GENETIC EPIDEMIOLOGICAL STUDIES
OF LATE ONSET AIRFLOW OBSTRUCTION

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Thesis submitted to the University of Leicester for the degree of
Doctor of Medicine
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Introduction

Airflow Obstruction is common in older persons and frequently under diagnosed (Bannerjee DK, 1987; Allen SC, 1998, Renwick DS, 1996). The term refers to a spectrum of chronic respiratory disorders characterised by cough, dyspnoea, mucus hypersecretion, wheeze and impaired pulmonary function tests. Patients may be diagnosed as having late onset asthma, chronic obstructive pulmonary disease (COPD), chronic bronchitis, emphysema or fixed airflow obstruction of the elderly. In the UK in 1992 COPD alone was the attributed cause of death in 26,033 individuals (Anderson N, 1993). It is also a major cause of morbidity, accounting for 680 hospital admissions, 9,600 in-patient hospital bed days in the average UK health district each year (OPCS, 1992).

Recent studies have used molecular genetic techniques to improve our understanding of a number of late onset disorders, most notably Alzheimer's disease (Corder EH, 1993) and non-insulin dependant diabetes mellitus (Risch N, 1992). These studies have the potential to yield new information about disease pathogenesis that may be of great importance for the development of future treatments.

My interest is in the use of molecular genetic techniques to investigate the spectrum of disorders that constitute 'late onset airflow obstruction'. Chapter 1 introduces key concepts and reviews the current literature and discusses methodological approaches and introduces my study design and methods. Chapter 2 reviews the study methodology. Chapter 3 includes data on the study population and its characteristics. In Chapters 4, 5 and 6 results from a series of six candidate gene studies are presented. Finally, I shall discuss implications for future study in the genetic investigation of late onset disorders, in particular Chronic Obstructive Pulmonary Disease (COPD), and present a pilot project that will be used in planning further extensions of the work.
Aims

The aim of this thesis was

(i) to establish that genetic epidemiological approaches are a feasible approach to the investigation of a chronic late onset disorder

(ii) to establish a collection of genetic samples from well characterised older adults with late onset airflow obstruction and appropriate controls for the purposes of genetic association studies

(ii) to use this DNA bank to begin investigations into the role of specific candidate genes in the pathogenesis of late onset airflow obstruction, COPD, and related phenotypes.

Hypotheses

I hypothesize that: -

(a) Late onset asthma and COPD are in part genetic diseases

(b) Genetic polymorphisms associated with the juvenile onset asthmatic phenotype are also important in the pathogenesis of late onset airflow obstruction.
PART A The spectrum of obstructive airways disease in the older patient

1.1 Disease Definitions

Disease definitions of airflow obstruction have a great potential for diagnostic confusion. Following the increased use of pulmonary function tests in the 1950's, the CIBA Symposium of 1958 attempted to precisely define emphysema, chronic bronchitis and asthma (CIBA guest symposium, 1959). Emphysema was defined on the basis of pathological lung changes; chronic bronchitis was defined by a cluster of symptoms. No agreement was reached regarding the definition of asthma, and this dilemma still exists. The symposium did result in the development of a new term 'generalised obstructive lung disease' which was used to distinguish emphysema and chronic bronchitis from asthma. Today the term chronic obstructive pulmonary disease (COPD) is more widely used to describe this group of individuals in whom the airflow obstruction is of uncertain aetiology. There is no international consensus for the definition of COPD (Table 1.1). It is characterised by airflow obstruction with limited reversibility and a progressive downhill course. Although a significant smoking history is usually present, it is not essential. Spirometry indicating airflow obstruction and reduced forced expiratory volume in 1 second (FEV1) is a core feature of the definitions but spirometric criteria differ. The publication of the Global Initiative for Chronic Obstructive Lung Disease (GOLD) workshop report may help to resolve some of the inconsistencies regarding COPD definition (National Heart, Lung and Blood Institute, 2001). However, none of the definitions address appropriate spirometric criteria for use in older populations.
<table>
<thead>
<tr>
<th>Organisation</th>
<th>Definition</th>
<th>Spirometric criteria</th>
</tr>
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<tbody>
<tr>
<td><strong>British Thoracic Society</strong></td>
<td>Chronic airflow obstruction which does not change over several months</td>
<td>FEV1 as percentage of predicted value (FEV1%)</td>
</tr>
<tr>
<td></td>
<td>slowly progressive disorder, <em>Usually</em> caused by tobacco smoke</td>
<td>60-79 = mild</td>
</tr>
<tr>
<td></td>
<td><em>May</em> be partially reversible</td>
<td>FEV1% 40-59 = moderate</td>
</tr>
<tr>
<td></td>
<td>Includes, chronic bronchitis, emphysema, and some cases of chronic asthma.</td>
<td>FEV1% &lt;40 = severe AND</td>
</tr>
<tr>
<td></td>
<td>Airflow obstruction due to chronic bronchitis or emphysema</td>
<td>FEV1/FVC &lt; 0.70</td>
</tr>
<tr>
<td><strong>American Thoracic Society</strong></td>
<td><em>Usually progressive</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Usually</em> significant smoking history present</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>May</em> be partially reversible</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>May</em> have airway hyperreacitivity</td>
<td></td>
</tr>
<tr>
<td><strong>European Respiratory Society</strong></td>
<td>Significant respiratory symptoms</td>
<td>FEV1% 50-90 = mild</td>
</tr>
<tr>
<td></td>
<td>Chronic bronchitis- cough with sputum production for most days for 3 months of year</td>
<td>FEV1% 50-69 = moderate</td>
</tr>
<tr>
<td></td>
<td>Emphysema-Pathological diagnosis</td>
<td>FEV1% &lt;50 = severe AND</td>
</tr>
<tr>
<td></td>
<td>FEV1/FVC% &lt; 88 (men)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FEV1/FVC% &lt;89(women)</td>
<td></td>
</tr>
<tr>
<td><strong>CIBA Symposium</strong></td>
<td>Obstructive spirometry</td>
<td></td>
</tr>
<tr>
<td><strong>Global initiative for Chronic Obstructive Lung Diseases (2001)</strong></td>
<td>Obstructive spirometry</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chronic cough, sputum, dyspnoea that is persistent and worse on exercise, or during respiratory infections. Exposure to key risk factors with diagnosis established by spirometry</td>
<td>FEV1% &lt;80 = mild</td>
</tr>
<tr>
<td></td>
<td>0.30 &lt; FEV1/ Predicted</td>
<td>FEV1 &lt; 0.80 = moderate</td>
</tr>
<tr>
<td></td>
<td>0.30 &gt; FEV1/Predicted</td>
<td>FEV1 = severe</td>
</tr>
<tr>
<td></td>
<td>FEV1/FVC &lt;70% predicted.</td>
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FVC = forced vital capacity
Disease definitions for asthma are also descriptive in nature and thus have a number of elements that are difficult to quantify in clinical practice. For example the World Health Organisation defines asthma thus: ‘Asthma is a chronic inflammatory disorder of the airways in which many cells play a role, in particular mast cells, eosinophils and T-lymphocytes. In susceptible individuals this inflammation causes recurrent episodes of wheeze, chest tightness, and cough particularly at night and/or in the early morning. These symptoms are usually associated with widespread but variable airflow limitation that is at least partly reversible either spontaneously or with treatment. The inflammation also causes an associated increase in airways responsiveness to a variety of stimuli.’ (National Heart, Lung and Blood Institute, 1996). Clearly such definitions are dependent on an overall assessment of many patient characteristics.

In general definitions of asthma generally include the presence of three core features:

- Chronic airway inflammation that in susceptible individuals causes recurrent wheeze, dyspnoea and cough.
- Widespread but variable airflow limitation (either spontaneously or with treatment).
- Bronchial hyperresponsiveness to a variety of stimuli.

However it is not clear which characteristics in combination are necessary for diagnosis. Associations with the atopic phenotype may support a diagnosis of extrinsic asthma but are neither sensitive nor specific.

Amongst older patients the current definitions are likely to result in a group of highly heterogeneous individuals whom may be labelled as having COPD or asthma. It is accepted that these diagnostic labels cover a range of clinical phenotypes that share a clinical picture of airflow obstruction. It has been demonstrated that a variety of pathological changes can occur...
in the lungs of patients with COPD and late onset asthma (Jeffrey PK, 1994; Glynn AA, 1960; Salvato G, 1968). Variable responses to treatments and disease progression have also been observed (Burrows B, 1987 and 1991; Braman SS, 1991; Lee HY, 1972; Burr M, 1979). What remains unclear is whether airflow obstruction is the result of a limited lung response to a range of stimuli, such as the environmental effects of tobacco, the effects of early childhood respiratory tract infection, extrinsic allergens, or inherited factors, or if asthma and COPD share pathogenic mechanisms that produce a variable response in the lung. Thus the two models for asthma and COPD, British model and the Dutch hypothesis.

1.2 Defining and diagnosing asthma and COPD in older populations

The diagnosis of airflow obstruction in older persons is particularly problematic for a number of reasons (Ahmed T, 1997):

- The differential diagnosis of wheeze and dyspnoea in older persons is broad
- Elderly patients frequently perceive symptoms of breathlessness differently to younger subjects
- Older patients are more likely to attribute their symptoms to normal ageing
- Older patients symptoms may be masked by a reduction in physical activity occurring for other reasons
- Co-morbidity is common in older subjects and the presence of a second condition may mask the presence of airflow obstruction

It is not therefore surprising that the diagnosis of airflow obstruction is frequently overlooked in this patient group.

Pulmonary function tests are useful in the diagnosis of both asthma and COPD. There is considerable evidence that they can be reliably performed in older subjects. In the Cardiovascular Health Study of 1993 spirometry was carried out in 5201 individuals aged
between 65-85 years (Enright PL, 1993). In only 6% of cases was spirometry inadequate. Studies of elderly British populations have achieved similar success rates (Renwick DS, 1996, 1997).

Although they can be reliably performed there are difficulties in interpreting the results of pulmonary function tests in the elderly. For example it is standard practice to adjust predicted pulmonary function test values for the age, sex and height of the population. However, reference equations used to determine the 'normal' values of pulmonary function for older populations (aged 75 and over) are based on very small numbers of subjects and hence their accuracy must be questioned (Ghio AJ, 1990). In the Cardiovascular Health Study the measured spirometric values for normal older adults differed from the standard spirometric reference values in common use by up to 20%. In middle-aged patients an FEV1/FVC ratio below 70% indicates 'obstruction'. However, Enrights study of a sub group of over 750 healthy older non-smokers found that predicted FEV1/FVC values declined from 75%-67% with increasing age and the lower limit of normality ranged from 64%-56% respectively (Enright PL, 1993).

There are however inconsistencies in patterns of lung function decline with age and a number of studies, all involving older populations, report far less decline in the FEV1/FVC ratio in certain age/sex/ height groups (Knudson RJ, 1983; Crapo RO, 1981; Dockerly DW, 1985; Morris JF, 1988).

A tendency for the FEV1/FVC ratio to decrease for purely physiological reasons is to be expected. Ageing results in a decrease in airway calibre of the small airways (Neiwoemer DE, 1974). This results in reduced flows at low lung volumes. As a consequence the FEV1, which represents the average flow during the first second, and includes flows over most of the vital capacity, is also reduced. The decline in FVC is less than that of FEV1 and hence the FEV1/FVC ratio is also reduced.
Given these considerations it is unwise to rely on spirometry alone in older patients to
diagnose airflow obstruction, since it would yield a high false positive rate.

1.2.1 Distinguishing asthma from COPD.

Not only is it difficult to establish the diagnosis of airflow obstruction in older people, but it
may also be difficult to distinguish chronic asthma from COPD.

The diagnosis of COPD is generally made on the basis of typical symptoms (dyspnoea,
cough, with or without sputum production, and wheeze, and confirmatory objective evidence
using spirometric tests. However, there may still be a degree of diagnostic uncertainty as has
been highlighted in a study by Pride NJ et al (1989). To establish the degree of diagnostic
concordance between specialists over 100 respiratory specialists from Europe and North
America reviewed four model case histories and spirometry of patients with airway
obstruction. The study showed that there was marked variation in the diagnostic labels applied
to each of the four cases, although no evidence was found that these semantic differences
resulted in differences in treatment. The latter is in contrast to clinical studies that have
shown that different diagnostic labels may have an effect on subsequent treatment (Anderson
HR 1981; Burrows B, 1987). These effects may be of particular importance in older patients
or those with atypical disease.

Asthma is typically characterised by the presence of airway hyperresponsiveness and
bronchodilator reversibility, and peak flow variability. The presence of atopy and eosinophilia
also suggest a diagnosis of asthma, and there are pathological differences between the two
conditions. These features may be used to distinguish asthma from COPD. However, this
distinction may be simplistic, particularly when applied to older subjects.
The methacholine challenge test is a standardised measure of airway hyper-responsiveness, used in both clinical practice and in research. Large numbers of tests have been carried out safely in older subjects, with and without mild COPD (Tashkin DP, 1992; O’Connor GT, 1989; Connolly MJ, 1988). Their use in those with a significant degree of airways obstruction is contra-indicated. The prevalence of bronchial hyper-reactivity is greater in older populations (Peat JK, 1992) and this may not be a reflection of increased pulmonary pathology but a result of the changes in elastic recoil that occur with ageing. Poiseulle’s Law states that the resistance to flow in a tube is inversely proportional to the radius of the tube to the power of four. Hence, in normal older adults the decline in airway calibre that occurs with ageing results in increased airway resistance. As the airway becomes even smaller, there is a proportionally greater increase in resistance. This may be one explanation as to why bronchial hyperresponsiveness might be expected to increase with age. Despite, clinical observations of increasing bronchial hyperresponsiveness there are no recommendations to increase the threshold values at which a test is deemed positive for older adults (Peat JK, 1992).

Another marker of bronchial hyperresponsiveness is the presence of reversibility to bronchodilating agents. The effects of ageing on bronchodilator reversibility are not clear. A study of over 2500 individuals aged between 7 and 75 found that age had no effect on FEV1 response to beta agonists (Dales RE, 1986). A second large study of over 1000 individuals showed no correlation between bronchodilator response and age (Eliasson O, 1985). In contrast several studies have suggested higher levels of bronchodilator reversibility in older persons (Hopp RJ, 1985; Lang DM, 1987; Burney PGJ, 1987). Similarly the presence of 'significant' reversibility is not uncommon in older subjects both with and without airflow obstruction.
However, despite these differences, studies of elderly and young asthmatics patients have shown that age is not a predictor of acute response to bronchodilators (Kradjen WA, 1992). In those with COPD, bronchodilator reversibility has been shown to occur in up to 30% of patients on a single testing and up to 68% if multiple tests are used over time (Anthonisen NR 1986).

Thus bronchodilator reversibility testing may be of little use in distinguishing asthma from COPD in older subjects and defining the criteria for significant reversibility in the very elderly therefore warrant special consideration.

Variable peak flow rates can be supportive of a diagnosis of asthma, given good compliance with regular readings (at least 3 readings a day for >2 weeks). However they are less sensitive and specific than bronchial challenge tests (Hunter CJ, 2002) and short and long term repeatability is poor (Boezen HM, 1994; Higgins BG, 1989 and 1993). Older subjects may find that poor vision and lack of manual dexterity hamper peak flow monitoring and hence this useful diagnostic aid is often not available. However, more significantly peak flow variability measurements do not appear to distinguish those with asthma from those with COPD in older populations (Brand PLP, 1991).

Another feature, which characterises asthma in younger subjects, is the presence of atopy, as demonstrated by elevated total and specific IgE levels, and positive skin tests to common aeroallergens. However all markers of the atopic phenotype decline with normal ageing, limiting their usefulness (Barbee RA, 1981, 1987). Dow and colleagues (Dow L, 1992) have shown that total serum immunoglobulin E levels (IgE) are greater in older subjects with airflow obstruction than in the general population, a finding confirmed by other studies in subjects with late onset airflow obstruction (Burrows B, 1989). A significant number of
studies have not shown such a relationship between asthma and atopy in older persons 
(Braman SS, 1991; Vollmer WM, 1986). The relationship between airflow obstruction and 
IgE in adults is confounded by the effects of smoking, which is known to elevate IgE levels 
(Gerrard JW, 1980; Burrows B, 1981). However patients with increased levels of total IgE, 
and eosinophilia are more likely to be subsequently diagnosed as having asthma rather than 
COPD, regardless of their smoking history. There is a complex relationship between IgE 
levels, smoking and bronchial hyperresponsiveness in older adults, which will be discussed in 
greater detail in Chapter 5.

Significant eosinophilia is not demonstrable in the majority of elderly asthmatics, regardless 
of age of onset of symptoms, however some studies have shown significant blood 
eosinophilia in this group (Burrows B, 1991), and associations between blood eosinophil 
level and FEV1 (Kaufmann F, 1986)
In general atopic markers are of limited use in distinguishing between asthma and COPD in 
older subjects. Whilst eosinophilia and elevated total and specific IgE levels favour a 
diagnosis of asthma they lack sensitivity and specificity.

Pathologically some distinction may be made between asthma and COPD. The airways in 
asthma are characterised by increased numbers of CD4+ lymphocytes, eosinophils, and mast 
cells (Corrigan CJ, 1992),, with basement membrane thickening in nearly all cases (Crepea 
SB, 1955). Induced sputum samples show a prominence of activated eosinophils. During 
acute exaserbations the small airways show increased muscle cell mass with mucus plugging 
(Huber HL, 1922; Cardell BS, 1959). Chronic asthma is characterised by parenchymal and 
alveolar tissue inflammatory changes and eosinophil infiltrates. However there is little data 
relating to pathological changes in subjects with late onset asthma.
By contrast the prominent cells in COPD are CD8+ lymphocytes, although CD4+ cells are also increased. There is prominent neutrophil and macrophage infiltrate in induced sputum samples. Sputum eosinophilia may be a feature in up to a third of patients. The small airways may also show an increased muscle cell mass and mucus hypersecretion but there is also evidence of fibrosis and obliteration of the small airways (Wright JL, 1992).

In summary, asthma and COPD may be difficult to distinguish and recognising that the diagnostic labels of 'asthma' and COPD are inconsistently applied is important, particularly in older subjects, in whom multiple environmental factors and physiological changes as a result of normal ageing have great effects on the phenotype.

1.2.2 Defining disease status for genetic studies.

It is particularly important to clearly define both asthma and COPD for the purposes of genetic studies. Phenotypic heterogeneity is a particular problem for these conditions. It can hamper interpretation of individual studies and limit comparisons between studies. The calculation of phenotypic ‘scores’ may ultimately prove the most attractive approach in genetic studies (Wilkinson J, 1998; Panhuysen CIM, 1998).

1.3 The Dutch Hypothesis

The evidence presented above suggests two basic models regarding the pathogenesis of COPD and asthma.

British and American physicians have in general regarded asthma and COPD as distinct conditions with separate pathogenic factors and different clinical courses. This model is supported by the clear pathological differences between the two conditions, which have
already been discussed. In addition although airway hyperresponsiveness may be a feature of COPD it could be related to geometric factors as a consequence of bronchial wall thickening (James AL, 1989). There is also epidemiological evidence to suggest that individuals with non-atopic emphysematous disease follow a quite different disease course to those with late onset asthma (Burrows B, 1987). The same authors however also describe atopic smokers with COPD who have many overlapping features with the asthmatic group thus highlighting that the evidence in support of this model is by no means consistent.

The alternative model, known as the “Dutch Hypothesis” (Orie NGM, 1960), regards asthma and COPD as two aspects of the same basic process, and there is considerable evidence in support of this model. In accepting this latter hypothesis it becomes apparent that genetic and environmental factors that determine the development of chronic inflammation, airflow obstruction, and the atopic phenotype in young asthmatics, might also be expected to have a role in the development of COPD and late onset asthma. Such a hypothesis need not exclude the importance of some non-asthma related factors in the development of COPD (such as alpha-1-antitrypsin deficiency), and does not assume that the relative importance of factors is the same for the two conditions. However, it does provide an alternative basis on which to investigate the spectrum of airflow obstruction in older patients, in light of conflicting and incomplete evidence of the role of atopy, bronchial hyperresponsiveness, and pathological changes. A possible model for the development of late onset airflow obstruction is shown in Figure 1.1. For the purposes of this study I have chosen to consider late onset asthma and COPD according to the Dutch Hypothesis, that is sharing some pathogenic mechanisms, rather than as discrete disease entities. This approach has the additional advantage of resolving some of the difficulties in distinguishing between asthma and COPD in older populations, whilst allowing a narrow definition of certain phenotypic characteristics.
Figure 1.1 Proposed model for the Dutch Hypothesis

Smoking  
\[\rightarrow\]  
Extrinsic Allergens  
\[\rightarrow\]  
Chronic Inflammation  
\[\rightarrow\]  
Airflow Obstruction  
\[\rightarrow\]  
Asthma  
\[\rightarrow\]  
COPD
PART B Genetic and environmental determinants of airflow obstruction

1.4 Environmental factors.

In recent years there has been a sharp increase in the prevalence of asthma and allergic disease in children and clearly this cannot be explained by genetic factors. There are a large number of environmental factors recognised as of importance in the development of asthma and COPD.

1.4.1 Cigarette Smoking

Cigarette smoking is the most important factor determining the development of COPD. However, Fletcher and Peto in 1977 suggested that only 10-15% of chronic heavy smokers would ever develop clinically significant airflow obstruction suggesting that other factors, both genetic and environmental are important (Fletcher CM, 1977). Amongst those smokers susceptible to the effects of tobacco smoke the disease severity and future prognosis appears to be correlated to total pack-year history, but this is not consistent in all studies (D Lomas, personal communication). Burrows showed that total pack year history, current number of cigarettes smoked, average number of cigarettes smoked and age, only explained a small percentage (estimated at 15%) of the variability of FEV1 observed within the general population (Burrows B, 1977). Evidence is accumulating that active smoking prior to the achievement of maximal lung function may be a particularly important in the accelerated decline in lung function in adult smokers (Kerstjens HAM, 1997).

1.4.2 Allergens and Atopy.

The response to environmental allergens in childhood is greatly influenced by possession of the atopic phenotype, which has a strong genetic determinant. In children, atopy is a predictor
of increased bronchial hyperresponsiveness and asthma. It is uncommon to find clinical provocation by aeroallergens in older subjects. Atopy may, however, be important in some late-onset asthmatics.

The relationship between IgE levels, smoking and lung function is complex. Subjects with late-onset asthma have higher serum IgE levels compared to those with COPD. In subjects with COPD those with elevated IgE levels have a better prognosis, with a more gradual decline in lung function. A recent longitudinal study by Palmer et al (Palmer LJ, 2001) of over 2,000 individuals, with a 29-year follow-up has shown that serum IgE levels are predictive of subsequent development of not only asthma, but also COPD. Burr M et al (1979) reported that 50% of elderly asthmatics have a family history of allergy. Braman SS (1991) has subsequently observed that although a history of atopy was common in elderly asthmatics that acquired their disease in childhood, it was virtually absent in those with late-onset disease. A study of skin testing in older subjects has shown a similar pattern of results with low rates in those with late-onset asthma and high positive rates in those developing asthma before 40 years of age (Reed C, 1997). In summary although atopy may be a factor in pathogenesis amongst some subjects evidence of provocation by aeroallergens for the majority is probably uncommon.

1.4.3 Occupation

The role of workplace allergens in the development of asthma has long been recognised, and not infrequently, symptoms persist after exposure to the initial sensitising agent has ceased. It is estimated that between 5-15% of all cases of persistent late onset asthma relate to initial sensitisation within the workplace (Berstein DI, 1993). Established occupations considered as high risk for occupational asthma include, printers, rubber and plastic workers, electronic
assembly, spray painters, sawmill workers, hairdressers, metal treatment workers and those involved in detergent manufacture.

In considering the development of COPD smoking dominates all other aetiological factors. However chronic occupational dust exposure may be of importance in determining the severity of disease (Hnizdo E, 1992). The link between coal mining and COPD is well recognised in the United Kingdom. Fishwick D et al (1997) identified a number of occupations with increased prevalence of COPD, in their population based study. A history of prior or current work with vapours, gases, dust or fumes was significantly associated with chronic bronchitis.

1.4.4 Respiratory tract infections

Early childhood respiratory tract infections are associated with both transient and persistent wheeze in childhood. However there is conflicting data concerning the role of viral infections and the subsequent development of atopy and asthma (Martinez FD, 1995; Von Mutius E, 1994; Schwarze J, 1997; Sigurs NR, 1995). There is some evidence to suggest that such infections may in fact be protective by reducing the airways sensitivity to environmental allergens (Martinez FD, 1995; Von Mutius E, 1994).

Approximately 25% respiratory tract infections in childhood are caused by viral infections. Fifty percent are due to respiratory syncytial virus, and 14% due to adenovirus. A number of epidemiological studies have suggested that such infections are a risk factor for the development of COPD. Following acute infection viruses, and in particular adenovirus, may persist in a latent form and it is hypothesized that in doing so they amplify the effects of tobacco induced lung injury (Hogg JC, 1999; Vitalis TZ, 1998).
1.4.5 Environmental Pollutants

The importance of environmental pollutants in the development of juvenile onset asthma is currently an area of much research interest. Older subjects have been exposed to a range of environmental pollutants, industrial, urban and domiciliary. The relative importance of each of these has changed over the course of the 20th century. The 1952 London smog was associated with an increase in respiratory mortality, with older people particularly susceptible (Logan WPD, 1953). It is established that pollution can increase morbidity and mortality in patients with existing lung disease. What is less clear is or whether it can produce disease de novo.
1.5 Genetic Factors

1.5.1 Heretability

The first step in considering the genetic analysis of a complex disease is to estimate the magnitude of the genetic component contributing to the phenotype. The larger the genetic contribution the higher the likelihood of detecting the disease causing genes.

The estimate of the genetic contribution to a disease may be expressed as a risk ratio (\( \lambda \)). The risk ratio is the prevalence of the disorder amongst first-degree relatives divided by the prevalence of the disorder in the general population. The higher the value of \( \lambda \) the greater the genetic contribution. The risk ratios for some common conditions are shown in Table 1.2. In some instances the \( \lambda \) decreases with ageing. This effect has been observed with death from myocardial infarction and Alzheimer's disease (Marenberg ME, 1994; Corder EH, 1993).

The \( \lambda \) for juvenile asthma is relatively low and to some extent this reflects the difficulties with phenotypic definition.

In preliminary genetic studies, groups with the highest \( \lambda \) are sometimes selected, but there are limitations with this approach, particularly because this usually involves the study of families with rare severe early onset forms of disease. For example the initial investigation into the genetic basis of Alzheimer's disease concentrated on families with a strong familial early onset form of the disorder. Autosomal Dominant genetic mutations in the genes for amyloid precursor protein (APP), presenilin-1 and presenilin –2 were described. These mutations however may be of little importance in the development of Alzheimer's disease in older populations where a genetic risk still persists. Similarly, when the genetic basis of emphysema was examined in families with an early onset severe form of the disease the alpha-1-antitrypsin gene mutation was identified. Further studies have consistently found that
alpha-1-antitrypsin deficiency accounts for only 2-3 % of all cases of emphysema. Hence although important as a major genetic mutation it is not significant as a cause of airflow obstruction in the majority of the population with COPD.

An alternative measure of the genetic contribution to a disease is heretability (H). This is defined as the phenotypic variance that may be attributed to genetic factors (often expressed as %). This measure will correct for the population prevalence of the trait.

Both family data and twin studies are used to measure λ and H. Frequently there is wide variation in the estimates given, often this occurs due to a failure to control for significant environmental factors and difficulty in establishing if concordance is due to shared environment or shared genes.
<table>
<thead>
<tr>
<th>Condition</th>
<th>Risk Ratio ($\lambda$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cystic Fibrosis</td>
<td>500</td>
</tr>
<tr>
<td>Schizophrenia</td>
<td>8.6</td>
</tr>
<tr>
<td>Late-onset Alzheimer's</td>
<td>4 – 5</td>
</tr>
<tr>
<td>NIDDM</td>
<td>3.5</td>
</tr>
<tr>
<td>Fatal MI &lt;65 years</td>
<td>15 (women) 7 (men)</td>
</tr>
<tr>
<td>Fatal MI &gt;65 years</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Asthma</td>
<td>Estimated 2-6</td>
</tr>
</tbody>
</table>
1.5.2 Evidence supporting the genetic basis of COPD.

The possibility that hereditary factors may determine the development of obstructive lung disease was initially proposed over 150 years ago (Louis P, 1837).


It is possible that the genetic factors determining FEV1 in a general population sample are quite distinct from those determining FEV1 in families with COPD. However there is specific evidence regarding correlations in pulmonary function in families with a COPD index case (Larson RK, 1970; Cohen HB, 1975; Kueppers F, 1977; Rybrick BA, 1990).

Although accurate estimates of the genetic heretability of COPD are difficult, the hereditibility of FEV1 in these families may be as high as 77%. Childhood asthma by contrast, has a hereditibility of between 30 and 60% (Dewar JC, 1996). Twin–twin studies show that there is a closer similarity in spirometric function between monozygotic versus dizygotic twins and that in monozygous twins who smoke there is a higher risk that both will develop airflow obstruction, even if raised apart, compared to dizygotic twins (Redline S, 1989; Hubert HB, 1982; Webster PM, 1979). There is evidence of familial clustering and an increased prevalence of COPD amongst relatives of patients with COPD compared to the relatives of controls and decreased prevalence of COPD with increased genetic distance.
Larson RK (1970) showed that abnormalities of pulmonary function were higher in 156 relatives of 61 subjects with COPD than their spousal controls. They suggested that hereditiy and smoking contributed equally to the likelihood of developing COPD. Khoury et al, showed that first-degree relatives of patients with COPD had significantly higher rates of airflow obstruction compared with first degree relatives of non-pulmonary patients with some of the familial aggregation due to shared genetic factors (Khoury MJ, 1985). Recently the same group found that within the COPD families there was significant evidence for a major gene effect (most probably many genes acting in unison rather than a single locus) contributing to pulmonary function that was absent in non-pulmonary families (Rybricki BA, 1990).

1.5.3 Evidence for the genetic basis of asthma

As with studies of COPD, the initial evidence supporting a genetic component to the development of asthma has come from family and twin studies. There is sufficient data to show that childhood asthma has a significant genetic component (Sandford AJ, 1996). Estimates of the heretability of TlgE are around 60% (Dewar JC, 1996). In a study by Sibbald B et al (1979) it was suggested that the inheritance of atopic asthma was under greater genetic control than non-atopic asthma. This group also suggested that asthma and atopy could be separately inherited and concluded that the manifestations of asthma may be enhanced in those with atopy.

There has some attempt to discriminate between other asthmatic subtypes. Recently, a polymorphism of the leukotriene C4 synthase gene has been associated with to aspirin intolerant asthma (Sanak M, 2000). For late onset disease it is unlikely that genetic factors
are identical to those causing childhood atopic asthma, but there may still be a significant genetic overlap.

In conclusion early studies have resulted in much interest in determining the genetic contribution to the development of airflow obstruction. In view of the evidence from family studies a search for the genetic influences determining late onset airflow obstruction is logical.

A large number of candidate gene studies and genome scans have investigated the genetic basis of juvenile-onset asthma. Far fewer studies have focused on late onset disease or COPD. The results of some of these studies are summarised in sections 1.6 and 1.7.
1.6 Genetics of COPD

Until recently there had been no published linkage data for COPD and related phenotypes. However Silverman and colleagues have recently published linkage data for early-onset COPD (Silverman EK, 2002) from their ongoing genetic epidemiological study based on the collection of sibling pairs. They demonstrated linkage to chromosomes 12, 19, 22, 17, and 2 for a variety of COPD phenotypes. Joost and colleagues (Joost O, 2002) examined quantitative markers of pulmonary function in a general population sample and reported linkage to chromosome 6q and 21p in their population. The relationship between genetic determinants of pulmonary function in the general population and that in COPD is unknown. The former may be of particular importance in lung growth and development rather than the development of COPD.

1.6.1 Alpha-1-antitrypsin deficiency

Alpha-1-antitrypsin deficiency, first described by Laurell and Eriksson in 1963 (Laurell CB, 1963) is the only inherited risk factor that is unequivocally associated with an increased risk of emphysema (Kalsheker N, 1987). It is an example of a Mendelian trait, with allelic heterogeneity, as a result of a mutation in the alpha-1-antitrypsin gene. It has been estimated that homozygosity, resulting in substantially reduced levels of alpha-1-antitrypsin, accounts for only 3% of cases of emphysema in Caucasian populations. There is still some debate as to the importance of heterozygosity at the locus and current evidence seems to suggest that such individuals, although they may have decreased serum levels of alpha-1-antitrypsin, remain at minimal or little increased risk of developing emphysema (Kalsheker N, 1990; Turino GM, 1996; Alvarez-Granda L, 1997).
1.6.2 *A-1- antichymotrypsin*

Alpha-1-antichymotrypsin is another proteinase inhibitor whose target proteinase is Cathepsin G. Cathepsin G is involved in lung injury mechanisms at the bronchial and lower respiratory tract level. Two mutations in the alpha-1-antichymotrypsin gene have been described in association with decreased serum levels of alpha-1-antichymotrypsin and COPD (Poller W, 1992 and 1993). However, the prevalence of the mutation appears to vary widely in different populations. Recently, Matsuse and colleagues found a weak association between a polymorphism in the signal peptide of the gene and COPD, although the study was small (Matsuse IT, 2000). An inherited deficiency which is autosomal dominant has also been described but appears to be extremely rare (Eriksson S, 1986).

1.6.3 *Cystic Fibrosis transmembrane regulator*

Cystic Fibrosis (CF) is the commonest Mendelian disorder in those of Northern European ancestry. Multiple mutations in the cystic fibrosis gene, which lies on chromosome 14q have been described. The ΔF508 mutation is the commonest and accounts for approximately 70% of all cystic fibrosis mutations in Britain.

It has been hypothesised that cystic fibrosis heterozygotes could be at increased risk of respiratory disease (Di Sant‘Agnese P, 1992). It has been shown that a group of F508 heterozygotes do have an increased incidence of wheeze, decreased FEV1 and increased reactivity to methacholine. However, subsequent work has suggested that heterozygosity may actually predispose to disseminated bronchiectasis rather than COPD (Pignatti PF, 1995; Gervais R, 1993). In addition, studies using a cystic fibrosis locus marker have failed to show linkage to COPD phenotypes.
1.6.4 Cytochrome P4501A1

Cytochrome P4501A1 is found throughout the lung and possibly has a role in the activation of procarcinogens to their carcinogenic forms. There is some evidence to suggest that there is an association between COPD and lung cancer that is independent of smoking history and age, suggesting that there may be some shared genetic predisposing factors (Cohen BH, 1977). A polymorphism that causes a functional increase in Cytochrome P4501A1 has been associated with an increased risk of lung cancer and emphysema. The polymorphism however does not appear to be of importance in determining the development of emphysema or lung cancer in isolation (Cantlay HM, 1995)

1.6.5 Microsomal Epoxide hydrolase

Microsomal epoxide hydrolase (mEPHX) is a xenobiotic enzyme that is involved in the metabolism of highly reactive intermediates that may be formed in cigarette smoke. Smith and Harrison (1997) demonstrated in a case-control study that homozygosity for a polymorphism of the mEPHX gene, resulting in slow enzyme activity, is associated with COPD. Independent replication of this finding is awaited.

1.6.6 Glutathione -S-transferase supergene family

The glutathione–s-transferase (GST) genes are a large supergene family with genes located across a number of chromosomes. These enzymes are critical in the metabolism of reactive oxygen species. These substances are produced in excess as by products of tobacco smoke. From a theoretical standpoint they are attractive candidate genes for COPD. Matsuse and colleagues (Matsuse IT, 1999) report an exon 5 polymorphism of GSTP1 to associate with COPD susceptibility in a Japanese population, a finding replicated by He JQ, et al (He JQ, 2002). Another study has failed to confirm these findings (Harries LW, 1997)
1.6.7 *Vitamin D binding protein*

Vitamin D binding protein (VDBP) has some influences on the intensity of inflammatory reactions by action as a macrophage-activating factor and by enhancing neutrophil chemotaxis via actions on the complement system (*Metcalf JP, 1991*). A number of polymorphism's involving the VDBP gene have been described. One common polymorphism results in a limited ability to form macrophage-activating factor. Association studies have suggested that this isoform of the VDBP gene is protective for the development of COPD (*Schellenberg D, 1998*). This finding awaits replication in further studies.

1.6.8 *Blood group antigens*

Recurrent respiratory tract infections in childhood are a risk factor for COPD. The ABO, secretor and Lewis systems are all involved in the adhesion of infectious agents and hence they have a plausible role in disease pathogenesis. An epidemiological study by *Cohen HB*, et al (1975) first described an association between ABO blood group A and impaired lung function. They also reported in a longitudinal study that Group A individuals had a greater decline in lung function (*Beaty TH, 1984*). Other groups however have not been able to replicate these findings consistently (*Higgins MW, 1982; Krazyzanski M, 1987*). There has also been a suggestion that ABH secretor status, which is dependant on ABO blood group and the Lewis blood group are additional risk factors with non-secretor status predisposing to the development of COPD (*Cohen BH, 1980; Haines 1982; Horne SL, 1985*). The majority of these studies are small in size and once again consistent replication of results is absent.
1.6.9 **Tumour Necrosis Factor**

Tumour Necrosis factor is a pro-inflammatory cytokine increased levels of which have been demonstrated to occur in bronchial-alveolar lavage fluid from COPD patients. A number of polymorphisms in and around the promoter region of the tumour necrosis factor gene on chromosome 6 have been described, one of which produces a functional increase in TNF levels (Wilson AG, 1997; Louis E, 1998). This is an Arginine to Glycine transition at codon 308. Recently a Taiwanese group reported on an association between this polymorphism and self reported chronic bronchitis (Huang SL, 1997). However this study involved only small numbers of subjects and thus must be interpreted with caution. A second larger study, also of an Asian population, described an association between the rarer G allele and COPD (Sakao S, 2001). Two subsequent studies, both involving those of European origin have failed to show any association, although they were also of limited size (Gile LS, 1998; Higham MA, 2000). In a case-control study by Keatings et al (Keatings VM, 2000) an association was found between the AA homozygotes, more severe disease, and a poorer prognosis. This finding is in contrast to the Japanese data. These studies are discussed in greater detail in Chapter 6.

1.6.10 **Beta adrenoceptor polymorphisms**

During the course of this study one paper has been published examining the role of beta-adrenoceptor polymorphism as disease modifiers in COPD (Ho L, 2001). This study of a Chinese population comprised 61 cases with COPD and 41 controls. Its results must therefore be interpreted with caution, although a significant association between susceptibility to COPD and Arg16 genotype was reported. The significance of this study is discussed in greater detail in Chapter 4.
<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Candidate(s)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>Alpha-1-antitpsin</td>
<td>Protease inhibitor</td>
</tr>
<tr>
<td>11q</td>
<td>CC16</td>
<td>Clara cell secretory protein.</td>
</tr>
<tr>
<td>12p</td>
<td>Alpha-2-macroglobulin</td>
<td>Protease/antiprotease functions</td>
</tr>
<tr>
<td>1q</td>
<td>Microsomal epoxide hydroxyalase</td>
<td>Metabolism of intermediate products of tobacco smoke</td>
</tr>
<tr>
<td>11q,</td>
<td>Detoxifying enzymes eg. Glutathione-s-transferase supergene family</td>
<td>Metabolism of intermediate products of tobacco smoke</td>
</tr>
<tr>
<td>4q</td>
<td>Vitamin –D-binding globulin</td>
<td>Possible action as macrophage activating factor</td>
</tr>
<tr>
<td>7q</td>
<td>Cystic Fibrosis F508</td>
<td>Heterozygotes may be at risk of disseminated bronchiectasis</td>
</tr>
<tr>
<td>6p</td>
<td>Tumour Necrosis Factor</td>
<td>Pro-inflammatory cytokine</td>
</tr>
<tr>
<td>5q31-33 and others</td>
<td>Bronchial hyperresponsiveness and atopy genes (see Table 1.4)</td>
<td>Up-regulation of mast cells, IgE, eosinophils, bronchodilation, etc.</td>
</tr>
</tbody>
</table>
1.7 Genetic Factors in late onset asthma

There has been considerable progress in determining the genetic factors that contribute to the atopic and juvenile onset asthmatic phenotype. To date there have been six published whole genome linkage scans for asthma and atopy (Daniels SE, 1996; CSGA, 1997; Ober C, 1998, 1999; Wjst M, 1999; Dizier MH, 2000, Van Eerdewegh P, 2002). Although they were carried out in distinct populations, and used some different diagnostic criteria for a number of the phenotypes, there were some areas of linkage identified that were common to a number of studies. The 12q area showed linkage in all studies and 5p, 11p, 13q, 6p and 7p areas were common to four studies. In addition there is considerable evidence for linkage for 11q, 5q and to a lesser extent 16p from non-genome linkage studies. The following regions have also been identified in linkage studies, 2q, 12p, 13q, 14q, 17p, 19q and 21q. The presence of plausible candidate genes at the 11q, 5q, 16p 6p, and 12q areas has meant that there are a greater number of association studies examining these areas. Some of the candidate genes for these loci are shown in Table 1.4. Whilst the importance of these chromosomal regions is clear there remains a large number of loci identified through linkage studies which have yet to be fully evaluated, perhaps in part due to paucity of information regarding candidate genes at these loci. The recent study by Van Eerdewegh et al (2002), describes a locus on chromosome 20p in linkage with asthma and related phenotypes. They subsequently identified the ADAM33 gene as a novel candidate gene for asthma. This study is of great interest and further replication of these findings in an independent population is awaited.
<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Candidate(s)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>5q31-33</td>
<td>IL-3, IL-4, IL-5, IL-9, IL-13, GM-CSF B$_2$adrenoceptor</td>
<td>Cytokines (upregulation of mast cells, eosinophils, IgE etc) Bronchodilation</td>
</tr>
<tr>
<td>6p</td>
<td>HLA</td>
<td>Antigen presentation</td>
</tr>
<tr>
<td></td>
<td>TNF</td>
<td>Pro-inflammatory cytokine</td>
</tr>
<tr>
<td>11q</td>
<td>CC16, FceRI, GSTP1</td>
<td>Mast cell signalling</td>
</tr>
<tr>
<td>12q</td>
<td>Interferon $\gamma$ Mast cell growth factor, nitric oxide synthetase</td>
<td>Inhibition of Th2 cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inflammatory mediators</td>
</tr>
<tr>
<td>14q</td>
<td>T-cell receptor</td>
<td>T cell activation</td>
</tr>
<tr>
<td>20p</td>
<td>ADAM 33</td>
<td>Membrane anchored metalloprotease, multiple functions including shedding cytokines and cytokine receptors</td>
</tr>
</tbody>
</table>
1.8 Summary

The genetic risk factors for late onset asthma have yet to be investigated but are the focus of current studies. Inevitably given the difficulties with disease definitions already discussed there may be some overlap with the genetic risk factors for COPD. The Dutch Hypothesis would also suggest that COPD and asthma share genetic risk factors.

It is clear that of all genetic factors thus far described alpha-1-antitrypsin deficiency is the only factor that has been shown to be unequivocally associated with an increased risk of developing emphysema. It explains only a small percentage of the genetic susceptibility to the development of late onset airflow obstruction. Other candidate genes are beginning to be examined in subjects with COPD and reflect the wide number of pathogenic mechanisms involved.
PART C Genetic epidemiological approaches to the investigation of late-onset disorders.

1.9 Methodological Approaches

Late onset asthma and COPD are both examples of complex genetic disorders and genetic epidemiological study design must reflect this. For any complex phenotype there are four main methodological approaches to cross-sectional study design that one may adopt:

- Linkage analysis;
- Allele-sharing methods;
- Association studies and
- Experimental crosses.

1.9.1 Linkage Studies

The aim of linkage analysis is to map the chromosomal locus of a gene responsible for a disease and subsequently to identify that gene through the techniques of positional cloning. Family data is used to follow the transmission of genetic information through generations. In the initial stage of linkage analysis DNA markers of known chromosomal location are examined for cosegregation with the disease phenotype.

DNA markers are highly polymorphic with between 4-10 allelic variants at each locus ensuring heterogeneity both within and between individuals making them ideal for use in linkage studies. The use of rapid polymerase chain reaction techniques enables multiple markers to be tested in a short space of time.

These markers enable one to calculate the odds of inheriting a particular allele together with the phenotype, within a pedigree. This calculation is dependent on the specification of the gene frequency, penetrance and mode of inheritance. A transmission model for a particular
marker allele to explain the inheritance of the disease in pedigrees is constructed and this model is accepted or rejected depending on the statistical likelihood of linkage vs. non linkage, usually expressed as a lod score.

Once linkage is established to a particular chromosomal locus the next stage is to isolate and identify the disease-causing gene using positional cloning techniques. Alternatively, the data from such linkage studies may be used to direct a search for candidate genes in an association analysis.

Traditional linkage analysis is a relatively robust technique in certain circumstances. Its main advantage is that it requires no *a priori* knowledge of likely disease causing mechanisms. Furthermore, the whole genome may be screened using approximately 300 markers, rapidly and reliably.

Parametric linkage approaches do have a number of limitations when applied to late onset diseases.

Firstly, the majority of cases of COPD and asthma arise due to multiple genetic and environmental influences, that is to say they are genetically complex disorders. Linkage studies however are best suited to monogenic conditions where a mutation, or a few mutations, gives rise to the phenotype. In the early stages of linkage analysis estimates of penetrance, gene frequency and mode of inheritance are specified, and with increasing genetic complexity, these specifications are more prone to error.

A further limitation, for linkage studies of late onset airflow obstruction, is that frequently relatively little genetic material can be obtained from any one family pedigree. Parental DNA is rarely available, and material from siblings may also be unavailable due to death or geographical isolation. This is in stark contrast to linkage studies involving younger subjects,
where for example in the case of juvenile onset asthma, genetic material from at least two generations is usually available in a single household.

1.9.2 Allele sharing methods.

Allele sharing methods also utilise family data but adopt a non-parametric approach and thus overcome some of the limitations of traditional linkage methods. The aim of allele sharing methods is to show that the inheritance of a particular allele is not consistent with random Mendelian segregation. Thus affected relatives are more likely to inherit a particular chromosomal locus that expected by chance (Lander ES, 1994). The affected sibling pair study is probably the most widely used and simplest form of the allele sharing method. In this instance one would predict that a sibling pair would be concordant for 0 or 1 or 2 alleles at any one loci in a ratio of 25:50:25 according to Mendelian segregation, thus any deviation from this expected ratio can then be measured.

Allele sharing methods are probably the method of choice for mapping complex disorders. A candidate gene or genome scan approach may be used. The methods are robust against incomplete penetrance, phenocopy, genetic heterogeneity and ideal for polygenic conditions. The major limiting factor for such approaches is that compared with a correctly modelled linkage study they may lack considerable power. Assuming a fully informative marker with zero recombination events approximately 1000 affected sibling pairs are needed to obtain a 90% power to detect linkage. However by selection of individuals at the extreme of the phenotype the power to detect linkage may be greatly enhanced so that < 100 sibling pairs may be required with similar power (Risch N, 1995).

Despite this limitation, allele-sharing methods remain a useful approach for complex disorders.
There are a number of further limitations with respect to allele sharing methods. There is still a reliance on the collection of family data.

Common to both forms of linkage analysis is the notion that false positive results are frequent if normal levels of statistical significance are adopted. It has been suggested that more stringent thresholds should be applied before concluding significant linkage (Lander ES, 1995). This approach undoubtedly provides low rates of false positive result but it also produces high rates of false negative results. One solution is to use a two-stage approach. Here liberal thresholds are adopted (e.g. p=<0.05 or 0.01), but the analysis is carried out in two independent sets of families, with positive results from the first population being retested in the second (Tordov AA, 1997). New analytical methods, such as multipoint analysis and the transmission disequilibrium test, overcome some of the flaws of traditional analytical methods.

There are a number of solutions to some of the problems raised by linkage studies of complex disorders.

One solution is to carry out the linkage studies in a subset of families who have an extreme form of early onset disease and this may have the added advantage of enabling collection of genetic material from a larger number of individuals within the family pedigree. It must always be remembered however that such a group of individuals may be very unrepresentative of the general population of late onset airflow obstruction.

Silverman and colleagues have recently adopted this approach (Silverman EK, 1998). They describe a group of individuals and awaiting lung transplantation with a diagnosis of COPD. None had alpha-1-antitrypsin deficiency. First-degree relatives of these individuals who were current or ex smokers were found to have significantly lower FEV1 and FEV1/FVC values than a control population with a similar pack year history. These observations suggested that
there may be a genetic risk factor for COPD that is expressed in response to cigarette smoke amongst these individuals. However the probands were all younger than 53 years and 79.6% of them were females making them rather unrepresentative of the COPD population as a whole.

Finally, it may be possible to carry out the study in an isolated population. Such groups are likely to be genetically homogenous and often have similar lifestyles and diets. Ober and colleagues have recently carried out a genome wide scan for asthma in the Hutterites, an Anabaptist community of North America. This community is descended from approximately 900 individuals and they share a unique lifestyle (Ober C, 1998, 1999). If accessible, such populations may be useful for genetic epidemiological studies. However there are ethical considerations involved in undertaking mass genetic screening of large populations.

1.9.3 Association Studies

Association studies in contrast to the methods already described, make no use of family data. They are simply a case-control studies in which one demonstrates that the gene of interest occurs more frequently in a group of affected individuals than in a group of unaffected individuals. Association studies are only meaningful if one selects a polymorphism that appears to be of functional importance to the phenotype under investigation. In the case of late onset airflow obstruction, particularly if one considers those with asthma as well as COPD, there are clearly a huge number of potential candidate genes that one could consider (Tables 1.3 and 1.4) and thus the chances of finding a positive association diminish. Some authors would suggest that association studies are only carried out to localise the disease genes that have already been identified by linkage analysis. In addition to having a plausible role in terms of disease pathogenesis the candidate gene, or it's controlling elements, should
contain a mutation which alters gene expression or function. This mutation should occur relatively frequently in the general population.

There are a number of limitations regarding association studies when compared to linkage approaches. Firstly the finding of a positive association does not necessarily imply a causative role for that allele since it may lie in linkage disequilibrium with the true disease-causing gene. The effects of linkage disequilibrium may explain the failure of certain positive associations to be reproduced in independent studies. This effect is more likely to occur in genetically young populations where there have been fewer recombination events over time. More worryingly however false positive associations may occur due to population stratification effects. This is particularly problematic in studies carried out in ethnically diverse populations. As with linkage methods one way which one may overcome such effects is to carry out studies in genetically isolated communities.

Given the difficulties in selecting controls for association studies due to ethnic ancestry another solution is the use of 'internal' controls. These individuals are relatives of the index cases (ideally parents) and thus of the same ancestral origin. Such a family based association study overcomes some of the limitations of traditional linkage and case control based study designs.

As with linkage studies, previous evidence from association studies of psychiatric disorders suggests that far more stringent values of significance are adopted before claiming a role for a polymorphism in disease pathogenesis. It has been shown, for example, that if only 5 genes were implicated in a polygenic condition, and 20,000 candidate genes were studied, 99.5% of the polymorphism identified would be false associations (at the p<0.05 level)(Paterson AD, 1997). In reality however, the examination for candidate genes is usually more directed and focuses on candidates genes of pathobiological significance or in known linkage areas. It has been suggested that higher thresholds of significance are adopted (Risch N, 1996).
Following recent reports of genetic associations that subsequently have not been reproduced, Nature Genetics (Editorial, 1999) have published guidelines with respect to the reporting of experimental results from association studies. In addition to the criteria of candidate genes already discussed, they also state that association studies should contain initial study with independent replication and that the association be observed in both family-based and population-based studies. These recommendations will place an increased emphasis on multi-centre collaborative research.

1.9.4 Quantitative Trait analysis

All three of these methods can be used to examine the relationship of genotypes to binary phenotypes (for example, asthma yes/no). For such analyses, the effects of misclassification of individuals are important. Individuals classified as normal may in fact go on to develop disease some years later with no clinical test available to identify these individuals at an early stage. In addition, pulmonary function tests which are an essential part of the diagnosis of COPD, can be difficult to interpret in older subjects although they can be reliably performed. An alternative approach is quantitative trait analysis. This approach may be used by all of the study designs. Quantitative traits are continuous variables which contribute to the overall phenotype. This approach may be particularly useful in a condition such as late onset asthma where measurements of TlgE levels, eosinophil counts, FEV1 or FEV1/FVC ratio would be of particular interest. Each of these traits alone has only a small contribution to the overall phenotype.

A related approach is to calculate an asthma (or COPD) 'score'. This approach avoids the difficulties associated with binary definition of affected or unaffected status about which there is still no international consensus. The approach is based upon the idea that any similarity
phenotypically between two relatives is related to the number of alleles shared at the trait causing locus of interest (Morton N, 1998).

1.9.5 Experimental crosses

Animal models are widely used in biomedical research. Using experimental animals to study ageing processes carries particular methodological challenges to do with maintaining equivalence between young, unaffected and old (either affected or unaffected) animals drawn from different birth cohorts. The principles are well described but not always applied. Animal experiments have the major advantage that the genetic conditions can be controlled – for example by the amplification of classical crossbreeding techniques to produce large numbers of genetically homogenous progeny for analysis. Furthermore non-genetic environmental “noise” can be minimised if needed. This method has the advantage of enabling the dissection of quantitative traits into discrete genetic factors. In recent years, specific target genetic manipulation has become possible and the production of transgenic mice possessing specific alterations or additions to the genome is now commonplace. These methods have begun to be used to explore the COPD phenotype (Zheng T, 2000).

1.9.6 Summary

It is clear that the genetic evaluation of late onset airway obstruction will require a combination of genetic epidemiological approaches. Linkage studies are difficult in older populations but important as they can provide useful information from which to direct a search for candidate genes. Thus studies of early onset severe forms of the disease for genetic linkage are necessary along with association studies adopting a candidate gene approach. These approaches are both necessary and complementary to one another. The development of phenotypic scores for genetic analysis could assist in comparisons between studies and enable
multi-centre collaborative research. For candidate gene approaches the selection of rigorously matched controls is essential. This is particularly true for late onset disorders since a number of factors that may alter the interpretation of studies in this group as will now be discussed.
1.10 Specific problems of association studies in older populations

There are a number of general issues that make the genetic epidemiology of late onset disorders particularly complex. The limitations, in terms of study design, due to lack of family data have already been alluded to. Those factors relating to ageing epidemiology will now be discussed.

1.10.1 Selective Survival

With any late onset condition differential survival may alter the gene pool by producing a selection bias. Such an effect has been observed with genetic studies of late onset Alzheimer's. There is much evidence that the Apo E gene on chromosome 19 is a risk factor for Alzheimer's disease. The Apo E 4 allele appears to be a major predisposing gene for Alzheimer's disease but it has also been linked to higher total cholesterol levels and premature cardiovascular disease. A number of investigators have reported that there is a lower frequency of Apo E 4 alleles in older populations compared to middle age. This effect leads to estimates that 80-90% of the apo E 4 alleles have been eliminated from the population gene pool by the age of 80. Hence those Apo E 4 individuals remaining appear to be resistant to the premature mortality associated with possession of the Apo E 4 allele and thus may differ in other ways genetically which makes them more prone to developing late onset Alzheimer's disease.

1.10.2 Co-morbidity

Co-morbidity is common in older subjects. The main concern in genetic association studies is that the presence of a second condition (or treatment of it) may alter the phenotypic expression of the condition under study and could either diminish or enhance the association. In addition there could also be a detection bias where the presence of one condition would
make a second condition more likely to be diagnosed. An apparent association could therefore then be made between the second condition and a genetic marker whilst the true association may be with the first condition. New analytical methods developed for use in psychiatric genetics can theoretically differentiate the degree of association between a specified gene and two comorbid conditions in the setting of a case control association study. These theoretical statistical models method have yet to be widely applied to real data (Wickramaratne PJ, 1998).

1.10.3 Risk Factors.

The susceptibility to chronic disease increases with age and this is a reflection of both physiological changes and cumulative environmental and genetic risk factor exposure. Risk factors however may have different effects at different ages. For example, given the same total tobacco consumption, a history of smoking before the age at which maximal lung function has been achieved will have quite different effects on lung function at aged 60 years than a history of smoking between the ages of 30 and 40 years. The association between a risk factor and a disease may differ across ages for a number of reasons. The population of individuals exposed to a risk factor will contain those individuals who are both susceptible and non-susceptible to the risk factor. As the population ages the pool of individuals who are susceptible and exposed to the risk factor diminishes so that those exposed to the risk factor begin to resemble the unexposed population in terms of risk of developing the disease. Some risk factors are associated with a fatal outcome and thus the impact of this risk factor could be hidden. In other circumstances where the majority of the exposed population develops the condition the ability of the risk factor to predict the disease is also limited.
2 CLINICAL AND LABORATORY METHODOLOGY FOR THE CANDIDATE GENE ASSOCIATION STUDY

2.1 Methodology

2.1.1 Study design.

I designed a series of case-control candidate gene association studies. I investigated the role of a number of polymorphisms, associated with juvenile onset asthmatic phenotypes, in a population of subjects with late onset airflow obstruction (asthma and COPD) and appropriate control individuals.

Initially I selected a limited number of candidate genes for study:

(i) The beta-2-adrenoceptor polymorphisms, Arginine16→Glycine16, and Glutamine 27→Glutamic acid 27 (chromosome 5q31-33).
(ii) The high affinity IgE receptor polymorphisms of intron 2 and exon 7 (chromosome 11).
(iii) The Lymphotoxin alpha NcoI polymorphism and the Tumour Necrosis Factor-308 polymorphism which both lie within the TNF locus on Chromosome 6

These candidates were selected because

a) they all lie in chromosomal areas with reported linkage to asthmatic phenotypes

b) they are all genes with potential pathobiological significance in the development and course of late onset airflow obstruction.

c) Most produce a nucleotide change which results in a change in the encoded amino acid
d) the frequency of the polymorphisms in the Caucasian population is sufficiently high to yield reasonable statistical power, given the intended sample size

2.1.2 Subject recruitment

Case recruitment

Cases were recruited from the outpatient Departments of Respiratory Medicine at Glenfield Hospital. A second group of patients were recruited from the outpatient Department of Medicine for the Elderly at Manchester Royal Infirmary. This subset was recruited to establish if there were any geographical differences in genotype distribution unique to the Leicester population. This is of potential importance in future collaborative extensions of our work.

If clinically indicated, for example if there was a strong family history of emphysematous lung disease, early onset disease, or predominately emphysematous disease, alpha-1-antitrypsin genotype was determined. Individuals who were ‘zz’ homozygotes were excluded.

Inclusion criteria

- Definite airflow obstruction with a clinical diagnosis of asthma or COPD
- Symptom onset after 45 years of age
- Caucasian origin

Exclusion criteria

- Bronchiectasis
- Uncontrolled medical condition, malignancy
- Dementia - defined as a Hodkinson mini-mental test score less than 7/10 (Hodkinson HM, 1972).
- Recent respiratory tract infection
Control recruitment

For each case recruited the subjects General Practitioner was approached and asked to generate an age and sex matched list. This list excluded, all non-Caucasians, all those with known malignancy or uncontrolled medical conditions, and those with dementia. A diagnosis of a respiratory disease was not an exclusion criteria. The first three individuals from this (non-alphabetical) list were contacted and all agreeable responders were included in the study. Where the General Practitioner declined to participate, the principal of the General Practice that was closest geographically to the cases home address was subsequently approached. This method of control recruitment was employed in an attempt to match as accurately for age, sex and also for geographical area. Control assessments were carried out on a single visit to the outpatients department, or where requested by the volunteer, on a visit to their home.

2.1.3 Data collection

All cases and controls completed a doctor-administered questionnaire with the author (Appendix A).

This contained details of occupation, current symptomatology and age at onset, medication use, details of past history of asthma, eczema and hay fever, with age at onset. In particular, specific response was recorded to the question “Has a doctor ever told you that you have asthma?” Presence of other co-morbid conditions was recorded. A family history of presence or absence of asthma, bronchitis or emphysema was recorded for all first-degree relatives.

Occupational History

A single occupation was recorded for each individual, this was the major occupation during working life. Where women had not been in regular employment, occupational history of
spouse was not recorded. In addition data on occupational history was not collected from the Manchester subjects, and thus data on occupational history was incomplete.

Occupation was classified according to the Registrar General classification (I-V).

Smoking History

Information on smoking history was recorded as follows

- current smoking status (never/ex/current).

For all ex-smokers and current smokers the total pack-year history was recorded. The pack-year history was categorised into the following groups, which in all cases represented the best estimate of lifetime tobacco exposure.

1 = < 5 pack-years
2 = 5-9 pack years
3 = 10-19 pack-years
4 = 20-29 pack-years
5 = 30-39 pack-years
6 = 40-49 pack-years
7 = 50-59 pack years
8 = >60 pack-years

Exact pack-years were not recorded, since although some smokers, particularly current smokers, were able to accurately recorded total number of years smoking and average number of cigarettes smoked/day, many ex smokers were not able to recall to the nearest year total number of years smoking.

Spirometry

All spirometry was performed in accordance with American Thoracic Society guidelines. The manoeuvres were performed with the subject seated in a standard manner. Each subject performed a minimum of three forced vital capacity manoeuvres, and the best value was selected. If subjects did not perform an acceptable manoeuvre, they were asked to make a further attempt, up to a maximum of eight times. A test was deemed acceptable if the FEV1
values showed a variability of +/- 5%. Spirometry was carried out on a Micolab Medical ML3300 portable spirometer or using the Morgan Medical Pulmolab system. Eleven subjects were tested on both machines to ensure comparability of results. The Morgan Medical Pulmolab system was calibrated daily and the portable Micolab Medical ML3300 spirometer was maintained according to the manufacturers instructions. Predicted values were based on European Community Coal and Steel Estimates (ECCS).

**Bronchodilator reversibility testing**

All cases underwent reversibility testing to nebulised bronchodilators. Where this had been carried out in the preceding 12 months it was not repeated. Cases were instructed to abstain from bronchodilator use for 12 hours before testing, unless they developed significant respiratory symptoms necessitating bronchodilator use.

Spirometry was performed on arrival after a short rest period. Nebulised salbutamol (2.5mg) was administered via a face-mask and spirometry repeated after 15 minutes. A positive bronchodilator response was regarded as an increase in FEV1 of 200mls and at least 15%, or an increase FVC of 330mls or at least 15%, occurring 20 minutes after administration of nebulised salbutamol.

**IgE assays**

A ten millilitre clotted sample was taken for estimation of total immunoglobulin E (IgE) levels. Specific radio-immuno-assay testing and skin prick testing were not routinely performed.
Full blood count and eosinophil counts

All cases and controls individuals had a full blood count and a differential white cell count to establish the degree of eosinophilia.

2.1.4 Deoxyribonucleic acid (DNA) extraction and storage

DNA extraction, and genotyping for four polymorphisms, was carried out in Leicester principally by a senior laboratory technician (Mrs M Hill), who was responsible for training the author in the procedures. DNA was extracted from whole blood collected in EDTA bottles using a Puregene DNA extraction kit (Flowgen, Ashby de la Zouche, Leicester).

Method for DNA extraction

1.35mls of blood was mixed with 4.5mls of red blood cell lysis solution and centrifuged for 10 minutes. The supernatant was discarded and the pellet re-suspended in 1.35mls of cell lysis solution and then incubated in a water bath at 37°C to ensure it had completely dissolved. An excess of protein precipitation solution was then added followed by centrifugation. The supernatant containing DNA was collected into a clean tube and 100μl of isopropanolol added to precipitate the DNA. After a short centrifugation the supernatant was discarded and the tubes drained on clean paper.

The final stage involved washing of the DNA sample with a 70% ethanol solution to remove any remaining isopropanolol. The sample was then air dried and then re-suspended in DNA hydration solution. The sample was stored at -20°C until required for genotyping.
Purity and yields of DNA samples

Purity of the DNA samples was assessed using optical densities. The optical density (OD) of the sample was measured at 260nm and 280nm using an UV spectrometer.

For pure DNA samples the ratio OD 260nm/OD 280nm should be 1.8.

DNA samples recording a ration of between 1.6 and 2.0 are also acceptable. Impurity of the DNA sample was expected if the ratio fell below the lower limit of 1.6. In order to assess purity further each DNA sample was electrophoresed on a 0.8% agarose gel, both pre and post digestion with HIND III. Where there was no evidence of digestion (or minimal digestion) in combination with a low OD ratio, protein contamination was suspected and DNA extraction was repeated.

Calculation of total DNA yield

Yields were calculated by measurement of optical density of the DNA sample at 260nm and 280nm

\[
\text{Yield of DNA(µg)} = \frac{\text{Total volume of DNA (µl)}}{\text{Volume DNA in test sample(µl)}} \times \text{OD}260 \\
\]

\[
0.02
\]

2.2 Genotyping

Details of the techniques used for genotyping of each of the polymorphisms can be found in Chapters 5 and 6 for the methods used in Leicester, and in Appendix B for the methods used in Nottingham.

Details for basic laboratory procedures are found in Appendix B
2.3 Primary hypotheses

Seven primary hypotheses were postulated and examined in the statistical analysis. Secondary exploratory analysis was also carried out for a number of outcome variables depending on the genotype under investigation. For the primary hypotheses, a p value of equal or less than 0.05 was accepted as a level of statistical significance. A limitation on the number of primary hypotheses tested to a total of seven is in keeping with this level of significance. The pre-determined primary hypotheses are presented here but discussed in greater length in Chapter 4 (for hypotheses (i)-(ii)), chapter 5 (for hypotheses (iii)-(iv)). And Chapter 6 (for hypotheses (v)-(vi)).

Primary Hypotheses.

(i) The beta-2-adrenoceptor polymorphisms at codons 16 and 27 are associated with bronchodilator reversibility in individuals with late onset airflow obstruction.

(ii) The beta-2-adrenoceptor polymorphisms at codons 16 and 27 are associated with disease severity in subjects with late onset airflow obstruction.

(iii) The high affinity IgE receptor polymorphisms of intron 2 and exon7 are associated with susceptibility to late onset airflow obstruction.

(iv) The high affinity IgE receptor polymorphisms of intron 2 and exon7 are associated with atopy and eosinophilia.

(v) The tumour necrosis factor polymorphisms, TNF-308 and LTαNcoI, are associated with susceptibility to late onset airflow obstruction.

(vi) The tumour necrosis factor polymorphisms, TNF-308 and LTαNcoI are associated with severity of disease in subjects with late onset airflow obstruction.
2.4 Statistical methodology

Characteristics of the case and control populations were compared. For binary variables Pearson's chi-test was used (and Fisher's exact test for small cell counts). For quantitative variables, Student's t test or Mann Whitney U test was used.

The statistical methodology used to examine the relationships between genotypes and the various outcome variables is described in the methods for Chapter 4, 5, and 6.

2.4.1 Power calculations and sample size

Power calculations for the purposes of genetic studies are notoriously sensitive to assumptions. However it was estimated that a sample size of 200 cases and 200 controls would be associated with for example with the ability to detect a difference for susceptibility of 2% and 9% between cases and controls for the TNF308 polymorphism (2,2) and 11% and 22% for the lymphotoxin alpha polymorphism (1,1) (p 0.05, β = 0.2). These differences in susceptibility are in the order of magnitude reported in studies with juvenile onset asthma for these particular genotypes.

Data was stored on a Microsoft Access database and analysed using a statistical package, SPSS version 9.0 and STATA.

2.5 Ethical considerations

All participating individuals gave informed written consent and gave specific consent for the storage of a DNA sample for future genetic analysis by named researchers. A local and a multi-centre research ethics committee approved the protocol.
Consent from individuals to participate in any study involving the collection and storage of genetic material involves particularly important ethical considerations.

Currently there are no UK guidelines specifically relating to DNA collections from human populations, although this may soon change. The proposed UK Biomedical Population Collection will have specific regulatory guidelines, probably based on the Medical Research Councils guidelines on biological samples and personal medical information. The Icelandic experience has led to an increased public awareness and concern over the establishment of databases and their potential for misuse of data by insurers, employers and other third parties.

For the purposes of our studies we did obtain consent for the storage of DNA samples for future genetic studies in the field of airflow obstruction by our group. Any other uses of our genetic material were not permissible without a fresh approach to the subject, by ourselves, being made. The consent form used for the study is included in Appendix A.
3 CHARACTERISTICS OF THE STUDY POPULATION

3.1 Cases

A total of 297 cases consented to enter the study, completed a questionnaire and provided a blood sample for genotyping. 257 cases were recruited from the Department of Respiratory Medicine at Glenfield Hospital Leicester and 40 from the Department of Medicine for the Elderly in Manchester. All the Leicester cases, and nine of the Manchester cases were recruited and assessed by the author. Dr M Connolly recruited the remaining cases from Manchester.

3.2 Population Controls

General Practitioners from 51 practices within Leicestershire had cases included in the study. All these practices were contacted and 33 practices agreed to take part. Each practice was visited and a list of age and sex matched controls was produced, (matched for each case registered at the practice). From these lists a total of 347 individuals were invited to participate in the study. 160 (46%) agreed to take part and full assessment was completed in 154 of these individuals. The six excluded volunteers had a contraindication to study entry (non-Caucasian, unknown dementia), or withdrew consent. 41% of those individuals contacted failed to respond to our letter and 13% declined to participate. We did not receive ethical committee approval to contact non-responders a second time. All the control subjects were recruited and assessed by the author.
Table 3.1 Characteristics of the case and control populations

<table>
<thead>
<tr>
<th></th>
<th>Case</th>
<th>Control</th>
<th>‘p’ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of subjects</strong></td>
<td>297</td>
<td>154</td>
<td>-</td>
</tr>
<tr>
<td><strong>Mean Age (with SD)</strong></td>
<td>68.1(10.6)</td>
<td>68.5(8.6)</td>
<td>0.685*</td>
</tr>
<tr>
<td>range 45-91</td>
<td></td>
<td>range 45-81</td>
<td></td>
</tr>
<tr>
<td><strong>Sex (% male)</strong></td>
<td>55.4 male</td>
<td>59.1 male</td>
<td>0.454†</td>
</tr>
<tr>
<td><strong>Personal history of hay fever (%)</strong></td>
<td>11.4</td>
<td>14.3</td>
<td>0.112†</td>
</tr>
<tr>
<td><strong>Personal history of eczema (%)</strong></td>
<td>9.4</td>
<td>7.8</td>
<td>0.351†</td>
</tr>
<tr>
<td><strong>Additional diagnosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (%)</td>
<td>36.7</td>
<td>38.3</td>
<td></td>
</tr>
<tr>
<td>One (%)</td>
<td>31.3</td>
<td>32.5</td>
<td>0.426†</td>
</tr>
<tr>
<td>Two (%)</td>
<td>20.9</td>
<td>22.1</td>
<td>0.275‡</td>
</tr>
<tr>
<td>Three or more (%)</td>
<td>11.1</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td><strong>Mean Total pack year history (with SD)</strong></td>
<td>4.5 (2.48)</td>
<td>2.7(2.53)</td>
<td>&lt;0.001‡</td>
</tr>
<tr>
<td>- also see text and Table 3.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Family history of asthma or COPD (%)</strong></td>
<td>58.9</td>
<td>41.2</td>
<td>&lt;0.001‡</td>
</tr>
<tr>
<td><strong>Current clinician diagnosis of asthma/COPD (%)</strong></td>
<td>100</td>
<td>5.8</td>
<td>&lt;0.001‡</td>
</tr>
<tr>
<td><strong>Use of asthma medication (%)</strong></td>
<td>98</td>
<td>5.8</td>
<td>&lt;0.001‡</td>
</tr>
</tbody>
</table>

* Students-Test for equivalence of means
† Pearsons Chi square test (or Fishers exact for cell counts less than 5)
‡ Chi square test for linear trend
3.3 Characteristics of cases vs population controls

3.3.1 General

A total of 451 individuals were recruited, comprising of 297 cases, 154 population derived controls. The characteristics of the case and population controls are presented in Table 3.1. There were no significant differences in age or sex.

3.3.2 History of atopy

A self-reported history of hay fever or eczema was sought from all individuals. This appeared to be uncommon in both the case and control populations, with no significant differences in prevalence between the cases and controls. 14 individuals were uncertain if they had a history of hay fever and 12 individuals did not know if they had a history of eczema.

3.3.3 Comorbidity

Self reported co-morbidity was common, with 63% of all cases reporting at least one co-existing medical condition, and 62% of controls reporting at least one medical condition. There was no statistically significant difference in the overall mean number of co-morbid conditions between the case and control groups, nor was there a statistically significant trend for the cases to have a greater number of co-existing conditions compared to controls ($\chi^2$ test for trend 1.19, $p=0.275$). Co-morbidity data was collected on the basis of self-reported symptoms and review of medication. Further validation was not sought from medical notes or General Practitioner records.
3.3.4 **Occupational history**

Data on occupational history was also collected. Data was available for 237 cases (80%) and 141 controls (92%). There were some differences in Social class between the case and control groups. Amongst the controls, 23% were from Class I or II, compared with 12% amongst cases, and 28% of controls from classes IV and V compared with 45% of cases. Occupational histories reflected the major industries within the Leicester area, with particularly large numbers employed in the hosiery trade and engineering industries. Six individuals (four controls and 2 cases) had spent some time as coal miners, although none were miners for the majority of their working lives (this was an exclusion criteria for entry into the study).

3.3.5 **Smoking status**

Current smoking status was recorded for all subjects. Amongst the cases, 23.6% were current smokers and 63.3% were ex smokers. Only 11.1% of subjects with airflow obstruction had never smoked. Data was missing for the remaining 2% of cases. In contrast 29.9% of the controls had never smoked, 53.2% were ex-smokers and 16.2% current smokers (data missing from 0.6%). The lifetime total tobacco exposure was recorded as pack-year history in nine categories, as previously described in Chapter 2. Smoking history for the study population is shown in Table 3.2. There was no statistically significant difference in being a current smoker, compared to an ex or never smoker, between the case and control groups. However the case and control populations had a highly significant difference in their total lifetime tobacco exposure ($\chi^2$ test for trend 47.2, $p<0.001$). Given the method of control recruitment these differences had been anticipated.
Table 3.2 Smoking habits for case and control populations

<table>
<thead>
<tr>
<th></th>
<th>Case</th>
<th>Control</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number (%)</td>
<td>Number (%)</td>
<td>Total (%)</td>
</tr>
<tr>
<td><strong>Current</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>6 (2)</td>
<td>1 (0.6)</td>
<td>7 (1.6)</td>
</tr>
<tr>
<td><strong>smoking</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>33 (11.1)</td>
<td>46 (29.9)</td>
<td>79 (17.5)</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>188 (63.3)</td>
<td>82 (53.2)</td>
<td>270 (59.9)</td>
</tr>
<tr>
<td>Current</td>
<td>70 (23.6)</td>
<td>25 (16.2)</td>
<td>95 (21.1)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>297 (100)</td>
<td>154 (100)</td>
<td>451 (100)</td>
</tr>
</tbody>
</table>

|                  |            |            |            |
| **Total pack-**  |            |            |            |
| year history     |            |            |            |
| >5               | 52 (17.5)  | 62 (40.3)  | 114 (25.3) |
| 5-9              | 5 (1.7)    | 15 (9.7)   | 20 (4.4)   |
| 10-19            | 27 (9.1)   | 23 (14.9)  | 50 (11.1)  |
| 20-29            | 43 (14.5)  | 16 (10.4)  | 59 (13.1)  |
| 30-39            | 50 (16.8)  | 10 (6.5)   | 60 (13.3)  |
| 40-49            | 53 (17.8)  | 12 (7.8)   | 65 (14.4)  |
| 50-59            | 34 (11.4)  | 8 (5.2)    | 42 (9.3)   |
| >60              | 33 (11.1)  | 8 (5.2)    | 41 (9.1)   |
| **Total**        | 297 (100)  | 154 (100)  | 451 (100)  |
3.3.6 Family history

A family history of asthma, bronchitis or emphysema, but not atopy, was sought for all first-degree relatives of subjects and a limited family tree drawn. 59.7% of all cases compared with 41.1% of controls reported at least one first degree relative with asthma, emphysema or bronchitis, and this difference was statistically significant ($\chi^2$ test 13.4, p=0.001 (n= 412)). 39 individuals (9%) were adopted, or had no contact with siblings and/or offspring and thus were unable to provide accurate family data. Further confirmatory evidence to confirm family history was not available.

3.3.7 Medication use

A large proportion of cases were taking regular salbutamol, either by inhaler or nebulised form (91.6%), and 44.9% were taking ipratropium. The majority of subjects were also taking inhaled corticosteroids 78.4%, but long term use of oral corticosteroids was unusual 7.1%. All cases had developed their disease after the age of 45 years, as specified in the entry criteria, but the duration of symptoms was variable. The mean duration of symptoms at time of recruitment was 8.3 years (SD 7.56, range 0-39).

3.3.8 Symptoms/diagnosis

By definition all cases had a clinical diagnosis of asthma, emphysema or chronic obstructive pulmonary disease, and all were symptomatic.

The most frequent symptom amongst the cases was breathlessness, which was reported by 92%. Wheeze and chronic cough were also common (62% and 49% respectively). Cough with sputum production on most days was reported by 28% and chest tightness in association with breathlessness by 32%. The presence of nocturnal symptoms was less common (12%).
The control population was unselected with respect to respiratory disease, and a significant proportion reported some respiratory symptoms (24%), particularly breathlessness (16%). However, only 6% had a clinical diagnosis of asthma or were on regular bronchodilator therapy.

Amongst the cases 34%, (n=101) reported that a physician had diagnosed asthma (as opposed to COPD) at some stage during their illness (although some individuals were subsequently diagnosed as having COPD). Fifteen individuals were unable to recall if they had been given a diagnosis of asthma or not. Those with a diagnosis of asthma were more likely to have been never smokers ($\chi^2 14.4$, $p=0.002$) and there was a strong inverse relationship between total pack year history and likely diagnosis of asthma ($\chi^2$ test for trend 29.8, $p=<0.001$). The asthmatics were also more likely to be female ($\chi^2 13.2$, $p=0.001$). The mean age of the asthmatics compared to the COPD subjects was not significantly different (67(SD10.3) years vs 69 years (SD 10.6), students t-test, $t=-1.65$, $p=0.1$), but the asthmatics developed their initial symptoms at a slightly younger age (57 years (SD 9.9) vs 60 years (SD 12.5), Students t-test, $t=2.14$, $p=0.033$). These results are summarised in Table 3.3.
<table>
<thead>
<tr>
<th>Cases</th>
<th>Doctor diagnosed</th>
<th>Doctor diagnosed</th>
<th>'p' value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>asthma</td>
<td>COPD</td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>101</td>
<td>181</td>
<td>-</td>
</tr>
<tr>
<td>Mean Age (SD)</td>
<td>66.9(10.3)</td>
<td>69.1(10.6)</td>
<td>0.1*</td>
</tr>
<tr>
<td>Sex (% male)</td>
<td>42.6%</td>
<td>64.1%</td>
<td>0.001*</td>
</tr>
<tr>
<td>Mean total pack year history</td>
<td>3.6(2.62)</td>
<td>5.0(2.22)</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Bronchodilator reversibility</td>
<td>61.2%</td>
<td>68.0%</td>
<td>0.258‡</td>
</tr>
<tr>
<td>Age at onset of symptoms</td>
<td>57.4(9.9)</td>
<td>60.5(12.5)</td>
<td>0.033*</td>
</tr>
</tbody>
</table>

Table based on response to question 'has a doctor (GP) ever told you that you have asthma?'

*Students t-test
†Chi square test for linear trend
‡Pearson's Chi square test
3.3.9 Summary

The case and control populations were well matched for age and sex, critical factors that may confound genetic association studies of late onset disorders. Not surprisingly, there were statistically significant differences in the lifetime total tobacco exposure, presence of family history of respiratory disease and social class between the case and control subjects. Co-morbidity was common, but surprisingly given the differences in smoking history between the case and control subjects, the prevalence of co-morbidity was fairly similar between the two groups, with no statistically significant differences between the case and control populations. The prevalence rate of 5.9% for self-reported asthma amongst our population controls is similar to that described in large epidemiological surveys of older populations (Enright PL, 1994). This suggests that our population controls are reflective of a general population sample, from which the cases have been drawn. Furthermore, the method of population control recruitment automatically resulted in matching of the two groups by geographical area.
3.4 Outcome variables

A large number of outcome variables were collected from all subjects. In this section I shall describe the characteristics of some of the key outcome variables in the study population.

3.4.1 DNA extractions and genotyping

From a total of four hundred and ninety nine subjects recruited to the study, DNA was successfully extracted from 476 individuals (95%). In the remaining 23 individuals two further attempts to extract DNA were made, after which no further DNA extraction was attempted. Failure to extract DNA from whole blood samples occurred in all three subjects groups (cases, siblings and population controls). The reasons for the failure of DNA extractions were not studied further.

Where DNA extraction was successful, subjects were all genotyped for three of the polymorphisms, the lymphotoxin alpha NcoI polymorphism, and the high affinity IgE receptor polymorphisms (intron 2 and exon 7). For the other polymorphisms genotyping was less successful and the proportion genotyped for each polymorphism is shown in Table 3.4.
Table 3.4 DNA extractions and genotyping

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Population controls</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number (%)</td>
<td>Number (%)</td>
<td>Number (%)</td>
</tr>
<tr>
<td>DNA Extraction</td>
<td>Yes</td>
<td>285 (96.0)</td>
<td>144 (93.5)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>12 (4)</td>
<td>10 (6.5)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>297 (100)</td>
<td>154 (100)</td>
</tr>
<tr>
<td>LTNcoI</td>
<td>Yes</td>
<td>282 (100)</td>
<td>144 (100)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>3 (0.7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>285 (100)</td>
<td>144 (100)</td>
</tr>
<tr>
<td>TNF 308</td>
<td>Yes</td>
<td>281 (98.6)</td>
<td>142 (98.6)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>4 (1.4)</td>
<td>2 (1.4)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>285 (100)</td>
<td>144 (100)</td>
</tr>
<tr>
<td>B2AR16</td>
<td>Yes</td>
<td>270 (95)</td>
<td>142 (99)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>15 (5)</td>
<td>2 (1)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>285 (100)</td>
<td>144 (100)</td>
</tr>
<tr>
<td>B2AR27</td>
<td>Yes</td>
<td>270 (95)</td>
<td>142 (99)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>15 (5)</td>
<td>2 (1)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>285 (100)</td>
<td>144 (100)</td>
</tr>
<tr>
<td>FceRI Intron 2</td>
<td>Yes</td>
<td>283 (99)</td>
<td>144 (100)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>2 (1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>285 (100)</td>
<td>144 (100)</td>
</tr>
<tr>
<td>FceRI Exon 7</td>
<td>Yes</td>
<td>283 (99)</td>
<td>144 (100)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>2 (1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>285 (100)</td>
<td>144 (100)</td>
</tr>
</tbody>
</table>
3.4.2 Spirometry

Spirometry was successfully carried out in 291 of the case subjects (98%), 153 of the population controls (99%) and 47 of the sibling controls (98%).

Comparison of laboratory vs home spirometry.

Spirometry was carried out using two different machines. In order to ensure comparability of results, a proportion of subjects, both cases and controls were invited to attend for two spirometry sessions, one on each machine.

A total of 11 individuals, randomly selected from the case and population control subjects, had spirometry carried out using both machines. The FEV1 was used to compare measurements between the two machines. The mean of the differences between the two machines was 46mls with 95% limits of agreement ranging from -408mls to +316mls. Given that the reliability of FEV1 measurements is -200mls to +200mls this difference was felt to be acceptable.

Spirometry for cases

The data for forced expiratory volumes amongst all the cases showed a normal distribution. The mean absolute FEV1 amongst the cases was 1.19L(SD 0.57, range 0.37-3.16L). A total of 120 cases (41%) had an FEV1 with an absolute value of less than 1.0L. The mean value for FEV1 as a percentage of its age, height and sex adjusted predicted value was 49%(SD20.8, range 11.6-117.6%).

Predicted FEV1 values were not available for 14 of the case subjects (all Manchester cases). From the remaining 277 cases, one hundred and two cases (36.8%) had a FEV1 of less than 40% of its predicted value, 93 cases (33.6%) an FEV1 of between 40 and 59% predicted and
55 cases (19.9%) an FEV1 of between 60 and 79% of predicted. Twenty seven cases (9.7%) had an FEV1 of greater than 80% of predicted value. All individuals with an FEV1 of greater than 80% of its predicted value had a clinical diagnosis of asthma, rather that COPD. The FEV1/FVC ratio showed a normal distribution amongst the population of cases. The mean FEV1/FVC ratio was 49% (SD 12.4, range 24-83). The ratio was also calculated as a percentage of the predicted age, height and sex adjusted FEV1/FVC ratio. The mean FEV1/FVC ratio as a percentage of the predicted value was 65%(SD 16.2, range 32-105). 279 cases (95.5%) had an FEV1/FVC ratio of less than 70% and 267 cases (92 %) a ratio of less than 65%. 12 subjects had a ratio of greater than 70% at the time of spirometry. All of these individuals had a clinical diagnosis of asthma, documentation of previous airflow obstruction, and/or a methacholine challenge test consistent with a diagnosis of asthma.

**Spirometry for controls**

The data for forced expiratory volumes amongst the controls showed a normal distribution. The mean absolute FEV1 was 2.33L(SD 0.65). The mean value for FEV1 as a percentage of its age, height and sex adjusted predicted value was 93.6(SD 18.3). The FEV1/FVC ratio showed also showed a normal distribution. The mean FEV1/FVC ratio was 75.8% (SD 0.09). The ratio was also calculated as a percentage of the predicted age, height and sex adjusted FEV1/FVC ratio. The mean FEV1/FVC ratio as a percentage of the predicted value was 100%(SD 0.11)

### 3.4.3 Diffusing capacity

Data on diffusing capacity was not routinely collected as part of the study protocol. However, wherever available from the medical records recent values (within the past 24 months) for lung diffusion were recorded. The total lung diffusing capacity for carbon dioxide (TLCO)
and alveolar diffusing capacity for carbon dioxide (KCO) were both recorded as a percentage of their predicted values. No data on CT evidence of emphysema was recorded, since this data was available for only a small number of subjects. Diffusing capacity data was available for 175 cases (59% of all cases, 82% of COPD subjects). The mean KCO (SD) as a percentage of its predicted value was 85.5 (29.9), TLCO 92.9 (27.1).

3.4.4 Bronchodilator reversibility testing
All case subjects were invited to attend for bronchodilator reversibility testing. This was completed in 276 of the cases, according to the study protocol as described in Chapter 2. A large proportion of cases were reversible to bronchodilators (66%). The presence of bronchodilator reversibility showed no relationship to patients reporting a physician diagnosis of asthma over COPD ($\chi^2$ 1.28, p=0.258, n=276, Table 3.3), but was related to total pack-year history ($\chi^2$ test for trend, p=0.013).

3.4.5 IgE
Total immunoglobulin E levels (TIgE) were obtained from 429 subjects (86%). The data on TIgE was not available from 70 subjects. Amongst the Leicester samples (number = 55) missing data was due to haemolysis of samples in 17 subjects, insufficient blood samples in 16 subjects, where the subject declined to allow a further attempt at phlebotomy, and for 22 samples a result could not be traced, despite documentation that an adequate sample was collected. Fifteen samples from Manchester had no data for TIgE, of these one individual refused blood sampling for TIgE. For the remaining fourteen Manchester samples the reasons for data omission were not stated. Serum IgE values showed a right-skewed distribution, median 45 (interquartile range 15-156, range 1.3-4535). Serum IgE levels were log-normally transformed and geometric values were
calculated. The geometric mean was 49.4, SD 4.95. Geometric mean was significantly higher amongst the cases compared to the population controls (66.5 vs 35.0, t=3.91, p<0.001). The relationship between serum IgE levels and smoking status, disease and genotypes is discussed in detail Chapter 6

3.4.6 Full Blood Counts

Full blood count data, including eosinophil counts, were available from 438 (88%) of subjects. Data for full blood counts and eosinophil counts were available for 431 (94%) of the Leicester subjects but only 7 (18%) of the Manchester cases.

3.4.7 Summary

The case population was representative of the broad spectrum of phenotypes that late onset airflow obstruction encompasses.

Spirometry demonstrated that our cases had, on average moderate airflow obstruction (based on the BTS criteria- Table 1.1). The control population had essentially normal spirometry with an average FEV1 of 93% predicted values and an FEV1/FVC ratio of 100% predicted values.

Bronchodilator reversibility was common in this older population, both in those with late-onset asthma and those with COPD as defined by the study criteria (see Chapter 4).

Our cases did show some evidence of atopy, with elevated total IgE levels compared to the population controls. There was evidence of considerable interaction between IgE levels and smoking and this data is presented and discussed in Chapter 5.
4 BETA-2-ADRENOCEPTOR POLYMORPHISMS AND LATE ONSET ASTHMA, COPD AND BRONCHODILATOR REVERSIBILITY.

4.1 Introduction

The Beta-2-adrenoceptor (B2AR) gene lies within the chromosome 5q31-33 area. Linkage of 5q markers to a range of asthmatic phenotypes has been reported by a number of authors in genome wide screens and also in non-genome wide genetic studies (Ober C, 1999; Marsh D, 1994; Postma DS, 1995; CSGA 1997; Doull JJ, 1996; Meyers DA, 1994). There are a number of candidate genes for asthma and atopy within this region, (I1-13, I1-4, Beta-2-adrenoceptor, I1-5, I1-3) and it is likely that all these loci contribute to the overall asthmatic phenotype.

The B2AR gene codes for an intronless 1239 base pair trans-membrane receptor. Beta adrenoceptors are widely distributed in airway smooth muscle, particularly the small airways, but also in other cells within the lung, such as mast cells and epithelial cells. Activation of the receptor induces airway relaxation. Beta agonists interact with the receptor, probably by binding to and stabilising the receptor in its activated form, thus producing airway smooth muscle relaxation and bronchodilation in patients with asthma (Johnson M 1992, 1995). It is also possible that the beta agonists amplify a low inherent receptor activity to convert to the activated state. Furthermore there is some evidence to suggest that this shift may result in sustained changes that persist even in the absence of beta agonist. In addition the receptor undergoes desensitisation, the extent of which is dependent on the degree and duration of the receptor/agonist response. Given the known properties of the beta adrenoceptor one might predict that polymorphisms of the receptor would act as disease modifiers or alter responsiveness to beta agonist therapy (Johnson M 1992, 1995).
A total of nine polymorphisms of the B2AR have been identified (Reishaus E, 1993). All of these variants result in a base pair change, but only four result in an amino acid change. Two of these are common in Caucasian populations. The first is the Arginine to Glycine substitution at codon 16(Arg16Gly), and the second is the Glutamine to Glutamate substitution at codon 27(Gln27Glu). Both of these polymorphisms lie within the extracellular domain of the receptor.

Both the Arg16Gly and the Gln27Glu appear to be functionally relevant (Green SA, 1993, 1994, 1995). In vitro studies have shown that cells expressing the Glu27 form of the receptor have attenuated down-regulation following beta agonist exposure (Green SA, 1994). Cells expressing the Gly 16 polymorphism show enhanced down-regulation. There is also some in vitro evidence to suggest that the polymorphisms may also have effect on desensitization.

Chong LK (2000) examined the effectiveness of beta agonists to inhibit histamine release from human lung mast cells. Long-term exposure to agonist reduces the effectiveness of the agonist to inhibit mast cell release, but this desensitization is highly variable. The Gly 16 and Glu 27 forms of the receptor were found to be resistant to desensitization compared with the Arg16 and Gln 27 forms.

The polymorphisms at codons 16 and 27 have been shown to correlate with a number of asthmatic phenotypes. The results from a number of these studies are summarised in Tables 4.1-4.3. Despite the evidence of linkage of asthma to markers at the 5q locus there have been no studies linking or associating either of the polymorphisms to asthma or atopy per se- that is no differences in distribution of genotype between case and control group has been shown.

A recent meta-analysis, using pooled data from linkage studies (Jacobs KB, 2001) has shown no evidence to support linkage of the chromosome 5q31-33 cluster to asthma susceptibility or atopy measured by IgE levels.
However a number of studies have focused on the relationship between the polymorphisms and components of the phenotype.

Considering the polymorphism at codon 27 first, *In vitro* studies of Chinese hamster fibroblasts suggest that the Glu 27 variant is resistant to beta agonist induced receptor downregulation (Green SA, 1995, 1994) The Glu 27 variant appears to be associated with less reactive airways (Hall IP, 1995; Ramsay CE, 1999) although this finding has not been repeated in all studies (Summerhill E, 2000). One group, (Dewar JC, 1997) have shown an association between the Gln 27 variant and elevated total serum IgE levels amongst asthmatic families, although a more recent study of atopic families failed to confirm this finding (Deichman KA, 1999).

The importance of the Arg → Gly 16 substitution is also unclear. Initial studies found associations of the Gly 16 variant to oral steroid use and the presence of nocturnal symptoms (Reihaus E, 1993; Turki J, 1995). In contrast to the effects of the Glu 27 polymorphism, *In vitro* studies have show that the Gly 16 variant shows increased downregulation of the receptor after beta agonist challenge. Subsequently the Gly 16 polymorphism has been associated with decreased responsiveness and desensitization to beta agonists (Tan S, 1997). Two studies have shown that individuals homozygous for Arg16 had greater bronchodilator responses to a single dose of inhaled bronchodilator (Martinez FD, 1997; Lima JJ, 1999). In the study by Martinez et al this effect was not confined to those with an asthmatic phenotype.

Recently two groups have shown the Arg16 variant to be associated with decreased lung function in adult populations, measured as FEV1 (Summerhill E, 2000) and peak expiratory flow rate (Israel E, 2000).

The relationship between phenotypes and genotypes has been further complicated by the observation that the Arg16Gly and the Gln27Glu lie in linkage disequilibrium. It is clear that
certain genotypic combinations are rare, for example the Arg16/Glu27 haplotype is not recorded at all, or present at very low frequencies in most Western populations (Weir TD, 1998; Uibrect M, 2000; D’Amato M, 1998; Tan S, 1997). Thus if an Arg 16 allele is present then it becomes extremely likely that Gln will be present at position 27. Conversely when Gly16 is present Glu27 is more likely although Gln 27 occurs not infrequently. The result from studies of haplotypes have suggested that the effects of the Arg 16 polymorphism are dominant over those of the Gln27. However, result of associations between haplotypes and phenotype have been inconsistent.

An extended haplotype including the SNP47, along with the arg16 and gln27 haplotypes has also been described in a number of studies. There has been a single published study examining the role of the arg16 and gln27 polymorphisms in COPD (Ho L, 2001). This study of 61 cases and 41 controls reported a significant association of the arg16 genotype to susceptibility to COPD in a Chinese population. This small study is of interest, but conflicts with the recent meta-analysis by Jacobs et al (2001), which convincingly demonstrated no evidence for linkage of the cytokine gene cluster to susceptibility to asthma.

I have examined the role of these polymorphisms in my population.
## Table 4.1 The Beta adrenoceptor Gln 27 polymorphism and disease phenotypes

<table>
<thead>
<tr>
<th>Author and location of study</th>
<th>Subjects</th>
<th>Disease definition</th>
<th>Number of subjects</th>
<th>Genotype frequency Gln 27</th>
<th>Glu 27</th>
<th>Disease Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hall IP (1995)</td>
<td>mild to moderate asthmatics</td>
<td>&gt;15% variability in PEFR or bronchodilator reversibility</td>
<td>65</td>
<td>0.45</td>
<td>0.55</td>
<td>Glu 27 associated with decreased airway reactivity in stable asthmatics</td>
</tr>
<tr>
<td>Dewar JC (1997) UK</td>
<td>Families recruited from asthmatic proband. Caucasians.</td>
<td>Asthma and atopy defined as semiquantiative traits based on scoring system</td>
<td>324 individuals from 60 families</td>
<td>0.52</td>
<td>0.48</td>
<td>Glu 27 polymorphism associated with elevated basal IgE in asthmatic families.</td>
</tr>
<tr>
<td>Hopes E (1998) UK</td>
<td>Children 5-15 years recruited from A + E</td>
<td>Asthma (24%) diagnosed by self report</td>
<td>425 children</td>
<td>0.52</td>
<td>0.48</td>
<td>Glu 27 associated with less self-reported asthma in children</td>
</tr>
<tr>
<td>Ramsay CE (1999) Australia</td>
<td>Caucasian families unselected for asthma</td>
<td>N/A</td>
<td>332 individuals from 76 families</td>
<td>0.59</td>
<td>0.41</td>
<td>Glu27 associated with decreased airway reactivity</td>
</tr>
<tr>
<td>Dewar JC (1998) UK</td>
<td>Population sample enriched for atopy and bronchial hyperreactivity</td>
<td>1/3 atopic by skin prick test 1/3 BHR- methacholine challenge 1/3 unselected</td>
<td>630 adults, from 2,400 selected at random from electoral register</td>
<td>0.52</td>
<td>0.48</td>
<td>No association with risk of developing asthma or allergic disease</td>
</tr>
<tr>
<td>Summerhill (2000) USA</td>
<td>Hutterites from 4 colonies with asthma prevalence rates of 10.9%</td>
<td>Asthma defined on basis of self reported symptoms, MCh provocation and reversibility studies</td>
<td>374 Caucasians, over 6 years</td>
<td>0.64</td>
<td>0.36</td>
<td>No linkage of polymorphisms to asthma or bronchial hyperreponsiveness</td>
</tr>
<tr>
<td>Author and location of study</td>
<td>Subjects</td>
<td>Disease definition</td>
<td>Number of subjects</td>
<td>Genotype frequency</td>
<td>Disease Association</td>
<td></td>
</tr>
<tr>
<td>-----------------------------</td>
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<td>--------------------</td>
<td>--------------------</td>
<td>-------------------</td>
<td>---------------------</td>
<td></td>
</tr>
<tr>
<td>Israel E (2000) USA</td>
<td>Asthmatics participating in NHLB asthma trial</td>
<td>'mild' asthmatics on basis of FEV1 and MCh test.</td>
<td>190-all mild asthma randomised to regular or as needed beta agonist</td>
<td>0.4 0.6</td>
<td>Arg homozygotes using regular beta agonist shown decline in pulmonary function (peak flow).</td>
<td></td>
</tr>
<tr>
<td>Holloway JW (2000) New Zealand</td>
<td>Asthma volunteer file. Source of controls not stated</td>
<td>Mild -no hospital admissions + low steroid use. Severe-at least 1 admission</td>
<td>92 controls 59 mild asthma 95 severe asthma</td>
<td>0.36 0.64</td>
<td>Gly 16 associated with severe rather than mild asthma.</td>
<td></td>
</tr>
<tr>
<td>Summerhill E (2000) USA</td>
<td>Hutterites from 4 colonies with asthma prevalence rates of 10.9%</td>
<td>Asthma defined on basis of self reported symptoms, MCh provocation and reversibility studies</td>
<td>374</td>
<td>0.45 0.55</td>
<td>No linkage of polymorphisms to asthma or bronchial hyperresponsiveness. Arg 16 associated with reduced lung function in adults but not children.</td>
<td></td>
</tr>
<tr>
<td>Martinez (1997) USA</td>
<td>Children from unselected population enrolled at birth</td>
<td>Bronchodilator reversibility and parental report of wheezing</td>
<td>269 children average age 11 years. Mixed ethnicity</td>
<td>0.39 0.61</td>
<td>Arg 16 allele produces a greater response to a single dose of inhaled beta agonist- in cases and controls – evidence for gene dosing effect.</td>
<td></td>
</tr>
<tr>
<td>Deichman (1998) Germany</td>
<td>Sib pairs with atopic index case</td>
<td>Atopy defined as elevated IgE or sensitization to Aero-allergen(s)</td>
<td>258 siblings from 105 families</td>
<td>Not stated Not stated</td>
<td>No relationship between Arg16Gly and IgE responsiveness.</td>
<td></td>
</tr>
<tr>
<td>Ramsay CE (1999) Australia</td>
<td>Caucasian families unselected for asthma</td>
<td>N/A</td>
<td>332 individuals from 76 families</td>
<td>0.46 0.54</td>
<td>No association to asthmatic phenotypes</td>
<td></td>
</tr>
<tr>
<td>Reihaus E (1993) USA</td>
<td>OPD respiratory medicine, non smokers</td>
<td>Asthma for &gt;2 years, continuous use of &gt;1 medication for asthma</td>
<td>51 patients with mod-severe asthma 56 normal controls</td>
<td>0.28 0.72</td>
<td>Gly 16 associated with oral steroid use</td>
<td></td>
</tr>
<tr>
<td>Author and location of study</td>
<td>Subjects</td>
<td>Disease definition</td>
<td>Number of subjects</td>
<td>Disease Associations</td>
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<td></td>
</tr>
<tr>
<td>-----------------------------</td>
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<td>---------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ho L (2001) China</td>
<td>Recruited from outpatient department of respiratory medicine. Origin of controls not stated.</td>
<td>Based on ATS criteria. All irreversible to bronchodilators</td>
<td>65 cases of COPD, 41 smoking controls</td>
<td>Associated with susceptibility to COPD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ulbrecht M (2000) Germany</td>
<td>Random sample from population census</td>
<td>BHR + &gt; 20% decrease in FEV1 on Methacholine challenge test.</td>
<td>152 bronchial hyperreactives, 295 normal controls</td>
<td>Gly16-Gln27-Th164 protective for development of bronchial hyperresponsiveness amongst females</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weir TD (1998) Canada</td>
<td>Severe cases recruited from inpatients. Origin of controls and mild-mod cases not stated.</td>
<td>Severe-fatal or intubated Moderate- &gt;400μg inh. steroid or FEV1pred.&lt;75% Mild-&lt;400μg inh. steroid or FEV1&gt;75% predicted</td>
<td>84 non-asthmatic/non-atopic, 81 severe, 86 mild/mod asthma</td>
<td>Gly16-Gln 27 associated with moderate rather than mild asthma.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D'Amato M (1998) Italy</td>
<td>White males aged 18-25, recruited from Italian Air Force</td>
<td>Bronchial hyperresponsiveness defined by constant positive response to serial MCh</td>
<td>248 individuals, All males 18-25 years.</td>
<td>Gly16-Gln 27 associated with bronchial hyperresponsiveness in males.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tan S (1997) UK</td>
<td>Origin not clearly stated Mod-severe stable asthmatics</td>
<td>FEV1 50-80% predicted. Significant bronchodilator reversibility</td>
<td>22 asthmatics</td>
<td>Gly 16 associated with decreased responsiveness and desensitization to beta agonists. Homozygous Gly 16 dominant effects over homozygous Glu 27</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.2 Methods

4.2.1 Study population

Details of the study population, data collection methods and measurement protocols have been presented in Chapter 3. In addition, specifically for this study, COPD was defined according to the following criteria: (i) forced expiratory volume in one second (FEV₁)/forced vital capacity (FVC) ratio of < 70%. (ii) onset of their respiratory symptoms after 45 years (iii) a physician diagnosis of COPD (iv) a total lifetime ‘pack-year’ smoking history of at least 20 years.

Eczema was defined as a positive response to the question ‘Has a doctor ever told you that you have eczema?’. Nocturnal asthma was defined as the presence of nocturnal cough or wheeze on a regular basis without other symptoms of a disease exacerbation.

4.2.2 Laboratory methods

DNA was extracted from blood samples using standard methods (Purgene DNA extraction kit, Flowgen, UK) as previously described. Genotyping for the Arg 16 and Gln 27 polymorphisms was carried out by a single individual at the University of Nottingham, using allele specific oligonucleotide hybridization (Newton CR, 1989). No genotyping for either polymorphism was carried out by the author. Details of genotyping are therefore included in the Appendix B.

4.2.3 Hypotheses

The primary hypothesis were that the Arg16 and Gln27 beta adrenoceptor polymorphisms have a disease modifying effects in subjects with late onset airflow obstruction. We hypothesised that the polymorphisms would determine severity of disease, as measured by quantitative markers of pulmonary function.
Primary hypotheses

(i) The beta-2-adrenoceptor polymorphisms at codons 16 and 27 do not determine disease susceptibility.

(ii) Beta adrenoceptor polymorphisms interact with smoking to determine lung function in older subjects.

(iii) The beta-2-adrenoceptor polymorphisms at codons 16 and 27 are associated with disease severity in subjects with COPD.

(iv) The beta-2-adrenoceptor polymorphisms at codons 16 and 27 are associated with bronchodilator reversibility in individuals with COPD.

Secondary hypothesis

(v) Beta adrenoceptor polymorphisms are associated with eczema, nocturnal asthma

4.2.4 Statistical methodology

Analysis for associations was carried out using contingency tables, analysis of variance, and generalized linear modelling (multiple and logistic regression analysis).

Contingency table analysis was used to examine the distribution of genotypes amongst our case and control population.

The effects of the polymorphism on lung function were examined using three quantitative variables related to pulmonary function. These primary response variables were [i] FEV₁ (as a percentage of its predicted value), [ii] FVC and [iii] FEV₁/FVC ratio. Where late onset airflow obstruction occurs smoking is clearly a key aetiological factor, despite the variable relationship between pack-year history and pulmonary function, hence the regression analysis included adjustment for smoking history. However, because of the importance of smoking in
determining lung function analysis involving these primary variables was carried out for two
groups based on high (greater than 19 pack-years) and low less (than 20 pack-years/never
smokers) lifetime tobacco exposure. The value of greater or less than 20 pack-years was
selected on the basis of the median pack-year history for the whole study population which
lay in the 20-29 pack-year category (Table 3.2)

For the analysis of subjects with COPD, the primary response variables were [i] FEV₁ (as a
percentage of its predicted value), [ii] FVC and [iii] FEV₁/FVC ratio, [iv] percentage (and [v]
total mls) change in FEV₁ in response to a nebulised bronchodilator. The primary analysis
also utilised a dichotomous response variable denoting reversibility to a bronchodilator.
Bronchodilator reversibility was defined as a post-dilator increase in FEV₁ of at least 200mls
and 15%.

Potential confounding variables considered included age as a continuous covariate, gender as
a binary covariate, pack year smoking history recorded as a categorical factor (1 = 20-29, 2 =
30 –39, 3 = 40-49, 4 = 50-59, 5 = >60). Smoking status was analysed both as a binary
covariate (current smoker vs. ex and never smokers) and as a 3-level categorical factor (never
smoker= 0, ex-smoker = 1, current = 2). Age, sex, pack-year history were included as
covariates in all models.

In order to ensure that primary conclusions were robust to modelling assumptions, genotype
was examined both as a 3-level factored variable (AA = 1, AB = 2, BB = 3) and as a binary
covariate relative to the AA genotype. This methodology primarily examines whether the
measured effects of a polymorphism are due the polymorphic variant having a linear effect on
a particular outcome (for the 3-level variable) or whether only when in the homozygous form,
a polymorphism exerts an effect, with heterozygotes displaying the ‘normal’ phenotype.

Statistical significance was taken at the 5% level.
4.3 Results

429 individuals had DNA successfully extracted, of these 412 (96.0%) were genotyped for the Arg16 and the Glu 27 beta adrenoceptor polymorphisms. The two polymorphism lay in strong linkage disequilibrium by contingency table analysis (Table 4.4).

The population consisted of 142 control subjects and 270 cases with late onset airflow obstruction. The population had a mean age (with standard deviation (SD)) of 68.2 years (9.4), 231 (56.1%) male. The median pack-year history was in the 10-19 pack-year category. There were no significant differences in age or sex between our case and control populations. Controls had significantly lower lifetime total tobacco exposure than cases (chi-square test for trend = 57.1, p < 0.001).

We hypothesized that these polymorphisms did not have a role in disease susceptibility but acted as disease modifiers. Thus, genotype frequencies for the case and control populations were examined and data shown in Table 4.5. There were no significant or important differences in genotype distribution between the case and control populations, for either the arg16 or gly27 polymorphisms.
Table 4.4. Genotype distribution for the Arg16 and Glu27 polymorphisms

<table>
<thead>
<tr>
<th>Glu27 genotype</th>
<th>Arg16 genotype</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arg16/Arg16</td>
<td>Arg16/Gly16</td>
</tr>
<tr>
<td>Glu27/Glu27</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Glu27/Gln27</td>
<td>0</td>
<td>150</td>
</tr>
<tr>
<td>Gln27/Gln27</td>
<td>54</td>
<td>44</td>
</tr>
<tr>
<td>Total</td>
<td>54</td>
<td>195</td>
</tr>
</tbody>
</table>

$\chi^2$ test = 301 <0.001

Table 4.5. Genotype frequencies for late onset airflow obstruction and control populations

<table>
<thead>
<tr>
<th></th>
<th>Case number (%)</th>
<th>Control number (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg16/Arg16</td>
<td>36(13.3)</td>
<td>18(12.7)</td>
<td>54(13.1)</td>
</tr>
<tr>
<td>Arg16/Gly16</td>
<td>126(46.7)</td>
<td>69(48.6)</td>
<td>195(47.3)</td>
</tr>
<tr>
<td>Gly16/Gly16</td>
<td>108(40.0)</td>
<td>55(38.7)</td>
<td>163(39.6)</td>
</tr>
<tr>
<td>Total</td>
<td>270(100)</td>
<td>142(100)</td>
<td>412(100)</td>
</tr>
</tbody>
</table>

Chi-square = 0.14, p = 0.932

| Glu27          |                  |                    |            |
| Glu27/Glu27    | 57(21.1)         | 23(16.2)           | 80(19.4)   |
| Glu27/Gln27    | 143(53.0)        | 79(55.6)           | 222(53.9)  |
| Gln27/Gln27    | 70(25.9)         | 40(28.2)           | 100(26.7)  |
| Total          | 270(100)         | 142(100)           | 412(100)   |

Chi-square = 1.46, p = 0.483
Full spirometric data was available for 388 (94%) subjects. Within the whole population of cases and controls, there was no apparent relationship between either polymorphism and pulmonary function, measured by FEV1 (as a percentage of its predicted value), FVC (as a percentage of its predicted value) or the FEV1/FVC ratio. Thus mean FEV1 (with standard deviation) for the Arg/Arg 16 was 64.2 (28.0), for the Arg/Gly was 64.5 (28.3) and for the Gly/Gly was 63.3(31.0). Corresponding values for mean FVC as a percentage of predicted value were 85.4(19.7), 85.0(21.7), and 83.6(23.5) respectively. Values for the FEV1/FVC ratio were Arg/Arg 57.3(17.4), Arg/Gly 58.2(16.9) and Gly/Gly 57.5(17.6). A one way analysis of variance showed that the relationship between genotype and FEV1, FVC and FEV1/FVC was not significant (one-way analysis of variance, p = 0.933, p = 0.877, p = 0.916, respectively). Because of the importance of age, sex and smoking history in determining lung function these variables were included as covariates in a regression analysis. This analysis confirmed that there was no relationship between the arg16 polymorphism and lung function in the whole population.

For the Gln27 polymorphism results were as follows. Mean FEV1 (with standard deviation) for the Glu/Glu 27 was 57.6(30.9), for the Gln/Glu was 65.7(28.6) and for the Gln/Gln was 65.3(29.2).Corresponding values for mean FVC as a percentage of predicted value were 79.9(23.6), 85.6(21.7), and 85.8(21.6), respectively. Values for the FEV1/FVC ratio were glu/glu 54.6(18.2), Gln/Glu 58.9(16.7) and Gln/Gln 58.0(17.3). A one way analysis of variance showed that the relationship between genotype and FEV1, FVC and FEV1/FVC was not significant (one-way analysis of variance, p = 0.105, p = 0.115, p = 0.169, respectively). Regression analysis showed that there was no significant relationship between Glu27 genotype and FVC or FEV1/FVC. The relationship between FEV1 and Glu27 genotype approached statistical significance (regression coefficient 7.12, 95% CI = -0.346-14.6 , p=0.062).
For the light smokers (number = 157) the polymorphisms had no apparent effect on pulmonary function.

However amongst cases and controls with a pack year history in excess of 20 pack-years (number = 231) there was an apparent relationship between various measures of pulmonary function and genotype. The results for mean FEV$_1$, mean FVC and mean FEV/FVC ratio by arg16 and glu27 genotype are shown in Table 4.6 and Table 4.7. A one way analysis of variance suggested that the relationship between arg16 genotype and FEV$_1$, FVC and FEV1/FVC was not significant (oneway, p = 0.134, p = 0.468, p = 0.119, respectively). In the regression analysis with age, sex and smoking history as covariates, a significant relationship was demonstrated between the FEV1/FVC ratio and a relationship of borderline significance between arg16 genotype and FEV1 was also suggested (Table 4.6).

For the Gln27 polymorphism, a one way analysis of variance showed that the relationship between genotype and FEV$_1$, FVC and FEV1/FVC was significant (one-way p = 0.022, p = 0.050, p = 0.037, respectively). The regression analysis confirmed that these relationships were significant and adjustment for covariates increased the strength of the associations.

Regression analysis for FEV1 suggested that Glu27 genotype was the most significant factor in determining pulmonary function in this group (regression coefficient = 12.0, 95%CI = 3.8 to 21.0, p = 0.009). In this analysis the glu27 genotype appeared to have a greater effect on pulmonary function than smoking (regression coefficient for smoking = -3.03, 95% CI = -5.47 to 0.593, p = 0.015). Corresponding values for FVC and FEV1/FVC are shown in Table 4.7.
Table 4.6 Effects of Arg16 genotype on primary outcomes for heavy smokers

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mean FEV₁ % predicted (standard deviation)</th>
<th>Mean FVC % predicted (standard deviation)</th>
<th>FEV₁/FVC (standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg16/Arg16</td>
<td>57.5(28.3)</td>
<td>80.5(17.1)</td>
<td>54.5(16.7)</td>
</tr>
<tr>
<td>Arg16/Gly16</td>
<td>54.1(25.1)</td>
<td>79.3(19.8)</td>
<td>52.6(16.3)</td>
</tr>
<tr>
<td>Gly16/Gly16</td>
<td>48.6(23.7)</td>
<td>76.4(20.2)</td>
<td>48.8(15.1)</td>
</tr>
<tr>
<td>Regression coefficient</td>
<td>-4.63</td>
<td>-2.21</td>
<td>-3.1</td>
</tr>
<tr>
<td>95% confidence intervals</td>
<td>-9.26 -</td>
<td>-5.95 – 1.52</td>
<td>-6.1 – -0.2</td>
</tr>
<tr>
<td>Significance</td>
<td>0.050</td>
<td>0.244</td>
<td>0.038</td>
</tr>
</tbody>
</table>
Table 4.7 Effects of Glu27 genotype on primary outcomes for heavy smokers.

<table>
<thead>
<tr>
<th></th>
<th>*Mean FEV₁ % predicted</th>
<th>*Mean FVC % predicted</th>
<th>*FEV₁/FVC (standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(standard deviation)</td>
<td>(standard deviation)</td>
<td></td>
</tr>
<tr>
<td>Glu27Glu27</td>
<td>44.1(24.0)</td>
<td>72.4(21.0)</td>
<td>46.4(15.1)</td>
</tr>
<tr>
<td>Glu27/Gln27</td>
<td>54.4(24.1)</td>
<td>79.6(19.3)</td>
<td>52.9(15.7)</td>
</tr>
<tr>
<td>Gln27/Gln27</td>
<td>55.6(24.5)</td>
<td>80.8(18.3)</td>
<td>52.8(16.6)</td>
</tr>
<tr>
<td>Regression coefficient</td>
<td>12.0</td>
<td>8.88</td>
<td>7.2</td>
</tr>
<tr>
<td>95% confidence intervals</td>
<td>3.08 - 21.0</td>
<td>1.66 -16.1</td>
<td>1.4-0.13.0</td>
</tr>
<tr>
<td>Significance</td>
<td>0.009</td>
<td>0.016</td>
<td>0.015</td>
</tr>
</tbody>
</table>
There were 193 cases with COPD. They had a mean age (with standard deviation (SD)) of 68 years (10), 115 (60%) male. The median pack-year history was in the 40-49 pack-year category, 63 (33 %) were current smokers, 130 (67%) ex smokers. Mean FEV\textsubscript{1} expressed as a percentage of its predicted value was 44.0(17.7), mean FVC as a percentage of its predicted value was 75.0(19.2), and the mean FEV\textsubscript{1}/FVC ratio was 46% (12). The median FEV\textsubscript{1} value for cases was 1.03 litres. Bronchodilator reversibility data was available from 192 subjects and full spirometry from 189.

There was no significant relationship between Arg16 genotype and lung function in this population (one way analysis of variance; for FEV\textsubscript{1} p = 0.363-, for FVC p = 0.622 and for FEV\textsubscript{1}/FVC ratio p = 0.460).

In the COPD subjects individuals homozygous for Glu/Glu 27 had significantly poorer lung function than the Gln27/Glu27 and Gln27/Gln27 individuals (Table 4.8). These differences in lung function were observed for measurements of FEV\textsubscript{1} as a percentage of its predicted value, and for FVC as a percentage of its predicted value. For example, mean FEV\textsubscript{1} (with standard deviation) for the Glu/Glu 27 was 38.0(18.3), compared with 45.9(18.1) for the Gln/Gln genotype. Corresponding values for mean FVC as a percentage of predicted value were 69.3(19.4), and 78.1(18.7) respectively. A one way analysis of variance showed that the relationship between genotype and FEV\textsubscript{1} was significant and that for FVC and FEV\textsubscript{1}/FVC approached significance, prior to adjustment for other confounders. (one way analysis of variance; p = 0.039 , p = 0.076, and p = 0.068 respectively). Regression analysis suggested that the relationship between Gln27 genotype and FEV\textsubscript{1} and FVC was highly significant (Table 4.8) Analysis using dummy variables suggested that glu27/glu27 homozygotes had significantly poorer lung function rather than the genotype exerting a linear effect.
The importance of these polymorphisms in determining bronchodilator reversibility was also examined. Contingency table analysis showed a significant relationship between the Glu27 polymorphism and bronchodilator reversibility when analysed as a binary covariate (Table 4.9). The degree of reversibility was also examined as a quantitative variable using millimetres change in FEV₁ and percentage reversibility. Average change in FEV₁ in millilitres and as a percentage change are shown in Table 4.8. One way analysis of variance using percentage reversibility suggested that this relationship may be of statistical significance but regression analysis was not significant.

The statistically significant difference between the Gln27 genotype and FEV₁ was preserved if post bronchodilator percent predicted values of FEV₁ were used, although the relationship was less strong (regression analysis, p = 0.030).

There was no apparent relationship between polymorphism at the codon 16 position and bronchodilator reversibility (chi-square= 2.3, p = 0.32). One-way analysis of variance for percent reversibility p = 0.26, and for mls reversibility p = 0.11.

There was no association between eczema and either the arg16 or glu27 polymorphisms. (contingency table analysis, p = 0.555 and p = 0.727 respectively) For nocturnal symptoms both Arg16 homozygotes and Gln27 homozygotes reported more nocturnal symptoms that heterozygotes or Gly16 and Glu homozygotes, although the relationship was not strong (contingency table analysis, for Arg16, chi square = 6.77, p = 0.034, for Gln27, chi-square = 9.97, p = 0.007 respectively).
Table 4.8 Effects of Glu27 genotype on primary outcomes for COPD subjects

<table>
<thead>
<tr>
<th></th>
<th>*Mean FEV₁ % predicted (standard deviation)</th>
<th>*Mean FVC % predicted (standard deviation)</th>
<th>*FEV₁/FVC (standard deviation)</th>
<th>*Reversibility to bronchodilators (percent) (standard deviation)</th>
<th>*Reversibility to bronchodilators (mls) (standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu27/Glu27</td>
<td>38.0</td>
<td>69.3</td>
<td>42.7(12)</td>
<td>25(13)</td>
<td>230(152)</td>
</tr>
<tr>
<td></td>
<td>(18.3)</td>
<td>(19.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu27/Gln27</td>
<td>45.1</td>
<td>76.2</td>
<td>47.8(12.8)</td>
<td>19(14)</td>
<td>186(137)</td>
</tr>
<tr>
<td></td>
<td>(16.8)</td>
<td>(19.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gln27/Gln27</td>
<td>45.9</td>
<td>78.1</td>
<td>45.8(12.4)</td>
<td>20(15)</td>
<td>210(162)</td>
</tr>
<tr>
<td></td>
<td>(18.1)</td>
<td>(18.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regression coefficient</td>
<td>9.70</td>
<td>10.1</td>
<td>4.6</td>
<td>5.5</td>
<td>13.3</td>
</tr>
</tbody>
</table>

95% confidence intervals

2.59 to 2.16 to -4.80 to 3.5
-10.9 - -16.2 - 42.9.

16.80 18.02
0.5

Significance p = 0.008 p = 0.013 p = 0.065 p = 0.074 p = .374

Means compared by multiple logistic regression analysis glu/glu compared with gln/gln and gln/glu

*n = 193
Table 4.9. Relationship between Glu 27 polymorphism and bronchodilator reversibility

<table>
<thead>
<tr>
<th>Bronchodilator reversibility*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>Glu27/Glu27</td>
<td>38(86%)</td>
</tr>
<tr>
<td>Glu27/Gln27</td>
<td>63(63%)</td>
</tr>
<tr>
<td>Gln27/Gln27</td>
<td>37(77%)</td>
</tr>
</tbody>
</table>

Chi square = 9.12, p = 0.011

Odds Ratio (95% CI) for glu/glu vs glu/gln and gln/gln = 3.04(1.18-7.82)

*positive bronchodilator reversibility defined as an increase in FEV₁ of 200mls or 15%, whichever greater.
4.4 Discussion

In this study I have examined the role of polymorphisms of the beta adrenoceptor on pulmonary function in older populations stratified by smoking status, and bronchodilator reversibility and pulmonary function subjects with COPD. The secondary outcomes of nocturnal asthma and eczema were also examined.

As expected, I showed that the polymorphisms are in strong linkage disequilibrium and they do not determine susceptibility to disease in this study.

Considering the population as a whole, the polymorphisms appear to have no effect on pulmonary function. However there appears to be a significant effect within the subgroup with a high lifetime tobacco exposure. However since this group consisted mainly of cases it is difficult to determine if this observation is due to smoking or is a consequence of the large numbers of COPD patients in the group.

Amongst individuals who have COPD, according to the formal definition used in this study, we have shown that the Gln→Glu27 beta-adrenoceptor polymorphism is significantly associated with pulmonary function. This is true both for FEV₁ as a percentage of its predicted value and for FVC. Furthermore we have also shown that the polymorphism may be associated with reversibility to bronchodilators, whether analysed as a binary or as a continuous outcome. The glu27 homozygotes had poorer lung function and a greater response to bronchodilators than the gln homozygotes and the heterozygotes. These differences held true after regression analysis to adjust for potential confounding variables.

There was also a trend toward poorer lung function in individuals homozygous for Gly16 variant, with borderline statistical significance in those with a high total lifetime tobacco exposure.

The effects of the polymorphisms on lung function have not been widely examined in previous studies. Martinez and colleagues in their study of children showed no association
between beta adrenoceptor genotypes and lung function (Martinez FD, 1997). In contrast to our findings, Summerhill et al in a study of the Hutterite population showed that the Gly16 allele was associated with higher FEV\textsubscript{1} percent predicted and FVC percent predicted. They hypothesized that the beta adrenoceptor could have a role in lung growth and development and thus influence the size of adult lungs (Summerhill E, 2000). Their population was markedly different from the individuals that we describe. The majority of their subjects had normal lung function, were non smokers and were generally younger. A number of other investigators have shown the Gly16 allele to be associated with asthma severity, with FEV\textsubscript{1} generally lower in the severe asthmatic groups. These studies however did not examine FEV\textsubscript{1} by genotype as one of their primary outcome variable (Holloway JW, 2000; Reihaus E, 1993; Weir TD, 1998).

Alternatively it is possible that the effects of the beta-adrenoceptor polymorphisms on FEV\textsubscript{1} and FVC are only unmasked when there has been a loss of functional reserve, due to the effects of smoking in the case of COPD. This would explain why studies of younger asthmatics fail to show an effect in terms of FEV\textsubscript{1} and FVC. With this hypothesis you might still expect to observe an effect of the polymorphisms in determining bronchial hyperresponsiveness. Clearly this is speculative but worthy of further investigation.

In vitro studies have shown that the Glu27 and Gln27 variants of the beta adrenoceptor do not alter receptor binding or receptor mediated activation of cAMP messenger pathways. However the Glu27 variant protects the receptor from downregulation. In cultured smooth muscle cells, following prolonged beta agonist exposure the Glu27 variant downregulates to a lesser extent that the Gln27 form, as assessed by receptor numbers. Functionally therefore one might predict glu27 variants would be associated with less bronchial hyper-reactivity on
methacholine challenge testing, and that the glu27 variant would be associated with a greater response to beta agonists, as shown in this study.

Previous studies involving younger populations with classical asthma have examined bronchial hyper-responsiveness using methacholine challenge testing, rather than analysing bronchodilator reversibility. A number have shown that the glu27 variant to be associated with decreased bronchial hyper-responsiveness (Ramsay CE, 1999; Hall IP, 1995). Given their findings it would have been ideal to perform methacholine challenge tests in our subjects with COPD. This measurement may also have been informative in our population of normal controls. However, in selecting cases with unequivocal disease, the median value for FEVi was 1.03 litres making methacholine challenge testing unsafe in a large proportion of our patients. It would be of interest to examine the effects of these polymorphism on bronchial hyperresponsiveness in a group of COPD patients with less severe disease. Recruiting such subjects however may be problematic in a hospital setting since many present late in their disease course.

Our definition of COPD in this study was not dependant on the presence of irreversibility to bronchodilators, although all cases had some degree of permanent airflow obstruction with an FEV1/FVC ratio of <70%. In fact 72% of our cases had partial but significant (greater that 200mls or 15% whichever was greater) reversibility to bronchodilators. It could be argued that our COPD population is somewhat atypical, perhaps containing large numbers of late onset asthmatics, but other studies have shown similar levels of reversibility in COPD subjects. For this reason both the American Thoracic Society and the British Thoracic Society diagnostic criteria for COPD recognise that partial reversibility to bronchodilators may be present. Furthermore, all our cases had a pack-year history of at least 20 pack-years,
making a diagnosis of pure late-onset asthma over COPD less credible. We also excluded those with any other medical condition that might affect lung function, for example uncontrolled heart failure or bronchiectasis.

Although we have shown statistically significant results the effects that we report must be interpreted with caution. Although our positive findings were as predicted, given the known functional effects of the polymorphism, and appear consistent with a number of different statistical methodologies, the number of subjects in our study is relatively small. Our failure to demonstrate an association between the genotypes and the susceptibility to COPD was also as expected and in keeping with previous published studies of asthma. However, given our sample size one must be cautious before rejecting the hypothesis that these polymorphisms do not confer disease susceptibility. This holds particularly true since our control population was not matched for the major environmental factor determining the development of COPD, namely smoking.

4.5 Conclusions
This study is important as it demonstrates that disease modifying genes important in determining the phenotype of asthma in young people appear to have some shared effects in determining disease phenotype in subjects with late onset smoking related airflow obstruction, namely COPD. We have also demonstrated that these polymorphisms have a significant effect on pulmonary function. This has not been widely reported in other populations. It may have occurred due to the loss of functional reserve that is inevitable in our population of elderly smokers. In these circumstances the effects of the beta-adrenoceptor polymorphisms on determining lung function are accentuated. This hypothesis warrants further investigation.
The Dutch hypothesis suggests that COPD and asthma share some pathogenic mechanisms and this study provides molecular evidence to support this hypothesis. It provides a rationale for the study of other asthma associated polymorphisms in subjects with COPD.
5 POLYMORPHISMS OF THE HIGH AFFINITY IGE RECEPTOR BETA SUBUNIT IN LATE ONSET AIRFLOW OBSTRUCTION

5.1 Introduction

The high affinity IgE receptor (FceRI) is an attractive candidate for asthma, atopy and related phenotypes. The receptor consists of three subunits, the α and γ subunits, the genes for which lie on Chromosome 1, and the β subunit gene which lies in the chromosome 11q13 region. The FceRI-β is an attractive candidate gene for asthma, atopy and related phenotypes. There have been a number of studies that have shown linkage of the 11q13 locus with a range of asthma and atopy phenotypes. Cookson WOCM (1989), Young R (1992), Collee JM(1993) all report linkage of serum IgE responses to 11q. Sandford et al (1993) reported linkage to ‘atopy’ and Herwerden et al (1995) found linkage to bronchial hyper-reactivity, even in the absence of atopy. Linkage appears to be strongest for the inheritance of maternal alleles (Shirakawa T, 1994; Cookson WOCM, 1992). In addition three genome wide scans of asthma have highlighted this locus (Daniels SE, 1996; Ober C, 1999; Diezer M, 2000). Not all studies have confirmed this linkage (Hizawa N, 1992; Brereton HM, 1994; Marsh DG, 1994; Rich SS, 1992; Lympany P, 1992). This is probably due to a number of reasons including small sample size, inconsistencies in phenotype definition between studies, and population admixture effects. It seems most likely that linkage is dependant upon two factors; (i) maternal derivation of alleles (ii) a phenotype which consists of those with clinical signs of atopy and/or severe disease. However inconsistent result from candidate gene studies involving polymorphisms of the FceRI-β gene have cast further doubts as to whether the β-subunit is the prime candidate at this locus. Alternative candidates include the clara cell secretory protein gene (CC16) (Laing, 1998) and a polymorphism of a gene from the
glutathione-S-transferase family (GSTPI). Despite these concerns there is sufficient evidence
to suggest that the FceRI-β is a key candidate for atopy in some populations to make its study
valid.

Atopy is a pivotal component of the juvenile onset asthmatic phenotype. It is characterised by
enhanced and prolonged IgE responses to aeroallergens. The FceRI is responsible for binding
IgE to mast cells (Dombrowicz D, 1993). Mast cell bound IgE then binds allergen leading to
the cellular release of inflammatory mediators and subsequent mucosal inflammation. The
FceRI also has roles β lymphocyte and mast cell differentiation and direct regulatory effects
on IgE synthesis via II-4 production (Galli SJ, 1993; Plaunt M, 1989; Paul WE, 1992; Ben
Sasson SZ, 1990). Polymorphism of the FceRI-β which have the potential to alter receptor
expression or sensitivity for example, clearly may have effects on atopic responses. There is
good evidence from the literature as discussed in Chapter 2 to suggest that atopy lies under a
strong degree of genetic control (heredability estimates of between 50-80 %), (Hanson B,
1991; Hopp R, 1984). However this is unlikely to be an effect of a single gene. The data
from Palmer LJ (1999) suggests that at most the mutations of the FceRI-β have only modest
effects on total and specific IgE levels, accounting for about 10% of the variability. Other
authors have suggested that the factors, environmental and genetic, that determine allergen
specific responses, are distinct from those determining generalised allergic responses.

Five polymorphisms of the FceRI-β have been frequently studied. However three of these,
the E237G polymorphism described by Hill et al (1996), and two coding polymorphisms in
exon 6 (Shirikawa T, 1994), are not consistently found in Western Caucasian populations.
The final polymorphisms are two restriction enzyme polymorphisms, Rsal in intron 2, and
Rsal in exon 7. Both polymorphisms are biallelic, and common in Caucasian populations:-
allele B of Rsal intron 2 has an allelic frequency of ~ 60% and allele B of Rsal 7 a
frequency of ~ 30%. The polymorphisms are in strong linkage disequilibrium with allele A of intron 2 occurring with allele B of exon 7. There is little evidence to suggest that either of these polymorphisms is functional and it is likely that they serve as markers being in linkage disequilibrium with the true causative polymorphism as yet unidentified.

There have been fewer studies examining the role of the Rsal intron 2 and exon 7 polymorphisms. Current studies are summarized in Tables 5.1 and Table 5.2. This may be primarily because neither is regarded as functional in contrast to the E237G and Leu181/183 variants. Shirikawa T (1996) examined the distribution of the Rsal intron 2 polymorphism in a total of 600 subjects comprising 100 population based controls and 100 subjects from each of the following groups: eczema, allergic rhinitis, intrinsic asthma, childhood allergic asthma, adult allergic asthma. Significant associations were found between possession of allele B of Rsal intron 2 and allergic asthma, eczema and allergic rhinitis, but not with intrinsic asthma. There was also a significant association with atopy, defined as elevated total serum IgE or a positive RAST to at least one allergen. The Rsal exon 7 polymorphism was not examined in this study. Trabetti E (1998) also examined the role of the Rsal intron 2 polymorphism, and found a weak association with bronchial hyper-responsiveness and possibly atopy in an Italian population ascertained via a child with atopic asthma. Their published allele frequencies were strikingly different from those reported by Shirikawa (0.44 vs 0.82 for allele A). However, as with the Japanese population, the association was with allele B. Palmer LJ (1997) described a second Rsal polymorphism in exon 7 and examined its distribution in a group of endemically parasitised Australian Aboriginal families. Allele A of the Rsal exon 7 polymorphism was associated with elevated IgE levels. Furthermore there was an additive effect across the three genotypes. The authors estimated that the polymorphism accounted for approximately 12% of the residual variation in IgE levels in this population. Palmer LJ (1999) subsequently examined the role of both the Rsal polymorphisms in an Australian
Caucasian population. In keeping with the earlier studies they showed that allele B of Rsal intron 2 and that allele A of Rsal exon 7 were associated with higher total mean adjusted IgE levels, with a linear effect across the three genotypes. For the Rsal exon 7 a significant relationship was also shown with eosinophilia and for the Rsal intron 2 with physician diagnosed asthma. The published allele frequencies for Rsal intron 2 in this study were similar to those reported in the Italian population (0.36 for allele A). Cox H(1998) also examined both Rsal polymorphisms in families recruited via a child with atopic dermatitis. They showed an association of atopic dermatitis to both polymorphisms. They also reported an association with asthma although the criteria for diagnosis was not clearly defined. We have examined the role of the intron 2 and exon 7 high affinity IgE receptor polymorphisms in our population.
Table 5.1 Rsa intron 2 polymorphisms and disease phenotypes

<table>
<thead>
<tr>
<th>Author and location</th>
<th>Subjects</th>
<th>Disease definition</th>
<th>Number of subjects</th>
<th>Rsa intron 2</th>
<th>Disease associations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shirikawa T (1996), Japan</td>
<td>Subject recruited via hospital outpatients</td>
<td>Atopy defined as enhanced IgE responsiveness</td>
<td>600 subjects (100 controls &amp; 500 cases)</td>
<td>0.82 0.18</td>
<td>Allele B associated with atopy, extrinsic asthma, eczema and rhinitis.</td>
</tr>
<tr>
<td>Trabetti E (1998), Italy</td>
<td>Subjects ascertained via child with atopic asthma</td>
<td>Atopy-positive SPT or elevated T1gE Asthma-bronchial hyperresponsiveness on methacholine challenge</td>
<td>659 subjects from 168 families</td>
<td>0.44 0.66</td>
<td>Allele B weakly associated with bronchial hyper-responsiveness.</td>
</tr>
<tr>
<td>Palmer LJ (1999), Australia</td>
<td>Caucasian, ascertained via child with severe asthma</td>
<td>Dr diagnosed Requiring inhaled corticosteroid</td>
<td>134 subjects from 26 families</td>
<td>0.36 0.64</td>
<td>Allele B associated with total serum IgE and physician-diagnosed asthma. Evidence for a linear effect for IgE levels across three genotypes</td>
</tr>
<tr>
<td>Author and location</td>
<td>Subjects</td>
<td>Disease definition</td>
<td>Number of subjects</td>
<td>Disease associations</td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
<td>----------</td>
<td>--------------------</td>
<td>--------------------</td>
<td>---------------------</td>
<td></td>
</tr>
<tr>
<td>Palmer LJ (1997), Australia</td>
<td>Endemically parasitised Aboriginies</td>
<td>N/A</td>
<td>234 subjects</td>
<td>Allele A associated with higher total IgE levels. Evidence for a linear effect for IgE levels across three genotypes.</td>
<td></td>
</tr>
<tr>
<td>Palmer LJ (1999), Australia</td>
<td>Caucasian with child with severe asthma</td>
<td>Dr diagnosed Requiring inhaled corticosteroid</td>
<td>134 subjects from 26 families</td>
<td>0.68 0.32</td>
<td>Allele A associated with higher IgE levels and eosinophil counts. Evidence for a linear effect for IgE levels across three genotypes</td>
</tr>
</tbody>
</table>
5.2 Materials and Methods

5.2.1 Study population

The characteristics of the study population and methods of recruitment and data collection for this project have already been discussed in Chapter Three. Eczema was defined as previously described.

5.2.2 Laboratory methods for genotyping the intron 2 and exon 7 polymorphisms

DNA was extracted from whole blood as described in chapter 2 using the Purgene DNA extraction kit (Flowgen, Ashby de la Zouche, Leicester).

Genotyping for intron 2

The PCR for intron 2 was performed as previously described using the following primers based on the published gene sequences:

Intron 2A 5' CAGAATGTTCATGACTGAATTG 3'
Intron 2B 5' CAAGTACAGAGCAGACAACTG 3'

For each sample PCR was set up as follows:

1.5 μl 10x biolin buffer
2.25 μl 10mMgCl
3 μl 1mMdNTP’s
0.69 μl Exon 2A primer
0.58 μl Exon 2B primer
4.98 μl PCR water
1 μl DNA (200ng)
1 μl Biotaq dilution (0.5units)
(for the negative control 5.5 µl PCR water was used in place of DNA and 4.5 µl of PCR water)

PCR programme and restriction enzyme digest

94°C for five minutes, then 60°C for one minute, then 72°C for two minutes for one cycle. Followed by 94°C for 30 seconds, 60°C for thirty seconds and 72°C for one minute, for 34 cycles.

Following completion of PCR, samples may be stored at 4°C if immediate digest is not planned. Care must be taken to carefully seal the samples. A number of assays were repeated due to evapouration of PCR product. To confirm that PCR was successful 2 µl of PCR product, 8 µl distilled water, 2 µl PCR loading buffer, were run on a 2% agarose gel made in 1x TAE + 1 µl 10mg/ml ethidium bromide. This was run in 1x TAE running buffer at 100V for about one hour and thirty minutes. A sample of 5 µl of PCR marker and 5 µl of distilled water was included on each gel. A 748bp product was visualised. PCR products were then digested with Rsa1. The digest reaction was set up as follows:-

For each sample the digest reaction was set up as follows:-

5 µl PCR product

3µl distilled water

1µl Rsa 1 (x 5 final concentration) (Invitrogen)

1 µl React 1 (Invitrogen)

Samples were placed in a water bath at 37°C for one and a half hours. Two µl of loading buffer were added to each sample following digest and the samples were loaded on to a 2% agarose gel (for method see appendix). Allele A produces a 700bp band, and Allele B produces a 450 and a 250bp band. A negative and positive controls of known genotype,
verified by sequencing on an ABI prism sequencer (PE Applied Biosystems) were included on each gel. Results were recorded on Polaroid film. Assessment of genotype was made by visual inspection of the film by an observer blinded to clinical phenotype. A random selection of samples were sequenced to confirm genotype. No errors were detected by sequencing. All genotyping for the intron 2 polymorphism carried out by the author was supervised by Mrs M Hill.

**Genotyping for exon 7**

The PCR for the *RsaI* exon 7 polymorphism was performed as previously described using the following primers based on the published gene sequences.

Exon 7A 5' TCACTGTGTATCATGCTAAGC 3'
Exon 7B 5' TGATACAATACTGCATCGTG 3'

For each sample a PCR reaction was set up as follows:-

1.5 µl 10x biolin buffer
3 µl 10mM MgCl
3 µl 1mM dNTP's
0.5 µl Exon 7A primer
0.5 µl Exon 7B primer
4.5 µl PCR water
1 µl DNA (200ng)
1 µl Biotaq dilution (0.5 units)

(for the negative control 5.5 µl PCR water was used in place of DNA and 4.5 µl of PCR water)

**PCR programme and enzyme digest**

94°C for one minute, then 60°C for one minute, then 72°C for one minute for 32 cycles.
Success of the PCR reaction was confirmed as described for intron 2. When PCR product was run on an agarose gel a 481bp product was visualised. PCR products were then digested with Rsal.

The digest reaction was carried out according, with appropriate controls and verification of genotype as described for intron 2.

For the exon 7 polymorphism, Allele A produces an undigested 481bp band, and Allele B produces a 295 and a 187 bp band.

An example of our genotyping for the exon 7 and intron 2 polymorphisms is shown in Figure 5.1 and 5.2. All my assessments of genotype were verified by a second observer, who also supervised any genotyping performed by the author (M C Hill).

5.2.3 Hypotheses

Primary hypotheses

Our primary hypotheses for the intron2 and exon 7 polymorphisms were

(i) The high affinity IgE receptor polymorphisms of intron 2 and exon7 are associated with susceptibility to late onset airflow obstruction.

(ii) The high affinity IgE receptor polymorphisms of intron 2 and exon7 are associated with atopy and eosinophilia.

Secondary hypothesis

(iii) The High affinity IgE receptor polymorphisms of intron 2 and exon 7 are associated with severity of late onset airflow obstruction

5.2.4 Statistical methodology

Thus, the primary continuous response variables for the association analysis were total serum IgE and peripheral blood eosinophil count. The primary dichotomous response variables were
the presence of late onset airflow obstruction, self reported eczema (as defined above). Both serum IgE levels and eosinophil counts exhibited right skew distribution and were loge transformed prior to analysis.

In our secondary analysis we examined the relationship between both polymorphisms and quantitative markers of lung function including FEV1 and FVC (both expressed as a percentage of their predicted values), and the FEV1/FVC ratio. We also examined IgE status as a dichotomised variable (high >122iu/l vs. low < 122iu/l).

Analysis for associations, confounding variable and genotype modelling was carried out as previously detailed in Chapter 4.2.4.

Because of the previously reported interaction between smoking and IgE levels, a stratified analysis was also carried out for two subgroups based on high and low total lifetime pack-year smoking history. The low tobacco exposure group comprised individuals with a total lifetime pack-year smoking history of less than 20 pack-years. The high tobacco exposure group comprised individuals with a total lifetime pack-year smoking history of equal to or greater than 20 pack-years.
Allele A produces a 700 base-pair band

Allele B produces a 450 base pair and 250 base-pair band

\( a = A,A \) homozygote

\( b = B,B \) homozygote

\( c = A,B \) heterozygote

Photograph provided by M Hill
Allele A produces an undigested 481 base-pair band

Allele B produces a 295 base-pair and a 187 base-pair band

a = A,B heterozygote

b = B,B homozygote

c = A,A homozygote
5.3 Results

297 cases and 154 controls were recruited to the study. DNA was extracted from 285 cases and 144 controls. 427 subjects consisting of 283 cases and 144 controls were successfully genotyped for the intron 2 and exon 7 polymorphisms.

The characteristics of the population successfully genotyped for these polymorphisms are shown in Table 5.3. Overall the mean age was 68 years (SD 9.4, range 45-91), and the population consisted of 247 (56%) males. 92 subjects (22%) were current smokers, 255 (60%) ex-smokers and 75 (18%) never smokers (5 (1%) unknown). The median pack-year history fell in the 20-29 pack year category. Total serum IgE (n = 374) exhibited a marked right skew distribution (mean 180 iunits/l, median 48 iunits/l) and was logarithmically transformed. Eosinophil counts (n = 379) also showed a right skew distribution (mean 0.22 x 10^9, median 0.18 x 10^9) and were also log transformed prior to analysis. Missing IgE and eosinophil count data arose due to numerous reasons including, loss in transit to the laboratory, haemolysis of samples and refusal to allow further blood collection.

Mean FEV1 as a percent of age and sex adjusted predicted values was 64.2 (SD 29.0, range 11.6-143) and mean FVC as a percentage of adjusted predicted value was 84.5 (22.8). Mean FEV1/FVC ratio was 58.0 (SD 17, range 21.4-94.7). Cases and controls were well matched for age and sex. The cases showed significantly poorer lung function and significantly higher lifetime total tobacco exposure ($\chi^2$ for trend = 43.3, $p < 0.001$). Eosinophil counts were similar in the two groups.

Total IgE levels were significantly elevated amongst the cases compared to the controls (geometric mean for cases 66.7 vs, 35.9 for controls, a ratio of 1.86). This relationship was not seriously confounded by age, sex, or current smoking status, or total pack-year history
(adjusted ratio of geometric means 1.75, 95% confidence intervals 1.24 to 2.49, \( p = 0.002 \)) (Table 5.4). The apparent elevation of IgE levels in subjects with late onset airflow obstruction was observed only in the subgroup with low lifetime tobacco exposure (Table 5.4). However the control subjects in the high exposure subgroup had significantly higher IgE levels than the non/low smoking controls (mean low tobacco exposure group controls = 26.1 (SD 1.583), mean high tobacco exposure group controls = 61.0 (SD 1.31), \( p < 0.001 \)).

Current smoking status did not confound the associations.
Table 5.3. Clinical features of the case and control populations.

<table>
<thead>
<tr>
<th></th>
<th>Cases n =283</th>
<th>Controls n =144</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Age</td>
<td>68</td>
<td>68</td>
<td>t = 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p = 0.973</td>
</tr>
<tr>
<td>Male sex</td>
<td>155 (54.8%)</td>
<td>86 (59.7%)</td>
<td>OR 1.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>95% CI 0.81-1.84, p = 0.329</td>
</tr>
<tr>
<td>Current smokers</td>
<td>67 (23.7%)</td>
<td>25 (17.4%)</td>
<td>OR 1.49,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>95% CI 0.89-2.49, p=0.12</td>
</tr>
<tr>
<td>Total pack years smoking</td>
<td>30-39</td>
<td>10-19</td>
<td>OR 1.30,</td>
</tr>
<tr>
<td>(median)</td>
<td></td>
<td></td>
<td>95% CI 1.2-1.4,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p=&lt;0.001</td>
</tr>
<tr>
<td>Mean FEV1 as percent</td>
<td>49.0</td>
<td>93.3</td>
<td>t = 21.4</td>
</tr>
<tr>
<td>predicted</td>
<td></td>
<td></td>
<td>p &lt;0.001</td>
</tr>
<tr>
<td>Mean FEV1/FVC</td>
<td>49.1</td>
<td>75.5</td>
<td>t = 22.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p &lt;0.001</td>
</tr>
<tr>
<td>Geometric Mean Total</td>
<td>0.192</td>
<td>0.192</td>
<td>t= 0.05</td>
</tr>
<tr>
<td>eosinophillog</td>
<td></td>
<td></td>
<td>p=0.958</td>
</tr>
</tbody>
</table>
Table 5.4 Total serum IgE levels by tobacco exposure for case and control populations

<table>
<thead>
<tr>
<th>Tobacco Exposure</th>
<th>Cases Geometric mean total serum IgE levels</th>
<th>Controls Geometric mean total serum IgE levels</th>
<th>Adjusted ratio of means (95% confidence interval)</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>High tobacco exposure</td>
<td>70.8 (172)</td>
<td>59.7 (52)</td>
<td>1.29 (0.79-2.11)</td>
<td>p = 0.303</td>
</tr>
<tr>
<td>N = 224</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low tobacco exposure</td>
<td>56.3 (65)</td>
<td>26.1 (85)</td>
<td>2.45 (1.47-4.06)</td>
<td>p = 0.001</td>
</tr>
<tr>
<td>N = 150</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>66.7 (237)</td>
<td>35.9 (137)</td>
<td>1.75 (1.24-2.49)</td>
<td>p = 0.002</td>
</tr>
</tbody>
</table>
5.3.1 Genetic Analysis

All individuals were successfully genotyped for both polymorphisms. Allele A of exon 7 was strongly associated with Allele B of intron 2 ($\chi^2 = 723.2, p = <0.001$, Table 5.5). Genotype frequencies for the case and control population are shown in Table 5.6. Contingency table analysis showed that there was no association between late onset airflow obstruction status (case/control status) and genotype in this population. (Table 5.7). Because of the possibility of undiagnosed airflow obstruction in our population of controls, and misdiagnosis in our population of cases, the relationship between quantitative markers of lung function and genotype was also examined in our secondary analysis. For intron 2, mean FEV1 as a percentage of its predicted value (SD) was 62.0(30.4) for genotype AA, 63.7(28.9) for genotype AB and 66.4(29.3) for genotype BB. One way analysis of variance, and analysis adjusted for potential confounding variables was not significant ($p = 0.540$, adjusted ratio $p = 0.371$). Mean FVC (SD) was 82.8(22.1) for AA, 84.8(23.5) for AB and 84.9(22.2) for BB. (one-way analysis of variance, $p = 0.811$). Values for FEV1/FVC ratio were 56.2 for AA, 57.3 for AB and 59.7 for BB (one-way analysis of variance, $p = 0.300$).

Corresponding values for the exon 7 genotype for mean FEV1 as a percentage of its predicted value (SD) were 66.4(28.8) for genotype AA, 63.6(29.2) for genotype AB and 61.5(30.4) for genotype BB. One way analysis of variance, and analysis adjusted for potential confounding variables was not significant ($p = 0.488$, adjusted ratio $p = 0.346$). Mean FVC (SD) was 85.5(21.6) for AA, 84.3(23.9) for AB and 82.7(22.3) for BB(one-way analysis of variance, $p = 0.700$). Values for FEV1/FVC ratio were 59.4(16.9) for AA, 57.5(17.0) for AB and 55.8(17.3) for BB (one-way analysis of variance, $p = 0.322$)
None of the relationships between airflow obstruction and genotypes changed following analysis using the two pre-identified subgroups based on high and low lifetime tobacco exposure.

The primary hypothesis also stated that the polymorphisms would associate with atopy measured by total serum IgE levels, and eosinophilia. Our population of cases had significantly elevated total serum IgE levels compared to controls as has already been demonstrated. However there were no significant associations between either genotype and IgE levels in the population. Geometric mean serum IgE levels (SD) for intron 2 AA were 56.3(1.69), for AB, 51.9(1.61), and for BB, 53.5(1.63). One-way analysis of variance showed that there was no significant difference, and linear regression analysis, with age, sex and pack-year history included as covariates confirmed this (p = 0.908 and p = 0.633 respectively). Corresponding values for exon 7 were 54.6(1.63) for AA, 49.9(1.6) for AB and 62.2(1.72) for BB. There were no significant differences in IgE levels between the exon 7 genotypes (one-way analysis of variance, p = 0.667). Analysis based on the dichotomous outcome variable high versus low IgE also demonstrated no significant relationship (Table 5.7). Because of the importance of smoking as a confounding factor, and the potential inaccuracies in adjusting for this in a regression model, analysis was also carried out for two groups based on high and low total lifetime tobacco exposure. IgE levels did not differ with genotype in the high or low tobacco exposure groups. Statistical significance from linear regression models was as follows: - for the low tobacco exposure group; intron 2, p = 0.121, exon 7, p = 0.189; for the high tobacco exposure group, intron 2, p = 0.104, exon 7 p = 0.117.

Mean eosinophils levels were the same in the case and control population. There were no associations between in eosinophil levels and either the intron 2 or the exon 7 polymorphisms. Mean eosinophil levels (SD) by genotype for the intron 2 polymorphism were as follows AA,
Corresponding values for exon 7 were AA, 0.20(0.12), AB, 0.22(0.18), and BB, 0.22(0.15), one-way analysis of variance, p = 0.523.

Finally, the relationship between both polymorphisms and self-reported eczema was examined (Table 5.7). There was no relationship between intron 2 and self reported eczema ($\chi^2 = 2.75$, p= 0.252). However, an association between allele A of exon 7 and doctor diagnosed eczema was demonstrated ($\chi^2 = 6.89$, p= 0.032). This relationship was not confounded by age, sex, or current smoking status. Further analysis treating genotype as a 3-level variable (see methods), suggested that the allele A homozygotes were the 'high risk' genotype ($\chi^2 = 6.22$, p=0.013, odds ratio for AA homozygotes compared with heterozygotes and homozygotes, 2.27 (95% confidence interval = 1.17-4.38) p=0.015).

Analysis for associations within the two subgroups showed that the relationship between the polymorphism and self reported eczema was of borderline statistical significance in the high tobacco exposure group ($\chi^2 = 5.70$, p= 0.058). There were no differences between exon7 genotype and self reported eczema in the low tobacco exposure group ($(\chi^2 = 2.47$, p= 0.290). However the number of individuals reporting eczema in this group was small (n=14), and thus there was limited statistical power to allow valid analysis for this group.
Table 5.5 Genotype distribution for intron 2 and exon 7 genotypes

<table>
<thead>
<tr>
<th>Exon 7 genotype</th>
<th>A,A</th>
<th>A,B</th>
<th>B,B</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (%)</td>
<td>0</td>
<td>18(4.2)</td>
<td>143(33.5)</td>
<td>161</td>
</tr>
<tr>
<td>A,A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A,B</td>
<td>3(0.7)</td>
<td>196(45.9)</td>
<td>4(0.9)</td>
<td>203</td>
</tr>
<tr>
<td>B,B</td>
<td>62(14.5)</td>
<td>1(0.2)</td>
<td>0</td>
<td>63</td>
</tr>
<tr>
<td>Total</td>
<td>65</td>
<td>215</td>
<td>147</td>
<td>427</td>
</tr>
</tbody>
</table>

$\chi^2$ test = 723, p < 0.001.
Table 5.6 Genotype frequencies for cases and control populations

<table>
<thead>
<tr>
<th></th>
<th>Case number (%)</th>
<th>Control Number (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intron 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A,A</td>
<td>44(15.5)</td>
<td>21(14.6)</td>
<td>65(15.2)</td>
</tr>
<tr>
<td>A,B</td>
<td>145(51.2)</td>
<td>70(48.6)</td>
<td>215(50.4)</td>
</tr>
<tr>
<td>B,B</td>
<td>94(33.2)</td>
<td>53(36.8)</td>
<td>147(34.4)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>283(100)</td>
<td>144(100)</td>
<td>427(100)</td>
</tr>
</tbody>
</table>

|                |                 |                    |                |
| **Exon 7**     |                 |                    |                |
| A,A            | 105(37.1)       | 56(38.9)           | 161(37.7)      |
| A,B            | 134(47.3)       | 69(47.9)           | 203(47.5)      |
| B,B            | 44(15.5)        | 19(13.2)           | 63(14.8)       |
| **Total**      | 283(100)        | 144(100)           | 427(100)       |

for intron 2 p = 0.761, for exon 7, p = 0.800
Table 5.7 Genotype frequencies and primary outcome variables

<table>
<thead>
<tr>
<th></th>
<th>% of each genotype with phenotype</th>
<th>Late onset</th>
<th>Eczema</th>
<th>Elevated serum IgE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>airflow</td>
<td></td>
<td>obstruction</td>
</tr>
<tr>
<td>FceRI-β Rsal</td>
<td>A,A</td>
<td>67.7</td>
<td>9.2</td>
<td>31.4</td>
</tr>
<tr>
<td>intron 2</td>
<td>A,B</td>
<td>67.4</td>
<td>7.6</td>
<td>30.0</td>
</tr>
<tr>
<td></td>
<td>B,B</td>
<td>64.0</td>
<td>12.9</td>
<td>30.1</td>
</tr>
<tr>
<td></td>
<td>p=0.761</td>
<td>p=0.252</td>
<td></td>
<td>p=0.981</td>
</tr>
<tr>
<td>FceRI-β Rsal</td>
<td>A,A</td>
<td>65.2</td>
<td>14.3</td>
<td>31.2</td>
</tr>
<tr>
<td>exon7</td>
<td>A,B</td>
<td>66.0</td>
<td>6</td>
<td>28.3</td>
</tr>
<tr>
<td></td>
<td>B,B</td>
<td>69.9</td>
<td>9.5</td>
<td>34.0</td>
</tr>
<tr>
<td></td>
<td>p=0.800</td>
<td>p=0.013*</td>
<td></td>
<td>p=0.699</td>
</tr>
</tbody>
</table>

* Odds Ratio AA vs (AB and BB) = 2.27 (95%CI 1.17-4.38)
5.4 Discussion

With respect to our primary hypothesis we have not shown the intron 2 and exon 7 high affinity IgE receptor polymorphisms to be associated with late-onset airflow obstruction or atopy, as measured by total serum IgE levels and eosinophilia. Amongst those with disease, the polymorphism affect neither severity of lung function nor bronchodilator reversibility.

We have shown that cases with late onset airflow obstruction have significantly higher total serum IgE levels compared to age and sex matched controls. This relationship was not confounded by total lifetime cigarette consumption, current smoking status, age or sex. Analysis by tobacco exposure status showed that serum IgE levels were significantly elevated, compared to controls, amongst cases with low exposure to tobacco, but not significantly so amongst cases with high exposure to tobacco. One reason for this could be that IgE levels amongst control subjects in the high tobacco exposure group were much higher than IgE levels in control subjects in the low tobacco exposure group, suggesting an effect of cigarette smoking on serum IgE levels. This effect has previously been described in smokers without asthma (Jensen EJ, 2000), smokers with asthma (Orjzszezyn MP, 2002), and older patients with asthma and COPD (Motegi T, 1999). It is therefore possible that smoking induced elevation of IgE levels will distort the analysis of the late onset airflow obstruction phenotype. Alternatively, it may be that in order to develop COPD in the absence of high exposure to tobacco, it is necessary to have an additional atopy-related risk factor reflected in a high total serum IgE. This is speculative, but clearly worthy of further investigation.

We also showed a significant association between eczema (as defined) and the Rsal exon 7 polymorphism. This finding replicates that of a previous study, although our definition of
eczema was less rigorous (Cox H, 1998). Further modelling suggested that A,A homozygotes were the ‘high-risk’ genotype, rather than the polymorphism exerting a linear additive genetic effect.

The lack of associations between the polymorphisms described and the various phenotypes described in our population may have arisen for a number of reasons as shall now be discussed.

Firstly neither of the polymorphism has been shown to be functional. This could be regarded as a flaw in their selection as candidate genes in this study. However, associations have been reported to a number of asthmatic phenotypes and thus whilst it is debatable that these are the true candidate genes, they may lie in linkage disequilibrium with other polymorphisms as yet undescribed.

In previous published data, regarding both the intron 2 and the exon 7 polymorphism, the population recruited has been significantly younger. The index case frequently being a child with asthma or atopy. It is entirely possible that determinants of asthma and related phenotypes are different in older populations.

We found no evidence associating either polymorphism with susceptibility to late onset airflow obstruction. The evidence from the linkage studies suggests that IgE responses, atopy, particularly for maternally derived alleles, and severe disease are the key phenotypes at this locus rather than the presence or absence of disease. However for these particular candidates there has been some evidence to suggest that they may have susceptibility effects (Cox H, 1998; Palmer LJ, 1999; Shirikawa T, 1996)

The strongest and most frequently reported associations of these polymorphisms are with serum IgE levels, a finding not demonstrated in our population. It is possible that the relative
importance of the FcεRI-β polymorphisms in determining IgE levels declines with ageing. We have shown that high tobacco exposure can elevate IgE levels and this will confound any relationship between genotype and total IgE. Palmer has estimated that, within his population, the likely contribution of polymorphisms at this locus to total serum IgE variability is small. Given that our population is significantly older (with a tendency for IgE levels to fall with ageing), and has a greater potential for confounding from the effects of tobacco smoke, the contribution of these polymorphism to IgE variability is likely to be even less than the 12% he calculates. Given our sample size, this study has relatively low power to detect such small genetic affects.

Eosinophilia was associated with the exon 7 polymorphism in only one of the studies (Palmer LJ, 1999), although examined for in all. There are no strong reports of linkage of eosinophil counts to the locus and thus this finding may be a spurious one. There is considerably more evidence supporting other markers of atopy such as total and specific IgE levels.

5.5 Conclusion

In conclusion, we have shown that serum IgE levels are significantly elevated in subjects with late-onset airflow obstruction. Elevations in serum IgE do not appear to be confined to those with the late onset asthma phenotype, but occur in subjects with COPD as well. There is evidence however of significant interaction with smoking which may confound further analysis. The elevations in serum IgE levels recorded do not appear to be associated with the intron 2 and exon 7 polymorphisms of the FcεRI β gene. There are numerous explanations for the lack of an association both biological and methodological, as has been discussed. To fully explore the role of these polymorphisms in late onset asthma and COPD, further large studies are needed. The potential gene by environment interactions, in particular
between smoking, IgE levels, and polymorphisms of the FcεRI-β, warrants further investigation.
6 THE ROLE OF THE TUMOUR NECROSIS FACTOR GENE COMPLEX IN LATE ONSET AIRFLOW OBSTRUCTION.

6.1 Introduction

The tumour necrosis factor gene and the lymphotoxin alpha gene are in strong linkage disequilibrium within the human major histocompatibility complex III on chromosome 6p. The genes lie close to the human leukocyte antigen (HLA) loci and are in linkage disequilibrium with a number of HLA polymorphisms. The 6p region has been reported as linked to asthmatic phenotypes in a number of genome wide scans (Daniels SE, 1996; CSGA, 1997; Wjst M, 1999; Ober C, 1999), although evidence of linkage is not consistent.

Tumour necrosis factor alpha (TNF) is a soluble homotrimer of 17kD subunits each synthesized from 26kD trans-membrane pro-peptides (Kreiger M, 1988). Initially described for its cytotoxic and anti-tumour activities, it is also known to have potent immunomodulating and pro-inflammatory effects. Its roles include adhesion of neutrophils to endothelial cells, enhanced IL--2 response, and induction of GM-CSF. It also appears to have effects on cell apoptosis (Allen RD, 1999). Its varied roles in inflammatory processes makes it a key candidate gene for immune and inflammatory responses and recently it has been proposed as a candidate gene for COPD. TNF is involved in early phase of bronchoconstriction via smooth muscle contraction and late phase via up-regulation of adhesion molecules and influx and activation of inflammatory cells. In addition, increased levels are found in induced sputum from patients with COPD (Keating V, 1996).

Lymphotoxin alpha (LTα) is a 25-kb soluble protein, synthesized primarily by stimulated T cells. Lymphotoxin alpha has a similar range of immunoregulatory effects as TNFα, although a broader range of bioactivities has been described for TNFα.
The TNF-308 polymorphism is a biallelic restriction fragment length polymorphism (RFLP) originally described by Wilson AG (1992). The common variant, termed TNF1 and the rare variant TNF 2. The polymorphism is an amino acid change (guanine to adenosine) in the promotor region of the TNFα gene at position 308 relative to the site of initiation. The less common TNF-308 allele has been associated with higher baseline and induced expression of TNF-α (Wilson AG, 1997).

The Ncol polymorphism lies in the first intron of the lymphotoxin alpha gene is also biallelic. Messer G (1991) showed that the polymorphism was associated with a nucleotide substitution at position 26 with allele 1 associated with asparagine and allele 2 with threonine. It is debated if the NcoI polymorphism is of any functional significance in terms of alteration in both lymphotoxin and TNFα production or activity. Messer G (1991) have shown that in vitro LTα*1 was associated with increased level of lymphotoxin response, but no association was found with respect to TNFα production. Conversely the LTα*2 allele has been associated with different TNFα secretory phenotypes in other studies. Both Molvig J (1990) and Pociot F (1993) have shown LTα*2 allele to be associated with higher TNFα levels. Despite these inconsistent findings the NcoI restriction site polymorphism continues to be a useful marker for disease predisposition in association and linkage studies.

Wilson and colleagues (Wilson AG, 1993) describe an extended haplotype for the TNF-308/LTNcoI/HLA al/B8/DR3. To date there have been few studies examining the role of extended haplotypes in disease causation and modification (Moffatt M, 1997; Sandford AJ, 2001). Several studies have examined the role of both of these polymorphisms, particularly the-308 polymorphism, in subjects with asthma, atopy and latterly, COPD. The results of some of these studies are summarised in Table 6.1 and Table 6.2.
<table>
<thead>
<tr>
<th>Author and location of study</th>
<th>Subjects</th>
<th>Disease definition</th>
<th>Number of subjects</th>
<th>Allele frequency</th>
<th>Disease Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Huang SL (1997), Tawain</td>
<td>Cases and controls from OPD at Vetrans hospital</td>
<td>FEV1 &lt; 80% (pred) + FEV1/FVC &lt; 69% + chronic bronchitis</td>
<td>42 chronic bronchitis, 42 matched controls</td>
<td>0.95 0.05</td>
<td>TNF1 associated with chronic bronchitis</td>
</tr>
<tr>
<td>Keatings V (2000), Ireland</td>
<td>Cases from OPD, controls from patients admitted for angiography</td>
<td>FEV1 &lt; 70%(pred) + FEV/FVC &lt; 70% + smokers + irreversible</td>
<td>106 COPD, 99 control smokers</td>
<td>0.76 0.24</td>
<td>TNF2 homozygotes had a greater all cause mortality at ~20 months follow-up.</td>
</tr>
<tr>
<td>Hingham MA (2000), UK</td>
<td>Cases from OPD, origin of control smokers not stated, Blood donor population controls</td>
<td>FEV1/FVC &lt; 80% predicted, irreversible + smokers</td>
<td>86 COPD, 63 controls smokers, 199 population controls</td>
<td>0.83 0.17</td>
<td>TNF genotype does not influence risk of developing COPD or severity of disease</td>
</tr>
<tr>
<td>Patuzzo C (2000), Italy</td>
<td>Hospital outpatients, Control volunteers from clinical staff</td>
<td>FEV1 &lt; 70% (pred) + irreversible airflow obstruction</td>
<td>66 COPD, 98 controls</td>
<td>0.84 0.16</td>
<td>No relationship to TNF genotypes</td>
</tr>
<tr>
<td>Sakao S (2001), Japan</td>
<td>Cases from OPD, Smoking controls from hospital health checks, Blood donor population controls</td>
<td>FEV1 &lt; 80%(pred) + FEV1/FVC &lt; 70%</td>
<td>106 COPD, 110 smokers, 129 population controls</td>
<td>0.89 0.11</td>
<td>TNF2 associated with COPD</td>
</tr>
<tr>
<td>Sandford AJ (2001), Canada</td>
<td>Selected participants of Lung Health Study</td>
<td>Current smokers. Rapid decliners &gt; 3% fall in FEV1/year</td>
<td>283 rapid decliners, 308 non decliners</td>
<td>0.82 0.18</td>
<td>No associations between TNF 308 and rate of lung function decline</td>
</tr>
<tr>
<td>Sakao S (2002), Japan</td>
<td>As Sakao 2001 study + HRCT performed</td>
<td>As Sakao 2001 study</td>
<td>84 COPD</td>
<td>0.86 0.14</td>
<td>Non-significant trend to greater degree of emphysema with allele 2</td>
</tr>
<tr>
<td>Author and location of study</td>
<td>Subjects</td>
<td>Disease definition</td>
<td>Number of subjects</td>
<td>Genotype frequency</td>
<td>Disease Association</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>----------</td>
<td>--------------------</td>
<td>--------------------</td>
<td>-------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Campbell DA (1996), UK</td>
<td>Random population cohort from electoral register</td>
<td>Asthma - doctor diagnosed + bronchial-hyperresponsiveness Atopy - elevated IgE or +ve SPT</td>
<td>33 asthmatics, 100 atopes 90 normal individuals</td>
<td>Not stated Not stated</td>
<td>No significant associations between LT alpha genotypes and disease</td>
</tr>
<tr>
<td>Moffatt M (1997), Australia</td>
<td>Families from general population sample with two or more atopic sibs</td>
<td>Self reported doctor diagnosed</td>
<td>413 subjects from 88 families (92 asthmatics).</td>
<td>0.45 0.55</td>
<td>Allele <em>1/TNF-308</em>2 associated with asthma (questionnaire definition)</td>
</tr>
<tr>
<td>Albuquerque RV (1998), Australia</td>
<td>Unselected child cohort and asthmatics from paediatric OPD</td>
<td>Physician diagnosed</td>
<td>50 controls 74 cases</td>
<td>0.35 0.65</td>
<td>Homozyotes for allele *2 fivefold risk of doctor diagnosed asthma</td>
</tr>
<tr>
<td>Trabetti E (1999), Italy</td>
<td>Families ascertained through atopic child</td>
<td>ATS definition of asthma + SPT + BHR + IgE levels</td>
<td>600 subjects from 131 families with atopic asthma children</td>
<td>0.27 0.73</td>
<td>Allele *2 associated with positive skin-prick tests and increased IgE levels</td>
</tr>
<tr>
<td>Patuzzo C (2000), Italy</td>
<td>Hospital outpatients Control volunteers from clinical staff</td>
<td>FEV1 &lt; 70% (pred) + irreversible airflow obstruction</td>
<td>66 COPD 98 controls</td>
<td>0.29 0.71</td>
<td>No relationship between LT genotype and COPD</td>
</tr>
<tr>
<td>Sandford AJ (2001), Canada</td>
<td>Selected participants of Lung Health Study</td>
<td>Current smokers. Rapid decliners &gt; 3% fall FEV1/year</td>
<td>283 rapid decliners 308 non decliners</td>
<td>0.66 0.33</td>
<td>No associations between LT alpha genotype and disease</td>
</tr>
</tbody>
</table>
6.2 Methods

6.2.1 Study population

The details regarding recruitment of the study population have already been discussed in Chapter Three

6.2.2 Laboratory Analysis

DNA was extracted from whole blood using methods already described.

Genotyping for the lymphotoxin alpha NcoI polymorphism

The PCR for the NcoI polymorphism was performed as previously described, using the following primers based on the published gene sequences

NcoI Allele 1 5'CCGTGCTTCGTGCTTTGG ACT A 3'

NcoI Allele 2 5'AGAGCCTGGTGAGGACATGCTTG 3'

For each sample a PCR reaction was set up as follows:-

1.5μl 10x biolin buffer

1.5μl 10mM MgCl

3μl 1mM dNTP's

1μl LT-α 1 primer

1.5μl LT-α 2 primer

4.5μl PCR water

1μl DNA (200ng)

1μl Biotaq dilution (0.5 units)

(for the negative control 5.5 μl PCR water was used in place of 1 μl DNA and 4.5 μl of PCR water)
PCR programme and restriction enzyme digest.

A PCR programme was set up based on previously described methodologies. Amplification conditions were, 95°C for 6 minutes followed by 30 cycles of 95°C for 1 minute, 64°C for 1 minute, 72°C for 1 minute with a final extension of 72°C for 5 minutes (Moffatt M, 1997). To confirm that PCR was successful 2 µl of PCR product, 8 µl distilled water, 2 µl PCR loading buffer, were run on a 2% agarose gel (see previous laboratory methods for details). A 750bp product was visualised. 5µl of PCR product was digested with 1µl NcoI at 37°C for 90 minutes. Products were visualised on a 2% agarose gel.

The digest reaction was carried out according, with appropriate controls and verification of genotype as described for previously.

For the NcoI polymorphism, Allele 2 produces an undigested 750bp band, and Allele 1 produces a 500 and a 250bp band(Figure 6.1).

Genotyping for the TNF-308 polymorphism

There are several methods for detecting the TNF-308 polymorphism.

The TNF-308 polymorphism by sequence specific oligonucleotide probing

Our original methodology involved the use of the following primers

5'CAAACACAGGCCTCAGGATC 3' and 5'AGGGAGCGTCTGCTGGCTG 3'. The presence of the 308 polymorphism was detected by sequence specific oligonucleotide probing. Two probes were used, 5'AGGGGCATGGGGACGGG 3' for TNF allele 1 (probe 1) and 5'AGGGGCATGAGGACGGG 3' for allele 2 (probe 2).

Briefly, denatured PCR products were dot blotted onto two duplicate filters baked at 120°C for 25 minutes. Hybridisation with radio-labelled probes was performed for probe 1 and probe 2 on the duplicate filters. The filters were then washed with 3M TMAC at 62°C. The filters were then exposed to a photographic plate and results recorded. Radio labelling with
probe 1 produced a strong blot for 1,1 homozygotes a faint/absent blot for 2,2 homozygotes and an intermediate signal for the heterozygotes. By contrast radio labelling with probe 2 produced a faint/absent blot for 1,1 homozygotes and a strong blot for 2,2 homozygotes. DNA samples of known genotype, determined by sequencing, were included on each filter to enable comparison with the observed blots to be made.

During the course of the study, the technique for genotyping the TNF 308 polymorphism by this method failed. Essentially it became impossible to distinguish between the different alleles after probing. This difficulty occurred with both the probes. Typically a picture as illustrated in Figure 6.2 was produced. Following incubation with probe 2, instead of 2,2 homozygotes appearing as bold blots, heterozygotes and 1,1 homozygotes as faint/absent blots, the genotypes look the same as each other. A similar lack of distinction between the genotypes was also found with probing for allele 1. Initially it was felt that further washes were needed. Despite more stringent washing and reducing the exposure time of the photographic plate it became impossible to remove the probe to any meaningful extent.

For this reason all samples were genotyped by an alternative method based on restriction fragment digest as described below.

The TNF-308 polymorphism by restriction enzyme (Sty 1)

The following primers for the TNF-308 enzyme digest were used

Allele 1  5’AGGCAATAGGTTTTGAGGGCCATG 3’

Allele 2  5’ACACACAAGCATCAAGGATACC 3’

The primer for Allele 1 introduces a Sty 1 Restriction site into allele 1 enabling restriction fragment digest

For each sample the PCR reaction is as follows

100ng DNA

0.1μM of each primer
200μM dNTP’s mix
1.5mM MgCl
1x Biolin Buffer
0.5 U Bioline Taq
PCR water to final volume of 20μl

A PCR programme was set up as follows: - 30 seconds at 94°C followed by 59°C for 30 seconds then 72°C for 10 seconds, for 35 cycles.

Following PCR products were run on an agarose gel. Enzyme digest using the restriction enzyme Sty1 was carried with 10μl of PCR product with 10 units of Sty1 incubated at 37°C for 4 hours. Digest products were run on a 3% agarose gel at 75V for 2 hours. Allele 2 produces an undigested 143bp band and allele 1 a 20bp and 123 bp band.

An example of our genotyping for the NcoI lymphotoxin alpha polymorphism and the original TNF-308 polymorphism genotyping are shown in Figure 6.1 and 6.2.

All my assessments of genotype were verified by a second observer (M Hill), who also supervised any genotyping performed by the author. Genotyping for the TNF polymorphism using the Sty1 enzyme digest was carried out by N Neale (under supervision of M Hill) and M Hill.

All the data presented below for the TNF-308 polymorphism uses genotyping based on the Sty1 restriction enzyme method.

6.2.3 Hypotheses

Primary Hypotheses

Our primary hypotheses were:-
The tumour necrosis factor polymorphisms, TNF-308 and LTαNcoI, are associated with susceptibility to late onset airflow obstruction.

The tumour necrosis factor polymorphisms, TNF-308 and LTαNcoI are associated with severity of disease in subjects with late onset airflow obstruction.

Secondary hypotheses

The tumour necrosis factor polymorphisms, TNF-308 and LTαNcoI are associated with emphysema in subjects with late onset airflow obstruction.

6.2.4 Statistical analysis

Analysis used conventional statistical methodology as has been previously described. For the primary hypotheses the outcome variables were binary disease affectation. The continuous variables FEV1, FVC and FEV1/FVC ratio were examined as quantitative markers of disease severity. For the secondary analysis, a limited amount data on gas transfer was available from subjects with COPD. This was analysed as a surrogate marker of emphysematous disease and examined for relationships to TNF 308 and LT alpha genotypes.

Analysis for associations, confounding variable and genotype modelling was carried out as previously detailed in Chapter 4.2.4.

As previously analysis was also carried out for two subgroups based on high and low lifetime tobacco exposure.

6.3 Results

426 individuals were genotyped for the LT NcoI polymorphism (144 controls and 283 cases) and 423 individuals for the tumour necrosis factor polymorphism (142 controls and 281 cases).
They had a mean age of 68.3 (9.44), range 45-91, 239 (56.5%) male. There were no statistically significant differences in age and sex distribution between the case and control populations. Lifetime pack-year history was significantly higher in the cases compared to the controls (median cases, 30-39 pack years, median control 10-19 pack years, p <0.001).

The two polymorphisms lie in linkage disequilibrium (Chi square$^4$ = 174, p <0.001).

Contingency table analysis showed that there was no difference in genotype distribution between the case and control populations for either polymorphic variant (Table 6.3).

We also hypothesised that the polymorphism might have an effect on disease severity as measured by pulmonary function. Tables 6.4 and Table 6.5 show the relationship between pulmonary function and genotype for these primary outcome variables. Briefly, amongst cases, mean FEV1 (SD) was 52.6 (15.4) for the LTαNcoI 1,1 homozygotes, compared with 48.1 (22.4) for 2,2 homozygotes; for FVC was 85.8 (19.3) for 1,1 and for 76.6 (23.1) for 2,2. Corresponding values for TNF-308 1,1 homozygotes were, for FEV1, 48.8 (21.6) compared with 46.8 (22.9) for TNF-308 2,2 homozygotes; for FVC 77.6 (21.9) for TNF-308 1,1 compared with 75.4 (24.1) for TNF-308 2,2 homozygotes. One-way analysis of variance suggested that there was no significant effect of either genotype on pulmonary function and this was confirmed with regression analysis. As with previous studies the importance of smoking was recognised and for this reason analysis was repeated for those individuals with a significant past smoking history. No significant change in the relationships between pulmonary function and genotype was observed.

Secondary hypothesis sought to explore the relationship between the degree of emphysema and the two genotypes. Gas transfer measurements (KCO and TLCO) were available for 159 cases with COPD (82%). These were examined as a quantitative variable, as a percentage of their age and sex matched predicted values. There were no statistically significant differences in KCO measurements between the TNF-308 genotype or the lymphotoxin alpha genotype.
The mean KCO (as a percentage of its predicted value was) 85.5 (30.9) for the TNF-308 1,1 genotype compared with 72.4 (22.8) for the 2,2 genotype. There were only three individuals with the TNF-308 2,2 genotype, hence this apparent difference was of no statistical significance, on either one-way analysis of variance or multiple linear regression analysis. Similarly there was an apparent trend for lower values of KCO amongst individuals homozygous for the 1,1 lymphotoxin alpha NcoI polymorphism (mean KCO 78.3, (SD 85.5)) compared to the 2,2 genotype(mean KCO 85.5(SD 30.4). Relatively few individual were genotyped as 1,1 homozygotes and thus, as before, these differences in KCO were not statistically significant. Analysis was also performed using TLCO (as a percentage of its predicted value). As with KCO, a non-significant trend in TLCO was observed. Mean TLCO as a percentage of its predicted value (SD) was 70.5 (24.6) for TNF-308 1,1 homozygotes compared with 67.0 (20.9) for heterozygotes and 50.4 (8.1) for TNF-308 2,2 homozygotes. On regression analysis with adjustment for covariates these differences approached statistical significance (p = 0.087). The relative differences in gas transfer measurements were less striking for the lymphotoxin alpha polymorphism and were not statistically significant.
Table 6.3 Genotype frequencies for cases and control populations

<table>
<thead>
<tr>
<th></th>
<th>Case number (%)</th>
<th>Control Number (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LT(\alpha)NcoI</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,1</td>
<td>24 (8.5)</td>
<td>20 (13.9)</td>
<td>44 (10.3)</td>
</tr>
<tr>
<td>1,2</td>
<td>129 (45.7)</td>
<td>64 (44.4)</td>
<td>193 (45.3)</td>
</tr>
<tr>
<td>2,2</td>
<td>129 (45.7)</td>
<td>60 (41.7)</td>
<td>189 (44.4)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>282 (100)</td>
<td>144 (100)</td>
<td>426 (100)</td>
</tr>
</tbody>
</table>

| **TNF 308**          |                 |                    |             |
| 1,1                  | 204 (72.6)      | 98 (69.0)          | 302 (71.4)  |
| 1,2                  | 71 (25.3)       | 40 (28.2)          | 111 (26.2)  |
| 2,2                  | 6 (2.1)         | 4 (2.8)            | 10 (2.4)    |
| **Total**            | 281 (100)       | 142 (100)          | 423 (100)   |

LT\(\alpha\)NcoI \(\chi^2 = 3.06, p = 0.216\), for TNF 308 \(\chi^2 = 0.66, p = 0.720\)
Table 6.4 Primary outcome variables by LTαNcoI genotype

<table>
<thead>
<tr>
<th>LTαNcoI</th>
<th>Mean FEV₁ % predicted (standard deviation)</th>
<th>Mean FVC % predicted (standard deviation)</th>
<th>FEV₁/FVC (standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,1</td>
<td>52.6 (15.4)</td>
<td>85.8 (19.3)</td>
<td>49.4 (10.1)</td>
</tr>
<tr>
<td>1,2</td>
<td>49.6 (21.4)</td>
<td>77.5 (21.1)</td>
<td>50.5 (13.1)</td>
</tr>
<tr>
<td>2,2</td>
<td>48.1 (22.4)</td>
<td>76.6 (23.1)</td>
<td>47.9 (12.8)</td>
</tr>
</tbody>
</table>

Regression coefficient

-0.91 -2.18 -0.68

95% confidence intervals

-4.60 to 2.79 -6.20 to 1.85 -2.85 to 1.49

Significance

0.629 0.288 0.536
<table>
<thead>
<tr>
<th></th>
<th>Mean FEV(_1) % predicted (standard deviation)</th>
<th>Mean FVC % predicted (standard deviation)</th>
<th>FEV(_1)/FVC (standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-308 1,1</td>
<td>48.8 (21.6)</td>
<td>77.6 (21.9)</td>
<td>48.6 (13.1)</td>
</tr>
<tr>
<td>TNF-308 1,2</td>
<td>50.4 (21.3)</td>
<td>78.9 (22.5)</td>
<td>50.1 (12.0)</td>
</tr>
<tr>
<td>TNF-308 2,2</td>
<td>46.8 (22.9)</td>
<td>75.4 (24.1)</td>
<td>49.0 (10.9)</td>
</tr>
<tr>
<td>Regression coefficient</td>
<td>0.247</td>
<td>0.117</td>
<td>0.82</td>
</tr>
<tr>
<td>95% confidence intervals</td>
<td>-4.43 to 4.92</td>
<td>-5.05 to 5.29</td>
<td>-1.95 to 3.6</td>
</tr>
<tr>
<td>Significance</td>
<td>0.917</td>
<td>0.965</td>
<td>0.560</td>
</tr>
</tbody>
</table>
6.4 Conclusions

The LT Ncol polymorphism and the TNF 308 polymorphism occurred relatively infrequently in our population. Allele frequencies for LT Ncol allele 1 were 0.33 and for TNF 308 allele 2 were 0.15. These frequencies are the same order of magnitude as reported in other studies involving Western Caucasian populations (Hingham M, 2000; Keating V, 2000; Patuzzo C, 2000; Sandford AJ 2001). The low frequency of the alleles significantly reduces the power of our study to detect a difference in susceptibility or disease severity, unless they exert a major effect on overall disease phenotype. Neither polymorphism exhibited an effect on disease susceptibility or severity for either late onset airflow obstruction (asthma and COPD), or for COPD alone in our population. This is in contrast to the findings of a number of smaller association studies (Huang SL, 1997; Sakao S, 2001, 2002; Keatings V, 2000). These studies have shown the TNF-308 polymorphism to be associated with both disease susceptibility and disease severity. However three recent studies have failed to show associations (Hingham M, 2000; Patuzzo C, 2000; Sandford AJ, 2001).

A possible trend towards a more emphysematous disease picture was observed for those individual who were homozygous for the rare TNF-308 allele in our population. A recent study by Sakao S (2002) had similar findings. Eighty four patients with COPD were scored for the presence of emphysematous changes observed on high resolution computerised topography scan of the chest (HRCT). A visual scoring system to quantify the degree of emphysema was developed using the scoring method of Goddard and co-workers (Goddard PR, 1982). There was a strong correlation between the visual scores and FEV1/FVC ratio. Gas transfer measurements were not available. A trend towards higher emphysema scores was shown with possession of allele 2, but conventional levels of statistical significance were not reached.
From a pathobiological perspective one would predict that this variant would be associated with more severe disease, and particularly a tendency towards emphysema. The TNF-308 rare allele is associated with increased baseline and induced expression of TNF from macrophages in vitro, although in vivo effects have not been demonstrated (Wilson AG, 1997). It is possible that homozygousity for the rare allele may accelerate inflammatory processes, in the presence of cigarette smoke. In vitro experiments have shown that TNF- can cause an increase in apoptosis in alveolar epithelial cells in culture, further accelerated by exposure to ultraviolet light. It is possible therefore that TNF can induce cellular changes making alveolar cells more prone to apoptosis by any stressor, including tobacco smoke. These cells are pivotal in the repair of alveolar injury.

There are a number of explanations for the inconsistent findings between the various studies. Firstly it is possible that there are population stratification effects that account for the differences. It is of interest that two of the statistically significant studies, although small in size, were both carried out in populations of Asian extraction, suggesting that population stratification effects may indeed be important (Huang SL, 1997; Sakao S, 2001, 2002). The gene frequencies in one of these Asian populations was also strikingly different, for example the allele frequency for the TNF-308 allele 2 was 0.05.

The potential lack of power has already been discussed. It is also possible that the inconsistent results from the studies are a consequence of different, as yet undescribed, candidate genes in linkage disequilibrium with the TNF-308 polymorphism. The human leukocyte antigen alleles are possible alternative candidates. Population stratification effects may then explain why the results are less frequently observed in Caucasian population such as ours.

Common to all the studies are less striking associations with the lymphotoxin alpha NcoI polymorphism. This is a less attractive candidate gene than the TNF-308 polymorphism,
since there is less evidence to support its functional effects in vivo. Although we do not describe any significant effects, any trends towards differences in lung function between the TNF-308 genotype groups were less marked in the lymphotoxin alpha genotypes.

In summary we have not shown the TNF gene-complex polymorphisms to play a significant role in determining disease susceptibility in subjects with COPD, although consistent trends to poorer pulmonary function were observed, particularly with allele 2 of the TNF-308 polymorphism. The polymorphic variants occur relatively infrequently in Caucasian populations and these non-significant trends observed warrant further investigation in a larger population of subjects with COPD.
Allele 2 produces an undigested 750bp band

Allele 1 produces a 500bp and a 250 bp band

a = 1,2 heterozygote

b = 2,2 homozygote

c = 1,1 homozygote
Figure 6.2 Genotyping for the TNF alpha-308 polymorphism

TNF PROBE ALLELE 2. 6/21/00

2 MINS EXPOSURE.

UNABLE TO SEPERATE 1,1, 1,2.

2,2 CONTROLS.

2,2 homozygote

1,1 homozygote
7 A PILOT STUDY OF A SIBLING PAIR APPROACH TO THE INVESTIGATION OF THE GENETIC BASIS OF COPD.

7.1 Introduction

The epidemiological evidence suggesting that COPD and associated physiological traits, such as decreased FEV1, cluster within families has been discussed in Chapter 2. Briefly, numerous large studies have shown significant familial correlations in FEV1, in those with COPD (Larson RK, 1970; Kueppers MD, 1977; Khoury MJ, 1986; Rybicki BA, 1990), those without COPD (Gilvelber RJ, 1998; Tager IB, 1976; Chen Y, 1996; Coul tas DB, 1991) in smoking and non smoking populations. Recently, very high risks to first degree relatives of patients with severe early onset emphysema have been shown, but only for those first degree relatives who smoke (Silverman EK, 1998).

This study was primarily designed to assess the feasibility of performing a sibling-pair study to investigate the genetic epidemiology of a late onset disorder, namely COPD. One aim therefore was to review data on the screening and recruitment patterns within the population. The study was not powered to detect genetic associations of candidate genes.

I hypothesized that:

(i) patients with late onset airflow obstruction come from families with a greater prevalence of obstructive airways disease than population controls.

(ii) siblings of cases would have poorer lung function, and higher rates of airflow obstruction than population controls.

7.2 Methods

All cases recruited were asked if they had any first degree relatives (parents, siblings or children) with asthma, bronchitis, emphysema or COPD. Where cases were unable to provide
full information on all first degree relatives the data was recorded as missing. Population controls also provided identical information about first degree relatives.

From our case population a sample of individuals with a definite diagnosis of COPD were invited to participate in the sibling–pair pilot study. Cases with siblings living locally were asked if they were willing for an approach to their sibling(s) to be made. If siblings did not take part in the study the reason for this was recorded (death or no siblings, no contact between cases and siblings, case refusal, sibling refusal, sibling ineligible). All siblings were contacted by letter initially. If they failed to respond to my initial letter they were not contacted again. Siblings were seen in their own homes or at the outpatients clinic, which ever was more convenient. They completed the same questionnaire as the cases and population controls and all underwent spirometry. A blood sample was collected from all siblings for genetic analysis.

7.2.1 Statistical Analysis

The primary dichotomous outcomes were, family histories of COPD or asthma (self reported) in our case and control populations and the use of bronchodilators in our sibling and control populations. Contingency table analysis and binary logistic regression was used for analysis of dichotomous outcomes with adjustment for potential confounding variables, including age, sex, (and smoking history, where appropriate). Further clarification of the self-reported family histories was not sought.

Continuous outcome variables were FEV1 and FVC as a percentage of their predicted values in our sibling and control populations. A one-way analysis of variance, and regression models were used to examine for differences between the sibling and control populations. Secondary outcome variables were the FEV1/FVC ratio, and physician diagnosis of asthma for the sibling and control populations.
7.3 Results

148 subjects with COPD (rather than late onset airflow obstruction) were screened for inclusion in the sibling study. Figure 7.1 shows the outcome of this screening procedure. 39(26%) had no siblings, 8(5%) had no contact with siblings and 3(2%) were adopted.

98(66%) individuals had siblings. Fifty COPD cases with siblings living locally (within a ten mile radius) were selected and invited to take part in the study. This represented 34% of the original cases screened, and 51% of those cases with siblings. A total of 85 siblings were contacted, of these 36(42%) declined to take part, or did not respond to our letter. We did not have approval to contact non-responders a second time. Of those siblings consenting to participate (number = 49) 47 (96%) were eligible for inclusion. These siblings came from 30 families.

The characteristics of the sibling population in comparison with the control population are shown in Table 7.1. Forty-seven siblings, 22 male, with a mean age of 64 years, were recruited. They were significantly younger than both the case and population control groups (p=0.003 and p=0.001 respectively), with range 42-79 years. There were no significant differences in sex distribution, between the sibling and other two groups. In common with the population controls, total pack year history was lower amongst the siblings compared to the cases, but was no different to the control group.
Table 7.1 Characteristics of the sibling and control populations

<table>
<thead>
<tr>
<th></th>
<th>Sibling</th>
<th>Control</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>48</td>
<td>154</td>
<td>N/A</td>
</tr>
<tr>
<td>Mean Age (SD)</td>
<td>63.7(10.3)</td>
<td>68.5(8.6)</td>
<td>p = 0.001*</td>
</tr>
<tr>
<td>Sex.</td>
<td>22 (45)</td>
<td>91 (59)</td>
<td>p = 0.106†</td>
</tr>
<tr>
<td>Number(%) male</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean pack-year history</td>
<td>3.1(2.57)</td>
<td>2.7(2.53)</td>
<td>p = 0.388‡</td>
</tr>
</tbody>
</table>

* students t-test
† chi-square test
‡ chi-square test for trend

Table 7.2 Family history of respiratory disease in case and control populations

<table>
<thead>
<tr>
<th>Positive family history of asthma, bronchitis, COPD or emphysema in first degree relative</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Number (%)</td>
<td>Number (%)</td>
</tr>
<tr>
<td>149 (58.2)</td>
<td>107 (41.8)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>63 (41.5)</td>
<td>89 (58.5)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>212 (52.0)</td>
<td>196 (48.0)</td>
</tr>
</tbody>
</table>

Chi square = 10.7, p = 0.001
256 (86.2%) cases and 152 (98.7%) controls were able to provide information on family history. A family history of asthma, bronchitis or emphysema, but not atopy, was sought for all first-degree relatives of subjects. The results are shown in Table 7.2. 58.2% of all cases compared with 41.5% of controls reported at least one first degree relative with asthma, emphysema or bronchitis, and this difference was statistically significant ($\chi^2$ test 10.7, $p=0.001$ (n = 408)). 43 individuals (9.5%) were adopted, had no contact with siblings and/or offspring, or did not have information on family history collected, and thus were unable to provide accurate family data.

A higher proportion of our sibling controls were taking regular medication for airflow obstruction (27.7%), compared to the population controls (5.9%), and this difference was significant ($\chi^2= 17.6, p < 0.001$) (Table 7.3). Furthermore, 16 (34%) of siblings reported that they used bronchodilators or had a diagnosis of asthma, compared with 9 (5.9%) of population controls. This difference was also highly significant ($\chi^2 = 25.5, p < 0.001$). A greater proportion of siblings reported a diagnosis of asthma or COPD than controls (14.6% compared to 5.8%) although this difference did not reach statistical significance.

Mean FEV1 (SD), expressed as a percentage of its predicted value, was compared in the control and sibling population. Amongst the siblings the mean FEV1 value was 84.2 (20.9). Corresponding value for controls was 93.6(18.3). One way analysis of variance suggested that this difference was significant ($p= 0.003$). Regression analysis adjusted for age, sex and smoking history, showed that although pack-year history was important in determining FEV1, having a sibling with COPD was highly significant (regression coefficient 11.9 (95% confidence intervals 17.9-5.8), $p < 0.001$) (Table 7.3). Thus confirming that siblings of patients with COPD have significantly poorer lung function than population controls. In fact,
although only 13 (28%) of siblings were taking a bronchodilator, 17 (36%) had an FEV1 of less than 80%, although not all of these had obstructive spirometry.

Despite the marked differences in FEV1 between population controls and siblings there were no significant differences in FVC between the control and sibling population. Mean FVC (SD) as a percentage of its predicted value for controls was 97.7(16.8) compared to 101.1(23.9) for siblings. This difference was not significant (Table 7.3).

Finally, in the secondary analysis the FEV1/FVC ratio was examined. The mean ratio for the controls was 75.8 (8.8) compared with a ratio of 67.9 (10.1) for the siblings. These differences were statistically significant, after adjustment for age, sex and smoking history (regression coefficient 9.7, 95% confidence intervals 6.9-12.6), p < 0.001).

Table 7.3 Pulmonary function of siblings compared to population controls

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Siblings</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>number = 152</td>
<td>number = 47</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Use of bronchodilators.</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number (%)</td>
<td>9(5.9)</td>
<td>13(27.7)</td>
<td>p &lt; 0.0005†</td>
</tr>
<tr>
<td>Mean FEV1 (SD)</td>
<td>93.6(18.3)</td>
<td>84.2(20.9)</td>
<td>p &lt;0.0005‡</td>
</tr>
<tr>
<td>Mean FVC (SD)</td>
<td>97.7(16.8)</td>
<td>101.1(23.9)</td>
<td>p = 0.706‡</td>
</tr>
<tr>
<td>Mean FEV1/FVC (SD)</td>
<td>75.8(8.8)</td>
<td>67.9(10.1)</td>
<td>p &lt;0.0005‡</td>
</tr>
</tbody>
</table>

† χ² = 17.6
‡ after adjustment for age, sex and pack-year history.

7.4 Discussion

We have shown that it is possible to construct a sibling pair study for the investigation of the genetic epidemiology of COPD. Our preliminary findings suggest that approximately two-
thirds of subjects with COPD will have siblings and of these about 40% were recruited. For a study with adequate power for candidate gene analysis a sample size of 1000 sib-pairs would be associated with sufficient power to detect all but the smallest genetic effects, depending on gene frequency and on the understanding that such power calculation are notoriously difficult and subject to numerous assumptions. It should be noted that at least 2000 cases would need to be screened to recruit this number of sibling pairs, based on this pilot data. Recruiting sibling pairs to a late onset disease study would be expected to be harder than for a juvenile onset condition, although screening rates are not quoted in sibling-pair studies of asthma. In the case of a late onset disease siblings may be geographically isolated, not in regular communication or unavailable due to death or co-existing morbidity, problems which rarely hamper sibling-pair studies involving younger subjects.

The family history data collected from the whole population demonstrates that patients with COPD have a greater recall of a family history of respiratory disease amongst their first degree relatives than age and sex matched population controls. This observation is supported by the data analysis of lung function amongst the population controls and siblings of patients with COPD. We have shown that siblings have significantly worse lung function, as measured by FEV1 as a percentage of its predicted value and the FEV1/FVC ratio, but not FVC compared to controls. These differences were highly significant and robust following adjustment for differences in age, sex and smoking history between the two populations. The apparent strength of these differences was surprising given our small sample size and needs to be repeated in a larger population. It is interesting that the differences in pulmonary function were observed for FEV1 but not FVC. There has been a considerable body of data (already discussed in Chapter 2) to suggest that FEV1 has a high degree of genetic hereditability, both amongst the general population and amongst relatives of patients with COPD. Our study
design did not allow us to examine these findings precisely, but our data would be consistent with these observations.

There are a number of limitations in interpreting the data presented in this pilot study. Firstly, the information on family history was provided by participants and thus subject to considerable recall bias. Furthermore, in comparing the family histories of our cases and control populations we were unable to adjust for differences in smoking history between these groups. It has been shown in a number of large epidemiological surveys that smoking histories are conserved amongst family members. Thus, one of the strongest factors in determining whether an individual smokes is whether their parents and/or siblings smoke. The consequences of this observation on the interpretation of the family history data is important since our controls (and by inference their first degree relatives) had significantly lower lifetime tobacco exposure than our cases (and by inference their first- degree relatives). We know that smoking is the major environmental determinant of COPD and minimising, or correcting for, differences in exposure is important. Paradoxically, recruited siblings of cases, appeared to have lifetime tobacco exposures that were closer to the population controls than the cases. We do not know if this is a consequence of a ‘survivor’ effect, with smoking siblings already dead from a smoking related disease.

Another concern is that only 49 from 85 siblings contacted (58%) agreed to participate in the study. Hence, it is possible that our findings are subjects to recruitment bias with siblings who had respiratory disease or symptoms more likely to respond to a request to participate.

7.5 Conclusions

It is possible to recruit subjects to a sibling-pair study for the investigation of a late onset disease such as COPD, although a larger number of subjects will need to be screened compared to a younger population. Despite some limitations, we have shown that siblings of patients with COPD have significantly lower FEV1 than population controls and that these
differences cannot be accounted for by differences in age, sex or smoking history. This suggests that there are other factors determining FEV1 in individuals. These factors could be genetic or could equally relate to early environmental or in-utero exposures.

This pilot data has been important in planning, designing and successfully obtaining funding for further work. A national study to investigate the genetic epidemiology of COPD is underway, based on a sibling pair design.
Figure 7.1. Recruitment patterns for cases and siblings for a pilot project.

148 cases of COPD

Any siblings? NO

98 (66%) had siblings

Siblings living NO

50 (34%) cases had siblings living locally
Total number of siblings

Siblings agreed to take part? NO

49 (58%) siblings agreed to take part

Siblings eligible? NO

47 siblings completed study (55% of those contacted).

39 (26%) No siblings/siblings dead
3 (2%) Adopted

48 (32%) siblings not living locally/location unknown

36 (42%) siblings declined to take part

1 sibling not eligible
1 refused spirometry
8 GENETIC STUDIES OF COPD – CONCLUSIONS, WORK IN PROGRESS AND FUTURE WORK.

8.1 Concluding comments

8.1.1 Genetic epidemiological approaches to late onset airflow obstructions

Obstructive airways disease is common in older adults and a major cause of morbidity and mortality. There is a spectrum of disorders with overlapping clinical and pathological features including late-onset asthma, COPD and emphysema. Defining these conditions for the purposes of genetic studies is problematic.

There is a considerable amount of work to suggest that COPD and related phenotypes have a genetic component that may determine susceptibility or modify disease severity. However, other than alpha-one anti-trypsin deficiency, strong candidate genes have not yet been described. Phenotypic heterogeneity may be one reason why the results from genetic association studies are often not replicated.

An area for future research is the development of phenotypic scores for COPD, for use in genetic association studies.

For studies of the genetic basis of late onset airflow obstruction, and COPD in particular, the specific problems of ageing must be considered. In particular the presence of co morbidity, and selective survival assume greater importance as a population ages. Although statistical solutions to some of these problems have been developed, they have yet to be applied to real populations.

The field of genetic epidemiology of complex diseases is rapidly evolving and it is likely that in the near future advances in molecular techniques and bio-informatics will significantly alter the approaches that are adopted to investigate the genetic basis of such complex disorders.

The preliminary studies that have been presented here illustrate two possible approaches to the investigation of COPD and its phenotypes – the case-control genetic association study and
the sibling-pair association study (pilot study). Both are feasible but large numbers of subjects are required to generate sufficient power for many outcomes.

The lack of linkage data, from which the selection of candidate genes might be guided, is beginning to be addressed by at least one ongoing study. Preliminary published data from Sililvermans group has shown linkage to quantitative spirometric phenotypes in pedigrees ascertained through severe early onset COPD phenotypes. Suggestive evidence for linkage was demonstrated for chromosomes 2q (lod 4.14), 1, 12p and 17, 22, 21 and 19 (Silverman EK, 2002). Further linkage data to confirm these initial findings is awaited.

The candidate genes selected in our study do not lie in any of these chromosomal regions.

8.1.2 The Dutch Hypothesis and candidate gene analysis

The results presented neither support or refute the ‘Dutch Hypothesis’. I have shown some interesting potential areas for future study, in an extended population.

Late onset airflow obstruction is associated with some atopic features, and significant bronchodilator reversibility is common even in subjects with COPD. Evidence that juvenile-onset asthma associated polymorphism determine susceptibility to late-onset airflow obstruction was not demonstrated. However a number of disease modifying effects, particularly for the beta-adrenoceptor polymorphisms, were demonstrated.

Some interesting associations were found between the polymorphisms of the beta adrenoceptor and pulmonary function, in particular FEV1. The gln27 polymorphism appears to be associated with disease severity, and possibly bronchodilator reversibility, although this relationship was not as strong. It is not clear, from this data if the modifying effects that the polymorphism has on FEV1 is important for all smokers or just those with COPD. Further studies in a population of smokers with and without disease would clarify this question.

Recently analysis for the beta-adrenoceptor polymorphisms has focussed on extended haplotypes, including the SNP47 polymorphism, not originally genotyped in our sample.
Extended haplotypes, to include the SNP47 polymorphisms, are therefore being examined in this population (Dr A. Wheatley and Prof I. Hall, University of Nottingham).

Subjects with late onset airflow obstruction did have some features of atopy, particularly elevated IgE levels, although this did not appear to be related to polymorphisms of the beta subunit of the high affinity IgE receptor genotype. Thus are the genetic factors determining atopy different in older subjects or are our findings simply a reflection of the limited sample size and cumulative effects of age and interactions with smoking?

In contrast to a growing number of studies, the polymorphisms of the tumour necrosis factor showed no significant associations to any of the disease phenotypes hypothesised. There have been a considerable number of similarly sized studies that have shown interesting associations and so our results were disappointing. However, the measured allele frequencies of the TNF-308 and the lymphotoxin alpha polymorphisms were low in our population and thus we have insufficient power to exclude a role for these polymorphisms. It is of note that the statistically significant studies both involved Asian populations suggesting that there may be important ethnic differences. Furthermore our population is considerably older than others reported in the literature. The polymorphisms of the TNF gene complex have also been linked to cardiovascular disease and it is possible that as the population ages individuals homozygous for the rare allele are removed from the population.

The failure of my studies to demonstrate multiple significant genetic associations with late-onset asthma and COPD phenotypes was not surprising, despite the fact that numerous much smaller studies of juvenile onset asthma have yielded statistically significant results. The failure to demonstrate significant associations is probably multi-factorial. Many of these factors such as the effects of age, phenotypic and genetic heterogeneity, and the difficulties
in selection of candidate genes have already been discussed. Issues relating to study design and sample size have also been highlighted.

It is possible that the genetic basis of COPD is distinct from that of asthma and that research need to focus on alternative candidate genes, such as those involved in oxidant/antioxidant pathways. However it is of interest that the preliminary linkage data for COPD highlights some shared loci with asthma linkage studies (Silverman EK, 2001). Furthermore, since a number of the results discussed in this thesis could be interpreted in support of the 'Dutch Hypothesis', further work is needed before rejecting this disease paradigm.

8.1.3 Criticisms

I collected data from a large population of subjects with obstructive airways disease and a group of well-matched control individuals. However, although almost 500 subjects were recruited in total, this is still a relatively small study for the purposes of examining candidate genes.

One criticism of the study population is that the phenotype was relatively broad, including individuals with late onset asthma, as well as those with COPD (with and without bronchodilator reversibility). It is possible that by narrowing the phenotype definition, or by focusing on those with an early onset severe form of disease, the probability of finding a positive association would be increased. Such a population may not be wholly representative of the 'normal' late-onset airflow obstruction phenotypes. However, the vast majority of individuals developing airflow obstruction later in life have a significant lifetime tobacco exposure and there is thus much rationale in restricting the phenotype to exclude those subjects with no past smoking exposure.

Furthermore the control population was not well matched for smoking history, the key environmental exposure and thus statistical adjustment for differences in lifetime tobacco exposure...
exposure was made during analysis. It is clear that the ideal control population would have a similar smoking history but recruiting such a population was difficult for practical reasons (all controls were initially contacted via General Practitioners who often had incomplete information on pack-year history).

Finally, throughout this thesis a standard ‘p-value’ of 0.05 has been adopted to define statistical significance. This differs from the criterion p<0.01, the value proposed by Lander and Kruglyak when there is a prior hypothesis. However, the concept of rigid guidelines for the arbitrary concept of ‘statistical significance’ that are appropriate in all cases, flies in the face of rational statistical philosophy. Regardless what reasonable criterion for statistical significance are used, all analysis presented is to some extent be exploratory and all findings require confirmation in independent data.

Many of these criticisms will be addressed in a further candidate gene study that aims to recruit a large population of cases with COPD and related (smoking) sibling controls. This project has already been funded and depending on numbers of sibling pairs recruited a family based method retains the advantage of future linkage analysis (Section 8.2.1).

An alternative and complimentary approach would be to collect a population of smokers and examine quantitative markers of pulmonary function. This has some merits in particular likely ease of recruitment and the opportunity to examine other phenotypes/genotypes of interest such a smoking cessation and addictive genes.
References


24. **Brereton HM**, Ruffin RE, Thompson PJ, et al. Familial atopy in Australian pedigrees; adventitious linkage to chromosome 8 is not confirmed nor is there evidence of linkage to the high affinity IgE receptor. Clin Exp Allergy 1994;24(9):868-877


34. **Campbell DA, Li Kam WA E, Britton J et al.** Polymorphism at the tumour necrosis factor locus and asthma. Monogr Allergy. Basel, Karger 1996;33:125-137


39. **Ciba Guest Symposium.** Terminology, definitions, and classifications of chronic pulmonary emphysema and related conditions. Thorax 1959;14:286-299


45. Collee JM, ten Kate PL, Riley GA et al. Allele sharing on chromosome 11q in sibs with asthma and atopy. Lancet 1993;342:936


64. Diezer MH Genome screen for asthma and related phenotypes in the French EGEA study. Am J Respir Crit Care Med. 2000;162(5):1812-8


91. Hall IP. β2-adrenoceptor polymorphisms: are they clinically important? Thorax 1996; 51:351-353


113. Huber HL, Koessler KK. The pathology of bronchial asthma. Arch Intern Med 1922; 30:689-760

161


119. Jeffrey PK. Comparative morphology of the airways in asthma and chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1994; 150:S6-13


139. Lander ES, Schork NJ. Genetic dissection of complex traits. Science 1994;265:2037-2048

144. Lee HY, Stretton TB. Asthma in the elderly. BMJ 1972;4:93-95


Invest. 1998;102:1927-1932


necrosis factor locus: an NcoI polymorphism in the first intron of the human TNFβ 
genere correlates with a variant amino acid in position 26 and a reduced level of TNFβ 

161. Metcalf JP, Thompson AB, Gossman GL. Gc globulin functions as a chemotaxin in the 
lower respiratory tract. A potential mechanism for lung neutrophil recruitment in cigarette 

162. Meyers DA, Postma, DS, Panhasysen CIM, et al. Evidence for a locus regulating total 
serum IgE levels maps to chromosome 5. Genomics 1994;23:464-470

163. Moffat MF and Cookson WOCM. Tumour necrosis factor polymorphism and asthma. 


165. Molvig J, Pociot F, Baek et al. Monocyte function in IDDM patients and healthy 


167. Morton NE. Quantitative scores for asthma and atopy. Clin Exp Allergy 
1998;28:S1:95-97

168. Motegi T, Kida K. A clinical study of serum IgE concentrations in elderly patients with 
bronchial asthma and chronic obstructive pulmonary disease. Nihon Kokyuki Gakkai 
Zasshi 1999;37(8):608-13


initiative for asthma. Global strategy for asthma management and prevention. 

171. National Heart, Lung and Blood Institute, World Health Organisation. Global strategy 
for the diagnosis management and prevention of chronic obstructive pulmonary disease. 
NHLBI/WHO Workshop Report 2001;2701


192. Pociot F, Briant L, Jongeneel CV et al. Association of TNF and class II major histocompatability complex alleles with the secretion of TNFα and TNFβ by human mononuclear cells; A possible link to IDDM. Eu J Immunol 1993(a);23:224-231


196. Pride NB. Smoking, allergy and airways obstruction; revival of the " Dutch hypothesis". Clin Allergy, 1986;16: 3-6


213. Salvato G. Some histological changes in chronic bronchitis and asthma. Thorax 1968; 23:168-172


218. Schellenberg D, Pare PD, Weir TD. Vitamin D binding protein variants and the risk of COPD. Am J Respir Crit Care Med 1998;157:957-961


231. Summerhill E, Leavitt SA, Gidley H et al. β-2 adrenergic receptor arg16/arg16 is associated with reduced lung function, but not with asthma in the Hutterites. Am J Respir Crit Care med 2000;162:599-602


237. Todd J. Interpretation of results from genetic studies of multifactorial diseases. Lancet 1999:354 S1


239. Tordov AA, Rao DC. Trade off between false positives and false negatives in the linkage analysis of complex traits. Genet Epidemiol 1997;14:453-464
255. Wilson AG, de Vries N, Pocoit F et al. An allelic polymorphism within the human
tumour necrosis factor alpha promoter region is strongly associated with HLA A1 B8 and

256. Wilson AG, Symons JA, McDowell TL. Effects of a polymorphism in the human
tumour necrosis factor gene on transcriptional activation Proc Natl Acad Sci
1997;94:391-399

257. Wjst M, Fischer G, Immervoll T et al. A genome wide search for linkage to asthma.
German Asthma Genetics Group Genomics 1999;58(1):1-8

258. Wright JL, Cagle P, Churg A et al. State of the art; Disease of the small airways. Am
Rev Respir Dis 1992;146:240-262


bronchial susceptibility to cigarette smoke. Chest 1981;80:57S

metalloproteinase and cathepsin dependant emphysema Clin Invest 2000;106:1080-1093

262. Zhu S, Chan yeun, M, Becker AB, et al. Polymorphims of the IL-4, TNF-α and FceRIβ
genes and risk of allergic disorders in at risk infants. Am J Respir Crit Care med
2000;161:1655-1659
Appendices

Contents

Appendix A  Data Collection form, Consent form
Appendix B  Additional laboratory techniques
Appendix C  Publications and presentations
Appendix A

Data collection form

Genetic studies of late onset airflow obstruction

DATE [ ] [ ] [ ] [ ] [ ] [ ]

STATUS INDEX CASE [ ]

SIBLING [ ]

CONTROL [ ]

DOB [ ] [ ] [ ] [ ] [ ] [ ]

AGE [ ] [ ]

SEX M [ ] F [ ]

AGE AT ONSET [ ] [ ]

HEIGHT (metres) [ ] [ ] [ ]

OCCUPATION __________________________

PAST MEDICAL HISTORY

____________________________________

____________________________________

____________________________________

____________________________________

A. Do you have any of the following symptoms

COUGH [ ]

SPUTUM PRODUCTION [ ]

WHEEZE [ ]

NOCTURNAL SYMPTOMS [ ]

BREATHLESSNESS [ ]

NONE [ ]

B. Have you ever been diagnosed with the following

ASTHMA [ ]

HAY FEVER [ ]

ECZEMA [ ]
AGE AT DIAGNOSIS

Under 10 years □ 10-20 years □
20-45 years □ Over 45 years □

C. SMOKING HISTORY

CURRENT SMOKING STATUS

Current □ Ex □ Never □

LIFETIME PACK-YEAR HISTORY

LESS THAN 5 □ 30-39 □
5-9 □ 40-49 □
10-19 □ 50-59 □
20-29 □ >60 □

D. MEDICINES & INHALERS

INDICATE WHICH MEDICINES ARE CURRENTLY IN USE

INHALERS
a. Beta agonist □
b. Ipratropium □
d. Inhaled Steroid □
e. Theophyllines □
f. Oral Steroids (maintenance) □
g. Oxygen (concentrator or cylinder) □
h. Nebulisers □
Data collection form

Genetic studies of late onset airflow obstruction

OTHER MEDICATION

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

E. YOUR FAMILY

Family History of Respiratory Disease?

Yes □ No □

Specify if known

Mother □ Father □

Sibling □ Offspring □

NONE □
Data collection form

Genetic studies of late onset airflow obstruction

BRONCHODILATOR REVERSIBILITY TESTING.  Yes  No

AGENT:
- SALBUTAMOL
- ATROVENT
- NEITHER

RESULTS

<table>
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<th>PREDICTED</th>
<th>PRE</th>
<th>POST</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SALBU-TAMOL</td>
<td>A TROVENT</td>
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<tr>
<td>FEV1</td>
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<td></td>
</tr>
<tr>
<td>FVC</td>
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<tr>
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</tr>
</tbody>
</table>

| HB         |               | PLT       |               |
| WCC        |               | EOS       |               |
| TOTAL IG E |               |           |               |
Appendix A

Consent form

Genetic studies of late onset airflow obstruction

The patient should complete the whole of this sheet himself/herself. (Please circle one)

1. Have you read the patient information sheet? YES/NO

2. Have you had chance to discuss this study and ask any questions? YES/NO

3. Have you had satisfactory answers to all of your questions? YES/NO

4. Have you been given enough information about the study? YES/NO

5. Who has explained the study to you?

Dr ...............................................

6. Do you understand that you are free to withdraw from the study:

   At any time?

   Without having to give a reason?

   Without affecting your future medical care? YES/NO

7. All information that you give us in the study will be treated confidentially.

   The doctor coordinating the study will have access to the information and blood tests that you give us during the course of the study but not your medical records.

   Do you give permission for the study coordinator to have access to the information collected? YES/NO

8. We would like to store a sample of your blood for use in our further genetic studies of lung disease. We will not release this sample to other researchers without your prior written permission and your blood sample will not be used for commercial or patent purposes.

   Do you agree that your blood samples may be stored for future use in these genetic studies? YES/NO
Consent form

Genetic studies of late onset airflow obstruction

9. Has the doctor talked about compensation? YES/NO

10. If you have private medical insurance have you checked with your insurance company about whether your status will be affected by taking part in this study? YES/NO

11. Have you had time to come to your decision? YES/NO

12. Do you agree to take part in this study? YES/NO

13. Do you agree with an approach to your siblings being made YES/NO

--------------------------------------------------------------------------------

PATIENT

Signed ......................................................................... Date ..............................................

Name (BLOCK LETTERS) ......................................................

I have explained the study to the above patient and he/she has indicated his/her willingness to take part.

INVESTIGATOR

Signature .......................................................... Date........................................................

Name (BLOCK LETTERS) ......................................................
Appendix B  Additional laboratory techniques

Genotyping and DNA extraction was carried out primarily by Mrs M C Hill who was responsible for training Dr C Ruse in the laboratory methods used for DNA extraction and genotyping for the high affinity IgE receptor polymorphisms, the tumour necrosis factor polymorphism and the lymphotoxin alpha polymorphisms. Dr C Ruse carried out some DNA extractions and some genotyping for the polymorphisms described above.

Genotyping for the beta-adrenocpetor polymorphisms

Genotyping for the beta adrenoceptor polymorphisms was carried out by Dr A Wheatley, in the Institute of Molecular Biology and Cell Signalling, University of Nottingham.

Briefly, beta adrenoceptor genotype was determined by allele-specific oligonucleotide hybridisation (ASO). PCR was used to generate a 234 base pair fragment spanning the codon 27 and codon 16 polymorphisms. A PCR reaction was set up as follows:-

1µl DNA, 34µl distilled water, 5µl PCR buffer (x10), 1.5mmol/L MgCl₂, 2 µmol/L each primer and 200 µmol/L deoxynucleotide and 1 unit Taq. PCR product was applied to duplicate Hybond N plus filters with a dot-blot apparatus. To determine genotypes one filter was hybridised as appropriate with unlabelled ASO wild-type probe, followed by ³²P labelled probes, and the duplicate filter to probes applied in the reverse order. Probed filters after washes with SSPE were then exposed to X-ray film – and result recorded by an individual blinded to clinical phenotype. Direct sequencing was carried out on a random selection of samples. No errors were detected by sequencing.

Preparation of agarose gels

For a 2% agarose gel
2g agarose in 100ml of 1xTAE (20ml 50xTAE from stock + 980ml dH₂O). Heat for thirty seconds to dissolve. Add 1μl of ethidium bromide. The gel is now ready for use.

**Preparation of TAE**

<table>
<thead>
<tr>
<th>Stock solutions</th>
<th>Final concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris HCl</td>
<td>10mM</td>
<td>1ml</td>
</tr>
<tr>
<td>0.5M EDTA pH 7.6</td>
<td>1mM</td>
<td>200μl</td>
</tr>
<tr>
<td>dH₂O</td>
<td></td>
<td>98.8ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100mls</td>
</tr>
</tbody>
</table>
Appendix C  Abstracts, Papers and meeting presentations

Published work

Papers and Reviews

1. **Ruse CE and Parker SG.** Molecular Genetics and age related disease. Age and Ageing 2001. 30(6) 449-454

2. **Ruse CE, Hill MC, Burton PB et al.** Associations of polymorphisms of the high affinity IgE receptor (FcerI -β) in older populations. JAGS 2002. Accepted for publication

Abstracts


Letters


2. Ruse CE, Parker SG. Genetic susceptibility to COPD. Thorax 2000. 55(8) 723

Invited Presentations at International Meetings (excluding abstracts)

April 2000

Ageing Lung Conference, Palermo, Sicily  The genetics of COPD

October 2001

BGS Autumn Meeting, London  The genetics of COPD

August 2002

ECGC Framework 6 Meeting, Leicester  UK COPD Genetics Network