PREVALENCE OF GENETIC POLYMORPHISMS IN ATOPIC FAMILIES

By

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ABSTRACT

Genetic susceptibility to atopy and asthma is due to multiple genes interacting with each other and with environment factors. Variants in regulatory or coding regions of specific genes have been associated with atopy and asthma. Polymorphisms at position -308 in the promoter region of the TNFα gene, -590 in the promoter region of the IL4 gene and amino acid position 237 of the FceRIβ gene have been reported with increased frequency in asthmatic as opposed to control subjects.

The aim of this thesis was to determine the predictive value of these polymorphisms in the development of childhood atopy and asthma and whether any of these polymorphisms were associated with atopy and asthma in adult subjects. A prospective cohort including 493 infants born into atopic families was selected. Cord blood was collected at birth. Each infant was assessed at 12 months for respiratory symptoms and skin prick tests for 14 common allergens were performed. DNA was extracted for infants and their parents. Polymerase chain reaction based techniques were used to genotype all the infants and their parents for TNFα-308, IL4-590 and FceRIβ polymorphisms. In whites, there was a significant association of the IL4-590 polymorphism with "probable asthma"; there were no association of the TNFα-308 and FceRIβ polymorphisms with asthma or atopy in either the parents or their children. Therefore, IL4-590 polymorphism may be a risk factor for the development of asthma while TNFα-308 and FceRIβ polymorphisms had no predictive value for atopic diseases in whites. In the whole infant population, we found the allele frequency of the IL4-590 polymorphism was significantly higher than that in the non-atopic, non-asthmatic controls (p=0.04) or in the general population (p=0.01). The prevalence of atopy was significantly higher in infants with the T allele of IL4-590 polymorphism (IL4-590*T) than in those without this allele (p=0.002). The allele frequency of IL4-590*T was significantly higher in Orientals than that in whites (p<0.0001). To avoid a false
positive association caused by population admixture, the transmission/disequilibrium test was performed. A borderline association of IL4-590*T with atopy (p=0.07) was detected. This adds power to our suggestion that IL4-590*T may play a role in the pathogenesis of atopy.
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<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ARMS</td>
<td>amplification refractory mutation system</td>
</tr>
<tr>
<td>ASPCR</td>
<td>allele-specific PCR</td>
</tr>
<tr>
<td>BHR</td>
<td>bronchial hyperresponsiveness</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Der f</td>
<td>dermatophagoides farinae</td>
</tr>
<tr>
<td>Der P I</td>
<td>dermatophagoides pteronyssinus allergen I</td>
</tr>
<tr>
<td>Der P II</td>
<td>dermatophagoides pteronyssinus allergen II</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleoside triphosphates</td>
</tr>
<tr>
<td>DZT</td>
<td>dizygotic twins</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>FcεRIβ</td>
<td>beta subunit of the high-affinity IgE receptor</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>IL4</td>
<td>interleukin 4</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine activation motif</td>
</tr>
<tr>
<td>IU</td>
<td>international unit</td>
</tr>
<tr>
<td>Lod Score</td>
<td>log of the odds score</td>
</tr>
<tr>
<td>MZT</td>
<td>monozygotic twins</td>
</tr>
<tr>
<td>NO₂</td>
<td>nitrogen dioxide</td>
</tr>
<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RAST</td>
<td>radioallergosorbent test</td>
</tr>
<tr>
<td>RPM</td>
<td>revolution per minute</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>RR</td>
<td>relative risk</td>
</tr>
<tr>
<td>RSV</td>
<td>respiratory syncytial virus</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SO₂</td>
<td>sulphur dioxide</td>
</tr>
<tr>
<td>SSO</td>
<td>sequence-specific oligonucleotides</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell antigen receptor</td>
</tr>
<tr>
<td>TD PCR</td>
<td>touchdown PCR</td>
</tr>
<tr>
<td>TDT</td>
<td>linkage disequilibrium test</td>
</tr>
<tr>
<td>Tm</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TMAC</td>
<td>tetramethylammonium chloride</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor alpha</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) aminomethane</td>
</tr>
</tbody>
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CHAPTER 1

INTRODUCTION

Asthma and other wheezing respiratory illness are responsible for a significant proportion of acute and chronic illness in childhood. The prevalence of childhood and adult asthma is increasing and currently between 5-10% of children have asthma (Bierman et al. 1990). Variation in asthma prevalence occurs between countries and over time (Bierman et al. 1990). Therefore, the risk factors associated with the development of wheezing illness and asthma have been major targets for research.

It is known that asthma is a complex genetic disease. Multiple factors contribute to the phenotypic expression of asthma. Genetic susceptibility, environmental exposures and their interaction are the major components (Figure 1). Although environmental factors play an important role in the expression of asthma phenotype, genetic factors and atopy are considered as important primary determinants.

Atopy is the single strongest risk factor for the development of asthma. Therefore, the study of the relationship between genetic polymorphisms of candidate genes and atopy may aid the understanding of the pathogenesis of atopy and asthma.

1.1 ATOPY AND ASTHMA

1.1.1 Definition of atopy

Atopy is a common familial syndrome characterized by increased specific or total serum IgE responsiveness and underlies allergic asthma, rhinitis and atopic dermatitis. It affects up to
Genetics

• Polygenic
• Genetic heterogeneity
• Gene-gene interaction
• Ethnic origin

Environment

• Aero-allergens: house-dust mite, pollens, animal danders
• Viral infections
• Air pollutants
• Maternal smoking

Modifying asthma phenotype expression

• Asthma, eczema and rhinitis
• Increased specific IgE, total serum IgE and BHR
• Time-dependent expression of genes

Figure 1. Summary of the pathogenic mechanisms in atopy and asthma.
40-50% of some populations. The term atopy was proposed by Coca and Cooke in 1923 to identify what were commonly known as allergies involving reaginic or skin-sensitizing antibodies that were subject to hereditary influence. Atopy predisposes individuals to be sensitive to environmental substances or allergens and is associated with diseases, such as allergic rhinitis, food allergy and asthma. The definition of atopy can be (1) clinical conditions associated with the presence of an identifiable total reaginic serum antibody (IgE); or (2) the presence of a specific skin-sensitizing IgE antibody regardless of the presence of an identifiable clinical condition. This type of inappropriate immune response is termed type I or immediate hypersensitivity.

Type I hypersensitivity is characterized by an allergic reaction immediately following contact with innocuous environmental antigens, such as pollen, house-dust mites or animal dander. The term ‘allergy’, meaning ‘changed reactivity’ of the host when meeting an allergen on a second or subsequent occasion, was originally coined in 1906 by Von Pirquet who referred to adverse immune reactions (Von Pirquet et al. 1906). It is a sensitivity possessed by some people and not by others. Allergens trigger IgE-sensitized mast cells to release pharmacological mediators that produce the inflammatory responses with symptoms such as asthma, eczema and hay fever.

Skin prick tests are the most commonly used method of defining the presence of atopy. Allergen in a glycerol solution is placed on the epidermis of the subject, and the skin surface is lightly pricked with a sterile needle. In sensitized individuals, the allergen interacts with IgE molecules attached to skin mast cells and induces release of histamine. This results in a wheal and flare reaction within 5-15 minutes. The wheal diameter is measured at 15 minutes, and compared to the response of a negative control (saline or the buffer used to dilute allergen). A
positive skin test is usually defined as a wheal diameter that is 2-3mm greater than the negative control.

1.1.2 Definition of asthma

Many definitions of asthma have now been proposed by expert panels and by individuals (Toelle et al. 1992). Early definitions authored during the 1950s and 1960s emphasized bronchoconstriction and those from the 1970s and 1980s added hyperresponsiveness of the airways. In the 1990s, inflammation has become central in the definitions of the expert panels on asthma convened by the National Heart, Lung, and Blood Institute and the World Health Organization (National heart 1991,1993). Based on these definitions, with the addition of specific cellular functions, asthma is described as a disorder characterized by: increased responsiveness of the tracheobronchial tree to a variety of stimuli; symptoms of episodic wheezing, dyspnea, cough and chest tightness, which may vary from mild and almost undetectable to severe and unremitting; reversibility to bronchodilators and corticosteroids; variable airways obstruction that changes in severity either spontaneously or as a result of therapy and evidence of airway inflammation in which eosinophils, mast cells and lymphocytes together with a multitude of cytokines have important pathogenic roles (Sears et al. 1997). Among children, it is difficult to diagnose asthma particularly in infants. There are different forms of wheezing which require different definitions, and very likely have different pathogenesis. Martinez et al. (1995) differentiated early transient wheezing with symptoms occurring before age 3 that do not persist to age 6, from wheezing which persisted beyond age 6. The former children have smaller airways and are more at risk from viral infections and maternal cigarette smoking and have less history of maternal asthma or allergy, whereas the latter are more likely to
have a family history of asthma, elevated serum IgE, and other manifestations of atopy.

There are two forms of asthma: allergic and nonallergic based on the presence or absence of allergic skin-test reactivity to one or more aeroallergens. Allergic asthma is also called extrinsic, which is hypersensitivity to foreign substances; nonallergic is referred to as intrinsic. Extrinsic asthma appears to be the predominant form of the disease among children and young adults, and intrinsic asthma is more common among older subjects (Dolovich et al. 1983).

1.1.3 The relationship of atopy to asthma

The role of allergy as a determinant of persistent bronchial inflammation in asthma has been extensively studied. It is widely recognized that most childhood asthma is related to allergy. Recently, attention also has been paid to the factors that determine and maintain the non-specific bronchial hyperresponsiveness (BHR) that is characteristic of most subjects with asthma.

Most subjects with asthma have increased total serum IgE levels and are known to be sensitized to environmental allergens. Burrows et al. (1989) showed that the prevalence of physician-diagnosed asthma after the age of 6 years was closely related to total serum IgE levels. No asthma was present in the subjects who had the lowest log IgE values (< 0.32). This led to the conclusion that asthma is almost always associated with some form of IgE-mediated reaction in the airways, and therefore has an allergic basis. The close correlation of asthma rates to total serum IgE has been confirmed in another population sample by Sears et al. (1991). In their study, they found that the prevalence of diagnosed asthma was strongly related to the total serum IgE level. No asthma was reported in children with IgE levels < 32 international unit (IU) per milliliter (ml), whereas 36% percent of those with IgE levels > 1000 IU per ml were reported to have asthma. BHR to a methacholine challenge also correlated very highly with serum IgE
levels, even in those who had never wheezed or had no clinical expression of allergy. Peat et al. (1996) also investigated the relationship between total serum IgE in early adulthood (aged 18-20 years) and a history of symptoms, BHR and atopy during childhood. They found that recent wheeze, BHR, and allergic sensitization all had a positive relationship to serum IgE. This finding indicated the important role of IgE-mediated responses in the natural history of BHR throughout childhood and into adulthood.

*Aeroallergen sensitization*

Zimmerman et al. (1988) selected 142 children with asthma aged 6 years or older and grouped them according to the severity of their symptoms. They found more than 82% of all patients with asthma had at least one positive skin test to common local aeroallergens, and all of the patients with the most severe asthma had three or more positive skin tests. Therefore, an allergic inflammatory reaction occurring in the airways is almost always necessary for the development of asthma in children more than 5 years of age, and the severity of asthmatic symptoms may be directly associated with the degree of sensitization to aeroallergens.

Exposure to an allergen is necessary to induce IgE antibody formation. Aeroallergens derived from the house dust mite are powerful inducers of allergic responses in children. The majority of school children with asthma in the United Kingdom are sensitized to the house-dust mite, 80% of children with asthma show skin hypersensitivity to the house-dust mite, as compared with 30% in a control population (Smith et al. 1969). Evidence of cat allergen is found in a high percentage of the homes of patients who had asthma in Vancouver and Winnipeg. Sensitization to cat dander was the most prevalent of all allergens tested in both children (66.7%) and adults (54.7%) (Quirce et al. 1995). In a prospective study of children at risk for asthma, Sporik et al. (1990) showed a strong correlation between wheezing at the age of 11 and
sensitization to the house dust mite, a weak correlation at the age of five and no apparent correlation before that time. The quantity of dust mite recovered from the homes of the children also correlated with severity of asthma; as 16/17 children with asthma requiring daily medication had been exposed to > 10mg Der pI per gram of dust during infancy. This indicated that individuals who had early sensitization to high exposures of dust mite antigen were at greater risk of more severe asthma.

Allergies to cockroach, and furred pets have also been shown to influence the development of asthma in children. In one study of inner city Atlanta children, those with asthma were nine times more likely to have been sensitized to cockroach antigen than nonasthmatic controls. Therefore, exposure to Bla g 1 or 2 antigen can be a potent sensitizer leading to BHR and influencing the development of asthma (Call et al. 1992). Children who were both allergic to cockroach allergen and exposed to high levels of this allergen was hospitalized more frequently (37%) than non-allergic children (11%), and had 2.56 unscheduled medical visit for asthma per year compared with 1.43 (Rosenstreich et al. 1997). Lindfors et al. (1995) performed skin tests to dander from four furred animals, dust mites, birch, and grass on asthmatic and control children between the ages of 12 and 48 months. There was clearly a trend towards increased prevalence of asthma in children exposed to cats or dogs, even at this young age. When older children were studied by Sears et al. (1989), sensitivity to cat and dog, as well as to mites and aspergillus, served as independent risk factors for the presence of asthma by age 13, while grass and tree pollens did not. These two studies suggest that early exposure to furred pets correlate with allergic sensitization and subsequently the development of asthma during childhood.

**Food allergen sensitization**

Food allergy and eczema in an infant are often the first manifestation of allergic disease in
the atopic individual. They are predictive of other manifestations of allergy, e.g. asthma. This means that the presence of food allergy and eczema indicate that the individual is atopic and at risk to develop other allergic manifestations later in life. Food allergy is less common after 3 years of age when sensitization to inhaled allergens dominates (reviewed by Björkstén 1997). To study the relationship between early positive skin test to food and later aeroallergen sensitivity, Sigurs et al. (1994) studied 324 children followed up from birth for 4 to 15 years. Serum sample were obtained at various ages and were analyzed for IgE antibodies against egg white, cows milk, wheat, animal dander and house dust mite. Eighty two percent of the children with positive skin tests to food allergens eventually developed atopic diseases. Thus, they concluded that sensitization to foods in infants was usually associated with appearance of IgE antibodies to inhalants later in life. In a similar study, Burr et al. (1997) selected a cohort of high risk children that was followed from birth to the age of 7 years. They found that positive skin test to egg in the first year of life was strongly associated with eczema, asthma, mite sensitivity and serum IgE at the age of 7 years.

In another prospective study, the levels of serum IgE to egg white, cows milk and inhalant allergens (pollen, danders and mite) were monitored from birth to 12 years of age in 84 children unselected for a family history of atopy. IgE to egg white and cows milk reached peak prevalence at 8 months of age with high prevalence only in atopics and disappeared subsequently during childhood. Similarly, high concentrations of IgE to inhalant allergens, without a tendency to decline, were seen only in atopics defined by symptoms such as eczema, rhinitis and asthma. High responders (high-grade IgE response) to egg white antigen during infancy were also usually high responders to inhalant allergens during childhood. Low levels of IgE produced by tolerance to these allergens were seen in non-atopics (Hattevig et al. 1993).
All these studies indicated that food allergen sensitization in infancy is predictive of aeroallergen sensitization in later life.

1.1.4 Childhood wheeze and asthma

Wheeze is a common occurrence in early childhood, most often following upper respiratory tract infections, but is also triggered by inhalant allergies. Nearly 20% of infants under the age of one year, 30% of children under the age of three, and almost 50% of six years old will have developed lower respiratory tract illnesses characterized by wheezing at some time in their lives (Martinez et al. 1995). Most of these episodes are caused by viral pathogens and are self-limited. About 15% of these children will wheeze repeatedly with each viral illness and will be diagnosed with asthma. Predicting who will develop recurrent symptoms and deciding who might benefit from early intervention is not yet possible. Several studies have prospectively followed infants with wheeze until school age and have tried to identify those infants whose symptoms are most likely to persist.

Martinez et al. (1995) studied a non-selected cohort of 826 infants born between 1980 and 1984 for evaluation at both three and six years of age. Of the total cohort, nearly 50% of the children wheezed at some point by the age of six years. Of these children, 20% wheezed only transiently in infancy, 15% had their first episode of wheeze after their third birthday, and 13% wheezed persistently throughout infancy and early childhood. Those who wheezed throughout the study period were significantly more likely to come from atopic families or have atopic symptoms themselves. Similarly, Kuikka et al. (1994) followed 83 patients with bronchiolitis. All patients were less than two years of age at entry to the study and were monitored for two to four years. Allergic symptoms, skin tests and IgE measurement were recorded at the end of the
study period. Twenty-five percent of the children had recurrent episodes of wheezing and were found to be more atopic, have an increased prevalence of noninfectious rhinitis and eczema and significantly higher IgE levels than the group who stopped wheezing. These two studies suggested that atopy was a predictor of persistent wheezing in non-selected children. In a highly selected prospective study, children born to atopic parents were assessed annually for the first five years of life and then at age 11 (Sporik et al. 1991). Their results showed 63% of the children wheezed at least once during the period of study, but only 25% of early wheezers continued to wheeze at age 11. Those children who developed asthma were more likely to be atopics compared with those who stopped wheezing when they were at school age. Bronchial reactivity also correlated with atopy since children with BHR were also more likely to have a positive skin test. Fifty-two percent of the children continued to acquire new positive skin tests from birth through age 11. Comparing children with early-onset atopy to those who had later onset, the former had more respiratory symptoms, more recent wheezing and more frequent bronchial hyperresponsiveness.

1.1.5 Additional exogenous risk factors for atopy, wheezing and asthma in childhood

A number of additional factors are associated with wheezing and asthma symptoms in children, including exposure to environmental tobacco smoking, environmental allergen exposure, and viral infections. The risk factors can be divided into two general categories: exogenous (environmental) and endogenous (genetic).

Among the exogenous risk factors, viral infection, maternal smoking and air pollution are generally accepted as major determinants of wheezing and asthma. Cofactors in the pathogenesis of asthma, such as viral infection and maternal smoking are called “inducers”. Factors that can
trigger asthma are called “inciters”. Some risk factors such as viral infection can act as both an inducer and an inciter.

**Viral infections**

The role of viral infections in the subsequent development of childhood allergic disease and asthma is complex. However, it is well established that viral infections trigger and aggravate asthma in already sensitized individuals and increase BHR in normal and allergic individuals. The first reports of asthma being aggravated by viral infection were in 1957 and 1958 when asthma attacks were observed during influenza epidemics that affected two large populations (Podosin et al. 1958, Rebhan et al. 1957). The rate of viral detection during exacerbation of asthma is much higher than the rate found during asymptomatic periods in asthmatics and non-asthmatics (10-50% VS 3%) (Horn et al. 1979, Jennings et al. 1987, Hudgel et al. 1979). Rhinovirus infection preferentially increased lower airway responsiveness in allergic subjects but not in non-allergic subjects (Gern et al. 1997). By contrast, the rate of bacterial infection in the respiratory tract of asthmatics is similar during asymptomatic and symptomatic periods. There is no correlation between asthma exacerbation and bacterial infection detected in cultured transtracheal aspirates (Hudgel et al. 1979, McIntosh et al. 1973 and Berman et al. 1975).

In addition, an association between the severity of the wheezing illness or asthma and viral infections has been studied. Viruses were detected in 49% of all episodes of wheezing bronchitis in children, and in 64% of severe episodes requiring corticosteroids (Horn et al. 1979). Similarly, it has been documented that 36% of severe exacerbations of asthma are related to viral infection in adult asthmatics (Beasley et al. 1988).

However, epidemiological studies of the relationship between infections and manifestations of allergic disease are complicated by the fact that symptoms like runny nose,
wheezing, and cough may all be caused by either an infection or an allergic reaction, therefore, it is not easy to identify the etiology of the symptoms. There are studies indicating that at least some viruses, for example, respiratory syncytial virus (RSV) enhance sensitization to inhaled allergen (Welliver et al. 1981). Animal experiments showed an enhanced IgE production in virus infected dogs after allergen exposure (Frick et al. 1983, Holt et al. 1988). Evidence that RSV protein and genomic RNA persist in the lungs and alveolar macrophages of guinea pigs for at least 60 days after infection suggests that viral infection may contribute to the pathogenesis of chronic bronchiolar inflammation (Hegele et al. 1994).

On the other hand, an inverse relationship between infection and sensitization has been observed in studies of the prevalence of asthma. Anderson et al. (1978) observed that respiratory infections were more common among young children in the highlands of New Guinea, where asthma prevalence was low, than in the coastal regions of the country, where asthma occurred more frequently. Similarly, Von Mutius et al. (1994) found that atopic sensitization, hay fever, current asthma and BHR were considerably more frequent in West German children than those in East Germany, while bronchitis was more prevalent in East Germany. This finding suggests that respiratory infections, mostly viral infections, in early life may play a role in the prevention of allergic sensitization which leads to a lower prevalence of asthma and allergies.

Air pollution

The role of air pollution in either the expression or severity of asthma is still unclear. There are a number of pollutants such as sulphur dioxide (SO₂), nitrogen dioxide (NO₂), particulate matter and ozone that may cause specific environmental effects related to asthma morbidity. Chronic exposure to SO₂ causes bronchoconstriction and asthma-like symptoms, even at low levels (Pierson et al. 1984; Sheppard et al. 1988.). Nitrogen dioxide has been associated
with an increase of respiratory symptoms and may affect pulmonary function (Neas et al. 1991). Ozone exposure has been reported to increase bronchial reactivity and asthma-associated symptoms and to cause a decrease in lung function (Pierson et al. 1984; Castillejos et al. 1992; and Romieu et al. 1996). An elevated level of respirable particulate matter has been shown to be associated with an increase in hospital admissions for asthma and bronchitis in some areas (Pope et al. 1989; Romieu et al. 1996 and Von mutius et al. 1995).

Exposure to multiple pollutants resulted in the highest risk of developing upper respiratory illnesses in childhood (Von mutius et al. 1995). However, other studies have shown that hospital admissions for asthma were unrelated to pollution levels (Dawson et al. 1983 and Ribon et al. 1972). Therefore, the findings that relate to asthma morbidity and pollution are inconclusive.

The air pollutants mentioned above are also associated with increased serum IgE levels in animal studies (Holt et al. 1995, Osebold et al. 1988, Nilsson et al. 1993). However, the relationship to human allergy is not clearly understood. When children living near an air-polluting paper factory were compared to those living in a forested unindustrialized area in Sweden, BHR and pollen allergy were both more common in the former children (Andrae et al. 1988). The prevalence of BHR and pollen allergy was further increased when the parents smoked at home, suggesting an additive effect of the two pollutants.

**Exposure to smoke**

Tobacco smoking is a well-recognized respiratory irritant. Children whose parents smoke have been reported to have more problems with wheezing, lower respiratory infections, and asthma than children of parents who do not smoke especially in the first year of life (Gortmaker et al. 1982, Neuspiel et al. 1989, Burchfiel et al. 1986 and Fergusson et al. 1981). Maternal
smoking is considered an important risk factor for childhood asthma morbidity (Chilmonczyk et al. 1993).

Tobacco smoke is also strongly associated with allergic sensitization (Ronchelti et al. 1990). Increased serum IgE levels and an increased prevalence of positive skin tests toward occupational allergens have been reported in smokers versus non-smokers in several studies (Ronchelti et al. 1990, Kunz et al. 1989, Halken et al. 1991).

Smoking rats exposed to antigen in aerosol develop higher IgE responses than subcutaneously immunized animals and nonsmoking aerosol-immunized controls. This suggests that smoke may induce an inflammatory reaction in the airways, which facilitates the penetration of allergens or, alternatively stimulates antigen-presenting cells (Zetterström et al. 1985).

**Other factors**

Strachan et al. observed a striking inverse relation between the prevalence of allergic rhinitis and the number of older siblings in a large population sample from the UK. As the effect was much stronger for older than for younger siblings, he suggested that exposure to infectious diseases early in life may have a protective effect on the development of atopic diseases (Strachan 1989).

The prevalence of asthma is different among racial groups. Blacks in the United States have more asthma than whites (Gergen et al.1988, Turkeltaub et al. 1991). Puerto Rican children in the New York City have some of the highest rates of asthma, while Mexican-American children in the Southwest have some of the lowest (Carter-Pokras et al. 1993). The mechanism for the differences in asthma morbidity between ethnic groups is unknown.

Breast-feeding in infancy may reduce the risk for wheezing lower respiratory tract illness (Wright et al.1989).
There are several primary determinants classified as endogenous host risk factors, which are mainly genetic and are expressed in family history of allergic conditions, atopy and ethnic grouping. I will review the genetic basis of asthma and atopy in detail in the next section.

1.2 GENETIC BASIS OF ATOPY AND ASTHMA

It has long been known that asthma and atopy run in families. The genetics of atopy and asthma has been studied using four general approaches: twin studies, segregation analysis, linkage analysis and association studies.

1.2.1 Twin studies

The development of asthma is complex and likely to be a function of genetic susceptibility and environmental exposure. Studies of twins have attempted to separate the issues of heredity and environment. Monozygotic twins (MZT) are genetically identical whereas dizygotic twins (DZT) share on average only 50% of their genes. Therefore, a disease that is at least partially determined genetically is expected to show a higher frequency in a monozygotic twin than in a dizygotic twin of an affected person. The degree of reduction from 100% concordance among monozygotic twins is an indication of the degree to which environmental factors contribute to the disease. Investigating twins reared apart is thus important for separating genetic effects from those due to common family environment. The proportion of phenotypic variance in a population contributed by genes can be estimated using twin pairs and is known as heritability (Gerrard 1978).

Various twin studies have identified a substantial familial contribution to the etiology of
atopic disease. Bazaral et al. (1974) concluded that there was a genetic effect on total IgE levels with a heritability of 0.79. Hopp et al. (1984) found that total IgE levels had a correlation coefficient of 0.82 for MZT and 0.52 for DZT that yielded a heritability estimate of 0.61. Skin tests were also analyzed, the total skin test scores had a correlation coefficient of 0.82 in MZT and 0.46 in DZT, with a heritability of 0.72. They concluded that both total IgE levels and total skin test reactivity were traits mainly determined by genetic factors.

Whthrich et al. (1981) found a similar result for specific IgE as measured by the RAST method. When one twin showed at least one positive RAST the concordance was 60% in MZT and 23% in DZT. If the specific allergen was considered the concordance in MZT was no better than in DZT.

The first study to investigate twins reared together and apart was by Hanson et al. (1991). For total IgE, significantly higher correlations were obtained for MZT compared to DZT. However, similar correlations were found in MZT raised apart and together, which argues against the rearing environmental having a large effect on total IgE levels.

Edfors et al. (1971) studied 7000 Swedish twin pairs and found concordance of 19% for asthma and its related symptoms in MZT versus 4.8% in DZT. A more recent twin study evaluated concordance for asthma/wheezing among twins from Australia and found that the concordance rate of the disease was higher in MZT (65%) than in DZT (25%)(Duffy et al. 1990).

Thus, twin studies have confirmed a major genetic contribution to atopy and asthma, but they are unable to provide information on the mode of inheritance. To determine the pattern of inheritance of atopy and asthma, segregation analysis has been performed.
1.2.2 Segregation analysis

Segregation analysis is a method of determining the mode of inheritance of a disorder by observing how it is distributed within families. In this manner, evidence for the transmission of a gene with a major effect can be documented and linkage analysis can be used to locate a major gene or genes. This approach has been used to establish Mendelian inheritance for single-gene disorders (Elston 1981) and has also be used in an attempt to understand the mode of inheritance of complex genetic diseases such as asthma and other allergic diseases.

In segregation analysis, the distribution of a disease is studied in multiple families or single large families, and the observed frequency of the disorder in the offspring is compared to the distribution expected with various modes of inheritance. If the distribution is significantly different than expected, that mode of inheritance is rejected. The model that cannot be rejected is considered the most likely.

Penetrance refers to the connection of genotypes to the corresponding phenotypes, and is defined as the probability that an individual with a given genotype will express the associated phenotype. Incomplete penetrance of a disorder complicates segregation analyses.

Three types of families may be studied in segregation analysis: nuclear families consisting of children and their parents; extended pedigrees including other family members such as aunts, uncles, grandparents, and cousins as available; or large extended pedigrees from isolated populations where there is extensive inbreeding.

The systematic study of the genetics of allergic conditions was started by Cooke and Van der Veer in 1916, who obtained family histories from 504 allergic and 76 non-allergic subjects. They found that the incidence of positive allergic family histories was three times higher in allergic than in non-allergic individuals (48% vs 14%). Children with a bilateral family history of
atopy were generally found to develop allergy before puberty, whereas children with a unilateral history developed allergy later in life. They also suggested that children inherit an 'allergic predisposition', but not specific allergies, equally from both parents. They concluded that allergy was inherited as an autosomal dominant trait (Cooke and Van der Veer 1916). Two other studies (Weiner et al. 1936, Schwartz 1952) observed a similar pattern but proposed incomplete penetrance of the disease to explain families with atopic children from normal parents. Cookson and Hopkin (1988) proposed a similar model of dominant inheritance with incomplete penetrance for their "atopy" phenotype, however, their subsequent studies (Cookson 1992, Dizier 1995) are not consistent with such a simple model.

Elevated total IgE is correlated with atopy and asthma (Burrows et al. 1989). Therefore, the results from segregation analysis of total serum IgE levels are relevant to determine the role of genetics in asthma. The first analysis concluded that the distribution of IgE levels was consistent with atopy as a dominant trait with partial penetrance in the heterozygote, as proposed by Weiner et al. (Bazaral et al. 1971).

Total serum IgE has been the most extensively studied phenotype in segregation analyses. Autosomal recessive, dominant, co-dominant, and polygenic models have all been suggested in these studies (Bazaral et al.1971). Marsh et al. (1974), Gerrard et al. (1978) and Meyers et al. (1987) have reported that a major recessive gene contributes to the inheritance of high levels of IgE. Blumenthal et al. (1986) analyzed data from 7 large pedigrees. They found strong evidence of genetic heterogeneity. When all their family data were pooled, a dominant model of inheritance of high total serum IgE gave the best fit. However, when the pedigrees were analyzed individually, two families (106 individuals) showed a best fit to a dominant model, while five families (105 individuals) gave a good fit to a recessive mode of inheritance. In the Amish
population, evidence for co-dominant inheritance was found (Meyers et al. 1982), while evidence for polygenic inheritance was found in the study of Mormon population (Hasstedt et al. 1983).

BHR is one of the phenotypic traits of asthma. Postma et al. (1995) selected 303 children and grandchildren of 84 probands with asthma from a homogeneous population in the Netherlands. They found serum total IgE levels were strongly correlated with BHR in affected sibling-pairs, suggesting these traits are co-inherited. Asthma prevalence among siblings tends to increase as the number of parents with asthma increase (Van Arsdel et al. 1959, Kjellman 1977).

A number of genetic mechanisms have been proposed for asthma and atopy: dominant, recessive, or polygenic with incomplete penetrance (Van Arsdel et al. 1959, Kjellman 1977, Edfors 1971). Sibbald et al. (1980) hypothesized that the tendencies for asthma and atopy are inherited independently, but the presence of atopy enhances the genetic susceptibility to asthma.

A complex segregation analysis of asthma related phenotypes was performed on a random cohort of families with three or more children using eight phenotypic traits (log serum IgE, bronchial response, skin prick tests, wheeze, eczema, hay fever, atopy and asthma). The data favored a two-locus model (Lawrence et al. 1994).

Thus, the results of these segregation analyses are conflicting and it has become evident that no simple inheritance pattern can be assigned to asthma or IgE levels. The reason for this is that genetic susceptibility to asthma and atopy is almost certainly due to multiple genes. The relative contribution of these genes is likely to be different in different populations or in different families. Therefore, no specific mode of inheritance will consistently emerge from segregation analysis of atopy and asthma. Other factors such as different environmental exposures will also confound these analyses.
1.2.3 Linkage analysis

Linkage analysis is a common method used to map genes to specific chromosomal locations. It is performed using a maximum likelihood method (lod scores) based on the genetic model for the disorder obtained from the segregation analysis.

The principle of the approach is simple: a disease locus and a genetic polymorphic locus often called ‘genetic marker locus’ that are physically close together on the same chromosome tend to be inherited together. The inheritance of the marker can be followed in an affected family. Genetic markers can be any identifiable locus where the DNA sequence is highly variable. The most common genetic markers are ‘microsatellite markers’, short lengths of DNA in which the nucleotide sequence consists of repeats of specific base pairs. Microsatellite markers are found scattered throughout the human genome.

The distance of a marker to a disease-causing gene can be estimated by measuring the number of recombination events between them. Recombination is a process where two homologous chromosomes align themselves, overlap and exchange chromosome segments by crossing over during meiosis. The combinations of the different alleles of the loci produced by crossing over are termed recombinants. The closer the two loci are on a chromosome, the lower the probability of recombination. When a parent is observed to produce only non-recombinant offspring two loci are said to be completely linked; when a parent produces both recombinant and non-recombinant offspring in equal proportions the two loci are unlinked. In order to distinguish recombinants and non-recombinants, at least one of the two parents must be doubly heterozygous. Most DNA markers used in current mapping studies have heterozygosity values greater than 60%.

If a specific marker allele is found to be transmitted with the disease phenotype more
frequently than should occur by chance, then the disease-causing gene must be close to that specific marker locus.

The statistical significance of the linkage is measured by the lod score, which is the log of the ratio of the likelihood of observing the data given that the two loci are linked, to the likelihood of observing the data given that the two loci are not linked. A lod score critical value of 3.0 or greater at a given recombination fraction is used as evidence in favor of linkage, while a lod score of -2 or less at a given recombination fraction has been used as significant evidence against linkage (Morton 1955).

Considerable success has been achieved using linkage analysis to map single-gene disorders onto the human genome, but the progress in the complex diseases like asthma has been slower. Incomplete penetrance, environmental factors and genetic heterogeneity all complicate and limit the power of linkage analysis of complex diseases. Therefore, the interpretation of lod scores for complex traits remains controversial (Risch 1992). Association studies have a greater statistical power than linkage studies in defining genetic risk factors for complex traits (Risch et al. 1996).

1.2.4 Association studies

**Case control studies**

Association studies are often case-control studies that are based on a comparison of unrelated affected and unaffected individuals from the same population. The purpose of this analysis is to test whether a particular allele (variant) occurs at a higher frequency among individuals affected with a particular disease than among unaffected individuals. If the number of patients with the allele is a, and the number of patients without the allele is b; the number of
unaffected controls with the allele is c, and the number of the unaffected controls with the allele is d; the odds ratio is then calculated as \((a+b)/(c+d)\). If the variant is more frequent in subjects with the disease than those without it, this variant is said to be associated with the disease. Associations can arise for three reasons: the allele contributes to the pathogenesis of the disease, the result is an artifact of population admixture and the allele is in linkage disequilibrium with the disease gene.

Linkage disequilibrium is a statistical measure that provides evidence that the genetic marker allele and the disease allele occur together on the same chromosome more often than predicted by chance alone. The study of linkage disequilibrium provides an estimate of the distance between marker allele and disease allele. In general, the stronger the disequilibrium, the closer the two loci are on the chromosome. Therefore, linkage disequilibrium can be used to map a gene.

The biggest potential pitfall of case-control studies is the choice of a control group. In a mixed population, any trait present at a higher frequency in an ethnic group will show positive association with any allele that also happens to be more common in that group. To prevent problems due to population admixture, association studies should be performed within relatively homogeneous populations. The application of the transmission disequilibrium test (TDT) can also avoid false associations arising from admixture.

**Transmission/disequilibrium test (TDT)**

The TDT evaluates whether an allele is transmitted to an affected offspring from heterozygous parents more often than is expected by chance alone. In general, when a positive association has been found, TDT can be applied to detect linkage. TDT does not require data either on multiple affected family members or on unaffected sibs unlike conventional tests for
linkage. The method of TDT has been used as a test for linkage between type I diabetes and the insulin gene region and in other disease-marker studies. Multiple alleles at the marker locus associated with disease and their linkage can also be detected by TDT. Therefore, TDT is a powerful test for linkage in a wide variety of diseases that have a genetic component (Spielman et al. 1996).

1.3 PREVIOUS GENETIC STUDIES OF ATOPY AND ASTHMA

Recently, breakthroughs in modern technology have led to a dramatic increase in the understanding of the biological functions of genes in human physiology, pathophysiology and immunology. The use of these techniques has allowed a number of genes to be identified in the pathway leading to atopy and asthma. These genes can now be tested for association to atopy and asthma using candidate gene analyses (figure 2).

1.3.1 Human leukocyte antigen (HLA)

The human leukocyte antigen (HLA) complex genes are located on the short arm of human chromosome 6 and are divided into three groups of loci—class I, class II and class III. Each locus is highly polymorphic and can be used in HLA disease association studies.

The HLA class II antigens (DR, DQ and DP) play a key role in antigen presentation and therefore influence the specificity of the immune response. The first human gene to be implicated in an allergic disease was the HLA loci (Levine et al. 1972). HLA-DR restriction of IgE reactions to allergens is well-documented (Marsh et al. 1982, Young et al. 1994).

In general, significant HLA associations are found only with highly purified simple allergens and not with complex common ones. The most definitive and reproducible findings
Figure 2. Schematic illustration of various candidate genes implicated in the pathogenesis of atopy and asthma. The candidate genes are shown in the boxes.
have been for associations of a specific HLA type to simple allergens which contain only one major epitope e.g. *Amb a 5* from short ragweed pollen (Marsh *et al.* 1982) or a limited number of epitopes e.g. *Amb a 6* (Marsh *et al.* 1987) and rye grass *Lol p 3* (Ansari *et al.* 1991). A clear causal relationship between the expression of specific T cells to the antigenic epitope on *Amb a 5* and the presence of the HLA-DR2 type, specifically DR (*αβ1*1501) was found (Marsh *et al.* 1990).

The complex allergens of house dust mites (*Der p* and *Der f*) have not been associated with a specific HLA type. Only specific epitopes of *Der p* have been associated with specific HLA-DR and DQ gene products (Verhoef *et al.* 1993). The two most important allergens are *Der p I* (25.4 kD) and *Der p II* (14.1 kD). A significant association of HLA- DRβ1*1501 with higher *Der p II* specific IgE titers has been documented (Moffatt *et al.* 1997). Hsieh *et al.* also found an association of house dust mite reactivity to the HLA-DQw2 in Chinese children who had asthma (Hsieh *et al.* 1991). The haplotype B7, SC01, DR3 was found almost only in asthmatic subjects, while the haplotype B7, SC31, DR2 was more prevalent in the non-asthmatics (Blumenthal *et al.* 1992).

**1.3.2 The T cell antigen receptor (TCR)**

Linkage of specific IgE responses to the TCR-α/β locus on chromosome 14 in two independent sets of families was established using affected sibling-pair methods. No linkage of IgE serotypes to TCR-β was detected (Moffatt *et al.* 1994). The TCR-α/β region is complex and contains many elements that might influence specific antigen recognition (Arden *et al.* 1995). Wedderburn *et al.* (1993) demonstrated that Vα8 may be in excess in T cell clones reacting to
house dust mites. A significant association was found between allele 2 of Vα8.1 (Vα8.1*2) and higher IgE titers to Der p II, and a weak association was seen to Der p I. In order to understand the relationship between Vα8.1 and HLA-DRB1 polymorphisms, Moffatt et al. typed all the subjects for the six most common HLA-DR types and the Vα8.1 polymorphisms. They found that both Vα8.1*2 and HLA-DRB1*1501 were positively associated with IgE titers to Der p II (Moffatt et al. 1997). This suggests that polymorphisms within the TCR genes interact with particular HLA-DR types to modify an individual's ability to respond to specific antigens.

### 1.3.3 Tumour necrosis factor (TNF) α

Tumour necrosis factor (TNF) α is a potent pro-inflammatory cytokine that is found in increased concentration in asthmatic airways and in lavage fluid from asthmatic lungs (Virchow et al. 1995). The genes for TNFα and lymphotoxin α (LTα or TNFβ) are located within the class III region of the MHC between the HLA-B and HLA-D regions on chromosome 6p. This location has prompted much speculation about the role of TNFα and LTα in the etiology of MHC-associated diseases. Jacob et al. showed that HLA-DR3 positive individuals produce higher levels of TNFα (Jacob et al. 1990, 1992). The level of LTα secretion is associated with allele 1 of a NcoI polymorphism in the first intron of the LTα gene (LTαNcoI*1). LTαNcoI*1 is associated with another polymorphism which causes an amino acid substitution at position 26 (Messer et al. 1991). Linkage disequilibrium may exist within the TNF locus and the MHC. A polymorphism at position -308 in the promoter region of TNFα, is of particular interest because of the strong genetic association of its rarer allele (TNF-308*2) with the HLA-A, -B8, -DR3 haplotype known to predispose to various autoimmune diseases (Wilson et al. 1992, 1993). This allele has also
been identified as a risk factor for disease severity. Individuals homozygous for the TNF-308*2 allele carried a seven fold higher risk of death or severe neurological sequelae from cerebral malaria (McGuire et al.1994). Another group demonstrated that TNF-308*2 homozygotes were present in primary, but not secondary progressive multiple sclerosis patients (Braun et al. 1996). Recently, Moffatt et al. selected 413 subjects in 88 nuclear families from a general population sample to investigate the possible association of asthma and the TNF-308 polymorphism. They found that asthma was significantly more common in subjects possessing LTα NcoI*1 and TNF-308*2 alleles. The association was confined to the LTα NcoI*1/TNF-308*2 haplotype, and therefore the causal allele could not be identified. However, only the TNF-308 polymorphism has been associated with increased TNF levels. Log IgE levels did not show association with the LTα NcoI*1 /TNF-308*2 haplotype, indicating that the association of these polymorphisms with asthma is independent of atopy (Moffatt et al. 1997).

In some TNF enhancer/promoter reporter gene studies, no significant difference was found in the level of TNFα transcript and inducible reporter gene expression between TNF-308*1 and TNF-308*2 alleles (Brinkman et al. 1996, Stuber et al. 1996). These results suggest that increased production of TNFα does not result from TNF-308*2 alone. Combinations of functionally distinct alleles of one or more genes in the MHC, or in the narrower region surrounding the TNF genes may be responsible for interindividual differences in TNFα levels. However, other groups have shown that TNF-308*2 was associated with higher secretion of TNF \textit{in vitro} and \textit{in vivo}. A six to seven fold higher level of reporter gene transcription from TNF-308*2 in both stimulated and unstimulated cells was first reported by Wilson et al. (1992). Bouma et al. selected 30 patients with inflammatory bowel disease and 12 healthy controls to
investigate the secretion of TNFα in relation to the TNF-308 polymorphism. They found that cells from individuals carrying the TNF-308*2 produced significantly higher levels of TNFα compared to individuals without TNF-308*2. When peripheral blood mononuclear cells (PBMC) were activated with T-cell specific activators, TNFα secretion was found to be significantly higher in TNF-308*2 homozygous individuals as compared to those homozygous for allele 1 (Bouma et al. 1996). The association of higher TNFα production with TNF-308*2 was confirmed by Braun et al.

1.3.4 The high-affinity immunoglobulin E receptor (FceRI-β)

Cookson and colleagues first reported genetic linkage of atopy to chromosome 11q13 marker D11S97 in seven extended families (Cookson et al. 1989) and then replicated the result in 60 nuclear families (Young et al. 1992, Moffatt et al. 1992). The linkage was only detected in maternally derived alleles (Cookson et al. 1992). Confirmation by other groups showed linkage to chromosome 11q13 in families with severe atopy (Collee et al. 1993, Shirakawa et al. 1994). A number of negative linkage studies have also been reported (Hizawa et al. 1992, Rich et al. 1992, Amelung et al. 1992). Investigations continued with the mapping of the 11q13 region to identify candidate genes. The gene encoding the beta subunit of the high-affinity IgE receptor (FceRI-β) was proposed as the candidate gene located to this region (Sandford et al. 1993).

The first reported FceRI-β polymorphisms were within exon 6 and were known as Leu 181 and Leu 183. These polymorphisms were highly predictive of severe atopy when inherited maternally (Shirakawa et al. 1994). However, other groups have failed to identify a single Leu 181 or Leu 183 carrier (Thomas et al. 1998). A further search through the coding regions of
FceRI-β has identified a coding polymorphism in exon 7 (Hill et al. 1996). An adenine to guanine substitution changes amino acid residue 237 in the cytoplasmic tail of the protein from glutamic acid to glycine (E237G). This polymorphism is also associated with atopy and asthma but with no maternal effect. E237G is predicted to introduce a hydrophobicity change within the C-terminus of FceRI-β. It is adjacent to the immunoreceptor tyrosine activation motif (ITAM) and may affect the intracellular signaling capacity of FceRI-β. The population frequency of E237G is 5.3% and 6% in unselected Australian and Japanese populations, respectively (Hill et al. 1996, Shirakawa et al. 1996). E237G positive subjects had elevated skin-test responses to grass and house dust mites, increased RASTs or specific IgE to grass, and bronchial reactivity to methacholine. The relative risk of an individual with this variant having asthma compared to subjects without the variant was 2.3. However, the function of this variant remains to be elucidated.

1.3.5 The chromosome 5 cytokine gene cluster

The cluster of cytokine genes on chromosome 5q31-q33 has made this region of the genome an attractive candidate in the pathogenesis of asthma and in the regulation of IgE. The cytokine gene cluster includes the cytokine cluster (IL-3, IL-4, IL-5, IL-9, IL-13, and the granulocyte/macrophage colony-stimulating factor) and the β2-adrenergic receptor (ADRB2) (reviewed by Sandford et al. 1996). Recently, evidence has been found for linkage of this region to both total serum IgE and BHR (Marsh et al. 1994, Meyers et al. 1994 and Postma et al. 1995).

Marsh et al. first reported a linkage of total serum IgE levels to the IL-4 gene locus in 11 large Amish families. The evidence for linkage was strongest when subjects with high specific IgE were excluded from the analysis, suggesting that specific IgE responsiveness is a
confounding factor in the analysis of the genetics of total serum IgE. A second group also reported linkage to chromosome 5q of total IgE as well as BHR in 92 Dutch families. The linkage was centered surrounding the ADRβ2 gene using both sibling pair analysis and the lod score approach (Meyers et al. 1994). In the same population, evidence for linkage of an asthma phenotype to 5q has also been obtained (Postma et al. 1995).

The 5' region of the IL-4 gene was of great interest because of the relationship of IL-4 to the IgE isotype switching in allergic inflammation. Rosenwasser and colleagues (Rosenwasser et al. 1995) obtained evidence in families with asthma that a polymorphism in the IL-4 promoter is associated with elevated total IgE levels. This polymorphism is a C→T transition. Subjects with the T variant at position -590 had significantly higher geometric mean IgE levels and higher IL-4 gene expression than subjects with the C allele. In an *in vitro* function study, the IL-4 T allele was associated with greater transcription factor binding activity and greater reporter gene expression. Thus, the association of this polymorphism with the IL-4 gene promoter activity seems to correlate with elevated total IgE *in vivo* and may be significantly linked to asthma. However, Walley *et al.* found that the -590 C to T IL-4 promoter polymorphism (IL4-590*T) was only weakly associated with certain measures of asthma and atopy in some subjects. It was not associated with serum IgE concentration or asthma in an Australian and an UK population (Walley *et al.* 1996). More recent study has shown that IL4-590*T was associated with asthma but not with total and specific serum IgE levels in Japanese children (Noguchi *et al.* 1998).

### 1.4 AIMS OF THESIS

The previous candidate gene studies of the TNF-308, IL4-590 and FceRIβ
polymorphisms have indicated that these polymorphisms are associated with atopy and asthma. However, several issues remain to be addressed. Firstly, TNF-308*2 was associated with asthma in British and Australian Whites but no other study has confirmed this association. Secondly, the results of studies of the IL4 and FceRIβ polymorphisms have often been contradictory and therefore remain controversial. Thirdly, none of the previous studies were prospective.

Therefore, a prospective cohort study was designed to determine the predictive value of the TNF-308, IL4-590 and FceRIβ polymorphisms in the development of childhood atopy and asthma. A case-control study was also designed to determine whether any of the polymorphisms are associated with asthma, atopy or total serum IgE levels in adult subjects.

There were three specific aims in this study. The first aim was to determine whether the prevalence of the TNF-308, IL4-590 and FceRIβ polymorphisms would be increased in infants born into allergic families compared to the general population. The second aim was to determine whether there was a higher prevalence of atopy and asthma in infants with these polymorphisms compared to those with the wild types. The relative risk of the polymorphisms for atopy and asthma could then be determined. The third aim was to determine whether any of the polymorphisms were associated with asthma, atopy or total serum IgE levels in the parents from these families.

To address these aims, 373 infants at high risk for allergic diseases were recruited. Cord blood was collected at birth. Each infant was assessed at 12 months for respiratory symptoms and skin prick tests for 14 common allergens were performed. DNA was extracted for each infant. DNA was also extracted from peripheral blood samples for the parents of these infants. Polymerase chain reaction (PCR) based techniques were used to genotype all the infants and their...
parents for TNF-308, IL4-590, and FcεRIβ polymorphisms. The presence of disease association with these polymorphisms was also assessed by TDT.
CHAPTER 2

MATERIAL AND METHODS

2.1 REAGENTS

All chemicals were purchased either from Sigma and/or BDH except where stated in the text. Agarose was obtained from Eclipse Molecular Biologicals. Taq DNA polymerase was obtained from Gibco BRL. All restriction enzymes were purchased from New England Biolabs.

2.2 SOLUTIONS

0.5X TBE: 
- 0.045M Tris-borate
- 0.001M EDTA (pH 8.0)

20X SSC: 
- 3.0M sodium chloride
- 0.3M sodium citrate

TE: 
- 10mM Tris-HCl (pH 8.0)
- 1mM EDTA (pH 8.0)

6X Loading buffer: 
- 0.25% bromophenol blue
- 0.25% xylene cyanol FF
- 15% Ficoll (Type 400; Pharmacia)

10X PCR buffer: 
- 200mM Tris-HCl pH 8.4
- 500mM KCl

Digestion buffer: 
- 100mM NaCl
- 10mM Tris (pH 8.0)
- 10mM EDTA
- 1.0% SDS

Denaturing solution: 
- 1.5M NaCl
- 0.5M NaOH
Neutralizing buffer 1.5M NaCl
0.5M Tris.Cl
0.001M EDTA pH 7.2

2.3 PREPARATION OF HUMAN GENOMIC DNA

2.3.1 DNA Extraction from Human Blood

Human genomic DNA samples were prepared from whole blood or clotted blood by standard techniques (Sambrook et al. 1989) with some modifications or by QIAamp blood mini kits (Qiagen). The procedure yielded 0.5-1.0mg of DNA for PCR amplification.

**DNA extraction from whole blood**

Twenty ml of unclotted whole blood was transferred to a 50ml centrifuge Falcon tube and the glass tube was rinsed with sterile water. The sample was made up to 50ml with sterile water and was mixed thoroughly to ensure complete lysis of the red blood cells. The tube was spun in a BECKMAN Model TJ-6 Centrifuge at 2300 RPM (1100g) for 20 minutes to pellet the white cells. The supernatant was discarded and the cell pellet was washed with 20 ml of 0.1% nonidet 40 or isotonic saline. The tube was spun at 2300 RPM for 20 minutes and the supernatant removed. The cell pellet was resuspended in 10 ml of digestion buffer and 75µl of 20mg/ml proteinase K and was incubated overnight at 42°C with shaking at 250 RPM. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to each sample and mixed completely, then spun at 2700 RPM for 20 minutes. The upper layer was transferred to a new tube. One-tenth volume of 3M sodium acetate and 2 volumes of 95% ethanol were added to precipitate the DNA. The solution was mixed very gently but thoroughly and a sterile pipette tip was used to spoon the DNA into 1 ml of 70% ethanol. DNA was collected by centrifugation at 2700 RPM for 30 minutes. The supernatant was discarded and the pellet was air-dried under Kimwipes. Finally, The
DNA pellets were dissolved in 300 µl TE Buffer at room temperature.

**DNA extraction from clotted blood**

Thawed clotted blood was transferred to a 50ml centrifuge tube containing 10ml digestion buffer and 75µl proteinase K (20mg/ml). The sample was incubated overnight at 42°C in a shaking incubator at 250 RPM. The rest of the procedures were the same as whole blood DNA extraction.

**Purification of genomic DNA by QIAamp blood mini kit**

QIAamp blood mini kits (Qiagen) were used to purify DNA from those samples that were not pure enough for PCR after the regular DNA extractions (Sambrook *et al.* 1989). DNA purification was carried out according to the protocols suggested by the manufacturer. A 40µg sample of previously extracted DNA was transferred to a new 1.5ml Eppendorf tube and made up to 200µl with PBS. To further purify the DNA, 25µl of Qiagen protease stock solution and 200µl of Buffer AL were added and mixed thoroughly by vortexing for 15 seconds. The mixture was incubated at 70°C for 10 min. A 210µl aliquot of 100% ethanol was added to the samples and mixed again by vortexing. The solutions were carefully transferred to the QIAamp mini column and centrifuged at 8000 RPM in an Eppendorf centrifuge for 1 min. During this brief centrifugation, DNA samples were adsorbed onto the QIAamp silica membrane. Salt and pH conditions in the lysate ensure that protein and other contaminants, which may have inhibited PCR and other enzymatic reactions were not retained on the membrane. If the solution had not completely passed through the membrane, it was centrifuged again at a slightly higher speed and the filtrate was discarded. The sample was first washed with 0.5 ml of Buffer AW and spun at 8000 RPM for 1 min. The column was then washed with 0.5 ml of Buffer AW and spun at 12000 RPM for 3 min and the filtrate was discarded. The column was placed in a clean centrifuge tube.
A 300μl aliquot of Buffer AE or distilled water preheated to 70°C was added to the column and incubated at 70°C for 5 min. The tube was spun at 12000 RPM for 3 min, the collected filtrate was reloaded onto the column and spun to obtain the maximum concentration of DNA. The eluate containing the purified DNA was stored at -20°C.

2.3.2 DNA Quantification

To determine the concentration of a DNA solution, the optical density of the sample was measured at a wavelength of 260 and 280nm using an ultraviolet spectrophotometer. Generally, a 100-fold dilution of the original DNA solution was measured. An OD$_{260}$ unit of 1 corresponds to a double stranded DNA concentration of 50 μg/ml. Proteins or RNA also absorb UV light at 260nm and 280nm. Therefore, the purity of DNA samples can be estimated by the ratio A$_{260}$/A$_{280}$. A ratio of 1.8 is indicative of a pure DNA sample.

2.4 GENOTYPING METHODS

2.4.1 DNA Amplification using the Polymerase Chain Reaction

The polymerase chain reaction (PCR) is an artificial, rapid means of replicating a short DNA sequence, so that millions of copies of the sequence are made. Two oligonucleotides are used as primers for a series of synthetic reactions that are catalyzed by a thermostable DNA polymerase such as Taq DNA polymerase. These oligonucleotides are complementary to sequences that lie on opposite strands of the template DNA and flank the segment to be amplified. PCR can also be used to detect variation at the DNA level.

The genomic DNA is first heated to a high temperature (~95°C) to denature the double-
stranded molecules. The PCR amplification is carried out in a solution containing an excess of each of the two primers and the four deoxynucleoside triphosphates (dNTPs). The reaction mixture is then cooled to a temperature (45 to 65 °C) which allows the primers to anneal to their target sequences. The DNA is then heated to an intermediate temperature (70 to 75°C) and the annealed primers are extended from their 3' termini by Taq DNA polymerase. The cycle of denaturation, annealing and primer extension is repeated up to 40 times using a thermal cycler. The products of one round of amplification serve as template for the next. Therefore, each successive cycle approximately doubles the amount of the desired DNA. This exponential amplification eventually ceases when the amount of active Taq DNA polymerase becomes limiting, by which time an amplification level of approximately $10^6$ has been obtained.

2.4.2 Primer Design

In order to achieve an efficient DNA amplification in PCR reactions, all primers that I designed for this study were based on the following principles (Todd et al. 1990).

1. The length of both the sense and antisense primers was between 18 to 22 nucleotides.

2. Where possible primers contained a GC-type sequence pair (such as CC, GG, GC or CG) at their 3' terminus to facilitate the initiation of complementary strand formation by the Taq polymerase. The sequence of the antisense primer was converted to the complementary form.

3. Each primer had a GC-type sequence content of between 45 to 55% to obtain similar melting temperatures (Tm). Tm was calculated from the standard equation:

$$T_m = 2 \times (A+T) + 4 \times (G+C)$$

4. The target region amplified by the sense and antisense primer pairs was 100 to 600 base pairs (bp) in length.
5. No primer contained more than four contiguous bp of homology to itself or to its respective
   sense or antisense counterpart.

6. All primer selection was checked by the OLIGO4 primer analysis software (Rychlik et al
   1989). Allele-specific primer designing required an extra rule that will be stated in the next
   section.

2.4.3 Allele-Specific PCR [Amplification Refractory Mutation System (ARMS)] Primer
Design

This technique requires that the terminal 3'-nucleotide of a PCR primer be allele-specific. The
primer is synthesized in two forms. The wild type form is refractory to PCR on "variant"
template DNA and the "variant" form is refractory to PCR on "wild type" DNA. Additional
deliberate mismatches were added near the 3'-end of appropriate primers to destabilize the
primer/template complexes to increase the specificity of the PCR. Two flanking primers serve as
an internal control (figure 3).

2.4.4 Analysis of PCR Products by Restriction Endonucleases

Restriction endonucleases are purified from bacteria and they recognize short stretches of
double-stranded DNA sequence as targets for cleavage. Each restriction enzyme has a particular
target in duplex DNA, usually a specific sequence of four to eight nucleotides. The enzyme cuts
the DNA at this target sequence to produce DNA fragments of defined sizes that are known as
restriction fragments. A point mutation may destroy or create a restriction recognition site, so the
presence or absence of the site can be used to detect DNA polymorphism.

PCR products were digested with a specific restriction enzyme. The reaction was usually
Figure 3. Schematic illustration of allele-specific PCR primer design for FcεRIβ polymorphism. B7M1 is an allele specific primer for the variant allele. B7W2 is an allele-specific primer for the wild type allele. B7A1 and B7A2 are flanking primers. The underlined bases are deliberate mismatches added to destabilize the primer/template complex to increase the specificity of the PCR.
performed in a final volume of 40μl using the enzyme buffer supplied by the manufacturer. The sample was incubated for at least 4 hours at the indicated temperature.

2.4.5 Agarose Gel Electrophoresis

PCR products or digested samples were run on agarose gels to determine the size of a fragment. The gels were usually made with 2-3% agarose and were cast and run in 0.5 X TBE. Ethidium bromide was included in the gel at a concentration of 0.5 μg/ml. A final concentration of 1X loading buffer was added into DNA samples before they were loaded into the wells. DNA fragments were separated by a Horizon™ 20.25 gel electrophoresis apparatus at a constant voltage of 130V for 2 hours. The results were examined on a 312nm ultraviolet transilluminator and then photographed by an Eagle Eye II image capture equipment.

2.5 DETECTION OF TNFα-308 POLYMORPHISM

2.5.1 Genotyping by Sequence-Specific Oligonucleotide (SSO) Hybridization

Amplification of TNFα-308 polymorphism

To type the TNFα-308 polymorphism, the region was amplified by PCR and was hybridized with sequence-specific oligonucleotides. Two allelic forms referred to as TNF-308*1 and TNF-308*2 were typed that are identical apart from a single base transition (G to A) at position -308 of the TNFα gene. The primers were designed for PCR of the TNFα-308 polymorphism according to the principles described in section 2.4.2. The sequences of the primers were:

TNF1: 5'-AAGGAAACAGACCACAGACCTG-3'
TNF$_2$: 5'-ACACACAAGCATAAGGATACC-3'

The predicted size of the amplification product was 182bp. Genomic DNA was used as a template. The appropriate reaction conditions were determined by optimizing reactions over a range of different Mg$^{2+}$, dNTP and primer concentrations, annealing temperatures and the number of cycles. The final reaction conditions used were: 0.1µg of genomic DNA in a total volume of 20µl of reaction mixture containing 0.1µM of each primer, 200µM of each dNTP, 20mM Tris-HCl (pH 8.4), 50mM KCl, 1.5mM MgCl$_2$ and 0.5 units of Taq DNA polymerase. After 35 cycles at 94°C for 30 seconds, 59°C for 30 seconds, 72°C for 10 seconds, a final 10min extension at 72°C was included. The reaction was made up to a final volume of 20µl, overlaid with light mineral oil and was carried out in a Hybaid thermal cycler (Interscience). Template-free and known genotype controls were included as controls in each experiment.

**Dot blot**

Two sheets of Hybond N+ (Amersham) nylon membrane and two sheets of Whatman 3MM paper were soaked in 10X SSC solution. The dot blotting apparatus was then set up. This consisted of two 96-well plates with the nylon membrane and the Whatman 3MM paper between. The nylon membrane was placed on the top of Whatman 3MM papers and was labeled with pencil. The apparatus was connected to a vacuum. The PCR product was denatured at 95°C for 10min and then dot blotted equally onto 2 nylon membranes via the vacuum manifold. The blot was then dismantled. The DNA on the membrane was denatured by immersing it in denaturing solution for 5min, transferred to neutralizing buffer for 1 min and then dried on Whatman 3MM paper overnight. The DNA was fixed by placing it on UV Stratalinker 1800 (Stratagene) through auto cross-linking for 3 min and the membrane was then wrapped with Saran Wrap.
3'-End labeling of oligonucleotides with digoxigenin-11-ddUTP

The allele-specific oligonucleotides 5'-AGGGGCATGGGGACGGG-3' (TNF*1) for the TNF-308*1 allele and 5'-AGGGGCATGAGGACGGG-3' (TNF*2) for the TNF-308*2 allele were taken from a previously described method (McGuire et al. 1994) and end-labeled using the DIG Oligonucleotide 3'-end Labeling Kit (Boehringer Mannheim). The labeling reaction was set up according to the protocol provided by the manufacturer. The 20μl reaction mixture for each oligonucleotide included 4μl of 5X reaction buffer, 4μl of 25mM CoCl₂, 1μl of 1mM DIG-11-ddUTP, 10μl of 10μM oligonucleotide and 50 units of terminal transferase. The reaction was incubated at 37°C for 15 minutes and was stopped by addition of 2μl of glycogen (10mg/ml) and 1μl of 0.2M EDTA (pH 8.0). The reaction mixture was then precipitated with 2.5μl of 4M LiCl and 75μl of prechilled 99% ethanol and placed at −20°C for 2 hours. The pellet was collected by centrifugation at 4°C at 13,000g for 15 minutes. The supernatant was discarded and the pellet was air-dried under Kimwipes. Finally, the labeled oligonucleotide pellets were resuspended in 20μl sterile water and stored at −20°C.

Hybridization

The membranes to be hybridized were placed in Hybaid MICRO-4 tubes and 50ml preheated hybridization buffer was added. The hybridization solution consisted of 0.5M NaPO₄ pH 7.0, 7% SDS, and 1mM EDTA pH 8.0. The membranes were pre-hybridized for at least 1 hour at 52°C. Prehybridization prevents non-specific binding of the probe to the nylon membrane.

The 20μl of labeled oligonucleotide was then added to the tubes. The membrane was hybridized for 1 hour at 52°C.
Membrane washes

Membranes were removed from the tube and were rinsed in a solution of 2X SSC, 0.1% SDS. Usually the membranes were then washed twice at room temperature for 15 minutes. Subsequent high stringency washes with 3M TMAC (tetramethylammonium chloride) were carried out at 58-62°C for 1 hour.

Detection

After hybridization and washes, the membranes were briefly rinsed in Washing Buffer, which consists of Buffer 1 (0.1M maleic acid, 0.15M NaCl) and 0.3% Tween 20 pH 7.5. Then the membranes were incubated with 50 ml of Buffer 2 (10% (w/v) Blocking Agent in Buffer 1) for 30 minutes. After blocking, the membranes were incubated with 20 ml of antibody solution (75 mU/ml anti-digoxigenin, Fab fragments–alkaline phosphatase (AP) conjugate, in Buffer 2) for 30 minutes and then washed twice for 15 minutes with 100 ml of Washing Buffer. After equilibrating for 5 minutes in 20ml Detection Buffer (0.1M Tris-HCl, 0.1M NaCl, pH 9.5), the membranes were incubated with 2ml Detection Buffer containing 250μM of CSPD (a chemiluminescent substrate for alkaline phosphatase) in a hybridization bag for 5 minutes. Excess liquid was removed by placing the membranes on a Whatman paper and the membranes were sealed in a hybridization bag. The membranes were further incubated for 10 minutes at 37°C to enhance the luminescent reaction and were then exposed to X-ray film for 20 minutes at room temperature. Heterozygotes were indicated by a signal on both membranes, wild type homozygotes produced a signal only on the membrane labeled with TNF*1, while variant homozygotes produced a signal only on the membrane labeled with TNF*2 (figure 4).
Figure 4. Dot blot analysis of TNFα-308 polymorphism hybridized with allele-specific oligonucleotide probes.

A: Dot blot after hybridizing with TNF1, specific to TNFα-308*1 allele. B: Dot blot after hybridizing with TNF2, specific to TNFα-308*2 allele. Allele-specific hybridization was not achieved with this assay. a1 and b3 are template-free negative controls. The genotypes of the samples (determined by restriction enzyme digestion) are as follows: a2, a3, a4 and b4 are homozygous wild types; a5 and b2 are heterozygotes; b1 and b5 are homozygous variants.
2.5.2 Genotyping the TNFα-308 Polymorphism by Restriction Enzyme Digestion

TNFα-308 genotypes were also determined by PCR-based restriction enzyme digestion with BsmF I. The primers selected for the amplification of TNF-308 polymorphism were the same as for SSO genotyping. The predicted size of the PCR product, which contained a naturally occurring BsmF I restriction site, was 182bp. A 20μl aliquot of digestion mixture containing 10 units of BsmF I, 4 μl of 10X digestion buffer 4 (NEB buffer 4, New England Biolabs) and 0.4 μl of Bovine Serum Albumin (BSA) (10mg/ml) was added to 20 μl of PCR product, mixed gently, and incubated at 65°C for at least 4 hours. After BsmF I digestion three fragments (104bp, 66bp, and 12bp) were produced from the wild type allele, whereas two fragments (174bp and 8bp) were produced from allele 2. Digested fragments were separated in a 3% agarose gel.

Because of the high cost of BsmF I, I designed a new upstream primer (TNFpmc: 5'-AGGCAATAGGTTTTTGAGGGCCATG-3') which creates an artificial Sty I restriction site on the wild-type allele as a result of a mismatch around the TNFα-308 polymorphism (figure 5). The downstream primer for SSO typing (TNF2) was used for this PCR. PCR optimization and final reaction conditions were as stated in section 2.5.1 except 0.5μM of each primer was used in the reaction.

The size of the PCR product was 143bp. A 20 μl aliquot of digestion mixture containing 10 units of Sty I, 4 μl of 10X digestion buffer 3, and 0.4 μl of BSA (10mg/ml) was added to 20 μl of PCR product and mixed gently, then incubated at 37°C for at least 4 hours. After digestion with Sty I, two fragments (123bp, 20bp) were produced from the wild-type allele, whereas allele 2 remained uncut. Digested fragments were separated in a 3% agarose gel (figure 6).
Figure 5. Restriction enzyme analysis for the the TNFα-308 polymorphism using an introduced restriction site. The underlined C was introduced into the PCR product, so that the wild type allele contained a \(Sty\) I recognition site (CCATGG). The G → A transition (bases in bold) abolishes the recognition site and the PCR product is not cut.
Figure 6. *Sty* I digestion for the TNFα-308 polymorphism. M: 100bp DNA molecular size marker. “-ve” represents template-free negative control. The homozygous variant is labeled by *, heterozygotes are labeled by arrows. Other lanes are homozygous wild types.
2.6 DETECTION OF THE FcεRIβ POLYMORPHISM

2.6.1 Genotyping the FcεRIβ polymorphism by Allele-specific PCR

Allele-specific PCR of the FcεRIβ polymorphism was performed as previously described (Hill et al. 1996). The method uses 2 E237G-specific ARMS primers (B7M1 and B7W2) and 2 non-ARMS control primers (B7FA1 and B7FA2). The sequences of the primers for the amplification were as follows:

**Upstream control primer: B7A1**

5'-CACGTGATTCTTATAATCAATGGGAGGAGCAATT-3'

**Downstream control primer: B7A2**

5'-GGACCATATTAAGGTGGACAGAAGCAGCAG-3'

**Upstream ARMS primer: B7M1**

5'-ATTCAGCTACTTACAGTGAGTTGGAAGAGCCAGGCGG-3'

**Downstream ARMS primer: B7W2**

5'-CACGTGATTCTTATAATCAATGGGAGGAGCAATT-3'

The predicted size of the amplification product was 446 bp for the control fragment, 280 bp for the wild type fragment and 238 bp for the allele 2 fragment. PCR optimization and final reaction conditions were as stated in section 2.5.1. except: 2mM MgCl₂, flanking primers at 0.6 μM, variant primer at 0.25μM, wild-type primer at 0.5μM, 1 unit Taq DNA polymerase, and a final volume of 40μl. The PCR profile was 35 cycles of: 30 seconds denaturation at 94°C, 30 second annealing at 62°C and 1 second extension at 72°C. PCR products were then separated on a 2% agarose gel (figure 7).
Figure 7. Allele-specific PCR for the FcεRIβ polymorphism. M: 100bp DNA molecular weight marker. Homozygous variants are labeled by ↓. Heterozygotes are labeled by *. The other lanes are homozygous wild types. Template-free negative control is represented by “-ve”.
2.6.2 Genotyping the FceRIβ polymorphism by Restriction Enzyme Digestion

The genotype of E237G of FceRIβ was confirmed in a subset of samples by PCR-based restriction enzyme digestion with Bsm I. The primers designed for the amplification were as follows:

**Upstream primer:**

EGU 5'-'GGTTCCAGAGGATCGTTTTATG-3'

**Downstream primer:**

EGD2 5'-'CTTATAAATCAATGGGAGGAGGTTATG-3'

The downstream primer was designed with a mismatch to produce a Bsm I site. The PCR reaction conditions were optimized by the same techniques as stated in section 2.5.1, except 0.5 μM of each primer and 1 unit Taq DNA polymerase. The PCR was programmed as 35 cycles of: 30 seconds denaturation at 94°C, 30 seconds annealing at 57°C and 60 seconds extension at 72°C. A final extension at 72°C for 5 minutes was included.

The predicted size of the PCR products was 101bp. A 20 μl aliquot of digestion mixture containing 10 units of Bsm I and 4μl of 10X digestion buffer 2 was added to the 20 μl of PCR product, mixed gently, and then incubated at 65°C for at least 4 hours. After digestion with Bsm I, the allele 2 was cut into 81bp and 20bp fragments, while the wild-type allele remained uncut. The digested fragments were separated in a 3% agarose gel (figure 8).

2.7 DETECTION OF THE IL4(-590) POLYMORPHISM

2.7.1 Genotyping the IL4-590 polymorphism by allele-specific amplification

Screening for the IL4-590 variant was carried out using allele-specific PCR. The primers
Figure 8. Restriction enzyme (Bsm I) digestion for the FcεRIβ polymorphism. M: 100bp DNA molecular weight marker. "-ve" represents template-free negative control. Heterozygotes are indicated by *. The other lanes are homozygous wild types.
for the amplification of IL4-590 were first designed as shown in figure 3 with the Oligo primer analysis software. The following primers were used:

**Downstream primer (PW): Specific to wild type allele with one mismatch**

\[5' -\text{CTCCTACCCAGCACTGGCGG-3'}\]

**Upstream primer (PM): Specific to allele 2 with one mismatch**

\[5'-\text{ACACTAAACTTGGAGAACATTCTT-3'}\]

**Upstream control primer (P3F):**

\[5'-\text{TAGACCTACCTTGCCAAGGGC-3'}\]

**Downstream control primer (P3R):**

\[5'-\text{TGCATAGAAGGGAGGCCAC-3'}\]

The expected sizes of the amplified products were: 350bp from P3F and P3R, 150bp from P3F and PW and 250bp from P3R and PM. However, the reproducibility of this PCR was low. Even after optimizing the ratio of the primers, Mg\(^{2+}\) concentration, annealing temperature and the number of cycles, only three primers worked. Primer PM had a high AT content, and failed to compete with other primers to produce the variant fragment. Therefore, a second set of allele-specific primers was designed. The primer PM was replaced by PW\(_2\), while PW was replaced by PM\(_2\). However, the two control primers remained the same. Since PW\(_2\) also had high AT content, the wild type and variant reactions were performed in separate tubes. The primers were:

**Upstream primer (PW\(_2\)): Specific to wild type allele with one mismatch**

\[5' -\text{CTCAAAACACTAAACTTGAGAGACATTCTC-3'}\]

**Downstream primer (PM\(_2\)): Specific to allele 2 with one mismatch**

\[5' -\text{AGACTCTCTCCTACCCAGCACTGGCGA-3'}\]

P3F and P3R produced a 350bp control band irrespective of the genotype at the –590
position. PW2 was complementary to the wild type sequence and produced a 260bp fragment with P3R only when the wild type sequence was present. PM2 was complementary to the variant sequence and produced a 148bp band with P3F only when the allele 2 was present. I first screened for the allele 2 in all subjects. P3F, P3R and PM2 were needed for this PCR reaction. The appropriate reaction conditions were optimized by the same principles as described in section 2.5.1. The optimized PCR was performed in a 40μl volume containing the same conditions as stated above except 0.32μM of each primer. The cycling parameters were 35 cycles of 94°C for 30s, 60°C for 30s, 72°C for 1s and final 10min incubation at 72°C. The reaction products were analyzed by electrophoresis through 2% agarose stained with ethidium bromide (figure 9A). As expected, a 350bp product was amplified with P3F and P3R in all subjects, as well as a 148bp product with P3F and PM2 when the allele 2 was present in a subject. However, it was not known whether these subjects were variant homozygotes or heterozygotes. Subjects who were homozygous for the wild-type allele only displayed a control band from this PCR.

In order to differentiate the homozygous variants and the heterozygotes, a second PCR was performed for subjects who had the allele 2. P3F, P3R and PW2 were required for this PCR. This PCR was difficult to optimize even after altering the annealing temperature, Mg2+ concentrations, dNTP concentrations, template concentrations and adding enhancers such as DMSO. Therefore a touchdown (TD) PCR was used (Hecker et al. 1996). The optimization of TD PCR is accomplished by focusing on a single variable, the annealing temperature. To perform a TD PCR, the annealing temperature of the initial two cycles is designed to be well above the estimated Tm of the primer-template complexes. This helps to ensure a competitive advantage for the target amplicon. The annealing temperature of each subsequent two cycles is run at 1°C less than the proceeding cycles. At high annealing temperatures only perfectly matched primer-template
complexes can form. This ensures specificity of the PCR reaction. As the cycling program progresses, annealing temperatures well below the $T_m$ can serve to significantly increase yields. In this PCR, the optimization of primer ratios was achieved by conventional methods. The reaction was carried out in a 40μl volume containing 0.1μg genomic DNA, 50mM KCl, 20mM Tris-HCl (pH 8.4), 0.32μM PW2, 0.64μM P3R, 0.08μMP3F, 200μM of each dNTP, 1.5mM MgCl2 and 0.5 units Taq DNA polymerase and was overlaid with 20μl of mineral oil. The TD PCR was programmed as follows: the annealing temperature started from 63°C, then progressively decreased every second cycle by 1°C to 57°C, followed by 30 cycles at 56°C. Each annealing temperature was set for 30s, a denaturation step (94°C for 30s) and extension step (72°C for 30s) were also included for each cycle. PCR products were analyzed by electrophoresis through 2% agarose. As expected, a 350bp product with P3F and P3R was amplified in all subjects and a 248bp product from P3R and PW2 was shown only when the wild type allele was present (figure 9B). If only the control fragment was present, this was an indicative of a homozygous variant.

2.7.2 Genotyping the IL4-590 polymorphism by restriction enzyme digestion

In a subset of samples, the genotype of the IL4-590 polymorphism was confirmed by PCR-based restriction enzyme digestion with BsmFI. The primers selected for the amplification were as follows:

Upstream primer: 5'-CCTCAGAATAGACCTACCTTGC-3'  
Downstream primer: 5'-GCATAGAGGCAGAATAACAGGC-3'

The appropriate conditions were optimized by the same techniques as described in section 2.5.1. The final reaction conditions used were: 0.2μg template DNA, 2mM MgCl2, 50mM KCl, 20mM Tris-HCl (pH 8.4), 200μM of each dNTP, 1.7μM of each primer, 10% DMSO and 1 unit of Taq
Figure 9. Allele-Specific PCR for the IL4-590 polymorphism using two separate reactions. A: ASPCR with variant primer. B: ASPCR with wild type primer. Both PCR reactions were carried out for each subject. M: 100bp DNA size marker. The arrow indicates a homozygous variant. The * indicates the heterozygotes. Other lanes are homozygous wild types.
DNA polymerase. The reaction was made up to a final volume of 30µl. The cycling conditions consisted of 5 min initial denaturation at 94°C, then 35 cycles of: 30 second denaturation at 94°C, 30 second annealing at 54°C and 30 second extension at 72°C. A final extension at 72°C for 5 minutes was included.

The predicted size of the PCR product was 178bp. A 15 µl aliquot of digestion mixture containing 10 units of BsmF I and 3 µl of 10X digestion buffer 4 (NEB buffer 4, New England Biolabs) was added to the 15 µl of PCR product, mixed gently and then incubated at 65°C for at least 4 hours. After digestion with BsmF I, two fragments (102bp, 76bp) were produced from the allele 2, three fragments (76bp, 64bp and 38bp) were produced from the wild type allele and four fragments (102bp, 76bp, 64bp and 38bp) from heterozygotes. Digested fragments were separated in a 3% agarose gel (figure 10).

2.8 SCORING THE GENOTYPES

Genotypes were scored without knowledge of the phenotypes. Another member of our laboratory independently checked all the genotypes. If there was any disagreement concerning the genotyping the samples were re-genotyped.

2.9 SUBJECTS

This was a prospective cohort study. Five hundred and forty infants at high risk for asthma were recruited from two centres: Vancouver and Winnipeg (principle investigators: Dr. Moira Yueng and Dr. Alan Becker, respectively). The infants were originally recruited for a study
Figure 10. *BsmF* I digestion for the IL4-590 polymorphism. M: 100bp DNA molecular size marker. Template-free negative control is indicated by "-ve". Each subject is represented by two lanes: the lane to the left contains the undigested PCR product (178bp); the lane on the right contains PCR product digested with *BsmF* I. Heterozygotes are labeled by arrows. Other lanes are homozygous wild types. Homozygous variants are expected to produce two bands of 102bp and 76bp.
designed to find out whether an intervention program instituted during the first 12 months of life protected against the development of asthma and other atopic disorders. The definition of high risk infants was those who had at least one first degree relative with a history of asthma or two first degree relatives with a history of other allergic disorders such as: allergic rhinitis, atopic dermatitis and food allergy. Exclusion criteria from the study were: infants who required ventilatory assistance, prematurity as defined by less than 10 percentile weight by gestational age, any significant congenital abnormalities, an apgar score less than 5 at five minutes, mothers who were alcohol or drug abusers and mothers who planed to move from their city of residence within two years. The final sample comprised 493 infants.

Recruitment to the study took place between November 1994 and June 1996. At the time of enrollment, a research nurse explained the study to all pregnant women in each centre during their third trimester visit. A circular with information about the study and a self-administered questionnaire was distributed. The questionnaire enquired whether the potential mother, father or siblings had asthma and other allergic disorders. Five hundred and forty families agreed to take part in the study and gave informed consent. Parents completed a questionnaire about their history of asthma and allergic diseases, smoking habits and ethnicity. The history of allergic disease (previously diagnosed asthma, atopic eczema, allergic rhinitis, or food allergy) in the mothers, their partners or their children were documented. The ethnicity was recorded by questioning of the parents as to their ethnic origin.

The presence of disease was determined by responses to a standardized self-completed questionnaire. The presence of asthma was indicated by an affirmative response to the question, “Have you ever had asthma or wheezing or whistling in the chest” that occurred “apart from
colds,” or had more than rare “attacks of shortness of breath with wheezing.” Allergy was indicated by the positive answer on self-administered questionnaire to the presence of atopic dermatitis, allergic rhinitis and urticaria that is related to food. Allergy skin prick tests using 16 common allergens were performed for both parents. The responses were compared with a positive (histamine phosphate test 1mg/ml) and a negative (normal saline) control. A positive reaction was recorded if the diameter of the skin wheal was ≥ 3 mm larger than that of the negative control.

Total serum IgE was measured for mothers. Peripheral blood was taken from parents and cord blood from infants was collected during delivery. Home visits were carried out before delivery and every three months for 12 months and again at 24 months. A questionnaire was administered to both parents.

Assessment of the infants at 12 and 24 months was done by pediatric allergists for respiratory symptoms and other allergic symptoms such as rhinitis, atopic dermatitis, and food allergy. It is not possible to diagnose asthma at the age of 12 months. Therefore, “possible asthma” and “probable asthma” were defined for infants. “Possible asthma” was defined as at least 2 distinct episodes of cough each lasting for 2 or more weeks; or at least 2 distinct episodes of wheeze each lasting for one or more weeks; or in the absence of a cold, at least one of the following: nocturnal cough (at least once a week), hyperpnoea induced cough, exercise-induced cough. “Probable asthma” was determined by at least 2 distinct episodes of cough each lasting for one or more weeks; or at least 2 distinct episodes of wheeze each lasting for one or more weeks; plus at least one of the following: nocturnal cough at least once a week in the absence of a cold, hyperpnoea induced cough or exercise-induced cough or wheeze at any time, response to treatment with beta-agonist and/or anti-inflammatory drugs. The definition of rhinitis was 2 or
more episodes of runny nose and sneezing without apparent colds. Atopy was defined by the presence of a positive skin test reaction to one or more of the allergens tested. Skin-prick tests were performed with 12 common allergens including cat dander, Der p I, cows milk, eggs and controls at 12 months of age. Completed phenotypic data for infants at the age of 12 months were obtained for the analyses contained in this thesis.

We also recruited a group of 43 non-atopic, non-asthmatic controls. These subjects had no history of asthma and had negative skin tests (<1mm wheal greater than saline) to common aeroallergens (from cat, dog, house dust mite, grass, molds, trees). All subjects in these groups were of white ancestry. Furthermore, we determined the prevalence of the polymorphisms in 119 random population controls recruited from a blood donor clinic who were unselected for atopy or asthma. Their ethnic backgrounds were unknown.

2.10 Statistical data analysis

The frequencies of the alleles and genotypes between groups were compared by chi square analyses for 2 x 2 contingency tables. The genotype and allele frequencies for TNFα-308 and FcεRIβ polymorphisms between Oriental parent study groups were analyzed by Fisher’s exact test. The mean log IgE between genotypes of the IL4-590 and FcεRIβ polymorphisms were compared by t tests. Since race, smoking and age are considered as confounding factors, potential associations of log IgE with the IL4-590 and FcεRIβ polymorphisms were analyzed using multiple regression. The associations of asthma, atopy and rhinitis with the TNFα-308, IL4-590 and FcεRIβ polymorphisms were analyzed by logistic regression.
Transmission/Disequilibrium Test (TDT)

The TDT is a method originally designed to detect linkage between any marker and a complex disease in the presence of association. TDT is also valid as a test of association with a particular allele in a sample from unrelated simplex families or trios of affected offspring and parents from multiplex families. The association detected by TDT is not confounded by ethnic status.

To perform a TDT, affected children and their parents ('trios') are recruited. Families that are used in the analysis are those in which at least one of the parents is heterozygous. TDT determines if the risk allele is transmitted more often to the affected individual from heterozygous parents than would be expected by chance. The TDT statistic is \((b-c)^2/(b+c)\), where \(b\) is the total number of transmissions of \(M_1\) to affected children and \(c\) is the total number of transmissions of the risk allele \(M_2\) (Figure 11). If the marker allele is not associated with the disease, the expected number of transmissions of \(M_1\) and \(M_2\) is equal. If there is linkage between marker and disease as well as allelic association, \(b\) and \(c\) will be different. The statistical significance of the TDT is tested by \(X^2\) analysis for goodness of fit (Spielman et al. 1996).

Power analysis

In order to test the power of this study, a power analysis was performed. Given the numbers of infants in the study the minimum relative risk (RR) which could be detected with 80% power for TNFα-308*2, IL4-590*T and FcεRIβ*G alleles for a variety of phenotypic frequencies is shown in table 1.
Figure 11. Transmission of a putative risk allele in nuclear families which are used for the TDT. Affected children are shown as closed symbols with the genotype of each individual at a risk locus shown below. $M_2$ is hypothesized to be the risk allele and $M_1$ the normal allele. Families of Type A illustrate the risk allele $M_2$ being transmitted to the affected child from a parent. Type B families illustrate non-transmission of the risk allele $M_2$ to the affected child from a parent.
CHAPTER 3

RESULTS

3.1 CHARACTERISTICS OF STUDY SUBJECTS

The total cohort of 540 families consisted of 411 asthmatic, 612 non-asthmatic and 41 parents of unknown asthmatic status; 665 atopic, 330 non-atopic and 67 parents of unknown atopic status. Asthmatic mothers had the highest mean total serum IgE levels compared with the non-asthmatics, atopic and non-atopic mothers. Non-asthmatic mothers had the lowest mean total serum IgE levels (figure 12). Of the 540 families, we were able to phenotype 493 infants at 12 months of age. Cord blood was collected for 373 infants during delivery. Of the 373 families, 289 families including 6 single mothers and 283 dual parents had DNA available for both parents (parent) and infants. The ethnic background of these subjects is shown in table 2.

Five hundred and seventy two parents from the 289 families were divided into 6 categories: non-asthmatics, asthmatics, non-atopics, atopics and unknown asthma or atopy status (Table 3), these categories are not mutually exclusive. In this study population, 82% were whites, 5.6% were Oriental, first nation 1.6% east indian 1.7%, blacks 0.7% and others 3.1%.

Allergic disorders had developed in some of the infants by the age of 12 months. Of the 490 infants, 22% had rhinitis; 7% had probable asthma; 11% had possible asthma. The prevalence of positive skin-prick tests in infants to a range of allergens including home-dust mites, cows milk, egg, peanut wheat, cat, dog and grass pollen, etc. was 21%. The most prevalent positive skin tests were to food allergens at 18% of the infants. Oriental infants have higher prevalence of dermatitis and positive skin tests compared to the white infants (Table 4). Of the 373 infants and
Figure 12. Cumulative frequency distribution of log IgE among non-atopic non-asthmatics, non-atopic asthmatics, atopic non-asthmatics and atopic asthmatics.
572 parents, the number of both genotypes and phenotypes available for each polymorphism is shown in table 5. Subjects who did not have the phenotypic data or whose PCR genotyping failed for any polymorphism were not included in the data analysis.

3.2 COMPARISON OF DIFFERENT TECHNIQUES FOR DETECTING POINT MUTATIONS

Three techniques were applied to detect the TNFα-308, IL4-590 and FcεRIβ E237G polymorphisms. Restriction enzyme digestion was the simplest technique compared with single-strand oligonucleotide hybridization (SSO) and allele-specific PCR (ASPCR). Restriction enzyme digestion was easy to optimize and had very high reproducibility. However, some enzymes can be very expensive. Partial digestion can give false genotypes.

ASPCR was the fastest and cheapest technique compared with the other two. Once the conditions were optimized, good reproducibility was obtained. However, this optimization process was time consuming for the IL4-590 polymorphism. Optimization for ASPCR is dependent on the sequence of the amplified region. The region surrounding the IL4-590 polymorphism was AT-rich, which made primer design difficult. The first set of primers designed for IL4-590 produced a product that required restriction enzyme digestion. This restriction enzyme (BsmF I) is very expensive and is not suitable for large population screening. Therefore, new primers were designed for ASPCR. In this reaction the ratio of the primers, annealing temperature and magnesium concentration were altered to try to optimize the PCR. Expected PCR products for the allele 2 were not obtained even in a separate reaction. However, the control fragment and wild type fragment worked well in the absence of allele-specific variant primer. Therefore, a second
pair of allele-specific primers was designed which proved to be suitable for ASPCR. The new allele-specific primer for the allele 2 was switched to the position where the first wild type allele-specific primer annealed. The length of the new allele-specific primer for the wild type allele was increased to 31 bp. Even with these primers the amplification reaction of the wild type and allele 2 could not be performed in a single tube. In contrast, ASPCR was easily optimized for E237G of FcεRIβ since the region where the allele-specific primers were located contained a balanced AT and GC composition. However, this set of primers was very sensitive to magnesium concentration. When samples were prepared differently, PCR required further optimization, particularly magnesium concentration.

SSO hybridization was the most expensive and time-consuming technique. The optimization for stringent hybridization and washing was difficult. Therefore, the first two techniques were chosen to genotype all the subjects. The presence of each variant was confirmed by at least two different techniques in 20 individuals.

The comparison of the three techniques was summarized in Table 6.

3.3 FREQUENCIES OF POLYMORPHISMS IN INFANTS

3.3.1 TNFα-308 polymorphism

In the whole or white infant population, the prevalences of allele 2 of TNFα-308 were not significantly different compared with that in the non-atopic, non-asthmatics or the general population (table 7 and 8). The prevalence of “probable asthms” or “possible asthma” and positive skin tests was not different between white infants with TNFα-308*2 and white infants without
this allele (table 9). In the Oriental infants, the prevalences of "probable asthma" or "possible
asthma" and atopy were not different between infants with allele2 and without allele2 (data not
shown). The genotype and allele frequencies of allele2 of TNFα-308 were not different between
whites and Orientals (data not shown). Therefore, TNFα-308 allele2 was not a risk factor for the
pathogenesis of asthma or atopy in either ethnic group by age 1.

3.3.2 FceRIβ polymorphism

The prevalence of E237G of FceRIβ in the whole or white infants population was not
significantly different compared with non-atopic, non-asthmatic controls or the general population
(table 10 and 11). In order to determine the relative risk of the FceRIβ*G allele for the
development of asthma, rhinitis and atopy, white infants were divided into two groups
(heterozygous EG and homozygous wild type EE). The prevalence of positive skin tests was the
same between infants with EE and those with EG. However, the prevalence of "probable
asthma", "possible asthma" and rhinitis were lower in EG than EE infants, but not significantly
different (table 12). The genotype and allele frequencies of FceRIβ polymorphism were not
different between white and Oriental infants.

3.3.3 IL4-590 polymorphism

Interestingly, we found the allele frequency of IL4-590*T was significantly increased in
the whole infant population compared with the non-atopic, non-asthmatic controls (p=0.04) or the
general population (p=0.01) (table 13). The prevalence of atopy was significantly higher in infants
with IL4-590*T than in those without this allele (table 14). The allele frequency of IL4-590*T in white infants was significantly different from the Oriental infants (table 15). Similarly, the allele frequency of this allele was lower in white than in other ethnic groups. However, these differences were not statistically significant. The genotype and allele frequencies of IL4-590 polymorphism were not different between Oriental and Black infants. Because of these differences in genotype and allele frequencies between ethnic groups we limited our association study in whites and Orientals in which we had the largest number of subjects.

The genotype and allele frequencies of IL4-590 polymorphism in white infants were not significantly increased compared with the non-atopic, non-asthmatic controls or the general population (table 16). In order to determine the relative risk of IL4-590*T allele for the development of asthma, rhinitis and atopy, the white infants were divided into two groups (heterozygous CT and homozygous variant TT vs homozygous wild type CC). The proportion of CT/TT white infants that developed "probable asthma" was significantly higher than that of CC white infants. However, the proportion of CT/TT white infants that developed rhinitis and atopy was not different from CC white infants (table 17).

The proportion of Oriental CT/TT infants that developed atopy was 62%, however, only one CC infant was found in this group. Therefore, the relative risk for this polymorphism for the development of atopy in Orientals could not be accurately determined (table 17).
3.4 FREQUENCIES OF POLYMORPHISMS IN THE PARENT POPULATION

To determine whether the TNFα-308, IL4-590 and FcεRIβ polymorphisms were associated with asthma and atopy, the parents were divided into atopics and non-atopics; asthmatics and non-asthmatics.

3.4.1 TNFα-308 polymorphism

There were no significant differences in the prevalence of the TNFα –308 polymorphism in white asthmatics compared to the non-asthmatic group (Table 18). In the Oriental parents, the allele frequency of TNFα-308 allele 2 was increased in atopics compared to non-atopics, and asthmatics compared to the non-asthmatics, but these differences were not significant (table 19).

3.4.2 FcεRIβ polymorphism

There were no significant differences in the prevalence of the FcεRIβ polymorphism between non-atopics (4%), atopics (6%), non-asthmatics (5%) and asthmatics (5%) in whites (table 20).

In the Oriental parents, FcεRIβ*G was more prevalent in atopics (16%) and asthmatics (18%) compared to non-atopics (7%) and non-asthmatics (12.5%). However, these differences were not statistically significant (table 21).

In order to investigate the role of FcεRIβ*G in determining log IgE levels, subjects were
divided into two groups. In whites, the mean log IgE for homozygous wild type (EE) was lower than that for heterozygous (EG) and homozygous variant (GG) subjects (1.52 ± 0.58 vs 1.67 ± 0.76 p=0.45). Similarly, in Orientals, the mean log IgE for EE subjects was lower than that for subjects with EG and GG (1.70 ± 0.47 vs 2.08 ± 0.44, p=0.13. table 22). Interestingly, the mean log IgE was significantly higher in subjects with the FcεRIβ*G allele than those without this allele in the whole parent population (table 22). The cumulative frequency distribution of log IgE is shown in figure 13.

To determine whether total skin test reactivity was associated with the FcεRIβ*G allele, log total skin test in whites with this allele was compared with those with the wild type alleles. No significant difference was detected (figure 14).

3.4.3 IL4-590 polymorphism

There was no significant difference in the prevalence of the IL4-590 polymorphism in the white non-asthmatics compared to the asthmatic parents. Similarly, no difference was detected between atopics and non-atopics (table 23). In the Oriental parent study groups, the prevalence of IL4-590 polymorphism was also not significantly different between any groups (table 24). However, we noticed that the allele frequency of the IL4-590*T allele was significant higher in Orientals than in whites (77% vs 16% p<0.001) (table 25). No wild type homozygotes for the IL4-590 polymorphism were detected in the Oriental population. The same result was found in Blacks. The frequency distribution of the IL4-590 allele 2 in different ethnic groups is shown in figure 15.

There was no significant difference in mean log IgE between white wild type homozygotes
Figure 13. Cumulative frequency distribution of log IgE levels in FcεRIβ genotypic groups in White and Oriental mothers.
Figure 14. Cumulative frequency distribution of log total wheal size in FcεRIβ genotypic groups in Whites.
Figure 15: Prevalence of the IL4-590 variant allele in different ethnic groups.
and white heterozygotes / variant homozygotes for IL4-590. The mean log IgE for Oriental parents was 1.8 that is higher than that for whites (table 26). The cumulative frequency distribution of log IgE in whites with IL4-590*T allele was not different from those with the wild type allele (figure 16).

3.5 TRANSMISSION/ DISEQUILIBRIUM TEST (TDT)

As described in section 3.3.3 and 3.4.3, the prevalence of IL4-590 polymorphism was increased in Oriental atopic infants and parents. To further test for association of this polymorphism with atopy and to control for ethnic diversity, the TDT was used.

To perform TDT, 29 families were selected. All infants had at least one positive skin test and at least one of their parents was heterozygous for the IL4-590 polymorphism. We found that allele T was transmitted more often than non-transmitted, but this difference was not significant at the 0.05 level (table 27). No association of TNFα-308*2 and FcεRIβ*G with atopy and asthma was found by TDT (data not shown).
Figure 16. Cumulative frequency distribution of log IgE levels in IL4-590 genotypic groups in White and Oriental mothers.
CHAPTER 4

DISCUSSION

4.1 THE INFANT POPULATION

4.1.1 Results of association studies

The results of this study show that IL4-590*T may be a risk factor for the development of asthma but not atopy in the white infant population at one year of age. The evidence was that the prevalence of “probable asthma” in white infants with IL4-590*T was significantly higher than that of those with the wild type allele. The prevalence of atopy was not different between white infants with IL4-590*T and those with the wild type allele.

The results also indicate that TNFα-308*2 and FceRIβ*G were not risk factors for the development of atopy or asthma in the white infant population at one year of age. This conclusion was drawn from two types of study. Firstly, the prevalence of TNFα-308*2 and FceRIβ*G were not significantly increased in the high-risk white infant population compared with non-atopic, non-asthmatic white controls or the general population. Secondly, the prevalence of “probable asthma” or “possible asthma” and atopy were not significantly different between infants with and without TNFα-308*2 and FceRIβ*G in whites.

Three important findings were also revealed in this study. Firstly, the allele frequency of the IL4-590*T was significantly higher in the whole high-risk infant population than in the non-atopic, non-asthmatic white controls or the general population. Secondly, the allele frequency of IL4-590*T was significantly higher in Oriental infants than that in white infants. Thirdly, we
found that the prevalence of atopy was significantly higher in the entire infant population with IL4-590*T than in those without this allele. All of these findings have relevance to understanding the role of these genes in atopy and asthma, and will be discussed in the following sections.

Previous studies have found associations between IL4-590*T and total serum IgE in American whites (Rosenwasser et al., 1995) but not in Australian and British whites (Walley et al., 1996). The latter only detected a weak association between IL4-590*T and specific IgE to house dust mite and wheeze (RR = 1.33). In both of these studies, no strong evidence supported the association of this polymorphism with asthma. More recent study has shown that IL4-590*T was associated with asthma but not with total and specific IgE levels in Japanese children (Noguchi et al. 1998). The results of this study are consistent with these data.

Contrary to our findings, a previous study of 413 subjects (age range 5-51) from a general white population found that asthma was significantly more common in subjects with the TNFα-308*2 and LTα Nco I*1 alleles. The RR for asthma associated with TNFα-308*2 was 1.5. The association was confined to the haplotype containing LTα Nco I*1 and TNFα-308*2 (Moffatt et al., 1997). This indicated that TNFα-308*2 was only associated with asthma in combination with LTα NcoI*1. The separate effects of the LTα Nco I and TNFα-308 alleles on the pathogenesis of asthma could not be differentiated since they are in strong linkage disequilibrium.

A previous study of 1004 Australian whites (age range 5-55) found that subjects with FceRIβ*G had increased skin test responses to aeroallergens and bronchial reactivity to methacholine. The RR of individuals with FceRIβ*G having asthma compared to subjects without the variant was 2.3 (Hill et al. 1996). A study of a Japanese population showed that the prevalence of FceRIβ*G was significantly increased in atopic asthmatics, particularly childhood atopic
asthmatics compared to non-asthmatic, non-atopic controls (OR = 3.92). The study also showed that the prevalence of FcεRIβ*G was not significantly increased in non-atopic asthmatic adults compared to non-atopic controls (Shirakawa et al. 1996). These two studies suggest that FcεRIβ*G is associated with atopy.

4.1.2 Reasons for lack of association

The lack of association of the TNFα-308, FcεRIβ and IL4-590 polymorphisms and asthma and atopy in the white infant population has several possible explanations. Firstly, since atopy and asthma are complex genetic traits, it is unlikely that every study will demonstrate the same associations. The lack of associations may reflect genetic heterogeneity in the pathogenesis of atopy and asthma.

The second reason for the lack of association in this study could be the difficulty of diagnosing asthma at the age of 12 months since infants who have respiratory illnesses with wheezing in the first year of life may stop wheezing in later life. This type of wheeze, called transient early wheezing, was significantly associated with maternal smoking in one study and was not associated with increased risk of asthma or allergies later in life (Fernando et al. 1995). In our population, Becker et al. (1998) from Winnipeg found that exposure to environment tobacco smoking was the most important determinant for the development of asthma (OR=2.6 for maternal smoking). Most episodes of transient early wheezing are precipitated by viral pathogens. Only those who have at least one lower respiratory tract illness with wheezing in the first three years of life and had wheezing at six years of age (persistent wheezing) are more likely to have rhinitis apart from colds, reduced lung function, sensitization to common aeroallergens and elevated serum IgE levels. Persistent wheezing is thought to predispose older children to asthma.
Therefore, the diagnosis of asthma at the age of 1 may be not an accurate predictor of later childhood or adult asthma. Thus the ability to detect associations in this study may have been weak.

The third reason for the lack of association in this study may be that the frequency of positive skin tests increases with age. An Australian study showed that the prevalence of atopy in the children aged 8 to 10 at the beginning of the study was 24%, while six years later it was 40% (Peat et al. 1990), indicating an additional 16% of children were sensitized to allergens during adolescence. It is well known that positive allergy skin tests in children correlate highly with types of exposure. Children at different ages have different skin test reactivity. At an earlier age children develop positive skin tests to foods more often than to inhaled allergens. Food allergy is less common after 3 years of age and sensitizations to inhaled allergens are more important after 18 months of age (Björkstén 1997). This reflects the amount, time, and route of exposure of allergens. Food allergy and eczema in an infant are often the first manifestation of allergic disease in the atopic individual. The presence of food allergy indicates the individual is atopic and is at risk for developing other allergic manifestations later in life. A previous study has shown that 82% of the children with positive skin tests to food eventually developed atopic diseases. However, only 32% of children who have IgE antibodies to inhalants at the age of 15 had antibodies to food allergens in infancy (Sigurs et al. 1994). This shows that the correlation of sensitivity to food and sensitivity to aeroallergens is not perfect. Negative skin tests in children under five years of age may not fully reflect their atopic status. A peak age of sensitization is between 15 and 25 years (Peat et al. 1990, Niemeijer et al. 1992, Barbee et al. 1976). Previous association studies used positive skin tests to aeroallergens to define atopy. In this study, most of the positive skin tests were to food allergens. Therefore, at the age of 1, the atopic status is not as
accurately determined as it will be in follow up studies.

Finally, the lack of association among white population in this study may be due to insufficient power in the study design. Assuming OR = RR, the minimum detectable RR for each polymorphism given the number of subjects in our study, and for different prevalences of phenotype is shown in table 1. Previous studies have shown an OR of 2.3 for asthma with TNFα-308*2 and 3.92 for atopic childhood asthma with FcεRIβ*G. Thus, we do not think that lack of power is an important reason for the negative results with these polymorphisms.

4.1.3 Ethnic Differences

Interestingly, we found the prevalence of the IL4-590*T was significantly increased in Oriental infants compared to white infants (table 15). In Oriental infants with IL4-590*T, the proportion of positive skin tests was 62% but was only 19% in white infants. The lack of association of IL4-590*T to atopy in the Oriental infants may be due to the small sample size. The prevalences of TNFα-308*2 and FcεRIβ*G were similar between white and Oriental infants. The occurrence of high levels of atopy (18/36) and increased allele frequency of the IL4-590*T in the Oriental infants is intriguing and suggests that the two may be causally linked. However, it was not possible to test this hypothesis due to the low prevalence of the IL4-590*C allele in this group.
4.2 THE PARENT POPULATION

4.2.1 Association studies

**TNFα-308 polymorphism**

Previous investigators have suggested that increased TNFα secretion is associated with the TNFα-308 polymorphism (Bouma et al. 1996, Wilson et al. 1992) and that TNFα-308*2 is associated with asthma and bronchial hyperresponsiveness (Moffatt et al., 1997; Campbell et al. 1996). The results of this study show that TNFα-308*2 was not a risk factor for asthma in both whites and Orientals. The allele frequency of TNFα-308*2 found in our asthmatic whites was lower than in non-asthmatics. However, in Orientals, a trend was found for increased frequency of TNFα-308*2 in atotics compared with non-atotics, and asthmatics compared with non-asthmatics.

The lack of association of TNFα-308*2 with asthma has several possible explanations. Firstly, the TNFα-308*2 allele may only be associated with the severe disease as found in other conditions. For example, McGuire et al. (1994) found that individual homozygous for TNFα-308*2 allele carry a 7-fold higher risk of death or severe neurological sequelae in malaria. Braun et al. (1996) discovered that homozygous TNFα-308*2 subjects were only detected in primary chronic progressive multiple sclerosis. In the same way, TNFα-308*2 may affect asthma severity. It is possible that the asthmatics in this study were mainly mild.

Secondly, it is known that there is strong linkage disequilibrium between the TNFα locus and the MHC locus. Moffatt et al. (1997) reported haplotype analysis from this region. The association of TNF-308*2 with asthma was only seen when this allele was in combination with LTα Nco I*1. We only tested the TNFα-308 polymorphism and therefore may have had less power...
to detect association.

Thirdly, the lack of association may reflect genetic heterogeneity in the pathogenesis of asthma, since atopy and asthma are complex diseases and different genes will be important in different populations. The lack of association of TNFα-308*2 with atopy and asthma reported by Campbell et al. (1996) supports this explanation.

Finally, the lack of association of TNF-308*2 with atopy and asthma in Orientals may due to the small sample size.

**FccRIβ polymorphism**

Contrary to our hypothesis, this study showed that E237G of FccRIβ was not a risk factor for atopy and asthma in both white and Oriental parents. We found that the prevalence of E237G was not different between non-atopics, atopics, non-asthmatics and asthmatics in whites. However, in Orientals, there was a trend for increased prevalence in atopics (16%) and asthmatics (18%) compared to non-atopics (7%) and non-asthmatics (12.5%), but this was not statistically significant. This may be due to the small numbers in Oriental study group.

The lack of association in this study differs from the previous results (Hill et al.1996) where it was found that the E237G allele was associated with atopy and bronchial hyperresponsiveness. However, in support of our finding, data from Sweden had shown that E237G was not a risk factor for atopy and asthma (Rohrbach et al. 1998). These differences may be due to variation in environmental exposure to allergens between recruitment areas, different definitions of atopy /asthma and different study designs. Since atopy and asthma are complex traits, several risk alleles may contribute to their expression. It is possible that different risk alleles may be important in different populations.

The allele frequencies in atopic and asthmatic Oriental parents were higher than the allele
frequencies reported by Shirakawa et al. (1996) in a Japanese population study. The allele frequencies in the non-atopic and non-asthmatic Oriental parents were also higher than that in Japanese non-atopics or non-atopic asthmatics (table 21). Moreover, in this study, there was a similar trend for increased frequency of FcεRIβ*G in atopics and asthmatics as in the Japanese study group. The lack of association of E237G of FcεRIβ with atopy and asthma in Orientals may be due to our small sample size. Another possible reason for the lack of association may be due to the differences between populations in amount or type of phenotypic expression to environmental factors.

In order to determine whether FcεRIβ*G was associated with total serum IgE levels, the mean log IgE levels of subjects with and without FcεRIβ*G were compared in both whites and in Orientals. The FcεRIβ*G was found not associated with increased total serum IgE in either population. Combined with the previous results (Hill et al. 1996 and Shirakawa et al. 1996), the lack of association in this study may reflect genetic heterogeneity in the pathogenesis of atopy and asthma.

**IL4-590 polymorphism**

In this study we have shown that the IL4-590 polymorphism was not a risk factor for atopy, asthma or elevated total serum IgE in both white and Oriental parents. We found that the genotype and allele frequencies of the IL4-590 polymorphism were not different between non-atopics and atopics, and between non-asthmatics and asthmatics in both whites and Orientals. Moreover, we found that the mean log IgE in white subjects with and without the T allele was not significantly different. However, we found that the allele frequency of IL4-590*T was significantly different between whites and Orientals.
Previous studies have found association between IL4-590*T and total serum IgE in American whites but not in Australian and British whites. In both studies, no strong evidence supported the association of this polymorphism with asthma (Rosenwasser et al. 1995, Walley et al. 1996). Our findings are consistent with the lack of association with asthma.

In this study there was no association of IL4-590*T with atopy or total IgE levels. There are several possible explanations. Firstly, atopy is a complex phenotype. Secondly, there may be differences between populations in time, amount or type of exposure to environmental factors. Finally, different study designs may produce different outcomes.

4.2.2 Ethnic Differences

An interesting finding in this study was the distinct differences in the frequencies of IL4-590*T between different ethnic groups. The variant genotype and allele frequencies were significantly higher in the Oriental population than in whites (table 25). The genotype and allele frequencies in other ethnic groups were also increased compared to whites (figure 15). This is the first report of these differences between ethnic groups. Two possible explanations of why the IL4-590*T allele frequency was increased in Orientals compared to the whites could be suggested. First, different allele frequencies among different ethnic groups may derive from selection or random genetic drift. Second, these allele frequency ethnic differences may contribute to differences in atopy severity and prevalence. It is possible that Oriental parents with severe allergic disease were recruited for this study compared with white parents.

No differences were found for TNFα-308*2 and FcεRIβ*G allele frequencies between ethnic groups. However, there were trends for increased TNFα-308*2 and FcεRIβ*G frequencies in Oriental atopic and asthmatic parents. These trends were not observed in the whites. One
explanation for this discrepancy is that association between these polymorphisms and asthma or atopy is only apparent in subjects who had severe disease. It is possible that more severe subjects were inadvertently recruited in the Oriental group. This may be because of cultural differences between Orientals and whites. For example, Orientals may be less willing to participate in a clinical study. Therefore, only those with severe disease will agree to take part in a study such as this. Another possibility is that genes may act differently in different populations due to interaction with other genes or with environmental factors.

4.3 POPULATION ADMIXTURE

The major difficulty with case-control studies is to adequately match the cases and controls for all the potentially confounding factors. In genetic case-control studies, it is important to ensure that both the cases and controls are selected from the same population which is genetically homogenous (i.e. has undergone no recent admixture). If there is unidentified population stratification this can potentially produce type 1 error. This was clearly illustrated by the results of this study. When the entire infant population was analyzed, we found that the prevalence of the IL4-590 polymorphism was significantly increased compared with non-atopic, non-asthmatic controls or the general population. We also found that the prevalence of atopy was significantly higher in infants with IL4-590*T than in those without this allele (table 14). After subgrouping the infant population into whites and Orientals, the prevalence of IL4-590*T was not different between white infants and non-atopic, non-asthmatic controls or the general population. There was no association of IL4-590*T with atopy in white. The apparent associations were due to the high level of IL4-590*T in the Oriental infants who also had a high prevalence of atopy.
In the total parent study group, the mean log IgE was significantly higher in subjects with FcεRIβ*G than in those without this allele. After subgrouping the parents into whites and Orientals, no association of FcεRIβ*G with mean log IgE was detected. The association was due to the high level of mean log IgE in the Oriental parents. To avoid false positive results, the TDT was used to test for associations. This test is not confounded by ethnic status.

4.4 TDT OF IL4-590 POLYMORPHISM

Using the TDT, a borderline association of IL4-590*T for atopy (p=0.07 table 27) was detected. This suggests IL4-590*T may be a risk factor for atopy. This finding supports previous association studies of this polymorphism with atopy (Rosenwasser et al. 1995, Walley et al. 1996). A significant association of IL4-590*T with atopy may be detected after increasing the number of affected families.

4.5 SUMMARY

In summary, the results of this study suggested the TNFα-308*2, FcεRIβ*G were not risk factors for the development of atopy and asthma in both white and Oriental infants and parents. The IL4-590*T may be associated with asthma in white infants. The allele frequencies of IL4-590*T were more prevalent in other ethnic groups than in whites. We showed evidence that IL4-590*T may be associated with atopy using the TDT.
4.6 FUTURE STUDY

The data presented here for the infants were based on the first year assessment. The infants need to be monitored for the development of atopy and asthma.

In this study there were trends for increased allele frequencies of TNFα-308*2 and FceRIβ*G in adult Oriental asthmatics/atopics compared to non-asthmatics/non-atopics. It is important to examine the prevalence of TNFα-308 and FceRIβ polymorphisms in a large sample of Orientals in order to determine whether these polymorphisms are risk factors for atopy and asthma in this population. Further TDT analysis will be performed.

New candidate genes for atopy and asthma can be studied with this population. There are many additional genes that have been implicated in the pathogenesis of atopy and asthma such as leukotriene C₄ synthase, IL4 receptor and IL-9.
**Table 1: Power analysis of each polymorphism in infant population**

<table>
<thead>
<tr>
<th>with % of phenotype</th>
<th>IL4-590*</th>
<th>TNFα-308*</th>
<th>FceRIβ*</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>2.19</td>
<td>4.34</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>2.24</td>
<td>4.36</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>2.34</td>
<td>4.49</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>2.51</td>
<td>4.82</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2.85</td>
<td>5.55</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3.78</td>
<td>7.69</td>
<td></td>
</tr>
</tbody>
</table>

* The genotype frequency for IL4-590 and TNFα-308 was 30%, while for FceRIβ was 6%.

**Table 2: The ethnic background of the infants recruited into the study**

<table>
<thead>
<tr>
<th>Ethnic background</th>
<th>Total number of infants</th>
<th>Number of families with both parents and infants in the study</th>
</tr>
</thead>
<tbody>
<tr>
<td>White1</td>
<td>281</td>
<td>225</td>
</tr>
<tr>
<td>Oriental2</td>
<td>22</td>
<td>12</td>
</tr>
<tr>
<td>Mixed3</td>
<td>48</td>
<td>30</td>
</tr>
<tr>
<td>Unknown</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>373</td>
<td>283</td>
</tr>
</tbody>
</table>

White1 represents white Caucasians. Oriental2 represents Asians. Mixed3 includes native Indians, East Indian Caucasians, blacks and others.

**Table 3: Subject characteristics**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Total</th>
<th>Whites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-asthmatics</td>
<td>346 (60%)</td>
<td>281 (60%)</td>
</tr>
<tr>
<td>Asthmatics</td>
<td>222 (39%)</td>
<td>186 (39.6%)</td>
</tr>
<tr>
<td>Unknown asthma</td>
<td>4 (1%)</td>
<td>2 (0.4%)</td>
</tr>
<tr>
<td>Non-atopics</td>
<td>190 (33%)</td>
<td>161 (34%)</td>
</tr>
<tr>
<td>Atopics</td>
<td>368 (64%)</td>
<td>299 (64%)</td>
</tr>
<tr>
<td>unknown atopy</td>
<td>14 (3%)</td>
<td>9 (2%)</td>
</tr>
</tbody>
</table>
Table 4: Infants’ phenotypic characteristics

<table>
<thead>
<tr>
<th>Phenotype</th>
<th># of positive</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>White</td>
<td>Oriental</td>
<td>Total</td>
</tr>
<tr>
<td>Probable asthma</td>
<td>12 (4%)</td>
<td>1 (4.5%)</td>
<td>35 (7%)</td>
</tr>
<tr>
<td>Possible asthma</td>
<td>29 (10%)</td>
<td>2 (9%)</td>
<td>53 (11%)</td>
</tr>
<tr>
<td>Atopy</td>
<td>47 (17%)</td>
<td>14 (64%)**</td>
<td>104 (21%)</td>
</tr>
<tr>
<td>Dermatitis</td>
<td>18 (6%)</td>
<td>7 (32%)**</td>
<td>47 (10%)</td>
</tr>
<tr>
<td>Rhinitis</td>
<td>63 (22%)</td>
<td>5 (23%)</td>
<td>108 (22%)</td>
</tr>
<tr>
<td>Food (GI)*</td>
<td>50 (18%)</td>
<td>0</td>
<td>72 (15%)</td>
</tr>
<tr>
<td>Urtifood*</td>
<td>13 (5%)</td>
<td>3 (14%)</td>
<td>27 (6%)</td>
</tr>
<tr>
<td>Food skin*</td>
<td>41 (15%)</td>
<td>9 (41%)</td>
<td>88 (18%)</td>
</tr>
</tbody>
</table>

Food (GI)* was defined as a history of vomiting, diarrhea, colic within 4 hours of ingestion of a recognized food allergen.

Urtifood* means urticaria with food triggers.

Food skin* means a positive skin test to at least 1 food antigen.

**P < 0.001 White VS Oriental

Table 5: Number of subjects with both genotypes and phenotypes available for each polymorphism

<table>
<thead>
<tr>
<th>Subjects</th>
<th>TNFα-308</th>
<th>FceRIβ</th>
<th>IL4-590</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Infants</td>
<td>373</td>
<td>371</td>
<td>365</td>
</tr>
<tr>
<td>White infants</td>
<td>281</td>
<td>280</td>
<td>275</td>
</tr>
<tr>
<td>Oriental infants</td>
<td>22</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>White parents</td>
<td>464</td>
<td>464</td>
<td>461</td>
</tr>
<tr>
<td>Oriental parents</td>
<td>63</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td>Non-atopic non-asthmatic</td>
<td>39</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>General population</td>
<td>119</td>
<td>119</td>
<td>119</td>
</tr>
</tbody>
</table>

Table 6: Summary of the three techniques

<table>
<thead>
<tr>
<th></th>
<th>Enzyme digestion</th>
<th>SSO hybridization</th>
<th>ASPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy</td>
<td>superior</td>
<td>good</td>
<td>moderate</td>
</tr>
<tr>
<td>Optimization</td>
<td>easy</td>
<td>difficult</td>
<td>variable</td>
</tr>
<tr>
<td>Time consuming</td>
<td>moderate</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Cost</td>
<td>variable</td>
<td>expensive</td>
<td>cheap</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>good</td>
<td>moderate</td>
<td>good</td>
</tr>
</tbody>
</table>
Table 7: Genotype and allele frequencies of the TNFα-308 polymorphism

<table>
<thead>
<tr>
<th>Population</th>
<th>11 (71%)</th>
<th>12 (28%)</th>
<th>22 (1%)</th>
<th>P value vs Infants</th>
<th>1 (85%)</th>
<th>2 (15%)</th>
<th>P value vs Infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>all Infants</td>
<td>266</td>
<td>104</td>
<td>3</td>
<td></td>
<td>636</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>Non-atopy, non-asthma</td>
<td>31 (79%)</td>
<td>8 (21%)</td>
<td>0</td>
<td>0.28</td>
<td>70 (90%)</td>
<td>8 (10%)</td>
<td>0.059</td>
</tr>
<tr>
<td>General population</td>
<td>82 (69%)</td>
<td>31 (26%)</td>
<td>6 (5%)</td>
<td>0.62</td>
<td>195</td>
<td>43</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Table 8: Genotype and allele frequencies of the TNFα-308 polymorphism

<table>
<thead>
<tr>
<th>Population</th>
<th>11 (70%)</th>
<th>12 (29%)</th>
<th>22 (1%)</th>
<th>P value vs Infants</th>
<th>1 (84%)</th>
<th>2 (16%)</th>
<th>P value vs Infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>White infants</td>
<td>196</td>
<td>82</td>
<td>3</td>
<td></td>
<td>474</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>Non-atopy, non-asthma</td>
<td>31 (79%)</td>
<td>8 (21%)</td>
<td>0</td>
<td>0.21</td>
<td>70 (90%)</td>
<td>8 (10%)</td>
<td>0.21</td>
</tr>
<tr>
<td>General population</td>
<td>82 (69%)</td>
<td>31 (26%)</td>
<td>6 (5%)</td>
<td>0.87</td>
<td>195</td>
<td>43</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Table 9: Genotype and phenotype frequencies for TNF-308 polymorphism in white infants.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Probable asthma</th>
<th>Possible asthma</th>
<th>Positive skin-prick test</th>
</tr>
</thead>
<tbody>
<tr>
<td>12+22 (n=85)</td>
<td>4 (5%)</td>
<td>11 (13%)</td>
<td>10 (12%)</td>
</tr>
<tr>
<td>11 (n=196)</td>
<td>8 (4%)</td>
<td>18 (9%)</td>
<td>37 (19%)</td>
</tr>
</tbody>
</table>

There were no significant differences between any groups.
### Table 10: Genotype and allele frequencies of E237G of FcεRIβ

<table>
<thead>
<tr>
<th>Population</th>
<th>EE</th>
<th>EG</th>
<th>GG</th>
<th>P value vs infants</th>
<th>E</th>
<th>G</th>
<th>P value vs infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Infants</td>
<td>341 (92%)</td>
<td>30 (8%)</td>
<td>0</td>
<td></td>
<td>712 (96%)</td>
<td>30 (4%)</td>
<td></td>
</tr>
<tr>
<td>Non-atopy, non-asthma</td>
<td>38 (91%)</td>
<td>3 (7%)</td>
<td>1 (2%)</td>
<td>0.75</td>
<td>79 (94%)</td>
<td>5 (6%)</td>
<td>0.41</td>
</tr>
<tr>
<td>General population</td>
<td>112 (94%)</td>
<td>6 (5%)</td>
<td>1 (1%)</td>
<td>0.43</td>
<td>230 (97%)</td>
<td>8 (4%)</td>
<td>0.64</td>
</tr>
</tbody>
</table>

### Table 11: Genotype and allele frequencies of E237G of FcεRIβ

<table>
<thead>
<tr>
<th>Population</th>
<th>EE</th>
<th>EG</th>
<th>GG</th>
<th>P value vs infants</th>
<th>E</th>
<th>G</th>
<th>P value vs infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>White infants</td>
<td>262 (94%)</td>
<td>18 (6%)</td>
<td>0</td>
<td></td>
<td>542 (97%)</td>
<td>18 (3%)</td>
<td></td>
</tr>
<tr>
<td>Non-atopy, non-asthma</td>
<td>38 (91%)</td>
<td>3 (7%)</td>
<td>1 (2%)</td>
<td>0.46</td>
<td>79 (94%)</td>
<td>5 (6%)</td>
<td>0.21</td>
</tr>
<tr>
<td>General population</td>
<td>112 (94%)</td>
<td>6 (5%)</td>
<td>1 (1%)</td>
<td>0.84</td>
<td>230 (97%)</td>
<td>8 (4%)</td>
<td>0.91</td>
</tr>
</tbody>
</table>

### Table 12: Genotype and phenotype frequencies for E237G of FcεRIβ in white infants.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Probable asthma</th>
<th>Possible asthma</th>
<th>rhinitis</th>
<th>positive skin-prick test</th>
</tr>
</thead>
<tbody>
<tr>
<td>EG (n=18)</td>
<td>0</td>
<td>1 (6%)</td>
<td>2 (11%)</td>
<td>3 (17%)</td>
</tr>
<tr>
<td>EE (n=262)</td>
<td>12 (5%)</td>
<td>28 (11%)</td>
<td>60 (23%)</td>
<td>44 (17%)</td>
</tr>
</tbody>
</table>

There were no significant differences between any groups.
Table 13: Genotype and allele frequencies of the IL4-590 polymorphism

<table>
<thead>
<tr>
<th>Population</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>P value vs infants</th>
<th>C</th>
<th>T</th>
<th>P value vs infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole infants</td>
<td>232</td>
<td>99</td>
<td>34</td>
<td>563</td>
<td>167</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(64%)</td>
<td>(27%)</td>
<td>(9%)</td>
<td></td>
<td>(77%)</td>
<td>(23%)</td>
<td></td>
</tr>
<tr>
<td>Non-atopy, non-asthma</td>
<td>31</td>
<td>11</td>
<td>0</td>
<td>0.19</td>
<td>73</td>
<td>11</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>(74%)</td>
<td>(26%)</td>
<td></td>
<td></td>
<td>(87%)</td>
<td>(13%)</td>
<td></td>
</tr>
<tr>
<td>General population</td>
<td>87</td>
<td>28</td>
<td>4</td>
<td>0.056</td>
<td>202</td>
<td>36</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>(73%)</td>
<td>(23.5%)</td>
<td>(3.4%)</td>
<td></td>
<td>(85%)</td>
<td>(15%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 14: Whole infant genotype and phenotype frequencies for IL4-590 polymorphism.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT+TT (n=133) 36%</td>
<td>Probable Asthma 14 (11%)</td>
</tr>
<tr>
<td></td>
<td>Possible asthma 8 (6%)</td>
</tr>
<tr>
<td></td>
<td>Rhinitis 31 (24.4%)</td>
</tr>
<tr>
<td></td>
<td>Positive skin test 40 (32%)</td>
</tr>
<tr>
<td>CC (n=232) 64%</td>
<td>Probable Asthma 9 (4%)</td>
</tr>
<tr>
<td></td>
<td>Possible asthma 25 (11%)</td>
</tr>
<tr>
<td></td>
<td>Rhinitis 50 (23%)</td>
</tr>
<tr>
<td></td>
<td>Positive skin test 38 (18%)</td>
</tr>
<tr>
<td>P</td>
<td>0.0023</td>
</tr>
</tbody>
</table>

Table 15: Genotype and allele frequencies for IL4-590 polymorphism in Oriental and white infants

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Genotype</th>
<th>Allele</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CT</td>
<td>TT</td>
</tr>
<tr>
<td>Oriental (n=22)</td>
<td>1 (5%)</td>
<td>7 (32%)</td>
<td>14 (64%)</td>
</tr>
<tr>
<td>White (n=275)</td>
<td>194 (71%)</td>
<td>69 (25%)</td>
<td>12 (4%)</td>
</tr>
</tbody>
</table>

Allele T of IL4-590 was more prevalent in Orientals compared to whites.
Table 16: Genotype and allele frequencies of the IL4-590 polymorphism

<table>
<thead>
<tr>
<th>Population</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>P value vs infants</th>
<th>C</th>
<th>T</th>
<th>P value vs infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>White infants</td>
<td>194 (71%)</td>
<td>69 (25%)</td>
<td>12 (4%)</td>
<td>457 (83%)</td>
<td>93 (17%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-atopy, non-asthma</td>
<td>31 (74%)</td>
<td>11 (26%)</td>
<td>0</td>
<td>0.66</td>
<td>73 (87%)</td>
<td>11 (13%)</td>
<td>0.38</td>
</tr>
<tr>
<td>General population</td>
<td>87 (73%)</td>
<td>28 (23.5%)</td>
<td>4 (3.4%)</td>
<td>0.61</td>
<td>202 (85%)</td>
<td>36 (15%)</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Table 17: Genotype and phenotype frequencies for IL4-590 polymorphism in white and Oriental infants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>*Probable asthma</th>
<th>Possible asthma</th>
<th>Non-asthma</th>
<th>rhinitis</th>
<th>Positive skin-prick test</th>
</tr>
</thead>
<tbody>
<tr>
<td>White</td>
<td>CT+TT (n=81)</td>
<td>7 (9%)</td>
<td>6 (7%)</td>
<td>68 (84%)</td>
<td>19 (23%)</td>
<td>15 (19%)</td>
</tr>
<tr>
<td>White</td>
<td>CC (n=194)</td>
<td>4 (2%)</td>
<td>23 (12%)</td>
<td>167 (86%)</td>
<td>41 (21%)</td>
<td>32 (16%)</td>
</tr>
<tr>
<td>Oriental</td>
<td>CT+TT (n=21)</td>
<td>1 (4.5%)</td>
<td>2 (9%)</td>
<td>19 (86.5%)</td>
<td>5 (24%)</td>
<td>13 (62%)</td>
</tr>
<tr>
<td>Oriental</td>
<td>CC (n=1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The prevalence of probable asthma in White infants with rare allele is significantly higher compared with those with wild type allele (p=0.015).

Table 18: Genotype and allele frequencies for TNFα-308 in the white parents

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Genotype</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Non-asthma</td>
<td>197 (71%)</td>
<td>72 (26%)</td>
</tr>
<tr>
<td>Asthma</td>
<td>137 (74%)</td>
<td>44 (24%)</td>
</tr>
</tbody>
</table>

There were no significant differences between the two groups.
Table 19: Genotype and allele frequencies for TNFα-308 in the oriental parents.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Genotype</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-atopy (n=7)</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>7 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>Atopy (n=25)</td>
<td>18 (72%)</td>
<td>7 (18%)</td>
</tr>
<tr>
<td>non-asthma (n=20)</td>
<td>17 (85%)</td>
<td>3 (15%)</td>
</tr>
<tr>
<td>Asthma (n=11)</td>
<td>7 (64%)</td>
<td>4 (36%)</td>
</tr>
</tbody>
</table>

There were no significant differences between any groups.

Table 20: Genotype and allele frequencies for FcεRIβ*G in white parents.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Genotype</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-atopy (n=160)</td>
<td>EE</td>
<td>EG</td>
</tr>
<tr>
<td></td>
<td>154 (96%)</td>
<td>5 (3%)</td>
</tr>
<tr>
<td>Atopy (n=297)</td>
<td>280 (94%)</td>
<td>17 (6%)</td>
</tr>
<tr>
<td>Non-asthma (n=280)</td>
<td>266 (95%)</td>
<td>13 (4.6%)</td>
</tr>
<tr>
<td>Asthma (n=184)</td>
<td>175 (95%)</td>
<td>9 (5%)</td>
</tr>
</tbody>
</table>

There were no significant differences between any groups.

Table 21: Genotype and allele frequencies for FcεRIβ*G in the oriental parents.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Genotype</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-atopy (n=7)</td>
<td>EE</td>
<td>EG</td>
</tr>
<tr>
<td></td>
<td>6 (86%)</td>
<td>1 (14%)</td>
</tr>
<tr>
<td>Atopy (n=25)</td>
<td>19 (76%)</td>
<td>4 (16%)</td>
</tr>
<tr>
<td>non-asthma (n=20)</td>
<td>16 (80%)</td>
<td>3 (15%)</td>
</tr>
<tr>
<td>Asthma (n=11)</td>
<td>8 (73%)</td>
<td>2 (18%)</td>
</tr>
</tbody>
</table>

There were no significant differences between any groups.
Table 22: The FcɛRIβ*G allele and logIgE levels in the mothers

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>Mean age</th>
<th>LogIgE</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Average</td>
<td>SD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EE (all parent)</td>
<td>183</td>
<td>33</td>
<td>1.54</td>
<td>0.59</td>
</tr>
<tr>
<td>EG +GG (all parents)</td>
<td>15</td>
<td>33</td>
<td>1.88</td>
<td>0.73</td>
</tr>
<tr>
<td>EE (White)</td>
<td>170</td>
<td>33</td>
<td>1.52</td>
<td>0.58</td>
</tr>
<tr>
<td>EG+GG (White)</td>
<td>10</td>
<td>33</td>
<td>1.67</td>
<td>0.76</td>
</tr>
<tr>
<td>EE (Oriental)</td>
<td>13</td>
<td>33</td>
<td>1.70</td>
<td>0.47</td>
</tr>
<tr>
<td>EG (Oriental)</td>
<td>5</td>
<td>34</td>
<td>2.08</td>
<td>0.44</td>
</tr>
</tbody>
</table>

There were no significant differences between any groups.

Table 23: Genotype and allele frequencies for IL4-590 in white parents.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>C</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-asthma (n=278)</td>
<td>193 (69%)</td>
<td>80 (29%)</td>
<td>5 (2%)</td>
<td>466 (84%)</td>
<td>90 (16%)</td>
</tr>
<tr>
<td>Asthma (n=183)</td>
<td>132 (72%)</td>
<td>45 (25%)</td>
<td>6 (3%)</td>
<td>309 (84%)</td>
<td>57 (16%)</td>
</tr>
<tr>
<td>Non-atopy (n=159)</td>
<td>110 (69%)</td>
<td>48 (30%)</td>
<td>1 (1%)</td>
<td>268 (84%)</td>
<td>50 (16%)</td>
</tr>
<tr>
<td>Atopy (n=295)</td>
<td>208 (71%)</td>
<td>77 (26%)</td>
<td>10 (3%)</td>
<td>493 (84%)</td>
<td>97 (16%)</td>
</tr>
</tbody>
</table>

There were no significant differences between any groups.
Table 24: Genotype and allele frequencies for IL4-590 polymorphism in Oriental parents.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Genotype</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CT</td>
</tr>
<tr>
<td>Non-atopy (n=7)</td>
<td>0</td>
<td>4 (57%)</td>
</tr>
<tr>
<td>Atopy (n=25)</td>
<td>0</td>
<td>11 (44%)</td>
</tr>
<tr>
<td>non-asthma (n=20)</td>
<td>0</td>
<td>10 (50%)</td>
</tr>
<tr>
<td>Asthma (n=11)</td>
<td>0</td>
<td>5 (45%)</td>
</tr>
</tbody>
</table>

There were no significant differences between any groups.

Table 25: Genotype and allele frequencies for IL4-590 polymorphism in Oriental and white parents

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Genotype</th>
<th>Allele</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CT</td>
<td>TT</td>
</tr>
<tr>
<td>Oriental (n=32)</td>
<td>0</td>
<td>15 (47%)</td>
<td>17 (53%)</td>
</tr>
<tr>
<td>White (n=454)</td>
<td>318 (70%)</td>
<td>125 (28%)</td>
<td>11 (2%)</td>
</tr>
</tbody>
</table>

Allele T of IL4-590 was more prevalent in Orientals compared to Whites.

Table 26: The IL4-590 polymorphism and log IgE levels in the mothers

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>Mean age</th>
<th>LogIgE</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC(White)</td>
<td>125</td>
<td>33</td>
<td>1.52</td>
<td>0.60</td>
</tr>
<tr>
<td>CT+TT(White)</td>
<td>53</td>
<td>33</td>
<td>1.56</td>
<td>0.56</td>
</tr>
<tr>
<td>CT+TT(Oriental)</td>
<td>18</td>
<td>33</td>
<td>1.80</td>
<td>0.48</td>
</tr>
</tbody>
</table>

* CC genotype not found in the Oriental population. There were no significant differences between any groups.
Table 27: Transmission /Disequilibrium Test for IL4-590*T.

<table>
<thead>
<tr>
<th>Allele T</th>
<th>Transmitted</th>
<th>Non-transmitted</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
<td>24</td>
<td>13</td>
<td>37</td>
</tr>
<tr>
<td>Expected</td>
<td>18.5</td>
<td>18.5</td>
<td>37</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td>0.07</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 5

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