A CLINICAL AND GENETIC STUDY

OF

ATOPIC DERMATITIS

Doctor of Medicine

University of London

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ABSTRACT

Atopic dermatitis is a chronic inflammatory skin disorder, sharing many pathogenic features with allergic asthma and rhinitis. It is a complex multifactorial disease, in which the underlying defect is still to be identified.

62 nuclear families (panel A) were recruited through a proband with active atopic dermatitis. The clinical genetics was defined and relevant genes predisposing to atopic dermatitis identified, using a candidate gene approach. Positive findings were tested in a second panel of 95 families (panel B).

Polymorphisms within the beta chain of the high affinity IgE receptor gene (FceRI-β) on chromosome 11q were found to strongly associate with atopic dermatitis when maternally inherited. Using the transmission disequilibrium test, significant sharing of maternal alleles was found with allele 2 of Rsal intron 2, (FceRI-β Rsal_in2*2) (p=0.0022) and allele 1 of Rsal exon 7 (FceRI-β Rsal_ex7*1) (p=0.0036) and atopic dermatitis. These findings were replicated in panel B. The combined significance of the association of atopic dermatitis to Rsal polymorphisms was p=0.0002 (Rsal_in2*2) and p=0.00034 (Rsal_ex7*1). The polymorphisms also showed association with asthma; p=0.0068 (Rsal_in2*2) and p=0.0018 (Rsal_ex7*1).

Analysis of a single polymorphism within the tumour necrosis factor (TNF) locus (LT-α Ncol) on chromosome 6, showed weak association to atopic dermatitis (p=0.032) and asthma (p=0.024). Atopic dermatitis failed however to associate significantly with a polymorphism within the promoter region of interleukin 4 on chromosome 5q.

This study demonstrates genetic similarities underlying the development of atopic dermatitis and asthma. Both FceRIβ and TNF were identified as potential candidate genes. Furthermore, supportive evidence for a maternal effect was demonstrated.
DECLARATION

I hereby declare that all the clinical and molecular genetic work represented in this thesis was undertaken by me. Dr Jennie Faux from the Asthma Genetics Group in Oxford, carried out the serum total and specific IgE assays, whilst the statistical work was done in collaboration with Dr Bill Cookson and Dr Mark Lathrop. Full and informed written consent was obtained from every family.

Helen Cox MBChB MRCP
ACKNOWLEDGEMENTS

I would like to acknowledge the input of my colleagues at Great Ormond Street Hospital for Children and the John Radcliffe Hospital for their stimulating and helpful discussion and guidance. I am particularly indebted to my supervisors, Dr John Harper and Dr Bill Cookson and to my laboratory colleagues, Dr Miriam Moffat, Dr Jennie Faux, Dr Andrew Walley and Dr Michael Hill. My thanks also to the computer department at the Institute of Child Health for their advice and finally to the children and families for their cheerful support and cooperation.
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<th>Description</th>
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<tr>
<td>AD</td>
<td>atopic dermatitis</td>
</tr>
<tr>
<td>ANE</td>
<td>sodium acetate/ sodium chloride/ disodium ethylene-diamine-tetra-acetate</td>
</tr>
<tr>
<td>AR</td>
<td>allergic rhinitis</td>
</tr>
<tr>
<td>ARMS</td>
<td>amplification refractory mutation system</td>
</tr>
<tr>
<td>APLs</td>
<td>altered peptide ligands</td>
</tr>
<tr>
<td>AS</td>
<td>asthma</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>cM</td>
<td>centimorgan</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP's</td>
<td>deoxyribonucleoside triphosphates</td>
</tr>
<tr>
<td>DZ</td>
<td>dizygotic</td>
</tr>
<tr>
<td>ECP</td>
<td>eosinophil cationic protein</td>
</tr>
<tr>
<td>EDTA</td>
<td>disodium ethylene-diamine-tetra-acetate</td>
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<tr>
<td>ELAM-1</td>
<td>endothelial leukocyte adhesion molecule</td>
</tr>
<tr>
<td>FcεRI</td>
<td>high affinity IgE receptor</td>
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<tr>
<td>FcεRIβ</td>
<td>beta chain of the high affinity IgE receptor</td>
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<tr>
<td>GAS</td>
<td>Genetic Analysis System</td>
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<tr>
<td>GOSH</td>
<td>Great Ormond Street Hospital for Children</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GRR</td>
<td>genotype relative risk</td>
</tr>
<tr>
<td>HLA</td>
<td>human leucocyte antigen</td>
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<tr>
<td>HDM</td>
<td>house dust mite</td>
</tr>
<tr>
<td>IBD</td>
<td>identical by descent</td>
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<tr>
<td>ICAM-1</td>
<td>intracellular adhesion molecule</td>
</tr>
<tr>
<td>ICH</td>
<td>Institute of Child Health</td>
</tr>
<tr>
<td>IRF-1</td>
<td>interferon releasing factor</td>
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<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>IgE</td>
<td>immunoglobulin E</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>JRH</td>
<td>John Radcliffe Hospital</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>LC</td>
<td>langerhans cells</td>
</tr>
<tr>
<td>LMP</td>
<td>low melting point</td>
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<tr>
<td>LT-α</td>
<td>lymphotoxin alpha</td>
</tr>
<tr>
<td>Mg</td>
<td>magnesium</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MZ</td>
<td>monozygotic</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
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<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>RAST</td>
<td>radio immunosorbent test</td>
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<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SPSS</td>
<td>statistical package for social sciences</td>
</tr>
<tr>
<td>SPT</td>
<td>skin prick test</td>
</tr>
<tr>
<td>STI</td>
<td>skin test index</td>
</tr>
<tr>
<td>TBE</td>
<td>0.09M Tris-Borate, 0.002M disodium ethylene-diamine-tetra-acetate</td>
</tr>
<tr>
<td>TDT</td>
<td>transmission disequilibrium test</td>
</tr>
<tr>
<td>Th-1</td>
<td>T helper 1 cell</td>
</tr>
<tr>
<td>Th-2</td>
<td>T helper 2 cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>VNTR</td>
<td>variable non tandem repeat</td>
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Chapter 1

INTRODUCTION
CHAPTER 1

INTRODUCTION

1.1 ATOPY

The term “allergy” was first used by von Pirquet in 1906 to describe the altered reactivity of a host when meeting an allergen on second or subsequent exposure. An explanation for this phenomenon was provided by Prausnitz and Kutsner in 1921 when they showed that the intradermal injection of fish extract into a fish allergic individual elicited an immediate wheal and flare reaction (Prausnitz C and Kustner H 1921). They were later able to demonstrate that this immediate hypersensitivity could be passively transferred to non-allergic individuals by intradermal injection of sera from the allergic subject. These observations were subsequently extended to other allergens.

In 1923 Coca and Cooke introduced the term “atopy”, meaning strange disease, to describe the familial syndrome of atopic dermatitis, asthma and allergic rhinitis. The atopic syndrome was typified by Type I immediate hypersensitivity to certain common innocuous environmental antigens such as house dust and pollens (Coca AF and Cooke RA 1923). Two years later they recognized the presence of 'reagins' as an important component of atopy (Coca AF and Grove E 1925). It was not until the 1960's however, that the atopic reagins were found to correspond to a new class of immunoglobulins, namely Immunoglobulin E (IgE) (Ishizaka K et al 1966) (Johansson S O and Bennich HH 1967).
All individuals are capable of producing IgE antibody in response to large quantities of certain antigens, such as helminthic gut infestations, where IgE plays an important role in the defence against such infections. In contrast, atopic individuals are capable of producing IgE antibody directed at otherwise common environmental allergens. It is this exaggerated propensity to form IgE antibody against environmental antigens that characterizes the atopic state (Ishizaka K 1971).

The syndrome of atopy, which describes the clinical features of atopic dermatitis, asthma and allergic rhinitis is phenotypically complex. There is little doubt that there is a hereditary contribution to these disorders, but as with other complex diseases such as diabetes mellitus and hypertension, they fail to follow simple Mendelian patterns of inheritance. It is likely to be related to more than one gene in the same individual (polygenic inheritance) or different combinations of genes in different individuals (genetic heterogeneity). It is the interaction of these genes with environmental factors that most likely determines the phenotypically complex syndrome of atopy.

Atopy is not synonymous with disease. The factors determining the onset of allergic manifestations are unclear. The reason why the skin should be selected as the target organ for expression of the atopic phenotype in some individuals whilst the lung in others is not known. Early life factors have been identified as important determinants of allergen sensitization, however it is not known how this sensitivity translates to disease expression, chronicity and severity. Genetic studies of atopic
dermatitis and asthma have the potential of increasing our understanding of the disease process and the important interactions between genes and environment. This may lead to earlier diagnosis and prevention and ultimately to the development of better therapies.

This thesis presents work in which the clinical and molecular genetic aspects of atopy and atopic dermatitis are explored in a population recruited through a proband with atopic dermatitis. The importance of a number of clinical determinants influencing disease expression is assessed. Using tests of linkage and association, specific genetic polymorphisms within several candidate gene regions are analyzed for their role in the genetic determination of atopic dermatitis and asthma.
1.2 ATOPIC DERMATITIS

"Atopic dermatitis is a chronically relapsing pruritic disorder in which the dermatitis (or eczema) is typically flexural and lichenified, occurring in individuals with a personal or family history of atopic diseases (that is, asthma, allergic rhinitis or atopic dermatitis)” (Archer 1986).

Historical background

Descriptions date back to the early 1800's, but it was only in 1892 that the syndrome of atopic dermatitis was differentiated from other eczematous and prurigo states by Besnier (Besnier E 1892). He noted its familial nature and association with asthma, hayfever and gastrointestinal disturbances. In 1933, Wise and Sulzberger introduced the term atopic dermatitis, citing nine cardinal features as being important to the diagnosis (Wise F and Sulzberger MB 1933). These included an atopic family history, a history of antecedent infantile eczema, localisation of the rash to flexural areas, grey-brown discoloration of the skin, an absence of vesicles, vasomotor instability, negative patch tests despite the presence of many contact irritants, numerous positive reactions of immediate wheal type to scratch or intradermal testing and the presence of reagins in the serum.

Various other names have since become synonymous with atopic dermatitis, the more notable of these being “neurodermatitis”, “asthma-eczema”, “eczema constitutionnel” and “eczema”. Eczema, derived from the Greek word “eczeo”, “a boiling over”, was originally linked to spongiosis, an important but not constant histiological feature of eczema (Ackerman AB and Ragaz A 1982). The term “atopic
dermatitis" (AD) is now more widely accepted as it describes all the cutaneous features of atopy including those which do not show spongiosis and are not therefore strictly speaking “eczema” (Civatte J 1982).

Natural history and prognosis

Atopic dermatitis is predominantly a disease of childhood with 65% of patients presenting within the first year of life and 93% before the age of 5 years (Rystedt I 1985)(Rajka G 1989)(Queille Roussel C et al. 1985). Most patients improve spontaneously, with 85% of affected individuals experiencing clearance of their lesions by 10 years of age (Vickers CHF 1977). Onset in adult life is rare (Rajka G 1989). Prediction of outcome is difficult as different members of the same family may vary widely in their presentation. Several factors however have been identified which appear to adversely influence prognosis. These include, early age of onset, severe widespread childhood dermatitis, a family history of AD and the coexistence of asthma or allergic rhinitis (Rystedt I 1985).

Diagnostic criteria

As no specific clinical or biological markers exist for atopic dermatitis, the criteria for diagnosis have greatly varied. Hanifin and Rajka compiled a list of 4 major and 23 minor criteria for the diagnosis of atopic dermatitis in 1980, which summarized the American and European points of view (Hanifin JM and Rajka G 1980). A diagnosis of atopic dermatitis depended on the presence of three or more ‘major features’ together with at least three ‘minor features’.
The major criteria included:

- Pruritis
- Typical morphology and distribution;
  - flexural lichenification or linearity in adults
  - facial and extensor involvement in infants and children
- Chronic or chronically relapsing dermatitis
- Personal and family history of atopy (asthma, allergic rhinitis, atopic dermatitis)

The 23 minor criteria listed were less specific for atopic dermatitis and occurred less frequently than the major features (Table 1). A number of doubts were raised regarding the specificity of the criteria with many of the minor features being found in normal individuals with no history of atopic dermatitis or skin disease. Furthermore in those patients with three or more major criteria, 95% were found to have more than six minor criteria (Rudzki E et al. 1994). Substantial between-observer variation was found to occur with eighteen separate physical signs, arguing against the continued use of these criteria in clinical and epidemiological studies (Williams HC et al. 1994).

In an attempt to overcome these difficulties, a UK working party of thirteen dermatologists, two family practitioners and a paediatrician compiled a minimum set of diagnostic criteria for atopic dermatitis, derived from over thirty clinically useful diagnostic features for atopic dermatitis (Williams HC et al. 1994). Using multiple logistic regression techniques, a set of six criteria was derived.
These proposed that an individual must have an itchy skin condition plus three or more of the following:

- history of flexural involvement
- onset of rash under the age of 2 years
- history of generalised dry skin in the past year
- visible flexural dermatitis
- a personal history of asthma/hayfever (or a history of atopic disease in a first degree relative in children younger than 4 years).

These criteria were validated both in an outpatient and hospital setting against a physician’s diagnosis of atopic dermatitis. The overall discrimination of these criteria achieved a sensitivity of 85% and specificity of 96% (Williams HC et al. 1994).

The UK working party’s set of validated criteria were used for the diagnosis of atopic dermatitis in this study.

**Association of atopic dermatitis with asthma, allergic rhinitis and atopy**

Atopic dermatitis is commonly associated with respiratory atopy, an observation which prompted clinicians in the early 1940’s to differentiate AD from other eczematous conditions (Levine B 1973). A personal or family history of asthma/allergic rhinitis is still included as one of the six main diagnostic features of atopic dermatitis. Hanifin and Rajka found that approximately 70% of patients with atopic dermatitis were aware of other family members with one or more manifestations of atopy (Hanifin J M and Rajka G 1980).
The onset of asthma and allergic rhinitis tends to be later than that of atopic dermatitis. Depending on the age of the population studied, between 30-80% of individuals with AD will have associated symptoms of respiratory atopy (Baer RL 1955)(Rajka G 1989). It is interesting to observe that in patients with a primary diagnosis of asthma, only 10-15% will develop atopic dermatitis (Rajka G 1975). Bronchial hyperresponsiveness, the hallmark of asthma, can be a physiopathological feature of AD even in the absence of clinical asthma. Fabrizi et al found that metacholine responders were more likely to be male than female and to show an earlier age of onset of AD. Neither skin test reactivity nor disease severity significantly predicted metacholine responsiveness (Fabrizi G et al. 1992).

Another Japanese study found that a low respiratory threshold to a histamine inhalation challenge significantly predicted the onset of asthma in children with AD between the ages of 2-6 years. Subjects who developed asthma were more likely to have elevated serum IgE levels and eosinophil counts as well as a positive RAST to *D. pteronyssinus* when they were less than 2 years of age (Kayahara M et al. 1994).

Total serum IgE levels are elevated in approximately 80% of patients with AD. The highest levels are found in those with severe skin disease and co-existing respiratory (Juhlin L et al. 1969)(Wittig HJ et al. 1980). The converse is also true in that patients with respiratory atopy and atopic dermatitis have higher levels of IgE than those with respiratory symptoms alone (Kumar L et al. 1971). In the absence of a personal or family history of asthma/allergic rhinitis, the majority of patients with ‘pure AD’ do not have elevated serum IgE levels and if present, the elevation is only slight to moderate (Uehara 1989).
<table>
<thead>
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<th>Must have 3 or more basic features:</th>
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<tr>
<td><em>Pruritis</em></td>
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<tr>
<td>Typical morphology and distribution;</td>
</tr>
<tr>
<td>- Flexural lichenification or linearity in adults</td>
</tr>
<tr>
<td>- Facial and extensor involvement in infants and children</td>
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<tr>
<td>Chronic or chronically relapsing dermatitis</td>
</tr>
<tr>
<td>Personal and family history of atopy (asthma, allergic rhinitis, atopic dermatitis)</td>
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<table>
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<tr>
<th>Plus 3 or more minor features:</th>
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<tr>
<td><em>Xerosis</em></td>
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<tr>
<td><em>Icthyosis palmar hyperlinearity/keratosis pilaris</em></td>
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<tr>
<td><em>Immediate (type I) skin test reactivity</em></td>
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<tr>
<td><em>Elevated serum IgE</em></td>
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<tr>
<td><em>Early age of onset</em></td>
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<tr>
<td>Tendency towards cutaneous infections (esp <em>Staph Aureus</em> and <em>Herpes Simplex</em>),</td>
</tr>
<tr>
<td><em>Impaired cell mediated immunity</em></td>
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<tr>
<td>Tendency towards non-specific hand or foot dermatitis</td>
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<td><em>Nipple eczema</em></td>
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<td><em>Cheilitis</em></td>
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<td><em>Recurrent conjunctivitis</em></td>
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<td><em>Dennie-Morgan infraorbital fold</em></td>
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<td><em>Keratoconus</em></td>
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<td><em>Anterior subcapsular cataracts</em></td>
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<td><em>Orbital darkening</em></td>
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<td><em>Facial pallor facial erythema</em></td>
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<td><em>Pityriasis alba</em></td>
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<td><em>Anterior neck folds</em></td>
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<tr>
<td><em>Itch when sweating</em></td>
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<tr>
<td><em>Intolerance to wool and lipid solvents</em></td>
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<tr>
<td><em>Perifollicular accentuation</em></td>
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<td><em>Food intolerance</em></td>
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<tr>
<td><em>Course influenced by environment/emotional factors</em></td>
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<tr>
<td><em>White dermographism/delayed blanch</em></td>
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Figure 1: Clinical patterns of atopic dermatitis

(i) Facial involvement

(ii) Generalised atopic dermatitis with erythema and excoriations of the trunk and flexural regions
(iii) Typical flexural distribution involving the back of the knees

(iv) Lichenification in chronic AD
There is little agreement on whether the degree of elevation of serum IgE correlates with disease severity. While some investigators have reported a positive relationship between serum IgE levels and the extent and severity of skin disease, others have found no correlation (Johnson EE et al. 1988)(Uehara 1989) (Juhlin L et al 1969) (Mackie RM et al 1979). Serum IgE levels do not appear to be a good marker of disease activity, with levels barely fluctuating during clinical flares and remissions. Johansson and co-workers have proposed that elevated serum IgE levels take at least 2 years to return to normal following total remission of disease (Johansson SGO and Juhlin L 1970). Levels are not influenced by short courses of corticosteroids or azathioprine (Johansson SGO and Juhlin L 1970).

It is not yet known why serum IgE levels are normal in approximately 20% of patients with otherwise typical AD (Juhlin L et al. 1969)(Stone SP et al. 1973). It has been suggested that high and low responders may be genetically determined at birth (Croner S et al. 1982).

**Other diseases with an eczematous component**

Some systemic diseases are associated with cutaneous changes resembling AD, irrespective of the presence or absence of atopy. These disorders can broadly be divided into immune and non-immune disorders. The former category includes Wiskott-Aldrich Syndrome, X-linked agammaglobulinaemia, ataxia telangiectasia and the hyper-IgE syndrome (Rajka G and Langeland T 1989). Non-immune disorders known to be associated with AD include phenylketonuria and anhidrotic ectodermal dysplasia. The reasons for these associations will hopefully become
clearer as we understand more about the complex genetic and immunopathogenic mechanisms underlying AD.

1.3 EPIDEMIOLOGY

Prevalence

There is a worldwide reported increase in the prevalence of AD. Ascertaining the true increase in prevalence however is difficult as methods of ascertainment have not been standardized. For example, some studies have used questionnaires completed by parents, whilst others have insisted on examination by a trained observer. In addition, diagnostic criteria have varied as have measures of disease frequency. Despite these difficulties, one cannot ignore the majority of studies which report an overall increase in the prevalence of AD.

In the 1950’s, prevalence rates for childhood atopic dermatitis ranged between 2 and 3%. (Eriksson-Lihr Z 1955)(Walker RB and Warin P 1956)(Freeman GL and Johnson S 1964). This increased to between 4-8% during the 1960’s (Arbeiter HI 1967)(Turner KJ et al. 1974)(Larsson PA and Liden S 1980). Most researchers in the 1970’s found that between 9-12% of the population developed atopic dermatitis during childhood (Fergusson DM et al. 1982)(Schultz Larsen F, 1986)(Taylor B et al. 1984)(Schultz Larsen F 1993). A Scottish study which recorded the prevalence of AD in children by a physician administered questionnaire was in agreement with this trend, reporting an increase in prevalence from 5.3% in 1969 to 12% in 1989 (Ninan TK and Russell G 1992).
Schultz Larsen looked retrospectively at a number of population studies from around the world, using a measure of disease frequency which included those individuals with a past or present history of AD at around 7 years of age. They confirmed the reported increase in disease frequency from pre-1960 to 1980 (Schultz Larsen F 1993). This was further supported by a rise in the cumulative incidence rate of AD in a Danish twin-study, from 3-9% in the 1960's to 12% in the 1970's and 1980's (Schultz Larsen F 1993).

Studies in the 1990’s, although not standardized for methods of ascertainment or measures of disease frequency, report significantly higher prevalence rates for AD from around the world. Using a physician administered questionnaire to diagnose atopic disease, the prevalence of AD was 18.8% in Southern Australian pre-school children, 17.3% in 8 year old German children, 20.1% in children aged 13-16 years in South East Asia and 25% in an adult Norwegian population (Volkmer RE et al. 1995)(Kuehr J et al. 1992)(Leung R et al. 1994)(Bakke P et al. 1990). In the UK Kay et al found the lifetime prevalence of atopic dermatitis in children aged 3-11 years to be 20% and prevalence in the last year, 12% (Kay J et al. 1994). Other studies have reported a point prevalence for AD of 14% in children aged 1-4 years (Neame R et al. 1995).

This implies that 1 in 7 children in the UK have atopic dermatitis and at least 1 in 5 children will have developed atopic dermatitis by their 11th birthday. Similar figures have been reported for asthma.
Population trends

There have been varying reports of differences in sex, race and social class influencing the development of atopic dermatitis.

Sex: There appears to be no major sex difference in the incidence of AD within the first 2 years of life (Dotterud L K et al. 1995). Later on however the proportion of girls with AD to boys is increased, with most studies reporting a female: male ratio of between 1.2:1 and 2.1:1. (Schnyder U 1960)(Smith LJ and Slavin R G 1988)(Rajka G and Langeland T 1989)(Schultz Larsen F et al. 1986)(Dotterud LK et al. 1995). Other studies have found no differences in the sex distribution at any age (Falk ES 1993).

Race: There is no agreement on whether ethnic variation influences the incidence of AD. In one study, London-born black Caribbean children were found to have a higher prevalence of AD (16.3%) compared with white children (8.7%) (Williams HC et al. 1995). In another UK study, no ethnic variation was found (Neame RL et al. 1995).

Social class: Various trends appear to have existed in relation to prevalence of AD and social class. In 1946 children from social classes I and II in the UK were less likely to have AD than children from lower classes (Taylor B et al. 1984). By 1958 the reverse was true, with children born into higher social classes having higher prevalence rates for eczema. This coincided with an increase in breast feeding. This trend has persisted within the UK, independently of breast feeding practices, with

Month of birth
The first few months of life appear to be an important period for allergic sensitization. Several studies have suggested a relationship between month of birth and the development of skin test responses to aeroallergens and the manifestations of atopic disease. Sensitization to ragweed pollen in college students was higher in those born immediately before the pollen season than in those born during or after the pollen season (Hagy GW and Settipane GA 1971). Several other studies have shown a higher incidence of sensitization to pollens in children born in March through to May (Bjorksten F and Suoniemi I 1976)(Businco L et al. 1988)(Aarlberse RC et al. 1992). Studies in the United Kingdom, Netherlands and Italy have also shown that birth during the Summer and Autumn months is associated with an increased risk of developing sensitization to house dust mite (Sporik R et al. 1990)(Businco L et al. 1988)(de Groot H et al. 1990). Other studies have reported no significant differences among the month of birth distributions in relationship to the development skin sensitivity (Schafer T et al. 1993).

Patients with atopic dermatitis and a positive skin prick test to house dust mite showed a significantly higher prevalence of birth in the interval of May to November. The effect was specific for atopic dermatitis and disappeared when patients with atopic dermatitis and concomitant asthma were analyzed (Beck HI and Hagdrup HK 1987). A similar effect was demonstrated in a German population with
atopic dermatitis with a marked increase in prevalence of AD occurring in patients born during the months August to October (Meffert H et al. 1990).

1.4 THE PHENOTYPE OF ATOPY

Selection of a particular phenotype, by which we mean the observed manifestations of a disease, for genetic studies is important. Differing phenotypes can greatly influence the type of genetic effects found in a population sample, especially when studying complex genetic diseases such as atopy, with a very variable or pleiotropic phenotype. When defining atopy as the presence of one or more positive skin prick tests to common allergens or the detection of elevated total serum IgE, the atopic phenotype is extremely common, occurring in approximately 50% of a young Western adult population. (Cline and Burrows 1989)(Holford Strevens V et al. 1984)(Peat JK et al. 1987). Alternatively, atopy may be defined at the level of the illness e.g. atopic dermatitis, asthma and allergic rhinitis or by the presence of symptoms such as itch and sleep disturbance for AD or wheezing and bronchial hyperresponsiveness for asthma.

Measurements of total and specific serum IgE are readily quantifiable, with well established normal values. For this reason these measures have been favored by geneticists for defining the atopic phenotype. This definition of atopy does not however take into account the 20% of AD sufferers with normal total serum IgE levels.
1.5 CHARACTERISTICS OF ALLERGENS

The majority of atopic patients develop IgE mediated reactions to environmental allergens, particularly in childhood and early adolescence. A wide variety of substances are capable of eliciting an allergic reaction either by inhalation, ingestion or on contact with the skin. These substances need not be present in large quantities with atopic individuals being capable of mounting a brisk type I immune response to minute quantities of antigen, often in the order of micrograms per year (Ishizaka K 1971)(Leung DYM and Geha RS 1986)(Platts-Mill TAE 1987).

Aeroallergens are typically complex mixtures of many proteins, the best characterized aeroallergens being house dust mite (HDM), grass pollen, animal dander (shedding from skin and fur) and fungal spores. The most commonly implicated ingested allergens include proteins found in eggs, cows milk, peanuts, fish, shellfish, soya and wheat.

Within each allergen source, a number of major allergens to which IgE responses are consistently found, have been identified. These include Der p I and Der p II from the house dust mite Dermatophagoides pteronyssinus, Alt a I from the mould Altenaria alternata, Can f I from the dog Canis familiaris, fel d I from the cat felis domesticus and Phl p V from Timothy grass, Phleum pratense.

Although sensitization to an allergen can occur at any time in life the likelihood of this occurring is dependent on a number of factors. These include the level of genetic susceptibility, the potency of the relevant allergen, the concentration and
duration of exposure and the degree of exposure to adjuvant factors. In addition the timing of allergen exposure appears to be crucial in determining sensitivity to that allergen in later life. The initial encounter with allergen in early infancy has been shown to be a critical time for primary sensitization to occur (Holt PG et al 1990). Conversely, later on in life the degree of susceptibility declines, although sensitization can still occur to an allergen encountered for the first time in adult life as occurs for instance in an occupational setting (Newman Taylor AJ 1980).

1.6 MEASUREMENT OF IgE RESPONSES

Measurement of total serum IgE

Since IgE was identified by Ishizaka and Johnson et al in the 1960’s, a large number of variables have been demonstrated to independently modify the IgE response, the most important of these being age, sex and cigarette smoking. Serum IgE levels rise towards the end of the first decade followed by a rapid decline in teenage years and a continuous decline throughout adult life. (Barbee RA et al. 1981)(Friedhoff LR et al. 1984). A similar age related decline was not observed in non-atopic adults (Holford Strevens V et al 984). Atopic males have consistently demonstrated higher IgE levels than females and this effect has been shown to occur independently of smoking (Barbee RA et al. 1987)(Friedhoff LR et al. 1984). The smoking-IgE relationship is now well documented with higher levels of IgE occurring in individuals who smoke. These observations have been taken into account when defining normal population values for serum IgE.
The paper radioimmunosorbent test (PRIST) is used to measure total serum IgE levels. This is a modification of the earlier radioimmunoassay, being more sensitive at detecting low levels of IgE (Kjellman N and Johansson SGO 1976). Serum is incubated with anti-IgE covalently coupled to a paper disc. After washing, enzyme labeled antibodies against IgE are added, which bind to the IgE on the paper disc. After washing, the disc is incubated with a developing agent, and the absorbency of a yellow product is measured. The absorbency is directly proportional to the concentration of IgE antibodies in the sample.

The total serum IgE is an attractive parameter for genetic studies in atopy, as it has well established normal values and correlates well with the presence of atopic dermatitis and asthma. However in genetic studies observing the inheritance of asthma, as much as 45% of the variation in total serum IgE has been attributed to specific IgE to HDM and grass pollen (CooksonWOCM et al. 1991). Furthermore, when multiple regressions are carried out on population data, asthma and bronchial hyper-responsiveness are found to relate to the specific IgE and fail to correlate with the residual IgE (obtained by removing the specific IgE). This may suggest that measurements of specific IgE are more suitable for genetic analysis in asthma. This does not necessarily apply to atopic dermatitis.

There are few studies which correlate atopic dermatitis and serum IgE to variation in IgE-RAST scores. Patients with positive specific IgE tests tend to have significantly higher serum IgE levels those with negative tests (Thompson RA and AG 1983). However, this is not always the case, with high specific IgE levels being found in
patients with as little as 40 IU/ml total IgE (Merrett TG et al. 1980). A Japanese study looking at the serum total IgE and IgE RAST scores to HDM and several food allergens in patients with AD, found the high values of total IgE to depend on the presence of specific IgE antibody to HDM at all ages and to cows milk and egg white during infancy (Fujisawa S et al. 1989). No studies in AD patients have looked at the correlation of AD to measurements of residual serum IgE.

**Measurement of specific serum IgE**

The prototype for in vitro analysis of IgE antibody in serum has been the radio-allergo-sorbent assay (RAST), first described by Wide et al in 1967 (Wide L et al. 1967). The first commercially available test was produced by Pharmacia Laboratories (Uppsala, Sweden) but has since been copied by several other laboratories. Differences between the various commercially available in vitro IgE tests have lead to a wide variation in results, making it difficult to compare the results of one assay with that of another. This is explained in part by differences in the allergen extracts used, the variability with which each technique insolubilizes the allergenic molecules in a mixture, the recommended incubation times, the anti-IgE antibody used in each assay and the threshold criterion recommended by each manufacturer (Statement P 1992). Standardization of RAST testing is of utmost importance in genetic studies.

RAST testing involves immobilising the allergen onto an insoluble matrix such as plastic, cellulose nitrate, cellulose or agarose beads and incubating the allergen with sera from allergic patients. IgE antibody then binds to the immobilised allergen and
the complex is detected by incubating it with a radioactively labeled antibody specific for IgE. The degree of IgE binding is determined by measuring the radiolabel (usually $^{125}$I). The greater the binding the greater the allergenicity (Holgate ST and Church MK 1993).

The prevalence of positive RAST tests in children with AD varies according to age. 63% and 58% of children under the age of 3 years demonstrate positive RAST tests to common inhalant and food allergens in comparison with 82% and 43% for children between 4 and 15 years. Thus the frequency of positive RASTs to food allergens decreases with age while the frequency of positive RASTs to inhalants increases. The same investigators showed that the coexistence of respiratory atopy with AD increased the prevalence of RAST positivity to inhalant allergens but not to food allergens (Rudzki E and Litewska D 1990).

The most common allergens giving rise to specific IgE antibody production in patients with AD are HDM, grass pollen and cat epithelium (Merrett TG, Pantin CFA et al. 1980)(Mackie RM et al. 1979). AD patients differ from patients with asthma and rhinitis in that they are more likely to develop multiple positive RASTs to common allergens and fail to show a seasonal variation in their RAST response to grass pollen (Mackie RM et al. 1979)(Kumar L et al. 1971)(Yunginger JW and Gleich GJ 1973).

**Skin prick testing**
A positive skin prick test is dependent not only on IgE antibody but on a number of allergen dependent and patient dependent factors. These include the biologic activity of the allergen extract used as well as the local integrity of mast cells and vascular and neural responsiveness. In practice however, a positive skin prick test is believed to reflect largely the presence of specific IgE antibody.

Methods for performing skin tests have varied. Scratch testing, intradermal puncture and prick testing have all been evaluated. The skin prick test (SPT) is generally accepted as the method of choice for the diagnosis of immediate hypersensitivity. In these tests a dilute solution of allergen, suspended in a glycerin solution, is placed on the skin together with a negative control. A sterile lancet is used to introduce the allergen in minute quantities into the dermis. In sensitized individuals, an immediate type I hypersensitivity response is elicited, following interaction with antigen and specific IgE attached to the surface of mast cells. This triggers mast cell degranulation and subsequent vascular permeability, local oedema and itching producing a wheal and flare reaction within 15 minutes. The minimum allergen dose required to illicit a skin test response is > 0.01pg of allergen extract. Extrapolating from studies which have looked at the minimum histamine dosed required to produce a 10mm wheal, the 0.01 pg limit means that for an allergen with a molecular weight of 25kd, at least 50 to 100 allergen molecules per mast cell are needed for a positive intracutaneous skin test result. This means in real terms that about 5000 to 10 000 mast cells must degranulate for the test to become positive (Witteman AM, Stapel SO et al. 1996).
The cut-off point for grading skin test positivity is critical. Many would only accept a wheal diameter of 3mm or more than the negative control, as being positive. Others would accept smaller responses in children under 2 years of age where the SPT reactivity is known to be diminished (Zeiger RS 1985). This most probably reflects their relative immunological immaturity or reduced exposure to environmental allergen. It has been shown that a true reaction diameter of 3mm represents a tenfold greater concentration of specific IgE than a true reaction diameter of 2mm. (Dreborg S 1988).

Most studies have found that skin test reactivity is acquired progressively during childhood, peaking between 15 and 25 years of age and declining gradually thereafter, until at 65 years of age it is half that at 30 (Barbee RA et al. 1987). There is no agreement on whether sex influences SPT reactivity with some studies reporting increased reactivity in males (Haahtela TMK et al. 1980)(Friedhoff LR et al. 1984). Seasonal variability of the SPT response was suggested by Kumar et al (Kumar L et al. 1971) but not supported by other studies which showed no variation in SPT reactivity according to the month of testing.

In the hands of experienced physicians, skin testing provides acceptable diagnostic information being sufficiently sensitive and specific to confirm the diagnosis of most inhalant allergens. The reproducibility of such tests however is variable with a 5mm diameter response having a 95% confidence interval of 3.1 to 6.9mm and a 4mm diameter having a 95% confidence interval of 2.3 to 5.7mm (Haahtela T 1989). Witteman et al found the skin test allergen threshold to differ by as much as a factor of 100 between patients with similar levels of specific IgE. They proposed the existence of a number of patient dependent factors contributing to the outcome of the
skin test response. These included the presence of a high total serum IgE, differences in mast cell releasability and the affinity of the IgE response. They proposed that only biologically standardized extracts should be used with known performance parameters (sensitivity, specificity, precision and reproducibility). This would reduce any discrepancies in IgE by a factor of 10 (Witteman AM et al 1996).

In patients with active atopic dermatitis the diagnostic value of skin prick testing has been questioned. In one study when patients with AD and allergic rhinitis were skin tested at different sites and at weekly intervals to a panel of common allergens, the overall reproducibility of prick tests was good in patients with mild to moderate skin disease but less reproducible in patients with severe skin disease (Wagenpfeil S et al. 1991). In patients with severe skin disease, the local release of inflammatory mediators was thought to have interfered with the skin test response leading to a number of false positives. Certainly oral antihistamines inhibit the skin test response and need to be discontinued 24-48 hours before testing (Slavin RG 1974).

Skin prick positivity is common with approximately 50% of the UK population demonstrating sensitivity to at least one allergen (Godfrey RC and Griffiths M 1976). Positive skin tests may be present before clinical manifestations of allergy have developed and persist long after clinical symptoms have subsided. Of those individuals testing skin prick test positive, at least half will have clinical symptoms of AD, asthma, allergic rhinitis or food allergy (Frew AJ 1992). One study has shown that clinically asymptomatic children with positive skin prick tests have a 50% risk of developing allergic rhinitis and a 5% risk of developing asthma when followed over a 5 year period (Horak F 1985).
Correlation between skin prick tests and RASTs

Positive skin tests correlate well with serum measures of specific IgE. A minimum wheal diameter of 5mm has been shown to predict RAST positivity (Frew AJ 1992). This clearly represents a much higher specific IgE concentration than the normally accepted 3mm cut-off. At higher specific IgE measurements the skin-test end point concentration tends to level off. Berg and Johansson found an overall agreement between skin prick testing and specific IgE in 92% of patients with SPT’s > 4mm and 37% for patients with SPT’s < 3mm (Berg TLO and Johansson SGO 1974). Other investigators have found SPTs and RASTs to similarly correlate (Godfrey RC and Griffiths M 1976)(Thompson RA 1983)(Holford Strevens V et al. 1984).

1.7 MECHANISM OF THE ALLERGIC RESPONSE

Regulation of IgE synthesis

Since the identification of IgE as a separate class of antibodies, there have been enormous advances in our understanding of the central role of this molecule in immediate hypersensitivity reactions and the mechanisms by which it is regulated. IgE is capable of binding to high affinity receptors, FceRI on basophils, mast cells and Langerhans cells, as well as low affinity receptors, FceRII(CD23) on a variety of effector cells (Table II)(Leung DYM 1994). These two receptors are both structurally and functionally distinct. Whilst FceRI appears to be of major importance in the initiation of the allergic response, the role of FceRII(CD23) is less well defined. It is thought to influence the regulation of IgE in allergic disease, although its exact functioning is unclear.
Crosslinking of antigen-specific IgE to FceRI triggers the IgE response, leading to the degranulation of mast cells and basophils with the release of preformed inflammatory mediators such as histamine, proteases, proteoglycans, chemotactic factors and cytokines (Serafin WE and Austen KF 1987)(Ishizaka T and Ishizaka K 1975). Once activated, mast cells and basophils help perpetuate the inflammatory response by providing a positive feedback loop through the release of a number of cytokines including interleukin 1 (IL1), IL3, IL-4, IL-5, IL-6, IL-10, IL-13, granulocyte colony-stimulatory factor (GM-CSF) and tumour necrosis factor-α (TNF-α), thereby maintaining antigen-specific IgE production. IL-4 is particularly important in regulating IgE synthesis, acting as an IgE-isotype specific switch factor controlling the differentiation of B lymphocytes from immature to mature cells (Galli SJ et al. 1991)(Plaut M et al. 1989). This occurs in conjunction with a variety of B cell activators and T cells (Vercelli D et al. 1989).
Role of T cells in the IgE response

The mechanism whereby T cells interact with B cells to induce IgE synthesis can be divided into cognate and non-cognate reactions. Cognate reactions depend on the recognition of the T cell receptor complex (TCR) of antigen bound to a molecule of the major histocompatibility complex (MHC-class II) on the surface of B cells. The antigen-MHC complex is presented to antigen specific CD4+ T cells by antigen presenting cells. Following engagement of the TCR the release of lymphokines, including IL-4 is stimulated leading to further B cell proliferation and production of antigen-specific IgE (Snapper CM and Paul WE 1987)(Pene J et al 1988).

Non-cognate reactions, in which the TCR does not recognise the B cell MHC-antigen complex, have also been shown to support IL-4 dependent IgE synthesis Parronchi P et al. 1990). These T cell-B cell interactions are mediated by the transmembrane receptor molecule CD40 and its ligand, expressed on activated T cells. Interestingly, B cells from patients with AD have increased expression of CD40 on their cell surface and can be activated by monoclonal anti-CD40 antibodies to produce IgE in vitro, even in the absence of IL-4 (Brodie C et al. 1992). Furthermore the absence of CD40-ligand on T cells in patients with hyper IGM syndrome, leads to the failure of IgE synthesis in the presence of IL-4 (Fuleihan R et al. 1986).

Several T cell independent B cell activators can act in combination with IL-4 to induce IgE synthesis. Examples of this include Epstein Barr Virus and
hydrocortisone, both of which appear to synergise with IL-4, inducing IgE synthesis (Thyphronitis G et al. 1989) (Jabara HH et al. 1991).

**Cytokine regulation of the allergic response**

From Mosmann’s initial studies in mice (Mosmann TR and Coffman RI 1989), T helper cells in humans have been designated as being either Th1-like or Th2-like depending on their cytokine-secretion profiles. Th2 clones secrete high amounts of IL-4, IL-5, IL-6 and IL-10 and low amounts of IFNγ and are associated with high and persistent antibody responses, particularly of IgE and eosinophilia. These responses are important for the defense of extracellular organisms, such as parasitic infections in healthy individuals. Th1 clones preferentially secrete IFNγ, IL2 and lymphotoxin but minimum amounts of IL-4 and are characterised by antibody production and strong delayed-type hypersensitivity reactions (Wierenga EA et al. 1990)(Wierenga EA et al. 1991)(Kapsenberg ML et al. 1991). This type of response is important in the pathogenesis of organ-specific autoimmune disorders, rejection of allotransplants and contact dermatitis. In allergic disorders such as AD, preferential expansion of Th2 cell clones occurs leading to the increased synthesis of IgE, mediated by IL-4 and to the differentiation and proliferation of eosinophils, mediated by IL-5. In vitro studies looking at the cytokine secretion profiles of CD4+ T lymphocytes specific for *dermatophagoides pteronyssinus* as atopen and tetanus toxoid and candida albicans as non-atopic antigens, have shown preferential secretion of IL-4, IL-5, IL-6 and TNFα but minimal amounts of IFN-γ and IL-2 from the *D. pteronyssinus*-specific T cell clones from AD patients. By contrast non-atopic T cell clones secreted substantial IFN-γ, IL-2, TNFα and GM-CSF but no IL-4 and
little IL-5 upon antigen stimulation (Wierenga EA et al. 1990)(Wierenga EA et al. 1991). Moreover the allergen specific Th-2 cells could induce IgE production by normal B cells in vitro.

Similar findings were obtained when skin infiltrating lymphocytes from biopsies of acutely eczematous skin in patients with AD were investigated. These cells secreted significant amounts of IL-4, GM-CSF and TNFα upon mitogen stimulation, but failed to secrete IFN-γ (Reinhold U et al. 1991). Random cloning of CD4+ T cells from lesional skin in patients with AD sensitised to D. pteronyssinus, revealed a high frequency of allergen-specific Th-2 like lymphocyte clones (Van der Heijden FL et al. 1991).

The actions of IL-4 and IFN-γ are antagonistic to one another with IFN-γ suppressing IL-4 synthesis and vice versa (Leung DYM 1994). In-vitro studies have shown that the addition of IL-4 to lymphocytes from AD patients undergoing activation results in down-regulation of IFN-γ production and increased IgE synthesis. Conversely, the activation of T lymphocytes in the presence of IFN-γ significantly reduced both IL-4 receptor expression and IL-4 production (Renz H et al. 1992)(Jujo K et al. 1992). PBMC from AD patients secrete reduced amounts of IFN-γ when stimulated (Reinhold U et al. 1990). It is therefore perhaps surprising that the percentage of IFN-γ producing cells in unstimulated cultures from AD patients compared with non-atopic controls is increased. This suggests in-vivo activation of the patients lymphocytes (Tang M and Kemp A 1994).
This altered balance of cytokine production has important functional consequences with the combined effects of elevated IL-4 synthesis and defective production of IFN-γ, promoting B cell synthesis of IgE, expansion of Th2 lymphocytes and the secretion of pro-inflammatory cytokines which further upregulate the allergic response.

**A pathogenic role for IgE in atopic dermatitis**

A significant advance in our understanding of the pathogenesis of AD was brought about by the discovery of FcεRI expression on the surface of human epidermal Langerhans cells (Bieber T and Ring J 1992). Subsequently, patients with AD were shown to have increased numbers of FcεRI-bearing Langerhans cells when compared with non-atopic individuals. It is recognised that these cells play an important role in allergen presentation, amplifying the immune response so that only very small amounts of antigen are needed to provoke an allergic response (Van der Heijden FL et al. 1993). Certainly it has been shown that only IgE positive Langerhans cells are capable of presenting house dust mite in patients with AD (Mudde GC et al. 1990; Mudde GC et al. 1992).

In the absence of antigen, activation of IgE bearing Langerhans cells does occur in patients with AD but through a different mechanism. These patients possess circulating autoantibodies to IgE which are capable of binding to IgE bearing Langerhans cells (Quinti I et al. 1986). This has been proposed as one mechanism whereby the inflammatory process is self-perpetuated in chronic AD.
1.7 MECHANISM OF CUTANEOUS INFLAMMATION IN AD

Skin reactions in atopic dermatitis

Intense pruritis and cutaneous reactivity is the hallmark of AD (Rajka G 1968). The pathogenesis of the pruritis is poorly understood but leads to a vicious cycle of itching, scratching and aggravation of the eczematous lesions. Skin reactions are typified by acute, subacute and chronic lesions. Acute skin reactions consist of pruritic erythematous papular vesicles overlying erythematous skin with associated excoriations, erosions and serous exudate. Subacute lesions are typified by scattered or grouped, less intense erythematous papules and plaques with excoriations and scaling. Chronic dermatitis on the other hand, is characterized by skin thickening secondary to chronic scratching and rubbing of the skin. All three stages frequently co-exist in the same patient. (Leung DYM 1992).

The distribution and pattern of cutaneous reactivity varies with age. In infancy the rash is more acute, involving predominantly the face, scalp, and extensor surfaces of the extremities. At about 18-24 months, the rash becomes more chronic in nature localising mainly to the flexural regions, affecting commonly the popliteal and cubital flexures, wrists, ankles and neck. Variant distribution patterns do occur with some individuals demonstrating predominantly extensor surface involvement, others exclusive involvement of their hands and feet and a small but distinct group who present with discrete discoid lesions (nummular eczema). The anatomical basis for these variances in distribution is unknown.
The inflammatory infiltrate

Differences are depicted in the type of cellular infiltrate seen in acute and chronic lesional AD skin. Biopsies of acute eczematous lesions demonstrate an infiltrate consisting predominantly of CD4+ T lymphocytes with occasional monocytes-macrophages and normal numbers of hypogranulated mast cells. The inflammatory infiltrate in chronic eczematous lesions on the other hand, is more typical of a delayed type hypersensitivity response with the mononuclear infiltrate consisting predominantly of CD4+ T cells, monocyte/macrophages and increased numbers of fully granulated mast cells (Mihm MC et al. 1976)(Soter NA 1989)(Sugiura H and Uehara M 1993).

Histologically chronic AD resembles allergic contact dermatitis apart from a number of important distinguishing features. Firstly, allergen specific T cells isolated from AD skin lesions are of the Th-2 type, secreting high amounts of IL-4 and no IFN-γ (Van der Heijden FL et al 1991). This is in contrast to the HLA-DR antigen expression seen in the keratinocytes of patients with contact dermatitis which determines IFN-γ production (Barker JNWN and MacDonald DM 1987). Secondly, AD skin lesions are associated with the extensive deposition of eosinophilic cationic protein (ECP), a feature not present in contact dermatitis. (Leiferman KM et al. 1985).

Observation of the inflammatory responses following patch testing in atopic individuals has given further insight into the pathogenic mechanisms underlying AD. T cells cloned within the first 12-24 hours are predominantly Th-2 in type, producing
IL-4 and IL-5 (Friedmann PS et al. 1995). In keeping with this observation is the detection of IL-4 and IL-5 by polymerase chain reaction up to 24 hours after intradermal challenge with house dust mite. At later time points however, Th-1 cells and their cytokines predominate (Friedmann PS et al. 1995). Thus it is postulated that Th-2 cytokines are critical for the initiation of AD, while Th-1 cytokines, such as IFN-γ, are important for the propagation of the inflammatory process (Kapp A 1995).

One study investigating the cytokine profiles of skin biopsies from acute and chronic skin lesions of patients with AD, demonstrated increased numbers of cells positive for mRNA IL-4 and IL-5 but not for IFN-γ when compared to normal controls (Hamid Q et al. 1994). Chronic lesions expressed significantly greater numbers of IL-5 mRNA bearing cells and fewer IL-4 mRNA cells as compared with acute lesions and increased amounts of activated EG2+ cells (a marker of eosinophil activation). Thus IL-4 driven responses dominated the initiation of acute skin inflammation, whereas increased IL-5 expression lead to the maintenance of chronic inflammatory changes with the infiltration of eosinophils.

Eosinophils are active proinflammatory cells found in increased numbers in the peripheral blood of patients with AD, their degree of activation correlating well with disease severity (Rajka G 1989). The development of an eczematous skin reaction following patch testing with inhalant allergens is associated with the infiltration of activated eosinophils. At odds with this is the lack of accumulation of eosinophils in the biopsies of chronic eczematous skin when using hematoxylin-eosin staining.
This is most likely due to the loss of morphological identity following eosinophil disruption (Leiferman KM et al. 1985) (Bosquet J et al. 1990).

Chronic AD is associated with extensive dermal deposition of eosinophil cationic protein (ECP), a toxic protein contained within the secondary granules of eosinophils (Leiferman KM et al. 1985). ECP is secreted during allergic inflammation and may contribute to the propagation of the allergic response by inducing the release of histamine from mast cells and mediating the destruction of epithelial cells and possibly keratinocytes (Leiferman KM 1991). Elevated serum levels of ECP correlate well with disease activity (Czech W et al. 1992) but there is no correlation between measurements of ECP and blood eosinophil counts (Kagi MK, et al. 1992). It is tempting to conclude that measurements of ECP reflect the activation state of the whole eosinophil pool and could therefore be useful in monitoring disease activity in patients with chronic AD (Kapp A 1995).

Another important component of the inflammatory process in chronic AD is the activation of leucocyte adhesion molecules such as ELAM-1, VCAM-1 and ICAM-1 by the cytokines IL-1 and TNF-α (Bochner B et al. 1990; Bochner BS et al. 1991). These molecules influence the migration of inflammatory cells to sites of allergic inflammation. The expression of ELAM-1 contributes to the migration of T helper lymphocytes with these cells selectively expressing the homing receptor, cutaneous lymphoid antigen (CLA), a ligand for ELAM-1. The expression of VCAM-1 is thought to play a role in attracting eosinophils to local tissue sites, with eosinophils selectively expressing VLA-4, the ligand for VCAM-1 (Rossiter H F et al. 1992).
The induction of these molecules is under the control of IL-1 and TNF. Antibodies which neutralize IL-1 and TNF have been shown to block the induction of leukocyte adhesion molecules in allergen stimulated skin explants (Leung DYM et al. 1991). Thus the release of such cytokines may represent an important regulatory event in the local accumulation of inflammatory cells at the site of allergic reactions.

Factors contributing to chronicity in atopic dermatitis

The chronic relapsing nature of AD cannot be explained entirely on the basis of abnormal IgE responses to environmental allergens, although repeated exposure to antigen with the triggering of receptor bound IgE and Th2 cell expansion is almost certainly a factor. Circulating autoantibodies capable of binding to IgE bearing Langerhans cells, have already been mentioned as a mechanism whereby the inflammatory response in the absence of antigen is perpetuated (Quinti I et al. 1986). Other investigators have looked for evidence of immune deficiency to account for the changes seen in chronic AD. Within lesional AD skin there certainly is evidence of decreased cell-mediated immunity (Bos JD et al. 1992). This is manifested as an increased susceptibility to skin infection with viruses and chronic dermatophyte infections, reduced responsiveness to contact allergens such as poison ivy, cutaneous anergy to intracutaneous tests with several allergens and a decreased sensitization to dinitrochlorobenzene. These defects however are confined to the skin with no evidence of any significant abnormality of systemic cell-mediated immunity. Furthermore, despite the partial defect in the effector responses of the skin immune
system, vigorous T-cell responses do occur within lesional AD skin serving to perpetuate the inflammatory response.

1.9 GENETIC STUDIES IN ALLERGIC DISORDERS

Research into the genetics of atopy began 80 years ago with the recognition of a familial component to allergic disease by Cooke and Van der Veer. Since then, the conflicting conclusions of a large number of family and twin-based studies has led to atopy being defined as a complex genetic disease with no simple Mendelian pattern of inheritance. This complexity is thought to represent the interaction of environmental factors in an individual genetically predisposed to the development of atopy.

Twin studies

Familial aggregation of disease is not necessarily genetic and may be influenced by environmental factors. In order to assess the relative influences of inheritance and environment, the concordance rates between monozygotic (MZ) and dizygotic (DZ) twins can be compared. A disease which is at least partially genetically determined is expected to show a greater degree of concordance in genetically identical MZ twins compared to DZ twins (Price B 1950). Any reduction in concordance from 100% in MZ twins is thought to reflect the degree to which environmental factors have contributed to the disease (Ott J 1991). Concordance of a trait in twins will also be influenced by the very similar environment that they share. One way to separate
the effects of shared genes and environment is to determine concordance in twins
reared together and apart.

Various twin studies have identified a substantial genetic contribution to the
aetiology of atopic disease. Concordance rates of 82% and 52% for total serum IgE
in MZ twins and DZ twins have been reported (Hopp RJ et al. 1984)(Wuthrich B et
al. 1981). Furthermore, no differences in total serum IgE were found in MZ twins
reared together and apart thus eliminating the confounding factors of a shared
environment (Hanson BM et al. 1991). Specific allergic sensitivities as measured by
skin prick tests or specific IgE assay (RAST) were found conversely to be governed
mainly by environmental factors (Wuthrich B et al. 1981).

Twin studies have on the whole supported the role of genetics in the aetiology of AD
with concordance rates of 77% and 15% in MZ and DZ twins respectively (Schultz
Larsen F et al. 1986). Several earlier studies reporting lower concordance rates for
AD in MZ twins and DZ twins had been criticized for being too small(Rajka G
1989). One large Swedish study which examined twin concordance for AD and
atopy in 7000 twin pairs, reported modest concordance rates of 15.4% and 4.5% for
MZ and DZ twins respectively (Edfors Lubs ML 1971). This study however was
carried out by mailed questionnaire and confined to adult twin pairs, where the
lifetime prevalence for AD was only 2.5%. These figures probably represent an
underestimation.
**Segregation analysis**

Segregation analysis is a method of determining the pattern of inheritance of a disorder by observing its distribution within families. Multiple families or single large families are used to examine the distribution of a trait and to compare the observed frequency of the trait in offspring and siblings to the distribution expected with various modes of inheritance. Both simple models of inheritance, such as autosomal dominant and recessive models, as well as more complicated models of inheritance, such as polygenic and multifactorial inheritance can be constructed. The analysis may be further complicated by the finding of incomplete penetrance with penetrance being defined as the probability of observing the phenotype given the specific genotype. When the penetrance of a trait is less than 1, incomplete penetrance is said to have occurred. Segregation analysis has been widely used in an attempt to determine the mode of inheritance of atopy, AD and asthma.

The initial proposal of a simple autosomal dominant Mendelian pattern of inheritance for allergy was based on the work of Cooke and Van der Veer, who found that if one parent was allergic then 50% of the offspring were affected, and that if both parents were allergic, then so too were 75% of their children. Their findings were disputed as they failed to explain the existence of families with atopic children and normal non-atopic parents. This led to the proposal of a model of dominant inheritance with incomplete penetrance for the syndrome of atopy (Schwartz M 1952)(Wiener A et al. 1936).
These early genetic studies looked at the inheritance of atopy as a whole syndrome, without reducing it to its component parts. Later studies which concentrated on the inheritance of specific phenotypes such as AD, asthma or measurements of specific or total IgE have been more difficult to define with autosomal dominant, dominant with incomplete penetrance, recessive and polygenic modes of inheritance all being suggested.

After the discovery that reaginic activity was mediated by IgE, an elevated total serum IgE rather than the presence of allergic symptoms, was used as a marker for atopy in genetic studies. This readily quantifiable measurement avoided the difficulties encountered with the use of clinical phenotypes, where the diagnosis was subject to observer variation and bias. Initial studies tried to distinguish between high and low IgE responders with attempts by some groups to control for the boosting effect of seasonal allergens. A recessive pattern of inheritance for high IgE levels was proposed by Marsh et al (Marsh DG et al. 1974) whilst Meyers (Meyers DA et al. 1994) suggested a major recessive locus acting together with a more polygenic component to account for the variances in IgE. Age, smoking and time of sampling with relation to seasons were all confounding variables. In addition no attempt was made to account for non-seasonal allergens such as house dust mite, known to significantly contribute to the elevation of total serum IgE. The work of Gerrard et al (Gerrard JW et al. 1978) also proposed a major locus controlling IgE levels with a recessive allele determining high IgE levels.
Deviating from previous nuclear family studies, Blumenthal et al (Blumenthal MN et al. 1981) were the first to study the inheritance of high levels of IgE in several large pedigrees. Taken together, their data failed to demonstrate either a monogenic or environmental component to the inheritance of this trait. However, when each of the seven families were looked at individually, the pattern of inheritance of high IgE levels was suggestive of a dominant model in two families whilst a recessive model best fitted the remaining five families. These results implied heterogeneity in the inheritance of atopy. Other genes were thought to be influencing the inheritance of the high IgE trait, thus accounting for its failure to inherit in simple Mendelian fashion.

Cookson and Hopkin in 1988 (Cookson WOCM and Hopkin J 1988) were the first group to study the inheritance of atopy defined by the presence of elevated total serum IgE levels and/or the presence of specific IgE to common allergens. This broader definition of atopy differed from previous definitions of atopy with as many as 50% of individuals in the Cookson study demonstrating positive specific IgE measurements to environmental allergens in the absence of elevated total serum IgE. Their study, which looked at 40 nuclear families and three extended pedigrees suggested that there was a major genetic component to atopy. The absence of atopic parents in 10% of their atopic subjects was attributed to dominant inheritance with incomplete penetrance.
Maternal effects

The possibility of a maternal effect in the transmission of atopy has been proposed by a number of investigators. Retrospective studies have found allergic disease to be transmitted twice as often from allergic mothers than from allergic fathers (Bray GW 1931) and asthmatic mothers to have more asthmatic children than asthmatic fathers (Happle R and Schnyder UW 1982).

Prospective studies have produced variable results. Croner et al screened 1701 newborn infants for the development of atopic disease concluding that maternal and paternal allergy had the same bearing on atopic disease within the first 18 months of life (Croner S et al. 1982). There was no attempt in this study to control for confounding variables such as breast feeding. Two reports on the predictive value of cord IgE concentrations noted higher concentrations in infants of atopic mothers (Magnusson CG 1988). In a study of school children and their parents, sensitization to four common inhalant allergens was found to occur significantly more frequently in the children of mothers who are sensitized to the same allergen whilst none of the paternal sensitization’s reached statistical significance (Kuehr J et al. 1993). Ruiz et al compared the risk of infantile atopic dermatitis posed by maternal atopy and paternal atopy in the infants from a birth cohort well matched for both environmental and feeding variables. Maternal atopy was found to carry a higher risk for infantile AD than paternal atopy (Ruiz G et al. 1992).

One possible explanation for the observed maternal effect is that maternal modification of the fetal immune response occurs in utero through placental transfer.
of antibodies. Further modification occurs subsequently during breast feeding. In rodents, but not in humans, maternal IgG has been shown to modify the infants subsequent IgE responses, by being passively transferred through the placenta. Alteration of the maternal immune responses following desensitization by antigen injection, has had uncertain effects on the subsequent immunological development of the fetus (Settipane RA et al. 1989). Another possible explanation for the finding of maternal inheritance is that of genomic imprinting, in which a paternal 'atopy gene' is suppressed during spermatogenesis (Hall JG, 1990).

Confounding factors

With such a high population prevalence, atopy is unlikely to be a single gene disorder as many of the population would have to be homozygous for that gene. It is far more likely that many genes confer genetic susceptibility and that in any large pedigree there will be several atopy genes introduced through different individuals (Cookson WOCM 1994). The high prevalence of atopy also means that at least one fifth of all marriages will be between two atopics. This needs to be taken into account when recruiting atopic families for genetic studies. An excess of atopics will result in considerable loss of power to detect linkage as seen in several previous studies where families were sought via media appeal or where the inclusion criteria have depended on the presence of two or more affected siblings (Moffatt MF et al. 1992)(Coleman R et al. 1993). To overcome this difficulty, recruitment should be from either random population samples (complete ascertainment) or through a defined proband with atopic disease.
The variability in penetrance or expression of a particular phenotype is important in genetic studies of atopy. This not only applies to the clinical phenotype but to measurements of specific and total serum IgE. In infancy the penetrance of both specific and total serum IgE is relatively low when compared to that found in early adult life where IgE responsiveness is at its greatest. Skin prick test positivity and measurements of IgE are at a maximum at 15 - 25 years of age. Thereafter IgE responsiveness steadily declines until at the age of 45 the serum IgE may be half of its value at the age of 15 (Cline MG and Burrows B 1989).

Measurements of total serum IgE are attractive parameters for genetic study as normal values are well established and correlate well with the presence of AD (Juhlin L et al. 1969)(Johnson EE et al. 1988). However approximately 45% of the variation in total serum IgE is attributable to the specific IgE (RAST) to house dust mite or grass pollen (Cookson WOCM et al. 1991). The same author has also shown by multiple regression analysis a relation between asthma and bronchial hyper-responsiveness to variation in the specific IgE, especially to house dust mite. The residual IgE, (obtained by removing the specific IgE) fails to correlate with the asthma phenotype (Cookson WOCM et al. 1991).

The genetics of complex disorders

The inability to demonstrate a consistent pattern of inheritance in atopic disorders is best explained by the interaction of several genes for any given atopic phenotype, where the expression of these genes is strongly influenced by environmental factors. Each individual locus may have a small effect or alternatively there may be a single
major locus with a multifactorial background. Simple Mendelian models of inheritance are inadequate and more complex models of inheritance are needed. The new approach to such complex disorders is the application of regressive models to segregation analysis. Using these models, genetic effects which independently modify the expression of closely correlated phenotypes such as symptoms, specific and total IgE, can be dissected out.

The above approach is capable of suggesting the existence of a major locus but is unable to identify the actual genes. The process of identifying major genetic susceptibility loci is done either by a candidate gene approach, where known genes or polymorphisms within them are tested for association with disease, or by using markers to localize genes to a particular chromosomal region. This process is known as genetic linkage and essentially examines the relationship between two loci. Once linkage is established, positional cloning of the region can be carried out to identify the gene. The gene is then mapped to a region on the chromosome.

1.10 METHODS OF IDENTIFYING DISEASE GENES

Genes are most easily identified by the discovery of an abnormal protein and its amino acid sequence, from which the DNA sequence of the mutated gene can be predicted. If the biochemical or physiological basis of a disease is unclear, then the gene may be found by one of two strategies. The first is the candidate gene approach where known genes are selected on the basis of their function as plausible candidates for being the disease gene and tested for association with disease. If one of these
candidates is found to be involved in the disease process, a search for the causative mutations can then be carried out. The second strategy involves searching all the human chromosomes using genetic markers to localize genes to a particular chromosomal region by a process known as genetic linkage. Once linkage is established, positional cloning of the region can be carried out to identify the gene. The gene is then mapped to a region on the chromosome.

**Genetic maps of the human chromosome**

The construction of genetic maps was not possible before the development of genetic markers. Furthermore, a minimum number of markers no greater than 20cM apart across the genome, were needed in order to reliably detect linkage to a disease trait. Genetic markers needed to be sufficiently polymorphic to give a reasonable chance that a randomly selected person would be heterozygous for the marker alleles. Throughout the genome, naturally occurring genetic variations in DNA sequence, such as point mutations and hypervariable regions are present. These DNA polymorphisms, resulting in a minimum of two distinguishable alleles, provided exactly the type of markers needed in order to start mapping disease genes. The transmission of markers through affected families could now be ascertained and compared to the transmission of disease. If a marker was found to co-inherit with disease, then the disease gene was likely to be in close genetic proximity to the marker on that chromosome. Thus the chromosomal location of the disease gene could be inferred from the location of the marker.
The first generation of DNA markers were restriction fragment length polymorphisms (RFLP’s), owing their existence to restriction site polymorphisms. Their informativeness as markers however was limited. Minisatellite markers or variable non-tandem repeats (VNTR) were an improvement, with most meioses being informative. The subsequent development of PCR greatly facilitated mapping leading to a new generation of markers, namely the microsatellite markers. Most of these markers are highly informative (CA)\textsubscript{n} repeats. Microsatellite markers have become the standard tools of linkage analysis, allowing the development of a comprehensive linkage map of the human genome, generating large numbers of highly polymorphic markers available for use in linkage studies (Weissenbach J et al. 1992).

Genetic markers however are not always informative with the heterozygosity of a marker (i.e. the proportion of heterozygotes in a population) determining its usefulness. Parents who are homozygous for a marker or share identical marker alleles with each other, limit the ability to identify the inheritance of alleles in offspring.

**Linkage analysis**

Linkage analysis uses family data to follow the transmission of genetic information between generations. The information is used to determine whether a genetic marker is close to (linked) or unlinked to a gene involved in the disease process that is being studied. The main aim of linkage analysis is to estimate the number of recombination events (recombination fraction) and hence the map distance between
genes. Recombination events occur during meiosis, when the chromosomes of a homologous pair align themselves, overlap, break and rejoin resulting in the exchange of genetic material between homologous chromosomes. Recombination events are random and therefore the closer two loci are, the lower the probability that they will be separated at meiosis. In a genome search, markers are tested sequentially until one is found that shows low levels of recombination with the disease. The marker is said to be linked to the disease gene.

When families are large and recombinants can be counted, analysis is simple. Unfortunately, with human diseases this is rarely the case. This has lead to the development of a number of statistical methods which attempt to extract linkage information from collections of ‘imperfect families’.

The lod score introduced by Morton in 1955 is the logarithm of the odds that the two loci are linked. A value of +3 (ratio of 1000/1) is traditionally taken as evidence for linkage and -2 (ratio of 1/100) as sufficient to exclude a disease gene from the area around the marker (Morton NE 1955). It is calculated by observing each meiosis in turn and comparing the likelihood of the observed genotypes on the alternative hypothesis of linkage or no linkage. Where the parental ‘phase’ is not known, the lod score must allow for either possible phase with equal probability. The overall probability of linkage in a group of families is the product of the probabilities in each individual. Therefore lod scores can be added up across families. (Ott J 1985).
The lod score approach has been used extensively in the mapping of single gene disorders. However the need to specify a precise genetic model, detailing the mode of inheritance, gene frequencies and penetrance of a genotype, makes it less useful for complex diseases such as atopy, where the mode of inheritance is not yet defined and penetrance variable. Interpretation of lod scores for complex disorders remains controversial (Risch N 1992).

**Sib-pair analysis**

Sib-pair analysis is a non-parametric test which enables an investigator to assess the evidence for linkage without having to define the mode of inheritance or other parameters (Shah S and Green JR 1994). Because both siblings are affected, the disease genes are assumed to have acted and non-penetrant individuals are excluded from the analysis. Sib pair analysis can therefore be performed without making any assumptions about the genetics of the disease. This method is able to detect linkage when a marker is linked to a disease gene, because affected siblings will share the marker alleles more often than expected by random assortment. (Emery AEH 1986).

If a marker is unlinked to a disease gene then there will be a 50/50 chance that each sibling pair will share a specific marker allele. Any significantly greater level of allele sharing than 50% indicates linkage of the marker to a disease gene. Thus selecting any chromosomal segment, pairs of sibs will be expected to share 0, 1 or 2 parental haplotypes with a frequency of 0.25, 0.5 and 0.25 respectively. If both sibs are affected by a genetic disease, then they will share the segment of chromosome carrying the disease locus. If the disease is dominant, they will share at least one parental haplotype, and if recessive they will share both haplotypes. If linkage exists
between a marker and disease, a distortion in the sharing of alleles from that expected will be seen. The significance of the distortion is determined by $\chi^2$ estimation. If dealing with a dominant disease, a sib-pair will exhibit excess sharing of 1 or 2 alleles. In a recessive disease they will show an excess sharing of two alleles.

**Genetic association**

Association studies are powerful statistical tools used in the identification of disease susceptibility genes once their chromosomal position has been determined by genetic linkage. This is especially important in the identification of genes of modest effect, which can be missed if using only tests of linkage to find disease susceptibility loci. Association studies look for significant differences in disease risk associated with different alleles of a gene by testing for different frequencies of a marker allele in patients and controls, matched for ethnicity and other factors (Parsian A et al 1991). The relative risk is then calculated as the ratio of a particular allele in the patient (affected) group compared to the control group. Association will arise if the marker allele is causally implicated in the disease, or if it is in linkage disequilibrium with a susceptibility locus. If an association is confirmed by studies in other population then the genotype involved may be causative for the condition.

Linkage disequilibrium occurs when haplotype combinations of alleles at different loci occur more frequently than would be expected from random association. Linkage disequilibrium may be generated because of a “founder effect”, which occurs when a large proportion of the people affected by a disease in a population is
descended from a common ancestor who carried the mutation. The mutation would have been originally surrounded by a particular set of marker alleles on the chromosome which after several generations would become separated from the mutation by recombination events. The proportion of recombinations between the marker and the locus will determine the rapidity with which linkage disequilibrium decays. Only those markers extremely close to the disease gene, with very low probabilities of recombination, will still be associated with the disease (Weeks DE and Lathrop GM 1995).

Association studies have several advantages over those of genetic linkage. Firstly they require a much smaller sample size, even allowing for a smaller significance level. They can be performed on single affected individuals or pairs of affected siblings and their parents and are stronger tests for detecting genes of modest effects. Their major limitation is that the actual gene(s) or polymorphisms within them must be defined and their chromosomal position known (Risch N and Merikangas K 1996). A number of factors affect the strength of a marker in testing for association. These include, the strength of the linkage disequilibrium between the disease and marker, the frequency of the disease mutation, the increase in risk attributable to the particular disease susceptibility locus under consideration, the penetrance of the disease genotypes and the recombination fraction between the disease and marker.

The transmission disequilibrium test of Spielman et al (Spielman R et al. 1993) examines the transmission of a particular allele at a locus from heterozygous parents to their affected offspring. It focuses on single affected children and their parents,
and assumes that under normal Mendelian inheritance, all alleles have a 50% chance of being transmitted to the next generation. If one of the alleles is associated with disease risk, it will be transmitted more often than 50% of the time.

Thus just as linkage is a relationship between loci, so association is a relationship between alleles. Allelic associations caused by linkage disequilibrium are seen only when a significant proportion of the apparently independent chromosomes examined in a population, are in fact copies of the same ancestral chromosomes. Alleles at two tightly linked loci will show associations only if they mark shared ancestral haplotypes. Linkage disequilibrium is not therefore an inevitable consequence of close genetic linkage. Apparently unrelated people share only very short chromosomal segments. For this reason, allelic associations reflecting sharing of ancestral chromosomes, are noticeable only over very short genetic distances, most likely within 1cM of each other. This has restricted its use to candidate chromosomal regions or loci, where there is strong reason to suspect that an association might exist (Risch N and Merikangas K 1996).

1.11 GENES INFLUENCING ATOPY AND ASTHMA

Most of the recent work on the molecular genetics of atopy to date has related to families with allergic asthma and rhinitis. A number of genes have been identified which fall into four separate classes: (Cookson WOCM 1996).

Class I: Genes predisposing in general to IgE mediated inflammation.
Class II: Genes influencing the specific IgE response.
Class III: Genes influencing bronchial hyper-responsiveness independently of atopy.
Class IV: Genes influencing non-IgE mediated inflammation.
Class I genes

Genes predisposing to generalized atopy have been identified to date on chromosomes 11 and 5.

Chromosome 11q12-13

The earliest report of genetic linkage to atopy was published by Cookson and Hopkin in 1989 (Cookson WOCM et al. 1989) demonstrating linkage to the marker D11S97 on chromosome 11q13. Almost all of the positive LOD score was attributable to a single family. Linkage was subsequently replicated in 60 nuclear families ascertained through a proband with asthma (Young RP et al. 1992). The study caused much controversy over its broad definition of atopy (Cookson WOCM and Hopkin JM 1988) and the failure to replicate linkage in other populations (Amelung PJ et al. 1992)(Hizawa NE et al. 1992)(Rich S et al. 1992). These studies however suffered from inadequate sample sizes which would have diminished the power to detect linkage. Furthermore, other confounding factors such as genetic heterogeneity, a high gene frequency and ascertainment bias affected the ability to replicate linkage in atopy (Moffatt MF et al. 1992). Subsequently linkage to chromosome 11 was confirmed by 2 independent groups in Japan and the Netherlands (Shirakawa T et al. 1994)(Collee JM et al. 1993).

Affected sib-pair analysis, the most robust method for detecting linkage, showed linkage to be exclusively maternal, with 62% of affected sib-pairs sharing the maternal 11q13 allele. This finding was in agreement with that of earlier clinical studies, suggesting possible maternal modification of the immune response or paternal imprinting (Shirakawa T et al. 1994)(Cookson WOCM et al. 1992). The discovery lead to better localization of the atopy locus centromeric to the original D11S97. 60% of families with symptomatic atopy were found to be influenced by the chromosome 11 atopy gene (Sandford AJ et al. 1993).
In the mouse, the B lymphocyte surface marker CD20, was found to show sequence homology to the beta chain of the high affinity receptor (FceRIβ) and to be located close to that gene on chromosome 19. This interesting observation lead to the discovery and localization of FceRIβ on chromosome 11 by Cookson et al (Sandford AJ et al. 1993). Due to the involvement of FceRIβ in the IgE pathway, it was considered a good candidate for the 11q atopy locus and was subsequently found to be in close genetic linkage to atopy (Sandford AJ et al. 1995).

Two coding polymorphisms were later identified within exon 6 of the FceRIβ gene, FceRIβ Leu 181 and Leu 181/183, and shown to associate strongly with atopy, when maternally inherited (Shirakawa T et al. 1994). A possible model for the action of this mutation is that it increases the signal transduction activity of the receptor thus enhancing the release of pro-inflammatory mediators from mast cells or increasing the mast cell expression of IL-4 and CD40. This change would stimulate local B lymphocyte IgE production. Shirikawa et al found the Leu 181 polymorphism to be associated with atopy in 15% of a British asthmatic population. Attempts to replicate this finding in an Australian asthmatic population have however been unsuccessful, with polymerase chain reaction failing to detect the Leu 181 variant. A Leu 181/183 variant however was found in 4.5% of the population (Hill MR et al. 1995). The reasons for this discrepancy are unclear but a possible explanation would be that of gene duplication.

Since these findings a new coding polymorphism within exon 7 of the FceRIβ locus, was found in 5.3% of an Australian general population sample (Hill MR and Cookson WOCM 1996). The variant, designated E237G, is the result of a single nucleotide substitution (adenine to guanine), changing glutamic acid to glycine at position 237. E237G positive subjects were found to have significantly elevated skin prick test responses to grass pollen (p=0.0004) and house dust mite (p=0.04) and significantly higher bronchial reactivity to metacholine stimulation (p= 0.0009). The
relative risk of individuals with this variant having asthma was 2.3. In a Japanese population of atopics a RsaI polymorphism situated within the second intron of FceRIβ has been shown to associate with atopic asthma, rhinitis and AD (Shirikawa T et al. 1995).

Although these variants are significantly associated with measures of atopy, they cannot explain the strength of linkage of atopy to chromosome 11q. As it stands, it is therefore not yet established whether the chromosome 11q atopy gene is FceRIβ or whether it is some other gene in linkage disequilibrium with the FceRIβ variants. It is most likely that other variants or mutations within the FceRIβ gene are present if it is indeed to be the atopy gene (Cookson WOCM 1996).

**Chromosome 5**

The IL4 cytokine-gene cluster on chromosome 5q31-33 contains a number of cytokines which play an important interactive role in the final expression of atopy, making this an interesting area for genetic linkage studies in allergy. Included in this dense genetic region are the IL4 and IL13 loci, regulating immunoglobulin heavy chain class switching to the e isotype and differentiation of naive T cells into TH-2 cells (Gauchat JF et al. 1990)(Mosmann TR and Coffman RI 1989)(Romagnani 1991). IL-5, IL-3 and GM-CSF mediating the eosinophilic response, and IL-3 and IL-9 responsible for mast cell proliferation (Paul WE et al. 1993)(Gauchat JF et al. 1990). Many of these genes are tightly linked, suggesting that they are to some extent co-regulated.

Using sib-pair analysis, Marsh et al (Marsh DG et al. 1994) demonstrated linkage to low levels of serum total IgE with 5 markers on chromosome 5q31.1 in an Amish population. Eleven families, with 170 subjects, were recruited on the basis of detectable serum IgE antibody to common inhalant allergens in at least one child. Significant linkage was demonstrated with the markers IL4-R1 (p=0.006), interferon
releasing factor, IRF-1 (p=0.04), IL9 (p=0.019), D5S393 (p=0.005) and D5S399 (p=0.002) to the total serum IgE in 349 sib pairs. No evidence of linkage for any of these chromosome 5 markers was demonstrated to specific IgE. Furthermore, exclusion of all siblings with positive specific IgE responses and analysis of only non-atopic sib-pairs, strengthened the evidence for linkage of IL-4 and IRF1 to total serum IgE (p=0.000004 and p=0.0007, respectively). These findings suggested a noncognate mechanism for IL-4 in the control of overall IgE production.

Meyers et al (Meyers DA et al. 1994) reported linkage in a Dutch population, ascertained through a parent with a diagnosis of asthma, to high total serum IgE levels with several markers on chromosome 5q31-33. Two of these markers included those previously tested by Marsh et al. Linkage was found to IL-9, D5S393, D5S436 and CSF-1R, the most significant being to D5S436 with a lod score of 3.56 (p<0.0005). Using segregation analysis they demonstrated recessive inheritance of high IgE levels to be the best fitting model.

In a random population sample of 131 families, where 41% of the parents and 46% of the children had at least one positive skin test to a panel of 14 common allergens, allelic association was demonstrated between total serum IgE and IL-9 on chromosome 5q (p<0.005) (Doull IJM et al. 1996). A number of studies have failed to show linkage to the chromosome 5 region in families recruited with asthma and atopy. In the largest study to date, no evidence of linkage to total IgE was found using chromosome 5 markers either by lod score or segregation analysis (Dizier MH et al. 1993). Using class D regressive models to account for the specific IgE response, evidence for linkage to a microsatellite repeat in IL-4 was demonstrated to the residual IgE. This supported work by Marsh et al (Marsh DG et al. 1994) in demonstrating linkage of a single marker on chromosome 5 to the low IgE phenotype. Linkage was not however found with any of the other polymorphic markers.
The suggestion that genes within the 5q31-33 region on chromosome 5 are under shared regulatory control, prompted one group of investigators to analyze polymorphisms within the 5’ promoter regions of five of these candidate genes in 20 asthmatic and 5 control families (Rosenwasser LJ et al. 1995). Three single stranded conformational polymorphisms (SSCP) were identified in the IL-3, IL-9 and IL-4 5’ regions respectively. Significant association of the IL-4 promoter polymorphism, (arising out of a single C to T base change at position 590 from the open reading frame of the IL-4 gene) with total serum IgE, was seen in asthmatic families. Analysis of this promoter polymorphism within a general Australian population sample however found only weak association with specific IgE to house dust mite, and wheeze but not to total serum IgE, bronchial hyperresponsiveness or asthma (Walley AJ and Cookson WOCM 1996). In a British asthmatic population, there were no significant associations with any measures of atopy or asthma.

In summary, no known polymorphisms have been identified in the coding region of any cytokine gene mapping to the chromosome 5 region, nor has any human disease state been linked to polymorphisms within the gene regulatory regions. The gene clustering in this region suggests that there is shared regulatory control. Linkage has been most clearly demonstrated to non-cognate IgE production and IL-4 in families recruited with asthma.

**Class II genes**

Individuals in the general population and within families differ in the way in which they react to different antigens. The risk of clinical disease is related to specific antigen responses with asthma sufferers tending to demonstrate sensitization to house dust mite but not to grass pollens(Cookson WOCM et al. 1991). It is therefore of interest to examine whether particular genes influence the restriction of IgE responses to specific allergens.
The two most likely candidates responsible for the genetic regulation of specific IgE responsiveness are the genes encoding the Human Leucocyte Antigen (HLA) proteins and the genes for the T cell receptor (TCR).

**Human leucocyte antigen (HLA)**

Human histocompatibility leucocyte antigens (HLA) play a central role in the regulation of the immune response and have been associated with a wide range of diseases with an immunological bias. The genes encoding the HLA molecules are situated on the short arm of chromosome 6, comprising the most polymorphic gene system known in man (Trowsdale J et al. 1991). This is due in part to the presence of several closely related genes at separate loci, but also to the existence of multiple alleles at each locus.

There are three major gene clusters within the HLA, designated class I, II and III. Classes I and II are important in influencing T cell expression (Schwartz RH 1989) and assume a central role in antigen presentation (Germain RN 1986). Antigens derived from the cytoplasm are processed by the endogenous pathway for presentation by HLA class I molecules. Class I molecules, denoted by the suffix HLA-A to HLA-L, are expressed on nearly all nucleated cells and present antigen to CD8+ T cells (Daar AS et al. 1984). Antigens arising from outside the cell are processed via the exogenous pathway for presentation on HLA class II molecules. Class II genes, denoted by the suffix HLA DR, DQ, DP, DO, DN or DM have a more limited distribution, being found on B cells, monocytes, macrophages, dendritic cells and activated T cells (Daar AS et al. 1984). They present antigen that has been internalized by phagocytosis or membrane bound molecules to primarily CD4+ T cells. The extensive molecular polymorphism of both class I and II molecules is derived from the particular properties of the peptide binding groove, forming the structural basis of HLA restriction in response to foreign antigens.
A presumed link between HLA restriction and the development of IgE responses has been documented for over a decade. Initial studies reported genetic associations between a number of HLA-DR2 haplotypes and IgE responses to aeroallergens. The most significant of these were DRB1*1501 alleles and ragweed pollen *Amb a V (Ambrosia artemisifolia) (Marsh DG et al. 1981). Other studies reported associations between DRB1*03 and DRB1*11 alleles and rye grass (*Lolium perenne) (Freidhoff LR et al. 1988)(Ansari AA et al. 1989) and DRB1, DRB3 and DRB5 alleles and house dust mite (*Dermatophagoides pteronyssinus)(O. Hehir RE et al. 1990). Numbers in these studies were often too small to establish an unequivocal HLA association and many of the results have yet to be replicated.

In the largest study of its kind, Cookson et al investigated HLA-DR and HLA-DP alleles in 431 subjects from 83 families and 300 controls, serotyped for IgE responses to 6 major purified allergens (Young R et al. 1994). HLA-DP genotypes failed to demonstrate any significant correlation with antigen responses thus excluding it as a likely candidate for the genetic regulation of specific IgE responsiveness. Weak associations were however shown between HLA-DR frequencies and IgE responses to common allergens. The most significant of these was the association of *Alt a I with HLA-DR4 (Odds ratio 1.9), and that of *Fel d I with HLA-DR1 (Odds ratio 2.0). Two smaller studies in Austrian and Danish subjects found a positive association between *Bet v I, the major allergen of birch pollen, and Drw52 (HLA- DRB3*0101) (Fischer GF et al. 1992) (Sparholt SH et al. 1994).

The 11th International Histocompatibility Workshop examined 1006 atopic patients in 13 population groups concentrating on genetic associations with the HLA-DRB1 gene, the most polymorphic HLA class II gene, to eight highly purified common aeroallergens (Marsh DG et al. 1991). The major cat allergen, *Fel d I, showed the highest rate of antibody responsiveness. It was associated with DRB1*15 in two of
the population groups. The most consistent association was found between IgE responses to the mould allergen *Altenaria Alt a I*, and HLA-DRB1*04 and DRB1*14 (Howell WM and Holgate ST 1995). Other associations confirmed the results of previous studies demonstrating an association between IgE responses to Amb a V allergen and DRB1*15, DRB1*04 and DRB1*03 in different population groups.

To date, only three associations seem secure: *Amb a V* and DRB1*15, *Alt a I* and DRB*04 and *Bet v I* and DRB3*0101. The lack of a significant positive association with the ubiquitous house dust mite in numerous population studies is surprising if HLA restriction is indeed a major determinant of specific antigen recognition. Furthermore in vitro studies of T cell reactions have shown that the same antigen-derived peptide may be presented by multiple class II molecules. In conclusion, the influence exerted by HLA class II restriction seems insufficient to account for individual differences in reactivity to common allergens.

*The T cell receptor (TCR)*

Since recognition of the specific antigen response is unable to be explained entirely by HLA restriction, the process whereby binding of MHC-peptide complexes, on the surface of antigen presenting cells, to T cells is likely to be important in modification of the immune response.

The T cell receptor (TCR) is a highly complex structure, which allows the T cell to differentiate one antigen from another. The TCR consists of a di-sulphide-linked heterodimer of two highly polymorphic glycoprotein chains, known as α and β. A second type of TCR, consisting of δ and γ chains exists less commonly in 1-10% of the population (Groh VS et al. 1989)(Inghirami GB et al. 1990). The receptor is non-covalently linked to CD3 molecules which are involved in TCR assembly and in signal transduction following antigen recognition by the TCR (Wegener AM et al.
1992). The diversity of the TCR arises out of its complex structure with many variable (V) and junctional (J) segments within the TCR loci contributing to antigen specificity as well as N region addition/deletion during rearrangement of T cell receptor genes. An additional source of variation contributing to further diversity of the TCR, is that of allelic polymorphism within the TCR gene segments (Reyburn H et al. 1993). Each T cell expresses only one species of TCR (Matsui K et al. 1991) thus necessitating a vast repertoire of T cell receptors for antigen recognition. Its unique structure allows for an estimated $10^{16}$ different receptor types (Davis MM and Bjorkman PJ 1988).

Determination of an individuals TCR repertoire is not a random event with individual variation being shown to occur in the use of the V and J segments of TCR-α and of the V segments in TCR-β. It is likely that differentiation of the T cell repertoire and hence the ability of an individual to respond to specific antigens while not to others, is under genetic control (Loveridge JA et al. 1991) (Moss PA et al. 1993) (Gulwani AB et al. 1995). Localization of the α-chain locus, and δ-chain locus within it, to the long arm of chromosome 14 and the β and γ chain locus to chromosome 7 has allowed investigators to test for genetic linkage to a number of human diseases.

T cell clonal studies have demonstrated restricted usage of TCR Vα and Vβ genes for a number of respiratory allergens (Moffatt MF and Cookson WOCM 1997). Genetic linkage studies, using microsatellite markers associated with T cell loci, have been used as an alternative approach to test the role of TCR genes in influencing specific IgE responses to allergens. Strong genetic linkage between the TCRα/δ locus and specific IgE responses was found within 2 large population groups, one British and one Australian, using affected sib pair analysis (Moffatt MF et al. 1994). Linkage was strongest with highly purified allergens (Der p I, Der p II and Fel d I) and in maternally derived alleles. Linkage to the total serum IgE was
also seen, although this was not as strong as that to specific IgE. Nevertheless, the close correlation between total and specific IgE makes it difficult to attribute the locus to the genetic control of either specific IgE or general IgE responsiveness. No evidence for linkage to the TCR-β locus was found.

Further work on these populations established a strong allelic association with a polymorphism within the Vα-8.1 gene and reactivity to Der p II, in the Australian subjects but not the British. (Moffatt MF et al. 1995) HLA-DRB typing of the families showed HLA-DRB1*02 to be positively associated with IgE titres to Der p II, consistent with the general hypothesis of interacting HLA-DR and TCR-α restriction of IgE responses to particular antigens.

Although genetic factors predominate, environment may also influence the T cell repertoire. Further genetic and functional studies are needed to elucidate the role of the TCR loci in determining IgE responses in asthma and atopic dermatitis and their interaction with HLA class II genes.

Class III genes

Bronchial hyperresponsiveness can be quantified through the use of challenge tests which measure airway lability. Common challenges include exercise, cold air or pharmacological bronchial spasmogens such as inhaled histamine or metacholine. Using such measures, airflow limitation can be detected in the absence of an asthma attack and therefore provides a highly sensitive measure of airway reactivity.

Currently there have been no genes identified which confer genetic susceptibility to bronchial hyperresponsiveness independently of atopy.
Class IV genes
Although IgE and its interaction with antigen plays a vital role in triggering the immune response, it does not explain the persistence of inflammatory changes so much a feature of atopic dermatitis and asthma. Furthermore, a number of asthma and AD patients are “non-atopic” with normal serum IgE measurements and negative skin prick test responses to common allergens.

Tumour necrosis factor alpha (TNF-α) has recently been identified as an important pro-inflammatory cytokine in the pathogenesis of asthma and AD. The genes encoding TNFα and TNFβ have been localized to chromosome 6. The relative importance of these genes in the inheritance of asthma and atopy is currently being evaluated.

Tumour necrosis factor (TNF)
Tumour necrosis factor α (TNF-α/cachectin) and lymphotoxin α (LT-α/TNF-β) have a number of similar immunoregulatory effects, binding to their target cells via cell surface receptor molecules TNF-R1 and TNF-R2 (Pfizenmaier K et al. 1992). While LT-α is mainly produced by stimulated T cells, TNF-α is secreted by a variety of cells, including monocytes, alveolar macrophages, T cell lymphocytes, neutrophils, epithelial cells and mast cells (Borish L et al. 1991)(Dubravec DB et al. 1990)(Gosset P et al. 1991).

Inflammatory changes in the airway lumen and wall are important factors in determining airway responsiveness in asthmatics. TNF-α has been found in the sputum of patients with acute attacks of asthma and increased numbers of TNF-α mRNA bearing cells have been isolated from the broncho-alveolar lavage fluid from stable asthmatics (Shah S et al. 1995). TNF-α also appears to play a key role in the pathogenesis of inflammatory changes in the skin. Human dermal mast cells contain sizable stores of biologically active TNF-α within its granules (Walsh LJ et al. 1991)
and plasma TNF-α concentrations are increased in patients with AD. Furthermore, in-vitro studies have revealed cytotoxic activity (indicative of TNF-α release) generated from human skin mast cells following IgE-dependent activation. (Benyon RC et al. 1991).

The genes governing the expression of TNF-α and TNF-β are situated in tandem on chromosome 6, within the MHC region, centromeric to HLA-B and telomeric to HLA class III genes. Unlike the highly polymorphic HLA genes, the coding portions of the TNF genes show only a low degree of polymorphism.

Two polymorphisms have been identified within the TNF gene region which are thought to be associated with allergic asthma. The first of these, an NcoI biallelic polymorphism (abbreviated as LTα NcoI*1), located within the first intron of lymphotoxin-α (TNF-β) has been shown to correlate with increased levels of LT-α production (Messer G et al. 1991) and with asthma (Campbell DA and Morrison JFJ 1995). A second polymorphism, situated within the promoter region of TNF-α at position 308 (abbreviated as TNF -308*2), is known to influence the secretion of TNF-α (Wilson AG et al. 1993). This polymorphism is likely to be important in the transcriptional regulation of TNF and appears to be associated with asthma.

The genetic proximity of TNF to the HLA genes on chromosome 6 may affect apparent associations between TNF alleles and asthma. The HLA-DR locus is known to influence IgE responses to allergens and increased secretion of TNF by peripheral blood lymphocytes and monocytes is associated with HLA-DRB1*03 and DRB1*04 genotypes (Jacob CO et al. 1990). Moreover, both LTα NcoI*1 and TNF -308*2 have been shown to be in linkage disequilibrium with HLA-DRB1*03 (Abraham LJ et al. 1991)(Wilson AG et al. 1993)(Brinkman BM et al. 1994). In a recent study investigating these two polymorphisms within a British asthmatic population and an Australian general population, similar linkage disequilibrium was
found with LTα NcoI*1, TNF -308*2 and HLA-DRB1*03. No HLA associations with asthma were found, suggesting that the relationship between the TNF -308*2/LT-α *1 haplotypes and asthma seen in both populations was real and not just an HLA-DR effect (Moffatt MF et al. 1995).

1.12 GENES INFLUENCING ATOPY AND ATOPIC DERMATITIS

The beta chain of the high affinity IgE receptor
The first genetic linkage study in atopic dermatitis, looked at several 11q13 markers, including D11S97 and CD20, in 95 nuclear families recruited on the basis of having at least two first degree relatives with active atopic dermatitis (Coleman R et al. 1993). The study was inconclusive, neither clearly demonstrating nor excluding linkage. A positive lod score of 0.8 in 19 families with unaffected fathers, raised the possibility of a maternal effect influencing the development of atopy.

Since then a single study conducted in atopic Japanese patients has demonstrated linkage of an intronic marker within FceRIβ, (RsaI allelic polymorphism), to atopy(p<0.01), allergic asthma (p<0.001) and AD (p<0.02) (Shirikawa T et al. 1995).

Mast cell chymase
Mast cell chymase is one of many serine proteases regulated by a cluster of genes on chromosome 14q11.2. It is secreted by the skin mast cells as part of the dermal inflammatory response and is thought to play an important role in the pathogenesis of atopic eczema. A BstXI polymorphism situated within the mast cell chymase gene has been found to associate significantly with eczema (odds ratio 2.17 [95%CI 1.21-3.88], p=0.009) (Mao XQ et al. 1996). No association was found with asthma or seasonal rhinitis. Additional studies are needed to assess the importance of other variants within this region in determining genetic risk for atopic eczema.
α subunit of the interleukin-4 receptor

The R567 allele of the interleukin-4 receptor has been found to be associated with atopy, atopic dermatitis and the hyper-IgE syndrome. It was present in 13 of 20 subjects with atopy compared with 5 of 30 patients without atopy (Hershey GK et al 1997). Its significance has not yet been tested in wider populations and its overall importance to atopic dermatitis has not been determined.

1.13 WHOLE GENOME SCREEN FOR ATOPY

The candidate gene approach has been successful in identifying a number of genes contributing to the development of atopy, asthma and atopic eczema. These genes do not however account for the high prevalence of atopy in the population as a whole. Furthermore segregation analysis is limited in that it fails to predict with any accuracy the number or nature of genes contributing to atopy. The discovery of highly informative microsatellite markers has allowed the development of a comprehensive linkage map of the human genome (Weissenbach J et al. 1992). This has offered the possibility of identifying many of the genes that raise susceptibility to common complex diseases such as insulin-dependent diabetes and Alzheimer’s disease (Clark RF and Goate AM 1993)(Hashimoto L et al. 1994).

Several groups are in the process of conducting large-scale genome screens for atopy with the aim of finding and cloning new genes responsible for the development of asthma and atopy. The Oxford group has recently published the results of their genome screen for atopy, in which 300 microsatellite markers spaced at approximately 10% recombination were used in 80 nuclear families selected with asthma and atopy (Daniels SE et al. 1996). They looked at linkage to four quantitative parameters, namely total serum IgE, the skin test index (STI), the peripheral blood eosinophil count and bronchial responsiveness to metacholine (slope). Atopy was used as the only qualitative parameter, based on a combination
of STI, RAST index and total serum IgE. Affected sib-pair methods were used to
detect linkage to atopy. The Haseman-Elston sib-pair technique was used for finding
linkage to the quantitative traits. Six regions of potential linkage (p<0.001) to
autosomal markers were detected with one or more phenotypes on chromosomes
4,6,7,11,13 and 16 respectively. On chromosome 11, a microsatellite marker in the
beta chain of FcεRIβ showed linkage to measures of IgE (p<0.0005) and the STI
(p<0.00005). IgE was also significantly linked to a marker on chromosome 16. The
regions on chromosome 4 and 7 were linked to bronchial responsiveness, whereas
the region on chromosome 6, in close proximity to the class I genes of the major
histocompatibility complex, was linked to eosinophil counts. Markers from
chromosome 13 showed evidence of linkage to the atopy phenotype.

The markers showing p<0.0001 for linkage were tested for replication in a second set
of families recruited with asthma and atopy (Daniels SE et al. 1996). In this study
FcεRIβ was shown to be linked to asthma (p=0.003), which was most clearly
demonstrated in maternally derived alleles (p<0.00001). Several other loci were
found to demonstrate a maternal effect. Interestingly, linkage to markers within the
chromosome 5q31 region was not demonstrated to either measures of IgE or
bronchial hyperresponsiveness.

The demonstration of linkage to various different measures of atopy emphasizes the
marked pleiotropy of the atopic phenotype. Using additional markers these regions
now need to be narrowed still further and tested for their association with disease.
Selected regions can then be identified for positional cloning. The results of other
genome screens are awaited and it is likely that agreement will soon be reached on
the number and nature of the most important loci causing atopic disease. A genome
screen for atopic dermatitis will help to identify further chromosomal linkages, which can then be tested for association with disease.

1.14 AIMS OF THIS THESIS

The main aims of this thesis are:

- To identify a panel of families recruited on the basis of a single proband with atopic dermatitis, fulfilling the updated UK diagnostic criteria for AD.

- To define the clinical genetics of atopic dermatitis within this panel of families.

- To identify relevant genes predisposing to atopic dermatitis based on a candidate gene approach.
Chapter 2

Materials and Methods
CHAPTER 2
MATERIALS AND METHODS

2.1 ASSIGNMENT OF CLINICAL PHENOTYPES

Subjects

Panel A: 61 nuclear families (276 individuals) were recruited from the Dermatology clinics at Great Ormond Street Hospital for Children, NHS Trust (GOSH), London through a single proband with active atopic dermatitis. Only those probands over the age of 12 months with at least one sibling > 1 year were included in the study. Children less than 12 months were excluded to avoid difficulties in differentiating between infantile seborrhoeic dermatitis and infantile AD. The study was approved by the GOSH Ethics Committee and written informed consent was obtained from each family.

Families attended a research clinic in the outpatients departments of GOSH where they were personally evaluated. A questionnaire was specifically designed for the study to include the diagnostic criteria for AD as defined by the UK working party and a set of questions based on the American Thoracic Society’s questionnaire for asthma and allergic rhinitis (Appendix I). Each person was examined for evidence of AD and a severity score assigned to those patients fulfilling the diagnostic criteria for AD.

Panel B: 95 nuclear families recruited by a previous investigator on the basis of at least two first degree relatives with active AD, from the paediatric dermatology outpatient clinics at GOSH (Coleman R 1993). DNA was available for analysis from 88 of these families. As with population A, a detailed physician administered questionnaire was completed for each individual and an examination carried out, documenting the extent and severity of skin involvement. The UK working party’s set of six diagnostic criteria were applied retrospectively to all individuals with AD,
who had previously fulfilled the requirements for a diagnosis of AD using the diagnostic criteria of Hanifin and Rajka. Only those patients fulfilling the UK working party's set of diagnostic criteria were regarded as having a definite diagnosis of AD.

**Diagnostic criteria for AD**

The diagnostic criteria for AD proposed by the UK working party were used (see page 22). The criteria were stringently adhered to with only those patients fulfilling the criteria being considered as having a positive diagnosis of atopic dermatitis.

**Severity criteria**

Severity of AD was assessed using the criteria proposed by Rajka and Langeland (Rajka G and Langeland T 1989) which broadly categorizes patients into mild, moderate and severe on the basis of surface area involvement, continuity of disease and nocturnal pruritis. (Table 3) Patients were scored on a scale of 0-9, 0 indicating the total absence of skin involvement, 3-4 indicating mild involvement, 4.5-7.5 indicating moderate involvement and 7-9 indicating severe skin involvement. A score of 9 was achieved when the atopic dermatitis involved greater than 36% of the surface area, followed a continuous course and caused nocturnal pruritis of sufficient severity to regularly disturb sleep.
Table 3. SEVERITY CRITERIA FOR ATOPIC DERMATITIS

1. **EXTENT**
   a) **Childhood and adult phase**
   - Less than approximately 9% of body area = 1
   - Involvement evaluated as > score 1 but < score 3 = 2
   - More than 36% of body area = 3
   b) **Infantile phase**
   - Less than approximately 18% of body area = 1
   - Involvement evaluated as > score 1 but < score 3 = 2
   - More than 54% of body area = 3

2. **COURSE**
   - More than 3 months remission during a year = 1
   - Less than 3 months remission during a year = 2
   - Continuous course = 3

3. **INTENSITY**
   - Mild itch, only exceptionally disturbing sleep = 1
   - Itch, > score 1, < score 3 = 2
   - Severe itching, usually disturbing sleep = 3

**SCORE SUMMATION:**
- Mild = 3-4
- Moderate = 4.5-7.5
- Severe = 8-9

(Rajka G and Langeland T 1989)
2.2 ASSIGNMENT OF LABORATORY PHENOTYPE

**Blood sampling**

Blood samples were taken from each individual (60mls from adults, 15 - 30mls from children). 5mls of blood was collected in a plain tube, and the rest in EDTA tubes. The clotted blood was refrigerated at 4°C for 4-12 hours before being centrifuged at 2500rpm for 20 minutes and the serum extracted for specific IgE analysis. 2mls of EDTA blood was sent to the Haematology laboratory at GOSH for an automated assay of the white cell and eosinophil count. The remaining EDTA blood was frozen at -70°C to be used later for DNA extraction.

**Skin prick testing**

Skin prick testing (SPT) was performed using a standard technique on all individuals. A dilute solution of allergen suspended in a glycerin solution was placed on the forearm and introduced into the dermis by the gentle use of a sterile lancet, sufficient to break the epidermis without causing bleeding. A separate lancet was used for each allergen.

A panel of seven allergen extracts from Dome-Hollister Stier were tested in addition to a positive control of histamine 1mg/ml and a negative control. Allergens included extracts of whole cows milk and egg white as well as highly purified major allergens *Der p I and Der p II* from the house dust mite, *Dermatophagoides pteronyssinus*, *Alt a I* from the mould, *Alternaria alternata*, *can f I* from the dog, *Canis familiaris*, *fel d I* from the cat, *felis domesticus* and *phl p V* from Timothy grass, *Phleum pratense*. 
Mean wheal diameters were recorded at 15 minutes as the number of millimeters greater than the negative control. A positive result was recorded as a wheal diameter measuring 3mm greater than the negative control.

**Total and specific serum IgE assays**

Total IgE was detected by fluorescent enzyme immunoassay (Pharmacia CAP system, Sweden). A raised serum total IgE in adults was defined as > 100 kU/l. A raised serum total IgE in children was defined as being greater than 1 SD from the mean geometric values for age (Table 4).

**Table 4. NORMAL VALUES FOR TOTAL SERUM IgE ACCORDING TO AGE**

<table>
<thead>
<tr>
<th>Age</th>
<th>Geometric mean ku IgE/l</th>
<th>+ 1 SD kU IgE/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.6</td>
<td>2.3</td>
</tr>
<tr>
<td>Months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>4.1</td>
</tr>
<tr>
<td>6</td>
<td>1.8</td>
<td>7.3</td>
</tr>
<tr>
<td>9</td>
<td>2.6</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>3.2</td>
<td>13</td>
</tr>
<tr>
<td>Years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5.7</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>8.0</td>
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<td>4</td>
<td>10</td>
<td>40</td>
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<td>12</td>
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<td>6</td>
<td>14</td>
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<td>20</td>
<td>78</td>
</tr>
<tr>
<td>10</td>
<td>22</td>
<td>85</td>
</tr>
</tbody>
</table>
The same method was used to measure serum specific IgE (Table 5). The panel of allergens included Der p I and Der p II from house dust mite, Alt a I from mould, fel d I from the domestic cat and phl p V from Timothy grass and extracts of cows milk and egg allergens. A specific radioallergosorbent test class 1 result (>0.35 KU/l) was considered positive.

Table 5. STANDARD MEASUREMENTS FOR SPECIFIC IgE

<table>
<thead>
<tr>
<th>RAST class</th>
<th>&gt; than</th>
<th>Level of specific IgE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>Undetectable</td>
</tr>
<tr>
<td>1</td>
<td>&gt;0.35</td>
<td>Low</td>
</tr>
<tr>
<td>2</td>
<td>&gt;0.7</td>
<td>Moderate</td>
</tr>
<tr>
<td>3</td>
<td>&gt;3.5</td>
<td>High</td>
</tr>
<tr>
<td>4</td>
<td>&gt;17.5</td>
<td>Very high</td>
</tr>
<tr>
<td>5</td>
<td>&gt;50</td>
<td>Very high</td>
</tr>
<tr>
<td>6</td>
<td>&gt;100</td>
<td>Very high</td>
</tr>
</tbody>
</table>

Criteria for the diagnosis of atopy

Atopy was diagnosed on the basis of a single positive skin prick test or a single positive RAST test and/or elevation of the total serum IgE greater than 1 SD above the geometric mean for age.

Clinical database

All data was entered into a clinical database designed in Microsoft ACCESS Version 2.0 by the investigator with the help of the computer department at the Institute of Child Health, London. Data was entered in the order of father, mother and siblings from the oldest to the youngest for each nuclear family. Families were numbered and not identifiable by name. The database included all clinical information obtained from the completed questionnaires as well as the marker allele frequencies for each of the loci tested. Relevant data was subsequently imported into a Microsoft EXCEL Version 5.0 spreadsheet.
2.3 STOCK SOLUTIONS AND REAGENTS

Enzymes
Restriction endonucleases were from New England Biolabs. Proteinase K was from Sigma Chemical Company Ltd and Taq DNA polymerase from Boehringer Mannheim UK Ltd and Bioline.

Buffers
Buffers for restriction enzymes
Restriction endonuclease digests were performed in the buffers supplied by the manufacturers.

Gel electrophoresis buffers
1x TBE
10x TBE is 108g Trizma base, 55g Boric acid, 40mls
0.5M EDTA pH 8.0, made up to 1000mls with distilled H₂O and autoclaved

Loading buffers for samples before electrophoresis
Gel loading buffer 5x
5 x TBE, 0.5M EDTA, 10%(v/v) Glycerol,
0.05%(w/v) Bromophenol Blue

Buffers for PCR
Boehringer Mannheim 10x reaction buffer, containing 500mM Kcl, 15mM MgCl₂ and 100mM Tris-HCl (pH 9.0), was used with Boehringer Taq polymerase.

Either Bioline NH₄ buffer containing or 10 x concentrated KCl buffer containing 0.1% NP40, 0.1% gelatin, 500mM KCl, 100mM Tris HCl pH 8.3, was used with Bioline Taq polymerase.
DNA molecular weight markers

DNA molecular weight marker VIII (Boehringer Mannheim UK LTD) and pUC Mix (MBI Fermentas) were used for agarose gel electrophoresis.

Marker VIII is a mixture of DNA fragments from the cleavage of pUCBM21 DNA with restriction endonuclease *Hpa* II and of pUCBM21 DNA with restriction endonucleases *Dra* I and *Hind* III. The DNA fragments are in base pairs, 1114, 900, 692, 501, 489, 404, 320, 242, 190, 147, 124, 110, 67, 37, 34, 34, 26 and 19bp.

The MBI pUC mix marker is a mixture of the digests of pUC19 and pUC57 DNA by *MspI* and *DraI* and *HindIII* respectively. The following discrete fragments are yielded in base pairs: 1116, 883, 692, 501, 489, 404, 331, 242, 190, 147, 111, 110, 67, 34, 34, 26, 19.

Oligonucleotides

Primers (oligonucleotides) for PCR were either commercially synthesized by R & D Systems Europe or synthesized on an Applied Biosystems DNA synthesizer.

Nucleoside Triphosphates

5mM and 2mM working stocks of dNTP were made from concentrated stocks of nucleoside triphosphates (Pharmacia), containing equimolar amounts of all four DNA precursors, namely dATP, dTTP, dCTP and dGTP.

Ethidium Bromide

0.5µg/ml working stock of ethidium bromide was used.
Solutions
Sterile distilled water was used throughout and where possible, all solutions were autoclaved for 20 minutes at 151b/sq in. For polymerase chain reactions (PCR), Analar grade water (BDH LTD) was used.

10% SDS
50g sodium dodecyl sulphate. Dissolve in 400ml distilled H₂O. Adjust pH to 7.2 by adding drops of concentrated hydrochloric acid. Adjust final volume to 500mls.

1M Tris-HCl
121.1g Trizma base. Dissolve in 800ml distilled H₂O. Adjust pH by adding concentrated hydrochloric acid.
For pH 7.5 Add 65mls conc HCl
For pH 7.6 Add 60mls conc HCl
For pH 8.0 Add 42mls conc HCl
Adjust final volume to 1000mls and autoclave.

0.5M EDTA - pH 8.0
186.1g disodium ethylene-diamine-tetra-acetate. 2 H₂O
Dissolve in 700mls distilled H₂O. Adjust pH immediately with NaOH pellets. Stir vigorously. Adjust final volume to 1000mls and autoclave.

1 x Lysis buffer
10mM Tris Hcl pH 8.0, 1M stock 10mls
10mM NaCl, 4M stock 2.5mls
10mM EDTA, pH 8.0, 0.5M stock 20mls
Make up to 1000mls with distilled H₂O and autoclave.
4M NaCl
232.2g NaCl. Dissolve in 800ml distilled H₂O. Adjust volume to 1000mls. Autoclave.

3M Sodium acetate
102.06g sodium acetate 3H₂O. Dissolve in 100mls distilled H₂O.
Adjust pH to 5.2 with acetic acid or adjust pH to 7.0 with dilute acetic acid.
Adjust volume to 250mls and autoclave.

**Preparation of Tris-saturated Phenol**
To a 500g bottle of solid phenol, add 1M Tris HCl, pH 8.0, to fill bottle and place in a 37°C water bath to dissolve. Mix by rolling or inversion. Allow the layers to separate and aspirate off the top layer of Tris HCl and replace with a fresh quantity of 1M Tris HCl, pH 8.0. Repeat the process. Once the second layer of Tris has been aspirated, estimate the amount of remaining Tris and add distilled water so as to dilute this volume of Tris 10 times to make 0.1M of Tris, pH 8.0. Check pH of the Tris layer with pH indicator paper. If acidic, the phenol will degrade any DNA it comes into contact with. Add 0.2-0.5g of hydroxyquinolone as preservative (to achieve a final concentration of 0.1%). This colours the phenol yellow when the phenol is fresh. If the phenol turns a pink/orange colour after standing for some time, it is likely to have become acidic and should be discarded.
2.4 PREPARATION OF GENOMIC DNA

Genomic DNA extraction - Proteinase K/SDS method

Frozen whole blood samples in EDTA tubes were allowed to thaw in a 37°C water bath, prior to extraction. Distilled water was then added in equal volume to the blood samples, to lyse the red blood cells. The samples were then centrifuged for 30 minutes at 3000rpm (1750g) at room temperature and the serum (supernatant) discarded, taking care to retain the nuclear pellet. The cells were then resuspended in 15-25mls distilled water, gently vortexed and centrifuged for a further 20 minutes at 3000rpm. After discarding the supernatant the second time, the resultant pellet was resuspended in an appropriate volume of lysis buffer, the volume varying in accordance with the initial starting volume of whole blood (10mls lysis buffer added to 50mls whole blood, 8mls to 15-20mls, 5mls to 5-15mls). Post vortexing the resuspended pellet, 500μl of 10% SDS solution was added to 10ml lysis buffer (or 400μl to 8ml lysis buffer, 250μl to 5ml lysis buffer) to disrupt the lipid bilayers. This was followed by the addition of proteinase K, the volume of which varied in accordance to the initial starting volume of whole blood (250μl to 50mls whole blood, 75μl to 30mls, 50μl to 20mls etc.). For badly haem contaminated samples, the amount of proteinase K was increased to a maximum of 200μg proteinase K per ml of whole blood. The sample was then incubated at 37°C for 16 hours in a water bath.

The addition of 1/5 volume of 5 x ANE buffer (0.1M NaCl, 0.01M NaAcetate, 1mM EDTA) protects the DNA by buffering against phenolic acidosis. It also enables the DNA to remain in the aqueous state by increasing the salt concentration. All samples were then organically extracted twice with a half volume of phenol/chloroform and twice with a half volume of chloroform. Organic and aqueous phases were separated by centrifugation at 3000rpm for 10 minutes at room temperature, and each time the organic layer removed, thus preserving the aqueous
layer. DNA was precipitated by the addition of 1/10 volume of 4M NaCl and 2 volumes of absolute ethanol. After storage at -70°C for 30 minutes or -20°C overnight, DNA was pelleted by centrifugation at 3000rpm for 30mins at 4°C. The supernatant was discarded and the pellet allowed to dry completely before resuspension in 0.5-1ml of sterile analar water depending on the size of the pellet seen.

**DNA Quantitation by spectrophotometry**

The DNA concentration of a solution was determined by measuring the optical density (OD) at 260nm and 280nm using quartz cuvettes and a UV light spectrophotometer (Camspec). A 200 fold dilution of the DNA sample was made prior to measurement. DNA concentration was then determined by using the standard of 1 OD$_{260}$ unit being equivalent to 50µg/ml od double stranded (ds) DNA and 40µg/ml single stranded (ss) DNA and taking into account the dilution factor involved.

\[
\text{Concentration of DNA} = \text{OD}_{260}\text{nm} \times 50\mu\text{g/ml} \times \text{dilution factor for DNA sample} \\
= \text{OD}_{260}\text{nm} \times 50\mu\text{g/ml} \times 100 \\
= \text{OD}_{260}\text{nm} \times 5000 \text{ (units are } \mu\text{g/ml)}
\]

The ratio between the OD$_{260}$ and the OD$_{280}$ gave an indication of the purity of the DNA. Pure preparations of DNA have an OD ratio of 1.8-2.0. A ratio of <1.6 indicates contamination with either protein or phenol.

**Preparation of working DNA stocks**

Working stocks of DNA were made at a concentration of 100µg/ml in a final volume of 250µl.
2.5 ASSIGNMENT OF GENOTYPES

Polymerase chain reaction
The polymerase chain reaction (PCR) was used as a rapid method for amplifying defined target DNA sequences within a source of DNA. This highly efficient method utilizes two oligonucleotide primers, typically 20-25 nucleotides long, to direct the synthesis of DNA. The primers are designed to flank the target DNA sequence, orientated so that replication in the 5’ to 3’ direction copies the target DNA. In order for this to occur, a plentiful supply of deoxyribonucleoside triphosphates is needed and the presence of a heat stable polymerase, Taq DNA polymerase. PCR proceeds by many rounds of denaturation, annealing primers and extension. Typically 20-30 cycles amplify the desired DNA fragment $10^5$ fold from 1μg of genomic DNA.

Each PCR reaction consisted of template DNA, Taq DNA polymerase, 10x Taq polymerase buffer, dNTP’s and sterile analar water to the appropriate final volume. All reactions were set up to include positive controls for each genotype and a control sample containing everything except DNA was used to monitor for contamination. The PCR mixtures were overlaid with mineral oil to reduce evaporation and transferred to either a Hybaid Omnigene or a Perkin Elmer Cetus thermal cycler.

Selection of Magnesium ion concentration
Titration of the MgCl$_2$ concentration was carried out for all PCR reactions to optimize the working of Taq DNA polymerase, a Mg$^{2+}$ dependent enzyme.

Concentration of DNA template
DNA concentration varied between 50ng - 200ng for each PCR reaction.
Annealing temperature
An initial denaturation of 94°C was routinely carried out and extension was always at 72°C. The annealing temperature was adjusted to suit the different oligonucleotide primers. Routinely the approximate Tm of each primer is calculated using the formula:

\[ T_m = 2x(A+T) + 4x(G+C) \]

The annealing temperature is then set at 5°C below the lower of the two predicted Tm's.
The initial anneal temperature in most reactions was 59°C.

Avoidance of contamination
Since PCR is able to amplify a single molecule of DNA, contamination is always a possibility. In addition to methods already outlined, several stringent principles were adhered to. A different set of Gilson pipettes was used for the preparation of a reaction as opposed to those used to handle the actual PCR product. Filter tips were used when dealing with template DNA, sterile solutions and when setting up a PCR reaction. Eppendorf tubes were autoclaved prior to use. A negative control containing no DNA was included with every reaction.

DNA digestion with restriction enzymes
Restriction endonucleases recognize short DNA sequences and cleave dsDNA at specific sites within or adjacent to the recognition sequences. It represents a crucial step in identifying the alleles present at a particular locus displaying a RFLP or VNTR type repeat. One can anticipate the generation of a set of discrete DNA fragments, following complete cleavage of DNA, that are bound by the restriction sites. Upon analysis by gel electrophoresis, the cleavage products should be visualized as sharp bands.
DNA was digested with the relevant restriction enzyme in the appropriate buffer for one hour at 37°C. Digestion was carried out in a solution containing 5µl of PCR product, 1µl of 10x restriction endonuclease buffer, 4-5U restriction enzyme and sterile water to give a final volume of 10µl. A 4-5 fold excess of restriction enzyme was sufficient to give complete digestion. The restriction was then stopped by the addition of 5µl of 5x agarose gel loading buffer.

**Amplification refractory mutation system (ARMS)**

The ARMS test is used to detect specific pathogenic mutations where target DNA sequences differ by a single nucleotide. Allele specific oligonucleotide primers are designed to differ at the nucleotide that occurs at the extreme 3' terminus. This is because DNA synthesis in a PCR reaction is crucially dependent on a correct base pairing reaction at the 3’end.

Paired PCR reactions are carried out. One primer (the common primer) is the same in both reactions, the other exists in two slightly different versions, one specific for the normal sequence and the other specific for the mutant sequence. An additional control pair of primes is usually included, which will amplify some unrelated sequence from every sample, as a check that the PCR reaction has worked.

**Agarose gel electrophoresis analysis of PCR product restriction digests**

Either a 2%(w/v) agarose gel or 4%(w/v) agarose gel (3g NuSieve: 1g LMP Agarose) was prepared with TBE electrophoresis buffer, depending on the size of the DNA fragments to be separated. The agarose was melted in a microwave oven and then cooled before the addition of 50ng/ml Ethidium Bromide. Gels were then poured into gel casting platforms to achieve a thickness of between 0.5 - 1cm. Gel combs were carefully placed and the gel allowed to set.
The gel casting platform, containing the set gel, was then placed in an electrophoresis tank and submerged in TBE buffer. 10-15μl of PCR digest mixture was loaded into each gel well and electrophoresed at 50-70V for 100-120 minutes. DNA molecular weight markers were loaded onto the gel for reference (Boehringer Mannheim Marker VIII). Fragments of DNA were then visualized by UV transillumination and photographed using a video image capture system and thermal printer.

2.8 LINKAGE ANALYSIS

Affected sib-pair analysis

In affected sib-pair analysis the assumption is made that sibling pairs will have inherited the same genotype at the locus (or loci) causing the disease. They are said to be identical by descent (IBD). If one parent has the marker alleles \(ab\) and the other parent \(cd\), the possible genotypes of the offspring are \(ac, ad, bc\) or \(bd\). The affected proportion of sibling pairs inheriting the same two parental chromosomes is 25%, for sharing a single parental chromosome 50%, and for sharing no parental alleles 25%. Linkage should result in a significant decrease in the proportion of sibling pairs sharing no parental alleles.

If linkage exists between a marker and disease, a distortion in the sharing of alleles from that expected will be seen. The significance of the distortion is determined by \(\chi^2\) estimation. If dealing with a dominant disease, a sib-pair will exhibit excess sharing of 1 or 2 alleles, if a recessive disease they will exhibit excess sharing of 2 alleles.

Alleles shared and not shared were counted using the Genetic Analysis System (GAS program), noting the parental origin and parental status for each of the phenotypes.
studied. All sibling pairs from multiple sib-ships were examined independently. Chi-square estimation was used to determine the significance of deviations from the expected proportion of shared alleles.

**Transmission disequilibrium test**

In the transmission disequilibrium test, the transmission of a particular allele at a locus from heterozygous parents to their affected offspring is examined. Under Mendelian inheritance, all alleles should have a 50% chance of being transmitted from one generation to the next. In contrast, if one of the alleles is associated with disease risk it will be transmitted more often than 50% of the time.

The GAS program (GAS v2.1) was used to perform association analysis between a marker and affectation locus using the transmission disequilibrium test. The proportion of maternally and paternally derived alleles transmitted more than 50% of the time, was assessed and a p value assigned.

**Power to detect genetic effects**

The ability of the affected sib-pair method to detect a disease susceptibility locus depends on the contribution the locus makes to the genetic variation of the trait. This can be measured using the risk ratio ($\lambda_4$), which is defined as the risk of disease in the siblings of an affected proband divided by the risk of disease in the general population, or by measuring the genotypic relative risk (GRR). The GRR is defined as the increased chance that an individual with a particular genotype has the disease and differs from $\lambda_4$ since it refers to a specific sequence variant while $\lambda_4$ refers to the combination of all involved genes. The higher $\lambda_4$ is, the easier it is to detect linkage (Risch N 1990). Similarly the larger the GRR and the more frequent the sequence variant (mutation or polymorphism) the greater the likelihood of linkage.
Whilst linkage analysis has been used effectively to find major genes, it has limited power for detecting genes of modest effect. In diabetes mellitus where the $\lambda_s$ is 15, the affected sib-pair method has been used to detect linkage to several polymorphisms with some success. In atopic disorders, where the $\lambda_s$ is $<2$ the power to detect linkage is considerably less for the same sample size. This would account for difficulties in replicating studies of linkage in atopic disorders eg to achieve 90% power, over 1000 sibling pairs will be required.

Tests of association utilising candidate genes, such as the TDT test of Spielman, have far greater power for finding genes in complex diseases where the gene effect is likely to be modest. Risch and Merikangas have estimated the sample size $N$ (number of families) necessary to obtain 80% power (the probability of rejecting the null hypothesis when it is false) (table 6) (Risch N and Merikangas K 1996). With a linkage approach and a disease gene with a GRR of 4 or greater, the number of affected sibling pairs necessary to detect linkage is 185 or 297 provided the allele frequency $p$ is between 5 and 75%. For a gene with a GRR of 2 or less, however, the sample sizes are generally well over 2000, beyond the reach of most genetic studies. In contrast, the required sample size for the association test is less than for linkage, especially for affected sibling pair families when the $p$ value is small. For a disease gene with a GRR of 4, the number of affected sibling pairs necessary to detect association is 48 or 61 for an allele of moderate frequency ($p$ is 0.1 to 0.5). (table 6).

Predicting what level of risks unknown disease susceptibility loci will have is difficult. Based on the calculations of Risch and Merikangas, most studies will require a sample size of at least 200, which represents a compromise between an attainable sample size and reasonable power. Using this sample number of parent-affected child trios, a candidate locus accounting for 5% of disease and conferring an elevated risk of between 4 and 10 times will have 90% power to detect association (Weeks D, Lathrop M 1995).
Table 6: Comparison of linkage and association studies. (Number of families needed for identification of a disease gene).

<table>
<thead>
<tr>
<th>Genotypic risk ratio</th>
<th>Frequency of disease allele A (p)</th>
<th>Probability of allele sharing (Y)</th>
<th>No of families required (N)</th>
<th>Probability of transmitting disease allele A (P(tr-A))</th>
<th>Proportion of heterozygous parents (Het)</th>
<th>(Het) (N)</th>
<th>(Het) (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>0.01</td>
<td>0.520</td>
<td>4260</td>
<td>0.800</td>
<td>0.048</td>
<td>1098</td>
<td>0.112</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.597</td>
<td>185</td>
<td>0.800</td>
<td>0.346</td>
<td>150</td>
<td>0.537</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>0.576</td>
<td>297</td>
<td>0.800</td>
<td>0.500</td>
<td>103</td>
<td>0.424</td>
</tr>
<tr>
<td></td>
<td>0.80</td>
<td>0.529</td>
<td>2013</td>
<td>0.800</td>
<td>0.235</td>
<td>222</td>
<td>0.163</td>
</tr>
<tr>
<td>2.0</td>
<td>0.01</td>
<td>0.502</td>
<td>296,710</td>
<td>0.667</td>
<td>0.029</td>
<td>5823</td>
<td>0.043</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.518</td>
<td>5382</td>
<td>0.667</td>
<td>0.245</td>
<td>695</td>
<td>0.323</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>0.526</td>
<td>2498</td>
<td>0.667</td>
<td>0.500</td>
<td>340</td>
<td>0.474</td>
</tr>
<tr>
<td></td>
<td>0.80</td>
<td>0.512</td>
<td>11,917</td>
<td>0.667</td>
<td>0.267</td>
<td>640</td>
<td>0.217</td>
</tr>
<tr>
<td>1.5</td>
<td>0.01</td>
<td>0.501</td>
<td>4,620,80</td>
<td>0.600</td>
<td>0.025</td>
<td>19,320</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.505</td>
<td>7</td>
<td>0.600</td>
<td>0.197</td>
<td>2218</td>
<td>0.253</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>0.510</td>
<td>67,816</td>
<td>0.600</td>
<td>0.500</td>
<td>949</td>
<td>0.490</td>
</tr>
<tr>
<td></td>
<td>0.80</td>
<td>0.505</td>
<td>17,997</td>
<td>0.600</td>
<td>0.286</td>
<td>1663</td>
<td>0.253</td>
</tr>
</tbody>
</table>

In this study, 61 families with 95 parent-affected child trios in panel A and 88 families with 176 parent-affected child trios in panel B were analyzed using both tests of linkage and association to identify the significance of several polymorphisms.
within three candidate gene regions in the genetic determination of atopic dermatitis and asthma.
Chapter 3

Clinical results
3.1 CLINICAL DETAILS OF STUDY GROUP (PANEL A)

276 individuals in 61 families were separated into 3 groups consisting of parents (n=122), siblings (n=93) and probands (n=61). The average number of children per family was 2.5. 90.5% of the population was of European extraction, the remaining individuals being of either African (3.9%), Asian (1%) or mixed racial origin (4.3%). An excess of male patients was present in the proband group (61%). The difference in sex distribution among all AD patients however was negligible (53 males vs 54 females). Most individuals in the study group were either children or adults less than 40 years of age. Only 12.6% of individuals were older than 40 years.

107 of 276 individuals (39%) fulfilled the diagnostic criteria for AD. In 24 individuals (20 adults; 4 children) the diagnosis of AD was made on historical grounds, with no current evidence of dermatitis. 13 members with a historical diagnosis of AD were excluded as they failed to fulfill the diagnostic criteria for AD. The most common reason for not meeting the criteria was a history of infantile eczema with subsequent resolution of symptoms. There were no false positive diagnoses using the UK diagnostic criteria.

Prevalence of atopic disease

185 of 276 (67%) individuals in the study were atopic. 107 (39%) fulfilled the diagnostic criteria for AD whilst 83 (30%) were diagnosed with asthma and 96 (35%) with allergic rhinitis (hayfever and/or allergic conjunctivitis). “Pure AD”, meaning atopic dermatitis in the absence of respiratory atopy, was present in 27 of the 107 individuals (25%). 61/107 (57%) individuals with AD suffered with asthma and 37/107 (34.5%) with allergic rhinitis or conjunctivitis. 10 individuals (9.3%) with AD were “non-atopic” with negative skin test responses to allergens, negative
RAST measurements and a normal total serum IgE. Similarly 9.6% and 7% of individuals with asthma and allergic rhinitis were similarly non-atopic.

A history of allergic disease in either sibling or parent was present in 80.3% (49/61) families. A further 8 families were found to have positive SPT’s in the absence of clinical disease, making the total family history of atopy up to 93.4% (57/61).

**Parents:** 73 of 122 parents (60%) in the study were atopic with 42% manifesting clinically with atopic disease. 21 parents (17%) had atopic dermatitis, 21 (17%) asthma, 32 (26%) hayfever and 32 (26%) allergic conjunctivitis. A greater proportion of fathers suffered with asthma (mothers:fathers 9:12) and allergic rhinitis (mothers:fathers; 9:23). There was no difference in the distribution of atopic dermatitis amongst the parents (mothers:fathers; 11:10).

**Probands:** 59 of 61 probands (96.7%) were atopic with as many as 80% of this group having associated asthma and/or allergic rhinitis/conjunctivitis. 36 probands (63%) had associated asthma, 18 probands (33%) suffered with hayfever and 25 (46%) suffered with allergic conjunctivitis. 2 of the probands were non-atopic as determined by negative SPT’s, negative RASTS and total serum IgE.

**Non-probands:** 53 of 93 siblings (57%) were atopic with 40% of them suffering with either AD or respiratory atopy. 34 siblings (37%) were diagnosed as having AD, 22 siblings (25%) with asthma, 15 siblings (17%) with allergic rhinitis and 18 siblings (21%) with allergic conjunctivitis.
Distribution of atopic disease with age

The prevalence of atopic disease is seen to decline progressively with age with the majority of atopic individuals being children under the age of 11 years. The pattern of distribution of atopic disease is also seen to vary with age. Whilst AD and asthma are more prevalent than allergic rhinitis in children under the age of 5 years, this trend is reversed later on in life, with allergic rhinitis occurring more frequently than AD or asthma in individuals over the age of 10 years (see figure 3.1).

Figure 3.1

AD=atopic dermatitis; AS=asthma; AR=allergic rhinitis; NA=not affected
Severity of atopic dermatitis

Children with AD were recruited from the dermatology outpatients clinics at Great Ormond Street Hospital for Children, which serves as a tertiary referral center for children with skin disease within the United Kingdom. As might be expected, most of the probands recruited into the study had moderate to severe atopic dermatitis (95%), scoring >4.5 on the severity scoring system of Rajka and Langeland. 37% of all patients fulfilling the diagnostic criteria for atopic dermatitis were classified as having severe disease, 32% were graded moderately severe and 8.5% had mild disease. (Table 6)

Table 7: Severity of atopic dermatitis in study group

<table>
<thead>
<tr>
<th>Severity of AD</th>
<th>All patients with AD</th>
<th>Probands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>24 (22.5%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Mild</td>
<td>9 (8.5%)</td>
<td>3 (4.9%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>34 (32%)</td>
<td>21 (34%)</td>
</tr>
<tr>
<td>Severe</td>
<td>40 (37%)</td>
<td>37 (60.6%)</td>
</tr>
</tbody>
</table>
Predictors of AD

Simple correlation analysis using the statistical package for social sciences (SPSS) was used to assess the value of clinical criteria in predicting the development of atopic dermatitis (Table 7). Pruritis, a flexural rash, current dermatitis and an early age of onset were the best predictors of AD. Asthma and allergic rhinitis were less good at predicting a diagnosis of AD. Eosinophilia correlated poorly with the presence of AD (r=0.28).

Table 8: Clinical signs as predictors of AD

<table>
<thead>
<tr>
<th>Clinical sign</th>
<th>r value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pruritis</td>
<td>.67</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>flexural rash</td>
<td>.76</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>age of onset</td>
<td>-.73</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>dry skin</td>
<td>.65</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>asthma</td>
<td>.45</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>hayfever</td>
<td>.19</td>
<td>.002</td>
</tr>
<tr>
<td>current dermatitis</td>
<td>.69</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Month of birth

116 patients with a confirmed diagnosis of atopic dermatitis (past or present) were analyzed according to month of birth. A slight Summer peak was seen, with 33% of births occurring during the months of July-September, compared with only 17% born in October-December.

The remaining 50% of births were evenly distributed through the first six months of January-June. (Table 8)

Table 9: Month of birth of AD individuals

<table>
<thead>
<tr>
<th>Time period</th>
<th>Month of birth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jan - March</td>
<td>28 (26%)</td>
</tr>
<tr>
<td>April - June</td>
<td>26 (24%)</td>
</tr>
<tr>
<td>July - September</td>
<td>35 (33%)</td>
</tr>
<tr>
<td>October - December</td>
<td>18 (17%)</td>
</tr>
</tbody>
</table>
Incidence of atopy and atopic dermatitis in offspring according to parental atopic status

The incidence of atopy in children was assessed with respect to parental atopy (Table 9). 124/154 (86.4%) children from panel A were suitable for analysis. Both parents were found to be atopic in 22 of the 62 families. Of their offspring, 36/44 (81%) were similarly atopic. In the presence of uniparental atopy, 39/52 (75%) offspring were atopic. Using Fishers exact test, the number of atopic children born to atopic mothers versus atopic fathers was assessed and found to be insignificant (Fi stat = 6.72; p=0.08).

The panel was then assessed for the incidence of atopic dermatitis in children born to parents with atopic dermatitis (Table 10). Out of a total of 150 children, 92 had atopic dermatitis. 54% of children with AD, 59/92 were born to parents with no history of AD. No significant difference was found between the incidence of children with AD born to mothers with AD versus fathers with AD (Fi stat=2.91; p=0.41).

Finally, we assessed the panel for the incidence of AD in offspring born to atopic parents. 66/81 (81.5%) children with AD were born to atopic parents.

A maternal effect was not demonstrated in this study. The small sample size and selection criteria are likely to have influenced this result, with atopic fathers being more willing to participate in the study than those without atopy.
Table 10: The incidence of atopy in the offspring according to parental atopic status

<table>
<thead>
<tr>
<th>Atopy in offspring</th>
<th>Maternal atopy Yes</th>
<th></th>
<th>Maternal atopy No</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Affected</td>
<td>Unaffected</td>
<td>Affected</td>
<td>Unaffected</td>
</tr>
<tr>
<td>Paternal atopy Yes</td>
<td>36</td>
<td>8</td>
<td>32</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>(29%)</td>
<td>(6.5%)</td>
<td>(26%)</td>
<td>(8.9%)</td>
</tr>
<tr>
<td>Paternal atopy No</td>
<td>7</td>
<td>2</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>(5.6%)</td>
<td>(1.6%)</td>
<td>(12%)</td>
<td>(10%)</td>
</tr>
</tbody>
</table>

Table 11: The incidence of AD in the offspring of parents with AD

<table>
<thead>
<tr>
<th>Maternal AD Yes</th>
<th></th>
<th>Maternal AD No</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Affected</td>
<td>Unaffected</td>
<td>Affected</td>
</tr>
<tr>
<td>Paternal AD Yes</td>
<td>3</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>(2%)</td>
<td>(0.67%)</td>
<td>(9.3%)</td>
</tr>
<tr>
<td>Paternal AD No</td>
<td>16</td>
<td>6</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>(11%)</td>
<td>(4%)</td>
<td>(39%)</td>
</tr>
</tbody>
</table>
Profile of “non-atopic” AD patients

10 individuals with AD from 9 families in panel A were non-atopic, as defined by the absence of a raised serum total IgE level, negative skin prick tests to a panel of six allergens and negative RAST tests to the same allergens (Table 11). The non-atopic AD group was made up of four adults and six children, their ages ranging from 3-11 years. Two of the children were probands and in one family, both mother and child suffered with AD and allergic rhinitis.

None of the individuals in this group fulfilled the criteria for a diagnosis of severe skin disease. The diagnosis of atopic dermatitis was made on historical grounds in 3 patients. The remaining individuals had moderate skin disease according to the severity criteria of Rajka and Langeland. The pattern of distribution varied as with other groups of AD patients, from a predominantly flexural distribution to predominant involvement of the extensor surfaces. 1 individual had discoid eczema. 5/10 individuals had associated respiratory atopy and in five families, another member was found to be highly atopic, with serum IgE levels in excess of 500kU/l. In all instances these individuals were children.

Table 12: Profile of ‘non-atopic’ AD patients

<table>
<thead>
<tr>
<th>Age of patient</th>
<th>Age of onset</th>
<th>Severity of AD</th>
<th>Total IgE</th>
<th>Asthma</th>
<th>Allergic rhinitis</th>
<th>Parental atopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 yrs</td>
<td>&lt;2 yrs</td>
<td>Moderate</td>
<td>11</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>4 yrs</td>
<td>2-5 yrs</td>
<td>Moderate</td>
<td>3</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>6 yrs</td>
<td>&lt;2 yrs</td>
<td>Moderate</td>
<td>24</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>6 yrs</td>
<td>2-5 yrs</td>
<td>Moderate</td>
<td>7</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>8 yrs</td>
<td>5-10 yrs</td>
<td>Mild</td>
<td>15</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>11 yrs</td>
<td>&lt;2 yrs</td>
<td>Moderate</td>
<td>72</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>32 yrs</td>
<td>&lt;2 yrs</td>
<td>Absent</td>
<td>26</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>36 yrs</td>
<td>&lt;2 yrs</td>
<td>Absent</td>
<td>46</td>
<td>No</td>
<td>Yes</td>
<td>-</td>
</tr>
<tr>
<td>44 yrs</td>
<td>&lt;2 yrs</td>
<td>Absent</td>
<td>33</td>
<td>Yes</td>
<td>No</td>
<td>-</td>
</tr>
</tbody>
</table>
3.2 SERUM IgE ANALYSIS

**Total serum IgE**

*Mean serum total IgE:* The serum IgE was raised in 96.6% of the probands and in 77 of 107 (72%) individuals who fulfilled the diagnostic criteria for AD. Of these individuals with AD, 45 (42%) demonstrated markedly raised serum IgE levels of >500KU/l and 30 (28%) massively raised IgE levels of >2000KU/l. A maximum serum IgE of 30 000KU/l and a mean serum IgE level of 1224.1 KU/l in this group was obtained.

Of the 78 remaining atopic individuals without a diagnosis of AD, 12 (15.3%) had serum IgE levels of >500KU/l, but only 1 individual had a serum IgE measuring >2000KU/l. 38 individuals (20.5%) found to be atopic on the basis of a positive RAST, SPT and/or elevation of the serum IgE, had no history of allergic disease. None of the latter group of patients had massive elevation of their serum IgE levels. 25% of the parents of probands were found to have raised serum IgE levels (mean serum IgE = 43KU/l). The mean serum IgE in the siblings of the proband was 41.6 KU/l.

*Variation of serum IgE with age:* Figure 3.2 shows a scatter plot of log IgE vs. age. The highest values of IgE fall within the younger age group of children < 10 years whereas in the adult age groups, clustering of IgE occurs in the lower serum IgE ranges.
Figure 3.2: Distribution of serum IgE with age
**Effect of coexistence of respiratory atopy on serum IgE levels in AD:**

Applying the student t test to the study data in the statistical package for social sciences (SPSS), a concomitant diagnosis of AD and respiratory atopy (i.e., asthma and/or allergic rhinitis/allergic conjunctivitis) significantly increased serum IgE levels in all children with AD (p=0.002). (Table 12)

Table 13: Effect of coexistence of AD and respiratory atopy on log IgE

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cases</th>
<th>Mean log IgE</th>
<th>t value</th>
<th>Degrees of freedom</th>
<th>2-tail probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD - resp atopy</td>
<td>27</td>
<td>4.71</td>
<td>-3.36</td>
<td>44.35</td>
<td>0.002</td>
</tr>
<tr>
<td>AD + resp atopy</td>
<td>80</td>
<td>6.33</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Serum total IgE as a predictor of AD:** Multiple regression analysis using SPSS was used to assess the value of log IgE in predicting the presence of atopic dermatitis in this study. The height of IgE was found to be a significant predictor of AD (p<0.001) as well as a significant predictor of AD severity (p=0.001).
Specific IgE

Agreement between RAST and skin prick test positivity

The agreement between RAST and skin prick test (SPT) positivity was assessed using the kappa statistic (κ) which measures chance-corrected proportional agreement (Altman DG 1991). Agreement was tested in all children with AD (n=89), in the remaining children without AD (n=53) and in adults (n=122).

In children with AD RAST testing was more sensitive than skin prick testing in detecting specific IgE to common antigens, with the exception of *Dermatophagoides pteronyssinus* where the agreement between the two methods was 0.8. This observation was most marked when comparing agreement between the food allergens, egg white and cows milk (table 13). Good agreement was seen between RAST and skin prick tests in children without AD for *Dermatophagoides pteronyssinus, Phleum pratense* and *Felis domesticus*. There were insufficient numbers of children in this group with specific IgE to cows milk, egg white and *Alternaria alternata* to test agreement between the two methods.
Table 14: Agreement (κ) between RAST and SPT positivity in all children with AD

<table>
<thead>
<tr>
<th></th>
<th>HDM</th>
<th>Grass</th>
<th>Cat dander</th>
<th>Altenaria</th>
<th>Egg white</th>
<th>Cows milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rast</td>
<td>Rast</td>
<td>Rast</td>
<td>Rast</td>
<td>Rast</td>
<td>Rast</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>SPT +</td>
<td>54</td>
<td>3</td>
<td>44</td>
<td>3</td>
<td>35</td>
<td>2</td>
</tr>
<tr>
<td>SPT -</td>
<td>3</td>
<td>18</td>
<td>10</td>
<td>21</td>
<td>13</td>
<td>27</td>
</tr>
<tr>
<td>K value</td>
<td>0.80</td>
<td>0.63</td>
<td>0.61</td>
<td>0.50</td>
<td>0.41</td>
<td>0.08</td>
</tr>
<tr>
<td>Strength of agreement</td>
<td>good</td>
<td>good</td>
<td>good</td>
<td>moderate</td>
<td>moderate</td>
<td>poor</td>
</tr>
</tbody>
</table>

Table 15: Agreement (κ) between RAST and SPT positivity in children without AD

<table>
<thead>
<tr>
<th></th>
<th>HDM</th>
<th>Grass</th>
<th>Cat dander</th>
<th>Altenaria</th>
<th>Egg white</th>
<th>Cows milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rast</td>
<td>Rast</td>
<td>Rast</td>
<td>Rast</td>
<td>Rast</td>
<td>Rast</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>SPT +</td>
<td>13</td>
<td>3</td>
<td>7</td>
<td>1</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>SPT -</td>
<td>3</td>
<td>32</td>
<td>2</td>
<td>40</td>
<td>1</td>
<td>37</td>
</tr>
<tr>
<td>K value</td>
<td>0.88</td>
<td>0.94</td>
<td>0.67</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Strength of agreement</td>
<td>very good</td>
<td>very good</td>
<td>good</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
In adults, skin testing was more sensitive than RAST testing in detecting specific IgE to all of the aeroallergens tested. This was particularly so for the allergens *alternaria alternata* and *felis domesticus*. There was good agreement between the two methods for detecting specific IgE to *dermatophagoides pteronyssinus* and *phleum pratense*. RAST testing to food allergens was not assessed in adults because of the expected low yield. In this group of patients only 5/117 adults tested positive to egg white and 2/120 tested positive to cows milk on skin prick testing.

Table 16: Agreement (κ) between RAST and SPT positivity in adults

<table>
<thead>
<tr>
<th></th>
<th>HDM</th>
<th>Grass</th>
<th>Cat dander</th>
<th>Altenaria</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RAST</strong></td>
<td>Rast</td>
<td>Rast</td>
<td>Rast</td>
<td>Rast</td>
</tr>
<tr>
<td><strong>SPT +</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>17</td>
<td>33</td>
<td>9</td>
</tr>
<tr>
<td><strong>SPT -</strong></td>
<td>1</td>
<td>67</td>
<td>3</td>
<td>76</td>
</tr>
<tr>
<td><strong>K value</strong></td>
<td>0.68</td>
<td>0.77</td>
<td>0.55</td>
<td>0.35</td>
</tr>
<tr>
<td><strong>Strength of</strong></td>
<td>good</td>
<td>good</td>
<td>moderate</td>
<td>fair</td>
</tr>
</tbody>
</table>
**House dust mite (Dermatophagoides pteronyssinus)**

57/89 (51%) children with AD demonstrated specific IgE to *Dermatophagoides pteronyssinus* on both RAST and skin prick testing. The majority of RASTS 49/57 (86%) were scored as class 4-6 and two thirds of children with class 4-6 RASTs had moderately severe or severe skin disease according to the criteria of Rajka and Langeland. A positive RAST to *Dermatophagoides pteronyssinus* however failed to significantly predict disease severity.

**Timothy grass (Phleum pratense)**

RAST and SPT positivity to timothy grass was found in 54/78 (69%) and 47 (60%) children with atopic dermatitis respectively. 52% of individuals had RAST scores of class 4-6 (n=41) and of these two thirds had moderately severe or severe disease. A positive RAST to *Phleum pratense* grass did not however predict disease severity.

**Cat dander (Felis domesticus)**

52/107 (48%) individuals with AD complained of symptoms following contact with animals ranging from itchy eyes, sneezing and wheezing to itching of the skin, erythema, urticaria and worsening of their AD. In 21 (20%) of these individuals cats were noted to specifically cause a reaction. Of the 77 children with AD tested, 48 (62%) had positive RASTS to *Felis domesticus* and 37 (48%) demonstrated positive skin prick tests. 47% of the RAST scores were class 1-3 and 53% class 4-6. Of these, only 20 individuals (26%) reported being regularly exposed to cats at home and elsewhere. A positive RAST to cat significantly predicted AD severity (p=0.026), although the class of RAST did not appear to be an important severity determinant. 22 children with class 1-3 RAST scores and 27 children with class 4-6 RAST scores had moderately severe to severe disease. Of the 53 patients with positive RASTs to cat dander, only 15 individuals (28%) were aware of their sensitivity to cats.
Altenaria (altenaria alternata)

41/77 (53%) AD children demonstrated positive RASTs and 30/77 (39%) positive SPTs to *altenaria alternata*. This compares strikingly to the group of adults and children without AD in which only 11% and 4% of individuals respectively demonstrate specific IgE to *altenaria alternata* by either RAST or skin testing. Specific IgE to *altenaria alternata* failed however to predict either the presence of AD or AD severity.

Egg white

A positive RAST to egg white was found in 28/71 (39%) children with atopic dermatitis and positive SPT in 14 (20%) children with AD. The agreement between these two methods of testing was 0.41. Most individuals with a positive RAST to egg had moderately severe (23%) or severe (67%) disease. A positive RAST to egg white however failed to predict disease severity (p=0.825). 12 children gave a history of egg allergy as manifested by immediate cutaneous and/or respiratory symptoms and/or vomiting within one hour post ingestion. All 12 children demonstrated positive RASTs to egg white.

Cows milk

RAST positivity to cows milk was present in 30/73 (41%) patients. A positive RAST to cows milk failed to predict disease severity (p=0.95). Out of the nine children who gave a history of having reacted immediately in the past to the ingestion of cows milk, eight tested positive on RAST testing to cows milk.
Predictors of AD

Multiple regression analysis was used to detect which measurement of total and specific IgE best predicted AD in children. Only the total serum IgE was independently associated with the presence or absence of AD. (Table 16) None of the measurements of specific IgE to allergens significantly predicted AD.

Table 17: Significance of log IgE as a predictor of AD

<table>
<thead>
<tr>
<th>Variable</th>
<th>B (SE)</th>
<th>T</th>
<th>sig T</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log E</td>
<td>0.102 (0.014)</td>
<td>7.3</td>
<td>.000</td>
<td>26.6</td>
</tr>
<tr>
<td>Constant</td>
<td>1.081 (0.082)</td>
<td>13.3</td>
<td>.000</td>
<td></td>
</tr>
</tbody>
</table>

Predictors of severity of RAST and SPT in AD: RAST and SPT positivity and the height of the serum IgE were assessed as predictors of severity of atopic dermatitis in both children (n=154) and probands (n=61). In the group of children, severity was significantly predicted by the height of the total serum IgE (p=0.001) and a positive RAST to cat (p=0.026) (Table 17). Log IgE was the only predictor of severity in the probands (p<0.001). Skin prick testing failed to predict severity in either group. The association of respiratory atopy with atopic dermatitis also failed to significantly predict severity of atopic dermatitis in the group of probands (p=0.354).
Table 18: RAST positivity as a predictor of severity of atopic dermatitis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Size</th>
<th>T value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat</td>
<td>.382</td>
<td>2.27</td>
<td>0.026</td>
</tr>
<tr>
<td>HDM</td>
<td>.045</td>
<td>.343</td>
<td>0.732</td>
</tr>
<tr>
<td>Grass</td>
<td>-.018</td>
<td>-.130</td>
<td>0.897</td>
</tr>
<tr>
<td>Altenaria</td>
<td>.196</td>
<td>1.63</td>
<td>0.107</td>
</tr>
<tr>
<td>Egg white</td>
<td>.026</td>
<td>.234</td>
<td>0.815</td>
</tr>
<tr>
<td>Milk</td>
<td>.009</td>
<td>.069</td>
<td>0.945</td>
</tr>
</tbody>
</table>

Variation of RAST and SPT positivity with age

Multiple regression analysis using the SPSS package was used to assess the effect of age on RAST and SPT positivity. Age was found to negatively correlate with eosinophilia and a positive RAST to egg and cows milk.

Eosinophilia as a predictor of AD and disease severity

The presence of eosinophilia on the peripheral blood smear was seen to correlate positively to Log IgE and to the six common allergens tested by both RAST and SPT (Table 18). It also correlated weakly to AD and asthma but not hayfever. Eosinophilia correlated negatively with age.
Table 19: Correlation between eosinophilia and other parameters in children with AD

<table>
<thead>
<tr>
<th></th>
<th>r value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-.336</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>AD</td>
<td>.278</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Asthma</td>
<td>.193</td>
<td>.002</td>
</tr>
<tr>
<td>Hayfever</td>
<td>.076</td>
<td>.227</td>
</tr>
<tr>
<td>Log IgE</td>
<td>.414</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>RAST HDM</td>
<td>.370</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>RAST Grass</td>
<td>.302</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>RAST Cat</td>
<td>.370</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>RAST Altenaria</td>
<td>.303</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>RAST egg</td>
<td>.280</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>RAST Cows milk</td>
<td>.321</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

Atopic dermatitis and food allergy

46/95 (48%) children gave a history of reacting to foods with 32 children reacting within the first hour of ingestion. 27 of these children (28%), gave a convincing history of acute IgE mediated allergy manifesting within 1 hour of food ingestion. These included features such as anaphylaxis, acute urticaria, angioedema, swelling of the lips or tongue, involvement of the respiratory tract or immediate vomiting. Children who gave a history of erythema, itching, worsening of their AD or non-acute gastrointestinal symptoms were not included in this group. In most cases (21/27), the food antigen had been identified as being egg (12), cows milk (9), nuts (12), fish (2) and wheat (1).
3.3 DISCUSSION

Within a population there exists a spectrum of ability to respond to allergen exposure which is largely genetically determined. In our study 75% of children with single parental atopy and 81% of children with bi-parental atopy were atopic as defined by the presence of raised serum levels of total IgE and/or positive SPT or RAST responses. This is higher than other studies quoting an incidence of 35% and 60% of allergy in children with uni and bi-parental allergy respectively. (Morton NE. 1992) Studies in AD however have shown a family history of at least one of the atopic disorders to be present in up to 90% of individuals with AD (Rudzki et al 1991)(Kang and Tian. 1987). 80.3% of children with AD in our study had a first degree relative with clinically apparent atopic disease and 93.4%, a sibling or parent with atopy as defined by SPT positivity.

This study emphasised the highly atopic nature of children with AD, with 80% having associated asthma, allergic rhinitis or conjunctivitis and 28% giving a convincing history of immediate food related allergic disease. IgE dysregulation was a prominent feature in this cohort of patients, with a raised serum total IgE being seen to significantly predict the presence of AD (p<0.001). Furthermore the height of the IgE (log IgE) was a significant predictor of AD severity (p=0.001). A striking feature in this study was the height of the mean serum IgE in the group of probands in panel A. In 42% of the probands the serum IgE levels were >500 KU/l and in 28% of probands the serum IgE levels were massively elevated, >2000 KU/l. The height of the serum IgE was seen to associate significantly with disease severity and was the only significant predictor of severity in the group of probands(p=0.001). Furthermore the height of the serum IgE was significantly associated with the coexistence of a personal or family history of asthma or allergic rhinitis. The coexistence of respiratory atopy did not however significantly predict disease severity(p=0.354). The serum IgE was seen to significantly decline with age.
Previous investigators have found the serum IgE to be raised in up to 82% of patients with AD. In agreement with our study the coexistence of a personal or family history of respiratory atopy appeared to significantly enhance the IgE producing potential (Uehara 1989)(Rudzki E and Litewska D 1990). Severity of disease however, has not always been associated with elevation of the serum IgE (Juhlin, Johansson et al. 1969)(Mackie RM, Cobb SJ et al. 1979) and levels have been found to be normal in approximately 20% of patients with otherwise typical AD. (Stone SP, Muller SA et al. 1973)(Gurewitch AW, Heiner DC et al. 1973). In our study 28% of individuals with AD had normal levels of serum IgE.

RAST and skin prick tests continue to be used in the diagnosis of allergic disease but the interpretation of these tests is still fraught with problems. Ideally a skin test should reflect only the degree of sensitization of a test subject. However, in practice, a number of additional factors are known to influence the test result. These include the cut-off chosen, the test technique, the type of allergen extract, the age, race and sex of the subject. A 3mm cut-off greater than the negative control, was used in this study as this has been shown to represent a ten-fold greater concentration of specific IgE than a true reaction of 2mm and is the recommended cut-off for epidemiological studies (Dreborg S 1988). A significant relationship exists between RAST and skin tests, with a 5mm skin test reaction diameter best predicting a positive RAST (Malling HJ, Dreborg S et al. 1986). In the study by Brown et al subjects with wheal diameters of <5mm were found to have raised specific IgE in 51% of cases, whereas those with wheal diameters of >5mm had raised IgE levels in 91% of cases. (Brown WG, Halonen MJ et al. 1979). They concluded that specific IgE levels were more specific for atopy, whilst skin prick tests were more sensitive. Of prick test negative subjects, 97.5% were also negative for specific IgE.

In this study RAST measurements were found to be more sensitive than skin prick testing in detecting specific IgE to five of the six allergens tested in children with
AD. This was particularly true for the food allergens egg white and cows milk and *alternaria alternata*. The reverse was true for adults where skin testing was more sensitive than RAST measurements for the four aeroallergens tested. In children without AD there was good agreement between RAST and skin testing to the antigens *dermatophagoides pteronyssinus, phleum pratense and felis domesticus*. RAST testing in this group did not appear to be more sensitive than skin testing in detecting specific IgE.

Severity of skin disease was shown by Wagenpfeil et al to adversely affect the reproducibility of skin test reactions in patients with AD (Wagenpfeil S, Grabbe J et al. 1991). In our study 60.6% of probands in panel A had severe skin disease and 34% moderately severe skin disease. The height of serum IgE was seen to significantly predict disease severity and indeed in 42% of probands the total serum IgE was in excess of 500KU/l and in 42% in excess of 2000KU/l. The inhibitory effect of total serum IgE on the skin test response is well documented. Eriksson tested 593 patients with hayfever and found that at each level of atopy (as defined by skin test), patients with a high total IgE had higher RAST values than those with low total IgE (Eriksson NE 1989). Others have reported lower allergenic reactivity in tropical environments with high total serum IgE levels secondary to helminthic infestations (Merrett TG, Merrett J et al. 1976) (Lynch NR, Hagel I et al. 1992). One explanation would be competition of the irrelevant IgE with specific IgE for binding to the IgE receptors on mast cells and basophils. Certainly in-vitro studies have been able to demonstrate inhibition of specific IgE binding through saturation of mast cell FcεRI receptors (Witteman AM, Stapel SO et al. 1996).

The RAST to egg white and cows milk in the study was shown to correlate negatively with age indicating that sensitivity to these allergens was confined predominantly to young infants and children. This is in agreement with the studies of Kjellman et al which have shown IgE antibodies to egg white and cows milk to
reach a peak prevalence at 8 months of age and to disappear successively during childhood. (Hattevig G, Kjellman B et al. 1993). IgE antibodies to inhalants on the other hand appeared from 2 years of age and increased in frequency during childhood. A positive SPT to cows milk and egg white failed to correlate negatively with age. One would anticipate this result not only due to the inhibitory effect of total serum IgE on skin test reactivity but also due to the progressive acquisition of skin test reactivity which occurs throughout childhood. The absence of a positive skin prick test to egg white and cows milk in the face of specific IgE to these allergens in the study may therefore simply reflect poor skin test reactivity in a young cohort of patients.

9.3% of individuals with AD in the study were non-atopic with normal serum IgE measurements and negative specific IgE responses to allergens. It has been shown previously that the Langerhans cells in skin biopsies from ‘non-atopic’ AD patients do not bear IgE (Kagi, Wuthrich et al. 1994). Hence the skin reactions due to irritants in this group of patients are not IgE-mediated. Kagi et al looked at the cytokine profiles of patients with non-atopic AD and compared these to patients with IgE responsive AD (Kagi, Wuthrich et al. 1994). The latter group of patients demonstrated a Th-2 related cytokine pattern with elevated levels of IL-4 and IL-5 whereas the non-atopic AD patients displayed high IL-5 but low IL-4 levels. A similar cytokine pattern was seen in lesional skin biopsies from these two groups of patients suggesting that these difference in cytokine production were largely T cell derived. This observation implies different immunological mechanisms responsible for the ongoing inflammation in the skin of patients with non-atopic AD. It is possible that this group of patients may represent a genetically distinct sub-population. Repeat testing of this population of “non-atopic” AD patients for the presence/absence of specific IgE against common allergens is necessary to establish whether these findings are consistent.
A relationship has been shown to exist between the variation in specific IgE to HDM and asthma and bronchial hyper-responsiveness (Cookson WOCM et al. 1991) and to grass pollen and allergic rhinitis. In this study no relationship was found between AD and variation in specific IgE to any of the six common antigens tested.
Chapter 4

Molecular genetic studies
in
atopic dermatitis
4.1 INTRODUCTION

A number of genes have been identified which are known to influence the development of asthma and atopy. There is considerable overlap in the pathogenesis of asthma and AD. Genetic similarities however have yet to be demonstrated. Using a candidate gene approach, the role of several genes known to influence asthma and atopy were assessed for their genetic contribution in the development of AD. Genetic linkage and association studies were used to assess the statistical significance of polymorphisms within the beta chain of the high affinity receptor gene and within the genes encoding TNF and IL-4.

4.2 THE BETA CHAIN OF THE HIGH AFFINITY IgE RECEPTOR

4.1 Introduction

IgE dysregulation is a major pathogenic feature of atopic dermatitis. Serum IgE levels are typically elevated and other IgE mediated allergic diseases, such as asthma and allergic rhinitis, are frequent accompaniments of this inflammatory skin disorder. It is postulated that contact of skin with environmental atopens triggers an allergen-specific immune response mediated via IgE. Allergen pre-complexed to IgE binds to the high affinity receptor for IgE (FceRI) on the surface of Langerhans cells thus initiating a series of events leading to the cellular release of inflammatory mediators.

Langerhans cells are the primary antigen-presenting cell of the epidermis (Maurer D and Stingl G 1995)(Klubal R and Kinet Mar 1997) The discovery of IgE binding structures on their cell surface provided an explanation for the enhanced functioning of these cells in the uptake, processing and presentation of allergen to T cells. Functional studies on monocyte enriched, lactic acid treated PBMC’s show that the targeting of allergens to monocytes by antigen-specific IgE critically depends on
FceRI expression. Moreover the ability to present allergen to T cells is increased 100-1000 fold if the allergen has been targeted to FceRI on these cells via allergen-specific IgE (Maurer D et al. 1995). Mudde et al showed that HDM antigens penetrating the epidermis of patients with AD, are only capable of being presented to T cells by IgE positive Langerhans cells (Mudde GC et al. 1990)(Mudde GC et al 1992). Recently the expression of FceRI on Langerhans cells has been shown to be increased in patients with AD compared to non-atopic patients (Jurgens M et al 1995). These findings together with other studies have proposed FceRI as the primary IgE binding structure on antigen presenting cells, providing a pivotal link between aeroallergens and antigen specific T cells infiltrating the skin lesion in AD.

Kinet et al have gone a long way to elucidating the structural and functional characteristics of FceRI (Ravetch JV and Kinet JP 1991). This multimeric receptor exists either as a \( \alpha \gamma_2 \) trimer or a \( \alpha \beta \gamma_2 \) tetramer in humans, with both forms being capable of activating haemopoeitic cells. This is in contrast to mouse studies where transfection with all three chains (\( \alpha, \gamma, \beta \)) is required for surface expression of the receptor. Complimentary DNAs have been isolated for the \( \alpha, \gamma \) and \( \beta \) chains of FceRI allowing their structural characteristics to be defined. The \( \alpha \) subunit, unlike the \( \gamma \) and \( \beta \) subunits, is a homologous member of the immunoglobulin superfamily. It represents the main IgE binding structure, acting independently of the other subunits. The \( \gamma \) subunit is a member of a group of related proteins (the zeta-dimers) that function as signal transducing components of both Fc receptors and the T cell antigen receptor (TCR). The contribution of each subunit to receptor signaling is still to be defined. The extracellular domain of the \( \alpha \) chain has been shown to function as the main IgE binding structure, attaching itself to the Fc portion of IgE. (Maurer D et al. 1995) The \( \gamma_2 \) dimer was thought to function as an autonomous activation module. A recent review however has proposed a model in which both \( \gamma_2 \) and \( \beta \) chains synergise in the initiation of Fc epsilon signal transduction (Lin S et al.
The predominant function of the β chain is that of a signal amplifier, providing a gain of 5-7 fold as measured by Syk activation and calcium mobilization.

Several polymorphisms have been defined within FcεRIβ showing association to the phenotypes of atopy and asthma. No studies however have been able to clearly demonstrate linkage of atopic dermatitis to FcεRIβ. This gene continues to be an important candidate region for study in AD, particularly since increasing evidence suggests the existence of similar pathogenic mechanisms underlying the development of atopic diseases. One of the main aims of this study was to analyze the putative role of FcεRIβ in the genetic determination of atopic dermatitis. This was achieved by looking for linkage and association of AD to two non-coding Rsal polymorphisms situated within intron 2 and the untranslated region of exon 7 respectively, in addition to a polymorphism within exon 7 of FcεRIβ coding for a single mutational base change.
Materials and Methods

Subjects: The study panel consisted of two groups:

Panel A: 61 nuclear families recruited through a single proband with AD with a total of 276 subjects and 37 affected AD sibling-pairs. (see chapter 3 for description of population)

Panel B: 88 multiplex families recruited through two affected siblings with AD by a previous investigator (Coleman R et al 1993).

Genotyping: For detection of the Rsal polymorphisms within intron 2 and the untranslated region of exon 7 of FceRI-β, specific PCR was carried out followed by restriction digestion of the PCR products. Amplification Refractory Mutation System (ARMS) was used to test for the presence of the E237G variant within exon 7 of the FceRI-β gene.

Rsai_in2: Rsai-1 restriction endonuclease introduces a restriction site in intron 2 of FceRIβ (Accession No: Genemblem file M89796). Amplification of genomic DNA (100ng) was performed in a Perkin Elmer Cetus thermal cycler, using 35 cycles of 94°C for 1 min, 61°C for 1 min, 72°C for 1 min. The reaction mix contained 0.3μM of each primer, 200μM dNTP’s, 0.1% (v/v) NP40, 0.1%(w/v) gelatin, 0.1M KCl, 0.1M Tris-HCl pH 8.8, 2mM MgCl₂ and 0.75 units of Taq DNA polymerase in a final volume of 15μl. Primers were 5’-TCT GTC TGT CGA GAA TGT TGC-3’ and 5’-CTG GTT AGA TCT GAG AAA GAG-3’.

Rsai_ex7: Exon 7 runs from 7224 - 10214 in the GENEMBLE File M89796 and is translated from 7224-7322. The Rsai-1 site in the untranslated region of exon 7 lies at position 9867. The recognition sequence of Rsai-1 is GTAC. The nucleotide change which abolishes the restriction site is documented:

9861 TTATAGTACG 9870 to 9861 TTATAGTATG 9870
Amplification of genomic DNA (200ng) was performed in a Hybaid Omnigene thermal cycler (block control) using 32 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. The PCR reaction mix contained 0.2μM of each primer, 200μM dNTP’s, 67mM Tris-HCl pH 8.8, 16mM (NH₄)₂SO₄, 0.01% Tween-20, 1.5mM MgCl₂ and 0.75 units Taq DNA polymerase in a final volume of 15μl. Primers were 5’ TCA CTG TGT ATC ATG CTA AGC 3’ and 5’ TGA TAC AAT ACT GCA TCG TGG 3’.

5μl of amplification products were digested with 4 units of RsaI enzyme (New England Biolabs, USA) together with the recommended buffer, in a final volume of 10μl, for 1 hour at 37°C. Digested products were resolved on a 2% (w/v) agarose gel containing 1mcg/ml ethidium bromide, run for 60 minutes at 70V in a BioRad wide sub cell. Restriction digestion of the intron 2 PCR product yielded 3 genotypes with an additional constant 148bp band being present as a result of the primers used. The 1,1 genotype was denoted by the presence of a 800bp and 148bp band pattern, the 2,2 genotype by the presence of a 500bp, 280bp and 148bp band pattern and the 1,2 genotype by a 800bp, 500bp, 280bp and 148bp band pattern. Digestion of the exon 7 polymorphism potentially gave rise to 3 genotypes: a single 481bp band denoting the 1,1 genotype, 481, 295 and 187bp band pattern denoting the 1,2 genotype or 295 and 187bp band pattern denoting the 2,2 genotype.

_E237G variant in exon 7:_ E237G lies within the C terminal cytoplasmic end of FceRIβ, within the coding region of exon 7. The polymorphism is an amino acid change at residue 237 from glutamic acid to glycine. Sequencing affected individuals confirms a nucleotide change from adenine to guanine at nucleotide residue 7297 of the published sequence of the file (GENEMBLE accession number M89796).
The following primers were used to test for the presence of the E237G polymorphism at nucleotide residue 7297 of the published sequence of the FceRI-β gene.\textsuperscript{14}

B7FA1: TGG CCA GCT AGT CTG GTT TGG TTT TCT GGA 30mer
B7FA2: GGA GCA TAT TAA GGT GGA CAG AAG CAG CAG 30mer
B7M1: ATT CAG CTA CTT ACA GTG AGT TGG AAG ACC CAG GCG G 37mer
B7W2: AC GTG ATT CTT ATA AAT CAA TGG GAG GAG ACA ATT 36mer

A total reaction volume of 50μl containing 1μM of each of the primers B7FA1, B7FA2, B7W2 and 0.5μM of the primer B7M1, 10x KCl buffer (1%(v/v) NP40, 1%(w/v) gelatin, 1M KCl, 1M Tris HCl pH 8.3), 2mM MgCl\textsubscript{2}, 200μM dNTP’s, and 50ng of genomic DNA was used. A hot start PCR was done with the addition of 2 units of \textit{Taq} DNA polymerase (Boehringer Mannheim, UK) after an initial 5 min denaturation at 95°C. PCR conditions then followed 35 cycles of 94°C for 1 min, 62°C for 2 min and 72°C for 2 min followed by a single cycle of 72°C for 10 min. Amplified products were then visualized in an ethidium bromide stained 4%(w/v) agarose gel (3:1 NuSieve:LMP agarose) and run for 120 min at 60V in a BioRad wide sub. The primers B7FA1-B7FA2 gave a 446bp control band, B7FA1-B7W2 a 280bp band in the presence of adenine at nucleotide position 7297 of the published sequence and B7M1-B7FA2 a 238bp band in the presence of guanine at position 7297.

Statistical analysis: The transmission disequilibrium test implemented in the GAS program (version 2.1)(A. Young, unpublished), was used to test for transmission of alleles at a particular locus from heterozygous parents to their affected offspring. The proportion of alleles transmitted from both mother and father was tested against a null hypothesis of 50% transmission by $\chi^2$ analysis.
Fig 4.1: *RsaI* restriction endonuclease digestion of the FcεRIβ intron 2 polymorphism

![Image of gel electrophoresis with markers and DNA bands](image)

**M**  
Marker VIII DNA molecular weight standards (Boehringer Mannheim)

**Lanes 1-14**  
Digested DNA of 14 individuals showing constant 148bp band as a result of primers used.

**Lane 1**  
Homozygous cut (*Rsa I*)  
500bp, 280bp and 148bp bands

**Lane 3**  
Homozygous uncut (*Rsa I*)  
800bp and 148bp bands

**Lane 4**  
Heterozygote (*Rsa I*)  
800bp, 500bp, 280bp and 148bp bands
Fig 4.2: *RsaI* restriction endonuclease digestion of the FceRIβ exon 7 polymorphism

<table>
<thead>
<tr>
<th>M</th>
<th>Marker VIII DNA molecular weight standards (Boehringer Mannheim)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lanes 1-5</td>
<td><em>RsaI</em> digested DNA of 5 individuals from a single family</td>
</tr>
<tr>
<td>Lane 1 and 5</td>
<td>Homozygous uncut single 481bp band</td>
</tr>
<tr>
<td>Lane 2 and 4</td>
<td>Homozygous cut 295bp and 187bp bands</td>
</tr>
<tr>
<td>Lane 3</td>
<td>Heterozygote 481bp, 295bp and 187bp bands</td>
</tr>
</tbody>
</table>
Fig 4.3a: ARMS testing for the FcεRIβ E237G variant

M
Marker VIII DNA molecular weight standards (Boehringer Mannheim)

Lanes 1-11
Digested DNA from 11 unrelated individuals showing constant 446bp control band.

Lane 1
Heterozygote E237G variant (A→G base substitution) - 280bp and 238bp bands

Lanes 2-11
Wild type sequence - 280bp band
Fig 4.3b: ARMS testing for the FeeRIβ E237G variant

Lanes 2-8: Digested DNA from 7 individuals from a single family.
Lane 1,2,7: Wild type sequence - 280bp band
Lane 3: Homozygote for the E237G variant - 238bp band
(A→G base substitution)
Lane 4,5,6,8: Heterozygote for the E237G variant - 280bp and 238bp bands
(A→G base substitution)
Results

*Genetic linkage of Rsa 1 FceRIβ polymorphisms to atopic dermatitis:*

Applying the Haseman-Elston regression algorithm implemented in the G.A.S. program (Young 1995-SW), each locus was analyzed for linkage to AD, log IgE and physician diagnosed asthma in population A.

Linkage of the *Rsa 1* polymorphism to AD within intron 2 (*Rsa1_in2*) and within the untranslated region of exon 7 (*Rsa1_ex7*) was tested by counting alleles shared by affected sib-pairs. Excess sharing of maternally derived alleles was seen for each marker *p*=0.0078 (*Rsa1_in2*), *p*=0.016 (*Rsa1_ex7*). Of the alleles that were paternal in origin no significant sharing was seen. The difference between the proportion sharing maternally derived alleles and that sharing paternally derived alleles was significant, Fisher’s exact test=6.08, *p*=0.06, (*Rsa1_in2*) and Fisher’s exact test=3.75, *p*=0.029 (*Rsa1_ex7*). Thus the detection of transmission of atopic dermatitis at this locus was only through the maternal line. Linkage was not demonstrated to either log IgE or asthma.
Table 20: Genetic linkage of FcεRIβ RsaI polymorphisms to AD in panel A

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Allele sharing 1</th>
<th>Allele sharing 0</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rsa 1 intron 2</td>
<td>Maternal 7</td>
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<td>0.0078</td>
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<td></td>
<td>Paternal 4</td>
<td>8</td>
<td>0.81</td>
</tr>
<tr>
<td>Rsa 1 exon 7</td>
<td>Maternal 6</td>
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<td>0.016</td>
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<td></td>
<td>Paternal 4</td>
<td>7</td>
<td>0.73</td>
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</table>

Table 21: Genetic linkage of FcεRIβ RsaI polymorphisms to asthma in panel A

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Allele sharing 1</th>
<th>Allele sharing 0</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rsa 1 intron 2</td>
<td>4</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>Rsa 1 exon 7</td>
<td>3</td>
<td>4</td>
<td>0.5</td>
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</tbody>
</table>

Table 22: Genetic linkage of RsaI FcεRIβ polymorphisms to log IgE in panel A

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Sibling pairs</th>
<th>Slope</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rsa 1 intron 2</td>
<td>83</td>
<td>-0.091</td>
<td>0.48</td>
</tr>
<tr>
<td>Rsa 1 exon 7</td>
<td>81</td>
<td>-0.42</td>
<td>0.43</td>
</tr>
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</table>
Genetic association of Rsa1 polymorphisms within FceRIβ to atopic dermatitis:

Allelic association of two non-coding Rsa1 polymorphisms within FceRIβ to atopic dermatitis, log IgE and physician diagnosed asthma was analyzed using the transmission disequilibrium test implemented in the G.A.S. program. 61 families within panel A and 88 families within panel B were examined for transmission of one of two alleles coding for each of the Rsa1 polymorphisms. In panel A subjects, transmission of allele 2 of the Rsa1 intron 2 polymorphism (Rsa1_in2*2) (p=0.0022) and allele 1 of the Rsa1 exon 7 polymorphism (Rsa1_ex7*1) (p=0.0036) to offspring with atopic dermatitis occurred significantly more frequently than expected by chance (Table 1). The difference between the proportion sharing maternal versus paternal alleles was highly significant; p=0.0019, \( \chi^2 = 9.66 \) (Rsa1_in2*2) and p=0.0031, \( \chi^2 = 8.78 \) (Rsa1_ex7*1). No significant association of these polymorphisms was found with asthma.

The association of the two Rsa1 polymorphisms with atopic dermatitis was confirmed in panel B families, in which an excess sharing of maternally derived alleles for both Rsa1_in2*2 (p=0.018) and Rsa1_ex7*1 (p=0.02) was found. Furthermore, an association with allergic asthma was present in maternally derived alleles; Rsa1_in2*2 (x transmitted:y not transmitted, 15:6, p=0.039) and Rsa1_ex7*1 (14:6, p=0.058).

Combining the data from panels A and B significantly strengthened the associations with atopic dermatitis and asthma. Significance was strongest when examining the TDT sharing of maternal Rsa1 alleles with atopic dermatitis (Table 2). The significance of transmission of Rsa1_in2*2 was p=0.0002 and Rsa1_ex7*1 p=0.00034. Transmission of these alleles was also significantly associated with asthma when maternally inherited; Rsa1_in2*2 (p=0.0068) and Rsa1_ex7*1 (p=0.018).
Table 23: (TDT): Sharing of FcεRIβ Rsal alleles with atopic dermatitis in panels A and B

<table>
<thead>
<tr>
<th>Panel</th>
<th>Rsal polymorphism</th>
<th>Allele</th>
<th>Paternal</th>
<th>Maternal</th>
<th>p</th>
<th>Trans</th>
<th>Not trans</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>in2</td>
<td>1</td>
<td>17</td>
<td>10</td>
<td>0.12</td>
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<tr>
<td></td>
<td></td>
<td>2</td>
<td>10</td>
<td>17</td>
<td>0.88</td>
<td>16</td>
<td>3</td>
<td>0.0022</td>
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<tr>
<td>A</td>
<td>ex7</td>
<td>1</td>
<td>9</td>
<td>16</td>
<td>0.89</td>
<td>17</td>
<td>4</td>
<td>0.0036</td>
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<tr>
<td></td>
<td></td>
<td>2</td>
<td>16</td>
<td>9</td>
<td>0.11</td>
<td>4</td>
<td>17</td>
<td>NS</td>
</tr>
<tr>
<td>B</td>
<td>in2</td>
<td>1</td>
<td>24</td>
<td>17</td>
<td>0.17</td>
<td>10</td>
<td>23</td>
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<tr>
<td></td>
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<tr>
<td>B</td>
<td>ex7</td>
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<td>21</td>
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<td>2</td>
<td>21</td>
<td>18</td>
<td>0.37</td>
<td>11</td>
<td>24</td>
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</tbody>
</table>

Table 24: TDT: Sharing of FcεRIβ Rsal alleles with atopic dermatitis in combined panels A and B

<table>
<thead>
<tr>
<th>Rsal polymorphism</th>
<th>Allele</th>
<th>Paternal</th>
<th>Maternal</th>
<th>p</th>
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<tbody>
<tr>
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<td>0.87</td>
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Table 25: TDT: Sharing of FcεRIβ RsaI alleles with asthma in combined panels A and B

<table>
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<th>RsaI polymorphism</th>
<th>Allele</th>
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<td>Not trans</td>
<td>p</td>
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<td>18</td>
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<td>25</td>
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<tr>
<td></td>
<td>2</td>
<td>22</td>
<td>16</td>
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</tbody>
</table>

No significant allele sharing was seen for either RsaI polymorphism when assessing the TDT probabilities for the phenotype of atopy, as defined by at least one positive skin prick test or RAST score and/or elevation of total serum IgE.

*ARMS testing for the E237G variant:* The E237G coding variant was present in 6.5% of subjects from panel A, but failed to associate significantly with atopic dermatitis (p=0.38) or asthma (p=0.5). For this reason the polymorphism was not examined in panel B subjects.

Table 26: TDT association of the E237G variant and AD in panel A

<p>| TDT association of the E237G variant and AD in panel A |</p>
<table>
<thead>
<tr>
<th>Source</th>
<th>Allele</th>
<th>Transmitted</th>
<th>Not transmitted</th>
<th>p value</th>
</tr>
</thead>
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<td></td>
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<td>2</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>Paternal</td>
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<td>4</td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>2</td>
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</table>
Discussion

In this study, genetic linkage and association of atopic dermatitis to two non-coding Rsα1 polymorphisms within FceRIβ on chromosome 11q was demonstrated. Inheritance was most clearly demonstrated in maternally derived alleles and no excess sharing of paternally derived alleles was seen. The results of this study were confirmed by testing for association in a second independent population, namely population B. Combining the data from the two populations A and B considerably strengthened the association between atopic dermatitis and Rsα1 intron 2*2 (p=0.0002) and Rsα1 exon7*1 (p=0.00034) in maternally derived alleles. In addition the maternally derived allele 2 of Rsα1 intron2 and allele 1 of Rsα1 exon 7 were significantly associated with physician diagnosed asthma. The results of this study suggest a putative role for FceRIβ in the transmission of atopic dermatitis and asthma.

Strict adherence to a set of six diagnostic criteria for atopic dermatitis, as defined by the UK working party, ensured an accurate diagnosis of AD. This set of criteria was applied prospectively to population A and retrospectively to population B. The latter population was recruited originally using the major and minor criteria for AD of Hanifin and Rajka. The diagnosis of asthma was made using the standard asthma questionnaire as set out by the American Thoracic Society.

Previously, a genetic study which looked at linkage of the AD phenotype to several markers within the chromosome 11q12-13 region, excluded linkage to this locus with location scores to -36 and lod scores to -7.8 (Coleman R et al. 1993). However when the data was reanalyzed taking into account parental atopic phenotype, a positive lod score of 0.8 was observed in the group with unaffected fathers. On this basis, a maternal pattern of inheritance could not be excluded. The markers analyzed namely, D11S97, PGYM and CD20, were situated some genetic distance telomeric
to the FcεRIβ locus, which may have accounted for their failure to unequivocally detect or exclude linkage to this locus when maternally inherited.

Several previous studies have suggested that maternal atopy increases the risk of atopy in subsequent offspring. Two reports on the predictive value of cord IgE concentrations noted higher concentrations in infants of atopic mothers than those of atopic fathers (Magnusson CGM 1988). Another study showed maternal atopy to pose a higher risk for infantile AD than paternal atopy (Ruiz G et al. 1992). Cookson et al were able to detect inheritance of the atopy locus on chromosome 11q12-13 only through the maternal line (Cookson WOCM et al. 1992). Similarly the transmission of a polymorphism within FcεRIβ coding for atopy and bronchial hyperresponsiveness, was shown to occur more frequently in maternally derived alleles (Hill MR et al. 1995). A maternal pattern of inheritance for AD was clearly demonstrated in this study, with the Rsa1 alleles being transmitted almost exclusively through the maternal line. This pattern of inheritance is consistent with either paternal genomic imprinting or with maternal modification of developing immune responses.

A high proportion of patients with atopic dermatitis from both study populations also suffered with asthma, reflecting previously published figures supporting the association of AD with respiratory atopy (Rajka G 1989). This association appears to boost IgE levels with far higher levels of IgE being present in AD patients with concomitant symptoms of asthma and allergic rhinitis compared with ‘pure AD’ or ‘pure asthma’ sufferers (Wittig HJ et al. 1980). It is possible that genetic regulation of the asthma/atopic dermatitis phenotype is occurring at the FcεRIβ locus, with a particular FcεRIβ genotype determining increased FcεRI receptor expression on the surface of Langerhans cells, mast cells and basophils and upregulation of the IgE response.
In this study allelic association was demonstrated to two non-coding polymorphisms situated at either end of the FcεRIβ gene and AD and asthma. Association was most strongly seen with the AD phenotype suggesting the possible existence of a major locus for AD situated somewhere within the FcεRIβ exons between these two polymorphisms. Analysis of the E237G coding variant in exon 7 of FcεRIβ however failed to show significant association with AD or asthma. This polymorphism which codes for an adenine to guanine substitution, has previously been shown to associate with asthma and total serum IgE levels in a Japanese population (Shirakawa T et al. 1996) and to asthma, bronchial reactivity and skin test responses to grass pollen and house dust mite in a general Australian population sample (Hill MR and Cookson WOCM 1996).

To date none of the polymorphisms identified within FcεRIβ sufficiently explain the chromosome 11q13 effect, whereby 60% of the populations studied demonstrate linkage to the marker D11S97 within the atopy locus. Novel structural variants within FcεRIβ need to be identified and tested for association to atopy, asthma and atopic dermatitis. The functional effect of these FcεRIβ variants on receptor expression and function is another important area for study. It is tempting to speculate that increases in β amplifier gain may be being influenced by structural variants within the FcεRIβ gene. An alternative theory is that FcεRIβ variants may be exerting an effect on the proportion of αγ2β receptors expressed, thereby increasing the strength of the FcεRI-mediated signal.

The discovery of new sequence variants within the FcεRIβ gene will have important implications for our understanding of how atopy-associated β polymorphisms participate in the pathogenesis of disease.
4.3 TUMOUR NECROSIS FACTOR

Introduction
Mechanisms involved in the initiation and maintenance of skin inflammation in atopic dermatitis are poorly understood. Why patients with AD fail to terminate their ongoing immune activation and cutaneous inflammation remains a difficult but critical question. Repeated antigen exposure triggering IgE and Th2 cell expansion is one possible explanation. However IgE dependent mechanisms are not a constant feature of disease, with up to 20% of patients failing to exhibit specific IgE responses to common allergens (Johnson EE et al. 1988)(Hoffman DR et al. 1975). In recent years it has become apparent that the pattern of cytokines expressed locally play a vital role in modulating the nature of tissue inflammation.

Tumour necrosis factor α, (TNF-α) is a powerful pro-inflammatory cytokine with a number of cytotoxic as well as immuno-modulatory activities. This pleiotropic cytokine has recently been implicated in the pathogenesis of allergic asthma with studies showing increased levels of TNF-α in the sputum of asthmatic patients (Taki F et al 1991) and in the fluid and cells obtained from broncho-alveolar lavage of asthmatic airways (Virchow JC et al. 1995). Secretion of TNF-α by alveolar macrophages is also increased in asthmatics exhibiting a late reaction when challenged with HDM antigen (Gosset P et al. 1991). Bronchial mucosal biopsies from symptomatic asthmatic individuals show greater numbers of cells positive for TNF-α and increased amounts of TNF-α within the mucosal mast cells compared with asymptomatic and control individuals (Ackerman V et al. 1994). In atopic dermatitis, levels of TNF-α have been shown to be increased both in the plasma and within human dermal mast cell granules (Walsh LJ et al. 1991). Furthermore, increased numbers of cells expressing mRNA for TNF-α in allergen induced late phase responses, have been found in the skin and nasal tissue of atopic subjects (Bradding P et al. 1994).
TNF-α contributes to the chronic inflammatory process underlying AD in a number of ways. Firstly, it is released during the cellular influx of granulocytes and mononuclear cells, which occurs as part of the late phase skin reaction following antigen challenge. This release of TNF-α, most probably from dermal mast cells, gives rise to increased expression of the vascular endothelial molecule ELAM-1 which binds to complimentary ligands on inflammatory cells, leading to the efflux of inflammatory cells into the surrounding tissues (Montefort S and Holgate ST 1991) (Schleimer RP et al. 1991). Secondly, TNF-α is capable of exerting a direct chemotactic effect on cells as well as activating a wide range of inflammatory cells such as eosinophils, neutrophils, macrophages and lymphocytes (Kips JC et al. 1993).

The human TNF locus, consisting of two genes arranged in tandem, has been located to the short arm of chromosome 6, situated within the major histocompatibility complex (HLA) between HLA-B and the HLA class III region (Dunham I et al. 1987) (Carroll MC et al. 1987). These genes, namely TNF-α and lymphotoxin-α (originally TNF-α and TNF-β), contain up to 10 polymorphic regions between them and are in close linkage disequilibrium with alleles of the MHC. The HLA-DRB1*03 haplotype in particular appears to be important in regulating this locus and has been previously associated with increased secretion of TNF by peripheral blood lymphocytes and monocytes (Jacob CO et al. 1990).

Two RFLPs within the TNF gene region have been shown to contribute to the development of asthma. The first of these, an NcoI biallelic polymorphism located within the first intron of lymphotoxin α (LT-α NcoI), codes for a guanine to adenine base change at the restriction site as well as the substitution of threonine for asparagine at position 26 (Messer G et al. 1991). The less frequent allele of this polymorphism, allele 1(LT-α*1), containing the NcoI recognition site has been shown to associate with the extended HLA haplotype HLA-A1,-B8,-DR3 (Wilson
AG et al. 1993). More recently it has also been associated with increased levels of LT-α secretion in PHA stimulated cells (Messer G et al. 1991) and with asthma (p=0.005) (Moffatt MF et al. 1994). No significant difference in TNF-α titres were reported in individuals homozygous for the LTα*1 allele in the study of Messers et al. Interestingly, homozygosity for allele 2 of LT-α in another study resulted in significantly higher amounts of TNF-α production by LPS stimulated monocytes (Campbell DA et al. 1996).

The second polymorphism known to associate with asthma is the -308 TNF-α RFLP situated within the promoter region of the TNF-α gene. This biallelic polymorphism results in a G to A base change at position -308 in the TNF2 alleles. Allele 2 of this polymorphism has been associated with increased levels of TNF-α secretion (Wilson AG et al. 1994), bronchial hyperreactivity (p=0.038) (Campbell DA et al. 1996) and asthma (p=0.004) (Moffatt MF et al. 1995). The latter association was confined to the LT-α*1/TNF-308*2 haplotype, making it difficult to differentiate between the individual effects of the LT-α and TNF-308 alleles. Importantly the HLA-DR locus was excluded as a cause of this association.

The TNF locus is an important candidate area for study in AD as mechanisms other than IgE dysregulation almost certainly play a major role in the pathogenesis of this chronic relapsing skin disease. To date there have been no reported allelic associations of polymorphisms within the TNF locus with atopic dermatitis. In this study, the contribution of a single RFLP within the TNF locus, LT-α NcoI, to the development of AD and asthma is assessed.
Materials and methods

Subjects: Subjects from panel A were analyzed for linkage and association to the LT-α NcoI polymorphism in intron 1 of the LT-α gene. See chapter 2.1 for description of population.

Methods: The primers used are those of Messer et al generating a PCR product of 750 bp.

5' primer BB28814 22mer
5' CCG TGC TTC GTG CTT TGG ACT A 3'

3' primer BB28815 22mer
3' AGA GCT GGT GGG GAC ATG TCT G 3'

The PCR was adapted for Falcon microtitre plates in the Hybaid Omnigene using Bioline Taq polymerase 0.45U, the NH4+ buffer, a final concentration of 1.0mM MgCl₂, 200µM dNTP’s, 1µM of each primer and 200ng of DNA in a final volume of 15µl. The following set of conditions were used: Initial denaturation of 95°C x 6 min followed by 30 cycles of 95°C x 1 min, 65°C x 1 min and 72°C for 1 min. Extension was completed with 5 min at 72°C.

Restriction digest of PCR products with NcoI restriction endonuclease 5U/PCR was carried out at 37°C for 1 hour. Restriction was stopped by the addition of 3x loading buffer. 15µl of digest product was then run on a 2% agarose gel for 45 min at 70V.
Fig 4.4  Restriction endonuclease digest of the TNFβ- NcoI polymorphism

M  Marker VIII DNA molecular weight standards (Boehringer Mannheim)
Lanes 1,2,8  Homozygosity for TNFβ1 allele (1,1)  500bp and 250bp
Lanes 3,4,5,6,9  Heterozygosity for TNFβ1 and TNFβ2 alleles (1,2)  250bp, 500bp and 750bp
Lane 7  Homozygosity for TNFβ2 allele (2,2)  750bp
Results

Linkage analysis: 61 families from panel A were analyzed using sibling-pair analysis implemented in G.A.S. program for linkage of the LT-α NcoI polymorphism within the TNF gene region to atopic dermatitis, asthma and log IgE. No significant linkage was found to either AD (p=0.5), asthma (p=0.81) or log IgE.

Association studies: Applying the transmission disequilibrium test implemented in G.A.S. program to subjects in panel A, allele 1 of LT-α was significantly associated with atopic dermatitis (p=0.032) and asthma (p=0.024). The association did not depend on inheritance through either the maternal or paternal line.

Table 27: Association of tumour necrosis factor LT-α NcoI polymorphism with AD and asthma

<table>
<thead>
<tr>
<th>Source</th>
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<th>Trans</th>
<th>Not trans</th>
<th>p value</th>
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<td>46</td>
<td>0.97</td>
<td>15</td>
<td>29</td>
<td>0.98</td>
</tr>
</tbody>
</table>
Discussion

Weak association of LT-α*1 was demonstrated to atopic dermatitis and asthma when observing transmission of alleles to affected offspring. The contribution of HLA-DRB1*03 to the association of LT-α*1 with AD was not assessed.

This was a small preliminary study which did not incorporate haplotypes of the MHC in its analysis. HLA restriction is presumed to be involved in the recognition of specific allergens in AD, although significant association with alleles of the MHC has not been demonstrated (Saeki H, Kuwata S et al. 1994). In the study of Moffat et al, the association of LT-α*1 and TNF-308*2 to asthma was shown to occur independently of HLA-DRB1*03. Both of these alleles were shown to be in strong linkage disequilibrium with each other (p<0.0001) and the LT-α/NcoI*1/TNF-308*2 haplotype was in strong disequilibrium with HLA-DRB1*03.

Further analysis of this population would involve examining the transmission of TNF-308 alleles to affected AD offspring and assessing the relationship of these alleles with LT-α haplotypes and alleles of the MHC. The full contribution of TNF in the genetic determination of asthma and AD is still to be determined. Analysis of other RFLPs and microsatellite markers within this region may shed further light on the intricate associations of these polymorphisms, not only with each other, but with neighboring MHC haplotypes.
4.4 INTERLEUKIN-4

Introduction
A large number of cytokines are produced by T cells, each cytokine having complex cellular effects. Two patterns of cytokine expression have been distinguished in humans, originating from Mossman’s initial studies of T cells in mice. Th1 cells synthesize IL-2, IFN-γ and TNFβ whereas Th2 cells synthesize IL-3, IL-4, IL-5 and IL-10 (Mosmann TR and Coffman RI 1989). Both cells are capable of producing TNF-α, GM-CSF and IL-6. In atopic subjects a number of factors favour the development of Th2 cell clones at the level of the target tissue, suggesting that the atopic state is a predisposition for atopen-specific CD4+ T cells to differentiate into Th2 cells.

Experimental data from studies have supported the view that the pathophysiology of allergic asthma, rhinitis and AD are very similar, sharing common inflammatory mechanisms. Both AD and allergic airways disease are characterized by IgE-dependent mast cell activation, the release of inflammatory mediators and the subsequent recruitment and activation of leucocyte populations, including mast cells, eosinophils and T cells. Furthermore, the allergic response in the skin and airways has been shown to be bi-phasic. In AD however, important differences are seen in the late phase response which differentiates this disease immunopathologically from allergic asthma and rhinitis. In the early response, skin infiltrating lymphocytes from biopsies of acutely eczematous skin show secretion of significant amounts of IL-4, GM-CSF and TNFα upon mitogen stimulation, but minimal amounts of IFN-γ (Reinhold U et al. 1991). Biopsies from chronic eczematous skin lesions are more typical of a delayed type hypersensitivity response consisting predominantly of Th1
CD4+ T cells, monocyte/macrophages and increased numbers of fully granulated mast cells. (Sugiura H and Uehara M 1993). Thus it is postulated that Th-2 cytokines are critical for the initiation of AD, while Th-1 cytokines such as IFN-γ are important for the propagation of the inflammatory process (Kapp A 1995).

Interleukin-4 is perhaps the most important pro-inflammatory cytokine in allergic disease, playing a key role in the initiation and maintenance of allergic inflammation. Primarily it acts by switching B lymphocytes to produce IgE, acting synergistically with IL-6 and TNF-α. A second role for this important cytokine is in influencing the differentiation and maturation of CD4+ T cells along the Th2 pathway, a function not shared by any other cytokine (Ricci M 1994). In this way IL-4 is able to positively regulate its own production. Thirdly IL-4 stimulates the expression of vascular endothelial adhesion molecule 1 (VCAM-1) leading to the recruitment of eosinophils to the site of allergic inflammation.

The factors which govern the skewing of T helper lymphocytes to either Th1 or Th2 cell clones are not fully understood. In vitro studies looking at the proliferative responses of lymphocytes from umbilical cord blood of infants born to atopic parents, suggest that this “skewing” is most probably an early event, occurring sometime during fetal life. IFNγ production by umbilical cord blood mononuclear cells is reduced in newborns with a family history of atopic disease (Warner JA et al. 1992). Furthermore, significant differences in the peripheral blood mononuclear cell (PBMC) proliferation responses to specific allergenic triggers at birth have been demonstrated in infants who subsequently develop allergic disease (Warner JA et al. 1994).

The specificity of the cytokine profile for certain allergens whilst not for others cannot be simply ascribed to a maturational delay in overall T cell function. Primary
sensitization of T cells is likely to be occurring in utero, to account for these altered immune responses at birth. Thus an infant at the time of delivery may already be programmed to respond in a certain way to antigens, with migration of specifically sensitized T cells to the target organ concerned following postnatal allergen exposure. The factors determining the primary sensitization of T cells are likely to be both genetic and environmental, with specific genes influencing the way in which T cells respond to allergen in utero. Geneticists have proposed that over-expression of the genes governing IL-4, IL-5, IL-9, IL-13 and GM-CSF is occurring in allergic individuals and that these cytokines could in turn be inducing the expression of their own genes, thus further amplifying the skewing of the antigen-specific T cell repertoire.

The genes encoding Th2 associated cytokines are in the main grouped together on the long arm of chromosome 5, between 5q 22 and 5q31. The IL-13 gene is centromeric and closely linked to the IL-4 gene whereas the IL-5 gene is mapped downstream from IL-4. Telomeric to these genes lies the IL-3, GM-CSF and IL-9 genes. Interestingly these genes are similarly arranged on mouse chromosome 11, suggesting some selective advantage for this conserved clustering. One hypothesis is that these genes are to some extent co-regulated.

The clustering of these cytokine genes on chromosome 5 have made this region an excellent candidate area for genetic study in allergic disease. Recently, a number of groups have been able to identify via linkage analysis genetic markers within and around 5q31-33 controlling total serum IgE and bronchial hyper-responsiveness in an American and Dutch population (Marsh DG et al. 1994) (Postma DS et al. 1995) (Xu J et al. 1995). Extending the hypothesis of co-regulation whereby a shared regulatory element governs the atopic state, investigation of the promoter regions of five genes within this region by single stranded conformational polymorphisms (SSCP) was carried out in asthmatic and control families. Three polymorphisms
were identified in the IL-3, IL-9 and IL-4 5' promoter regions (Borish L et al. 1994). The IL-4 base substitution involving a C to T exchange at position -590bp from the open reading frame, was present in 40% of the subjects studied and was significantly associated with enhanced IL-4 activity and increased serum IgE production in atopic asthmatic individuals. It was proposed that this promoter polymorphism may be important in the transcriptional regulation of the genetic mechanisms underlying the development of asthma and atopy. Subsequent analysis of this polymorphism in a British cohort recruited through an asthmatic proband failed to show significant association with any measures of asthma or atopy, whereas it was weakly associated with wheeze (p=0.029) and specific IgE to house dust mite (p=0.013) in a general Australian population (Walley AJ and Cookson WOCM 1996).

The purpose of this study was to analyze the significance of the IL4 promoter polymorphism in influencing the development of atopic dermatitis and atopy in a population recruited through a proband with AD.
Materials and methods

Subjects: 276 subjects from panel A were included in the analysis.

Methods: The following primers were used to amplify DNA containing the IL-4 promoter polymorphism at position -590, counting from the first ATG codon. (Genumble accession number: M23442).

5’ primer AW41A: 5’-ACT AGG CCT CACCTG ATA CG-3’
3’ primer AW41B: 5’-GTT GTA ATG CAG TCC TCC TG -3’

The PCR was optimized for Boehringer Mannheim Taq DNA polymerase in a final volume mix of 15μl: 1.5μl 10 x NH₄ reaction buffer, 1.5μl 500μM dNTP’s, 1.5μl of each primer and 0.6U Taq polymerase was added to 50 ng genomic DNA. PCR amplification was carried out in a Perkin Elmer Cetus Therma Cycler using the following conditions: 94°C x 5 min followed by 32 cycles of 30 seconds at 94°C, 57°C and 72°C and a final 5 min at 72°C. This resulted in a PCR product of 252bp spanning positions 522 to 774 in the interleukin-4 promoter sequence.

Restriction digest was carried out using 1 unit of BsmFI (New England Biolabs) and the appropriate buffer at 37°C for 1 hour. This temperature was chosen rather than the optimum temperature of 65°C for BsmFI activity, because of the undesirable Taq DNA polymerase activity at the higher temperature. At 37°C the restriction enzyme retained 50% of its activity. The reaction was stopped by the addition of 5 x loading buffer and run on a 2% agarose gel at 70V for 90 minutes.
Fig 4.5: *BsmFI* restriction endonuclease digestion of PCR products amplified from nucleotide 522 to 744 of the IL-4 gene

<table>
<thead>
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<th>Lane</th>
<th>Description</th>
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</thead>
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<td>M</td>
<td>DNA molecular weight marker (Boehringer Mannheim Marker VIII)</td>
</tr>
<tr>
<td>Lane 1</td>
<td>homozygote for the -590 C→T allele</td>
</tr>
<tr>
<td>Lanes 2,4,5,6,7</td>
<td>homozygote for the wild type allele</td>
</tr>
<tr>
<td>Lane 3</td>
<td>heterozygote for the -590 C→T allele and the wild type allele</td>
</tr>
</tbody>
</table>
Results

Linkage analysis: 13 of 37 affected sib-pairs had parents who were informative for transmission of the IL4 G to C base substitution at position -590. No significant sib-pair distortion for atopic dermatitis (p=0.5), asthma (p=0.77) or log IgE (slope 1.7) was demonstrated with this polymorphism.

Table 28: Linkage analysis for the IL-4 promoter polymorphism and AD by descent

<table>
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<tr>
<th>Source</th>
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<th>Alleles not shared</th>
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</tr>
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<tr>
<td>Pat &amp; Mat</td>
<td>7</td>
<td>6</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Association studies: The transmission dis-equilibrium test of Spielman, implemented in G.A.S. program, was used to test for the association of IL4 promoter polymorphism alleles with atopic dermatitis and asthma. Transmission of alleles did not occur more frequently than expected by chance for either atopic dermatitis or asthma.

Table 29: Allelic association of the IL-4 promoter polymorphism with atopic dermatitis

<table>
<thead>
<tr>
<th>Source</th>
<th>Allele</th>
<th>Transmitted</th>
<th>Not transmitted</th>
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</tr>
</thead>
<tbody>
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<td>0.93</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9</td>
<td>3</td>
<td>0.073</td>
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<td>Paternal</td>
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<td>2</td>
<td>8</td>
<td>9</td>
<td>0.5</td>
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<td></td>
<td>2</td>
<td>30</td>
<td>25</td>
<td>0.3</td>
</tr>
</tbody>
</table>
Discussion

Both tests of linkage and association failed to demonstrate a significant role for the IL4 promoter polymorphism in the genetic regulation of atopic dermatitis in this study.

There is increasing evidence supporting the role of transcription factors in the expression of inflammatory cytokines. It is thought that transcription factors may be important in influencing disease severity. Conversely, response to certain immunomodulatory therapies used in the treatment of atopic disease e.g. cyclosporin A, glucocorticoids, may exert their effect through interaction with transcription factors. Their importance in the regulation of allergic disease however is still unknown.

Polymorphisms have been identified within the first 0.5-1kB upstream from the open reading frame of three genes within the chromosome 5 gene cluster by SSCP. Of these polymorphisms, the -590 C-T IL4 promoter polymorphism generated particular interest as it was found to be functional for enhanced IL-4 activity in atopic asthmatics in one study. This finding was not supported in a British asthmatic population or in the current study investigating the role of the IL-4 promoter polymorphism in AD. It was however weakly associated with certain measures of asthma and atopy in an Australian population. Despite these negative findings it is still possible that the genetic control of cytokine expression in allergic disease and hence the skewing of the Th2 response, may be governed by transcriptional factor expression of polymorphisms within the promoter regions in more distal upstream or downstream enhancer sequences.

The switching of the inflammatory response within the skin from a Th2 to a Th1-cell mediated response may be important in the perpetuation of chronic dermal inflammation in AD. The factors which determine these observed changes are
unclear, but may well be genetically determined, with the pattern of cytokines secreted being centrally regulated by one or several transcription factors. Further work to confirm the existence and importance of polymorphisms within the promotor regions of the inflammatory cytokines is needed.
Chapter 5

Conclusion
Atopic dermatitis and allergic airways disease have increased in prevalence over the last three decades in most developed countries around the world. Up to 15% of children in the UK will develop AD and 9% atopic asthma. The diseases frequently co-exist with up to 60% of children with AD developing asthma and up to 80% of asthmatics developing allergic rhinitis. Increasingly AD is being recognised as a major risk factor for the development of atopic respiratory disease, representing the first step of the “allergic march” to asthma and allergic rhinitis. 80% of children with AD in our study had associated asthma or allergic rhinitis.

Although a familial component to allergic diseases has been recognised for over 80 years, inheritance of the atopic phenotype is complex, failing to inherit in simple Mendelian fashion. This complexity is thought to reflect the polygenic nature of the disease and the interaction of genes with environmental factors. Defining the genetics of atopy and allergic disease is a crucial step towards understanding these complex interactions and the identification of those infants most at risk for the development of allergic disease. In this study a candidate gene approach was used to investigate the putative role of a number of polymorphisms in the genetic determination of AD, based on our understanding of the complex pathophysiology of the disease.

IgE dysregulation is a key feature of AD, with most but not all patients demonstrating exaggerated IgE responsiveness to common allergens. In this study 96.6% of probands and 72% of all individuals with AD had raised serum IgE levels, with 42% demonstrating serum IgE levels in excess of 500KU/l. Furthermore, the height of serum IgE significantly predicted disease severity. Cross-linking of
allergen specific IgE to FceRI receptors has been shown to occur on the surface of human epidermal Langerhans and FceRI expression has been shown to be increased in lesional AD skin. (Bieber T and Ring J 1992). These cells play an important role in allergen presentation, amplifying the immune response so that only very small amounts of antigen are needed to provoke an allergic response triggering IgE production and Th2 cell expansion (Van der Heijden FL et al. 1993).

The gene encoding the β chain of FceRI (FceRIβ) on chromosome 11q was selected as a likely candidate gene for AD in this study and the role of several FceRIβ polymorphisms assessed in the genetic determination of AD. Linkage and association of atopic dermatitis to two non-coding RsaI polymorphisms within FceRIβ was demonstrated in maternally derived alleles, in a panel of families (panel A), recruited through a single proband with AD. These results were confirmed in a second independent panel, (panel B), recruited through two first degree relatives with AD. Combining the data from these two panels considerably strengthened the reported associations, showing significant sharing of maternal alleles for both atopic dermatitis and asthma. A coding polymorphism within the exon 7 region (E237G variant) was rare in our families and failed to associate significantly with AD or asthma.

Studies looking at the inflammatory infiltrate in skin biopsies from acute and chronic lesional AD skin and following atopy patch testing, have provided interesting insight into the pathogenesis of AD. As with asthma and allergic rhinitis, a biphasic response is seen to occur. However in AD, the late phase response appears to consist predominantly of Th1 cells whereas in asthma and allergic rhinitis both early and late phase responses are predominantly Th2 driven (Friedmann PS et al. 1995). Thus it
is postulated that Th-2 cytokines are critical for the initiation of AD, while Th-1 cytokines, such as IFN-γ, are important for the propagation of the inflammatory process (Kapp A 1995).

Interleukin-4 is perhaps the most important inflammatory cytokine in allergic disease, playing an important role in immunoglobulin class switching to IgE. It also influences the differentiation of CD4+ T cells along the Th2 pathway. The gene encoding IL-4 is grouped together with a number of other Th2 associated cytokines on chromosome 5q22-31. In this study a single promoter polymorphism within the IL4 region was analysed to assess its role in AD. Both tests of linkage and association failed to demonstrate any significant relationship of this polymorphism to AD. Weak association was however demonstrated between AD and a biallelic polymorphism within another important inflammatory Th2 cell derived cytokine gene, namely TNF β (NcoI-TNFβ). This polymorphism was also shown to associate weakly with asthma.

Both FcεRIβ and TNFβ were identified as potential candidate genes determining the phenotypic expression of atopic dermatitis. Polymorphisms within these genes were also shown to associate with asthma, suggesting the existence of genetic similarities underlying the development of atopic dermatitis and asthma. The exclusion of linkage and association to a polymorphism within the promoter region of IL-4 suggests that other polymorphisms within this important candidate gene region may be important in the genetic regulation of atopic dermatitis. Further studies are needed investigating the role of transcription factors and the genes that they regulate within the promotor regions of the inflammatory cytokine genes.
Tests of association in this study were more sensitive than linkage analysis in detecting disease genes, with significant transmission disequilibria being demonstrated for atopic dermatitis and asthma in the absence of significant linkage.

A maternal effect was clearly demonstrated in this study. This has previously been substantiated by a number of family and candidate gene based studies. Arshad et al found that a family history including maternal disease, posed a higher risk factor for the development of atopy than paternal disease (Arshad SH et al. 1993), whilst Happle et al found asthmatic mothers to have more asthmatic children than asthmatic fathers (Happle R and Schnyder UW 1982). Similarly maternal atopy was found to pose a higher risk for the development of atopic dermatitis than paternal atopy (Ruiz G et al. 1992). Investigation of cord blood IgE levels in the infants born to atopic mothers and fathers provides additional evidence for a maternal effect (Magnusson 1988)(Shirakawa T et al. 1993). Cookson et al showed transmission of atopy at the chromosome 11q locus to be occurring through the maternal line (Cookson WOCM et al. 1992).

The finding of a maternal effect poses an interesting question. Exactly how a mother exerts her influence on the subsequent development of the atopic response is not yet fully understood. In rodents maternal IgG has been shown to modify the IgE responses of offspring (Jarrett EE and Hall E 1983). It is possible that similar modification of the fetal and neonatal immune system is occurring in humans, either through placental transfer of IgG or through subsequent transmission of IgG in breast milk. Desensitization of atopic mothers by antigen injection however, has had uncertain effects on the subsequent immunological development of the human fetus (Jarrett EEE and Hall E 1983). Another explanation for the maternal influence on atopy is that of paternal genomic imprinting in which paternally derived genes are not expressed. A gene imprinted in this way would remain inactive through the life of an individual, although it could result in disease when passed through a female to
her children. Under this model of inheritance, substantial numbers of 'carriers' of atopy would exist. Cookson et al however have demonstrated the presence of atopy in the children of atopic fathers and non-atopic mothers and that the sharing of maternal alleles was not significantly greater than paternal alleles in these individuals (Cookson WOCM et al. 1992). Furthermore, that the risk of atopy in the offspring born to parents who are both atopic is greater than that if only one parent is atopic, suggests that both parents are capable of transmitting susceptibility genes for atopy. The recent finding of maternal linkage at several loci to measures of atopy favours immunological interactions between mother and child, rather than imprinting or anticipation (Daniels SE et al. 1996).

The discovery of raised proliferative responses to anti-CD3 and beta-lactoglobulin in the peripheral blood mononuclear cells of infants at birth, who subsequently develop AD, supports the theory of maternal-fetal interactions (Warner JA et al. 1994). Based on these findings it has been postulated that primary sensitization to allergen may in fact be an in utero event, with a genetically susceptible infant being 'programmed' to react in a certain way to antigen already at the time of birth. Development of the immune response thereafter is thought to be an evolutionary process, taking as long as 2 years to mature before allergen-specific IgE is produced. Certainly sensitization to allergen can occur at any time of life but the first encounter with antigen appears to be crucial in determining subsequent antigen sensitivity (Holt PG et al. 1990). Holt et al proposed the first few months of life as a critical period in determining primary allergen sensitization, especially given the relative immaturity of the immune system in early infancy and the possibility that transient deficiency of IgA during this period, may facilitate the penetration of allergens (Holt PG et al. 1992)(Taylor B et al. 1973). They were able to demonstrate quantitative differences in T cell numbers and maturation in the T cells from cord blood samples of infants at 'high risk' for the development of atopy. Disappointingly, breast feeding and maternal avoidance of allergenic foodstuffs during pregnancy have not been
found to reduce the prevalence of AD or respiratory allergy in children followed to 2 and 4 years (Arshad SH et al. 1993)(Zeiger RS et al. 1992).

The rise in prevalence of allergic disease around the world cannot be attributed solely to genetic factors. Environmental factors are more likely to be determining this increase. Whether the reported increase in prevalence of atopic disease however is as a result of increased exposure to the more potent allergens, or merely an increased susceptibility to allergens which have always been present, is not yet known. Certainly several studies have positively correlated mite exposure with an increased risk for the development of atopic dermatitis and asthma (Korsgaard J 1983)(Platts-Mills TAE et al. 1982)(Sporik R et al. 1990). It has also been speculated but not proven that changes in farming practices may have increased levels of *altenaria* and grass allergens. Diet is another frequently implicated risk factor in the development of allergic disease, although studies examining the benefits of allergen avoidance regimes on the incidence of allergic disease are lacking in agreement. (Chandra RK et al. 1986)(Falth-Magnusson K 1992). Much attention has been focussed in recent years on the effects of environmental pollution on the development of allergic disease. The effects of air pollution on total and specific IgE are well documented with tobacco smoke, rating as one of the most powerful adjuvant factors promoting sensitization to allergens. Passive smoking appears to trigger the IgE response in infants and maternal smoking can exert an effect on the IgE response in fetal life (Kjellman NIM 1981)(Magnusson CGM 1986). Post-natal exposure to tobacco smoke results in a higher incidence and an earlier age of onset of allergic disease (Wittig JH et al. 1978).

Whatever the mechanism, the factors determining allergen sensitization are likely to be complex, with sensitization being affected by genetic susceptibility, timing of allergen exposure, the potency and concentration of allergen as well as several other adjuvant factors. Understanding the factors which lead to the initial induction of an
immediate hypersensitivity response and the mechanisms which result in the re-orientation of an ongoing immune response will provide the challenge for the development of immunotherapeutic strategies of the future.

LOOK TO THE FUTURE
The recognition of infants or children genetically predisposed to atopic dermatitis and asthma is the first step, if preventative strategies are to be implemented. Although a number of genes have been identified which appear to be important in the development of atopic diseases, they most likely represent only a small proportion of the genes determining the phenotypic expression of asthma and atopic dermatitis. The Human Genome Project offers the possibility of identifying many of the genes that raise susceptibility to common complex diseases, as illustrated by recent advances in breast cancer (Miki Y et al. 1994), Alzheimer’s disease (Clark RF and Goate AM 1993) and insulin-dependent diabetes (Hashimoto L et al. 1994). A genome-wide search has already been undertaken in families recruited with asthma and atopy showing six potential new regions of linkage to autosomal markers on chromosomes 4, 6, 7, 11, 13 and 16 respectively (Daniels SE et al. 1996). Linkage was demonstrated to various different measures of atopy emphasizing the marked pleiotropy of the atopic phenotype. These regions now need to be tested for association with disease and selected regions identified for positional cloning. A genome wide search for atopic dermatitis will allow the identification of other genes contributing to the development of this common inflammatory skin disorder, which can then be tested for association with disease. It is hoped that these advances will enable a comprehensive estimation of genetic risk to be made at birth for each infant and in doing so, maximize the benefits of vaccination and environmental manipulation.
Interest in new pharmacological approaches have already lead to a number of studies investigating various therapeutic approaches aimed at modulating the inflammatory cytokine response. One approach has been to prevent the differentiation of precursor cells into Th2 cells by stimulating the production of Th1 cells using IL-12, IFNγ and TGFβ or by blocking the production of IL-4 using anti IL-4 antibodies or IL-4R (receptor) antibodies (Lord CJM and Lamb JR 1996). Although some success has been achieved in reducing IgE responses, their use in vivo has been hampered by toxic side effects and a short half-life. Interest is currently being generated in the application of these reagents to specific disease sites. Allergen-specific immunotherapy has proved effective in the treatment of some patients with allergic rhinitis or bee venom allergy. The exact mechanism of action of such therapy has not been fully characterized although some studies have shown altered cytokine responses with reductions in IL-4 and IL-5.

Peptide analogues termed altered peptide ligands (APLs) are peptides which have been structurally modified with a single amino acid substitution and although they bind normally to the MHC, they deliver incomplete signals via the TCR. The incomplete signal induce anergy and dissociation of effector function in T cells. Sloan-Lancaster have been able to modulate both Th1 and Th2 responses using APL’s (Sloan-Lancaster J and Allen PM). It is possible however that these peptides themselves may be immunogenic, capable of producing a T cell population cross-reactive with the original agonist and thus potentially capable of exacerbating disease. Other methods of inducing anergy are currently under investigation. These include the use of high doses of antigenic peptide to induce anergy in Th2 cells in vitro.

The ability of plasmid DNA containing reporter genes to be expressed in mouse muscle has raised the possibility of DNA vaccination for the prophylaxis of allergic disease(Moffatt MF and Cookson WOCM 1996). In the work of Hsu et al on the
Brown-Norwegian rat, injection of a plasmid expression vector containing the gene for the minor house dust mite antigen, Der p V, resulted initially in a transient IgG response in the absence of specific IgE production (Hsu CH et al 1996). Priming and subsequent challenge of the rats with Der p V protein resulted in a markedly diminished IgE response and reduced airway responsiveness. These results do suggest a number of new and interesting approaches to allergy prevention and treatment but are not without their difficulties or potential hazards.

Current management is directed almost exclusively at the treatment of established allergic disease. The results of the complete genome search for asthma has already identified a number of novel genes for asthma and polymorphisms within these genes are currently been tested for their association with atopy and asthma. A genome search for AD will similarly help to identify new genes for AD. In this way individuals most ‘at risk’ for allergic disease will be identified in infancy allowing a more targeted approach to environmental modification and immunomodulatory therapy. The latter will depend on an improved understanding of the complex factors determining allergen sensitization, T cell differentiation and anergy. It is hoped in the long term that screening for AD and asthma may provide an effective strategy for prevention of disease.
Publications arising from this work


Publications in preparation

Cox HE, Harper JI, Cookson WOC. Genetic association of atopic dermatitis and tumour necrosis factor α.

Cox HE, Harper JI, Cookson WOC. The predictive value of allergy testing in atopic dermatitis.


The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis. II. Observer variation of clinical diagnosis and signs of atopic dermatitis


QUESTIONNAIRE FOR CHILDREN UNDER 16 YEARS

Surname: _____________________ Date: ___________
Forename: ____________________ Hosp No _____________
Sex: Male/Female DOB: ___________
Index case Yes/No Age: ___________
Relationship to index ____________________

Family No: ________________ Member No: __________
Address: __________________________________________
____________________________________________________
____________________________________________________
Tel No ______________________

GP Name: ________________ Tel No ___________
Address: _________________________________________
____________________________________________________

Racial background: ___________ Mother ___________
Father ___________
1=Caucasian/Mediterranean 5=Arab
2=West Indian 6= Orthodox jew
3=Asian 7= African
4=Oriental 8=Mixed/other

Country of Birth _______________________

Length of time resident in the UK: ________________
1. ALLERGY HISTORY

1.1 ATOPIC DERMATITIS (Children under 16 years)

1. Has your child had an itchy skin condition in the last year - by itchy we mean scratching or rubbing the skin a lot?  
Yes / No

If you have answered 'No', please go to question 4.

2. How old was your child when this skin condition began?  
(does not apply to children under 4 years of age)

   Under 2 _____________ 2 to 5 _____________ 5 to 10 _____________

   Answer yes if onset under 2.  
Yes / No

3. Has this skin condition ever affected the skin creases in the past - by skin creases we mean fronts of elbows, behind the knees, fronts of ankles, around the neck, around the eyes, or on the cheeks?  
Yes / No

4a. Has your child ever suffered from asthma?  
Yes / No

4b. Has your child ever suffered from hayfever?  
Yes / No

4c. If under 4 years, does anyone in your child’s immediate family ever suffered from eczema, asthma or hayfever?  
Yes / No

5. In the last year, has your child suffered from a generally dry skin?  
Yes / No

6. Is there visible flexural dermatitis today?  
(or eczema involving the cheeks/forehead and outer limbs in children under 4 )  
Yes / No

Does this child have atopic dermatitis?  
Yes / No

Positive diagnosis = history of itch plus 3 or more othe above
1.2. INHALED ALLERGIES

1. Has your doctor ever told you that your child has hayfever?  Yes / No

2. Has your child ever had attacks of sneezing with an itchy, blocked or running nose?  Yes / No

3. Has this happened more than once in the last year?  Yes / No

4. Has this ever happened without a cold?  Yes / No

5. At what age did you first notice it?  

6a. Does this occur only during certain months of the year?  Yes / No

6b. If yes, which months?  Jan  Mar  May  Jul  Sep  Nov  Feb  Apr  Jun  Aug  Oct  Dec

7. Has your child ever had itchy, red or sore eyes?  Yes / No

8. Has this happened more than once in the last year?  Yes / No

9a. Does this occur only during certain months of the year?  Yes / No

9b. If yes, which months?  Jan  Mar  May  Jul  Sep  Nov  Feb  Apr  Jun  Aug  Oct  Dec
1.3. ASTHMA

1. Has your doctor ever told you that your child has asthma? Yes / No

2. Has this happened more than once in the last year? Yes / No

3. Has your child’s chest ever sounded wheezing or whistling? Yes / No

4. Has this happened more than once in the last year? Yes / No

5. At what age did it start? 

6. Has your child had this wheezing/whistling when he/she did not have a cold? Yes / No

7. Has your child ever had attacks of wheezing/coughing brought on by exercise? Yes / No

8. Has your child ever been kept awake at night by attacks of wheezing/coughing? Yes / No

9. Does this wheezing/whistling occur only during certain months of the year? Yes / No

10. If yes, which months? Jan Mar May Jul Sep Nov Feb Apr Jun Aug Oct Dec
1.4 INGESTED ALLERGENS

1a. Is your child allergic to any specific foods? Yes / No

1b. If yes, which ones?

1. Eggs
2. Wheat
3. Nuts
4. Cows milk
5. Fish
6. Other

2a. Has your child had a reaction to any specific foods? Yes / No

2b. If yes, which ones?

1______ 2______ 3______ 4______ 5______ 6______

2c. Describe reaction

2d. How soon did it occur after eating/drinking?

1.immediately?  2.< 60 minutes?  3. >60 minutes?

3a. Is your child allergic to any specific medicines? Yes / No

3b. If yes, which medicines?

3c. How does he/she react?
2. MEDICATIONS

**Current medication other than treatment for eczema**

1 = inhaled bronchodilators  
2 = inhaled steroids  
3 = cromoglycate  
4 = oral steroids  
5 = xanthines  
6 = digoxin  
7 = beta blockers  
8 = diuretics  
9 = antihistamines  
10 = others  
11 = None

**Eczema medication:**

<table>
<thead>
<tr>
<th>Item</th>
<th>Current</th>
<th>Previous</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Mild topical steroids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Moderate topical steroids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Potent topical steroids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. V. Potent topical steroids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Have you ever used steroid wet wraps?</td>
<td>Yes/No</td>
<td></td>
</tr>
<tr>
<td>5. Oral steroids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Other immunosuppressives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Oral antibiotics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. Topical antibacterials</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. Topical antifungals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. Anti - virals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11. Antihistamines/Sedatives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12. Emollients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13. Coal tar preparations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14. Icthammol preparations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15. KMNO4 soaks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16. Evening Primrose Oil</td>
<td></td>
<td></td>
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<tr>
<td>17. Chinese Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18. PUVA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19. UVB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20. None</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3. HOME ENVIRONMENT

Is there central heating in your home? Yes / No

Are there carpets in your child's bedroom? Yes / No

How old is your child's mattress?

< 1 year _______ 1 - 5 years _______
5 - 10 years _______ > 10 years _______

Does anyone in the household smoke? Yes / No

4. ANIMAL CONTACT

Are there any pets at home? Yes / No

If yes, what pet?

1 = cat  2 = dog  3 = rabbit  4 = hamster
5 = bird  6 = other

Does your child have frequent contact with pets elsewhere?
(i.e. at least once a week) Yes / No

If yes, what pet?

Do you notice any symptoms in your child following contact with animals? Yes / No

If yes, what symptoms?
### EXAMINATION

**Description of eczema**

1. Generalised atopic dermatitis
2. Predominance in the flexures
3. Extensor surface predominance
4. Facial predominance
5. Nummular/Discoid
6. Predominantly hands/feet
7. Follicular
8. Prurigo
7. SEVERITY INDEX

<table>
<thead>
<tr>
<th>Extent of eczema</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infantile phase:</td>
<td></td>
</tr>
<tr>
<td>Less than approx 18% body area</td>
<td>1</td>
</tr>
<tr>
<td>Between 18% and 54% body area</td>
<td>2</td>
</tr>
<tr>
<td>Greater than 54% body area</td>
<td>3</td>
</tr>
<tr>
<td>Childhood and adult phase:</td>
<td></td>
</tr>
<tr>
<td>Less than approx 9% body area</td>
<td>1</td>
</tr>
<tr>
<td>Between 9% and 36% body area</td>
<td>2</td>
</tr>
<tr>
<td>Greater than 36% body area</td>
<td>3</td>
</tr>
</tbody>
</table>

| Course                           |       |
| Greater than 3 months remission during 12 months | 1     |
| Less than 3 months remission during 12 months    | 2     |
| Continuous course                  | 3     |

| Intensity                        |       |
| Mild itch, rarely disturbing sleep| 1     |
| Itching > score 1 but < score 3  | 2     |
| Severe itching, usually disturbing sleep | 3     |

Score summation

<table>
<thead>
<tr>
<th>Level</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>3.0 - 4.0</td>
</tr>
<tr>
<td>Moderate</td>
<td>4.5 - 7.5</td>
</tr>
<tr>
<td>Severe</td>
<td>8.0 - 9.0</td>
</tr>
</tbody>
</table>

Score: ___
11. **SUMMARY**

Does this patient have:

Atopic eczema?

Asthma?

Hayfever?

Allergic conjunctivitis?
QUESTIONNAIRE FOR ADULTS AND CHILDREN
OVER 16 YEARS

Surname: _________________ Date: ______________

Forename: _________________ Hosp No: ____________

Sex: Male/Female DOB: ______________

Index case Yes/No Age: ______________

Relationship to index

Family No: ____________ Member No: ____________

Address: ______________________________________

________________________________________________

Tel No: ________________________________________

GP Name: _________________ Tel No: ______________

Address: ______________________________________

________________________________________________

Racial background: _________________ Mother: _________________

Father: _________________

1=Caucasian/Mediterranean 5=Arab
2=West Indian 6= Orthodox jew
3=Asian 7= African
4=Oriental 8=Mixed/other

Country of Birth

Length of time resident in the UK:

Occupation: ____________________________________
ALLERGY HISTORY

1.1 ATOPIC DERMATITIS (Adults and children over 10 years)

1a. Have you ever had an itchy skin condition? - by itchy we mean scratching or rubbing the skin a lot?  
Yes / No

If you have answered ‘No’ please skip to question 4.

1b. Have you had an itchy skin condition in the last year?  
Yes / No

2. When did this skin condition began?

Under 2 __________ 2 to 10 __________
10 to 16 __________ Over 16 __________

(Answer yes if under 2)  
Yes / No

3. Has this skin condition ever affected the skin creases in the past - by skin creases we mean fronts of elbows, behind the knees, fronts of ankles, around the neck, around the eyes, or on the cheeks?  
Yes / No

4a. Have you ever suffered from asthma?  
Yes / No

4b. Have you ever suffered from hayfever?  
Yes / No

5. In the last year, have you suffered from a generally dry skin?  
Yes / No

6. Is there visible flexural dermatitis today?  
Yes / No

Does this individual have a history of Atopic Dermatitis?  
Yes / No

History of itchy skin condition plus 3 or more of the above
1.2. INHALED ALLERGIES

1. Has your doctor ever told you that you have hayfever?  Yes / No

2. Have you ever had attacks of sneezing with an itchy, blocked or running nose?  Yes / No

3. Has this happened more than once in the last year?  Yes / No

4. Does this ever occur without a cold?  Yes / No

5. At what age did you first notice it?

6a. Does this occur only during certain months of the year?  Yes / No

6b. If yes, which months?

7. Have you had attacks when your eyes itch or water or swell and redden?  Yes / No

8. Has this happened more than once in the last year?  Yes / No

9a. Does this occur during certain months of the year?  Yes / No

9b. If yes, which months?

Jan  Mar  May  Jul  Sep  Nov
Feb  Apr  Jun  Aug  Oct  Dec
1.3. ASTHMA

1. Has your doctor ever told you that you have asthma? Yes / No

2. Has your chest ever sounded wheezing or whistling? Yes / No

3. Has this happened more than once in the last year? Yes / No

4. At what age did you first notice it?

5. If yes to above:
   Have you had this wheezing/whistling when you did not have a cold? Yes / No

6. Have you ever had attacks of wheezing/coughing brought on by exercise? Yes / No

7. Are you ever woken at night with attacks of wheezing/coughing? Yes / No

8a. Does this wheezing/whistling occur only during certain months of the year? Yes / No

8b. If yes, which months?

   Jan  Mar  May  Jul  Sep  Nov
   Feb  Apr  Jun  Aug  Oct  Dec

9. Has your chest ever felt tight or your breathing become difficult? Yes / No

10. Has this happened more than once in the last year? Yes / No
1.4 INGESTED ALLERGENS

1a. Are you allergic to any specific foods?

1b. If yes, which ones? Yes / No

1. Eggs
2. Wheat
3. Nuts
4. Cows milk
5. Fish
6. Other

2a. Have you had a reaction to any specific foods? Yes / No

2b. If yes, which ones?

2c. Describe reaction

2d. How soon did it occur after eating/drinking?

1. immediately? 2. < 60 min 3. > 60 min

3a. Are you allergic to any specific medicines? Yes / No

3b. If yes, which medicines?

3c. How do you react?
2. MEDICATIONS

**Current medication other than treatment for eczema**

<table>
<thead>
<tr>
<th>Number</th>
<th>Medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>inhaled bronchodilators</td>
</tr>
<tr>
<td>2</td>
<td>inhaled steroids</td>
</tr>
<tr>
<td>3</td>
<td>cromoglycate</td>
</tr>
<tr>
<td>4</td>
<td>oral steroids</td>
</tr>
<tr>
<td>5</td>
<td>xanthines</td>
</tr>
<tr>
<td>6</td>
<td>digoxin</td>
</tr>
<tr>
<td>7</td>
<td>beta blockers</td>
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<td>8</td>
<td>diuretics</td>
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<tr>
<td>9</td>
<td>antihistamines</td>
</tr>
<tr>
<td>10</td>
<td>others</td>
</tr>
<tr>
<td>11</td>
<td>None</td>
</tr>
</tbody>
</table>

**Eczema medication:**

<table>
<thead>
<tr>
<th>Medication</th>
<th>Current</th>
<th>Previous</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Mild topical steroids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Moderate topical steroids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Potent topical steroids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. V. Potent topical steroids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Have you ever used steroid wet wraps?</td>
<td>Yes/No</td>
<td></td>
</tr>
<tr>
<td>5. Oral steroids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Other immunosuppressives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Oral antibiotics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. Topical antibacterials</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. Topical antifungals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. Anti-virals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11. Antihistamines/Sedatives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12. Emollients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13. Coal tar preparations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14. Icthammol preparations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15. KMNO4 soaks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16. Evening Primrose Oil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17. Chinese Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18. PUVA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19. UVB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20. None</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3. **HOME ENVIRONMENT**

Is there central heating in your home?  
Yes / No

Are there carpets in your bedroom?  
Yes / No

How old is your mattress?  

< 1 year    ________    1 - 5 years    ________  
5 - 10 years ________    > 10 years    ________  

4. **ANIMAL CONTACT**

Are there any pets at home?  
Yes / No

If yes, what pet?  

1 = cat   2 = dog   3 = rabbit   4 = hamster  
5 = bird   6 = other

Do you have frequent contact with pets elsewhere?  
(i.e. at least once a week)  
Yes / No

If yes, what pet?  

Do you notice any symptoms following contact with animals?  
Yes / No

If yes, what symptoms?
5. **SMOKING**

Do you smoke regularly? (1 cigarette/day for 1 year)  
Yes/No

Have you ever smoked?  
Yes/No

How old were you when you started regularly?  

How many years ago did you stop?  

Does anyone in your household smoke?  
Yes/No

Does anyone in your workplace smoke?  
Yes/No

---

EXAMINATION

**Description of eczema**

1. Generalised atopic dermatitis  
2. Predominance in the flexures  
3. Extensor surface predominance  
4. Facial predominance  
5. Nummular/Discoid  
6. Predominantly hands/feet  
7. Follicular  
8. Prurigo
7. SEVERITY INDEX

**Extent of eczema**

<table>
<thead>
<tr>
<th>Infantile phase:</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than approx 18% body area</td>
<td>1</td>
</tr>
<tr>
<td>Between 18% and 54% body area</td>
<td>2</td>
</tr>
<tr>
<td>Greater than 54% body area</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Childhood and adult phase:</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than approx 9% body area</td>
<td>1</td>
</tr>
<tr>
<td>Between 9% and 36% body area</td>
<td>2</td>
</tr>
<tr>
<td>Greater than 36% body area</td>
<td>3</td>
</tr>
</tbody>
</table>

**Course**

| Greater than 3 months remission during 12 months | 1 |
| Less than 3 months remission during 12 months   | 2 |
| Continuous course                              | 3 |

**Intensity**

| Mild itch, rarely disturbing sleep           | 1 |
| Itching > score 1 but < score 3             | 2 |
| Severe itching, usually disturbing sleep     | 3 |

**Score summation**

| Mild       | 3.0 - 4.0 |
| Moderate   | 4.5 - 7.5 |
| Severe     | 8.0 - 9.0 |

**Score:**
11. SUMMARY

Does this patient have;

Atopic eczema?  

Asthma?  

Hayfever?  

Allergic conjunctivitis?
Association of atopic dermatitis to the beta subunit of the high affinity immunoglobulin E receptor

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Accepted for publication 2 October 1997

Summary

IgE dysregulation is a major pathogenic feature of atopic dermatitis and other IgE-mediated allergic diseases such as asthma and rhinitis. Allergen complexed to IgE binds to the high affinity receptor for IgE (FceRI) on the surface of epidermal Langerhans cells, mast cells and basophils, triggering the release of inflammatory mediators. The β subunit of FceRI has been localized to human chromosome 11q12–13, and variants within this gene have been shown to associate with asthma and measures of atopy. We have tested several polymorphisms within FceRI-β for association to atopic dermatitis in a panel of 60 families (panel A), recruited through a proband with atopic dermatitis. The findings were tested in a second panel of families (panel B). Significant sharing of maternal alleles was seen for atopic dermatitis and allele 2 of Rsal intron 2 (Rsal_in2*2) (P = 0.0022) and allele 1 of Rsal exon 7 (Rsal_ex7*1) (P = 0.0036) FceRI-β gene polymorphisms. These findings were replicated in Panel B, confirming the association of FceRI-β Rsal polymorphisms with atopic dermatitis. The combined significance of the association of atopic dermatitis to Rsal polymorphisms was P = 0.0002 (Rsal_in2*2) and P = 0.00034 (Rsal_ex7*1). The polymorphisms also showed association with asthma: P = 0.0068 (Rsal_in2*2) and P = 0.018 (Rsal_ex7*1). Polymorphisms within the FceRI-β gene are strongly associated with atopic dermatitis.

Atopic dermatitis is a chronic inflammatory skin disease affecting approximately 15% of children in the U.K.1 It is characterized by intense pruritus, a predilection for the flexural regions, a chronic relapsing course and a personal or family history of atopy. Total serum IgE levels are elevated in 80% of cases and most patients demonstrate positive immediate skin tests or specific IgE titres to common inhalant allergens.2 Atopic dermatitis is a complex multifactorial disease sharing many pathogenic features with allergic asthma and rhinitis. Twin studies have identified a substantial genetic contribution to the development of atopic dermatitis, but as with asthma, the mode of transmission does not conform to a simple Mendelian pattern of inheritance.3

In predisposed individuals, contact of the skin with environmental antigen leads to the formation of allergen–IgE complexes. These complexes bind to high affinity IgE receptors (FceRI) on the surface of epidermal Langerhans cells, triggering the release of preformed inflammatory mediators.4 Uprregulation of FceRI expression has been shown to occur in lesional skin of atopic dermatitis patients, facilitating the uptake and processing of antigen by Langerhans cells and the dermal inflammatory response.5

The gene for the β subunit of FceRI has been localized to chromosome 11q12–13 and is in close genetic linkage to atopy.6–9 This locus shows strongest linkage through maternally-derived alleles, consistent with the recognized pattern of maternal inheritance of atopy.10–13 Several polymorphisms within the FceRI-β gene have been shown to associate with skin prick test responses to allergens, bronchial hyper-reactivity and asthma. These include a coding variant E237G within exon 714 and three noncoding variants, a Rsal polymorphism in intron 2 (Rsal_in2*2).15
morphism in the untranslated region of exon 7 (Rsal_ex7). Other coding polymorphisms, 1181L and 1181L/V183L have proved difficult to detect consistently and are of uncertain status.

In order to test the importance of FcεRI-β in the genetic determination of atopic dermatitis, we analysed the association of FcεRI-β gene polymorphisms to atopic dermatitis and asthma in two independent panels of subjects, recruited through probands with active atopic dermatitis. Atopic dermatitis was rigorously defined according to the U.K. working party's set of validated diagnostic criteria. The transmission disequilibrium test (TDT) was used to explore the association of these polymorphisms to asthma and atopic dermatitis and to search for evidence of a maternal effect.

Materials and methods
Sixty nuclear families (Panel A) comprising 277 individuals, age range 1–50 years, were recruited from the dermatology clinics at Great Ormond Street Hospital for Children, NHS Trust (GOSH) through a single proband with active atopic dermatitis. Only those probands over the age of 1 year with at least one sibling were included in the study. Most individuals were Caucasian (90-5%), the remaining individuals being either Asian (1%), African (4%) or of mixed racial origin (4-5%). A questionnaire, which included the diagnostic criteria for atopic dermatitis defined by the U.K. working party, and a set of questions based on the American Thoracic Society's questionnaire for asthma and allergic rhinitis, was completed for each individual. Each family was examined for evidence of atopic dermatitis by the same doctor. A positive diagnosis of atopic dermatitis was made in individuals with a history of pruritus plus three or more of the following criteria: onset of rash under 2 years, history of flexural involvement, a personal history of asthma or hay fever (or respiratory atopy in a first degree relative in children under 4 years), a history of generalized dry skin or visible flexural dermatitis. Severity of atopic dermatitis was assessed using the scoring system of Rajka and Langeland which categorizes patients into having mild (3-0–4-0), moderate (4-5–7-5) or severe (8-0–9-0) skin disease on the basis of surface area involvement, continuity of disease and nocturnal pruritus.

Atopy was defined as the presence of a positive skin prick test response 3 mm greater than the negative control, or a positive specific IgE. raised total serum IgE, or any combination of these features. Skin prick testing (Dome-Hollister-Stier, Spokane, WA, U.S.A.) to house dust mite (Dermatophagoides pteronyssinus), timothy grass (Phleum pratense), alternaria (Alternaria alternata), cat dander (Felis domesticus), egg white and cow's milk was carried out on all individuals. Total and specific IgE to the same panel of allergens were measured by a fluorescent enzyme immunoassay (Pharmacia CAP system, Pharmacia, Uppsala, Sweden). A specific ELISA class I result (>0-35 kU/L) was considered positive. A raised total serum IgE level was taken to be greater than 100 kU/L in adults (mean + 2 SD) or greater than 1 SD above the geometric mean of the published normal values for children according to their age. Automated assays of eosinophil and white cell counts were conducted. DNA was isolated from peripheral blood leucocytes by standard phenol–chloroform extraction followed by ethanol precipitation.

All positive results were tested in a second independent panel of families (Panel B) of similar racial origin (Caucasian 87%, Asian 6%, African 5%, Oriental 2%). These families were recruited in a previous study, on the basis of at least two first degree relatives with active atopic dermatitis, from the outpatient clinics at GOSH. The first affected child from each of these pedigrees was chosen for TDT analysis. DNA was available for analysis from 88 of these families (402 individuals).

Genotype analysis
For detection of the Rsal polymorphisms within intron 2 and the untranslated region of exon 7 of FcεRI-β, a specific polymerase chain reaction (PCR) was carried out followed by restriction digestion of the PCR products. The amplification refractory mutation system was used to test for the presence of the E237G variant within exon 7 of the FcεRI-β gene.

For Rsal_in2, amplification of genomic DNA (100 ng) was performed in a thermal cycler (Perkin Elmer Cetus, Warrington, U.K.) using 35 cycles of 94 °C for 1 min, 61°C for 1 min and 72 °C for 1 min. The reaction mix contained 0-3 μmol/L of each primer, 200 μmol/L dNTPs, 0-1% (v/v) NP40, 0-1% (w/v) gelatin, 0-1 mol/L KCl, 0-1 mol/L Tris-HCl pH 8-8, 2 mmol/L MgCl2 and 0-75 units of Taq DNA polymerase in a final volume of 15 μL. Primers were 5' CAG AAT GTT CTC ATG and the untranslated region of exon 7 followed by restriction digestion of the PCR products. The amplification refractory mutation system was used to test for the presence of the E237G variant within exon 7 of the FcεRI-β gene.

For Rsal_ex7, amplification of genomic DNA (200 ng) was performed in a Hybaid Omnigene (Teddington, U.K.) using 35 cycles of 94 °C for 1 min, 61°C for 1 min and 72 °C for 1 min. The reaction mix contained 0-3 μmol/L of each primer, 200 μmol/L dNTPs, 0-1% (v/v) NP40, 0-1% (w/v) gelatin, 0-1 mol/L KCl, 0-1 mol/L Tris-HCl pH 8-8, 2 mmol/L MgCl2 and 0-75 units of Taq DNA polymerase in a final volume of 15 μL. Primers were 5' CAG AAT GTT CTC ATG and the untranslated region of exon 7 followed by restriction digestion of the PCR products. The amplification refractory mutation system was used to test for the presence of the E237G variant within exon 7 of the FcεRI-β gene.
Middlesex, U.K.) thermal cycler (block control) using 32 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. The PCR reaction mix contained 0·2 μmol/L of each primer, 200 μmol/L dNTPs, 67 mmol/L Tris-HCl pH 8·8, 1·6 mmol/L (NH₄)₂SO₄, 0·01% Tween-20, 1·5 mmol/L MgCl₂ and 0·75 units Taq DNA polymerase in a final volume of 15 μL. Primers were 5’ TCA CTG TGT ATC ATG CTA AGC 3’ and 5’ TGA TAC AAT ACT GCA TCG TGG 3’.

Five microlitres of amplification products were digested with 4 units of RsaI enzyme (New England Biolabs, Hitchin, Herts, U.K.), together with the recom­mended buffer, in a final volume of 10 μL, for 1 h at 37°C. Digested products were resolved on a 2% (w/v) agarose gel containing 1 μg/mL ethidium bromide, run for 60 min at 70 V in a BioRad (Hemel Hempstead, Herts, U.K.) wide subcell. Restriction digestion of the intron 2 PCR product yielded three genotypes with an additional constant 148 bp band being present as a result of the primers used. The 1,1 genotype was denoted by the presence of an 800 bp and 148 bp band pattern, the 2,2 genotype by the presence of a 500 bp, 280 bp and 148 bp band pattern and the 1,2 genotype by an 800 bp, 500 bp, 280 bp and 148 bp band pattern. Digestion of the exon 7 polymorphism potentially gave rise to three genotypes: a single 481 bp band denoted the 1,1 genotype, a 481 bp, 295 bp and 187 bp band pattern denoted the 1,2 genotype and a 295 bp and 187 bp band pattern denoted the 2,2 genotype.

For the E237G variant in exon 7, the following primers were used to test for the presence of the E237G polymorphism at nucleotide residue 7297 of the published sequence of the FceRI-β gene:14 B7FA1, TGG CCA GCT AGT CTG TGG TTT TCT GGA (30mer); B7FA2, GGA GCA TAT TAA GGT GGA CAG AAG CAG CAG (30mer); B7M1, ATT CAG CTA CTT ACA GTG AGT TGG AAG ACC CAG GCG G (37mer) and B7 W2, AC GTG ATT CTT ATA AAT CAA TGG GAG GAG ACA ATT (36mer). A total reaction volume of 50 μL containing 1 μmol/L of each of the primers B7FA1, B7FA2 and B7 W2 and 0·5 μmol/L of the primer B7M1, 10 KCl buffer (1% (v/v) NP40, 1% (w/v) gelatin, 1 mol/L KCl, 1 mol/L Tris-HCl pH 8·3), 2 mmol/L MgCl₂, 200 μmol/L dNTPs, and 50 ng of genomic DNA was used. A hot start PCR was done with the addition of 2 units of Taq DNA polymerase (Boehringer Mannheim, Lewes, U.K.) after an initial 5 min denaturation at 95°C. PCR conditions then followed 35 cycles of 94°C for 1 min, 62°C for 2 min and 72°C for 2 min followed by a single cycle of 72°C for 10 min. Amplified products were then visualized in an ethidium-bromide stained 4% (w/v) agarose gel (3: 1 NuSieve: LMP agarose) and run for 120 min at 60 V in a BioRad wide subcell. The primers B7FA1-B7FA2 gave a 446 bp control band, B7FA1-B7 W2 a 280 bp band in the presence of adenine at nucleotide position 7297 of the published sequence and B7M1-B7FA2 a 238 bp band in the presence of guanine at position 7297.

Statistical analysis

The TDT implemented in the GAS program (version 2.1) (A.Young, unpublished), was used to test for transmission of alleles at a particular locus from heterozygous parents to their affected offspring. The proportion of alleles transmitted from both mother and father was tested against a null hypothesis of 50% transmission by χ² analysis.

Results

The mean age of probands in Panels A and B was 6·4 and 8·1 years, respectively. Ninety-three per cent of probands in panel A were atopic and 63% asthmatic. The majority had moderate to severe skin disease with a mean severity score of 7·6 and mean geometric serum IgE of 1224·1 kU/L. Atopy and a history of asthma were present in 96 and 57%, respectively, of probands in Panel B. The mean severity score was 6·1 and mean geometric serum IgE 566·8 kU/L.

Genetic analysis

In Panel A subjects, transmission of allele 2 of the RsaI intron 2 polymorphism (RsaI_in2*2) (P = 0·0022) and allele 1 of the RsaI exon 7 polymorphism (RsaI_ex7*1) (P = 0·0036) to offspring with atopic dermatitis occurred significantly more frequently than expected by chance (Table 1). The difference between the proportion sharing maternal vs. paternal alleles was highly significant: P = 0·0019, χ² = 9·66 (RsaI_in2*2) and P = 0·0031, χ² = 8·78 (RsaI_ex7*1). No significant association of these polymorphisms was found with asthma.

The association of the two RsaI polymorphisms with atopic dermatitis was confirmed in Panel B families, in which an excess sharing of maternally-derived alleles occurred significantly more frequently than expected by chance (Table 1). The difference between the proportion sharing maternal vs. paternal alleles was highly significant: P = 0·0019, χ² = 9·66 (RsaI_in2*2) and P = 0·0031, χ² = 8·78 (RsaI_ex7*1). No significant association of these polymorphisms was found with asthma.

The association of the two RsaI polymorphisms with atopic dermatitis was confirmed in Panel B families, in which an excess sharing of maternally-derived alleles for both RsaI_in2*2 (P = 0·018) and RsaI_ex7*1 (P = 0·02) was found. Furthermore, an association with allergic asthma was present in maternally derived alleles: RsaI_in2*2 (x transmitted:y not transmitted, 15:6, P = 0·039) and RsaI_ex7*1 (14:6, P = 0·058).

Table 1. Transmission disequilibrium test: sharing of Rsal alleles with atopic dermatitis

<table>
<thead>
<tr>
<th>Panel</th>
<th>Rsal polymorphism</th>
<th>Allele</th>
<th>Transmitted</th>
<th>Not transmitted</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>in2</td>
<td>1</td>
<td>17</td>
<td>10</td>
<td>0-12</td>
</tr>
<tr>
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<td>16</td>
<td>9</td>
<td>0-11</td>
</tr>
<tr>
<td>B</td>
<td>in2</td>
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<td>24</td>
<td>17</td>
<td>0-17</td>
</tr>
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<td></td>
<td></td>
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<td>17</td>
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<tr>
<td></td>
<td></td>
<td>2</td>
<td>21</td>
<td>18</td>
<td>0-37</td>
</tr>
</tbody>
</table>

NS, not significant.

Combining the data from panels A and B significantly strengthened the associations with atopic dermatitis and asthma. Significance was strongest when examining the TDT sharing of maternal Rsal alleles with atopic dermatitis (Table 2). The significance of transmission of Rsal_in2*2 was P = 0-0002 and, for Rsal_ex7*1, P = 0-00034. Transmission of these alleles was also significantly associated with asthma when maternally-inherited: Rsal_in2*2 (P = 0-0068) and Rsal_ex7*1 (P = 0-018) (Table 3).

The positive results of this genetic association study are in contrast to the results of a previous linkage study in Panel B subjects, which failed either to detect or exclude linkage of atopic dermatitis to markers within the 11q12–13 region.23 This is probably because the markers tested were situated telomeric to the FceRI-3 gene. Furthermore, tests of association are more powerful than linkage studies in detecting genetic effects in complex disorders.24

The E237G coding variant was present in 6.5% of subjects from Panel A, but failed to associate significantly with atopic dermatitis (P = 0.38) or asthma (P = 0.5). For this reason, the polymorphism was not examined in Panel B subjects.

Discussion
This study has shown significant association of atopic dermatitis to two non-coding Rsal polymorphisms within the FceRI-3 gene. The association was demonstrated only in maternally-derived alleles. We were able to replicate these findings in a second independent panel of families and to show significant association of the polymorphisms to allergic asthma.

Strict adherence to a set of six diagnostic criteria, as defined by the U.K. working party,19,20 ensured an accurate diagnosis of atopic dermatitis. Children were selected as probands in view of the high rate of spontaneous remission of atopic dermatitis with age. The exclusion of infants under 1 year of age reduced the problems in differentiating infantile seborrhoeic dermatitis from atopic dermatitis.

The E237G coding variant was present in 6.5% of subjects from Panel A, but failed to associate significantly with atopic dermatitis (P = 0.38) or asthma (P = 0.5). For this reason, the polymorphism was not examined in Panel B subjects.

Table 2. Transmission disequilibrium test: sharing of Rsal alleles with atopic dermatitis in combined panels A and B

<table>
<thead>
<tr>
<th>Rsal polymorphism</th>
<th>Allele</th>
<th>Transmitted</th>
<th>Not transmitted</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>2</td>
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<td>27</td>
<td>0-13</td>
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</table>

NS, not significant.

Table 3. Transmission disequilibrium test: sharing of Rsal alleles with asthma in combined panels A and B

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<th>Allele</th>
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<th>P</th>
<th>Transmitted</th>
<th>Not transmitted</th>
<th>P</th>
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</thead>
<tbody>
<tr>
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<td>0.18</td>
<td>9</td>
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<td>NS</td>
</tr>
</tbody>
</table>

NS, not significant.

maternal line. This is in agreement with a number of studies showing strong maternal linkage of FceRI-β and other loci to measures of atopy and asthma.10–13 Foetal–maternal interactions are favoured as a cause for this phenomenon, with maternal modification of the infant’s developing IgE response occurring through the placenta or breast milk; however, genetic mechanisms such as genomic imprinting remain a possibility.12

FceRI-β polymorphisms have been associated previously with asthma and severe atopy.6–9 In a study of atopic Japanese adults, the Rsal_in2 polymorphism was shown to associate significantly with atopic asthma, rhinitis and eczema.15 The findings of our study suggest that the combination of severe atopic dermatitis and asthma may form a distinct clinical subtype of atopic disease, attributable to FceRI-β. However, the FceRI-β E237G coding variant was found not to be associated with atopic dermatitis. The functional polymorphism in FceRI-β responsible for atopic dermatitis is therefore still to be identified.

FceRI is a multimeric receptor expressed either as a αγ2 trimer or αγ2β tetramer.25 Functionally, the β subunit has been shown to act as a signal amplifier, providing a gain of five to seven-fold.26 Increases in β amplifier gain could be influenced by variants within the FceRI-β gene, thus potentiating allergic responsiveness. Alternatively, FceRI-β variants may increase the proportion of αγ2β receptors expressed, thereby increasing the strength of the FceRI-mediated signal.

Further studies of the FceRI-β locus and the identification of other relevant genes will hopefully lead to an improved understanding of the genetic basis of atopic dermatitis and its relationship to asthma.

References
8 Sandford AJ, Shirakawa T, Moffatt MF et al. Localisation of atopy and beta subunit of high-affinity IgE receptor (FceRI) on chromosome 11q. Lancet 1993; 341: 332–4.