UNIVERSITA' DEGLI STUDI DI VERONA

DIPARTIMENTO MATERNO INFANTILE E DI BIOLOGIA GENETICA

SEZIONE DI BIOLOGIA E GENETICA

DOTTORATO DI RICERCA IN BIOTECNOLOGIE

APPLICATE ALLE SCIENZE BIOMEDICHE

Ciclo XIX

SNP ANALYSIS OF THE PHF11 GENE IN ITALIAN FAMILIES WITH ALLERGIC ASTHMA

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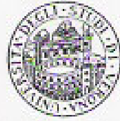

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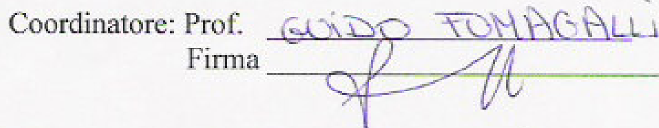

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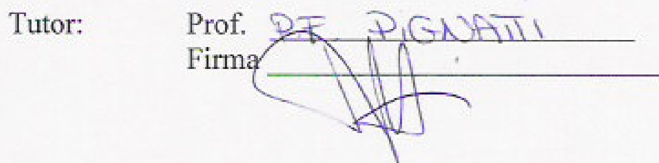

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Al Magnifico Rettore
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Oggetto: Presentazione del Collegio dei Docenti dell'attività scientifico-formativa svolta dalla dott.ssa Penelope Zorzi durante il Corso di Dottorato in Biotecnologie Applicate alle Scienze Biomediche e ammissione all'esame finale di dottorato decisa dal Collegio dei Docenti del Dottorato nella riunione del 22 dicembre 2006

La dott.ssa Penelope Zorzi ha partecipato, come dottoranda di ricerca, alle ricerche coordinate dalla dott.ssa Cristina Bombieri e dal Prof. Pier Franco Pignatti. Nei tre anni di dottorato la sua attività di ricerca si è focalizzata su analisi di associazione genica in alcune malattie polmonari. In particolare, la dott.ssa Zorzi si è occupata dell'allestimento di protocolli di analisi di mutazioni del DNA mediante minisequencing che ha poi utilizzato per l'analisi dei polimorfismi del gene PHF11 in famiglie con asma allergico che presentavano linkage con la regione cromosomica 13q14. Durante il corso, la dott.ssa Zorzi è diventata esperta in varie metodiche di biologia molecolare come estrazione di DNA da sangue, PCR, restrizione enzimatica, elettroforesi, sequenziamento e altre tecniche. La dott.ssa Zorzi ha lavorato con impegno ottenendo risultati interessanti che verranno descritti in dettaglio dalla candidata nella sua tesi di dottorato. Ha dimostrato buona capacità di lavorare in team e di interagire produttivamente con i colleghi. Il suo impegno è anche dimostrato dalla partecipazione e presentazione dei suoi dati a congressi nazionali ed internazionali e dalla pubblicazione, fino a questo momento, di un lavoro su rivista internazionale.

La dott.ssa Zorzi ha affrontato le sue tematiche di ricerca acquisendo competenze metodologiche e solide basi culturali su aspetti centrali di biologia molecolare, raggiungendo la professionalità e le competenze richieste per il conseguimento del dottorato.

Per concludere, la dottoressa Penelope Zorzi ha raggiunto la maturità scientifica adeguata al conseguimento del titolo di Dottore di Ricerca.

Il Coordinatore del Dottorato

Prof. Guido Fumagalli

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RIASSUNTO

L'asma è una malattia complessa delle vie aeree, caratterizzata da ostruzione reversibile del flusso respiratorio e da ipersensibilità bronchiale. L'asma viene definita come una malattia genetica con vari fenotipi, attribuiti ad un interazione tra fattori ambientali e geni multipli.

In una precedente scansione genomica per asma, condotta su 123 famiglie del nord est Italia caratterizzate per asma clinica, rinite, IgE seriche totali elevate, positività al test di cutireazione per aeroallergeni comuni e iperreattività bronchiale alla metacolina, è stata osservata la presenza di linkage tra la regione cromosomica 13q14 e livelli elevati di IgE seriche totali.

Due studi recentemente riportati in letteratura, Zhang et al., 2003, e Jang et al., 2005, condotti nei geni contenuti in questa regione, hanno identificato la presenza di associazione del gene PHF11 con IgE elevate e dermatite atopica, rispettivamente.

Lo scopo di questo lavoro era di sviluppare una tecnica di genotipizzazione multipla e di effettuare uno studio di associazione del gene PHF11 nelle 23 famiglie (per un totale di 144 individui) che avevano mostrato linkage positivo per IgE seriche totali elevate.

Sono state analizzate 7 SNP all'interno del gene PHF11, riportate in letteratura in associazione ad IgE elevate: rs2247119, nell'introne 3; rs2274276, nell'introne 4; rs2031532, nell'esone 2; rs1046295, nella zona 3'UTR; 185752b5_2, nell'introne 9; 185306b7_1, nell'introne 1; e 185752b4_2, nell'introne 5. Le prime quattro sono state riportate associate anche a dermatite atopica. I 7 polimorfismi sono stati prima confermati con alcuni controlli della stessa popolazione mediante sequenziamento o restrizione enzimatica. Le SNP rs2247119, e rs2274276 sono state analizzate tramite PCR e restrizione enzimatica con gli enzimi Fnu4 HI e Nsp I, rispettivamente. Per le altre 5 SNP è stato sviluppato un metodo di genotipizzazione multipla mediante la tecnica del ddNTP Primer Extension, utilizzando lo SNaPshot Multiplex kit (Applied Biosystems) analizzata dal sequenziatore ad elettroforesi capillare ABI PRISM 310 con il programma Genescan.

L'analisi statistica mediante il TDT test con il programma Merlin ha confermato la presenza di linkage tra la regione cromosomica studiata ed IgE seriche totali elevate, ma non ha mostrato presenza di associazione tra le SNP analizzate ed IgE in asma allergica. Nessuna associazione è stata identificata con altri fenotipi. L'associazione potrebbe quindi essere dovuta nella nostra popolazione ad altri polimorfismi presenti in questo gene o in geni vicini.

SUMMARY

Asthma is a disease of the lungs characterized by reversible airway obstruction and bronchial hyper-reactivity and is associated with pulmonary inflammation. Asthma has an important genetic component but no clear pattern of inheritance; its phenotypes, including bronchial hyper-responsiveness (BHR), atopy, and increased IgE, result from the interaction of multiple gene variants, each having a modest effect, and the environment.

In a previous genomic screen for asthma, conducted on 123 families from north east Italy, characterized for clinical asthma, rhinitis, elevated total serum IgE, positive Skin Prick Test, and bronchial hyper-responsiveness (BHR) to methacholine, it has been observed the presence of linkage between region 13q14 and elevated total serum IgE.

Two studies, recently reported in literature, Zhang et al., 2003, and Jang et al., 2005, identified an association of the PHF11 gene with elevated IgE and atopic dermatitis, respectively.

The aim of this study was to develop a multiple genotyping technique and to perform an association study of the PHF11 gene in the 23 families, for a total of 144 individuals, which demonstrated positive linkage to elevated total serum IgE.

7 SNPs have been analyzed in the PHF11 gene, reported in literature associated with elevated IgE: rs2247119, in intron 3; rs2274276, in intron 4; rs2031532, in intron 2; rs1046295, in the 3'UTR zone; 185752b5_2, in intron 9; 185306b7_1, in intron 1; and 185752b4_2, in intron 5. The first four SNPs have also been associated to atopic dermatitis.

The 7 polymorphisms have been first validated with 15 control samples of the same population by sequencing and enzymatic restriction. The SNPs rs2247119, and rs2274276 have been analyzed through PCR and enzymatic restriction with the enzymes Fnu4 HI and Nsp I, respectively. The other 5 SNPs have been analyzed in multiplex with the ddNTP Primer Extension technique, utilizing the SNaPshot Multiplex kit (Applied Biosystems) with the ABI PRISM 310 Genetic Analyzer using the Genescan program.

The genotyped SNPs have been analyzed through TDT Test with the Merlin program. This analysis confirmed the presence of linkage between region 13q14 and IgE, but a presence of association between the analyzed SNPs and IgE in allergic asthma was not observed. No association was identified with other asthma related phenotypes.

The analysis suggests that other polymorphisms or genes may be involved, and can interfere with the association. It may be appropriate to further extend the analyzed region to other neighbouring regions, which demonstrated positive linkage to asthma and related phenotypes.

1. INTRODUCTION

1.1 ASTHMA

The term *asthma* derives from the Greek word “άσμα”, which means “*heavy breath*.” Hippocrates, acknowledged as the “father of medicine” around 400 A.C., was the first to address asthma and to denominate it as a disorder (Trabalzini, 2005).

In the early 1900, asthma was considered a Mendelian inheritance disorder, where a single gene is responsible for a defect or an abnormality. It was only in 1954 that Tips proposed a polygenetic mode of asthma inheritance (Tips, 1954). This theory was further developed in 1967 by Leigh and Morley, who suggested that asthma is a multifactorial trait in which multiple gene loci interact with one or more external factors (Bias et al., 1978 and Marsh et al., 1992).

The prevalence of asthma has increased in the past two to three decades in both children and young adults. The reasons for this increase are still poorly understood. There has been speculation about the importance of changes in the indoor or outdoor environment, in the climate and in the lifestyles.

Today, asthma has reached epidemic proportions, affecting, globally, 300 million people of all ages and all ethnic backgrounds, touching annually health care costs of approximately 30 billion dollars (Selgrade et al., 2006)

1.2 ASTHMA CLASSIFICATION

The American Thoracic Society and the National Asthma Education and Prevention Program (NAEPP) define asthma as a chronic inflammatory disorder of airways characterized by reversible obstruction, bronchial hyper-reactivity and recurrent episodes of wheezing, chest tightness, or coughing. (**Figure 1.1**) Airway inflammation, smooth muscle contraction, epithelial sloughing, mucous hyper-secretion, bronchial hyper-responsiveness (BHR), and mucosal edema, are all responsible for the underlying pathophysiology of asthma (Lenchner et al., 2004).

Asthma can be classified according to its severity as atopic or extrinsic asthma, nonallergic or intrinsic asthma, mixed asthma, exercise-induced asthma, cough variant asthma, aspirin-induced asthma, and fatal asthma (Lenchner et al., 2004).

Allergic asthma, found in 60 to 75% asthma patients (McFadden et al.,1992 and Abbas et al., 1994), is caused in part by a predisposition to atopy, which is indicated with elevated levels of total serum IgE and hypersensitivity to certain

antigens called allergens. Atopic individuals produce abnormally high levels of IgE antibody in response to these allegens, express more high affinity IgE receptors than normal individuals, and have more of these receptors occupied by IgE (Corrigan et al.,1992 and Abbas et al., 1994).

Non-allergic or instrinsic asthma does not have an allergic cause. It usually develops later on in life, with a 10-33% incidence. It is associated with respiratory or sinus infections, pollution, and tobacco smoke, but it has also some family disposition.

Mixed asthma is a combination of allergic and nonallergic asthma, in which the patient can have asthma symptoms but no positive skin test for allergens.

Exercise-induced asthma may occur in cold and dry environments, and it can be present in patients with persistent or mild asthma.

Cough variant asthma presents, as a primary symptom, cough, and it can occur after respiratory infections, exercise, allergic or non-specific triggers.

Aspirin-induced asthma may be triggered within minutes or up to 3 hours after intake of an aspirin or other nonsteroidal anti-inflammatory medications (Lenchner et al., 2004)

The study of this thesis is referring to allergic asthma.

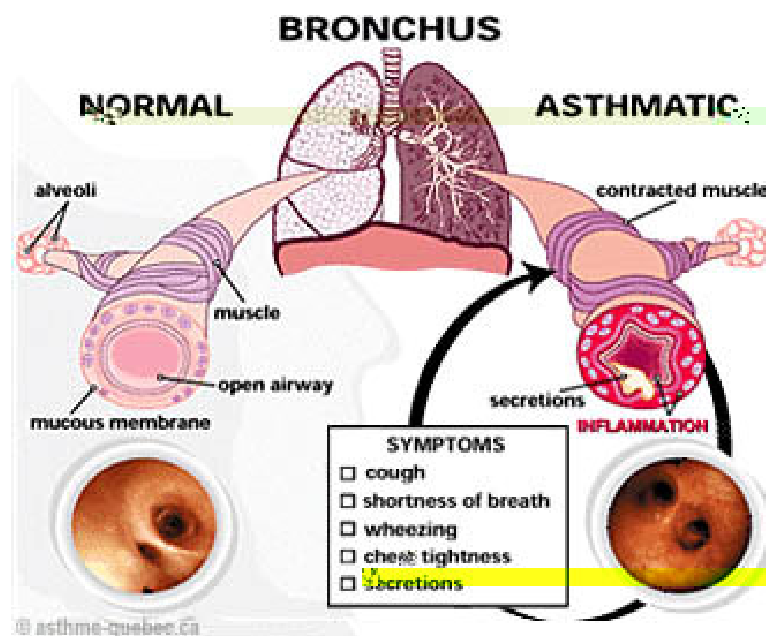

Figure 1.1 Image showing the differences in the lung structure of a normal and of an asthmatic individual.

1.3 ASTHMA RISKS

Allergic asthma is a very complex disorder. It usually manifests itself with the rising of allergies. Asthma is characterized by an elevated IgE production in response to common aeroallergens, such as pollen, dust mites, indoor and outdoor mold, animal dander, and cockroaches. Also tobacco smoke, indoor and outdoor air pollution, strong profumes, cleaning sprays, and chemical fumes can play an important role in triggering asthma as well as food preservative, milk, eggs, and shellfish.

An asthma episode is due to a sensitization to allergens. Once this event has occurred, all the successive exposures to that allergen will elicit a hypersensitive immune response. Sensitization to allergens may occur as early as in the developing fetus, resulting in allergens passing directly crossing the placenta or via maternal cells, carrying allergens into the fetal bloodstream (Holgate et al, 1995).

Allergen exposure in early life has been linked to allergic sensitization and later onset of asthma (Selgrade et al., 2006). Asthma affects for the majority children under 10 years of age. It has been observed that in the first decade of life, asthma is more frequent in males, with a ratio males to females 2.5:1, the ratio becomes about equal during adolescence, and in adults, female asthmatic patients outnumber males (Selgrade et al., 2006).

Allergic asthma is identified by skin prick test (STP) or by measurement of specific serum IgE titers against allergens or elevation of the total serum IgE concentration.

Because of its complexity, it has been very challenging to find a definite cure for asthma. The drugs used currently in the treatment are bronchodilators, corticosteroids, mediator antagonists, cytokine modulators, and specific anti-allergic drugs that inhibits IgE production.

Recently, it has been reported in the Seattle Puget Sound Business Journal, a new experimental cure for patients with moderate to severe asthma, called *bronchial thermoplasty*. *Bronchial thermoplasty* uses radiofrequency to reduce smooth muscles in the bronchial airways, bringing to the patient less frequent and severe asthma symptoms (MacNaughton, 2006).

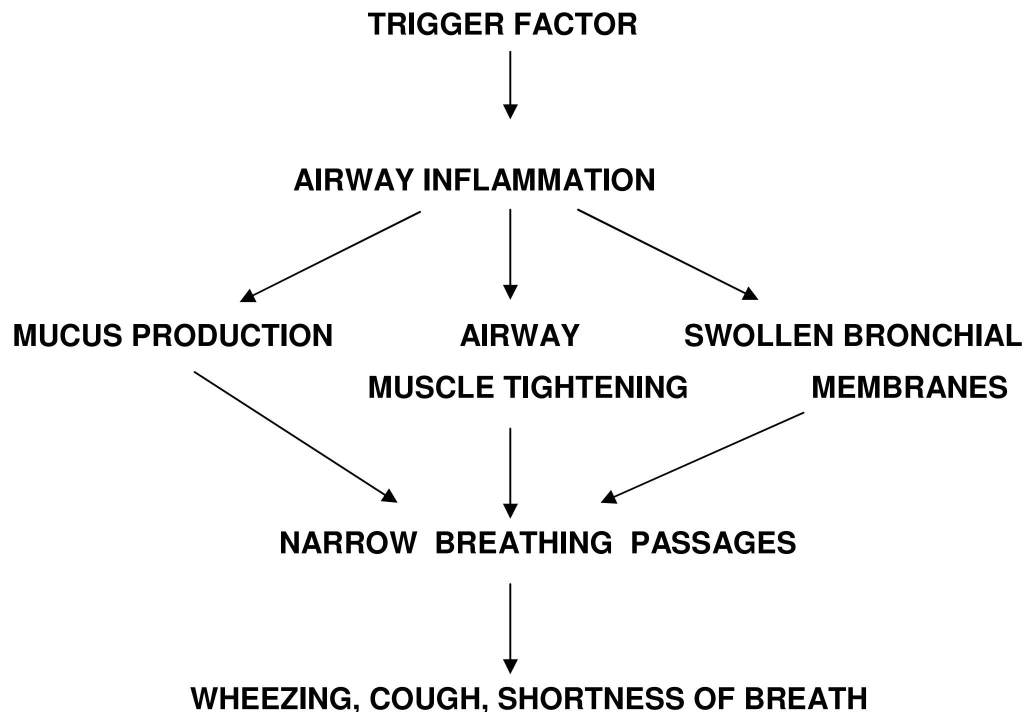

Figure 1.2 The diagram summarizes the events that lead to an asthma attack. A trigger factor as tobacco smoke or respiratory infections, initiates airway inflammation, which produces bronchial membrane swelling, muscle tightening, and mucus production. These events cause a narrowing of the passages initiating cough, wheezing, and shortness of breath.

1.4 PATHOGENESIS

Upon initial exposure to an allergen, CD4+ T helper cells begin to differentiate into Th1 or Th2 cells. Th1 cells mediate phagocytic immune responses to intracellular microbes, while Th2 cells mediate IgE-dependent immune responses to allergens. In addition to mediating immune responses, each of these T cell subsets produces cytokines that inhibit the proliferation of the other subset. Th1 cells produce high IFN- γ levels, which inhibits Th2 cell growth. Th2 cells produce IL-10, which inhibits Th1 cell growth, and IL-4, which promotes IgE production. In response to an antigen, these cell populations will competitively grow, and one subset will become dominant and differentiate into memory cells, that will determine future responses to the allergen. When the Th1 subset is dominant, the individual is able to inhibit IgE production, which effectively protects against atopy. This IgE

suppression may also be enhanced by allergen-specific CD+8 T cells that secrete IFN- γ. However, if the Th2 subset is dominant, IgE production is increased and the individual becomes atopic to the allergen (Holt, 1995, and Corrigan et al., 1992).

Once sensitization to an allergen has occurred, all the successive exposures to that allergen will elicit a hypersensitive immune response, as seen in asthma. The immune response begins with the binding, processing, and presentation of the allergen by dendritic cells in the lungs. In non asthmatic individuals, the bronchial epithelium provides a protective barrier against inhaled allergens that prevents these particles from reaching the antigen presenting dendritic cells beneath the epithelial layer. In asthmatic patients, however, it has been demonstrated that the bronchial epithelium is unusually permeable to certain allergens, allowing access to the subepithelial dendritic cells.

The initial response to allergen exposure is called the early phase asthmatic response (occurring 4 to 6 minutes after exposure), and is mediated by the IgE antibody network. Upon activation by allergen, the Th2 cell secretes IL-4 which induces the isotype-switching and allergen-specific IgE production by B lymphocytes. IgE binds to the allergen, and this complex in turn binds to certain high affinity IgE receptors (Sutton et al., 1993).

After binding to the mast cell, these allergen-IgE complexes, cross-linked with each other, stimulate the mast cell to release its intracellular granules. Such degranulation is also stimulated by histamine releasing factors secreted by T cells (Wilson et al., 1994).

Mast cell granule products, including histamine, leukotrienes, tryptase, prostaglandin, and platelet-activating factor, are major contributors to asthma clinical manifestation. These products produce the immediate bronchoconstruction, vasodilation, mucus secretin, and tissue destruction that characterize an early phase asthmatic response.

The second pathway in an asthmatic immune response is the direct Th2 cell-mediated eosinophil recruitment and lungs' infiltration in the late phase asthmatic response. This recruitment phase is begun by IgE-activated mast cells and is greatly enhanced by the Th2 cells' arrival to the allergen exposure site

(Holgate et al., 1995, and Makino et al. 1995). In addition to IL-4, Th2 cells secrete IL-3, IL-5, and GM-CSF, all of which result in the migration of eosinophils to the region of allergen exposure. As seen in mast cells, these cytokines promote the expression of certain adhesion molecules by local endothelial cells to increase eosinophil binding, and also function in eosinophil activation, differentiation, and survival (Drazen et al., 1996, Redington et al., 1995, and Corrigan et al., 1992). Eosinophils recruited to the site of allergen exposure become the major effector cells of the late phase asthmatic response. The most significant eosinophil response result in asthma, is the bronchial epithelium damage and death, which is directly correlated with increased airway hyper-responsiveness.

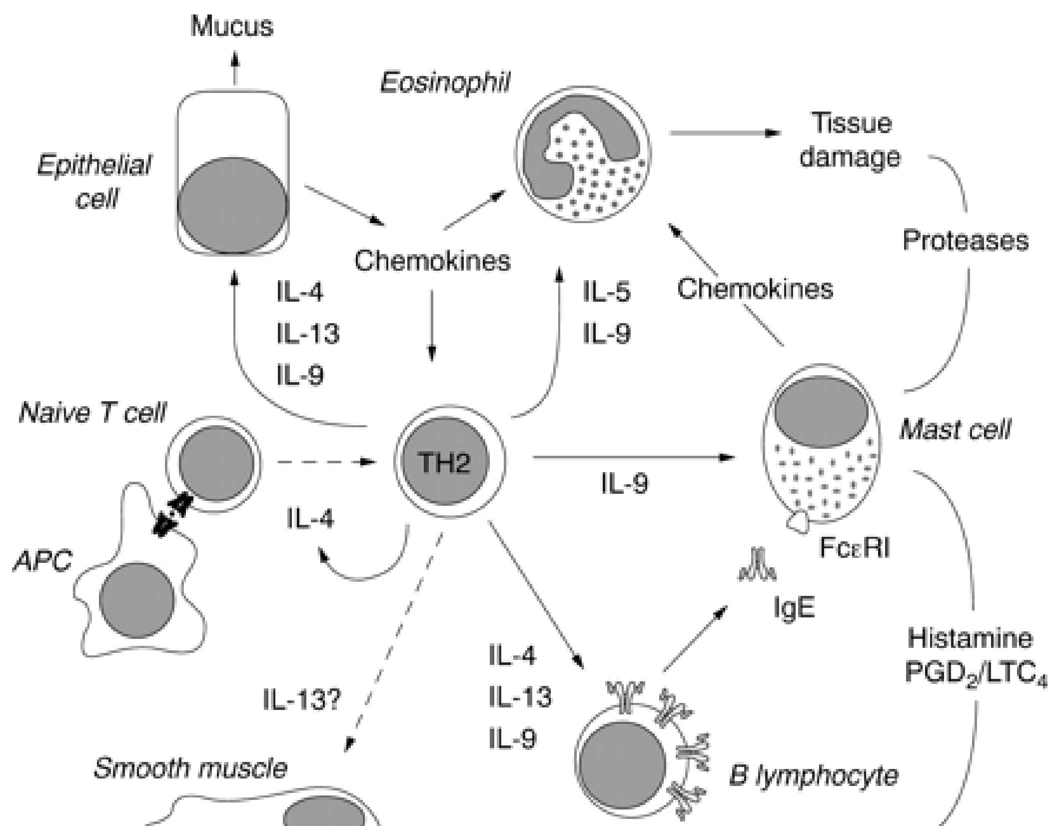

Figure 1.3 Principal mechanisms involved in the pathogenesis of allergic asthma. Antigen recognition from antigen presenting cells (APC) causes limphocytes activation and differentiation into TH2 cells, The activated cell stimulate B cells to produce IgE antibodies, in response to IL-4, and to a lower extend to IL-13 and IL-9. IgE stimulate mast cells granulation, and the release of histamine, which causes bronchospasm. Mast cells also release chemotactic factors for eosinophils. TH2 cell cause epithelial cells mucus production.

1.5 ALLERGIC ASTHMA GENETICS

Multifactorial diseases are complex genetic disorders with variable phenotypes, largely attributed to the interaction of the environment and multiple genes, each potentially having small effects (Hakonarson et al., 2001).

In the case of asthma, multiple environmental factors are known to play an important role in triggering and modulating asthma responses, whereas the genetic components that underline the susceptibility to react to these environmental factors still remain largely unknown.

In several studies, it has been reported that asthma has a higher frequency in first degree relatives than in non-related individuals. In fact, it has been seen that asthma phenotypes are present for 25% in children of asthmatic parents (Panhuysen et al., 1998). Also in twins studies, it has been observed that there is a greatest incidence of asthma and its phenotypes, in monozygotic twins comparing to dizygotic ones, given that monozygotic share 100% of the genes while dizygotic about 50%, as normal siblings (Panhuysen et al., 1998).

Moreover, an Australian twins study, conducted on 3800 twin-pair , showed a clear evidence of a genetic component in the development of asthma (Duffy et al., 1990).

Litonjua et al., in fact, has shown evidence for preferential inheritance of early-childhood asthma through the maternal line; while the development of asthma later in life have been observed to be preferentially inherited from the paternal line.

However, up until now, it has not been possible to identify a precise inheritance model for asthma because of its complex genetic factors' interaction, that confer with familiar disposition, and with environmental factors. It is likely that asthma inheritance model is polygenic, involving multiple genes, each one having a small distinct effect, and environmental factors, such as allergens, pollution and respiratory infections, which could act as causing elements in genetic susceptible individuals (Anderson et al., 1999; Cookson, 2002; Wills-Karp and Ewart, 2004).

IDENTIFICATION METHODS FOR ASTHMA'S SUSCEPTIBILITY GENES

Complex diseases are common and they represent the larger cause of morbidity in developed countries. The identification of genetic involved agents, represent today one of the principal objectives of genetic research, and will allow a better understanding of the pathogenetic mechanisms, new pathologies development, prevention strategies and diagnostic methods (King et al., 2002).
The strategies used to identify susceptibility genes for asthma include linkage analysis and association studies.
The linkage analysis takes into examination affected individuals' families and identifies the region that could contain the susceptible gene, showing a marker cosegregation with the disease. It can be carried out by the analysis of markers distributed all over the genome, known as a genome-wide screening. The genomic screenings have had an excellent success on Mendelian diseases' gene mapping, but they have been much more difficult to use for multifactorial diseases, despite the genotyping methods and statistical analysis, due to their complexity.
Linkage analysis can also be performed with the candidate gene, of which it is hypothesized the involvement of the disease (Altmüller et al., 2001; Sengler et al., 2001).

An association study, on the other hand, compares the relative frequencies of gene polymorphisms in unrelated affected individuals and unaffected controls; the presence of a significant difference indicates association between the allele and the disease (Gao et al., 2004). Candidate genes are chosen on the basis of functional or positional correlation to the disease.
A positive association can signify that the allele is the disease causing factor, since it influences the gene function, modifying for example the transcriptional properties, the splicing sites or the amino acidic sequence. But a positive association can also indicate that the allele is in *linkage disequilibrium* (LD) with the real locus of the disease (Hoffjan et al., 2003).
Genetic markers used for linkage and association studies are polymorphic sites.
Single nucleotide polymorphisms (SNP) are the markers mostly used in association studies. SNPs are biallelic, therefore less informative than other markers, such as microsatellites, but they are more numerous, usually 1every

1000 nucleotides, widely spread along the whole genome, and more easily managed for multiple genotyping techniques at advanced levels (Schork et al., 2000; Strachan & Read, 2004).
SNPs are arranged in LD blocks allowing the definition of chromosomal region by the use of a single SNP inside the LD block (tagged SNP).

1.6 LOCALIZATION TECNIQUES

The complexity of asthma genetics implies the analysis of a wide number of polymorphic markers. The susceptibility genes' research uses various techniques, which are divided into two major groups: the first group includes techniques that identify new sequence variation of the candidate gene, such as Single Strand Conformation Polymorphisms (SSCP), Denaturing Gradient Gel Electrophoresis (DGGE), and Denaturing High-Performance Liquid Chromatography (DHPLC). The second group includes techniques that analyze already known variations, such as RFLP Analysis, Reverse Dot Blot (RDB), Oligonucleotide Ligation Assay (OLA), Minisequencing or ddNTP Primer Extension, and techniques based upon DNA chips. Sequencing can be used for both groups (King et al.. 2002; Strachan & Read, 2004).

- The SSCP technique identifies the presence of a sequence variation in a Single Strand DNA molecule through alteration in eletrophoretic mobility in non-denaturing gel.

- The DGGE is a technique that allows to identify any sequence variation in Double Strand DNA molecules, on the basis of difference in T_m and gel eletrophoretic mobility in denaturing conditions of hetero or homoduplex molecules.

- DHPLC is a capillary chromatographic technique based on the same principles of DGGE.

- The polymorphisms length analysis of the restriction fragments (RFLP), is based upon the property of this polymorphism to eliminate or create a natural restriction site or an appropriate designed PCR primer (Gasparini et al,. 1992).

- The Reverse Dot Blot (RDB) technique is based upon the DNA hybridization with specific allele oligonucleotide primers.

- The OLA (Oligonucleotide Ligation Assay), is based upon the use of two oligonucletidic allele specific primers, which hybridize DNA in the sequence next to the variation to be analyzed, and the DNA ligase, which covalently binds to a third primer only in the presence of a perfect match. The analysis method is performed with automatic DNA sequencers.

- The minisequencing or ddNTP Primer Extension is based upon the ddTerminators' chemistry, and results from a Single di-deossinucletide-trifosphate ddNTP primer extension. The analysis method uses automatic DNA sequencers, or other technologies, such as MALDI-TOF Mass spectroscopy (Ross et al., 1998).

- DNA chip are based upon minisequencing or allele specific primer hybridization, described above, but with the use of automatic instruments. They are obtained, fixing oligonucletides on glass supports, through photolithography process. The oligonucleotides are marked with fluorescence, and they are analyzed with laser instruments (Carella et al., 2003).

1.7 IDENTIFIED REGIONS ASSOCIATED TO ASTHMA

A genome wide screen consists of a systematic search for genetic linkage to a disease across all human chromosomes. Eleven full genome screens have been reported for asthma and its associated phenotypes, and others have been carried out in industry (Cookson, 2002). Several of these screens have been performed in distinct European populations, such as German (Wjst et al., 1999), French (Dizier et al., 2000), Finnish (Laitinen et al., 2001), Icelandic (Hakonarson et al., 2002), Dutch (Koppelman et al., 2002), and Danish (Haagerup et al., 2002).

The replicated regions reported, for the asthma phenotype, have been on 1p, 2q, 4q, 5q, 6p, 12q, 13q, 14q, 19q, and 21q; for total serum IgE sites on 2q, 3q, 5q, 6p, 7q, 12q, 13q; atopy on chromosomes 3q, 4q, 6p, 11q, and 17q; and eosinophil counts on 15q (Hoffjan et al., 2002) **Table 1.1**

The loci most consistently identified by these screen will be further described underneath:

- ***1q32*** In this region, some studies have shown that Adenosine A1 receptor (A1AR) is a signalling molecule that plays a role in the regulation of inflammation by promoting IL-4 and IL-13 production, and stimulates mucus production by the regulation of CLCA1 (McNamara et al., 2004)

- ***2q14*** In this region, two independent groups recently reported two different candidate gene, IL-R1N and DPP10, both mapping in the region of the IL-1 cluster. DPP10, encoding a homolog of dipeptidyl peptidases (DPPs), shares features with members of the S9B family of DPP serine proteases, which includes DPP4, a widely expressed enzyme that plays a central role in chemokine processing as part of the innate immune response (Allen et al., 2003; Gao et al., 2004).

- ***5q34*** This region has been linked to total serum IgE concentrations, to eosinophil levels and to schistosomiasis resistance. The region contains several genes that modulate atopic responses, including IL-4, IL-13, IL-5, CD14, and granulocyte macrophage-colony stimulating factor (GM-CSF) (Cookson, 2002).

Table 1.1 Summary of asthma related genes and their chromosome location (Malerba et al., 2005)

Description	Gene	Chromosome	Genomic location from p-ter
1	2	3	4
Early growth response protein 1	*EGR-1*	1p34	45.5
Prostaglandin E receptor 3	*PTGER3*	1p31	71
Chloride channel calcium activated family member 1	*CLCA1*	1p31	86
Vascular cell adhesion protein 1 precursor	*V-CAM 1*	1p21	100.9
Glutatione-S-transferase	*GSTM1*	1p13	109.9
Adenosine A3 receptor	*A3AR;ADORA3*	1p13	111.7
Adenosine A1 receptor	*A1AR;ADORA1*	1q32	199.8
Transforming growth factor beta 2 precursor	*TGF-β-2*	1q41	214.9
Interleukin-1 receptor	*IL-1R1*	2q11	102.2
High affinity interleukin-8 receptor A	*IL-8RA; CXCR1*	2q35	218.9
Interleukin-1 receptor antagonist protein precursor	*IL-1RN*	2q13	113.6
Interleukin-1 alpha precursor	*IL-1α*	2q13	113.2
Interleukin-1 beta precursor	*IL-1β*	2q13	113.3
Dipeptidylpeptidase 10 isoform 1	*DPP10*	2q14	114.9
C-C chemokine receptor type 1	*CCR1*	3p21	46.2
Interleukin-8 precursor	*IL-8*	4q13	75.0
Aminopeptidase A	*APA*	4q25	111.8
Interleukin-5 precursor	*IL-5*	5q23	131.9
Interleukin-4 precursor	*IL-4*	5q23	132
Interleukin-13 precursor	*IL-13*	5q23	132
Interleukin-9 precursor	*IL-9*	5q31	135
Monocyte differentiation antigen CD14 precursor	*CD14*	5q31	140
Serine protease inhibitor Kazal-type 5 precursor	*SPINK5*	5q32	147.4
Beta-2 adrenergic receptor	*ADRB2*	5q32	148.2
Plasma glutathione peroxidase precursor	*GPX3*	5q33	150.4
SH2 domain-containing leucocyte protein	*SLP-2 LCP2*	5q35	169.6
Lymphocyte cytosolic protein 2	*SLP-76*	5q35	169.6
HLA class I histocompatibility antigen – alpha chain G precursor	*HLA G*	6p21	30
Major histocompatibility complex – class II – DR beta 1	*HLA-DRB1*	6p21	32.7
Tumor necrosis factor precursor	*TNF-α*	6p21	37.1
Pim-1 oncogene	*PIM1*	6p21	37.2
Peroxisome assembly factor-2	*PAF-2*	6p21	43
Arginase I	*ARG1*	6p23	131.9
Superoxide dismutase 2 mitochondrial	*SOD2*	6q25	160.1
Interleukin-6	*IL-6*	7p15	22.5
G-protein-coupled receptor for asthma susceptibility	*GPRA*	7p14	34.3
T cell receptor gamma	*TCRG*	7p14	38
Epidermal growth factor receptor precursor	*EGFR*	7p11	59.9
Plasminogen activator inhibitor-1 precursor	*PAI-1 o SEPRINE1*	7q22	100.4
Nitric-oxide synthase – endothelial	*eNOS; NOS3*	7q36	150.1
Peroxisome assembly factor-1	*PAF-1*	8q21	78.1

Table 1.1

1	2	3	4
Prostaglandin E synthase	*PTGES*	9q34	129.6
Mucin 2	*MUC2*	11p15	
Prostaglandin D2 receptor DP	*PTGDR*	11q	51.8
High affinity immunoglobulin epsilon receptor beta-subunit	*FcεRI*	11q12.1	59.6
Glutatione-S-transferase	*GSTP1*	11q	67.1
Early activation antigen CD69	*CD69*	12p13	9.8
Vitamin D3 receptor	*VDR*	12q13	46.5
Signal transducer and activator of transcription 6	*STAT6*	12q13	55.8
Interleukin-1 receptor-associated kinase 3	*IRAK3*	12q14	64.9
Interleukin-22 precursor	*IL-22*	12q15	66.9
Interferon gamma precursor	*IFNG*	12q15	68.8
Kit ligand precursor	*KITLG*	12q21	87.4
Nuclear transcription factor Y subunit beta	*NF-YB*; *CCAAT*-binding transcription factor subunit A	12q23	103
Nitric-oxide synthase type I	*nNOS; NOS1*	12q24	116.9
SET domain bifurcated 2	*SETDB2*	13q14	48.9
PHD finger protein 11	*PHF11*	13q14	49
Regulator of chromosome condensation	*RCBTB1*	13q14	49
Regulator of chromosome condensation	*RCC1; RCBTB1*	13q14	49
Prostaglandin E receptor 2	*PTGER2*	14q22	51.9
Arginase II	*ARG2*	14q24	67.2
Alpha-1-antichymotrypsin precursor	*AACT*	14q32	94.1
Extracellular signal-regulated kinase 3	*ERK-3*	15q21	50.1
Arachidonate 15-lipoxygenase	*ALOX15*	17p13	4.5
Nitric oxide synthase - inducible	*iNOS; NOS2*	17q11	23.1
Small inducible cytokine A2 precursor	*CCL2;MCP-1*	17q12	29.6
Small inducible cytokine A7 precursor	*CCL7;MCP-3*	17q12	29.6
Squamous cell carcinoma antigen 1	*SCCA-1 SerpinB4*	18q21	59.5
Low affinity immunoglobulin epsilon Fc receptor	*Fc-ε-RII; CD23*	19p13	7.7
Intercellular adhesion molecule-1 precursor	*ICAM-1*	19p13	10.2
Prostaglandin E receptor 1	*PTGER1*	19q13	14.4
Transforming growth factor beta 1 precursor	*TGF-β-1*	19q13	46.5
Disintegrin and metalloproteinase domain 33	*ADAM33*	20p13	3.6
Superoxide dismutase [Cu-Zn]	*SOD1*	21q22	32.0
Prostaglandin-E(2) 9-reductase	*CBR1*	21q22	36.4
Glutatione-S-transferase	*GSTT1*	22q11	22.7
Tissue inhibitor of metalloproteinase 1	*TIMP1*	Xq11	47.2
Synaptobrevin-like protein 1	*SYBL1*	Xq28	154.7
Signal transducer CD24 precursor	*CD24*	Yq11	19.5
Synaptobrevin-like protein 1	*SYBL1*	Yq12	57.6

Table 1.1

- ***6p21*** The human major histocompatibility (MHC) gene and other genes that play a role in regulating the immune system are mapped on chromosome 6p21 (Shiina et al., 2004). This region has shown strong linkage to atopic phenotype and asthma, and it is considered a major locus influencing allergic diseases (Cookson 2002; Hakonarson and Wjst 2001; Moffat et al., 2003). Other studies reported also association with the TFN-α (Shin et al., 2004). The susceptibility for asthma linked to this region is very complex and it may be influenced by maternal factors.

- ***7p14-15*** This region contains the GPRA gene, a G-protein-coupled receptor for asthma susceptibility, which is associated with high levels of IgE, clinical asthma, and atopy (Laitinen et al., 2004).

- ***9p21*** In Hutteries families, it has been reported an evidence of linkage with a short tandem repeat polymorphism (STRP) within the type I interferon (IFN) gene cluster on chromosome 9p21(Chan et al., 2006). This region has shown linkage to IgE and Skin Prick Test.

- ***11q13*** This region was first linked to atopy. It contains the β-chain of the high-affinity receptor for IgE (FcεRI-β). The FcεRI-β acts as the allergic trigger on mast cells and other types of cells; it is central to allergic response. The β-chain is not essential for FcεRI-β function, but it both stabilizes the surface expression of the receptor and acts as an amplifying element within it. Any variation in the level of the β-chain expression may therefore modify receptor function (Cookson, 2002).

- ***12q13-26*** In the long arm of the chromosome 12 are located many different candidate genes for allergic asthma and its phenotypes. STAT6 (Signal transducer and activator of transcription 6 gene), involved in the initiation of signals from Th2 cells, through IL-4 and IL-13 receptors; IFN-γ gene, and the nitric oxide synthase 1 gene (NOS1) (Malcolm, 2005).

- **13q14** This region was identified as associated to high levels of total serum IgE and atopic dermatitis. Chromosome 13 will be further described in the next chapter since chosen for the analysis of this study.

- ***14q*** In this region, recently, Mansur et al. reported linkage and association of the D14S63 marker with total serum IgE levels in asthmatic families (Mansur et al., 2004). The marker D14S63 is located 16 Mb from the prostaglandin D2 receptor (DP) gene (PTDGR). The PTDGR gene is present on mast cells and eosinophils, which generate the effector molecules of the asthmatic diathesis (Kabashima and Narumiya 2003).

- ***17q*** On chromosome 17, the predominant eosinophil chemoattractant is the gene for eotaxin (SCYA11), which is involved in allergic inflammation. SNPs, in its promoter region, have been found in many studies to be associated with asthma, eosinophil count, and lung function (Hoffjan et al., 2003). Shin et al. confirmed its association with asthma and total serum IgE (Shin et al.,2004).

- ***20p13*** In this region, it is located ADAM33 gene, associated with asthma and bronchial hyper-reactivity. It codes for a disintegrin and metalloproteinase that triggers intracellular and extracellular signalling by protein shedding (Werner et al., 2004). Expression analysis confirmed that ADAM33 is expressed in multiple tissues, such as the lung and lymph node. ADAM33 variants may directly impact lung architecture and function. On the other hand, since ADAM proteins (ADAM10 and ADAM17) appear to interact with inflammatory cytokines, it has been hypothesized that ADAM33 may also have important cytokine-stimulating effects, contributing to asthma susceptibility (Weiss et al., 2004).

1.8 CHROMOSOME 13

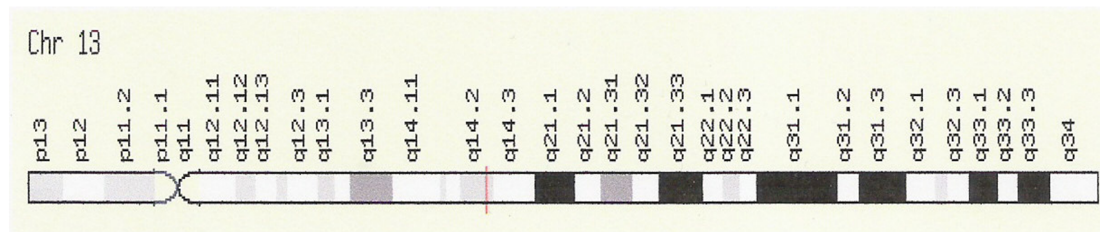

Figure 1.4 Chromosome 13

Chromosome 13 has been subject of many studies throughout the years.
In 1985 was reported by Eiberg et al., a first linkage of total serum IgE to the esterase D protein on chromosome 13q14 (Eiberg et al., 1985).
Association, on a segment of chromosome 13q14 that spanned 7.5cM, was then confirmed in a set of Australian families After this initial findings, investigators genotyped additional microsatellite markers within the peak region, and assessed each marker for association with total serum IgE. A strong association was detected with a novel marker USA24G1 (Anderson et al., 2002). After creating a dense SNP map over 1.5Mb region around USAT24G1 marker, Anderson et al. genotyped 54 common variants in 80 nuclear families. Significant association, extending over 100 kb region spanning to *linkage disequilibrium* blocks, identified PHF11 gene as a putative candidate gene (Zhang et al., 2003).

PHF11 gene

The PHF11 gene, PDH Finger Protein 11, encodes NY-REN-34, a protein that was first described in patients with renal cell carcinoma (Scanlan et al., 1999) and its transcripts are highly represented in stomach, tonsil and B cells (Zhang et al., 2003)
The gene product contains two PHD (plant homeodomain) zinc finger domains, which suggest involvement in chromatin-mediated transcriptional regulation (Aasland et al., 1995).
PHD fingers protein normally posses two Zn^{2+} coordinating groups that contain cysteine and histidine residues. The N-terminal (5') finger of the PHF11 finger pair, however, lacks one of the two coordinating groups. The arrangement of PHD finger in PHF11 is a characteristic of human proteins, such as ALL-1 and AF10,

whose genes are fused in some cases of acute lymphoblastic leukemia (Angioni et al., 1998). Analogy to AF10 and all PHD fingers suggests that the PHF11 PHD finger pair has a role in homodimerization, and protein binding or both.

The PHF11 gene contains 10 exons (**Figure 1.5**). PHF11 is flanked by SETDB2 and RCBTB1. SETDB2 is 4 kb proximal to PHF11 and it is transcribed in the same direction. It is a histone H3 methyltransferase, as it contains both active site and flanking cysteine residues, that are important for catalytic activity (Walker et al., 1996).

The close position of SETDB2 and PHF11 suggested that the two genes might be coordinately expressed (Zhang et al., 2003).

A study performed in European descent population from Busselton in Western Australia, has demonstrated association of this gene with elevated IgE (Zhang et al., 2003)

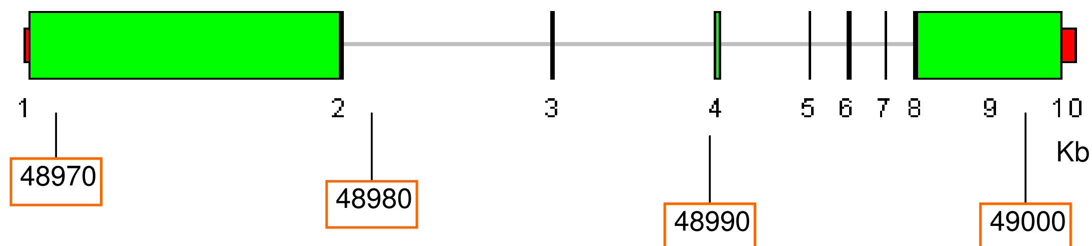

Figure 1.5 PHF11 gene

1.9 REGION INVESTIGATED

In this study, the region 13q14, located on the PHF11 gene, was investigated. This region was selected for its strong linkage to levels of total serum IgE resulting from a previous genomic screening.

The previous linkage analysis was conducted in families collected from the Pediatrics Section of the Mother and Child, and Biology-Genetic Department of the University of Verona (professor Boner), and from the Bolzano Hospital (Dr. Pescollderungg). The families were selected for the presence of at least 1 affected child with allergic asthma.

The sample included both nuclear families (parents and children), and more extended families, formed by dependent nuclear families. In particular, the sample was composed by 108 nuclear families, 8 families with 2 nuclei, 4 with 3, 1 with 4, 1 with 5, and 1 with 9 nuclei for a total of 154 nuclear families.
The studied individuals were characterized for the following phenotypes: clinical asthma, rhinitis, bronchial hyper-reactivity to metacholine (BHR), elevated total serum IgE, positive Skin Prick Test to common allergens (SPT), positive test to common allergens, such as dust mites, and atopy.
Families showing positive linkage for elevated total serum IgE with region 13q14, were selected for the present study.

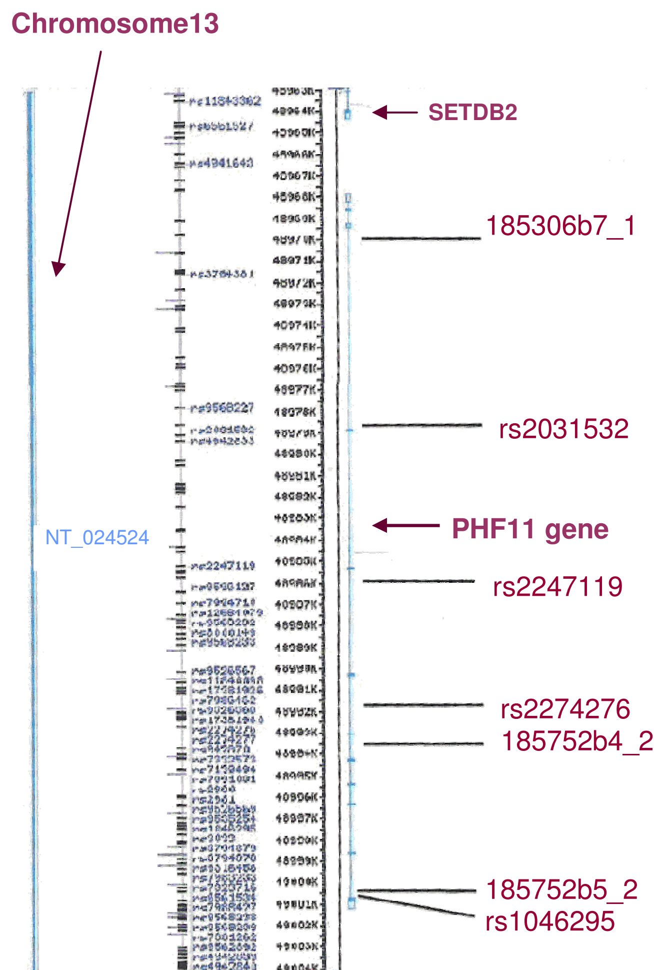

Figure 1.6 Location of the PHF11 gene and the 7 analyzed SNPs

2. AIM OF THE STUDY

The aim of this study was to perform an association analysis of the PHF11 gene Single Nucleotide Polymorphisms, in Italian families with allergic asthma, which previously demonstrated positive linkage to increased levels of total serum IgE.
This study also included the development of an efficient multiple genotyping technique.

3. MATERIALS AND METHODS

3.1 PATIENTS

The study of this thesis, was conducted on 23 asthmatic families (144 individuals) from North East Italy. The selected individuals, collected from the University of Verona Pediatrics Clinic, belonged to a bigger sample of patients, analyzed in a previous linkage study on asthma. All the enrolled subjects were characterized for the following phenotypes: clinical asthma, rhinitis, bronchial hyper-reactivity to metacholine (BHR), elevated total serum IgE, positive Skin Prick Test to common allergens (SPT), and atopy.

The families, selected for the study described in this thesis, showed positive linkage between the region 13q14 and elevated total serum IgE, in a previous wide genome screen (data not yet published).

The diagnosis for clinical asthma and rhinitis is based upon family and personal medical history, physical exam, lung function tests, and the completion of a survey prepared in line with the standards of the American Thoracic Society and the European Respiratory Society.

Bronchial hyper-reactivity to methacholine (BHR) is determined by progressive intake of increased doses of methacholine, and the response is measured by a spirometer. All the healthy and asthmatic individuals, respond to methacholine with a bronchial-construction, but they differ in the doses (low dose for the asthmatic, high dose for the healthy patients). The response to bronchial-construction is measured by FEV1 (force expiratory volume in one second). When the methacholine response causes a 20% FEV1 reduction (PC20), compared to the normal value, the patient is said to be positive. There are two PC20 values: 10 mg/ml (BHR10 constructed value), and 25 mg/ml (BHR25 standard value).

Patients are considered positive to Skin Prick Test if reactive to at least one of the tested aeroallergens.

IgE represent only the 0.004% of all immunoglobulin. The total serum concentration differs in adults non atopic individuals, from 10 to 200 kU/l
(1kU/l = 2.4 ng/ml); in infants the concentration is only a few kilo Units, and around 10 years of age, it augments reaching adult's levels.

An individual presents elevated total serum IgE when the IgE levels are superior of a determined relationship between age and IgE value, as seen in **Table 3.1**

AGE	IgE (kU/l)
0	20
1	50
2	60
3	85
4	130
5	130
6	160
7	160
8	180
9	180
10	200

Table 3.1 Threshold levels of IgE defined for age

Individuals were defined atopic when in presence of elevated IgE levels and /or positive SPT.

3.2 GENETIC ANALYSIS

The genomic DNA analyzed, came from a sample of whole peripheric blood, previously purified with the standard technique of “salting out.”

In brief, after the hemolisis through osmotic shock, the plasma membrane is lysed with the NONIDETP40 0.1% detergent. The nuclei are lysed with SDS and are incubated at 37˚C with proteinase K for 12 hours or over night. For the protein selective precipitation, a saturated solution of NaCl is added and the DNA is precipitated with absolute ethanol.

The purity of the extracted DNA is calculated by the relationship between the absorbance at 260 nm and 280 nm.

3.3 SNP SELECTION

SNPs, rs2031532, rs1046295, rs2247119, rs2274276, 185752b5_2, 185306b7_1, and 185752b4_2, located inside the PHF11 gene, previously associated with high IgE levels in Australian population (Zhang et al., 2003), were selected for the analysis. The first four SNPs have been also associated to atopic dermatitis (Jang et al., 2005). **Figure 3.1**

All the subjects enrolled in this analysis were genotyped for the 7 selected SNPs. Moreover, for polymorphism validation analysis, 15 control samples from the local book bank, were analyzed.

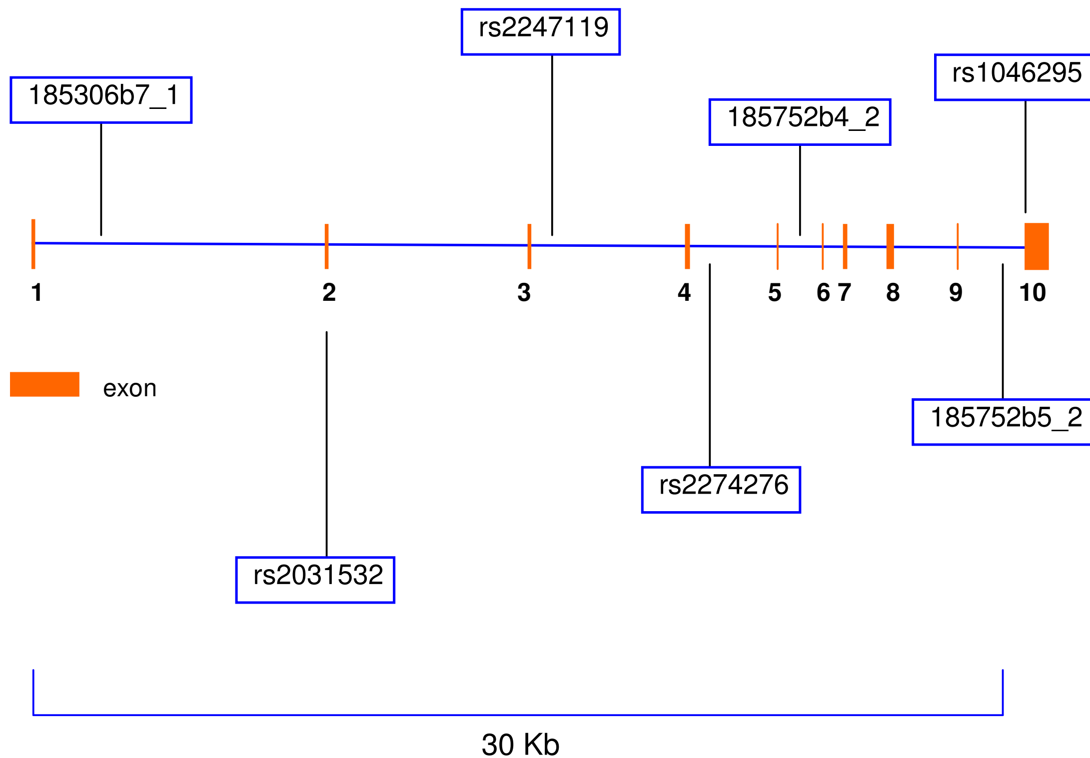

Figure 3.1 PHF11 gene – polymorphisms analyzed in this study

3.4 POLYMORPHISMS' VALIDATION

Initially a set of 15 control samples, from the same population of the asthmatic families, were genotyped for the selected polymorphisms, to assess if the markers were polymorphic enough. The methods applied to analyze allelic frequencies, and the amplification conditions are summarized on **Table 3.2**.

SNP / Markers	Primer sequence	Validation Method	PCR T ann. (°C)	PCR Product
rs2031532 G/A (2R_)	**F** AGACTGGAAGAGGAGTTAGTGA **R** TGTGTCCAAAGTGTTGTTTATC	Enzymatic Restriction RsaI /37°C	50°C	279bp
rs1046295 G/A (b5_3)	**F** TCATGCATCTCAACAAAGGT **R** CAAGAGTGTGCAAAGAGTGAT	Sequencing	52°C	308bp
185752b5_3 G/A	**F** TCATGCATCTCAACAAAGGT **R*** CATGACTCTTGGCTTAGGAGA	Enzymatic Restriction BsmAI /55°C	50°C	169bp
185752b5_2 C/T	**F** TTCTAAGTTTCCACAATTAAAA **R** AAGTCCCTTTGTATATCTTTGA	Enzymatic restriction AluI /37°C	47°C	218bp
185752b4_2 A/G	**F** CGTTTTTAATCACAAAAATAGACA **R** TCAAAGAGAATAGGTCTTGTTCAT	Sequencing	48°C	342bp
185752b4_2 A/G	**F*** GGGTTTTAAAGAAAGGTAAACAAT **R** TCAAAGAGAATAGGTCTTGTTCAT	Enzymatic Restriction MfeI /37°C	47°C	187bp
185306b7_1 A/C	**F** GTCCTCAGTGTGACTCCCAGAG **R** CAGTGCAGATGGATTAAAAACA	Sequencing	53°C	220bp
rs2247119 T/C	**F** TTAGGATAGGGGATCAGAC **R** TTGTGCATACTCTGGATTT	Enzymatic restriction Fnu4HI/ 37°C	55.2°C	270bp
rs2274276 G/C	**F** TTCATCCTTATTAGTCTGTTTT **R** ATCATAAGTGCTGCAAACA	Enzymatic restriction NspI /37°C	46°C	243bp

Table 3.2 Conditions utilized for the polymorphisms' validation

For the validation, the samples were amplified through PCR (Polymerase Chain Reaction). The PCR products were incubated for 3 hours with 3U of Restriction Enzyme at the enzyme reaction conditions temperature.

The genotypes of some samples to be used as positive controls were checked by sequencing (**Figure 3.2 and 3.3)**. After the PCR, the products were purified with Microspin HR Columns S-300, the purification was checked through the DNA Mass Ladder Dye on a 2% agarose gel. The sequencing reaction was performed with the ABI PRISM Big Dye Terminator V1.1 Cycle sequencing Ready Reaction kit, after which each reaction was purified with the AutoSeq G-50 columns, and analyzed by the Automatic Sequencer ABI PRISM 310 (Applied Biosystems).

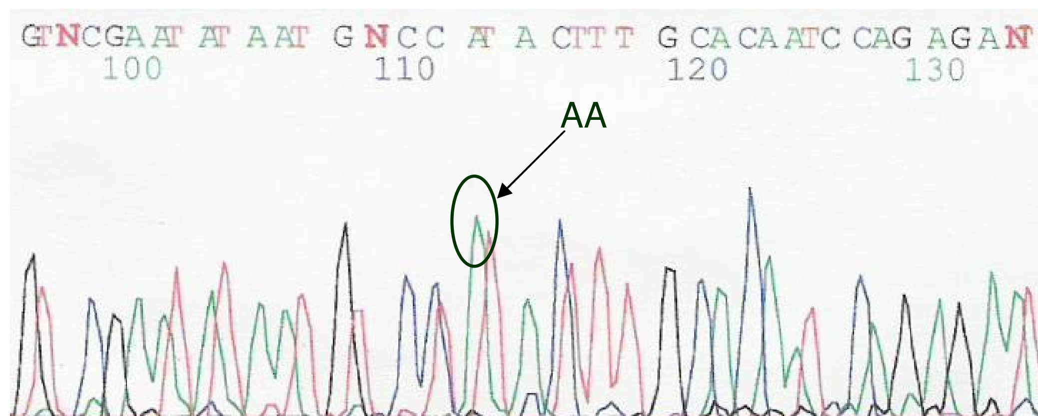

Figure 3.2 Example of sequence of an homozygote individual

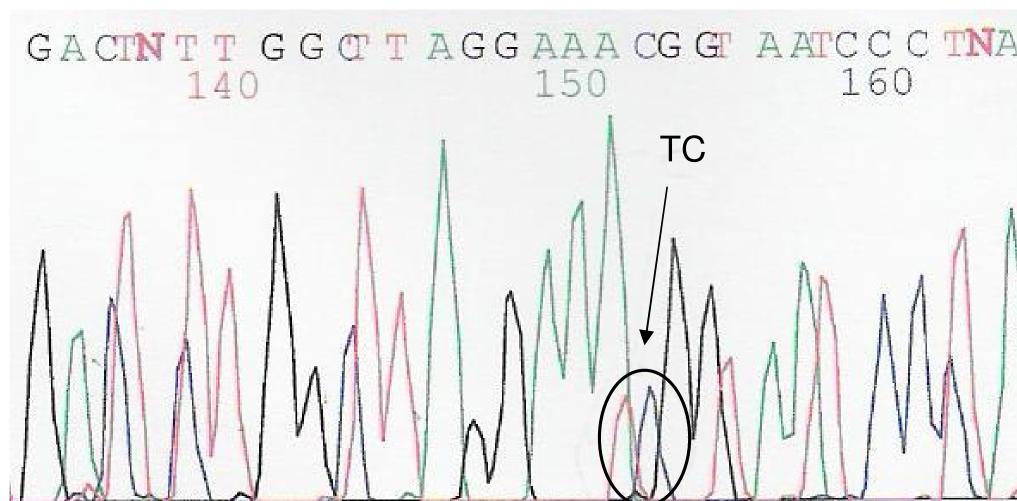

Figure 3.3 Example of sequence of an heterozygote individual

3.5 THE ddNTP PRIMER EXTENSION OR MINISEQUENCING TECNIQUE

The ddNTP Primer Extension technique is based on the dideoxy single-base extension of an unlabeled oligonucleotide primer (or primers). Each primer binds to a complementary template immediately before the SNP to be analyzed in the presence of fluorescently labelled ddNTPs and DNA Polymerase. The polymerase extends the primer by only one nucleotide, adding a single ddNTP to its 3' end.

The ddNTP Primer Extension technique identifies a specific mutation without the sequence of the whole amplified product.

3.6 THE ABI PRISM SNaPshot MULTIPLEX SYSTEM

The ABI PRISM SNaPshot multiplex system is a ddNTP Primer Extension technique developed by Applied Biosystems for ABI PRISM Genetic Analyzer.

The multiplex minisequencing protocol implicates five distinct steps:

1. **Polymerase Chain Reaction (PCR)** - each SNP target-containing region is amplified using primers that flank it. Different PCR product sizes for each selected SNP, are designed and optimized in a multiplex reaction.

2. **ExoSap Treatment** - after inspection of the PCR product in an agarose gel, the amplicons are treated with exonuclease I (ExoI) and shrimp alkaline phosphatase (Sap) to eliminate primers and dNTPs.

3. **Minisequencing reaction –** the technique is based on the annealing of a single probe adjacent to the polymorphic target site. The probe is extended by a DNA polymerase in a cycle sequencing reaction with a fluorescently labelled dideoxynucleotide (ddNTPs). The four different ddNTPs are labelled with different fluorescent markers, which can be detected by an automatic sequencer, allowing the identification of the incorporated ddNTPs. The product size varies according to the probe size. Tails of different size are added to each probe to allow the type resolution of several SNPs in the same reaction.

4. **Sap Treatment –** the product is purified with Sap enzyme to remove unincorporated ddNTPs, that will co-migrate with the interested fragments.

5. **Electrophoresis on Genetic Analyzer –** Minisequencing reaction products are separated by electrophoresis and analyzed on the ABI PRISM 310 Genetic Analyzer with the Genescan analysis software.

Figures 3.4 and 3.5 summarize the SNaPshot multiplex system reaction and procedure.

The SNaPshot Multiplex Kit allows to multiplex up to 10 loci in one reaction. It includes a Ready Reaction Mix, containing an AmpliTaq DNA Polymerase, fluorescently labelled ddNTPs, and a reaction Buffer.

The fluorescent dyes are assigned to the individual ddNTPs as shown in the table below:

ddNTP	Dye Label	Color of Analyzed Data
A	dR6G	Green
C	dTAMRA	Black
G	dR110	Blue
T (U)	dROX	Red

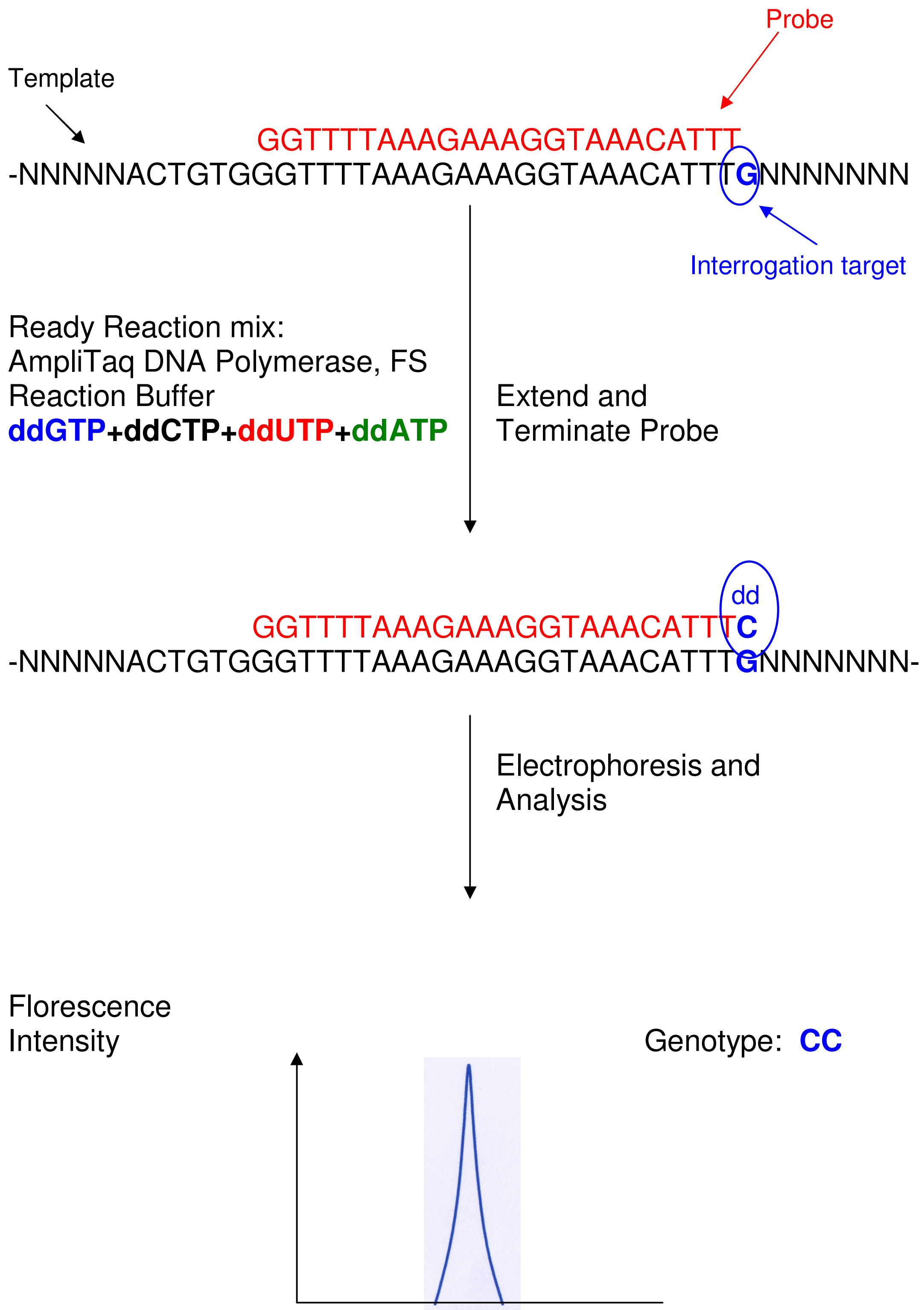

Figure 3.4 Diagram of the Primer Extension Reaction

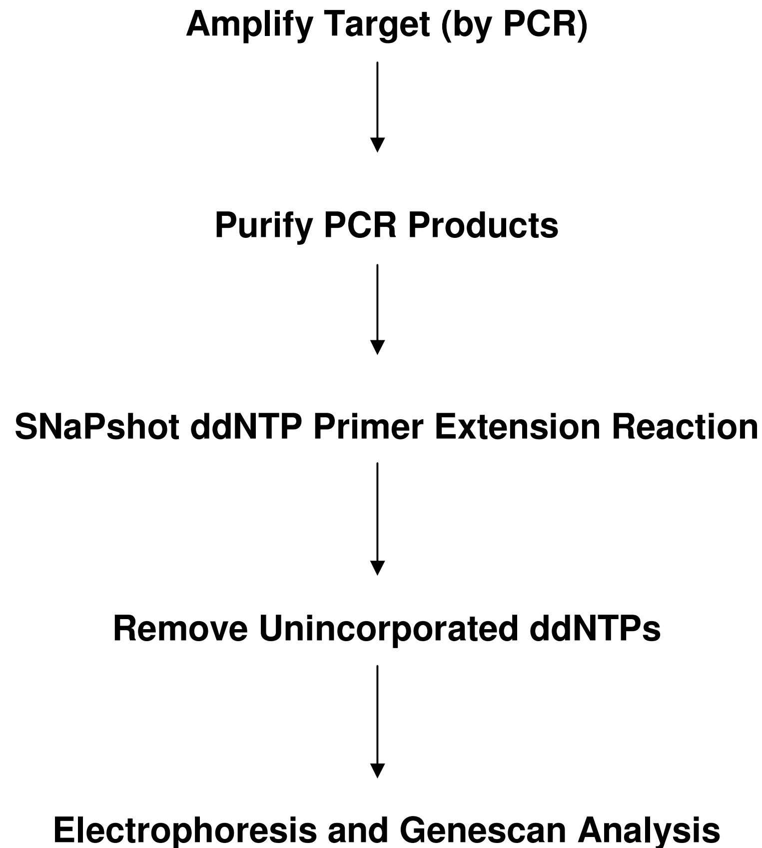

Figure 3.5 Single Base Extension reaction is performed after PCR amplification and post PCR clean up locus. Specific primers anneals upstream of SNP and dideoxynucleotide is incorporated at the 3' end with sequence specific terminator. The resulting SNaPshot multiplex product contains a mobility modifier at the 5' end. Samples are post extension and separated on a capillary electrophoresis platform. The resulting product is then differentiated by size and color. The size is a reflection of a locus specific mobility address while the color denotes the specific sequence context. Using both tools allows high multiplexed SNP analysis.

The SNaPshot Multiplex kit includes also a Size Standard, which enables SNP length normalization and allows comparison of data set for inter- and intra-run comparison. A Control Template containing an amplicon from CEPH DNA, and a Control Primer Mix with primers specific for 6 distinct SNPs are included in the kit to be used as positive control. (**Table 3.3**).

Multiplex Control Primer Mix	Length of Final Product (nt)	Signal color	Heterozygosity
20A primer	21	Green	Homozygote
28G/A primer	29	Blue/green	Heterozygote
36G primer	37	Blue	Homozygote
44T primer	45	Red	Homozygote
52C/T primer	53	Black/red	Heterozygote
60C primer	61	Black	Homozygote

Table 3.3 List of the six primers contained in the SNaPshot positive control and their characteristics.

In **Figure 3.6** is shown an example of electropherogram of a kit positive control Multiplex reaction.

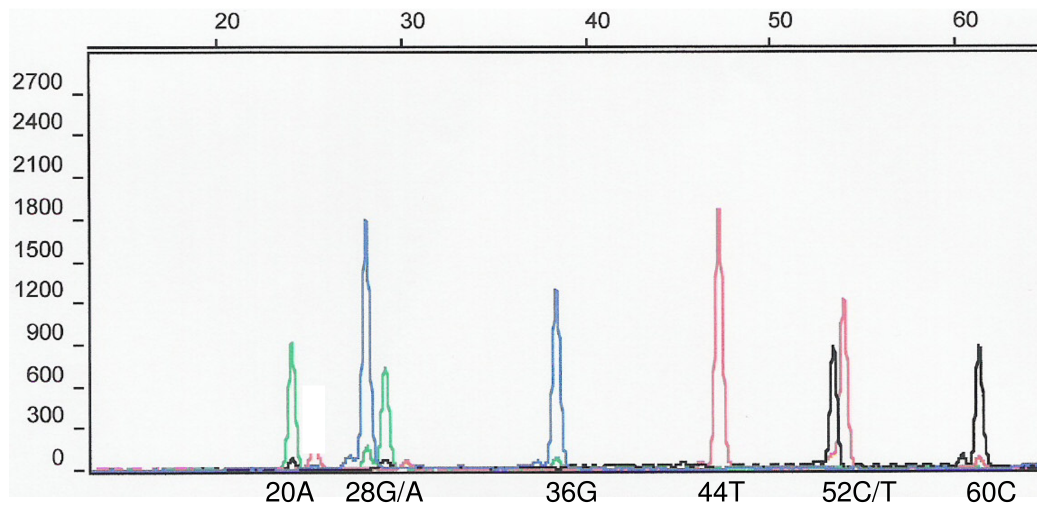

Figure 3.6

Each probe, for the minisequencing reaction, was designed following the SNaPshot probe recommendations:

- difference of 4-6 nucleotides between probe lengths
- temperature at least 50˚C
- absence of hairpin structures
- HPLC purification for probes longer than 30 nucleotides
- Use of 5' non-homologous tails with minimal secondary structures, such as poly (dA), poly (dGACT), poly (dC), and poly (dT).

The probes were designed with OLIGO Primer Analysis Program.
The melting temperature (Tm), temperature in which the filaments of a double helix DNA are half dissociated, was calculated using the Wallace Role:
4(G+C) + 2(A+T) (Thein and Wallace, 1986).
For the technique set up, several control samples, with known genotype, coming from the SNP validation analysis, were used together with the positive control included in the kit. The known genotypes allowed to observe the minisequencing reaction performance and the electropherogram quality, including the peaks' size and florescence color.

3.7 GENOTYPING ASSAY ANALYSIS

The seven SNPs selected for the study were divided into three groups of analysis in order to resolve some setting up problems, such as different annealing temperature, and primer residue.
Group A: SNPs 185306b7_1 (A1), rs1046295 (A2)
Group B: SNPs rs2031532 (B1), 185752b4_2 (B2), 185752b5_2 (B3)
Group C: SNPs rs2247119 (C1) and rs2274276 (C2)
Group A and B were analyzed through the ddNTP Primer Extension technique with the SNaPshot Multiplex Kit, while SNPs C1 and C2 were analyzed by single enzymatic restrictions.

In **Table 3.4** are listed all the designed SNaPshot probes, HPLC purified and synthesized at 0.05µmol, tested for the technique.

SNP	SNaPshot probe sequence	Name/ Strand	Length	Tail	Tm
rs2031532 G/A (B1)	$(T)_9$ AGATGTCGAATATAATGTCCT	2R-S1 Positive	30	9 T	56˚C
	$(T)_9$ AAAGATGTCGAATATAATGTCCT	2R-S2 Positive	32	9T	60˚C
185752b4_2 A/G (B2)	ACATTGTATTCCTTATCAGATTAAAA $(T)_{12}$	b4-S1 Negative	38	12T	64˚C
	ACATTGTATTCCTTATCAGATTAAAA $(T)_{14}$	b4-S2 Negative	40	14T	64˚C
	$(GACT)_4$ GGTTTTAAAGAAAGGTAAACATTT	b4-S3 Positive	40	12G ACT	60˚C
185306b7_1 A/C (A1)	$(T)_{21}$ AGAAGAGCCGAAATCCATTAAGAC	b7-S1 Positive	45	21T	68˚C
	GACACTATACCCCCCTAAATCGTC $(T)_{21}$	b7-S2 Negative	45	21T	72˚C
	$(GACT)_8$ AAGAGCCGAAATCCATTAAGAC	b7-S3 Positive	58	8GA CT	62˚C
rs1046295 G/A (A2)	CCTGTCTTATTAGGGATTACC	b5.3-S1 Positive	21	/	60˚C
	$(ACT)_1(GACT)_7$ CCTGTCTTATTAGGGATTACC	b5.3-S2 Positive	52	7GA CT	58˚C
185752b5_2 C/T (B3)	AAATACATTGTAATTTTTAACCTGTG TAAAAATC $(T)_{19}$	b5.2-S1 Negative	53	19T	82˚C
	$(T)_{23}$ CCTATTTCTAAATGAAACCAAAAGC	b5.2-S2 Positive	48	23T	66˚C
	AAATACATTGTAATTTTTAACCTG $(T)_{24}$	b5.2-S3 Negative	48	24T	58˚C

Table 3.4 List of all the designed Oligo Probes utilized for the preparation of the minisequencing technique, and their characteristics.

The SNaPshot technique development involved various tests in which were introduced continuously changes to the procedure; several sets of primers, probes, and reaction conditions, were tested to obtain the optimal conditions.
The preparation of a multiplex and a duplex PCR, with group B and A, respectively, was tested.

3.8 ASTHMATIC SAMPLE ANALYSIS

The following final protocol was used to genotype all the samples, belonging to the 23 asthmatic families, enrolled in the study.
The asthmatic samples were amplified through multiplex PCR.
The five SNPs were analyzed into two groups, Mix A and Mix B, respectively. In both protocols, 25ng of genomic DNA were used, with 0.55 Units of Taq DNA Polymerase (Platinum), 10x Buffer (50mM of KCl, 20mM Tris-HCl pH 8.4), 1.5mM of $MgCl_2$, 0.2mM of each dNTP, H_2O, and different primers' quantities in a final volume of 30µl.
In **Table 3.5** are reported the PCR amplification conditions specific for Mix A and Mix B.

PCR Mix	**PRIMERS**		**T ann.**	**PCR**
	Sequence	**quant.**	**(˚C)**	**Product**
MIX A	**A1-F:** GTCCTCAGTGTGACTCCCAGAG **A1-R:** CAGTGCAGATGGATTAAAAACA	9pmoli 15pmoli	53˚C	220bp
	A2-F: TCATGCATCTCAACAAAGGT **A2-R:** CAAGAGTGTGCAAAGAGTGAT	9pmoli 3pmoli		309bp
MIX B	**B1-F:** AGACTGGAAGAGGAGTTAGTGA **B1-R:** CAAGAGTGTGCAAAGAGTGAT	4pmoli 9pmoli	50˚C	279bp
	B2-F: CGTTTTTAATCACAAAAATAGACA **B2-R:** TCAAAGAGAATAGGTCTTGTTCAT	13pmoli 8pmoli		342bp
	B3-F: TTCTAAGTTTCCACAATTAAAA **B3-R:** CAAGAGTGTGCAAAGAGTGAT	16pmoli 11pmoli		218bp

Table 3.5

To eliminate non-specific PCR residue, it has been necessary to perform a touchdown PCR for both Mix A and B. The following diagram illustrates the cycles and program used for the DNA amplification.

MIX A

1. 3 minutes of initial denaturation at 94˚C
2. 20 cycles composed by:
   30 seconds at 94˚C
   30 seconds at 60˚C with a touchdown of -0.5 ˚C at each cycle
   30 seconds at 72˚C
3. 10 cycles composed by:
   30 seconds at 94˚C
   30 seconds at 53˚C (annealing temperature)
   30 seconds at 72˚C
4. 2 minutes at 72˚C for final extension

MIX B

1. 3 minutes of initial denaturation at 94˚C
2. 20 cycles composed by:
   30 seconds at 94˚C
   30 seconds at 60˚C with a touchdown of -0.5 ˚C at each cycle
   30 seconds at 72˚C
3. 20 cycles composed by:
   30 seconds at 94˚C
   30 seconds at 50˚C (annealing temperature)
   30 seconds at 72˚C
4. 2 minutes at 72˚C for final extension

After the PCR, the DNA was purified with SAP (Shrimp Alcaline Phosphatase) enzyme, which removes, from the dNTP, the phosphorous group in 5'; and ExoI (Exonuclease I) enzyme, which removes primer's residue. The purification is needed to eliminate unincorporated primers and dNTP before the SNaPshot reaction takes place. For the purification, 5 Units of SAP and 2 Units of ExoI enzymes were added to 15 µl of PCR product. These, were incubated for 1 hour at 37˚C, followed by 15 minutes at 75˚C to inactivate the enzymes.
A GeneAmp PCR System 9700 was used for PCR and products purification.

The minisequencing reaction for the samples was prepared as shown on **table 3.6**

ITEM	VOLUME (µl/ sample)
SNaPshot Multiplex Ready Reaction Mix	5
Pooled PCR Products	2
Pooled SNaPshot primers	2 (0.5-1µM)
Deionized water	1
Total µl	10

Table 3.6 Sample SNaPshot reaction mix

A positive and a negative control, with known fragments and probes, were added in each reaction.

The Pooled SNaPshot primer solution were composed by a mixture of all the five designed primers, while the Pooled PCR products were composed by a mixture of PCR with Mix A and PCR with Mix B. The specific quantities used for the two mixture are reported on **Table 3.7**

<table>
<tr><td rowspan="2">PCR Products
Total 2µl</td><td>Mix A</td><td rowspan="2">1 µl</td></tr>
<tr><td>Mix B</td></tr>
<tr><td rowspan="5">SNaPshot Designed Primers
Total 2µl</td><td>B7-S3 (A1)</td><td>1.2µM</td></tr>
<tr><td>B5.3-S2 (A2)</td><td>0.6µM</td></tr>
<tr><td>2R-S2 (B1)</td><td>1.3µM</td></tr>
<tr><td>B4-S3 (B2)</td><td>1.2µM</td></tr>
<tr><td>B5.2-S2 (B3)</td><td>2 µM</td></tr>
</table>

Table 3.7 Quantities used in the SNaPshot reaction mix

The thermal cycler program applied to the minisequencing reaction was:

25 cycles composed by 10 seconds at 96˚C
5 seconds at 50˚C
30 seconds at 60˚C

After the SNaPshot reaction, the samples were purified with 1-2 Units of SAP enzyme for 1 hour at 37˚C, and 15 minutes at 75˚C. A second purification prohibits the unincorporated ddNTPs to co-migrate with the fragments of interest causing interference.

3.9 CAPILLARY ELECTROPHORESIS ON ABI PRISM 310

The reaction products minisequencing analysis was performed on ABI PRISM 310 Genetic Analyzer, which separates the DNA fragments by size through capillary electrophoresis, a movement of charged molecules through a polymer in an electrical field.

Figure 3.7 ABI PRISM 310 Genetic Analyzer (Applied Biosystems)

During the analysis, the sample tubes are placed in a tray in the instrument's autosampler. The autosampler brings each sample successively into contact with the cathode electrode and one end of a glass capillary filled with polymer. An anode electrode at the other end of the capillary is immersed in buffer. A portion of the sample enters the capillary as current flows from the cathode to the anode. This is called electrokinetic injection. The end of the capillary near the cathode is then placed in buffer. Current is applied again to continue electrophoresis. When the DNA fragments reach the detector window in the capillary coating, a laser excites the fluorescent dye labels. Emitted fluorescence from the dyes is collected by a CCD camera. A matrix corrects the spectral overlap between colors on the CCD array. The software interprets the result, calculating the size of the fragments from the fluorescence intensity at each data point.

The ABI PRISM 310 can read up to five different wavelengths, allowing the labelling of the four dNTPs and of a GeneScan -120 LIZ size standard, specifically designed for short fragments (**Figure 3.8**).

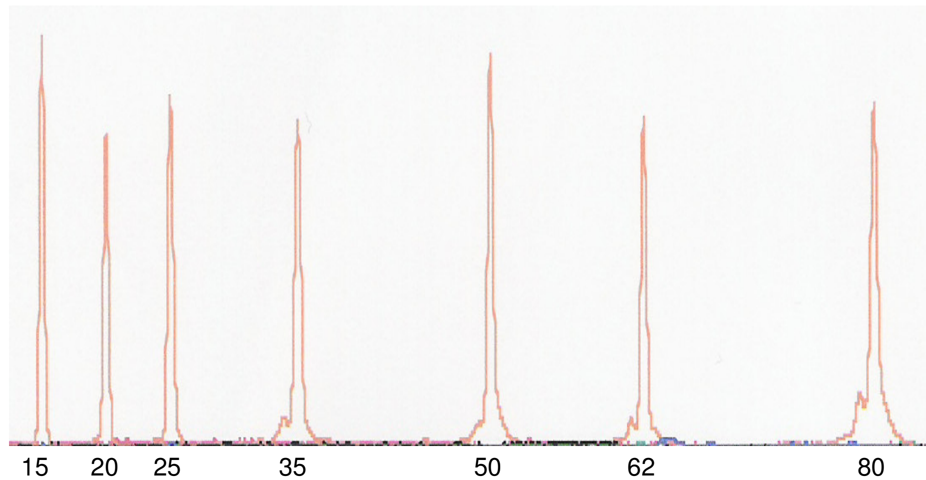

Figure 3.8 Elecropherogram of the GeneScan 120 Liz Size Standard

After the purification, the minisequencing products were prepared for capillary electrophoresis. To each tube were added 1.5µl of SNaPshot reaction product, 1.5µl of 1:2 LIZ size standard, and 12µl of Hi-Di formamide, for a total injection volume of 15µl. The samples were denatured for 5 minutes at 95˚C, and put on ice until ready to be loaded on the genetic analyzer.
The polymer used for the sample analysis was "POP-6 Polymer" in conjunction with "GS POP-4 (1mL) E5" module.

On **Table 3.8** are summarized all the parameters encoded for the E5 Run Module.

Parameter	**Control Module GS POP-4 (1mL) E5**
Injection time	5 seconds
Electrophoresis voltage	15 kV
Collection time	24 minutes
EP Voltage	15 kV
Heat plate temperature	60˚C
Syringe pump time	150 seconds
Preinjection EP	120 seconds

Table 3.8

The run time was shorten to 18-20 minutes, which was found to be enough to obtain the appearance of all the interested peaks.

3.10 MIX C SNP ANALYSIS

Differently from Mix A and B, Mix C SNPs, rs2247119 and rs2274276, were amplified singularly through PCR (see **Table 3.2** for primers, and PCR products), and analyzed with enzymatic restriction, since it was difficult to find common PCR conditions with the other SNPs.

Table 3.9 briefly summarizes the PCR and enzymatic restriction conditions use for Mix C SNP analysis.

<table>
<tr><td rowspan="3">rs2247119 (C1)</td><td rowspan="6">PCR</td><td>3’ at 94˚C</td><td rowspan="6">Enzymatic Restriction
at 37˚C
for 3 hours</td><td rowspan="3">Fnu4 HI
(3U)</td></tr>
<tr><td>35cycles of:
30s at 94˚C
30s at 55.2˚C
30s at 72˚C</td></tr>
<tr><td>2’ at 72˚C</td></tr>
<tr><td rowspan="3">rs2274276
(C2)</td><td>3’ at 94˚C</td><td rowspan="3">Nsp I (3U)</td></tr>
<tr><td>35cycles of:
30s at 94˚C
30s at 46˚C
30s at 72˚C</td></tr>
<tr><td>2’ at 72˚C</td></tr>
</table>

Table 3.9

In both PCR protocols, 200ng of genomic DNA were used, with 0.50 Units of Taq DNA Polymerase (Platinum), 10x Buffer (50mM of KCl, 20mM Tris-HCl pH 8.4), 1.25mM of $MgCl_2$, 0.2mM of each dNTP, H_2O, 15pmoli of primers, in a final volume of 30µl.

The PCR products were, then, incubated for 3 hours at 37˚C with 3 Units of the appropriate restriction enzyme.

3.11 STATISTICAL ANALYSIS

The statistical analysis of asthma and of all complexes diseases, is based on non parametric linkage analysis. The use of non parametric analysis is necessary, since there aren't any indications on the diseases' transmission method, on the number of involved genes, and on allelic frequencies.

Allele and genotype frequencies for each SNP were determined. The Hardy-Weinberg equilibrium was calculated. Genotyping data were analyzed by the TDT Test. This test compares the frequency with which a particular allele is transmitted from each parent to the affected child, with the frequency with which the allele is not transmitted. The test is performed on families with one or more affected child, in which one of the parents is heterozygote for the questioning allele. It is not important if the parent is affected or not. The estimation is completed on the all family progeny, in the entire sample families analyzed. Then, the chi-square test is used to observe if the difference between the two frequencies is significant. When both of the parents are homozygote or heterozygote, the family is eliminated from the calculation (Hoffjan, 2003).

The program used for the analysis is the UNPHASED Software (by Frank Dudbridge), available on the website:

http://www-bsu.cam.ae.uk/personal/frank/software/unphased

The haplotypes analysis was performed with the same program.

The advantages of this analysis method compared to the association study on the general population, is that, it reduces the risk of mistaken associations caused by population evolution and incorrect discovery.

4. RESULTS

4.1 SNP ANALYZED

The SNPs selected for the analysis, were previously reported in literature (Zhang et al., 2003; Jang et al., 2005) to be associated to elevated IgE levels and atopic dermatitis. The seven SNPs analyzed were: rs2031532, in exon 2; rs1046295, in the 3' UTR zone; rs2247119, in intron 3; rs2274276, in intron 4; 185752b5_2, in intron 9; 185306b7_1, in intron 1; and 185752b4_2, in intron 5.

4.2 POLYMORPHISM VALIDATION

A polymorphism validation analysis was performed in a set of 15 control samples from the analyzed population through sequencing and enzymatic restrictions (see **Table 3.2** in Material and Methods). The SNP genotype frequencies of the validation analysis are summarized on **Table 4.1**

SNP	Validation Allelic Frequencies (%)
rs2031532 G/A (B1)	G = 67 A = 33
185752b4_2 A/G (B2)	A = 50 G = 50
185752b5_2 C/T (B3)	C = 53 T = 47
185306b7_1 A/C (A1)	A = 33 C = 67
rs1046295 G/A (A2)	G = 40 A = 60
rs2247119 T/C (C1)	T = 22 C = 78
rs2274276 G/C (C2)	G = 42 C = 58

Table 4.1 Allelic Frequencies of the validation analysis

All the selected SNPs, after the validation analysis, resulted polymorphic, therefore, they were chosen to be analyzed in the population object of this study.

4.3 ANALYSIS SETUP RESULTS

The multiplex (triplex and duplex) PCR required various tests for a correct amplification product, since the selection of an annealing temperature that worked for all the sets of primers, was difficult to obtain. Also, the presence of non-specific fragments brought several obstacles to both PCR assembly.

After PCR preparation, the designed oligo probes, such as b5.2-S1, b5.3-S1, b7-S1, b7-S2, 2R-S1, b4-S1, summarized on **Table 3.4** in Materials and Methods, were used in the SNaPshot minisequencing reaction.

At first, the peaks, resulted from the reaction, were anomalous. They were joined together at the same position as shown in **Figures 4.1 and 4.2.**

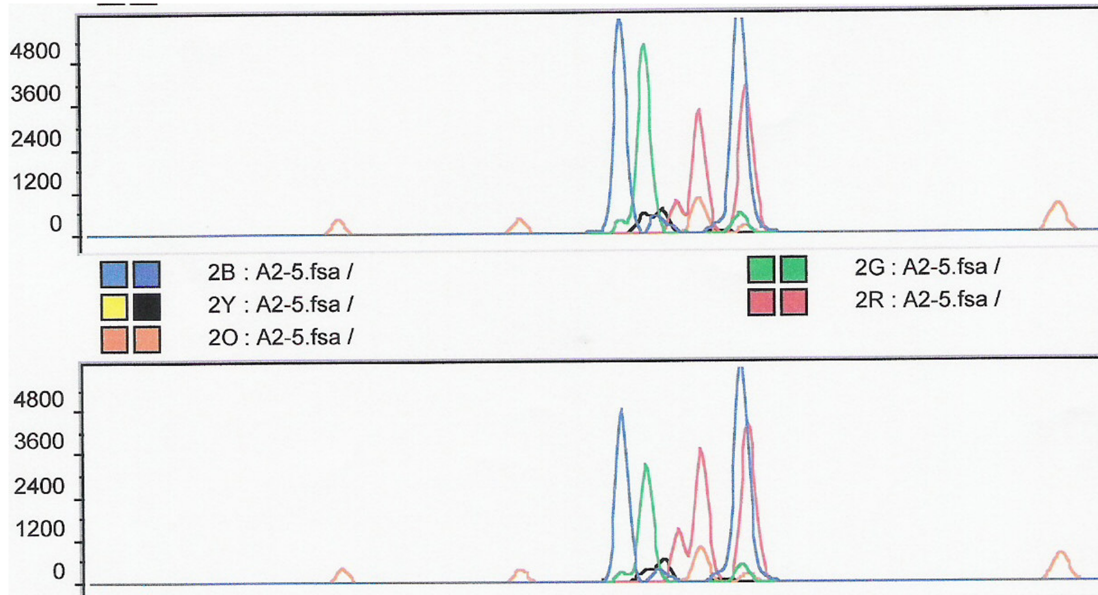

Figure 4.1 and 4.2 Anomalous peaks of the SNaPshot reaction

To improve the peaks, several tests with diverse reagents quantities, duplex and triplex PCR, separated and then united just before the SNaPshot reaction, were tested. At this point, the peaks improved, but still presented some irregularities in their position. (**Figure 4.3 and 4.4**).

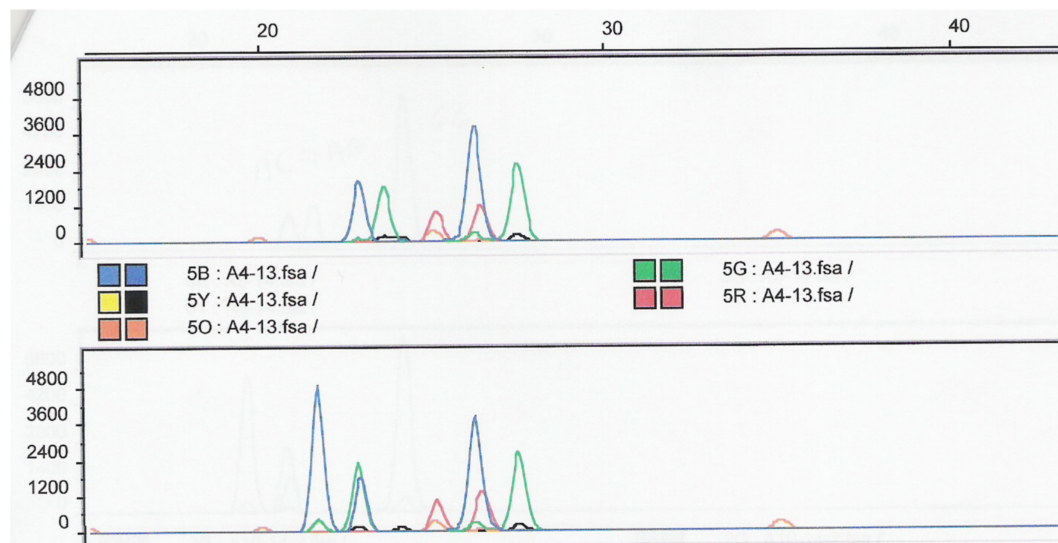

Figure 4.3 and 4.4 Electropherograms still presenting some irregularities.

Following these second series of tests, the probes were run on a 40% acrylamide gel to check their purification. This analysis showed that the probes were not synthesized properly as seen in **Figure 4.5**.

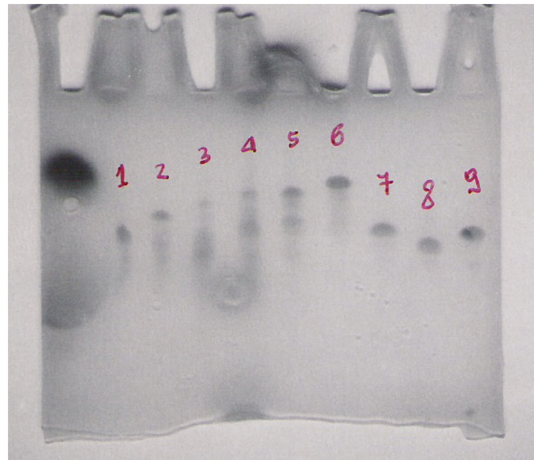

Figure 4.5 Acrylamide gel showing the probes purity. As seen on the gel, only probes 7, 8, and 9, were synthesized properly.

Then, a second set of probes, with different characteristics, was designed: b5.2-S2, b5.2-S3, b5.3-S2, b7-S3, 2R-S2, b4-S2. Several new tests were carried out with the new probes. The tests completed, first with single PCR, then with duplex and triplex PCR together, presented new electropherograms. The peaks were normal and in the correct nucleotide position, except for the probe b4-S2 peak, which failed to amplify. (**Figure 4.6 and 4.7**)

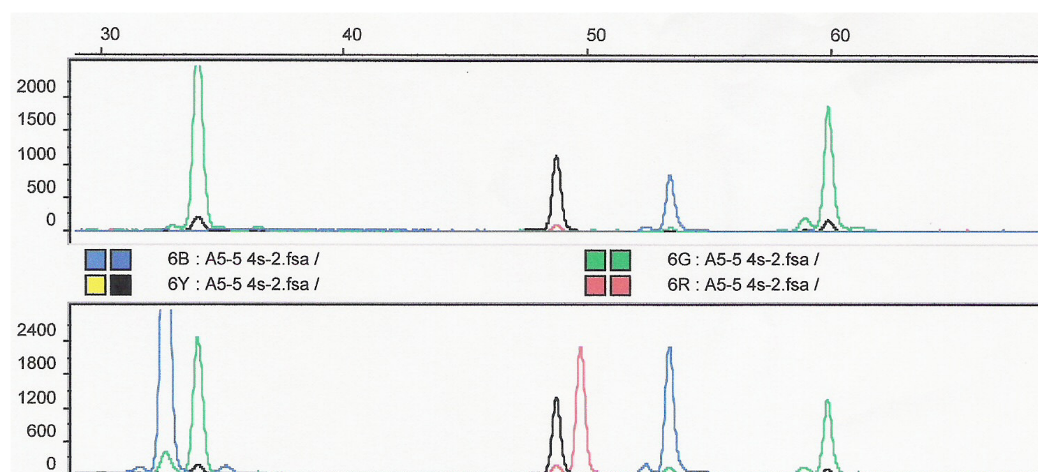

Figure 4.6 and 4.7 Electropherogram peaks of the new designed probes, probe b4-S2 is not showing.

Further tests were completed with probe b4-S2, but the problem with it persisted. The resulted genotype, in fact, did not correspond to the genotype obtained from the polymorphism validation. Therefore, a third probe for marker 185752b4_2 (b4-S3) was designed with a poly dGACT tail.

Various SNaPshot reaction, with diverse PCR and probe quantities, were carried out with the new synthesized b4-S3 probe; the obtained new peaks matched the genotype seen in the previous analysis. (**Figure 4.8**)

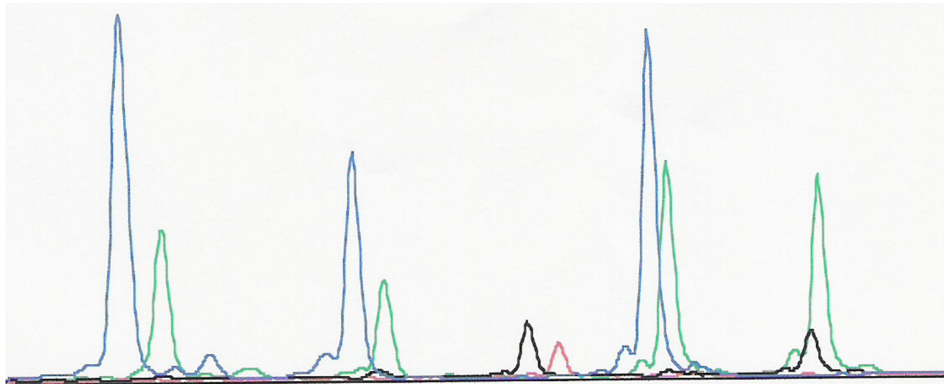

Figure 4.8 Final electropherogram peaks resulted from the preparation of the Primer Extension Technique.

The final probes used in the SNaPshot reaction to genotype all the asthmatic families, are summarized on **Table 4.2**.

SNP	PROBE SEQUENCE	LENGTH (nt)	TAIL
2R-S2 G/A rs2031532 (B1)	(T)9 AAAGATGTCGAATATAATGTCCT	32	9T
b4-S3 A/G 185752b4_2 (B2)	(GACT)4 GGTTTTAAAGAAAGGTAAACATTT	40	12GACT
b7-S3 A/C 185306b7_1 (A1)	(GACT)8 AAGAGCCGAAATCCATTAAGAC	58	8GACT
b5.3-S2 G/A rs1046295 (A2)	(ACT)1(GACT)7 CCTGTCTTATTAGGGATTACC	52	7GACT
b5.2-S2 C/T 185752B5_2 (B3)	(T)23 CCTATTTCTAAATGAAACCAAAAGC	48	23T

Table 4.2 Summary of final probes used for the analysis of the asthmatic families

7 SNPs, located on an overall area of 30 Kb, were amplified through PCR: SNPs rs2031532 (B1), 185752b4_2 (B2), and 185752b5_2 (B3), with a triplex PCR; SNPs rs1046295 (A2), and 185306b7_1 (A1) with a duplex PCR. 5 SNPs were analyzed simultaneously with the SNaPshot ready reaction mix kit (Applied Biosystems) on the ABI PRISM 310 Genetic Analyzer, and the SNPs rs2247119 (C1) and rs2274276 (C2), were analyzed by PCR, and restriction analysis.
(see **Table 4.3**)

<table>
<tr><th>GROUPS</th><th>SNP</th><th>PCR</th><th>ANALYSIS METHOD</th></tr>
<tr><td rowspan="3">GROUP A</td><td>rs2031532 (B1)</td><td rowspan="3">Triplex</td><td rowspan="5">ddNTP Primer Extension Technique</td></tr>
<tr><td>185752b4_2 (B2)</td></tr>
<tr><td>185752b5_2 (B3)</td></tr>
<tr><td rowspan="2">GROUP B</td><td>185306b7_1 (A1)</td><td rowspan="2">Duplex</td></tr>
<tr><td>rs1046295 (A2)</td></tr>
<tr><td rowspan="2">GROUP C</td><td>rs2247119 (C1)</td><td rowspan="2">Single</td><td rowspan="2">Single Enzymatic Restriction</td></tr>
<tr><td>rs2274276 (C2)</td></tr>
</table>

Table 4.3 Summary of the analysis performed in the study

4.4 ASTHMATIC FAMILIES ANALYSIS

In this study, 23 families with at least one affected child, have been analyzed. The selected families showed positive linkage to elevated total serum IgE. The characterization of 7 SNPs, located on the PHF11 gene, have been completed through the development of an efficient multiple genotyping method, based on the ddNTP Primer Extension technique

4.5 MINISEQUENCING ANALYSIS RESULTS

The results obtained from the analysis performed in this study are following.

In **Figure 4.9** is illustrated the image of the triplex PCR products completed on asthmatic individuals analyzed for SNPs rs2031532 (B1), 185752b4_2 (B2), and 185752b5_2 (B3) at 50°C annealing temperature. The electrophoresis was completed on a 2% agarose gel, and the PCR amplified fragments are 342bp (B2), 279bp (B1), and 218 bp (B3).

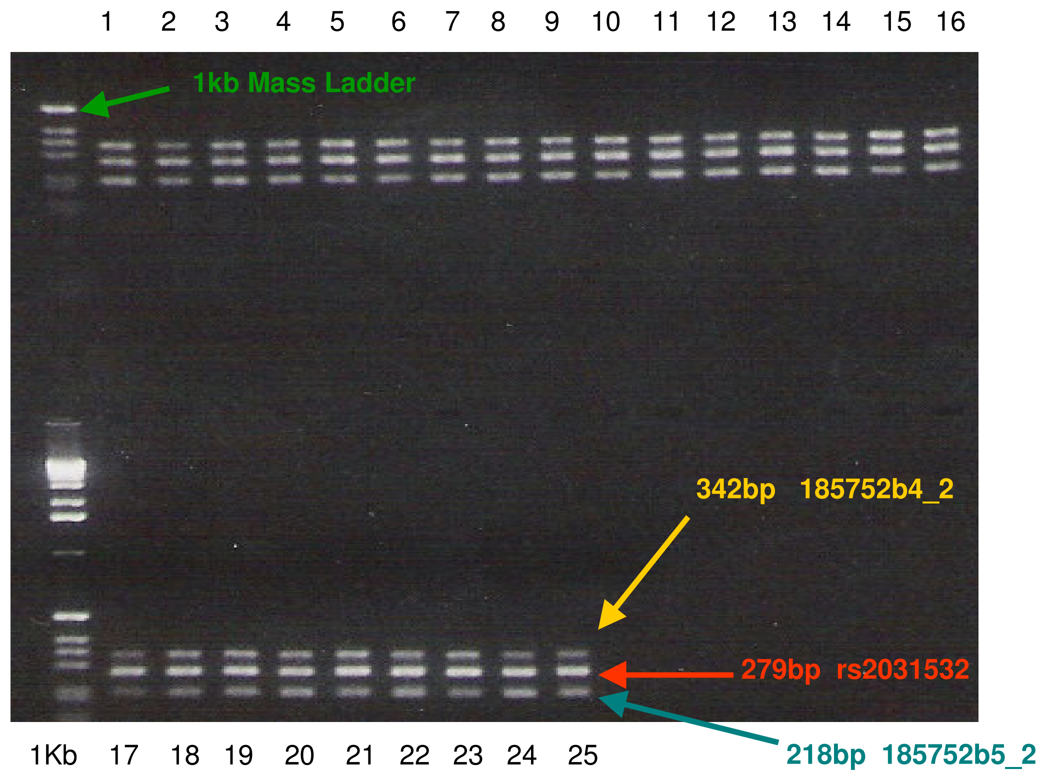

Figure 4.9 Amplification of SNPs 185752b4_2, rs2031532, and 185752b5_2 at 50°C annealing temperature.

In **Figure 4.10** is illustrated the image of the duplex PCR products completed for SNPs rs1046295 (A2), and 185306b7_1 (A1) at 53°C annealing temperature. The electrophoresis was completed on a 2% agarose gel, and the amplified PCR fragments are 309 bp (A2), and 220 bp (A1).

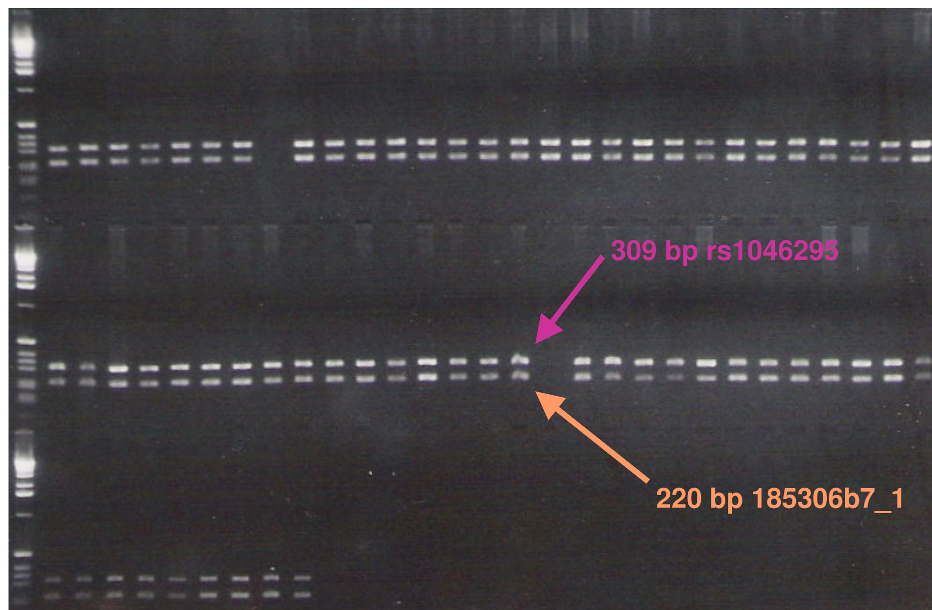

Figure 4.10 Image of the duplex PCR for SNPs rs1046295, and 185306b7_1 on a 2% agarose gel at 53℃ annealing temperature

The triplex and duplex PCR products, after purification with SAP and ExoI enzymes, were joined together to undergo a single SNaPshot reaction using the probes listed on **Table 4.2.** In **Figures 4.11, 4.12, 4.13, 4.14, 4.15, 4.16,** and **4.17** are illustrated the elecropherograms of the asthmatic families analyzed for SNPs A1, A2, B1, B2, B3, respectively located at 58, 52, 32, 40, and 48 nucleotides, with the corresponding genotype.

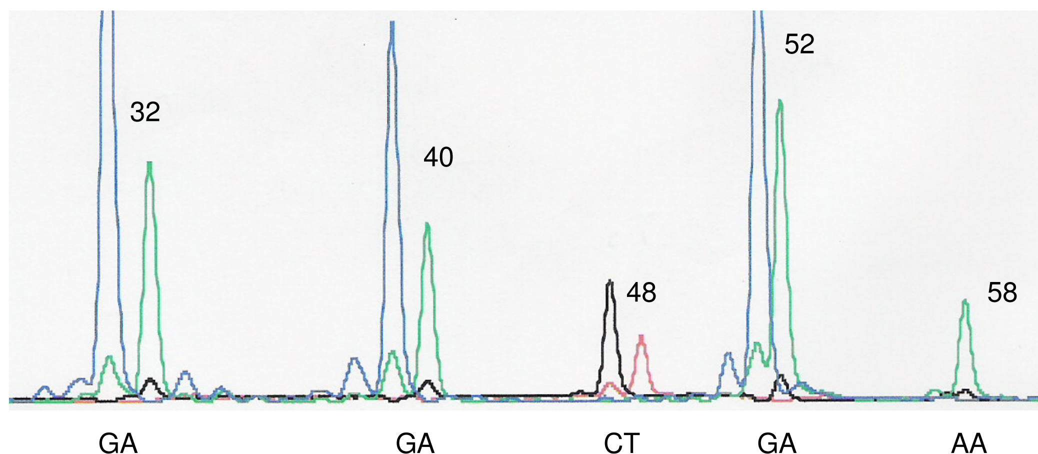

Figure 4.11 Family 2 individual's electropherogram of the 5 analyzed SNPs. The figure illustrates the nucleotide position and the genotype.

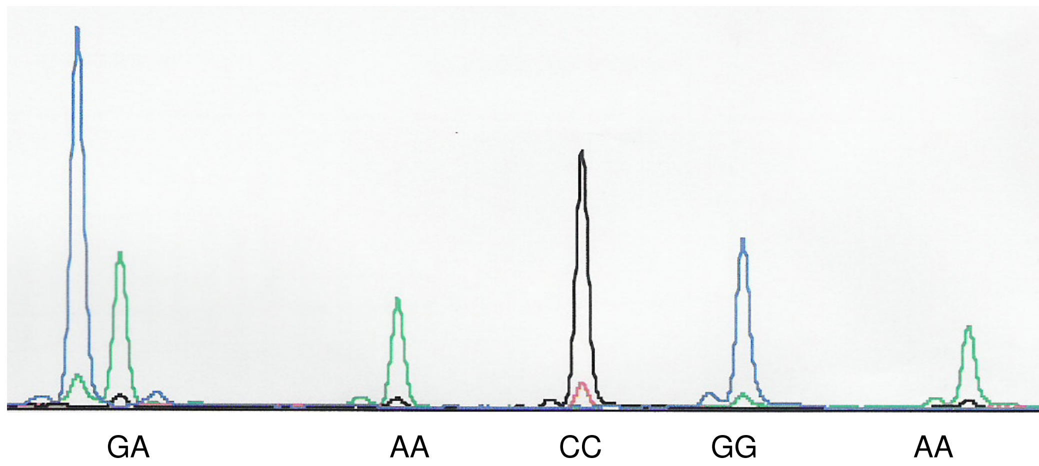

Figure 4.12 Family 63 individual's electropherogram of the 5 analyzed SNPs.

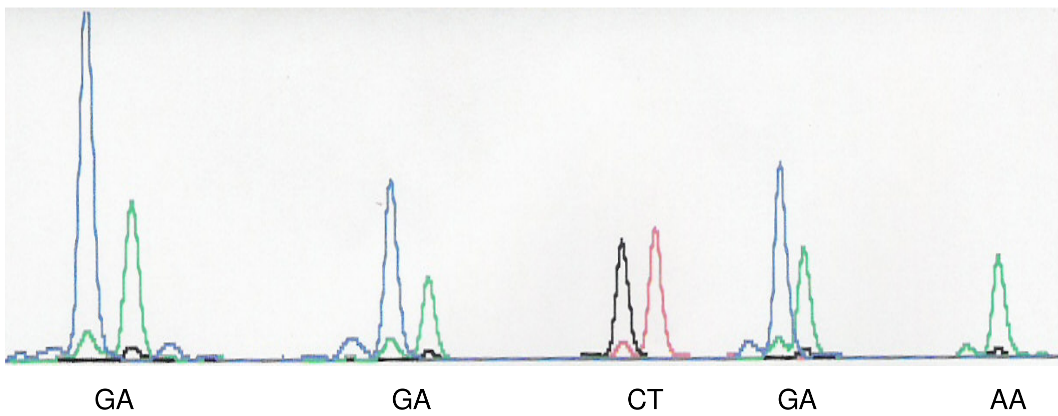

Figure 4.13 Family 51 individual's electropherogram of the 5 analyzed SNPs with the corresponding genotype.

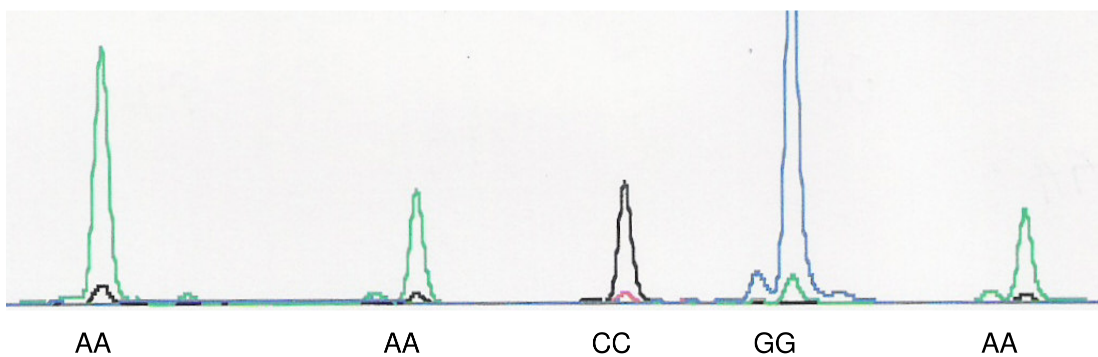

Figure 4.14 Family 105 individual's electropherogram

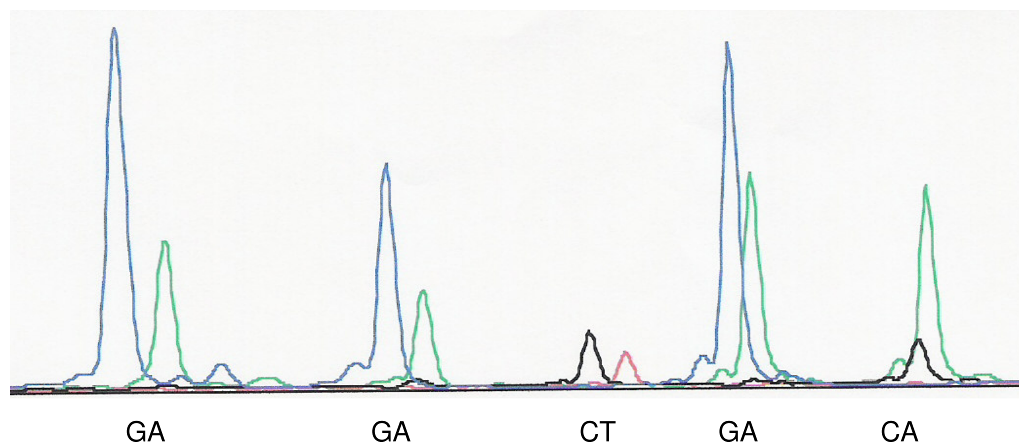

Figure 4.15 Family 65 individual's electropherogram

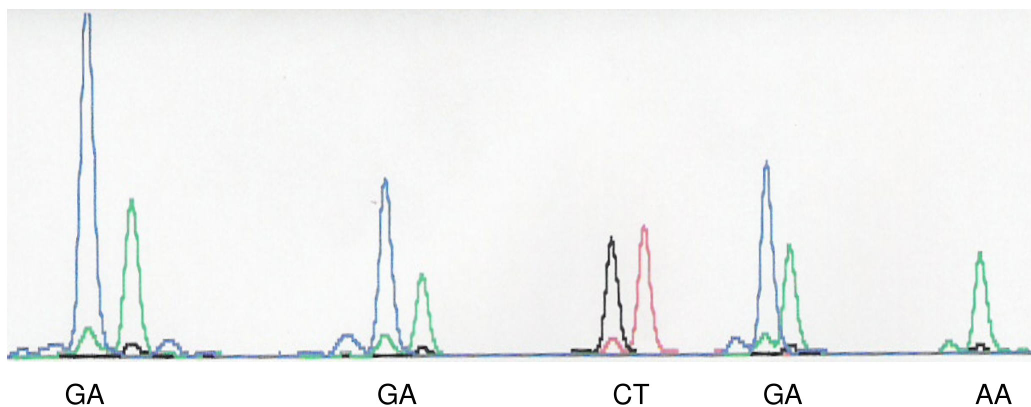

Figure 4.16 Family 106 individual's electropherogram

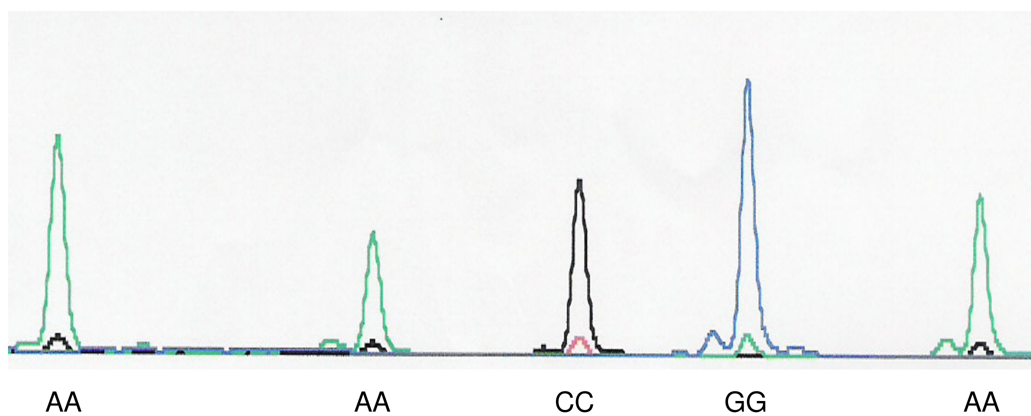

Figure 4.17 Family 79 individual's electropherogram

4.6 RESTRICTION ENZYME ANALYSIS RESULTS

SNPs C1 and C2 were analyzed through single PCR and Enzymatic Restriction. Beneath, are reported the two sequences for SNPs rs2247119 and rs2274276, with their forward and reverse primers in orange, and the point of the restriction site is market in green.

ATTGCTCAAAGCCCATTCCACACTTTTAGGATAGGGGATCAGACTGACCCAGCTTGGCATCAG
GGTTCCACCCAGGTCAATGAGTTATTAGAGGTCAGGATTACAAAGTATAAAACATGCCCGGAA
ACCAGGGTCATTCCCAGAGAAATGGGATGGCTTATGGTCTGCTCATCCACCCCCAAAGTTGCA
CTGTTTTACCCCCAGTCCATTTCCCACTCCTGGTTTGATTTTCCACTTTTGGTATGATTTTCTGA
CATGT/CAGCTTTCAATATTACTTTGTGCATACTCTGGATTTTTTTTTCCAATCTGCAGCTGTAT
CTTCAGGACTTGTGGAATGTGAGGATCAGGATCCACTTAATCCTGATAGAAGTTTTGATGTGG
AATCAGTAAAGAAAGAAATCCA

Figure 4.18 Sequence of SNP *rs2247119*. In orange are highlighted the forward and reverse primers, as well as the analyzed allele**.** The enzyme used in the restriction was *Fnu4 HI*.

TACAGATACTTCTTTAAATCAGTGTTTATTGACAGGGAAAAGCACCAGCAATACACACTTAACC
AAATCCTTGCAAATGTCATCTATTAAATATCTTCATCCTTATTAGTCTGTTTTACTTTGAATATCT
TCTGAGTGAAATTGAGTGCATTCCCATATCTTTTCACCAATTATATTTGTTTTCCTATGACCCAA
TTTGTTCATTTTTCTATTCAATGAACCCTCTCCCCAGAGAGTTCCG/CCATGTGCCAATTTTTCT
ACTCAATTATTTACCTGTTTTGCATTAAACTTATAATATCTTTTTTAAAAATTAACCCTTTATCATA
AGTGCTGCAAACACTTAGTTGAAGTTTGCCATATCTTTTGACTTTGTAAAAACTTTTGGCATATG
AGTTGTATATTTCATGTAGTCAAACAGTAAT

Figure 4.19 Sequence of SNP *rs2274276*. In orange are highlighted the forward and reverse primers, as also the analyzed allele. The enzyme used in the restriction was *Nsp I*.

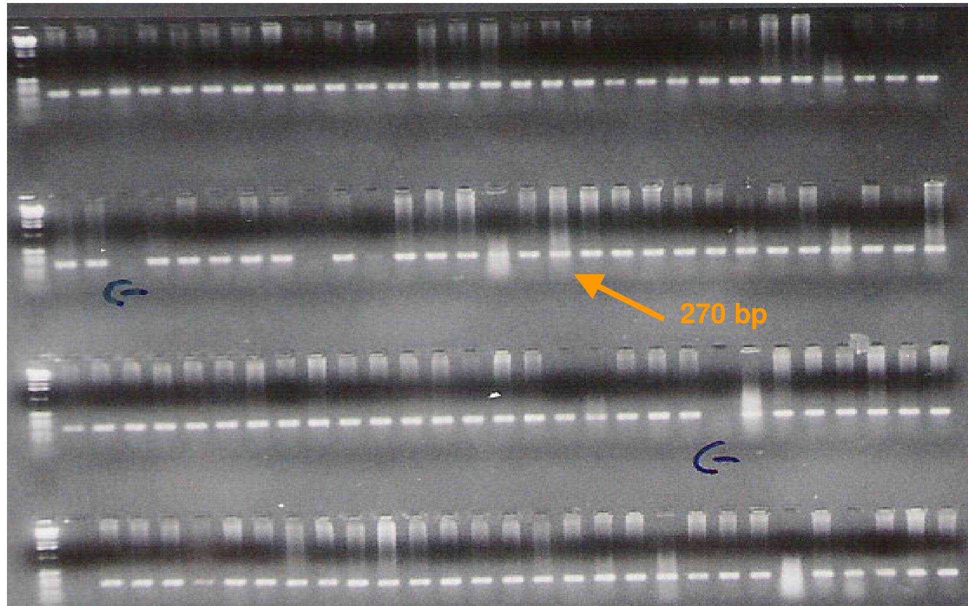

Figure 4.20

In **Figure 4.20** is illustrated the image of the PCR products for SNP *rs2247119* performed on all the asthmatic patients. The electrophoresis was completed on a 2% agarose gel, and the amplification fragment was 270 bp long.

Figures 4.21 and 4.22 illustrate rs2247119 T/C enzymatic restriction with *Fnu4 HI* enzyme, performed after PCR. The electrophoresis was completed on a 3.5% agarose gel. In lines 1, 2, 3, 4, are shown the normal homozygote patients eletrophoresis patterns (fragment 270bp); lines 5 and 6 show the affected homozygote patterns; and lines 7, 8, 9, and 10 show the heterozygote patterns (fragments 270bp and 235bp). In line 11, the 1kb molecular weight marker pattern, is present.

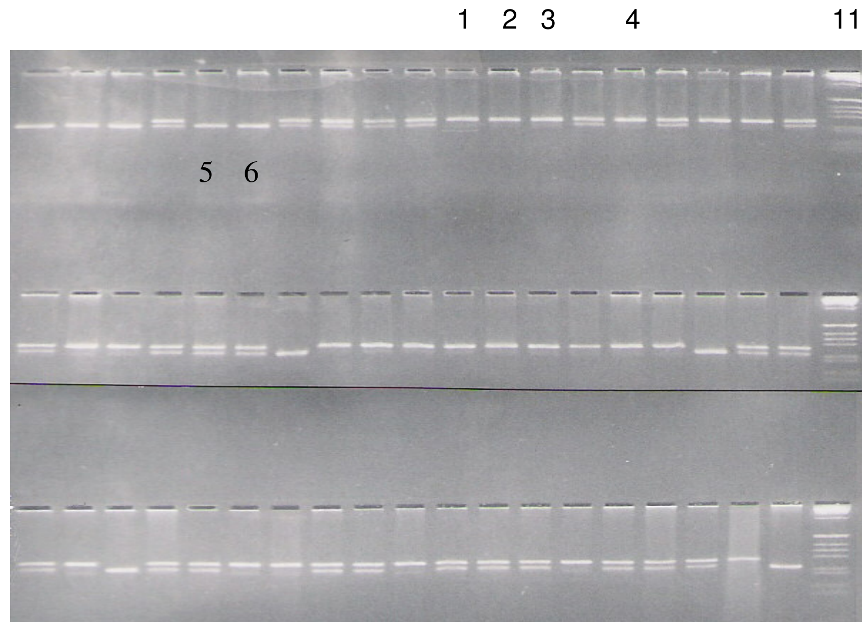

Figure 4.21

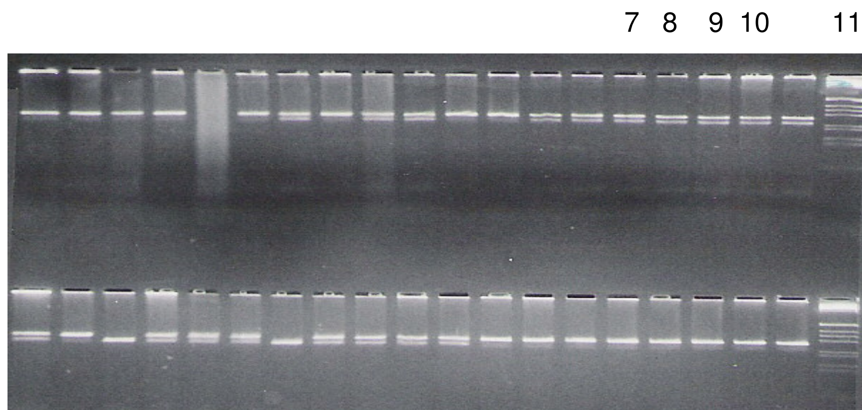

Figure 4.22

Figures 4.23 Illustrates a single PCR of SNP *rs2274276* performed on all the asthmatic patients. The electrophoresis was completed on a 2% agarose gel, and the amplification fragment was 243 bp long.

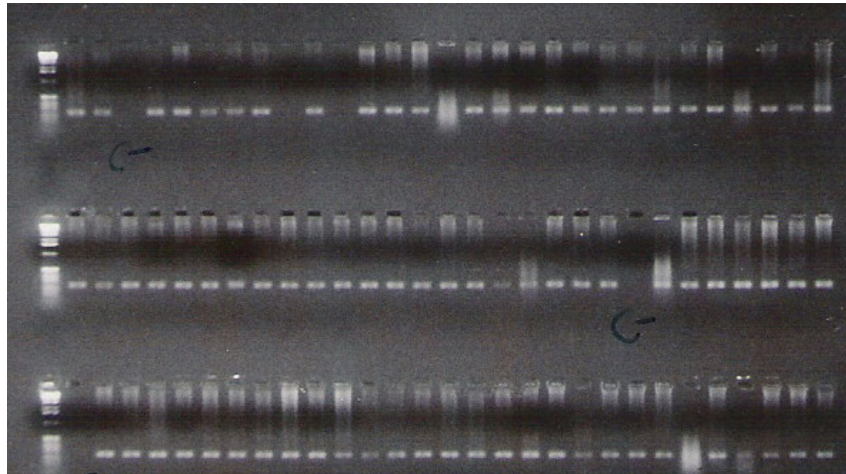

Figure 4.23

Figure 4.24 and **Figure 4.25** illustrate rs2274276 G/C enzymatic restriction with *Nsp I* enzyme, performed after PCR. The electrophoresis was completed on a 3.5% agarose gel. In lines 1, 2, 3, 4, are shown the affected homozygote patients eletrophoresis patterns (fragment 150 bp and 93 bp); lines 5, 6 and 7, show the normal homozygote patterns; and lines 8, 9, and 10 show the heterozygote patterns (fragments 243 bp, 150 bp, 93 bp).

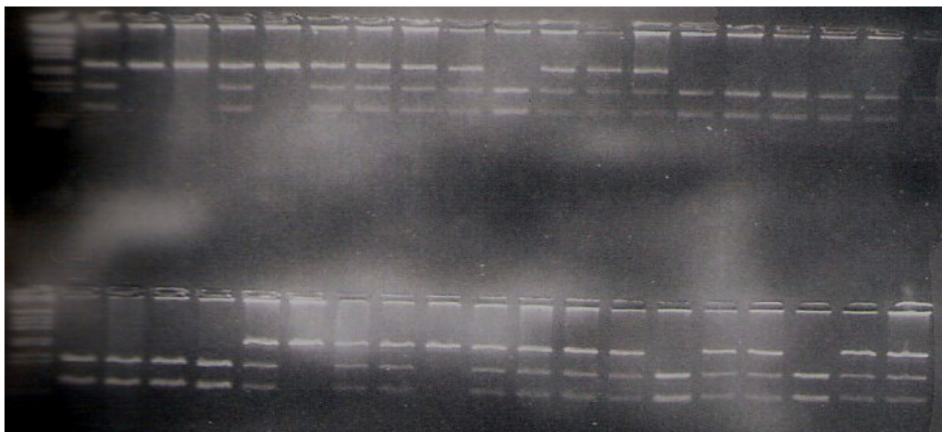

Figure 4.24

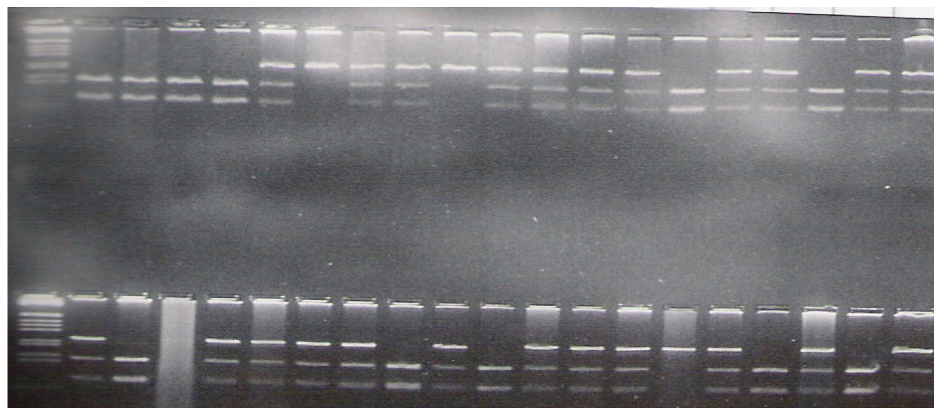

Figure 4.25

4.7 STATISTICS

Genotyping data were collected in a database and sent to the math laboratory for statistical analysis. Allele and genotype frequencies were calculated and the Hardy-Weinberg equilibrium was tested.

The allelic frequencies, calculated for the 46 founders of the analyzed population, are reported on **Table 4.4**.

SNP	ALLELE	FREQUENCY
rs2031532 G/A (B1)	G	37 %
	A	63 %
185752b4_2 A/G (B2)	A	46 %
	G	54 %
185752b5_2 C/T (B3)	C	39 %
	T	61 %
185306b7_1 A/C (A1)	A	41 %
	C	59 %
rs1046295 G/A (A2)	G	52 %
	A	48 %
rs2247119 T/C (C1)	T	30 %
	C	70 %
rs2274276 G/C (C2)	G	46 %
	C	54 %

Table 4.4

A non-parametric linkage analysis was completed for asthma, atopy, BHR, IgE, Rhinitis, and SPT phenotypes, using the Merlin program. The analysis results provided the p-value and the logarithm of odds (LOD). The results are reported on **Table 4.5.** As seen on the table, a significant p-value was observed only for atopy and IgE phenotypes, confirming previous finding of the presence of linkage with IgE for 13q14 region.

PHENOTYPE	LOD	p-value
Asthma	0.1	0.2
Atopy	2.03	0.001
BHR	0	0.6
IgE	1.78	0.002
Rhinitis	0.36	not significant
SPT	1.01	0.02

Table 4.5 The table illustrates the non-parametric linkage analysis results attained with the Merlin program in the asthma population sample (144 individuals) with the polymorphisms used. The LOD is the logarithm of odds; a LOD value equal or bigger than 3, indicates a linkage between the marker and the locus. A p-value equal or smaller than 0.05 indicates that the results are significant. These values were still significant after correction for multiple testing.

Subsequently, it was completed a qualitative association analysis with all the collected phenotypes and the analyzed SNPs through the TDT test. The analysis was performed on all the offspring (affected children 49 for asthma, 74 for atopy, 47 for BHR, 63 for IgE, 45 for rhinitis, and 66 for SPT) together for all the 7 markers analyzed. Then, it was carried out only on female offspring for all the 7 markers, and after only on male offspring for the 7 markers. The results of the qualitative association analyses are reported on **Table 4.6**

STATISTICAL ANALYSIS: TDT TEST			
PHENOTYPE	**All 7 markers All offspring**	**all 7 markers female offspring**	**all 7 markers male offspring**
Asthma	not significant	not significant	0.02 p-value marker 5
Atopy	not significant	not significant	not significant
BHR	not significant	not significant	not significant
Dust mites	not significant	not significant	not significant
IgE	0.09 p-value marker 5	0.07 p-value marker 5	not significant
Rhinitis	not significant	not significant	0.03 p-value marker 5
SPT	not significant	not significant	not significant

Table 4.6 Illustration of the results obtained from the TDT analysis. As seen on the table, IgE showed a 0.09 p-value on marker 5 for all offspring, a 0.07 p-value on marker 5 for female offspring. Asthma showed a 0.02 p-value on marker 5 for male offspring, and rhinitis showed a 0.03 p-value for male offspring. These values were not significant after correction for multiple testing.

Then, a qualitative analysis was completed on the haplotypes for all the 7 analyzed markers, with no significant data.

The analysis was also extended on all the markers for the 4 most common haplotypes (with a population frequency higher than 2%) on female offspring; reporting a 0.07 p-value for atopy, a 0.05 p-value for dust mites, and a 0.07 p-value for SPT. These values were not significant after correction for multiple testing.

A quantitative Ig analysis of the genotyped families did not show any significant association for single SNP or 7 markers haplotype analysis.

5. DISCUSSION

5.1 RESULTS HYPOTHESIS

Asthma is a multifactorial disease attributed to environment and multiple interacting genes, each contributing in the disease development, and having its own variable tendency to be expressed.

Asthma can be caused by an environmental allergen sensitization. Its interaction with the innate immune system, its uptake by antigen-presenting cells, and the subsequent T cell priming, leads to the stimulation of cytokines such as IL-4, IL-5, and IL-13. These cytokines interact with their receptors to stimulate IgE production and increase the numbers of eosinophils and mast cells, triggering inflammation in the respiratory tract.

Significant progress has been made in the field of asthma genetics in the past decade. Investigators have been studying the disease and its complexity, but still now it has been difficult to clearly determine the roles of the genes and the trigger factors associated with the disease expression.

Eleven full genomic screens reported chromosomes 1p, 2q, 3q, 4q, 5q, 6p, 7q, 11q, 12q, 13q, 14q, 15q, 17q, 19q, and 21q, to be associated to asthma and its phenotypes (Hoffjan et al., 2002).

Since our previous linkage of region 13q14 to total serum IgE levels and asthma; it was decided to further investigate this region in asthmatic patients from north east Italy.

Cookson reported in literature (Zhang et al, 2003) a positive linkage of region 13q14 to total serum IgE and related phenotypes. In the study, Cookson found 3 SNPs, rs1046295, in the 3' UTR zone; 185752b5_2, in intron 9, and 185752b4_2 in intron 5 (through TDT program analysis) of the PHF11 gene to have an independent effect in the preferential transmission of the allele associated to elevated IgE (rs1046295 with a 0.05 p-value; 185752b5_2 with a 0.0004 p-value; and 185752b4_2 with a 0.008 p-value).

In this study, the development of a multiple genotyping technique in a group of Italian asthmatic families, was set up and then used for the analysis of 5 SNPs (2 analyzed by enzymatic restriction) located on the PHF11 gene, in region 13q14.

The results of this study confirmed the presence of linkage with elevated total serum IgE levels in the region. The data observed from the analysis carried out with the Merlin program, showed significant associated p-values with atopy (0.001)

and elevated IgE (0.002) phenotypes. This indicates that the 13q14 region, as confirmed by the study of Cookson, is linked to total serum IgE levels, suggesting the possibility of the presence of a positive correlation.

On the other hand, the results obtained from the TDT analysis did not confirm association to asthma, as observed in the study of Cookson. Most likely this is because in the present study only 7 markers were analyzed, and perhaps it is necessary to analyze a wider number of markers in the PHF11 gene, extending the analysis in the neighbouring upstream and downstream genes, such as SETDB2 and RCBTB1. Also the number of the analyzed families was very small, even if they were selected for the presence of linkage, but in a bigger number of individuals, it would be simpler to find association. In fact, usually in wider analysis there is a larger probability to find positive and certain data.

Additionally, the association in the population, analyzed in this work, may possibly be altered by the presence of other polymorphisms in the PHF11 gene or in neighbouring genes, which may have had a small or modest effect on the analyzed disease. In fact, it has been observed in literature that consistency in replicated studies is difficult (Hakonarson et al., 2001). This suggests that there may be a wide range of variability in the allelic frequencies of these genes, and that the studied loci possibly could represent a false-negative/positive association or linkage.

It is important also to mention that environmental and lifestyle factors play a major role in atopy and asthma. Recent studies have discussed the so-called *hygiene hypothesis*, which states that the lack of intense infections in industrialized countries owing to improve hygiene, vaccination, and use of antibiotics may alter the human immune system such that it responds inappropriately to innocuous substances (Martinez, 2001). Besides, early life infections, which are considered negative, may instead have a protective effect on a later development of allergies.

In Germany, it was seen that children from Leipzig have an inferior and less common incidence of asthma, than children from Munich, suggesting that atopy and asthma are more common in westernized countries (von Mutius et al., 1992). Also, studies performed on farmers' children found that growing up on a farm confers significant protection against the development of atopy, allergic rhinitis,

and asthma (Von Ehrenstein et al., 2000; Braun-Fahrlander et al., 1999; Riedler et al., 2000; Ernst et al., 2000). This indicates that substances produced by farm animals, could play a role in the prevention of allergies.
The development of asthma could be possibly affected by the different lifestyles and the environmental factors that these people have experience during their childhood and later life.
Therefore, it is difficult to define a specific reason why in the present study the linkage with elevated total serum IgE, but not the association with the disease, was confirmed, since many factors could have had considerable importance.

5.2 THE ANALYSIS TECHNIQUE

The ddNTP Primer Extension technique was selected for the SNP investigation employed in the study, to test its efficiency and accuracy for future studies. The technique turned out to be very efficient in the analysis after the setting up procedure. In this study only 5 SNPs where analyzed with the technique and 2 with enzymatic restriction; definitely I suggest to analyzed more SNPs by minisequncing, the SNaPshot Multiple Reaction Kit can analyze up to 10 SNPs simultaneously. The disadvantages of the technique could be represented by procedure setting up. Finding the best condition for the multiplex PCR and minisequencing reactions require a lot of time and are a little expensive. But, once the best conditions are obtained, it is possible to test a large number of samples in a short period of time in an automatic way.
Overall, the ddNTP Primer Extension technique is an excellent method for large populations multiple SNP analysis. Moreover, in large investigations the costs will be amortized by the SNPs' number and samples analyzed.

5.3 CONCLUSION

In conclusion, 7 SNPs on the PHF11 gene, region 13q14, were analyzed in this study. The results, differently from what reported in literature, did not confirm association of elevated total serum IgE to asthma, but confirmed the presence of linkage with region 13q14.

The analysis suggests that other polymorphisms or genes may be involved, and can interfere with the association, as well as lifestyle and environmental factors may have considerable importance.

It may be appropriate to further extend the sample size and the analyzed region to other neighbouring regions.

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