect of body fat distribution on pulmonary function tests.* Chest, 1995. **107**(5): p. 1298-1302.
173. Canoy, D., R. Luben, A. Welch, S. Bingham, N. Wareham, et al., *Abdominal obesity and respiratory function in men and women in the EPIC-Norfolk Study, United Kingdom.* Am J Epidemiol, 2004. **159**(12): p. 1140-49.
174. Busetto, L. and G. Sergi, *Visceral fat and respiratory complications.* Diabetes, Obesity and Metabolism, 2005. **7**(4): p. 301-306.
175. Hogg, J., P. Pare, and R. Moreno, *The effect of submucosal edema on airways resistance.* Am Rev Respir Dis, 1987. **135**(6 pt 2): p. S54-6.
176. Hakala, K., B. Stenius-Aarniala, and A. Sovijarvi, *Effects of Weight Loss on Peak Flow Variability, Airways Obstruction, and Lung Volumes in Obese Patients With Asthma.* Chest, 2000. **118**(5): p. 1315-1321.
177. Inselma, L.S., A. Milanese, and A. Deurloo, *Effect of obesity on pulmonary function in children.* Pediatr Pulmonol, 1993. **16**(2): p. 130-7.
178. Shore, S.A. and J.J. Fredberg, *Obesity, smooth muscle, and airway hyperresponsiveness.* Journal of Allergy and Clinical Immunology, 2005.

115(5): p. 925-927.
179. FREDBERG, J.J., D. INOUYE, B. MILLER, M. NATHAN, S. JAFARI, et al., *Airway Smooth Muscle, Tidal Stretches, and Dynamically Determined Contractile States.* Am. J. Respir. Crit. Care Med., 1997. **156**(6): p. 1752-1759.
180. Turcotte, H., B. Simard, M. Boulay, and L. Boulet, *Influence of body mass index (BMI) on the effect of deep inspiration avoidance (DIA) on airway response to methacholine.* Am J Respir Crit Care Med, 2004. **169**: p. A246.
181. Shore, S.A., Y.M. Rivera-Sanchez, I.N. Schwartzman, and R.A. Johnston, *Responses to ozone are increased in obese mice.* Journal of Applied Physiology, 2003. **95**(3): p. 938-945.
182. Ricciardolo, F.L.M., B. Gaston, and J. Hunt, *Acid stress in the pathology of asthma.* Journal of Allergy and Clinical Immunology, 2004. **113**(4): p. 610-619.
183. Rajala, M. and P. Scherer, *Minireview: the adipocyte: at the crossroads of energy homeostasis, inflammation and atherosclerosis.* Endocrinology, 2003. **144**: p. 3765-73.
184. Tantisira, K. and S. Weiss, *Complex interactions in complex traits: obesity and asthma.* Thorax, 2001. **56**(Suppl 2): p. ii64-73.
185. Fantuzzi, G., *Adipose tissue, adipokines, and inflammation.* J Allergy Clin Immunol, 2005. **115**(5): p. 911-9; quiz 920.
186. Maffei, M., J. Halaas, E. Ravussin, R.E. Pratley, G.H. Lee, et al., *Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects.* Nat Med, 1995. **1**(11): p. 1155-61.
187. Lord, G.M., G. Matarese, J.K. Howard, R.J. Baker, S.R. Bloom, et al., *Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression.* Nature, 1998. **394**(6696): p. 897-901.
188. Guler, N., E. Kirerleri, U. Ones, Z. Tamay, N. Salmayenli, et al., *Leptin: Does it have any role in childhood asthma?* Journal of Allergy and Clinical Immunology, 2004. **114**(2): p. 254-259.
189. Rosenbaum, M., M. Nicolson, J. Hirsch, S.B. Heymsfield, D. Gallagher, et al., *Effects of gender, body composition, and menopause on plasma concentrations of leptin.* J Clin Endocrinol Metab, 1996. **81**(9): p. 3424-7.
190. Chen, Y., R. Dales, D. Krewski, and K. Breithaupt, *Increased effects of smoking and obesity on asthma among female Canadians: the National Population Health Survey, 1994-1995.* Am J Epidemiol, 1999. **150**(3): p. 255-62.
191. Mai, X.-M., M.F. Bottcher, and I. Leijon, *Leptin and asthma in overweight children at 12 years of age.* Pediatric Allergy and Immunology, 2004. **15**(6): p. 523-530.
192. Matsuda, K., Y. Nishi, Y. Okamatsu, M. Kojima, and T. Matsuishi, *Ghrelin and leptin: a link between obesity and allergy?* J Allergy Clin Immunol, 2006. **117**(3): p. 705-6.
193. Lu, F.L., R.A. Johnston, L. Flynt, T.A. Theman, R.D. Terry, et al., *Increased pulmonary responses to acute ozone exposure in obese db/db mice.* Am J Physiol Lung Cell Mol Physiol, 2006. **290**(5): p. L856-65.
194. Shore, S.A., I.N. Schwartzman, M.S. Mellema, L. Flynt, A. Imrich, et al.,

Effect of leptin on allergic airway responses in mice. Journal of Allergy and Clinical Immunology, 2005. **115**(1): p. 103-109.
195. Shore, S.A., R.D. Terry, L. Flynt, A. Xu, and C. Hug, *Adiponectin attenuates allergen-induced airway inflammation and hyperresponsiveness in mice.* J Allergy Clin Immunol, 2006. **118**(2): p. 389-95.
196. Nawrocki, A. and P. Scherer, *The delicate balance between fat and muscle: adipokines in metabolic disease and musculoskelatal inflammation.* Curr Opin Pharmacol, 2004. **4**(3): p. 281-9.
197. Shum, B.O., C.R. Mackay, C.Z. Gorgun, M.J. Frost, R.K. Kumar, et al., *The adipocyte fatty acid-binding protein aP2 is required in allergic airway inflammation.* J Clin Invest, 2006. **116**(8): p. 2183-2192.
198. Forbes, L., *Asthma and atopy: endocrine or metabolic conditions?* Thorax, 2005. **60**(10): p. 793-794.
199. Sears, M.R., J.M. Greene, A.R. Willan, E.M. Wiecek, D.R. Taylor, et al., *A Longitudinal, Population-Based, Cohort Study of Childhood Asthma Followed to Adulthood.* N Engl J Med, 2003. **349**(15): p. 1414-1422.
200. Anderson, H.R., A.C. Pottier, and D.P. Strachan, *Asthma from birth to age 23: incidence and relation to prior and concurrent atopic disease.* Thorax, 1992. **47**(7): p. 537-42.
201. Kaplowitz, P., *Delayed puberty in obese boys: comparison with constitutional delayed puberty and response to testosterone therapy.* J Pediatr, 1998. **133**(6): p. 745-9.
202. Cooper, C., D. Kuh, P. Egger, M. Wadsworth, and D. Barker, *Childhood growth and age at menarche.* Br J Obstet Gynaecol, 1996. **103**(8): p. 814-7.
203. Cāmārgō, C.A., Jr, S.T. Weiss, S. Zhang, W.C. Willett, and F.E. Speizer, *Prospective Study of Body Mass Index, Weight Change, and Risk of Adult-onset Asthma in Women.* Arch Intern Med, 1999. **159**(21): p. 2582-2588.
204. Troisi, R.J., F.E. Speizer, W.C. Willett, D. Trichopoulos, and B. Rosner, *Menopause, postmenopausal estrogen preparations, and the risk of adult-onset asthma. A prospective cohort study.* Am J Respir Crit Care Med, 1995. **152**(4 Pt 1): p. 1183-8.
205. Varraso, R., V. Siroux, J. Maccario, I. Pin, F. Kauffmann, et al., *Asthma Severity Is Associated with Body Mass Index and Early Menarche in Women.* Am. J. Respir. Crit. Care Med., 2005. **171**(4): p. 334-339.
206. Gomez Real, F., C. Svanes, E.H. Bjornsson, K. Franklin, D. Gislason, et al., *Hormone replacement therapy, body mass index and asthma in perimenopausal women: a cross sectional survey.* Thorax, 2006. **61**(1): p. 34-40.
207. Degano, B., P. Mourlanette, S. Valmary, S. Pontier, M.C. Prevost, et al., *Differential effects of low and high-dose estradiol on airway reactivity in ovariectomized rats.* Respir Physiol Neurobiol, 2003. **138**(2-3): p. 265-74.
208. Salem, M.L., G. Matsuzaki, K. Kishihara, G.A. Madkour, and K. Nomoto, *b-Estradiol Suppresses T Cell-Mediated Delayed-Type Hypersensitivity through Suppression of Antigen-Presenting Cell Function and Th1 Induction.* Int Arch Allergy Immunol, 2000. **121**(2): p. 161-9.
209. Griffith, J.S., S.M. Jensen, J.K. Lunceford, M.W. Kahn, Y. Zheng, et al.,

Evidence for the genetic control of estradiol-regulated responses. Implications for variation in normal and pathological hormone-dependent phenotypes. Am J Pathol, 1997. **150**(6): p. 2223-30.
210. Hamano, N., N. Terada, K. Maesako, G. Hohki, T. Ito, et al., *Effect of female hormones on the production of IL-4 and IL-13 from peripheral blood mononuclear cells.* Acta Otolaryngol Suppl, 1998. **537**: p. 27-31.
211. Svanes, C., F.G. Real, T. Gislason, C. Jansson, R. Jogi, et al., *Association of asthma and hay fever with irregular menstruation.* Thorax, 2005. **60**(6): p. 445-450.
212. Lawlor, D.A., S. Ebrahim, and G. Davey Smith, *Associations of measures of lung function with insulin resistance and Type 2 diabetes: findings from the British Women's Heart and Health Study.* Thorax, 2004. **59**(3): p. 199-203.
213. Van Heek, M., D.S. Compton, C.F. France, R.P. Tedesco, A.B. Fawzi, et al., *Diet-induced Obese Mice Develop Peripheral, but Not Central, Resistance to Leptin.* J. Clin. Invest., 1997. **99**(3): p. 385-390.
214. Hallstrand, T.S., M.E. Fischer, M.M. Wurfel, N. Afari, D. Buchwald, et al., *Genetic pleiotropy between asthma and obesity in a community-based sample of twins.* J Allergy Clin Immunol, 2005. **116**(6): p. 1235-41.
215. Large, V., L. Hellstrom, S. Reynisdottir, F. Lonnqvist, P. Eriksson, et al., *Human Beta-2 Adrenoceptor Gene Polymorphisms Are Highly Frequent in Obesity and Associate with Altered Adipocyte Beta-2 Adrenoceptor Function.* J. Clin. Invest., 1997. **100**(12): p. 3005-3013.
216. Dewar, J.C., J. Wilkinson, A. Wheatley, N.S. Thomas, I. Doull, et al., *The glutamine 27 beta2-adrenoceptor polymorphism is associated with elevated IgE levels in asthmatic families.* J Allergy Clin Immunol, 1997. **100**(2): p. 261-5.
217. Hall, I.P., A. Wheatley, P. Wilding, and S.B. Liggett, *Association of Glu 27 beta 2-adrenoceptor polymorphism with lower airway reactivity in asthmatic subjects.* Lancet, 1995. **345**(8959): p. 1213-4.
218. Albuquerque, R., C. Hayden, and L. Palmer, *Association of polymorphisms within the tumour necrosis factor (TNF) genes and childhood asthma.* Clin Exp Allergy, 1998. **28**: p. 578-84.
219. Moffatt, M., A. James, G. Ryan, A. Musk, and W. Cookson, *Extended tumour necrosis factor/HLA-DR haplotypes and asthma in an Australian population sample.* Thorax, 1999. **54**: p. 757-61.
220. Norman, R., C. Bogardus, and E. Ravussin, *Linkage between obesity and a marker near the tumor necrosis factor-alpha locus in Pima Indians.* J Clin Invest, 1995. **96**: p. 158-62.
221. Barsh, G.S., I.S. Farooqi, and S. O'Rahilly, *Genetics of body-weight regulation.* 2000. **404**(6778): p. 644-651.
222. Garenc, C., L. Perusse, Y. Chagnon, T. Rankinen, J. Gagnon, et al., *HERITAGE Family Study. Effects of beta2-adrenergic receptor gene variants on adiposity: the HERITAGE Family Study.* Obes Res, 2003. **11**: p. 612-8.
223. Zhang, S., H. Smartt, S.T. Holgate, and W. Roche, *Growth factors secreted by bronchial epithelial cells control myofibroblast proliferation: an in vitro co-culture model of airway remodeling in asthma.* Lab Invest, 1999. **79**: p. 395-

405.
224. Tantisira, K.G., A. Murphy, M.E. Soto-Quiros, L. Avila, C. Liang, et al., *Genome-wide linkage analysis of body mass index in families of children with asthma in Costa Rica.* Am J Respir Crit Care Med, 2007. **175**: p. A459.
225. Dijkstra, A., T.D. Howard, J.M. Vonk, E.J. Ampleford, L.A. Lange, et al., *Estrogen receptor 1 polymorphisms are associated with airway hyperresponsiveness and lung function decline, particularly in female subjects with asthma.* Journal of Allergy and Clinical Immunology, 2006. **117**(3): p. 604-611.
226. Barker, D.J., K.M. Godfrey, C. Fall, C. Osmond, P.D. Winter, et al., *Relation of birth weight and childhood respiratory infection to adult lung function and death from chronic obstructive airways disease.* BMJ, 1991. **303**(6804): p. 671-5.
227. Hediger, M.L., M.D. Overpeck, R.J. Kuczmarski, A. McGlynn, K.R. Maurer, et al., *Muscularity and fatness of infants and young children born small- or large-for-gestational-age.* Pediatrics, 1998. **102**(5): p. E60.
228. Brooks, A.M., R.S. Byrd, M. Weitzman, P. Auinger, and J.T. McBride, *Impact of low birth weight on early childhood asthma in the United States.* Arch Pediatr Adolesc Med, 2001. **155**(3): p. 401-6.
229. Svanes, C., E. Omenaas, J. Heuch, L. Irgens, and A. Gulsvik, *Birth characteristics and asthma symptoms in young adults: results from a population-based cohort study in Norway.* Eur Respir J, 1998. **12**(6): p. 1366-1370.
230. Lawlor, D.A., S. Ebrahim, and G. Davey Smith, *Association of birth weight with adult lung function: findings from the British Women's Heart and Health Study and a meta-analysis.* Thorax, 2005. **60**(10): p. 851-858.
231. Young, J.B. and S.F. Morrison, *Effects of fetal and neonatal environment on sympathetic nervous system development.* Diabetes Care, 1998. **21 Suppl 2**: p. B156-60.
232. Tataranni, P.A., J.B. Young, C. Bogardus, and E. Ravussin, *A low sympathoadrenal activity is associated with body weight gain and development of central adiposity in Pima Indian men.* Obes Res, 1997. **5**(4): p. 341-7.
233. Burney, P.G., L.A. Laitinen, S. Perdrizet, H. Huckauf, A.E. Tattersfield, et al., *Validity and repeatability of the IUATLD (1984) Bronchial Symptoms Questionnaire: an international comparison.* Eur Respir J, 1989. **2**(10): p. 940-5.
234. Frank, P., M. Hazell, J. Morris, M. Linehan, and T. Frank, *A longituinal study of changes in respirtory status in young adults, 1993-2001.* Int J Tuberc Lung Dis, 2007. **11**(3): p. 338-43.
235. Sistek, D., K. Wickens, R. Armstrong, W. D'Souza, I. Town, et al., *Predictive value of respiratory symptoms and bronchial hyperresponsiveness to diagnose asthma in New Zealand.* Respir Med, 2006. **100**(12): p. 2107-2111.
236. Burney, P. and S. Chinn, *Developing a new questionnaire for measuring the prevalence and distribution of asthma.* Chest, 1987. **91**(Suppl): p. 79s-83s.
237. ECRHS, *The European Community Respiratory Health Survey. "Medicine and Health"*, in *EC Directorate General XIII.* 1994, Office for Official

Publications: Luxembourg. p. L-2920.
238. Baena-Cagnani, C., G. Rossi, and G. Canonica, *Airway remodelling in children: when does it start?* Curr Opin Allergy Clin Immunol, 2007. **7**(2): p. 196-200.
239. ATS, *Standardisation of spirometry; 1994 Update.* Am J Respir Crit Care Med, 1994. **152**: p. 1107-36.
240. Dupont, W. and W. Plummer, *Power and sample size calculations for studies involving linear regression.* Controlled Clin Trials, 1998. **19**(6): p. 589-601.
241. Rothman , K., *No adjustments are needed for multiple comparisons.* Epidemiology, 1990. **1**: p. 43-46.
242. Hall, I. and J. Blakey, *Genetic association studies in Thorax.* Thorax, 2005. **60**(5): p. 357-9.
243. Colhoun, H.M., P.M. McKeigue, and G.D. Smith, *Problems of reporting genetic associations with complex outcomes.* The Lancet, 2003. **361**(9360): p. 865-872.
244. Excoffier, L. and M. Slatkin, *Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population.* Mol Biol Evol, 1995. **12**: p. 921-7.
245. Slatkin, M. and L. Excoffier, *Testing for linkage disequilibrium in genotypic data using the Expectation-Maximisation algorithm.* Heredity, 1996. **76**: p. 377-83.
246. *R Development Core Team. A language and environment for statistical computing*. 2004, Vienna.
247. Heinzmann, A., S.P. Jerkic, K. Ganter, T. Kurz, S. Blattmann, et al., *Association study of the IL13 variant Arg110Gln in atopic diseases and juvenile idiopathic arthritis.* J Allergy Clin Immunol, 2003. **112**(4): p. 735-9.
248. DeMeo, D., C. Lange, E. Silverman, S. JM, J. Drazen, et al., *Univariate and multivariate family-based association analysis of the IL-13 ARG130GLN polymorphism in the Childhood Asthma Management program.* Genetic Epidemiology, 2002. **23**(4): p. 335-348.
249. Tarazona-Santos, E. and S.A. Tishkoff, *Divergent patterns of linkage disequilibrium and haplotype structure across global populations at the interleukin-13 (IL13) locus.* Genes Immun, 2005. **6**(1): p. 53-65.
250. Madhankumar, A.B., A. Mintz, and W. Debinski, *Alanine-scanning Mutagenesis of alpha -Helix D Segment of Interleukin-13 Reveals New Functionally Important Residues of the Cytokine.* J. Biol. Chem., 2002. **277**(45): p. 43194-43205.
251. Reich, D. and E. Lander, 17 (), pp., *On the allelic spectrum of human disease.* Trends Genet, 2001. **17**: p. 502-510.
252. Hackstein, H., H. Hofmann, A. Bohnert, and G. Bein, *Definition of human interleukin-4 receptor alpha chain haplotypes and allelic association with atopy markers.* Hum Immunol, 1999. **60**(11): p. 1119-27.
253. Grize, L., M. Gassner, B. Wuthrich, B. Bringolf-Isler, K. Takken-Sahli, et al., *Trends in prevalence of asthma, allergic rhinitis and atopic dermatitis in 5-7-year old Swiss children from 1992 to 2001.* Allergy, 2006. **61**(5): p. 556-62.
254. Riedi, C.A., N.A. Rosario, L.F. Ribas, A.S. Backes, G.F. Kleiniibing, et al.,

Increase in prevalence of rhinoconjunctivitis but not asthma and atopic eczema in teenagers. J Investig Allergol Clin Immunol, 2005. **15**(3): p. 183-8.
255. Palmer, L.J., P.R. Burton, J.A. Faux, A.L. James, A. William MUSK, et al., *Independent Inheritance of Serum Immunoglobulin E Concentrations and Airway Responsiveness.* Am J Respir Crit Care Med, 2000. **161**(6): p. 1836-1843.
256. Lebowitz, M.D., R. Barbee, and B. Burrows, *Family concordance of IgE, atopy, and disease.* J Allergy Clin Immunol, 1984. **73**(2): p. 259-64.
257. Mitsuyasu, H., Y. Yanagihara, X.Q. Mao, P.S. Gao, Y. Arinobu, et al., *Cutting edge: dominant effect of Ile50Val variant of the human IL-4 receptor alpha-chain in IgE synthesis.* J Immunol, 1999. **162**(3): p. 1227-31.
258. HapMap, *The International HapMap Consortium. The International HapMap project.* Nature, 2003. **426**(6968): p. 789-96.
259. Oryszczyn, M., E. Bouzigon, J. Maccario, V. Siroux, R. Nadif, et al., *Interrelationships of quantitative asthma-related phenotypes in the Epidemiological Study on the Genetics and Environment of Asthma, Bronchial Hyperresponsiveness, and Atopy.* J Allergy Clin Immunol, 2007. **119**: p. 57-63.
260. Abecasis, G., E. Noguchi, A. Heinzmann, J. Traherne, S. Bhattacharyya, et al., *Extent and distribution of linkage disequilibrium in three genomic regions.* Am J Hum Genet, 2001. **68**: p. 191-97.
261. Lin, H.J., F.J. Tsai, P. Hung, W.C. Chen, H.Y. Chen, et al., *Association of E-Cadherin Gene 3'-UTR C/T Polymorphism with Primary Open Angle Glaucoma.* Opthalmic Res, 2006. **38**(1): p. 44-8.
262. Kuersten, S. and E.B. Goodwin, *The power of the 3[prime] UTR: translational control and development.* Nat Rev Genet, 2003. **4**(8): p. 626-637.
263. Kay, A.B., *The role of T lymphocytes in asthma.* Chem Immunol Allergy, 2006. **91**: p. 59-75.
264. Smit, J.J. and N.W. Lukacs, *A closer look at chemokines and their role in asthmatic responses.* European Journal of Pharmacology, 2006. **533**(1-3): p. 277-288.
265. Matsukura, S., C. Stellato, S.N. Georas, V. Casolaro, J.R. Plitt, et al., *Interleukin-13 Upregulates Eotaxin Expression in Airway Epithelial Cells by a STAT6-Dependent Mechanism.* Am. J. Respir. Cell Mol. Biol., 2001. **24**(6): p. 755-761.
266. Fujisawa, T., Y. Kato, H. Nagase, J. Atsuta, A. Terada, et al., *Chemokines induce eosinophil degranulation through CCR-3.* Journal of Allergy and Clinical Immunology, 2000. **106**(3): p. 507-513.
267. Shinkai, K., M. Mohrs, and R.M. Locksley, *Helper T cells regulate type-2 innate immunity in vivo.* Nature, 2002. **420**(6917): p. 825-9.
268. Lee, J.J., D. Dimina, M.P. Macias, S.I. Ochkur, M.P. McGarry, et al., *Defining a Link with Asthma in Mice Congenitally Deficient in Eosinophils.* Science, 2004. **305**(5691): p. 1773-1776.
269. Zhu, Z., R.J. Homer, Z. Wang, Q. Chen, G.P. Geba, et al., *Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production.* J Clin Invest, 1999. **103**(6): p. 779-88.

270. Zimmermann, N., G.K. Hershey, P.S. Foster, and M.E. Rothenberg, *Chemokines in asthma: cooperative interaction between chemokines and IL-13.* J Allergy Clin Immunol, 2003. **111**(2): p. 227-42; quiz 243.
271. Kelly-Welch, A., E. Hanson, M. Boothby, and A. Keegan, *Interleukin-4 and interleukin-13 signaling connection maps.* Science, 2003. **300**: p. 1527-8.
272. Conne, B., A.M. Stutz, and J. Vassalli, *The 3' untranslated region of messenger RNA: A molecular 'hotspot' for pathology?* Nat Med, 2000. **6**(6): p. 637-41.
273. Di Marco, S., Z. Hel, C. Lachance, H. Furneaux, and D. Radzioch, *Polymorphism in the 3'-untranslated region of TNF alpha mRNA impairs binding of the post-transcriptional regulatory protein HuR to TNF alpha mRNA.* Nucl. Acids Res., 2001. **29**(4): p. 863-871.
274. Niwa, R. and F. Slack, *The evolution of animal microRNA function.* Curr Opin Genet Dev, 2007. **17**(2): p. 145-50.
275. Stutz, A.M. and M. Woisetschlager, *Functional synergism of STAT6 with either NF-kappa B or PU.1 to mediate IL-4-induced activation of IgE germline gene transcription.* Journal of Immunology, 1999. **163**(8): p. 4383-4391.
276. Strachan, D., H. Wong, and T. Spector, *Concordance and interrelationship of atpic diseases and markers of allergic sensitization among adult female twins.* J Allergy Clin Immunol, 2001. **108**(6): p. 901-907.
277. Hytonen, A.M., O. Lowhagen, M. Arvidsson, B. Balder, A.L. Bjork, et al., *Haplotypes of the interleukin-4 receptor alpha chain gene associate with susceptibility to and severity of atopic asthma.* Clin Exp Allergy, 2004. **34**(10): p. 1570-5.
278. Burchard, E., E.K. Silverman, L. Rosenwasser, L. Borish, C. Yandava, et al., *Association between a sequence variant in the IL-4 gene promoter and FEV(1) in asthma.* Am J Respir Crit Care Med, 1999. **160**(3): p. 919-22.
279. *ALLHAT Officers and Coordinators for the ALLHAT Collaborative Research Group. Major outcomes in high-risk hypertensive patients randomized to angiotensin-converting enzyme inhibitor or calcium channel blocker vs diuretic: The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT).* JAMA, 2002. **288**(23): p. 2981-97.
280. Engstrom, G., B. Hedblad, and L. Janzon, *Reduced lung function predicts increased fatality in future cardiac events. A population-based study.* Journal of Internal Medicine, 2006. **260**(6): p. 560-67.
281. Vercelli, D., *Genetics of IL-13 and functional relevance of IL-13 variants.* Curr Opin Allergy Clin Immunol, 2002. **2**(5): p. 389-93.
282. Kumar, R.K., C. Herbert, M. Yang, A.M. Koskinen, A.N. McKenzie, et al., *Role of interleukin-13 in eosinophil accumulation and airway remodelling in a mouse model of chronic asthma.* Clin Exp Allergy, 2002. **32**(7): p. 1104-11.
283. Lee, J.H., N. Kaminski, G. Dolganov, G. Grunig, L. Koth, et al., *Interleukin-13 induces dramatically different transcriptional programs in three human airway cell types.* Am J Respir Cell Mol Biol, 2001. **25**(4): p. 474-85.
284. van der Pouw Kraan, T.C., M. Kucukaycan, A.M. Bakker, J.M. Baggen, J.S. van der Zee, et al., *Chronic obstructive pulmonary disease is associated with the -1055 IL-13 promoter polymorphism.* Genes Immun, 2002. **3**(7): p. 436-9.

285. Sadeghnejad, A., D. Meyers, E. Bleecker, and J. Ohar, *IL13 promoter polymorphism enhances the adverse effect of smoking on lung function.* Am J Respir Crit Care Med, 2007. **175**: p. A563.
286. Rosenwasser, L., D. Klemm, J. Dresback, J. Inamura, J. Mascali, et al., *Promoter polymorphisms in the chromosome 5 gene cluster in asthma and atopy.* Clin Exp Allergy, 1995. **25**(suppl 2): p. 74-78.
287. Nickel, R., V. Casolaro, U. Wahn, K. Beyer, K. Barnes, et al., *Atopic dermatitis is associated with a functional mutation in the promoter of the C-C chemokine RANTES.* **J** Immunol, 2000: p. 1612-16.
288. Stephens, J.C., J.A. Schneider, D.A. Tanguay, J. Choi, T. Acharya, et al., *Haplotype Variation and Linkage Disequilibrium in 313 Human Genes.* Science, 2001. **293**(5529): p. 489-493.
289. Risch, N. and K. Merikangas, *The future of genetic studies of complex human diseases.* Science, 1996. **273**(5281): p. 1516-17.
290. Bernstein, D.I., N. Wang, P. Campo, R. Chakraborty, A. Smith, et al., *Diisocyanate asthma and gene-environment interactions with IL4RA, CD-14, and IL-13 genes.* Ann Allergy Asthma Immunol, 2006. **97**(6): p. 800-6.
291. Faffe, D., T. Whitehead, P. Moore, S. Baraldo, L. Flynt, et al., *IL-13 and IL-4 promote TARC release in human airway smooth muscle cells:role of IL-4 receptor genotype.* Am J Physiol Lung Cell Mol Physiol, 2003. **285**(4): p. L907-14.
292. DoH, *Health Survey for England.* 2004.
293. WHO, *Controlling the global obesity epidemic.* 2001.
294. Deane, S. and A. Thomson, *Obesity and the pulmonologist.* Arch Dis Child, 2006. **91**(2): p. 188-191.
295. Kim, S. and J. Camargo, Carlos A., *Sex-race differences in the relationship between obesity and asthma: the behavioral risk factor surveillance system, 2000.* Annals of Epidemiology, 2003. **13**(10): p. 666-673.
296. Von Behren, J., R. Kreutzer, and A. Hernandez, *Self-reported asthma prevalence in adults in California.* The Journal Of Asthma: Official Journal Of The Association For The Care Of Asthma, 2002. **39**(5): p. 429-440.
297. Shaheen, S.O., J.A. Sterne, S.M. Montgomery, and H. Azima, *Birth weight, body mass index and asthma in young adults.* Thorax, 1999. **54**(5): p. 396-402.
298. Luder, E., R.I. Ehrlich, W.Y.W. Lou, T.A. Melnik, and M. Kattan, *Body mass index and the risk of asthma in adults.* Respiratory Medicine, 2004. **98**(1): p. 29-37.
299. Chinn, S., D. Jarvis, and P. Burney, *Relation of bronchial responsiveness to body mass index in the ECRHS.* Thorax, 2002. **57**(12): p. 1028-1033.
300. Pistelli, F., M. Bottai, G. Viegi, F. Di Pede, L. Carrozzi, et al., *Smooth Reference Equations for Slow Vital Capacity and Flow-Volume Curve Indexes.* Am J Respir Crit Care Med, 2000. **161**(3): p. 899-905.
301. Celedon, J.C., L.J. Palmer, A.A. Litonjua, S.T. Weiss, B. Wang, et al., *Body Mass Index and Asthma in Adults in Families of Subjects with Asthma in Anqing, China.* Am J Respir Crit Care Med, 2001. **164**(10): p. 1835-1840.
302. Chen, Y., D. Rennie, Y. Cormier, and J. Dosman, *Sex Specificity of Asthma Associated With Objectively Measured Body Mass Index and Waist*

Circumference: The Humboldt Study. Chest, 2005. **128**(4): p. 3048-3054.
303. Kronander, U.N., M. Falkenberg, and O. Zetterstrom, *Prevalence and incidence of asthma related to waist circumference and BMI in a Swedish community sample.* Respir Med, 2004. **98**(11): p. 1108-16.
304. Del-Rio-Navarro, B.E., G. Fanghanel, A. Berber, L. Sanchez-Reyes, E. Estrada-Reyes, et al., *The relationship between asthma symptoms and anthropometric markers of overweight in a Hispanic population.* J Investig Allergol Clin Immunol, 2003. **13**(2): p. 118-23.
305. Garcia-Marcos, L., I. Miner Canflanca, J. Batlles Garrido, A. Lopez-Silvarrey Varela, G. Garcia Hernandez, et al., *The relationship of asthma and rhinoconjunctivitis with obesity, exercise and Mediterranean diet in Spanish schoolchildren 6-7 years old.* Thorax, 2007.
306. Rizwan, S., J. Reid, Y. Kelly, P. Bundred, M. Pearson, et al., *Trends in childhood and parental asthma prevalence in Merseyside, 1991-1998.* J Public Health (Oxford), 2004. **26**: p. 337-42.
307. Flaherman, V. and G.W. Rutherford, *A Meta-analysis of the Effect of High Weight on Asthma.* Arch Dis Child, 2006. **91**(4): p. 334-9.
308. Ford, E.S., D.M. Mannino, S.C. Redd, A.H. Mokdad, and J.A. Mott, *Body mass index and asthma incidence among USA adults.* Eur Respir J, 2004. **24**(5): p. 740-744.
309. Xu, B., J. Pekkanen, J. Laitinen, and M.-R. Jarvelin, *Body build from birth to adulthood and risk of asthma.* Eur J Public Health, 2002. **12**(3): p. 166-170.
310. Guerra, S., D.L. Sherrill, A. Bobadilla, F.D. Martinez, and R.A. Barbee, *The Relation of Body Mass Index to Asthma, Chronic Bronchitis, and Emphysema.* Chest, 2002. **122**(4): p. 1256-1263.
311. Beckett, W.S., J. Jacobs D.R., X. Yu, C. Iribarren, and O.D. Williams, *Asthma is associated with weight gain in females but not males, independent of physical activity.* American Journal of Respiratory and Critical Care Medicine, 2001. **164**(11): p. 2045-2050.
312. Huovinen, E., J. Kaprio, and M. Koskenvuo, *Factors associated to lifestyle and risk of adult onset asthma.* Respiratory Medicine, 2003. **97**(3): p. 273-280.
313. Chen, Y., R. Dales, M. Tang, and D. Krewski, *Obesity May Increase the Incidence of Asthma in Women but Not in Men: Longitudinal Observations from the Canadian National Population Health Surveys.* Am. J. Epidemiol., 2002. **155**(3): p. 191-197.
314. Beuther, D.A. and E.R. Sutherland, *Overweight, obesity, and incident asthma: a meta-analysis of prospective epidemiologic studies.* Am J Respir Crit Care Med, 2007. **175**(7): p. 661-6.
315. Litonjua, A.A., D. Sparrow, J.C. Celedon, D. DeMolles, and S.T. Weiss, *Association of body mass index with the development of methacholine airway hyperresponsiveness in men: the Normative Aging Study.* Thorax, 2002. **57**(7): p. 581-585.
316. Nystad, W., H.E. Meyer, P. Nafstad, A. Tverdal, and A. Engeland, *Body mass index in relation to adult asthma among 135,000 Norwegian men and women.* American Journal of Epidemiology, 2004. **160**(10): p. 969-976.
317. Jarvis, D., S. Chinn, J. Potts, and P. Burney, *Association of body mass index*

with respiratory symptoms and atopy: results from the European Community Respiratory Health Survey. Clinical & Experimental Allergy, 2002. **32**(6): p. 831-837.
318. Gilliland, F.D., K. Berhane, T. Islam, R. McConnell, W.J. Gauderman, et al., *Obesity and the Risk of Newly Diagnosed Asthma in School-age Children.* Am. J. Epidemiol., 2003. **158**(5): p. 406-415.
319. Gold, D., A. Damokosh, D. Dockery, and C. Berkey, *Body mass index as a predictor of incident asthma in a prospective ohort of children.* Pediatr Pulmonol, 2003. **36**(6): p. 514-521.
320. Mannino, D.M., J. Mott, J.M. Ferdinands, C.A. Camargo, M. Friedman, et al., *Boys with high body masses have an increased risk of developing asthma: findings from the National Longitudinal Survey of Youth (NLSY).* Int J Obes (Lond), 2006. **30**(1): p. 6-13.
321. Chinn, S. and R.J. Rona, *Can the increase in body mass index explain the rising trend in asthma in children?* Thorax, 2001. **56**(11): p. 845-850.
322. Hancox, R.J., B.J. Milne, R. Poulton, D.R. Taylor, J.M. Greene, et al., *Sex Differences in the Relation between Body Mass Index and Asthma and Atopy in a Birth Cohort.* Am. J. Respir. Crit. Care Med., 2005. **171**(5): p. 440-445.
323. Castro-Rodriguez, J.A., C.J. Holberg, W.J. Morgan, A.L. Wright, and F.D. Martinez, *Increased Incidence of Asthmalike Symptoms in Girls Who Become Overweight or Obese during the School Years.* Am J Respir Crit Care Med, 2001. **163**(6): p. 1344-1349.
324. Macgregor, A.M.C. and R.A. Greenberg, *Effect of surgically induced weight loss on asthma in the morbidly obese.* Obesity Surgery, 1993. **3**(1): p. 15-21.
325. Dhabuwala, A., R.J. Cannan, and R.S. Stubbs, *Improvement in co-morbidities following weight loss from gastric bypass surgery.* Obesity Surgery: The Official Journal Of The American Society For Bariatric Surgery And Of The Obesity Surgery Society Of Australia And New Zealand, 2000. **10**(5): p. 428-435.
326. Schachter, L.M., J.K. Peat, and C.M. Salome, *Asthma and atopy in overweight children.* Thorax, 2003. **58**(12): p. 1031-1035.
327. Huang, S., G. Shiao, and P. Chou, *Association between body mass index and allergy in teenage girls in Taiwan.* Clin Exp Allergy, 1999. **29**(3): p. 329-9.
328. Matricardi, P.M., C. Gruber, U. Wahn, and S. Lau, *The asthma-obesity link in childhood: open questions, complex evidence, a few answers only.* Clin Exp Allergy, 2007. **37**(4): p. 476-84.
329. Schachter, L.M., C.M. Salome, J.K. Peat, and A.J. Woolcock, *Obesity is a risk for asthma and wheeze but not airway hyperresponsiveness.* Thorax, 2001. **56**(1): p. 4-8.
330. Xu, B., M.R. Jarvelin, and J. Pekkanen, *Body build and atopy.* J Allergy Clin Immunol, 2000. **105**(2 Pt 1): p. 393-4.
331. von Kries, R., M. Hermann, V. Grunert, and E. von Mutius, *Is obesity a risk factor for childhood asthma?* Allergy, 2001. **56**(4): p. 318-22.
332. von Mutius, E., J. Schwartz, L.M. Neas, D. Dockery, and S.T. Weiss, *Relation of body mass index to asthma and atopy in children: the National Health and Nutrition Examination Study III.* Thorax, 2001. **56**(11): p. 835-838.

333. Wickens, K., D. Barry, A. Friezema, R. Rhodius, N. Bone, et al., *Obesity and asthma in 11-12 year old New Zealand children in 1989 and 2000.* Thorax, 2005. **60**(1): p. 7-12.
334. Gold, D.R., A.I. Damokosh, D.W. Dockery, and C.S. Berkey, *Body-mass index as a predictor of incident asthma in a prospective cohort of children.* Pediatric Pulmonology, 2003. **36**(6): p. 514-521.
335. Calvert, J. and P. Burney, *Effect of body mass on exercise-induced bronchospasm and atopy in African children.* Journal of Allergy and Clinical Immunology, 2005. **116**(4): p. 773-779.
336. Gallagher, D., M. Visser, D. Sepulveda, R. Pierson, T. Harris, et al., *How useful is body mass index for comparison of body fatness across age, sex, and ethnic groups?* Am J Epidemiol, 1996. **143**(3): p. 228-39.
337. Alberti, K.G.M., P. Zimmet, and J. Shaw, *The metabolic syndrome--a new worldwide definition.* The Lancet, 2006. **366**(9491): p. 1059-1062.
338. WHO, *Definition, diagnosis and classification of diabetes mellitus and its complications*, in *Report of a WHO consultation.* 1999.
339. WHO, *Measuring obesity: classification and distribution of anthropometric data.* 1989, World Health Organisation: Copenhagen.
340. Simard, B., H. Turcotte, P. Marceau, S. Biron, F.S. Hould, et al., *Asthma and sleep apnea in patients with morbid obesity: outcome after bariatric surgery.* Obes Surg, 2004. **14**(10): p. 1381-8.
341. McLachlan, C.R., R. Poulton, G. Car, J. Cowan, S. Filsell, et al., *Adiposity, asthma, and airway inflammation.* Journal of Allergy and Clinical Immunology, 2007. **119**(3): p. 634-639.
342. Irei, A.V., K. Takahashi, D.S.N.T. Le, P.T.N. Ha, N.T.K. Hung, et al., *Obesity is associated with increased risk of allergy in Vietnamese adolescents.* 2005. **59**(4): p. 571-577.
343. Sun, S., W. Chumlea, S.B. Heymsfield, H. Lukaski, D. Schoeller, et al., *Development of bioelectrical impedance prediction equations for body composition with the use of a multicomponenet model for use in epidemiological surveys.* Am J Clin Nutr, 2003. **77**: p. 331-40.
344. Wattanapenpaiboon, N., W. Lukito, B. Strauss, B. Hsuhage, M. Wahloyist, et al., *Agreement of skinfold measurement and bioelectrical impedance analysis methods with dual energy x-ray absorptiometry in estimating total body fat in Anglo-Celtic Americans.* Int J Obes Relat Metab Disord, 1998. **22**: p. 854-60.
345. Wannamethee, S.G., A.G. Shaper, and P.H. Whincup, *Body fat distribution, body composition, and respiratory function in elderly men.* Am J Clin Nutr, 2005. **82**(5): p. 996-1003.
346. Zhu, S., Z. Wang, W. Shen, S.B. Heymsfield, and S. Heshka, *Percentage body fat ranges associated with metabolic syndrome risk: results based on the third National Health and Nutrition Examination Survey (1988-1994).* Am J Clin Nutr, 2003. **78**(2): p. 228-235.
347. Egger, M., G. Davey Smith, M. Schneider, and C. Minder, *Bias in meta-analysis detected by simple graphical test.* BMJ, 1997. **315**: p. 629-34.
348. Sin, D.D., R.L. Jones, and S.F. Paul Man, *Obesity is a risk factor for dyspnea but not for airflow obstruction.* Archives of Internal Medicine, 2002. **162**(13):

p. 1477-1481.
349. Perez-Padilla, R., R. Rojas, V. Torres, V. Borja-Aburto, G. Olaiz, et al., *Obesity among children residing in Mexico City and its impact on lung function: a comparison with Mexican-Americans.* Arch Med Res, 2006. **37**(1): p. 165-71.
350. Lazarus, R., G. Colditz, C.S. Berkey, and F.E. Speizer, *Effects of body fat on ventilatory function in children and adolescents: Cross-sectional findings from a random population sample of school children.* Pediatric Pulmonology, 1997. **24**(3): p. 187-194.
351. Chen, R., H. Tunstall-Pedoe, C. Bolton-Smith, M.K. Hannah, and C. Morrison, *Association of Dietary Antioxidants and Waist Circumference with Pulmonary Function and Airway Obstruction.* Am. J. Epidemiol., 2001. **153**(2): p. 157-163.
352. Busetto, L. and G. Sergi, *Visceral fat and respiratory complications.* Diabetes Obes Metab, 2005. 7(4): p. 301-6.
353. Harik-Khan, R.I., J.L. Fleg, D.C. Muller, and R.A. Wise, *The effect of anthropometric and socioeconomic factors on the racial difference in lung function.* Am J Respir Crit Care Med, 2001. **164**(9): p. 1647-1654.
354. Lazarus, R., D. Sparrow, and S. Weiss, *Effects of obesity and fat distribution on ventilatory function: the normative aging study.* Chest, 1997. **111**(4): p. 891-898.
355. Sahebjami, H. and P. Gartside, *Pulmonary function in obese subjects with a normal FEV1/FVC ratio.* Chest, 1996. **110**(6): p. 1425-1429.
356. Rubinstein, I., N. Zamel, L. DuBarry, and V. Hoffstein, *Airflow limitation in morbidly obese, nonsmoking men.* Ann Intern Med, 1990. **112**(11): p. 828-32.
357. Mansell, A.L., N. Walders, M.Z. Wamboldt, R. Carter, D.W. Steele, et al., *Effect of body mass index on response to methacholine bronchial provocation in healthy and asthmatic adolescents.* Pediatr Pulmonol, 2006. **41**(5): p. 434-40.
358. De Lorenzo, A., C. Maiolo, E.I. Mohamed, A. Andreoli, P. Petrone-De Luca, et al., *Body Composition Analysis and Changes in Airways Function in Obese Adults After Hypocaloric Diet.* Chest, 2001. **119**(5): p. 1409-1415.
359. Nava, S., *Monitoring respiratory muscles.* Monaldi Arch Chest Dis, 1998. **53**(6): p. 640-3.
360. Do, M., H. Jeong, B. Choi, L. Hunter, S. Langley, et al., *Inflammatory gene expression patterns revealed by DNA microarray analysis in TNF-alpha-treated SGBS human adipocytes.* Yonsei Med J, 2006. **47**(5): p. 729-36.
361. Cottam, D., P. Schafer, G. Shaftan, L. Velcu, and L. Angus, *Effect of surgically-induced weight loss on leukocyte indicators of chronic inflammation in morbid obesity.* Obes Surg, 2002. **12**(3): p. 335-42.
362. Hashimoto, I., J. Wada, A. Hida, M. Baba, N. Miyatake, et al., *Elevated Serum Monocyte Chemoattractant Protein-4 and Chronic Inflammation in Overweight Subjects.* Obesity, 2006. **14**(5): p. 799-811.
363. Moerlosse, K., R. Pauwels, and G.F. Joos, *Short-term cigarette smoke exposure enhances allergic airway inflammation in mice.* Am J Respir Crit Care Med, 2005. **172**(2): p. 168-72.

364. Harrison, B., *Psychosocial aspects of asthma in adults.* Thorax, 1998. **53**(6): p. 519-25.
365. Akerman, M., C. Calacanis, and M. Madsen, *Relationship between asthma severity and obesity.* J Asthma, 2004. **41**(5): p. 521-6.
366. Chanez, P., S.E. Wenzel, G.P. Anderson, J.M. Anto, E.H. Bel, et al., *Severe asthma in adults: What are the important questions?* J Allergy Clin Immunol, 2007.
367. Wang, W., B. Barratt, D.G. Clayton, and J.A. Todd, *Genome-wide association studies: theoretical and practical concerns.* Nat Rev Genet, 2005. **6**: p. 109-118.
368. Niwa, N. and F. Slack, *The evolution of animal microRNA function.* Curr Opin Genetic Devel, 2007.
369. Andrews, A.-L., F. Bucchieri, K. Arima, K. Izuhara, S.T. Holgate, et al., *Effect of IL-13 receptor [alpha]2 levels on the biological activity of IL-13 variant R110Q.* Journal of Allergy and Clinical Immunology, 2007. **120**(1): p. 91-97.
370. Wenzel, S. and S.T. Holgate, *The Mouse Trap: It Still Yields Few Answers in Asthma.* Am. J. Respir. Crit. Care Med., 2006. **174**(11): p. 1173-1176.
371. Haitchi, H.M. and S.T. Holgate, *New strategies in the treatment and prevention of allergic diseases.* Expert Opin Investig Drugs, 2004. **13**(2): p. 107-24.
372. Zavorotinskaya, T., A. Tomkinson, and J.E. Murphy, *Treatment of experimental asthma by long-term gene therapy directed against IL-4 and IL-13.* Mol Ther, 2003. **7**(2): p. 155-62.
373. Karras, J.G., J.R. Crosby, M. Guha, D. Tung, D.A. Miller, et al., *Anti-Inflammatory Activity of Inhaled IL-4 Receptor-alpha Antisense Oligonucleotide in Mice.* Am. J. Respir. Cell Mol. Biol., 2007. **36**(3): p. 276-285.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS THESIS

1. G.A. Davies, J.M. Hopkin. Aetiology of Asthma. In: Johnson S, ed. Asthma: An Atlas of Investigation and Diagnosis. Oxford, UK: Clinical Publishing Ltd; 2007.

2. G.A. Davies, C.A. Thornton, M. Moller, D. Gopalakrishnan, P. Bikhchandani, S. Benjamin, A. Benton, M.B. Gravenor, J.M. Hopkin. IL-13 Signalling Polymorphisms Predict Asthma and Immune Phenotypes in an Unselected Population. Am J Resp Crit Care Med 2007, 175 (Abstracts Issue): A458.

3. G.A. Davies, M. Moller, D. Gopalakrishnan, P. Bikhchandani, S. Benjamin, M. Sansbury, M.B. Gravenor, J.M. Hopkin. IL-13 Signalling Polymorphisms Predict Lung Function in an Unselected Population. Am J Resp Crit Care Med 2007, 175 (Abstracts Issue): A832.

4. G.A. Davies, M. Moller, D. Gopalakrishnan, P. Bikhchandani, S. Benjamin, M Sansbury, M.B. Gravenor, J.M. Hopkin. IL-13 signalling polymorphisms predict asthma and atopy phenotypes in an unselected population. Thorax 2006; Dec (Abstracts Issue): S124 (Oral presentation).

5. G.A. Davies, C.A. Thornton, D. Gopalakrishnan, P. Bikhchandani, S. Benjamin, M.B. Gravenor, A. Benton, J.M. Hopkin. Adiposity associates with asthma, IgE and eotaxin levels. Thorax 2006; Dec (Abstracts Issue): S83 (Oral presentation).

6. G.A. Davies, M.B. Gravenor, D. Gopalakrishnan, P. Bikhchandani, S. Benjamin, M. Moller, J.M. Hopkin. Adiposity predicts asthma and pulmonary function in a young adult population. Thorax 2006; Dec (Abstracts Issue): P219.

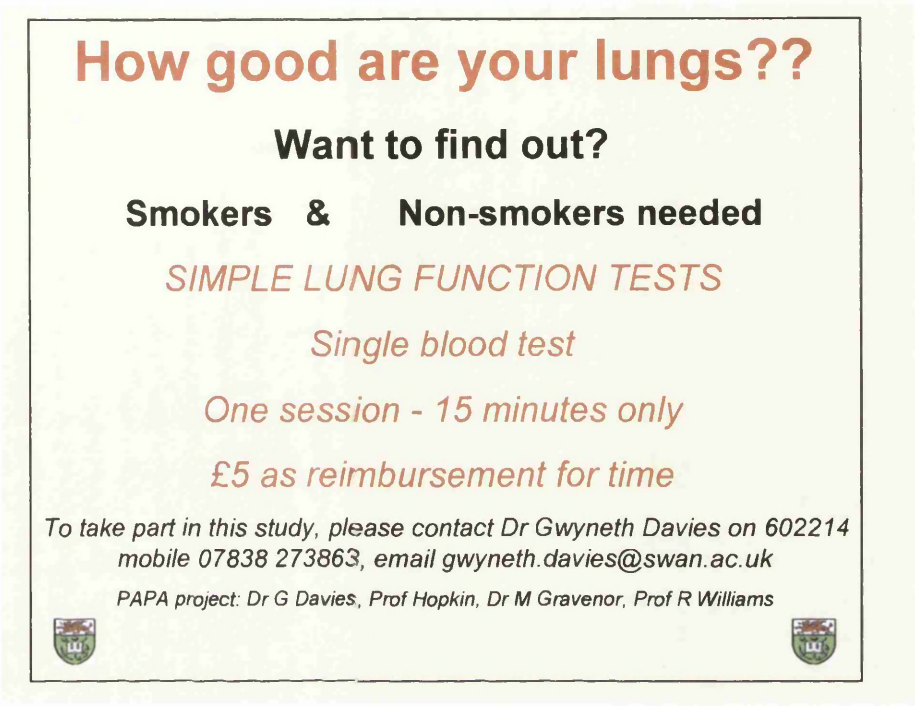
7. Davies GA, Gravenor MB, Gopalakrishnan D, Bikchandani P, Benjamin S, Williams R, Hopkin JM. Body fat predicts asthma and pulmonary function. *Proceedings of the American Thoracic Society* 2006; 3 (Oral presentation).

Other presentations

1. The role of adiposity in asthma and Th2 immunity. *Welsh Thoracic Society* October 2006.

2. Obesity and asthma. *Welsh Thoracic Society* April 2006.

APPENDIX IA **Poster advertisement**



APPENDIX IB Email advertisement

Dear Student Colleague,

How good are your lungs ?

You are invited to take part in a simple study which will take only around 15 minutes of your time. The purpose of the study is to investigate whether certain genetic markers can predict lung function and other markers of asthma in a young adult population.

v We are offering simple lung function tests and can discuss the results with you if requested

v We will check basic screening tests of weight and body mass index: we can discuss the results with you and let you know your optimum weight if requested

v A single blood test is needed

v We are recruiting all students (aged 18-30), whether you have asthma or not, whether you smoke or not

v All participants will be receive £5 as reimbursement for their time

Interested?

Please contact Dr Gwyneth Davies to arrange a convenient time or for further information. Tel 01792 602214 (or extension 2214 if phoning internally) or mobile 07838 273863 or email gwyneth.davies@swan.ac.uk.

We hope to hear from you soon!

Kind regards,

Dr G Davies Professor J Hopkin Dr M Sansbury Dr M Gravenor
PAPA, Poblogaeth Asthma Prifysgol Abertawe: School of Medicine, Swansea University

APPENDIX II

CONSENT FORM

Project Reference Number 04/WMW02/29
Version (2)
Date: 06/06/2005

Participant Identification number for this trial .....
N.B. Two copies should be made: (1) participant (2) researcher

Title of Project: ***The Genetic Prediction of Asthma***

Name of Researchers: ***Dr Gwyneth Davies, Professor Julian Hopkin Dr M Sansbury, Dr Divya Gopalakrishnan, Dr Silas Benjamin, Dr Mike Gravenor Professor Rhys Williams***

Contact Telephone Number: **01792 602214**

Please Initial

1. I confirm that I have read and understood the information sheet dated 06/06/05 (version 2) for the above study and have had the opportunity to ask questions. ☐

2. 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. ☐

3. I agree that my blood samples can be analysed for possible genes relating to asthma. ☐

4. I agree that my blood samples can be kept for 25 years for possible further analyses of genes, as more information becomes available. I understand that my samples are anonymous and I am not able to be identified from them. ☐

5. I understand that I may be asked to give a further blood sample on one occasion. ☐

6. I agree to take part in the above study. ☐

Name of Participant	**Date**	**Signature**
Name of Person taking consent	**Date**	**Signature**

APPENDIX III

DATA SHEET

Volunteer number	
Height (to 0.1cm)	**cm**
Waist circumference (to 0.1cm)	**cm**
Hip circumference (to 0.1cm)	**cm**
Weight (to 0.1 kg)	**Kg**
% Body fat	**%**

	MEASURED	**% PREDICTED**
FVC	**L**	**%**
FEV1	**L**	**%**
FEV1/FVC	**%**	

	Please tick
BLOODS	
QUESTIONNAIRE	
ASTHMA INTERVIEW QUESTIONNAIRE, IF APPLICABLE	

APPENDIX IV

MODIFIED IUATLD BRONCHIAL SYMPTOMS QUESTIONNAIRE

To answer the questions, please choose the appropriate box; IF YOU ARE UNSURE OF THE ANSWER, PLEASE CHOOSE 'NO'

Wheeze and tightness in the chest

1. Have you, at any time in the last 12 months, had wheezing or whistling in your chest? No [ ] Yes [ ]

   If no, go to question 2. If yes to 1:

   1a. Have you been at all breathless when the wheezing noise was present? No [ ] Yes [ ]

   1b. Have you had this wheezing or whistling when you did *not* have a cold? No [ ] Yes [ ]

2. 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

3. 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 [ ]

4. 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 [ ]

5. 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

6. Have you, at any time in the last 12 months, been woken at night by an attack of coughing?

No	Yes
[ ]	[ ]

7. Do you usually cough first thing in the morning?

No	Yes
[ ]	[ ]

If yes to 7:

7a. Do you have a cough like this most mornings for as much as 3 months per year?

No	Yes
[ ]	[ ]

7b. How many years have you had this cough?
_____YEARS

8. Do you usually bring up phlegm from your chest first thing in the morning?

No	Yes
[ ]	[ ]

If yes to 8:

8a. Do you have phlegm like this most mornings for as much as 3 months per year?

No	Yes
[ ]	[ ]

8b. How many years have you had this phlegm?
_____YEARS

Breathing

9. 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 [ ]

Hayfever and eczema

10. Do you have any nasal allergies including hayfever?

No [ ] Yes [ ]

11. Have you ever had eczema? _____

No [ ] Yes [ ]

If yes to 11:

11a) Do you still have it?

No [ ] Yes [ ]

Asthma

12. Have you ever had asthma?

No [ ] Yes [ ]

PLEASE LET US KNOW IF YOU ANSWER YES TO THIS QUESTION AS WE WILL NEED SOME FURTHER INFORMATION FROM YOU

If no to 12, go to question 13. If yes to 12:

12a) Do you still have it?

No [ ] Yes [ ]

12b) Was it confirmed by a doctor?

No [ ] Yes [ ]

12c) At what age did it start? _____ Age in years

12d. If you no longer have it, at what age did it stop? _____ Age in years

13. Have you had an attack of asthma at any time in the last 12 months?

No [ ] Yes [ ]

14. Are you currently taking any medicines (including inhalers, aerosols or tablets) for asthma?

No [ ] Yes [ ]

Other conditions

15. Do you have any medical conditions (apart from asthma/hayfever/eczema)?

No	Yes
[ ]	[ ]

If yes to 15:

15a) Please list medical conditions

_____
_____
_____
_____

16. Are you on any medication (apart from any for asthma)?

No	Yes
[ ]	[ ]

If yes to 16:

16a) Please list medications

_____
_____
_____
_____

Smoking

17. Have you ever smoked for as long as one year?

No	Yes
[ ]	[ ]

If yes to 17: Check one

18. Do (did) you usually smoke:

cigarettes? [ ]
pipe? [ ]
cigars? [ ]

Other (precise please)_____

19. How many cigarettes do (did) you smoke each day, on average?_____

20. Have you: Check one:

continued to smoke?	[ ]
given up smoking altogether, but less than 4 weeks ago?	[ ]
given up smoking altogether, at least 4 weeks ago?	[ ]

21. For how many years have you smoked (did you smoke) _____

Your family

22. Did your (natural) mother ever have asthma?

No	Yes	Don't know
[ ]	[ ]	[ ]

23. Did your (natural) mother ever have eczema, skin or nasal allergy or hayfever?

No	Yes	Don't know
[ ]	[ ]	[ ]

24. Did your (natural) father ever have asthma?

No	Yes	Don't know
[ ]	[ ]	[ ]

25. Did your (natural) father ever have eczema, skin or nasal allergy or hayfever?

No	Yes	Don't know
[ ]	[ ]	[ ]

26. How many brothers do or did you have? NUMBER

IF 'NONE' GO TO QUESTION 27, IF 'YES':

26.1 How many ***older*** brothers?

26.2 How many ***younger*** brothers?

26.3 How many of your brothers ever had asthma?

26.4 How many of your ***other*** brothers ever had eczema, skin or nasal allergy or hayfever?

27. How many sisters do or did you have? NUMBER

IF 'NONE' GO TO QUESTION 28, IF 'YES':
27.1 How many ***older*** sisters?
27.2 How many ***younger*** sisters?

27.3 How many of your sisters ever had asthma?
27.4 How many of your ***other*** sisters ever had eczema, skin or nasal allergy or hayfever?

More about yourself

28. When were you born day month year

29. Are you Male Female

30. What is today's date? day month year

31. How old are you Years

32. Are you a student (undergraduate/postgraduate)?
No [ ] Yes [ ]

33. 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)]_____

34 . Contact details:

NAME: _____ _____ _____
(Last) (First) (Middle initial)

CONTACT ADDRESS:

_____

_____

PHONE NUMBER: _____

E MAIL: _____

Original questionnaire prepared for the Respiratory Disease Committee of the International Union Against Tuberculosis and Lung Disease (UNION)

Study reference for UNION questionnaire and validation:
Burney PG, Laitinen LA, Perdrizet S, Huckauf H, Tattersfield AE, Chinn S, et al. Validity and repeatability of the IUATLD (1984) bronchial questionnaire : an international comparison. Eur Respir J,1989; 2: 940-5

APPENDIX V

MODIFIED ECRHS II INTERVIEW QUESTIONNAIRE

FOR VOLUNTEERS WITH ASTHMA

Volunteer number

Interviewer number

Date

DAY MONTH YEAR

I AM GOING TO ASK YOU SOME QUESTIONS ABOUT YOUR ASTHMA. WHEREVER POSSIBLE, I WOULD LIKE YOU TO ANSWER 'YES' OR 'NO'.

1. Have you had an attack of asthma *in the last **12 months***? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 1.3, IF YES

1.1 How many attacks of asthma have you had in the last ***12 months***? ___ ATTACKS

1.2 How many attacks of asthma have you had in the last ***3 months***? ___ ATTACKS
GO TO QUESTION 2

1.3 How old were you when you had your most recent attack of asthma? YEARS ☐☐

2. 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	☐

3. How often have you had trouble with your breathing because of your asthma

in the last ***3 months?***

	TICK ONE BOX ONLY	
continuously	1	☐
about once a day	2	☐
at least once a week, but less than once a day	3	☐
less than once a week	4	☐
not at all	5	☐

4. Do you have written instructions from your doctor on how to manage your asthma if it gets worse or if you have an attack?

NO	YES
☐	☐

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 NO' GO TO QUESTION 7, IF 'YES':

Which of the following have you used in the last ***12 months***?

	NO	YES
6.1 short acting **beta-2-agonist inhalers**	☐	☐

(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	☐
b) in short courses	2	☐
c) continuously	3	☐
d) not at all	4	☐

If answer to 6.1.4 is <u>when needed</u>:

6.1.5 Number of puffs per month ☐☐

If answer to 6.1.4 is in <u>short courses</u>

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 <u>continuously</u>

6.1.9 number of puffs per day ☐☐

6.2 **long acting beta-2-agonist inhalers** NO ☐ YES ☐

(Please include combinations that include beta 2 and steroids in section 76.5)

6.2.1 If used, which one? _____ ☐☐

6.2.2 What type of inhaler do you use?

6.2.3. What is the dose per puff (in micrograms)? ☐☐

6.2.4. In the last 3 months, how have you used them:

	TICK ONE BOX ONLY
a) when needed	1 ☐
b) in short courses	2 ☐
c) continuously	3 ☐
d) not at all	4 ☐

If answer to 6.2.4 is <u>when needed</u>:
6.2.5 Number of puffs per month ☐☐

If answer to 6.2.4 is <u>in short courses</u>
6.2.6 number of courses ☐☐

6.2.7 number of puffs per day ☐☐
6.2.8 average number of days per month ☐☐
If answer to 6.2.4 is <u>continuously</u>
6.2.9 number of puffs per day ☐☐

NO ☐ YES ☐

6.3 **non-specific adrenoreceptor agonist inhalers**

6.3.1 If used, which one? _____ ☐☐

NO ☐ YES ☐

6.4 **anti-muscarinic inhalers**

6.4.1 If used, which one? _____ ☐☐

6.4.2 What type of inhaler do you use?

6.4.3. What is the dose per puff (in micrograms)? ☐☐☐

6.4.4. In the last 3 months, how have you used them:

	TICK ONE BOX ONLY
a) when needed	1 ☐
b) in short courses	2 ☐
c) continuously	3 ☐
d) not at all	4 ☐

*If answer to 6.4 .4 is **when needed**:*

6.4.5 Number of puffs per month

*If answer to 6.4.4 is in **short courses***

6.4.6 number of courses

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

6.5 **inhaled steroids** NO YES

(*if combined B2 and steroid please insert inhaled steroid dose*)

6.5.1 If used, which one? _____

6.5.2 What type of inhaler do you use?

6.5.3. What is the dose per puff (in micrograms)?

6.5.4. In the last 3 months, how have you used them:

TICK ONE BOX ONLY

a) when needed	1
b) in short courses	2
c) continuously	3
d) not at all	4

*If answer to6.5.4 is **when needed**:*

6.5.5 Number of puffs per month

*If answer to 6.5.4 is in **short courses***

6.5.6 number of courses

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

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

TICK ONE BOX ONLY

a) when needed	1	☐
b) in short courses	2	☐
c) continuously	3	☐
d) not at all	4	☐

If answer to 6.6.3 is <u>when needed</u>:

6.6.4 Number of puffs per month ☐☐

If answer to* 6.6.3 *is in <u>short courses</u>

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 <u>continuously</u>

6.6.8 number of puffs per day ☐☐

6.7 **inhaled compounds** NO ☐ YES ☐

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 ☐

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

TICK ONE BOX ONLY

a) when needed	1	☐
b) in short courses	2	☐
c) continuously	3	☐
d) not at all	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** NO ☐ 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

TICK ONE BOX ONLY

a) when needed 1 ☐
b) in short courses 2 ☐
c) continuously 3 ☐
d) not at all 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** NO ☐ 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	1 ☐
b) in short courses	2 ☐
c) continuously	3 ☐
d) not at all	4 ☐

If answer to 7.3.3 is <u>when needed</u>:

7.3.4 Number of tablets per month ☐☐

If answer to 7.3.3 is in <u>short courses</u>

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 <u>continuously</u>

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

TICK ONE BOX ONLY

a) when needed	1 ☐
b) in short courses	2 ☐
c) continuously	3 ☐
d) not at all	4 ☐

If answer to 7.4.3 is <u>when needed</u>:

7.4.4 Number of tablets per month ☐☐

If answer to 7.4.3 is in <u>short courses</u>

7.4.5 number of courses ☐☐

7.4.6 tablets per day ☐☐

7.4.7 average number of days per month ☐☐

If answer to 7.4.3 is <u>continuously</u>

7.4.8 tablets per day ☐☐

7.5 **ketotifen** NO YES

7.5.1 If used, which one? _____

7.5.2. What dose of tablet?

7.5.3. In the last 3 months, how have you used them

TICK ONE BOX ONLY

a) when needed	1
b) in short courses	2
c) continuously	3
d) not at all	4

If answer to 7.5.3 is <u>when needed</u>:

7.5.4 Number of tablets per month

If answer to 7.5.3 is in <u>short courses</u>

7.5.5 number of courses

7.5.6 tablets per day

7.5.7 average number of days per month

If answer to 7.5.3 is <u>continuously</u>

7.5.8 tablets per day

8. Have you ever used inhaled steroids (show list)? NO YES

IF NO GO TO QUESTION 9, IF YES

YEARS

8.1. How old were you when you first started to use inhaled steroids?

8.2. Have you now stopped using inhaled steroids? NO YES

IF NO, GO TO Q8.3, IF YES

YEARS

8.2.1 How old were you when you stopped using inhaled steroids?

8.3 Have (did) you used inhaled steroids ***every year*** since you started using them? NO YES

IF NO GO TO QUESTION 8.4, IF YES

MONTHS

8.3.1. On average how many months each year have you taken them (or did you take them)?

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 ☐☐

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 ☐

IF NO GO TO 10, 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 ☐

IF NO GO TO Q11, 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 ☐☐

10.2 Have you spent a night in ITU because of asthma, shortness of breath or wheezing in the ***last 12 months***? NO ☐ YES ☐

IF NO GO TO Q11, IF YES

10.2.1 How many nights have you spent in ITU because of asthma, shortness of breath or wheezing in the ***last 12 months***? NIGHTS ☐☐

11. Have you been seen by a doctor because of asthma, shortness of breath or wheezing in the ***last 12 months***? NO ☐ YES ☐

IF NO GO TO Q12, IF YES

11.1 Have you been seen by a general practitioner because of asthma, shortness of breath or wheezing in the ***last 12 months***? NO ☐ YES ☐

IF NO GO TO Q11.2, IF YES

11.1.1 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 ☐☐

11.2 Have you seen a specialist (chest physician, allergy specialist, internal medicine specialist, ENT doctor) because of asthma, shortness of breath or wheezing in the ***last 12 months***? NO ☐ YES ☐

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 ☐

IF NO YOU HAVE FINISHED THE QUESTIONNAIRE, IF YES

12.1 How many days on average each month? ☐☐

END

APPENDIX VI

Table 9.1 Summary of variants where overall association with dichotomous outcomes approached significance with p<.05 for difference between individual pairs of genotypes.

	Location	*Outcome OR (95% CI)*	*P value*
IL13			
SNP 1	Promoter	Current eczema	.131[1]
	-1024C/T	2.19 (1.02-4.71)	.044[2]
SNP 10	Promoter	Current eczema	.121
	-1512A/C	2.28 (1.01-5.12)	.046
IL4RA			
SNP 12	Exon 12 Pro478Val	Asthma ever	.134
G/G vs A/A		1.40 (1.00-1.97)	.049
SNP 5	Exon 5	Hayfever	.134
	Ile50Val	1.38 (1.01-1.89)	.046
STAT6			
SNP 1	3'UTR	Asthma ever	.091
A/A vs G/G		1.75 (1.01-3.02)	.029
SNP 12	Intron 17	Eczema ever	.084
C/C vs T/T		1.52 (1.05-2.19)	.027
SNP 2	3'UTR	Current eczema	.125
G/G vs A/A		1.70 (1.02-2.83)	.043

[1] Overall p value for association at this locus, after adjusting for relevant covariates.

[2] P value between individual genotypes shown.

Table 9.2 Summary of variants where overall association with quantitative outcomes approached significance with p<.05 for difference individual pairs of genotypes.

	Location	*Outcome*	*P value*
IL13			
SNP 10	Promoter	FEV1	.067[1]
A/C vs A/A	-1512A/C		.021[2]
IL4RA			
SNP 7	Intron 8	Eosinophil count	.067
C/G vs C/C			.040
SNP 13	Exon 12	FEF 25-75	.102
A/G vs G/G	Arg551Gln		.034
		% FEF 25-75	.125
A/G vs G/G			.042
SNP 16	3'UTR	FEV1/FVC	.051
C/T vs C/C			.015
STAT6			
SNP 1	3'UTR	% FEV1	.128
A/G vs A/A			.044
G/G vs A/A		% FEF 25-75	.135
			.046

[1] Overall p value for association at this locus, after adjusting for relevant covariates.

[2] P value between individual genotypes shown.