Polymorphisms of Interleukin 13 (IL13) in

Local Asthmatic Population

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b. CERTIFICATE BY THE SUPERVISOR

It is hereby certified that thesis is based on the results of experiments carried out by *Dr Afia Hasnain* and that it has not been previously presented for *PhD* degree. Dr Afia Hasnain has done research under my supervision. She has fulfilled all the requirements and is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

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d. LIST OF ABBREVIATIONS:

A	Adenine
ACTH	Adrenocorticotrophic Hormone
AD	Accessory Device
AH	Airway Hyperresponsiveness
APC	Antigen Presenting Cells
Arg / R	Arginine
ASM	Airway Smooth Muscle
AVP	Arginine Vasopressin
BHR	Bronchial Hyperresponsiveness
BMI	Body Mass Index
Bp	Base Pair
BW	Body Weight
C	Cytosine
^{o}C	Centigrade
cM	Centi Morgans
Cm	Centimeters
CNS	Central Nervous System
COPD	Chronic Obstructive Pulmonary Disease
CRH	Corticotrophin Releasing Hormone
DNA	Deoxyribonucleic Acid
ECHRS	European Community Respiratory Health Survey

EDTA	Ethylene Diamine Tetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FcεRI	Fc Epsilon Receptor RI
FEV1	Forced Expiratory Volume In 1 Second
G	Guanine
GINA	Global Initiative For Asthma
Gln / Q	Glutamine
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HCl	Hydrochloric Acid
HPA	Hypothalamic Pituitary Adrenal Axis
ICS	Inhaled Corticosteroid
IgE	Immunoglobulin E
IL	Interleukin
IL-4Rα	Interleukin-4 Receptor α
ISAAC	International Study Of Asthma And Allergies In Childhood
IU	International Units
JIA	Juvenile Idiopathic Arthritis
Kb	Kilo Base
KCl	Potassium Chloride
kDa	Kilo Dalton
Kg	Kilograms
LTC4S	Leukotriene C4 Synthase
MDI	Metered Dose Inhaler

$MgCl_2$	Magnesium Chloride
MW	Molecular Weight
NCBI	National Center For Biotechnology Information
NIH	National Institutes of Health
OD	Optical Density
PCR	Polymerase Chain Reaction
PEF	Peak Expiratory Flow
RANTES	Regulated Upon Activation, Normal T-Cell Expressed, And Secreted
RBC	Red Blood Cells
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RSV	Respiratory Syncitial Virus
SEM	Standard Error Of Mean
SNP	Single Nucleotide Polymorphism
STAT	Signal Transduction-Activated Transcription Factors Family
STDEV	Standard Deviation
T	Thymine
T1D	Type 1 Diabetes
TBE	Tris Boric Acid EDTA
TH1	T-Helper Type 1
TH2	T-Helper Type 2
TMB	Tetramethylbenzidine

UN	United Nations
UTR	Untranslated Region
WHO	World Health Organization

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i. ABSTRACT

Asthma is a chronic inflammatory disorder characterized by wheezing, breathlessness, chest tightness and cough. The inflammation is responsible for bronchial hyperresponsiveness which renders the patient susceptible to certain environmental stimuli. The environmental factors alone are not responsible for these changes as they have an intricate interaction with genetic factors. In this study we determined the spectrum of symptoms and their correlation with serum IgE levels in asthmatic patients since no such data could be found for indigenous population. Cortisol and ACTH levels were determined to ascertain the status of hypothalamic pituitary adrenal axis. Interleukin 13 gene polymorphisms and their association with asthma and serum IgE levels were investigated. For the present study a detailed history was taken and peak expiratory flow measured on 164 asthmatic and 50 controls. Seventy five randomly selected patients underwent serology and SNP genotyping. Serum was analyzed for total IgE, cortisol and ACTH levels. Six SNPs of interleukin 13 gene were studied by PCR-RFLP. Four polymorphisms were from the promoter region C-1512A, T-1112C, A-646G and C-469T, one nonsynonymous polymorphism from the exon 4 A2044 G (Arg to Gln) and a 3'UTR polymorphism A2525G. The results of this research revealed that majority of patients belonged to moderate intermittent to severe persistent group. Exposure to dust was the most common triggering factor in our population. Serum IgE levels were directly proportional to the severity of asthma and were significantly correlated with history of allergy. However, no correlation was found between IgE levels and family history of asthma. Adrenal functions were

normal when compared with the control group. Two SNPs T-1112C and A2044G showed strong association with serum IgE levels and both the presence and the severity of asthma. C-1512A showed association with asthma and its severity and was the only polymorphism that showed an association with the family history of asthma. While A2525G had a weak association with serum IgE levels only. The other two polymorphisms were in very low frequency in our population and hence no association with phenotypes could be ascertained. It could be concluded from the present investigation that exposure to dust could be the most common precipitating factor of asthma in our population. The two previously studied SNPs T-1112C and A2044G are strongly associated with serum IgE levels and both presence and severity of asthma in our population sample.

1. INTRODUCTION

Asthma is defined as a chronic inflammatory disorder characterized by airway inflammation caused by many cells and cellular elements, in particular mast cells, eosinophils, T-lymphocytes, macrophages, neutrophils and epithelial cells [1]. This inflammation is usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation and airflow obstruction are responsible for the manifestation of asthma symptoms like recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or early in the morning in susceptible individuals. The inflammation is also responsible for an associated increase in the existing bronchial responsiveness to a variety of stimuli [2].

Asthma is considered to be the result of allergic inflammation leading to the production of immunoglobulin E (IgE). Allergens that enter the airway are presented by antigen presenting cells (APC) to the T cell which differentiates into T helper type 2 cells (TH2) in the presence of certain cytokines. TH2 cells secrete a number of cytokines including interleukin (IL)-4 and IL-13 acting on different target cells such as mast cells, eosinophils, epithelial cells, smooth muscle cells and lymphocytes [3]. The stimulated B cells start synthesizing IgE [4, 5]. All these changes in the airways in the form of inflammation lead to the airflow obstruction and thus asthmatic symptoms.

1.1 Epidemiology

According to World Health Organization (WHO), 300 million people suffer from asthma and this is expected to increase to 400 million by 2025. In the year 2005, estimated 255,000 people died of asthma worldwide. Morbidity and mortality associated with this disease are high despite available treatment that is effective in majority of the patients. Asthma accounts for one in 250 deaths worldwide [6].

In 2004, the Global Initiative for Asthma (GINA) conducted a study on the burden of asthma based on literature primarily published through the International Study of Asthma and Allergies in Childhood (ISAAC) and the European Community Respiratory Health Survey (ECHRS) [7]. According to this report an estimated 4.3% children suffer from asthma in Pakistan. These estimates might not be a true reflection of asthma prevalence in our population since majority of the cases are either not reported or the data is poorly managed.

1.2 Immunoglobulin E in asthma

Asthma and other allergic disorders like atopic dermatitis and allergic rhinitis are characterized by increased synthesis of IgE. Not only elevated levels of serum total IgE have been demonstrated to be associated with allergic asthma in several studies [8] but elevated cord blood IgE levels have also been linked to a predisposition to develop atopy in young children [9].

Antigen presenting cells present an allergen, in the form of an antigen, to naive T cells differentiating them into TH2 lymphocytes with the help of IL-4. TH2 lymphocytes are responsible for the release of a number of cytokines including IL-5 which is important for the recruitment and activation of eosinophils and IL-4/IL-13, both of which are critical to the production of IgE by B lymphocytes. IL-4 and IL-13 lead to the transcription and subsequent production of IgE in B lymphocytes [10].

Allergen-bound specific IgE binds to the high affinity IgE receptor, Fc epsilon receptor RI (FcεRI) mediating release of mast cell components resulting in increased mucus production and the constriction of airway smooth muscle thus starting infiltration of inflammatory cells. These events lead to an increase in the obstruction of airflow decreasing lung function [11].

1.3 Hypothalamic Pituitary Adrenal Axis in Asthmatics

The hypothalamic pituitary adrenal (HPA) axis in asthmatic patients is influenced by many factors. Stress, cytokines and exogenous corticosteroids play an important role in regulating the HPA axis and thus affecting the adreno corticotrophic hormone (ACTH) and cortisol levels among these patients [12-15]. It is well accepted that the HPA axis represents a major immuno-regulatory system that plays an important role in balancing the immune response especially under stressful conditions.

1.4 Role of Cytokines in Asthma

Cytokines and chemokines play a major role in mediating inflammation in asthmatic patients and have been found to be present in the broncho-alveolar lavage fluid in patients with asthma. These include elevated levels of TH2 type cytokines, such as IL-4, IL-5, IL-9 and IL-13. It has recently been shown that the imbalance between TH1 (interferon-γ and IL-2) and TH2 (IL-4, IL-5, IL-9, IL-13) lymphocytes is the fundamental underlying mechanism in asthma [4].

1.5 Interleukin 13

Studies conducted on these different cytokines and chemokines as mediators of asthma have shown that each of them has an important and specific role to play in the pathogenesis. Yet studies on IL-13 have emphasized a singular role for IL-13 in the regulation of allergic conditions. Several studies have reported an association between IL-13 genetic variants and asthma and related traits [16]. IL-13 has been shown to be consistently over expressed in the lungs of human asthmatics and the genetic polymorphisms in the IL-13 gene are considered to be major contributory factor to susceptibility to asthma and related traits.

Human IL-13 is a 17-kDa glycoprotein cloned from activated T cells [17]. The IL-13 gene is located on human chromosome 5q31 in what is known as the cluster of genes encoding IL-3, IL-4, IL-5, IL-9, and granulocyte-macrophage colony-stimulating factor (GM-CSF).

Genetic studies have demonstrated that multiple genes are involved in the development or the severity of asthma and related traits. The genes implicated in the pathogenesis of asthma have so far been found on chromosomes 5q31±33, 6p21.3, 11q13 and 12q14.3±24.1, and are considered to be major contributory factors to asthma phenotypes [18]. IL-13 gene has been the focus of many studies for the relationship between its variants and different asthma phenotypes [19-22].

It is important to identify the genetic polymorphisms in different geographic populations and ethnic groups since there are differences between populations in environmental exposure, their genetic background and in the analytical approaches between the various studies carried out in different populations. Such studies lead to a better understanding of gene-gene and gene-phenotype relationships in asthma and allergy. In addition , study of the variants not only offer a better understanding of the origin and mechanism of asthma but also identify novel diagnostic markers of susceptibility, severity and outcome. Hence, these variants can be important therapeutic targets and are suitable for patients requiring immunomodulatory treatments.

In the present study we described the spectrum of asthma and trigger factors specific to local population. Keeping in view the environmental conditions dust and smoke were expected to be the major trigger factors of asthma. Their serum total IgE levels were measured for the assessment of their atopy status and for comparisons with disease severity and family history of asthma. Serum IgE levels

were also used as one of the phenotypes for association with the polymorphisms studied. The status of hypothalamic pituitary adrenal axis was also investigated for a possible suppression by the inhaled corticosteroids taken by these patients. We sought to screen polymorphisms in the IL-13 gene, and evaluated the association of these variants with different phenotypes of asthma. The objective was to identify the IL-13 polymorphisms in local asthmatic population and compare them with a control group.

2. REVIEW OF LITERATURE

2.1 Asthma

Asthma and atopy are closely interrelated. Asthma is often preceded by atopy and there is a 10-20 fold increase in the risk of developing asthma in atopic individuals [23]. Individuals with atopic disorders have a genetic predisposition to produce IgE antibodies in response to common environmental allergens. Asthma, allergic rhinitis and atopic dermatitis are atopic disorders with increased IgE levels. A person becomes exposed to an environmental allergen typically in the first few years of life. Around 25-30% of these early sensitized individuals develop asthma later in life [24]. The association of asthma with sensitization to a particular environmental allergen mainly depends on the geographical distribution of the allergen. Specific allergens like house dust mites, animal dander, cockroach and pollen have variable distribution in different geographical populations [25]. The trigger factors for asthma have not been studied in detail in Pakistani population but the environmental pollution, exposure to dust, smoke and pollen are thought to be major contributory factors.

On the other hand patients with non atopic or intrinsic asthma present typically in adulthood with variable airway obstruction and reversibility of airway obstruction [26].

2.1.1 Asthma and Lung function

Asthma is characterized by two functional alterations, variable airway obstruction and bronchial hyperresponsiveness. The airway wall diameter in normal healthy individuals is dependant on its elastic properties and the tonicity of the smooth muscle in its wall, while the airway wall in asthmatic individuals is characterized by smooth muscle contraction, airway wall thickening, edema and increased secretion of mucus; all leading to airway narrowing [2].

The airways in asthmatics become over sensitive to certain stimuli leading to airway narrowing and obstruction. This over sensitivity of bronchi can be described as bronchial hyperresponsiveness (BHR).

Lung function in these individuals is assessed by performing forced expiratory tests and measuring the response of a specific stimulatory agent after inhalation. A comparison is made between the response by a healthy individual and an asthmatic with a resulting decrease in forced expiratory volume in asthmatics. Sustained stimulation of the airway shows a progressive decrease in lung function in asthmatics as compared to a consistent response by the normal airways [2].

2.1.2 Immunopathology of Asthma

Airway remodeling is an important feature of asthma in which there are structural changes in the cells and tissues [3]. These changes include thickening of all components of the airway wall, subepithelial fibrosis (thickening of the basement

membrane), deposition of collagen beneath the basement membrane, mast cell degranulation, and infiltration of the airway by lymphocytes and eosinophils [4]. The airway epithelium is thought to play an important role in airway remodeling and inflammation in asthma by producing cytokines and interacting with inflammatory cells [27, 28] and the extracellular matrix [29]. Their role in airway remodeling is further strengthened by the fact that IL-13 over expressing transgenic mice showed features consistent with immunopathological findings in asthmatic individuals [30].

As soon as an allergen enters the body it is encountered by the APC that are responsible for presenting the environmental allergens to T-lymphocytes. IL- 4 is responsible for differentiation of these T lymphocytes into TH2 cell which secrete a number of cytokines and inflammatory mediators. These include IL-4, IL-5, IL-9 and IL-13 acting on different target cells like epithelium, smooth muscles cell, neutrophils, basophils, eosinophils and mast cells [28]. These cytokines bind their respective receptors on the cell surface and act through the activation of transcription factors, such as nuclear factor-κB and members of the signal transduction-activated transcription factors family (STAT). The activation of transcription factors upregulate adhesion molecules, pro-inflammatory cytokines, chemokines and their receptors.

IL-4 is the major factor regulating IgE production by B cells, and is required for optimal TH2 differentiation. However, blocking IL-4 alone is not sufficient to

inhibit the development of asthma in experimental models. IL-5 is a key factor for eosinophilia and could therefore be responsible for some of the tissue damage seen in chronic asthma.

2.2 Epidemiology of Asthma

In 1989 the Global Initiative for Asthma (GINA) program was initiated with the U.S. National Heart, Lung, and Blood Institute, National Institutes of Health (NIH) and the World Health Organization (WHO) in an effort to raise awareness among public health and government officials, health care workers, and the general public about asthma [7]. In an effort to explore the worldwide prevalence of asthma, GINA in 2004 obtained data on the burden of asthma in 20 different regions worldwide from literature primarily published through the International Study of Asthma and Allergies in Childhood (ISAAC) and the European Community Respiratory Health Survey (ECHRS). The study regions have been grouped according to geographical, political, historical, and racial considerations based on official data from WHO, the United Nations (UN), and other sources, and to some extent, the availability of asthma epidemiological data within the study region.

According to this report asthma is one of the most common chronic diseases in the world. It is estimated that around 300 million people in the world currently have asthma. In the recent years asthma has become more common in both children and adults around the world. There is an increase in atopic sensitization

leading to increase in the prevalence of asthma and other allergic disorders such as eczema and rhinitis. A major part of this increase in asthma prevalence can be attributed to the western lifestyles adopted world wide and increasing urbanization. There is likely to be a marked increase in the number of asthmatics worldwide over the next two decades as the report shows an expected increase in urbanization of the world's population from 45% to 59% in 2025. It is estimated that there may be an additional 100 million persons with asthma by 2025.

It is estimated that asthma accounts for about 1 in every 250 deaths worldwide. Many of the deaths are preventable, being due to suboptimal long-term medical care and delay in obtaining help during the final attack.

According to this report an estimated 4.3% children suffer from asthma in Pakistan. These estimates might not be a true reflection of the asthma prevalence in our population since majority of the cases are either not reported or the data is poorly managed.

There is not enough data available on epidemiology of asthma in Pakistan. The studies that have been conducted in this population are either focused on the management of asthma or the knowledge, attitudes and perceptions of asthma patients towards the disease. In the year 2007 three studies were conducted, two were focused on the management and cost effectiveness of the treatment while one was related to the immigrant south Asian women suffering from asthma in

UK [31-33]. The report on management of asthma suggested that Metered Dose inhaler (MDI) with accessory device (AD) is an effective alternative to nebulizer for the treatment of children with acute asthma exacerbation in the emergency room [32].

Regarding the affordability of asthma treatment in Pakistan the study recommended that improving governance and management efficiency, and assessing local supply options, may improve availability [31]. A study on hospital based management of asthma reported deterioration in history and physical examination skills over a period of ten years, under use of peak flow readings, and poor pre-discharge instructions. Some aspects of improved care included frequent use of pulse oximeter, preference of inhaled over systemic bronchodilators and increased use of systemic steroids [34].

While a study on management of asthma by general practitioners showed that great majority of doctors were not aware of treatment options for persistent symptoms despite the use of preventive therapy (8% prescribed long-acting beta2-agonists, 6% high-dose inhaled corticosteroids and 13% theophyllines) and misconceptions about inhaler therapy and diet were found in majority of them [35]. Another study aimed at estimating the prevalence and identifying some risk factors of adult asthma in male leather tannery workers in Karachi showed high prevalence of asthma. The prevalence of asthma among this group was associated

with educational status, ethnicity, smoking, glove use, perceived to have allergy and duration of work [36].

2.3 Serum IgE levels in Asthma

IgE has been proven to play an important role in the pathogenesis of allergic disorders. Increased levels of total serum IgE have been implicated in the clinical expression of allergy and asthma [8, 37-39]. Serum IgE levels have been studied as the predictor of the severity of asthma and (BHR) bronchohyperresposiveness [8, 40, 41]. Several studies have been conducted showing serum IgE levels to be directly related to the severity of allergic symptoms. To better understand the precise mechanism of action and its role in the pathogenesis of allergic disorders several genetic studies have been conducted. The studies have identified a number of genes involved in the regulation of IgE and thus in the pathogenesis of allergic disorders [42].

IgE mediated mast cell activation results in the accumulation of leukocytes such as eosinophils and TH2 lymphocytes in the airway [43, 44]. TH2 lymphocytes are believed to play a crucial role in the allergic inflammatory response. They are responsible for the production of the inflammatory cytokines such as IL-4, IL-5 and IL-13 which have been linked to the asthmatic condition and inflammatory cell activation.

IgE has been identified as being closely associated in mediating hypersensitivity since its isolation and purification [45, 46]. Serum IgE concentrations in normal

individuals rarely exceed 150 IU/mL, being the least abundant antibody in human serum [47]. A good majority of patients with atopic dermatitis exhibit the extrinsic form of the disease with sensitization to environmental allergens and very highly elevated levels of IgE [48, 49]. Individuals with other atopic disorders have IgE levels up to 10 times greater than the normal individuals [50].

2.4 Hypothalamic Pituitary Adrenal Axis in Asthma

The hypothalamus is activated to secrete corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) by the central nervous system (CNS) in response to circadian stimuli [51]. In addition to stimulation by the circadian rhythm, other activators of the hypothalamus include neurosecretory and limbic signals as well as cytokines. The cytokines so far identified for their possible role in the stimulation of hypothalamus include IL-1, IL-6, and tumor necrosis factor-α (TNF α) [52]. The CRH and AVP thus secreted by the hypothalamus act synergistically on the anterior pituitary gland to secrete ACTH. This ACTH is responsible for increasing cortisol synthesis and release within 2-3 min [53]. The cortisol is synthesized only when there is demand in the body and very little amount of this cortisol is stored in the adrenals for future use. Thus, the cortisol concentration depends largely on the ACTH levels, decreasing when ACTH production is diminished. There is a negative feedback mechanism acting on hypothalamic and pituitary level, and possibly at higher centers in order to regulate the glucocorticoid secretion [53].

ACTH is secreted in a circadian pattern influencing the production and release of cortisol levels in the body. The levels are highest between 04.00 and 10.00 hours with a peak at 08.00 hours in the morning. Levels reach their lowest at midnight (00.00–03.00 hours). Episodic increases also occur at meal times [53, 54]. Negative feedback control ensures that the plasma cortisol concentration is kept at the appropriate level at all times.

This circadian rhythm and its negative feed back control mechanism can be disturbed by stress. In asthmatic individuals there is a possible dysfunction of the HPA axis leading to reduced levels of cortisol in response to psychosocial stress [55]. The role of HPA axis as a major immunoregulatory system in balancing the immune response especially under stressful conditions is well established. It has been proven by animal studies that an appropriate responsiveness of the HPA axis is necessary to control immunological processes, and to prevent an immune response from reaching a level that may damage the host [56, 57].

Cytokines have been shown to play an important role in the activation of HPA axis. So far three cytokines, TNF α, IL-1, and IL-6 have been described in HPA axis stimulating activity in plasma. TNF α is the first cytokine to appear followed by secretion of IL-1 and IL-6 [58-60].

The three cytokines stimulate their own secretion from the cells responsible for their secretion. In addition, they also regulate the stimulation and inhibition of

secretion of each other. TNF-α and IL-1 stimulate the secretion of IL-6, whereas IL-6 inhibits the secretion of TNF-α and IL-1 [61, 62].

These inflammatory cytokines are capable of activating the HPA axis independently or in combination [63-68]. They also mediate the stimulation of the HPA axis through bacterial lipopolysaccharides as it was proven by the use of antibodies against IL-6 completely inhibiting this effect [68]. IL-6 has also been shown to be capable of elevating plasma concentrations of corticotrophin and cortisol well above their normal range in humans.

Exogenous intake of steroids in the form of inhaled or oral steroids have been shown to alter the HPA axis. Cortisol production is inhibited at hypothalamic, pituitary and possibly at adrenal level in response to inhaled corticosteroids (ICS). The circadian rhythm of secretion of cortisol is disturbed and the total daily secretion of cortisol is reduced.

ICS have been in use for the treatment of asthma and other allergic disorders for the last three and a half decades. Their efficacy in controlling asthma symptoms and cost-effectiveness is well established world wide [69, 70]. Their role in controlling symptoms, reducing exacerbations, improving lung functions and quality of life is undisputed [71, 72]. Nowadays ICS are routinely being used in maintenance therapy in the management of asthma.

Concerns about side effects have been raised, but they have been proven to be efficacious and out weigh the risk of inadequately controlled asthma [73, 74, 75]. Suppression of the hypothalamic-pituitary adrenal axis (HPA) is a benign physiological response to exogenous corticosteroids [13, 14, 15]. According to one of the recent study survey, 2% of health care givers reported at least one case of adrenal crisis associated with ICS [76]. Yet another case series reported that the use of regular dose of budesonide with a MDI and a nebuhaler for 1 year was enough to cause symptomatic adrenal suppression [77].

2.5 Genetics of Asthma

Asthma is a complex genetic disorder in which the mode of inheritance does not follow the classical Mandelian patterns and thus cannot be classified as autosomal, dominant, recessive or sex-linked. Genetic factors alone are not responsible in the pathogenesis of asthma but there is a close interaction with the environmental factors.

Genetic studies conducted on asthmatic individuals have shown that chromosome 5 houses a major susceptibility locus for asthma and high IgE levels. Linkage and association studies were conducted for genotype-genotype and genotype-phenotype relationship. Genotype-genotype studies have found linkage between many cytokine genes and their receptor. Chromosome 12 has been found to be an important susceptibility locus for asthma containing potential candidate cytokine genes, including the gene encoding interferon γ, the prototypical TH1 cytokine

with inhibitory activities for TH2 lymphocytes. Genome wide studies point to chromosome 5, 6, 11 and 12 as major loci and TH2 cytokines, such as IL-4, IL-13, IL-5, and IL-9, as important targets for therapeutic applications in patients with asthma.

2.5.1 Chromosome 5q

Chromosome 5q31-q33 houses numerous candidate genes for asthma and atopy, such as a cluster of cytokine genes IL-3, IL-4, IL-5, IL-9, IL-13, CD-14, the β-chain of IL-12 and the genes coding for the β2- adrenergic receptor, the granulocyte macrophage colony stimulating factor and the corticosteroid receptor.

First study reporting a linkage between total serum IgE levels and chromosome 5q in US Amish population was conducted in 1994 [78]. Meyers et al. in the same year reported linkage between chromosome 5 and serum IgE levels among Dutch families [79]. Postma et al. in 1995 showed a linkage between airway hyperresponsiveness (AH) to histamine the same regions of chromosome 5q as serum total IgE. Thus pointing to a region of high susceptibility for airway hyperresponsiveness and serum IgE levels located on chromosome 5 [80]. Similar studies reporting a positive association between chromosome 5 and asthma were conducted in Japanese [81], British [82] and US [83, 84] populations also implicating chromosome 5q as a region containing one or more susceptibility genes for asthma. However, there are reports of no linkage found between

chromosome 5 and asthma or atopy from studies carried out in Australian [85], Finnish [86], British [87], German [88] and four US families, [89].

2.5.2 Chromosome 11q

Linkage of atopy on chromosome 11q was first reported by Cookson et al. in 1989 [90]. They looked for a linkage with either elevated serum total IgE, raised allergen specific IgE or the presence of one or more positive skin prick tests.

A study conducted in Netherland on 26 sib-pairs, linkage was found between 11q and asthma and atopy defined as the presence of two respiratory symptoms and elevated specific or serum total IgE levels [91]. A German study reported linkage between 11q and a clinical history of atopy in atopic dermatitis patients and an elevated serum total IgE level [92]. A Japanese study showed linkage between 11q and severe atopy in four selected families [93].

An Australian study showed no linkage between chromosome 11q and atopy, however, AH to methacholine appeared to be linked to 11q [94]. Several studies reported no linkage between this chromosome and asthma and related traits [95-101].

2.5.3 Chromosome 12q

Chromosome 12q is another region widely studied for linkage and association with asthma and atopy, because of several candidate genes, including interferon-γ (an inhibitor of IL- 4 production by TH2 lymphocytes), a mast cell growth factor,

and the ß subunit of nuclear factor-Y which possibly upregulates transcription of IL-4. Two different populations, an Afro-Caribbean population from Barbados and Caucasian Amish from Pennsylvania, USA, were studied for linkage between chromosome 12 and asthma and elevated serum IgE levels.

This study provided evidence for linkage and association to this chromosomal region for both elevated total serum IgE (Barbados and Amish) and for physician diagnosed asthma (Barbados) [102]. A similar study was conducted in German population for linkage of high serum IgE levels to 12q15-q24.1, showing a positive linkage between the two [103]. Further studies in British and the Hutterites in the USA reported linkage of this chromosome to asthma and high IgE levels [91]. Further studies are needed to ascertain a role of 12q in asthma and atopy as the regions of chromosome 12 that were studied were different in different populations.

2.5.4 Genome-Wide studies

Several genome-wide studies on asthma and atopy have been carried out so far to find out other chromosomal regions implicated in the pathogenesis of asthma and atopy. The goal of a genome-wide search is to detect unknown regions of interest for asthma and atopy. The advantage of such a study is that new regions with susceptibility genes can be identified. But it is equally important to replicate these studies in different population so that the role of that particular gene in the pathogenesis of the disease can be ascertained.

The first genome wide study was conducted by Daniels et al in 1996 to look for asthma susceptibility loci was conducted in an Australian and British sample. This study reported linkage on chromosome 4 and 7 for AH, on chromosome 6 for eosinophilia, chromosome 11 for skin test positivity and elevated serum total IgE levels and on chromosome 16 for elevated serum total IgE levels [104].

A collaborative study was carried out by CSGA in 1997 to conduct a genome-wide search in US population with 140 asthma families in which two or more siblings were affected with asthma. The study population comprised of three different racial groups, namely Hispanics, Caucasians and Afro- Americans [83]. This study concluded different regions being linked to these three racial groups. These regions included chromosome 5p and 17p for African Americans; 11p and 19q for Caucasians and 2q and 21q for Hispanic population for susceptibility to asthma and related traits.

Another genome-wide search was conducted by Ober et al in 1998 in the Hutterites, a homogeneous group of population originating from less than 90 ancestors. They divided the asthma phenotype for this study in two broad groups namely strict asthma and loose asthma. Those belonging to strict asthma group comprised of individuals with AH to methacholine and reported asthma symptoms and were linked to chromosome 19q and 21q. The other group with loose asthma had either AH to methacholine or reported asthma symptoms.

Regions linked to this group were 5q, 3p and 12q. The interesting finding was that multiple susceptibility genes were found even in a homogeneous population such as the Hutterites [84].

A genome wide screen for asthma susceptibility was performed in German families with asthma. In this study the phenotype was defined by clinical history and supported by questionnaire data of a history of at least 3 years of recurrent wheezing in children over age 3. The study concluded linkage at chromosome 2p, 6p, 9q and 12q. These regions were further compared with other phenotypes of asthma and atopy like AH, specific and total serum IgE, and eosinophilia. Chromosome 2 showed linkage with AH to methacholine, and elevated serum specific and total IgE levels. Chromosome 6p was linked to elevated total and specific IgE and eosinophilia while chromosome 9q and 2q showed linkage for elevated total and specific IgE [18].

Another study was conducted by Meyers et al in 2005 to carry out a genome-wide linkage screen for asthma and BHR and to determine the influence of passive tobacco smoke exposure during childhood on the results of genetic linkage studies to investigate gene-environment interactions. The study showed that strongest evidence for linkage was observed for chromosomes 3p and 5q [105].

Kurz et al in 2007 carried out a genome wide scan on Hutterites and an out-bred case control from German population. These results suggested that a broad region

on 5p, separated by >9 Mb, harbors at least 2 and possibly 5 asthma or BHR susceptibility loci [106].

These genome wide studies have further emphasized the role of chromosome 5q, 11q and 12q in the susceptibility for asthma and atopy. The replication of such studies in different geographic and ethnic populations is important since it not only confirms the role of a certain region or gene in the pathogenesis but also help identify the false positives. But comparison between different studies conducted on different population groups is difficult. The main problem in such a comparison is the definition of the phenotype by the investigators. Some studies rely on questionnaire based information while some perform serological tests or skin prick tests or a combination of these for the description of their phenotype and hence there remains no uniformity in these studies. Thus it is sometimes difficult to draw a conclusion from such studies, if the linkage was actually there or was just a false positive result due to poor description of the phenotype.

Another reason for varying results can be high degree of genetic heterogeneity. Different genes are implicated in different populations; and each of these genes is sufficient to express the phenotype.

Therefore, it is important that linkage studies are replicated by different investigators in different populations of sufficient size. Thereafter, confirmed regions can be studied in detail and candidate genes can be detected.

2.6 Role of IL-13

The genome wide studies pointed out some regions of interest for susceptibility to asthma and atopy and the studies were narrowed down to individual genes and their role in linkage and association with the phenotype. Several genes coding for cytokines, chemokines, inflammatory mediators, their receptors and ligands were screened for a possible association. Although each of the TH2 cytokines implicated likely contributes to immunopathological response directed against environmental antigens, a substantial body of evidence points to a singular role for IL-13 in the regulation of the allergic conditions. IL-13 tissue levels were shown to be associated with its genetic variants in individuals with asthma and atopy [16]. Recent studies have shown that genetic polymorphisms in the IL-13 gene may contribute to susceptibility to asthma and related traits.

2.6.1 IL-13 Structure and Location

Human IL-13 is a 17-kDa glycoprotein cloned from activated T cells [17]. The IL-13 gene is located on human chromosome 5q31 in the cluster of genes encoding IL-4, IL-3, IL-5, IL-9, and granulocyte-macrophage colony-stimulating factor (GM-CSF). The gene is composed of 2937 bp DNA and transcribes into a 1287 bp mRNA. The gene has four exons (Figure 1).

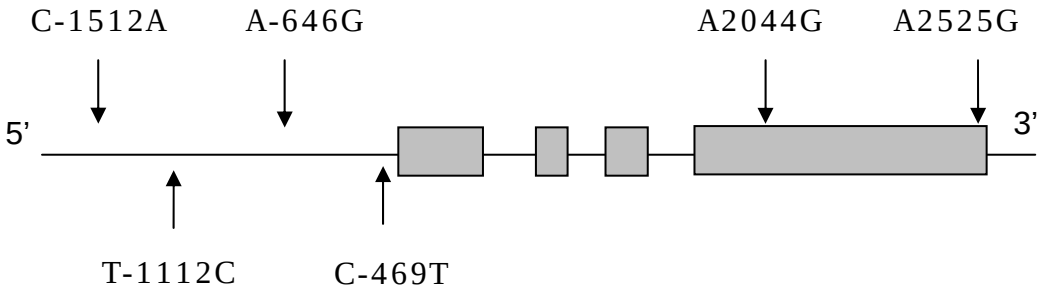

Figure 1: A map of IL-13 gene showing the 5' promoter region, 3' untranslated region (UTR) and the location of six polymorphisms studied.

The IL-13 and IL-4 genes are located on the same chromosome, in the same orientation and IL-13 being only 25 kb upstream to IL-4. They are thought to have originated se as a duplication event during evolution. Both of these proteins show many structural and functional similarities. The functional similarities between the two can be attributed to the fact that they share a receptor chain in their individual multimeric receptor complexes [107].

2.6.2 Functions of IL-13

IL-13 is predominantly produced by TH2 CD4+ T cells with small amounts being produced by a variety of cell types including both TH1 CD4+ T cells and CD8+ T cells. Akbari et al. [108] have recently demonstrated that natural killer T cells may also be an important source of IL-13 early in the allergic response. Several other studies conducted have shown that it is also produced by numerous other non-T-cell populations that are of particular importance to the allergic response such as mast cells, basophils, and eosinophils [109].

Once produced this cytokine acts via a complex receptor system located on cells namely B cells, monocytes/ macrophages, dendritic cells, eosinophils, basophils, fibroblasts, endothelial cells, airway epithelial cells, and airway smooth muscle (ASM) cells. The receptor complex is composed of the IL-4 receptor α (IL-4Rα) chain and at least two other IL-13 binding proteins that have been designated as IL-13Rα1 and IL-13Rα2 [110-112]. This heterodimeric complex formed by the IL-4Rα chain and the IL-13Rα1 chain is thought to constitute the functional IL-13 receptor. In cells lacking the common γ chain, this receptor complex serves as an alternative receptor for IL-4 (type II IL-4 receptor). The ability of both cytokines to bind this complex explains the similarities in function observed between IL-4 and IL-13.

IL-13 plays an important role in the immunopathogenesis of asthma, such as its well known roles in the regulation of IgE synthesis and induction of adhesion molecule and chemokine expression [113].

IL-13 functions were blocked by the administration of a soluble form of the IL-13Rα2 chain, which specifically binds to IL-13 and not IL-4. The allergen-challenged mice showed a reversal in airway hyperresponsiveness (AHR) and mucus production. Recent studies have shown that IL-4 is essential for the initiation of TH2 polarized immune responses to allergens, while IL-13 alone may mediate the main physiological consequences of disease, namely airway AHR, mucus hypersecretion, and subepithelial fibrosis [16, 114].

2.7 Interleukin 13 gene Polymorphisms

The evidence to date suggests that genetic variants of the IL-13 gene and/or its signaling components contribute at least in part to genetic susceptibility to allergic airway disease.

Genetic studies have demonstrated that multiple genes are found to be involved in the pathogenesis of asthma or related traits. At least four regions in the human genome, chromosomes 5q31±33, 6p21.3, 11q13 and 12q14.3±24.1, contain genes consistently found to be associated with asthma and its related phenotypes [19]. Several studies have reported associations of polymorphisms in the IL-13 gene with various features of the asthmatic phenotype [20-23].

To date, 31 single nucleotide polymorphisms have been identified in the human IL-13 gene. Most of these polymorphisms are in the 5' regulatory region or in the non coding intron region of the gene. So far only one nonsynonymous SNP has been identified which is located in the Exon 4 of the gene and changes the amino acid Arg to Gln at the position 2044. Functional studies have revealed the importance of location of some of these SNPs in the regulation of expression of IL-13 gene. The most widely studied SNPs are a promoter polymorphism T-1112C and a nonsynonymous polymorphism located in exon A2044G.

The results of these studies conducted in different geographic and ethnic populations vary since there are differences between populations in environmental

exposure, genetic differences and differences in analytical approaches between the various investigators.

The studies conducted in relation to asthma focused on finding out a possible relationship between polymorphism and the asthma phenotypes. The most widely studied phenotypes of asthma were the presence of asthma itself, Serum IgE levels and skin test positivity.

van der Pouw Kraan in 1999 studied a promoter polymorphism T-1112C for an association with allergic asthma [19]. They found it to be associated with allergic asthma (P= 0.002), altered regulation of IL-13 production (P=0.002), and increased binding of nuclear proteins to this region. Based on these results they postulated that the presence of this polymorphism predisposes to the development of allergic asthma.

Heinzmann et al in 2000 [21] conducted a study on more than 200 atopic subjects to identify a single variant A2044G in the terminal coding region of the gene for human IL-13 that results in a predicted amino acid change in residue 110 (Gln110Arg). They conducted case control studies in a British population (150 young adults with asthma and atopy, and 150 controls) and a Japanese population (100 young adults with asthma and atopy, 100 subjects with non-atopic asthma, and 100 controls). In the British population, the variant was significantly associated with asthma (P = 0.014), whereas it was associated with both atopic

asthma (P = 0.033) and non-atopic asthma (P = 0.047) in the Japanese population. When compared with the serum IgE levels, interestingly, they did not find an association between this genotype and serum IgE levels in either population (British, P = 0.10; Japanese, P = 0.508). These results suggest that SNP is associated with asthma rather than atopy.

In the year 2000, Graves et al., [20] conducted a study in which they associated a cluster of seven SNPs, out of which 6 were novel, with serum IgE levels in children from three different populations. They found a strong correlation between the A2044G (Arg130Gln) and serum IgE levels and the rest of the polymorphisms studied were in strong linkage disequilibrium with this SNP. In a subsequent study they assessed the relationship between total serum IgE and Arg130Gln and the two polymorphisms in the IL-13 promoter (–1112 and –1512) in three separate study populations of children (Tucson, P = 0.0023; Leipzig, P = 0.0081; Munich, P = 0.0069); when all subjects were considered, they found stronger associations in subjects with a negative skin test.

Another study conducted in the year 2000 in a Dutch population by Howard et al., IL-13 gene was sequenced in 20 probands and 20 unaffected spouses, and 10 polymorphisms were identified, four novel and six previously reported. Three SNPs were detected in the 5'-promoter region, two in intron 1, and five in exon 4. Only one of the exon 4 SNPs resulted in an amino-acid change (Arg130Gln). They analyzed three SNPs in IL-13 in an extended group of 184 probands and

their spouses: one in the promoter region 5' T-1112C, the Arg130Gln (A2044G), and a 3' untranslated region SNP. The most significant associations were observed to asthma (P = 0.005), bronchial hyperresponsiveness (P = 0.003), and skin-test responsiveness (P = 0.03) with T-1112C. These results provide evidence that variation in the IL-13 gene is involved in the pathogenesis of asthma and atopy [115]. Further investigation is required to determine which specific alleles or combination of alleles contribute to these phenotypes, and the possible downstream effects of the resulting change in IL-13 levels or activity.

There is enough evidence to prove the role of these polymorphisms in the regulation of asthma and at the same time there are studies showing contradictory results. These are in part due to differences in their genetic and environmental background and partly because of the different methods employed for the analysis of these polymorphisms. One major factor can be the difference in defining the phenotypes on the basis of history, examination and/ or specific tests.

The polymorphism A2044G when studied in a Chinese population by Leung et al in 2001 showed that it was associated with atopy but not with the presence of asthma in patients. They studied one hundred and fifty-seven patients and 54 control children. The polymorphism in the IL-13 gene was associated with elevated serum total IgE phenotype as well as sensitization to house dust mite, dog and cockroaches, but not the development or severity of asthma, in Chinese children [116].

Celedon et al in 2002 found no significant linkage between the polymorphism A2044G and the presence of asthma, serum IgE levels, or sensitization to allergens in a Costa Rican population [117].

Gene-gene interaction studies were conducted in which the combined effect of polymorphisms in IL-13 and its neighboring genes were conducted. Most of the studies investigated the role of IL-4, a neighboring gene with similar function and a shared receptor, in relation with IL-13. The receptor IL4Rα which is shared by IL-4 and IL-13, has been studied extensively along with other mediators and signaling molecules such as STAT 6, RANTES (regulated upon activation, normal T-Cell expressed, and Secreted) and LC4S (Leukotriene C4 synthase).

A study conducted in Germany in 2003, identified associations between total serum IgE levels and the 6 potentially functional variants within the genes IL-4, IL-13, and IL-4Rα. They found significant associations between increased total serum IgE levels and 2 variants in the IL-13 gene ($P < 0.005$ and 0.0002 for A2044G and T-1112C, respectively). These findings suggest that variants T-1112C and A2044G of the IL-13 gene play an important role in total serum IgE production in this study population [118].

Another study conducted in Korea in 2006, reviewed the possible role of IL-13 polymorphisms along with an IL-13 receptor SNP. Their findings indicated that

the IL-13 A2044G is associated with asthma development and the IL-13 and IL-13Rα1 polymorphisms may interact to enhance IgE production [119].

A study conducted in Germany in 2006 analyzed combined extended haplotypes involving IL-4, IL-13, and their shared receptor chain IL-4Rα, and the intracellular signal transducer and activator of transcription, STAT6, to assess the combined effect of single nucleotide polymorphisms in this important immunological signaling pathway. They concluded that only the combined analyses of genetic alterations in the IL-4/IL-13 pathway reveal its actual significance to the development of atopy and childhood asthma [120].

Another study looked into the combined role of IL-13, RANTES, and leukotriene C4 synthase (LTC4S) gene promoter polymorphisms with asthma and/or atopy in African Americans in the year 2005. They suggested that IL-13 and LTC4S SNPs can be used as predictive markers for asthma/atopy in African Americans [121].

Howard et al in 2002 looked at gene-gene interaction between IL-4Rα and IL-13 genes in association with asthma. Their results suggested that variations in IL-4Rα contribute to elevated total serum IgE levels, and interaction between IL-4Rα and IL-13 markedly increases an individual's susceptibility to asthma [122].

IL- 13 has been implicated in the pathogenesis of many other diseases besides asthma. The polymorphisms in IL-13 gene have widely been studied in context of other diseases.

Homma et al in 2006 conducted a study in which they found an association between the IL-13 polymorphism A2044G and chronic obstructive pulmonary disease (COPD) in Japanese population. They concluded that this polymorphism maybe useful in predicting susceptibility to COPD [123].

Heinzmann et al in 2003 conducted an association study of the IL-13 variant A2044G in atopic diseases and juvenile idiopathic arthritis (JIA). The results showed no association of the variant with JIA when compared with the control population. However, the variant was significantly less frequent in children with JIA compared with its presence in children with bronchial asthma. The results suggest that the same gene variant might protect from one disease and make an individual susceptible to the other [124].

Wang et al in 2003 studied an IL-13 SNP A2044G among Chinese atopy patients with allergic rhinitis. The results suggested a possible involvement of IL-13 SNPs in the regulation of IgE production in response to allergens in this Chinese population [125].

A study conducted in Filipino population by Bugawan et al in 2003, for an association and interaction of the IL-4R, IL-4, and IL-13 Loci with Type 1 Diabetes (T1D). Their data suggested that the risk for T1D is determined, in part, by polymorphisms within the IL-4R locus, including promoter and coding-sequence variants, and by specific combinations of genotypes at the IL-4R and the IL-4 and IL-13 loci [126].

Recently, there has been a focus on the functional relevance of these variants in association with asthma and related phenotypes. Arima et al in 2002 studied the functional properties of the A2044G (Arg130Gln) variant. They generated two types of recombinant IL-13 proteins, the amino acids of which at position 130 were arginine or glutamine, and analyzed the binding affinities with the IL-13 receptors, as well as the stability of the proteins. They further compared the relationship between the genotype and serum levels of IL-13. The variant showed a lower affinity with the IL-13 receptor alpha2 chain, a decoy receptor, causing less clearance. The variant also demonstrated an enhanced stability in both human and mouse plasma. The patients homozygous for the Gln110 variant showed high serum levels of IL-13. These results suggested that the variant might act as a functional genetic factor of bronchial asthma with a unique mechanism to upregulate local and systemic IL-13 concentration in vivo [127].

Another study looked into the functional consequences of the Q110 IL-13 variant in vitro and in vivo to determine whether it displays enhanced functional activity

compared with R110 IL-13, both in the context of an IL-4R alpha SNP (I50Q551) and of the atopy-associated variant IL-4R alpha SNP (V50R551). Either Q110 IL-13 variant or V50R551 IL-4R alpha variant has enhanced function alone, but the 2 together have a synergistic effect on IL-13-dependent gene induction [128].

Table 1: IL- 13 SNPs and their association with phenotype

	SNP	**Publication/Year**	**Ethnicity/ Population**	**Associated phenotype**
1.	A2044G (Arg130Gln)*	Hunninghake et al., (2007) [129]	Costa Rican / White Hispanics	Eosinophilia and high IgE levels
2.	A2044G (Arg130Gln)	Lopez et al., (2007) [130]	Mexican	No association
3.	A2044G (Arg130Gln)	Kim et al., (2006) [119]	Korean	Asthma
4.	A2044G (Arg130Gln)	Xi et al., (2004) [131]	Chinese	IgE
5.	A2044G (Arg130Gln)	Hoffjan et al., (2004) [132]	American	Increased IgE levels in infancy
6.	A2044G (Arg130Gln)	Liu et al., (2004) [118]	German	Serum IgE
7.	A2044G (Arg130Gln)	Wang et al., (2003) [125]	Chinese	High IgE levels

8.	A2044G (Arg130Gln)	Bugawan et al., (2003) [126]	Filipino	Type 1 diabetes
9.	A2044G (Arg130Gln)	Heinzmann et al., (2003) [124]	German	Serum IgE, weakly with asthma and not with juvenile idiopathic arthritis
10	A2044G (Arg130Gln)	Tsunemi et al., (2002) [133]	Japanese	Atopic Dermatitis
11	A2044G (Arg130Gln)	Celedon et al., (2002) [22]	Costa Rican	Not with serum IgE
12	A2044G (Arg130Gln)	Arima et al., (2002) [127]	Japanese	High Serum IL-13
13	A2044G (Arg130Gln)	Hoerauf et al., (2002) [134]	German	Onchocerciasis
14	A2044G (Arg130Gln)	DeMeo et al., (2002) [135]	American	Eosinophil counts, Allergy but not with BHR, asthma or severity of

				asthma
15	A2044G (Arg130Gln)	Leung et al., (2001) [116]	Chinese	Atopy but not asthma
16	A2044G (Arg130Gln)	Graves et al., (2000) [20]	White American	Serum IgE
17	T-1112C	Hunninghake et al., [129]	Costa Rican	Inversely associated with asthma
18	T-1112C	Puthothu et al., (2006) [136]	German	*RSV infection*
19	T-1112C	Kim et al., (2006) [119]	Korean	High IgE levels
20	T-1112C	Maier et al., (2005) [137]	British	Not with Type 1 Diabetes
21	T-1112C	Brown et al., (2005) [138]	American	Latex Allergy

22	T-1112C	Moissidis I et al., (2005) [121]	African American	Asthma
23	T-1112C	Schwartzbaum et al., (2005) [139]	American	*Inversely to Glioblastoma Multiforme*
24	T-1112C	Kouriba et al., (2005) [140]	Mali	*Schistosoma haematobium infections.*
25	T-1112C	Ohashi et al., (2003) [141]	Thai	Protection against malaria
26	T-1112C	Bugawan et al., (2003) [126]	Filipino	Type 1 diabetes
27	T-1112C	Wang et al., (2003) [125]	Chinese	Not with high IgE levels
28	T-1112C	Liu et al., (2003) [118]	German	Serum IgE

29	T-1112C	van der Pouw Kraan et al., (2002) [142]	Dutch	*COPD*
30	T-1112C	Howard et al., (2001) [115]	Dutch	Asthma, skin test responsiveness and BHR, but not with IgE
31	T-1112C	van der Pouw Kraan et al., (1999) [19]	Dutch	Allergic asthma
32	A2525G	Sun et al., (2003) [143]	Chinese	Serum IL-13 and Eosinophil cation protein

Abbreviations: SNP: single nucleotide polymorphism, Immunoglobulin E: IgE, RSV: respiratory sincitial virus, COPD: chronic obstructive pulmonary disease, BHR: bronchial hyperresponsiveness. *Nucleotide and/or amino acid change.

3. MATERIAL AND METHODS

3.1 Study Population

3.1.1 Sample size

The asthmatic patients participating in this study were from Asthma and Allergy Clinic, Gulab Devi Chest Institute Lahore. The patients, 164 in number, with variable severity of asthma were enrolled for the assessment of the spectrum of the disease and asthma trigger factors. Out of these 164 patients, 75 patients were randomly selected for serological and genetic analysis. Non asthmatic 50 controls belonging to the same age group and living conditions as patients were enrolled for the study. An informed written consent was taken from all subjects recruited in this study (Appendix I: patient's consent form). The study was approved by the Advanced Studies and Research Board of the University of Health Sciences, Lahore.

All the asthmatic subjects had specialist physician-diagnosed asthma with following three criteria: (i) recurrent breathlessness and chest tightness requiring ongoing treatment; (ii) physician documented wheeze; and (iii) documented labile airflow obstruction with variability in serial expiratory peak flow rate > 30%.

Fifty unrelated subjects without history of asthma and atopy, matched for age and sex with asthmatic patients, were randomly selected from general population to serve as control subjects for the association studies.

3.1.2 History and physical examination

For the assessment of the severity of asthma and their symptoms in response to different allergens a detailed history was taken based on a questionnaire designed for the study (Appendix: II) and undergo peak expiratory flow measurement. The questionnaire was designed in a way to cover different aspects of asthma symptoms which could help us in defining disease severity on the basis of history.

The frequency and exacerbation of their symptoms, physical activity and night time symptoms were recorded. The patients with a positive family history of asthma and / or any other allergic condition like atopic dermatitis, eczema and allergic rhinitis were identified. They were asked to identify allergens and the symptoms in response to them. For the study we included specific precipitating factors like exposure to dust and smoke keeping in view the local environmental conditions. Allergen induced symptoms included cough, wheeze, chest tightness, shortness of breath, and runny nose. The effect of seasonal variation on the exacerbation of allergy and symptoms was also noted. It also included a complete drug history with emphasis on oral and/or inhaled steroids.

A detailed history was taken from non-asthmatic controls to exclude previous history of asthma-like symptoms, allergic disease or family history of asthma and/or allergy. Their peak expiratory flow was recorded to assess their baseline pulmonary function.

Body weight (BW) in kilograms (Kg) and height in meters (m) were recorded for all patients. Body mass index (BMI) was calculated using the following equation:

$$BMI = \text{Body Weight (kg)/height (m)}^2$$

3.1.3 Peak expiratory flow (PEF)

Peak expiratory flow provides a simple, quantitative, and reproducible measure of the existence and severity of airflow obstruction. PEF can be measured with inexpensive and portable peak flow meters. Peak flow monitoring can be used for short-term monitoring, managing exacerbations, and daily long-term monitoring.

Peak expiratory flow was measured using standard Wright Peak Flow Meter (Clement Clarke Int, UK). The pointer on the gauge of the peak flow meter was adjusted to zero and the mouth piece was attached to it. The patient was asked to take a deep inspiration while standing and then expiring as fast as possible within 1 or 2 second. The maneuver was repeated three times and the highest value was recorded.

3.1.4 Exclusion criteria

Patients with a history of smoking or any illness involving lungs e.g., chronic bronchitis, emphysema, tuberculosis, pneumonia etc. were excluded from this study. The patients with a history of a disease altering the IL- 13 status including autoimmune rheumatoid arthritis, Hodgkin's disease, Graves's disease etc. were also excluded from our study.

The participants in control group were healthy individuals and exclusion criteria included asthma-like symptoms, known allergies, history of smoking and a positive family history of asthma and allergy. Also, controls with deranged PEF were excluded.

3.1.5 Phenotypes for asthma

In addition to the presence of asthma and serum IgE levels as phenotypes we have included two other parameters family history and severity of asthma, in our study for association with the polymorphism. Family history status was ascertained by taking a detailed history of asthma and allergy and thus dividing our patients into two broad groups with and without family history of asthma. On the basis of their history and PEF recordings, the severity of the disease was determined. The patients were divided into two groups of mild/moderate and severe asthma. The grouping was based on the recommendations of the National Institutes of Health; National Heart, Lung and Blood Institute, for the classification of asthma severity (Appendix III: classification of asthma severity) [144]. According to this classification the patients were divided into two broad groups: mild intermittent/persistent and moderate/severe persistent. In later discussions, they are referred to as mild and severe groups respectively. This grouping was later used in comparing the different genotypes among these patients.

3.2 Sample Collection

Blood samples were drawn by venipuncture of the cubital vein from each individual. All samples were taken between 8 and 10 am as they were to be analyzed for adrenal function. The samples for serum ACTH and Cortisol should ideally be drawn between this period due to circadian rhythm in their secretion and diurnal variation. Their blood levels peak between 8 and 10 in the morning reaching its lowest level by night.

Five mL blood was collected in tubes containing EDTA as whole blood for isolating leukocytes and extraction of DNA. For serum, 5 mL was taken in a separate tube, kept standing in a tube for a few minutes and centrifuged (Hettich, Germany) at 14,000 x g for 10 minutes and serum was separated. Three aliquots were made from each whole blood and serum sample and stored at -80 C till further analysis.

3.3 Serology

The serum was analyzed for total IgE, cortisol and ACTH levels by Enzyme linked immunosorbent assay (ELISA).

3.3.1 Total IgE levels

Serum total IgE levels were measured by ELISA using a commercially available kit (BioCheck Inc, Burlingame, CA, USA).

3.3.1.1 Method

The test was based on the sandwich principle and all samples were run in duplicate. Serum was added to the IgE monoclonal antibodies immobilized on polystyrene microtiter wells (solid phase) and incubated with the Zero Buffer. The wells were then washed to remove any residual sample, and goat anti-IgE in the antibody enzyme (horseradish peroxidase) conjugate reagent was added. The conjugate reagent bound immunologically to the IgE on the well, resulting in the IgE molecules being sandwiched between the solid phase and the enzyme-linked antibodies. After incubation at room temperature, the solid phase was washed with water to remove unbound labeled antibody. A solution of 3,3',5,5'-Tetramethylbenzidine (TMB) was added and incubated for 20 minutes, resulting in the development of a blue color. The color development was stopped and the resulting yellow color was measured spectrophotometrically at 450 nm with an automated EIA analyzer (Bio-Rad Laboratories, Hercules, CA, USA). The concentration of IgE was directly proportional to the color intensity of the test sample.

3.3.1.2 Calculation of Results

A standard curve was constructed by plotting the mean absorbance obtained for each reference standard against its concentration in IU/mL on graph paper, with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis. Using the mean absorbance value for each sample, the corresponding concentration of IgE in IU/mL was determined from the standard curve (Fig 2).

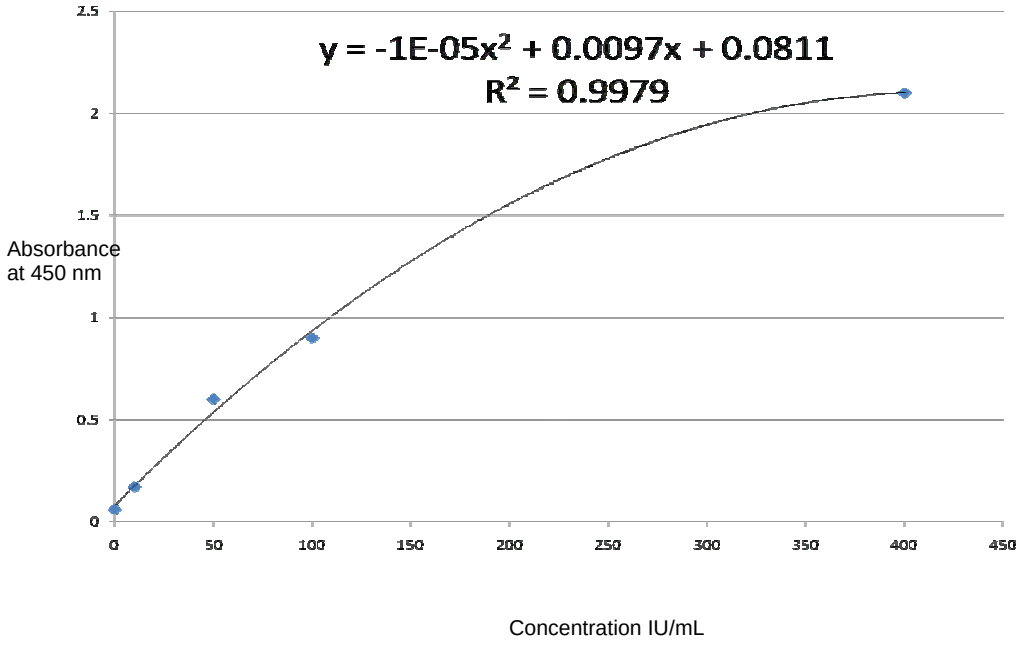

Figure 2 : Standard curve for the estimation of serum total IgE levels by ELISA.

3.3.1.3 Interpretation of serum IgE levels

The total IgE level in normal, allergy-free adults is less than 150 IU/mL in the serum. Serum total IgE levels have been shown to increase in patients with atopic allergic diseases such as atopic asthma, atopic dermatitis, and hay fever [145-147]. Elevated levels of IgE indicate an IgE-mediated hypersensitivity, responsible for allergic reactions. Parasitic infestations such as hookworm, and certain clinical disorders including aspergillosis, have also been demonstrated to cause high levels of IgE [148, 149]. Decreased levels of IgE are found in cases of hypogammaglobulinemia, autoimmune diseases, ulcerative colitis, hepatitis, cancer, and malaria [145].

3.3.2 Serum Cortisol Levels

Serum cortisol levels were measured by Enzyme linked immunosorbent assay (ELISA) using a commercially available kit (Diagnostic Systems Laboratories, Inc., Webster, Texas, USA).

3.3.2.1 Method

The procedure was based on the basic principle of enzyme immunoassay where there is a competition between an unlabeled antigen and an enzyme labeled antigen for a fixed number of antibody binding sites. The amount of enzyme labeled antigen bound to the antibody is inversely proportional to the concentration of unlabeled antigen present. Each Standard, Control and serum samples was added to the appropriate well and the Enzyme Conjugate Solution was added to each well. The Cortisol Antiserum was added to each well and incubated at room temperature (~25°C). The wells were aspirated and washed 5 times with the Wash Solution. Now the 3,3',5,5'-Tetramethylbenzidine (TMB) Chromogen Solution was added to each well and incubated the wells at room temperature (~25°C) for 10-15 minutes. The Stopping Solution was added to each well and reading was taken after shaking the plate for 5-10 seconds. The absorbance of the solution in the wells was read immediately using an automated EIA analyzer (Bio-Rad Laboratories, Hercules, CA, USA) set to 450 nm.

3.3.2.2 Calculation of Results

The mean absorbance for each Standard, Control and serum samples was calculated. Using a linear-log graph paper, the mean absorbance readings for each of the standards was plotted along the y-axis versus the cortisol concentrations in ng/mL along the x-axis. The cortisol concentrations of the Controls and samples were determined from the standard curve by matching their mean absorbance readings with the corresponding cortisol concentrations (Fig 3).

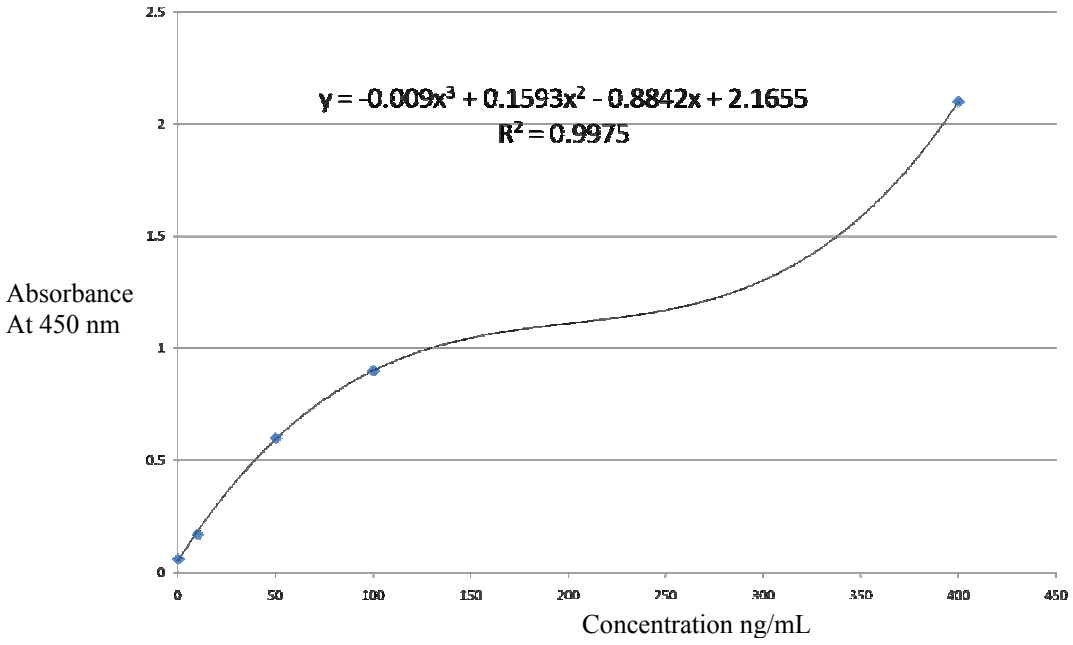

Figure 3: Standard curve for the estimation of serum cortisol levels by ELISA.

3.3.2.3 Interpretation of serum cortisol levels

Cortisol production has an ACTH-dependent circadian rhythm with peak levels in the early morning and a fall at night. Increased amounts of ACTH and cortisol are

secreted independently of the circadian rhythm in response to physical and psychological stress [150, 151]. Elevated cortisol levels and lack of diurnal variation have been identified in patients with Cushing's disease (ACTH hypersecretion) [152, 153]. Elevated circulating cortisol levels have also been identified in patients with adrenal tumors [154]. Low cortisol levels are found in primary adrenal insufficiency (e.g. adrenal hypoplasia, congenital adrenal hyperplasia, Addison's disease) and in ACTH deficiency [152-157].

3.3.3 Serum ACTH levels

Serum ACTH levels were measured by enzyme linked immunosorbent assay using commercially available kit (Biomerica Inc., Newport Beach, CA, USA).

3.3.3.1 Method

The ACTH Immunoassay is based on a two-site ELISA principle for the measurement of the biologically active 39 amino acid chain of ACTH. A goat polyclonal antibody to human ACTH and a mouse monoclonal antibody to human ACTH are specific for well defined regions on the ACTH molecule. One antibody is prepared to bind only the C terminal ACTH and this antibody is biotinylated. The other antibody is prepared to bind only the midregion and N-terminal ACTH and this antibody is labeled with horseradish peroxidase [HRP] for detection.

In this assay, calibrators, controls, and patient samples were simultaneously incubated with the enzyme labeled antibody and a biotin coupled antibody in streptavidin-coated microplate wells. At the end of the assay incubation, the microwells were washed to remove unbound components and the enzyme bound to the solid phase was incubated with the substrate, tetramethylbenzidine (TMB). An acidic stopping solution was then added to stop the reaction which converted the color to yellow. The intensity of the yellow color is directly proportional to the concentration of ACTH in the sample. The absorbance of the solution in the wells was read immediately using an automated EIA analyzer (Bio-Rad Laboratories, Hercules, CA, USA) set to 450 nm.

3.3.3.2 Calculation of results

Computer program using cubic spline was used for a point to point curve (Fig 4).

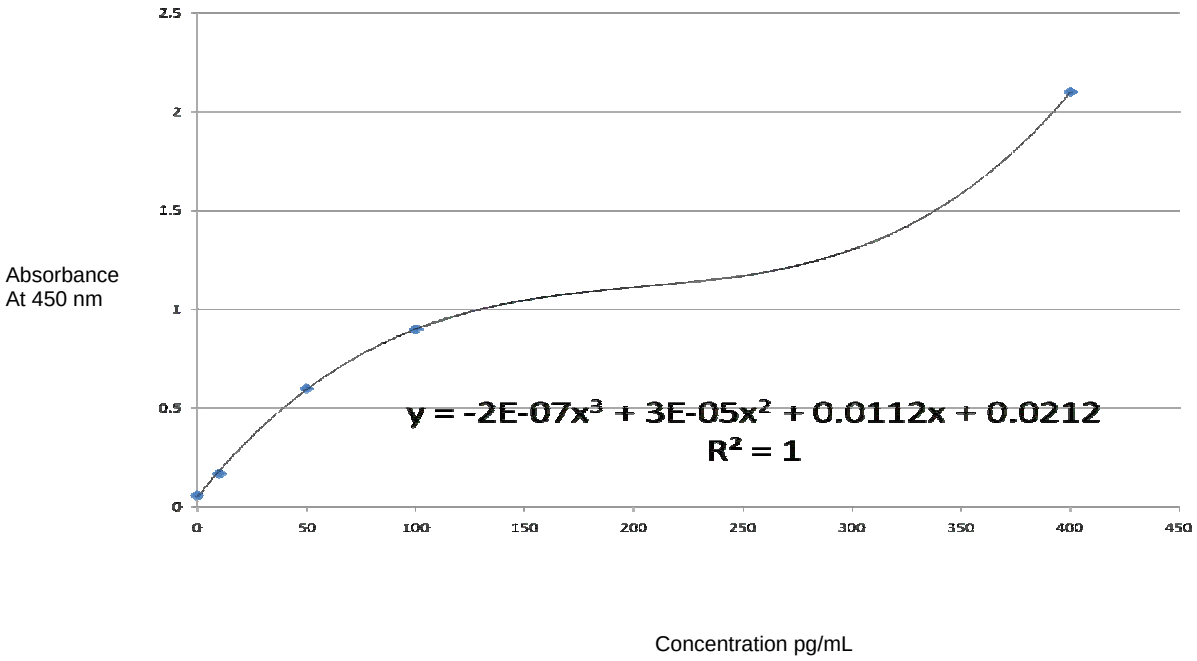

Figure 4: Standard curve for the estimation of serum ACTH by ELISA.

3.3.3.3 Interpretation of ACTH levels

ACTH is secreted in a pulsatile manner. These small pulses are superimposed on a characteristic diurnal fluctuation of greater amplitude. Plasma ACTH assays are useful in the differential diagnosis of pituitary Cushing's disease, Addison's disease, autonomous ACTH producing pituitary tumors (e.g. Nelson's syndrome), hypopituitarism with ACTH deficiency and ectopic ACTH syndrome. [158-162]. In patients with adrenal tumors, ACTH levels are low. High levels of ACTH are seen in patients with ectopic ACTH syndrome. Patients with bilateral adrenal hyperplasia will have ACTH levels inappropriately elevated for their degree of hypercortisolism, which should suppress ACTH.

3.4 Genotyping of IL-13 Polymorphisms

3.4.1 DNA Extraction

DNA extraction was performed on whole blood samples using PUREGENE Genomic DNA extraction kit by Gentra systems (Minnesota, USA).

3.4.1.1 Method

1. Cell lysis step: RBC lysis solution was added to the whole blood and incubated at room temperature for 10 minutes. After centrifuging the sample at 14,000 x g for 20 seconds, supernatant was removed leaving behind a white pellet of leukocytes. Cell lysis solution was added to re suspend the pellet. Proteinase K (20 mg/mL) (Sigma, USA) was added after this step to remove any protein contamination.

2. RNAse Treatment: RNase A solution (4mg/mL) was added to the lysate and mixed by inverting several times before incubating at 37 ^{o}C for 15-60 minutes. This additional step of RNase treatment was added to improve the quality of extracted DNA samples.

3. Protein Precipitation: Protein Precipitation solution was added to the cell lysate and mixed by vortexing at high speed for 20 sec. Centrifuged at 14,000 x g for 3 min**.**

4. DNA Precipitation: 100% Isopropanol was added to the supernatant containing DNA. Sample was mixed by inverting gently 50 times and centrifuged at 13,000-16,000 x g for 1 minute**,** the DNA was visible as a small white pellet.

DNA yield was augmented by using Glycogen 20 mg/mL (Sigma, USA) after precipitation with Isopropanol. The supernatant was removed and the DNA pellet was washed with 70 % ethanol twice.

5. DNA Hydration: The DNA pellet was air dried before adding the DNA hydration solution and Incubated at 65 ^{o}C for 5 min (up to 1 hr) to accelerate hydration. DNA stored at -80 ^{o}C.

Extracted DNA was quantified on spectrophotometer (Jenway, Essex, UK). The quality of DNA was assessed by taking ratio of absorbance obtained at 260 and 280 nm. Samples with ratio between 1.7 and 2.0 were considered pure. The samples were re confirmed on 0.8 % agarose gel for the quality and quantity of DNA.

3.4.2 SNP Genotyping

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is a classic and relatively inexpensive method of genotyping that is based on endonuclease cleavage. An SNP that alters a restriction sequence can be genotyped by this method. Amplification of a region surrounding a restriction enzyme site, followed by digestion with the appropriate enzyme allows rapid genotyping. For the detection of SNPs we employed the technique of PCR-RFLP. Genebank accession number AC004039 was used as the reference sequence for the IL-13 gene.

3.4.3 Polymerase Chain Reaction (PCR)

Subjects were genotyped for each polymorphism by PCR amplification of the region containing the polymorphism and selective restriction endonuclease digestion. PCR was performed on all extracted DNA samples to amplify the selected regions of IL-13 gene.

We studied six polymorphisms in our study. Four polymorphisms were from the promoter region C-1512A, T-1112C, A-646G and C-469T, one nonsynonymous polymorphism from the exon 4 A2044 G (Arg to Gln) and a 3'UTR polymorphism A2525G. The two polymorphisms T-1112C and A2044G have previously been studied in different populations while the 3'UTR polymorphism was previously described in one study. The two promoter polymorphisms A-646G and C-469T have so far not been studied for an association with asthma. These two polymorphisms were included in this study to find out a possible role of these sites in the regulation of IL-13 gene since they are in close proximity to the other two promoter polymorphisms previously studied which are also included in this study. The polymorphisms C-1512A, T-1112C, A2044 G and A2525G were amplified in such a way to create a restriction site for SNP analysis by modifying a nucleotide base in their primer.

3.4.3.1 Primer Design

A total of 5 primer pairs were used to amplify five specific regions of IL-13 gene containing six SNPs. Four polymorphisms C-1512A, T-1112C, A2044 G and

A2525G were amplified using primers with modified bases to create a restriction site as described by Graves et al. [20]. The two promoter polymorphisms A-646G and C-469T were analyzed by natural PCR RFLP using primers without any modification in their sequence [115]. The assay conditions for the amplification of all these SNPs along with their respective primers are listed in Table 2.

The primers were diluted at 100 μM in molecular biology grade water. The formula used for the calculation of dilution factor was:

$$X = \frac{MW/10}{OD \times 33}$$

Where MW is molecular weight, OD is optical density and X is the dilution factor. To dilute them to 100 μM, 1000 was divided by the dilution factor (X) calculated.

3.4.3.2 PCR Protocol

The PCR reactions were prepared in the PCR work station (Air clean systems, Lake Woodard Drive, NC, USA). PCR amplifications were carried out in 50 μL volumes containing 1X PCR buffer (10mM Tris-HCl pH 8.8, 50mM KCl, 0.8% Nonidet P40), 2 mM/L $MgCl_2$ (concentrations for each PCR reaction are given in Table 2), 200 μM/L of each deoxynucleotide triphosphate, 30 ng of each primer, and 0.6 units of Taq DNA polymerase (BioRad Inc, USA) in 25 ng of genomic DNA.

Samples were denatured at 94°C for 2 minutes followed by 35 cycles of 94°C for 40 seconds, annealing (temperatures given in table 3 for the respective primers) for 40 seconds, and 72°C for 50 seconds, and then a final extension for 10 minutes at 72°C (Thermo Scientific, USA).

3.4.4 RFLP analysis

Aliquots of the PCR amplified product were used for digestions at the site of each polymorphic nucleotide. For restriction analysis all PCR products were digested by addition of 0.25 units of the respective enzyme (New England BioLabs, Boston, USA) and 1X buffer in 10 µL of PCR product. The samples were incubated at 37°C for 2 hours. The restriction enzymes, $MgCl_2$ concentration and the annealing temperatures for each primer are given in Table 3. The digested PCR products were subjected to gel electrophoresis on 3% agarose gels to separate the fragments.

3.4.5 Gel Electrophoresis

The extracted DNA samples and PCR products were run on agarose gel. For genomic DNA 0.8 %, for PCR amplified products 1.5% and for restriction enzyme digested PCR products 3 % agarose gel was prepared. The agarose for routine use (Sigma, USA) was diluted in 1 X TBE (1M Tris, 1M Boric Acid, 20mM EDTA pH 8.3; Sigma, USA) buffer according to the strength required.

Gels were stained with ethidium bromide (10mM Tris-HCl, 1 mM EDTA, 1 mg/mL ethidium bromide; Sigma, USA).

DNA Samples were loaded on the gel along with 1 X bromophenol loading dye (0.25% bromophenol blue prepared in 40% sucrose solution; Sigma, USA). Electrophoresis was performed at 100 V for about 35-40 minutes. A 50 bp DNA ladder (MBI-Fermentas, England) was also used to verify sizes of the PCR products and enzyme digested samples.

Table 2: Primers and assay conditions for the amplification of IL-13 gene

SNP	Primers	Mg Cl_2 (μl)	Anne-aling temp-erature
C-1512A rs1881457	F- 5′-CAACCGCCGCGCCAGCGCCTTCTC-3′ R- 5′-CCGCTACTTGGCCGTGTGACCGC-3′	1.5	54
T-1112C rs1800925	F- 5′-GGAATCCAGCATGCCTTGTGAGG-3′ R- 5′-GTCGCCTTTTCCTGCTCTTCCCGC-3′	1.5	54
A-646G rs2069743	F- 5′-CCTAGGCAGGCAACATAGTG-3′ R- 5′-CTGGACCCTTCTCAATAAGT-3′	2.5	52
C-469T rs2069756	F- 5′-CCTAGGCAGGCAACATAGTG-3′ R- 5′-CTGGACCCTTCTCAATAAGT-3′	2.0	52
A2044 G rs20541	F- 5′-CTTCCGTGAGGACTGAATGAGACGGTC-3′ R- 5′GCAAATAATGATGCTTTCGAAGTTTCAGTGGA -3′	1.5	55
A2525G rs1295685	F- 5′-GGACAGGGACCCACTTCACAC-3′ R- 5′-GCTAACATATTTAATATTTATGTAC-3′	2.0	44

Table 3: Restriction Enzymes and assay conditions for RFLP analysis

Polymorphism	**Restriction enzyme**	**Alleles**	**Fragment sizes (bp)**
C-1512A	*Bst*UI	A C	214 192
T-1112C	*Bst*UI	C T	224 247
A-646G	*Drd*I	A G	549 126, 423
C-469T	*Acc*I	C T	549 303, 246
A2044 G	*Nla*IV	G A	178 210
A2525G	*Nhe*I	G A	390 253, 137

3.5 Statistical Analysis

The clinical characteristics between groups with different severity of asthma were compared with serum levels using student's t test. The serum IgE levels among patients were compared to their age and sex matched controls keeping in view their family history and allergy status. Individuals with high total serum IgE levels were defined as having total serum IgE $\geq$ 150 IU/ mL. The serum Cortisol and ACTH levels were compared between patients and controls.

Fisher's exact test was applied for Hardy Weinberg equilibrium for all SNPs. Differences of allele and genotype frequencies between cases and controls were compared using DeFinetti program. X^2 tests were used for comparing the allele frequencies between the two groups and the significance was measured in terms of Pearson's goodness-of-fit-chi-square. They were applied to ascertain a possible association between each SNP and phenotypes including presence of asthma, severity of asthma, serum IgE levels and family history of asthma.

4. RESULTS

4.1 Characteristics of Population Sample

The patients and the controls were from the same age group (mean= 52 years & 50 years, respectively), with a male to female ratio of 52:48. Their demographic characteristics, distribution on the basis of family history, history of allergy and seasonal variation in symptoms are given in Table 4. Detailed family history revealed a positive family history of asthma for 32%, history of any other allergy (eczema, allergic rhinitis) in the family for 6 % and no history of asthma, allergy or asthma like symptoms for 62 % (Table 4).

Table 4: Characteristics of population sample

Prevalence of Asthma symptoms: (n=164)		**Percentage**
By Gender	Male	52
	Female	48
Family History:		
No family history		62
Of Asthma		32
Of any other allergic condition (Eczema, allergic rhinitis)		6

They were further asked to specify any known allergen that triggered the asthma attack. It showed that majority of patients (88%) were allergic to dust while a small percentage of population being allergic to animal dander and pollen, grass, trees etc. A large proportion of patients had their symptoms exacerbated during winter and spring seasons. Their details are illustrated in Table 5.

Table 5: History of allergy and seasonal exacerbations due to asthma

History of Allergy	**Percentage**
Allergy to dust	88
No known allergy	7
Allergy to animal dander	3
Allergy to pollen, grass, trees	2
Prevalence of Asthma crisis by season	
No Seasonal Correlation	38
Winter	34
Spring	16
Summer	10
Autumn	2

A detailed account on the trigger factors was also taken and majority of patients had a combination of two or more of these trigger factors responsible for their exacerbations (Table 6). The principal trigger factors for asthma were exposure to dust, smoke and seasonal variation. Other factors included physical exercise, anxiety or stress, acute respiratory infection, food items, animals and aspirin

intake. Their percentages are given in table 6. The atopic manifestations of these allergens and trigger factors are given in Table 7 with cough, wheeze and shortness of breath being the commonest.

Table 6: Trigger factors for asthma

Trigger Factors for Asthma	Percentage
Exposure to dust	88
Smoke	62
Seasonal variation	62
Physical exercise	36
Aspirin	32
Food items	29
Acute respiratory infection	26
Anxiety or stress	24
Contact with animals	3

Table 7: Symptoms in response to the trigger factors

Atopic manifestations	Percentage
Cough	68
Shortness of breath	59
Wheeze	54
Runny, stuffy nose or sneeze	31
Itchy or watering eyes	23
Chest tightness	18

For the assessment of the severity of their symptoms they were asked about the frequency of symptoms, physical activity, exacerbations and night time symptoms. 41% of patients had daily symptoms of asthma while the physical activity was limited for 55% of the patients (Figure 5 and 6). Symptom exacerbations were frequent for 44% of the patients more than twice weekly for 40 % and brief for 14% (Figure 7). The night time symptoms were also found to be significantly frequent in majority of patients (Figure 8).

Majority of patients were taking a combination of inhaled corticosteroids, anti allergic and bronchodilators. All of the patients reported using steroid inhalers but none of them were taking oral steroids.

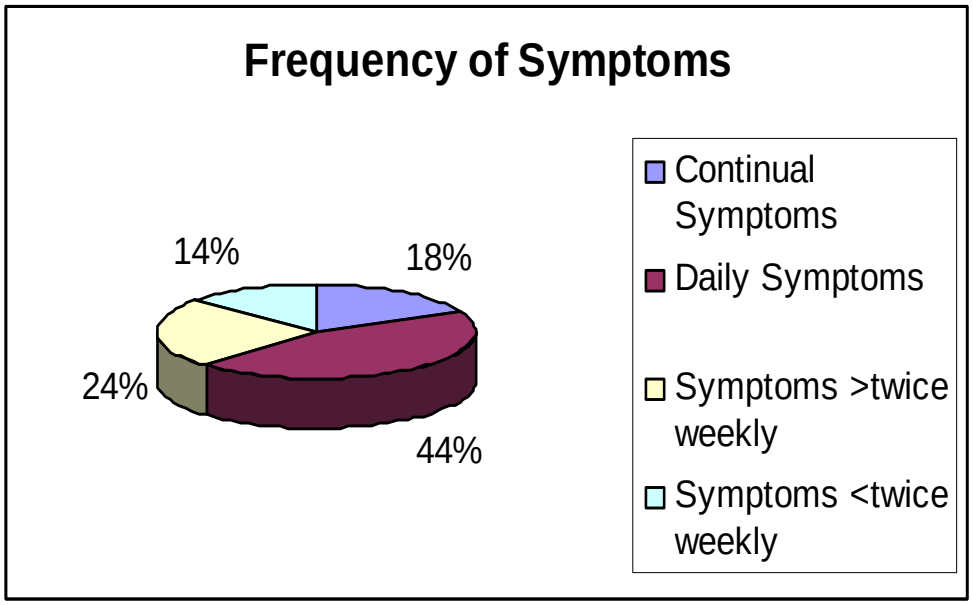

Figure 5: Frequency of Symptoms

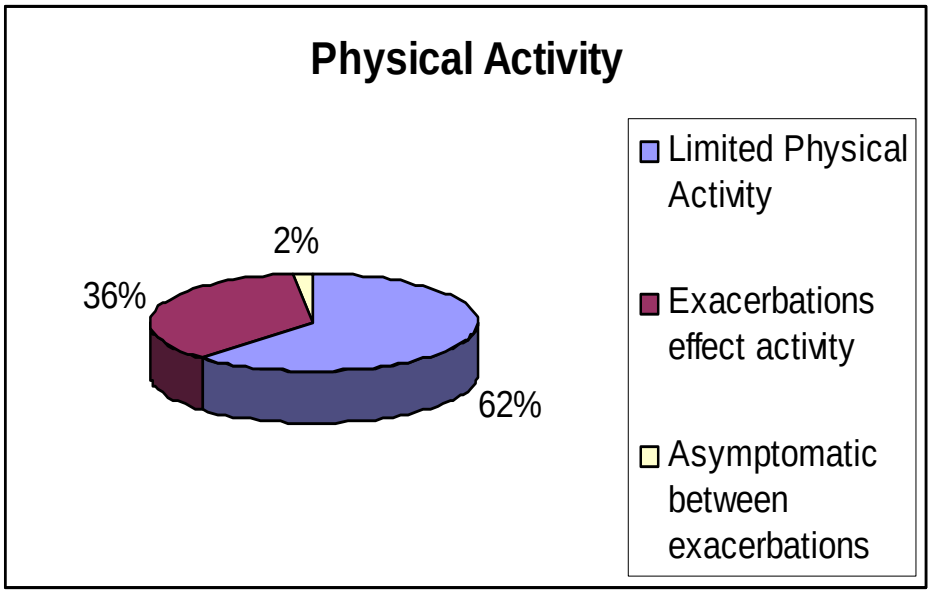

Figure 6: Physical Activity

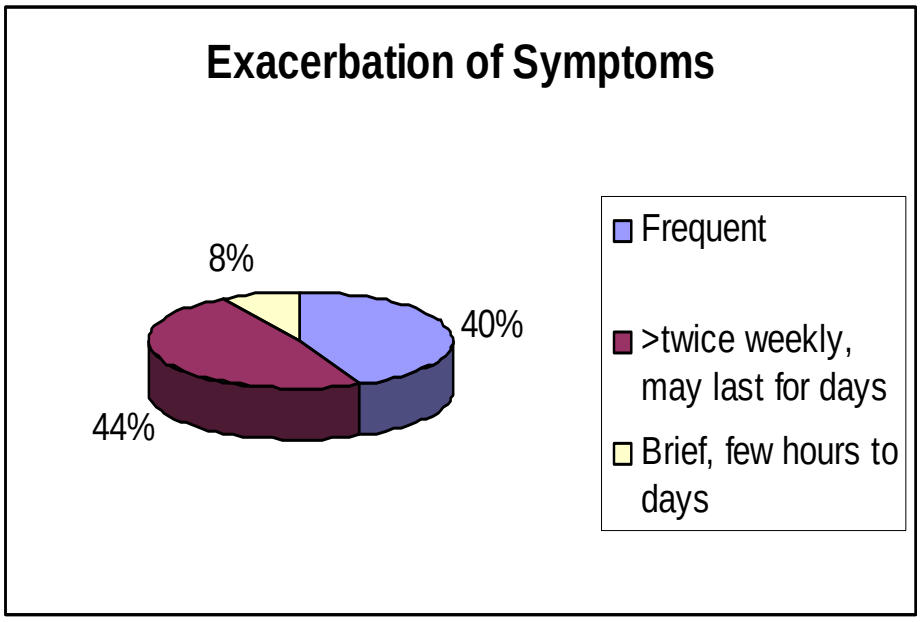

Figure 7: Exacerbation of symptoms

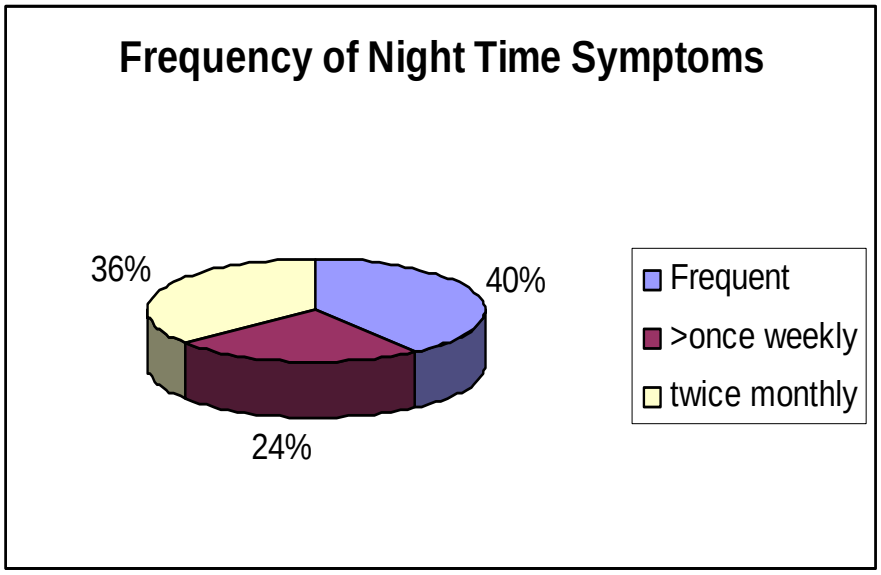

Figure 8: Frequency of night time symptoms

4.2 Serology

4.2.1 Total serum IgE levels

All samples were run in duplicate. As expected, the total serum IgE levels were found to be higher (p value 0.006) in asthmatic individuals as compared to the control group (Figure 9). When compared with the severity of their symptoms the levels were directly proportional to the severity of asthma (Figure 10). They were found to be higher (p value 0.037) in patients with a history of allergy than in non allergic patients (Figure 11). Interestingly, no correlation (p value 0.49) was found between patients with and without family history asthma in our population (Figure 12). The details of the results are illustrated in the Table 8.

Table 8: Total serum IgE levels in asthmatic and control individuals

	Asthmatic Patients (n=75)	**Controls (n=50)**
Total Serum IgE levels (150 IU/mL)	24-1955 IU/mL	15-512 IU/mL
Mean	816.00 IU/mL	245.00 IU/mL
± SD	689.63	155.97
±SE	114.90	49.30
P value	0.006	

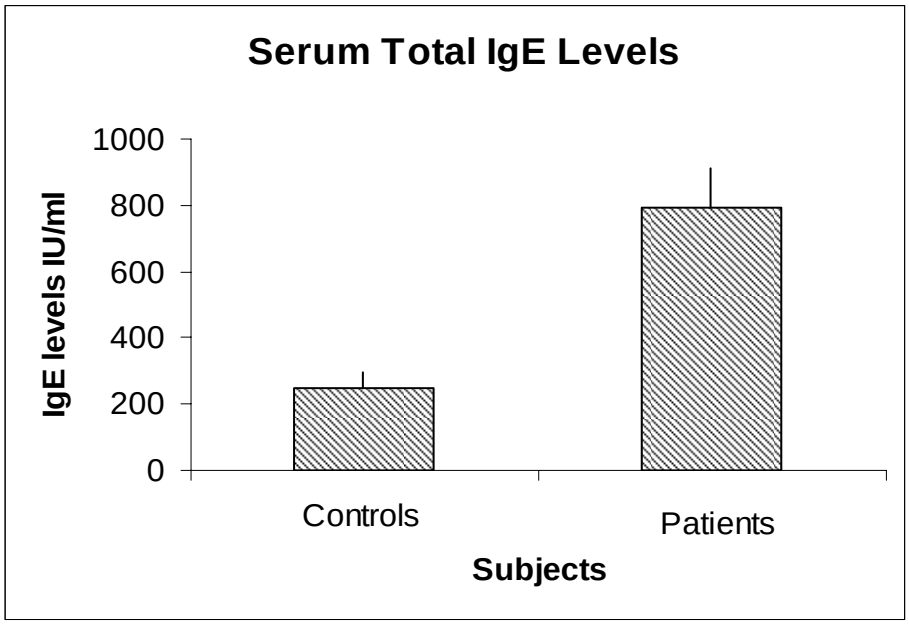

Figure 9: Serum IgE levels among control and asthmatic individuals.

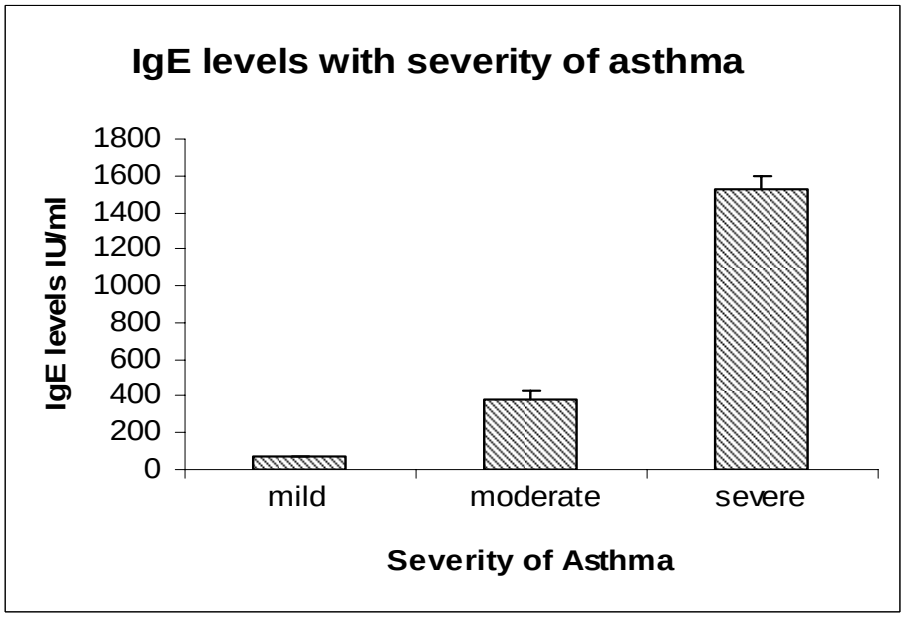

Figure 10: Serum IgE levels compared with the severity of asthma.

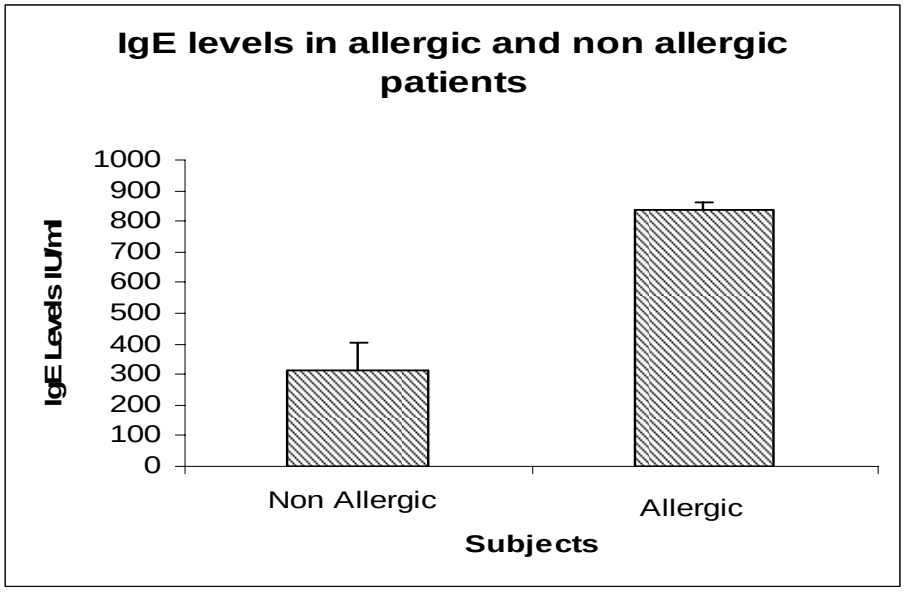

Figure 11: Serum IgE levels among patients with and without history of allergy.

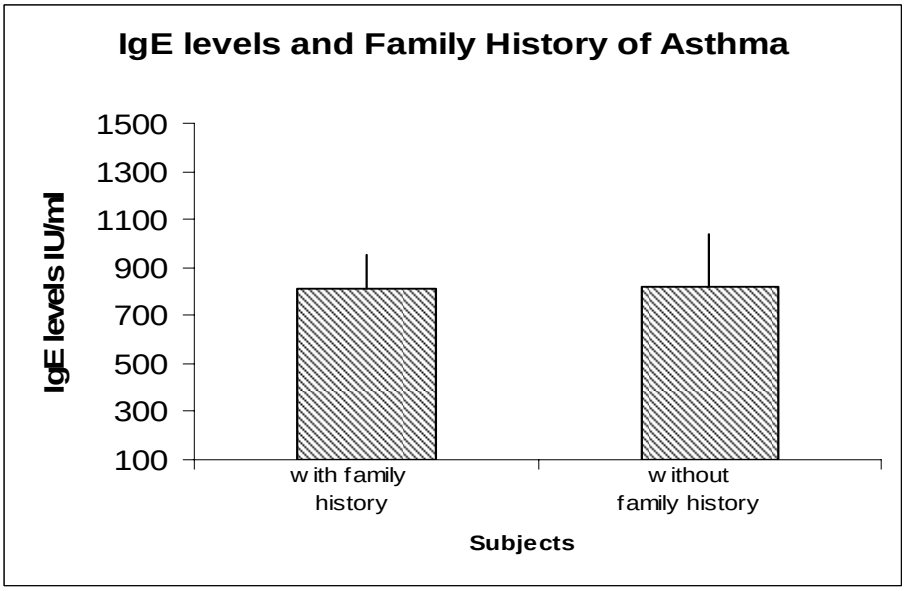

Figure 12: Serum IgE levels in patients with and without a family history of asthma.

4.2.2 Serum Cortisol Levels

All samples were run in duplicate. The correlation between serum cortisol levels in asthmatics and controls was not found to be significant (p= 0.43) Figure 13. Their mean values, standard deviations, standard error of means (SEM) and p value are given in table 9. When these levels were compared between patients with variable severity of asthma they were found to be significantly low in mild asthmatics compared to moderate (p value 0.01) and severe asthmatics (0.006) Figure 14.

Table 9: Serum cortisol levels in asthmatic patients and control subjects

	Asthmatic Patients	**Controls**
Serum Cortisol levels (ng/mL)	5-163(ng/mL)	42-160(ng/mL)
Mean	88.00(ng/mL)	85.30(ng/mL)
± SD	49.31	39.56
±SE	8.85	12.50
P value	0.43	

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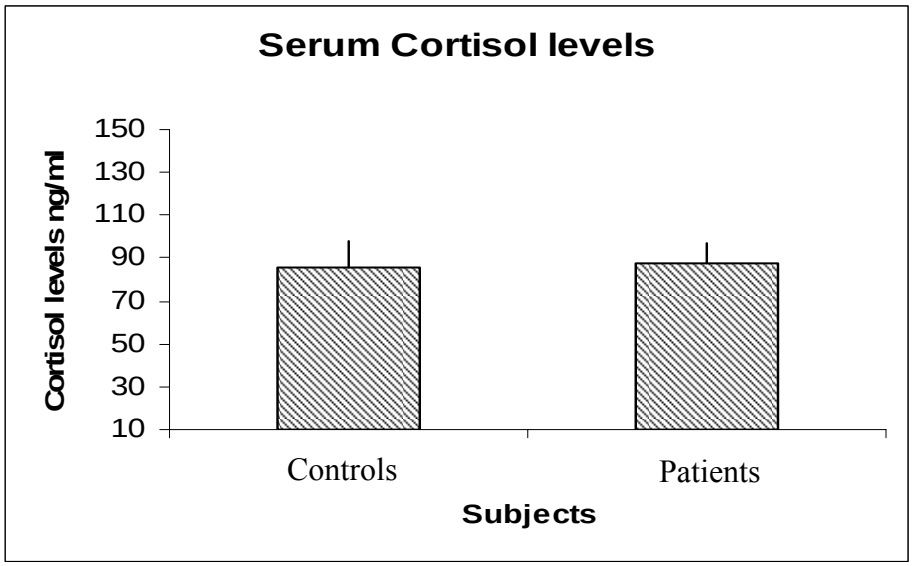

Figure 13: A comparison of serum cortisol levels among controls and asthmatic patients.

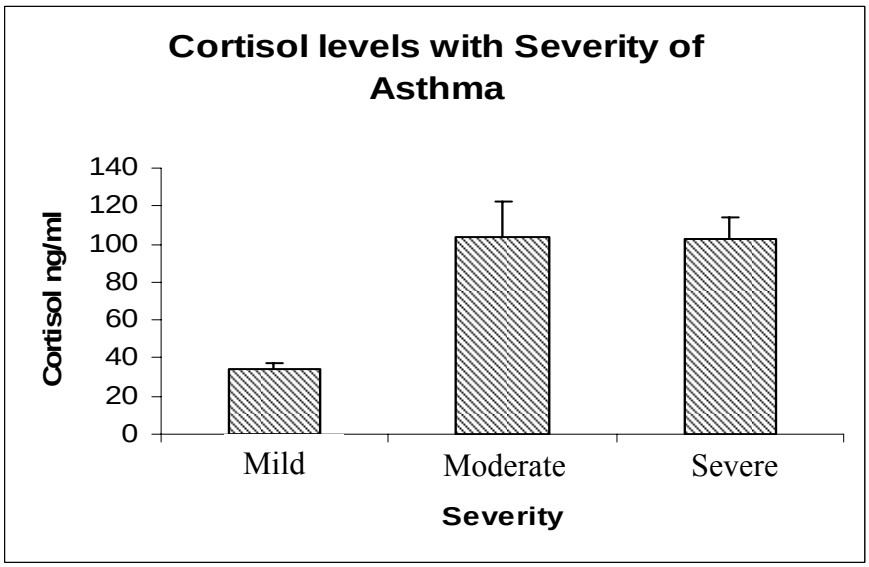

Figure 14: Serum cortisol levels compared with the severity of asthma.

4.2.3 Serum ACTH levels

All samples were run in duplicate. The correlation between the ACTH levels in asthmatics and controls was not found to be significant (p= 0.30), however the ACTH levels in controls were found to be on lower side of the normal Figure 15. In addition, the ACTH levels were not different in patients with variable severity of asthma, Figure 16.

Table 10: Serum ACTH levels in asthmatic patients and control subjects

	Asthmatic Patients	**Controls**
Serum ACTH levels (pg/mL)	0.50 -31.00(pg/mL)	1.90-19.00(pg/mL)
Mean	9.78(pg/mL)	8.31(pg/mL)
$\pm$ SD	8.25	5.10
$\pm$SE	1.37	1.61
P value	0.30	

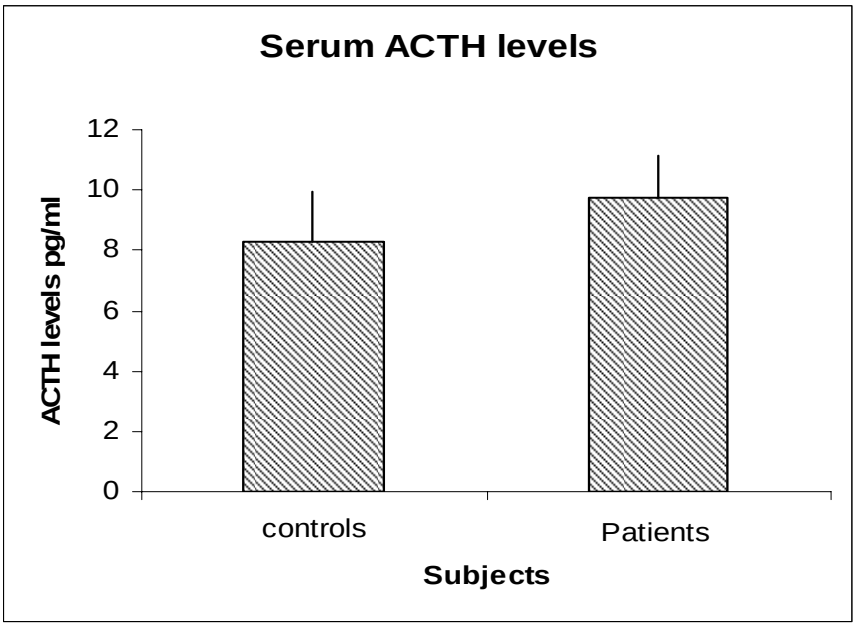

Figure 15: Serum ACTH levels compared between the controls and asthmatic patients.

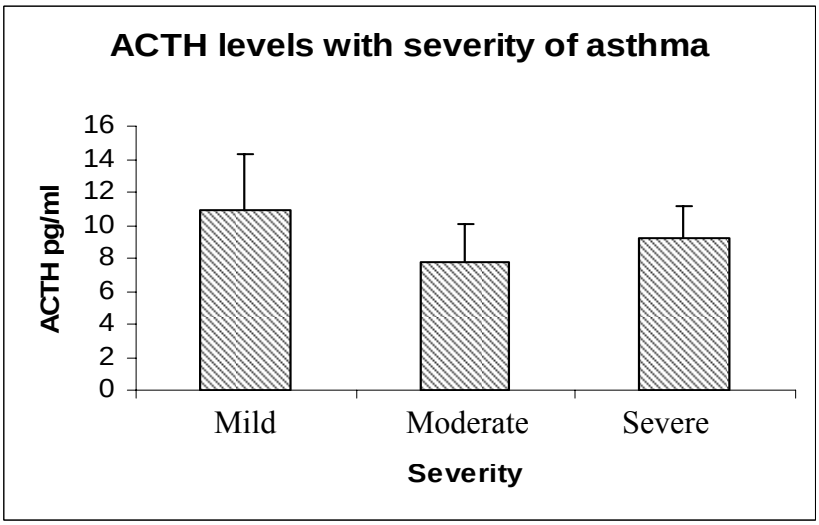

Figure 16: A comparison of ACTH levels among different severities of asthma.

4.3 Association of IL-13 SNPs with asthma

A total of 75 patients and 50 controls were genotyped for all six SNPs, four in the promoter region C-1512A, T-1112C, A-646G and C-469T, one in exon 4 A2044 G (Arg to Gln)and one in the 3'UTR A2525G. These SNPs were analyzed for an association with the presence of asthma, serum total IgE levels, severity of asthma and family history of asthma. The association of each of these polymorphisms with the asthma phenotypes is described in detail below:

4.3.1 5' promoter polymorphism C-1512A

The PCR-RFLP analysis was performed for this polymorphism. Homozygous for A alleles resulted in a 214 bp fragment on agarose gel. The other genotype C homozygous was seen as a 192 bp fragment. In case of heterozygous both 214 and 192 bp fragments were seen on the gel (Figure 17).

This promoter polymorphism was found to be in Hardy Weinberg equilibrium in our population. A significant association was seen between this polymorphism and the presence of asthma (p= 0.001). It was also shown to be significantly associated with the severity of asthma (p= 0.02) and family history of asthma (p= 0.03). However, no association was found with serum IgE levels (p= 0.57). The allele frequencies in comparison with each phenotype, the allele frequency difference and the risk for allele 2 are given in Table 11.

C-1512A

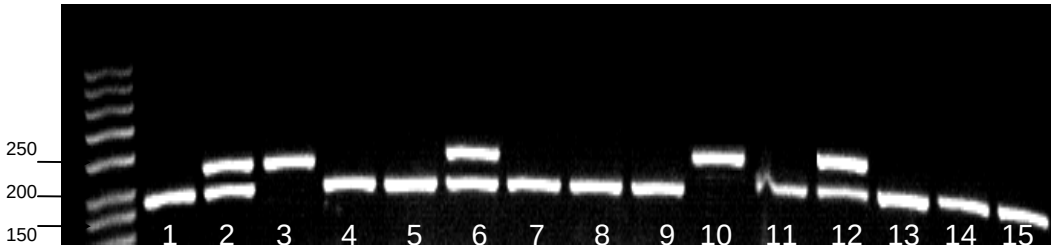

Figure 17: The 5' promoter polymorphism C-1512A on 3% agarose gel. Lanes 1, 4, 5, 7, 8, 9, 11, 13, 14, 15, showing a single band at 192 bp representing CC genotype. Lanes 2, 6 and 12 represent the heterozygous genotype CA with two bands at 192 and 214 bp respectively. Lanes 3 and 10 show a single band at 214bp representing AA genotype.

Table 11: C-1512A polymorphism and its severity association with asthma, serum IgE levels, family history and of asthma

5' promoter polymorphism C-1512A					
Phenotype	**Subjects**	**Allele 1**	**Allele 2**	**Allele Frequency Difference (p value)**	**Risk for allele 2 (p value)**
Asthma	Controls Cases	0.75 0.53	0.25 0.467	0.0005	0.001
Serum IgE levels	Low IgE (<150 IU/mL) High IgE (>150 IU/mL)	0.51 0.57	0.49 0.43	0.50	0.57
Family History	Negative Positive	0.67 0.46	0.33 0.54	0.01	0.03
Severity of Asthma	Mild Severe	0.68 0.45	0.32 0.55	0.004	0.02

4.3.2 5' promoter polymorphism T-1112C

The PCR-RFLP analysis resulted in a 224 bp fragment in case of CC genotype. A 247 bp fragment was generated where the genotype was TT. In case of a heterozygous both 224 and 247 bp fragments were seen on the gel (Figure 18).

This polymorphism was found to be in Hardy Weinberg equilibrium in this population. This polymorphism had a strong correlation with the presence of asthma (p= 0.0008), the severity of asthma (p= 0.003), weak association with serum IgE levels (p= 0.01). No correlation was found with the family history of asthma (p=0.9). The allele frequencies in comparison with each phenotype, the allele frequency difference and the risk for allele 2 are given in Table 12.

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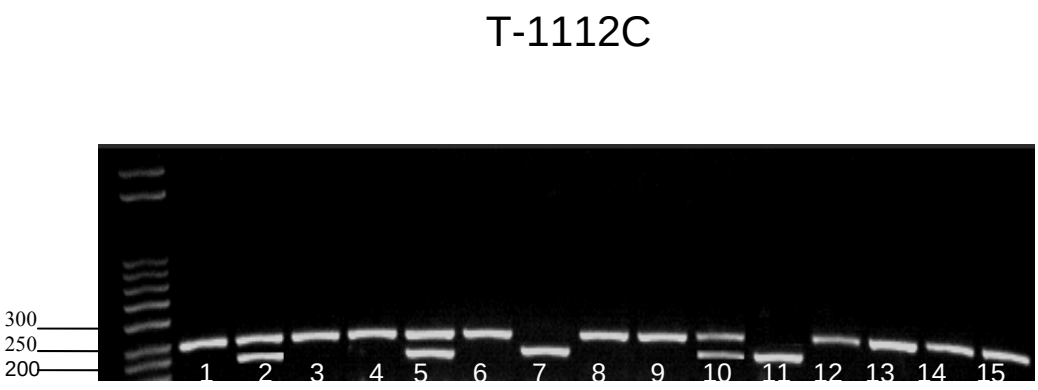


Figure 18: The 5' promoter polymorphism T-1112C on agarose gel after RFLP analysis. Lanes 1, 3, 4, 6, 8, 9, 12, 13, 14, 15 showing the genotype TT with a single band at 247 bp. Lanes 2, 5, and 10 showing heterozygous genotype CT with two bands at 247 and 224 bp. The homozygous CC is shown in lanes 7 and 11 with a single band at 224 bp.

Table 12: T-1112C polymorphism and its association with asthma, serum IgE levels, family history and severity of asthma

5' promoter polymorphism T-1112C					
Phenotype	**Subjects**	**Allele 1**	**Allele 2**	**Allele Frequency Difference (p value)**	**Risk for allele 2 (p value)**
Asthma	Controls Cases	0.78 0.55	0.22 0.45	0.0001	0.0008
Serum IgE levels	Low IgE (<150 IU/mL) High IgE (>150 IU/mL)	0.68 0.47	0.32 0.53	0.006	0.01
Family History	Negative Positive	0.54 0.55	0.46 0.45	0.80	0.90
Severity of Asthma	Mild Severe	0.71 0.44	0.29 0.56	0.0006	0.003

4.3.3. 5' promoter polymorphism A-646G

The PCR-RFLP analysis for this polymorphism revealed a 549 base pair fragment for AA genotype. The GG genotype resulted in two fragments 423 and 126 bp. All three bands were seen in case of a heterozygous (Figure 19).

This polymorphism was found in a very small frequency in our population and hence no association could be made with any of the phenotypes.

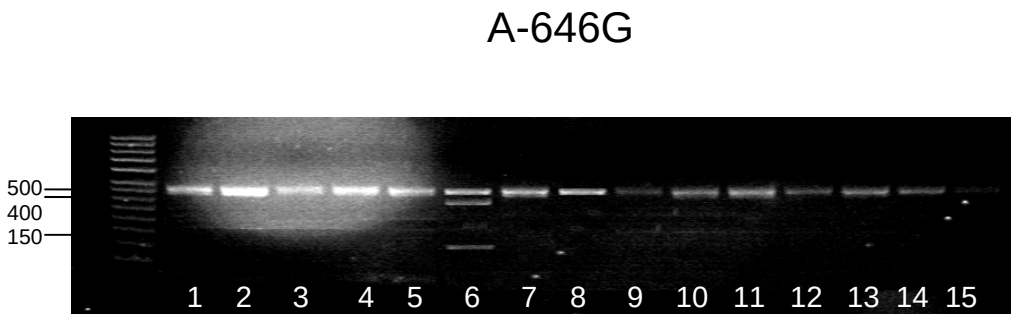


Figure 19: A-646G polymorphism on agarose gel after digestion with *DrdI* enzyme. Lanes 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14 and 15 showing uncut band at 549 bp representing AA genotype. Lane 6 shows three bands corresponding to 549, 423 and 126 bp representing the heterozygous genotype AG. No GG genotype was seen in this case.

4.3.4. 5' promoter polymorphism C-469T

The PCR-RFLP analysis for this polymorphism generated two fragments in case of TT genotype and the fragments were 303 and 246 bp long. In case of CC genotype a 549 bp band was seen (Figure 20).

All three bands could be seen in case of a heterozygous genotype. This polymorphism was found in a very small frequency in our population and hence no association could be made with any of the phenotypes.

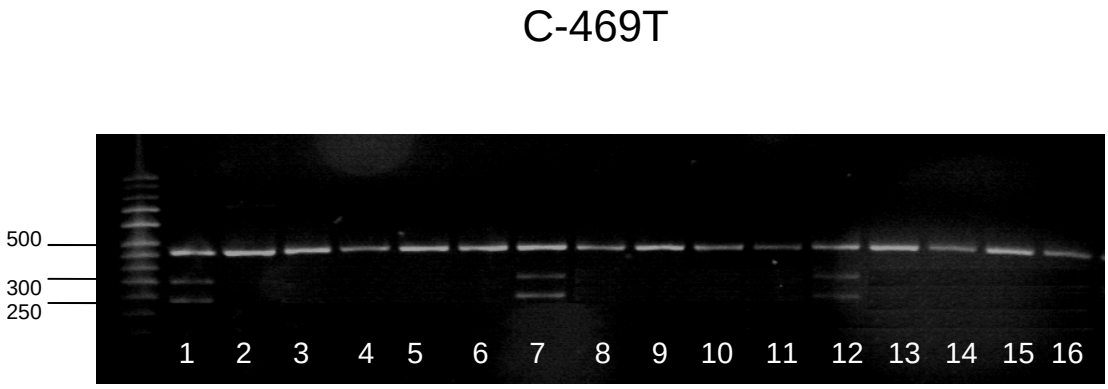


Figure 20: The promoter polymorphism C-469T on agarose gel after digestion by *AccI* enzyme. Lanes 2, 3, 4, 5, 6, 8, 9, 10, 11, 13, 14, 15 and 16 showing a single band at 549bp representing CC genotype. The lanes 1, 7, and 12 show three bands at 549, 303 and 246 bp representing heterozygous genotype CT. None of the samples showed TT genotype.

4.3.5. Exon 4 nonsynonymous polymorphism A2044 G

The GG genotype resulted in a 178 bp long fragment on PCR-RFLP analysis. A 210 bp fragment was seen in case of an AA genotype. Both fragments were seen when the genotype was heterozygous (Figure 21).

This polymorphism was found to be in Hardy Weinberg equilibrium in this population. A strong correlation was observed between this polymorphism and the serum IgE levels (p= 0.009). It was also strongly associated with the presence of asthma (p= 0.004) and the severity of asthma (p=0.007). No association was found with the family history of asthma (p= 0.8). The allele frequencies in comparison with each phenotype, the allele frequency difference and the risk for allele 2 are given in Table 13.

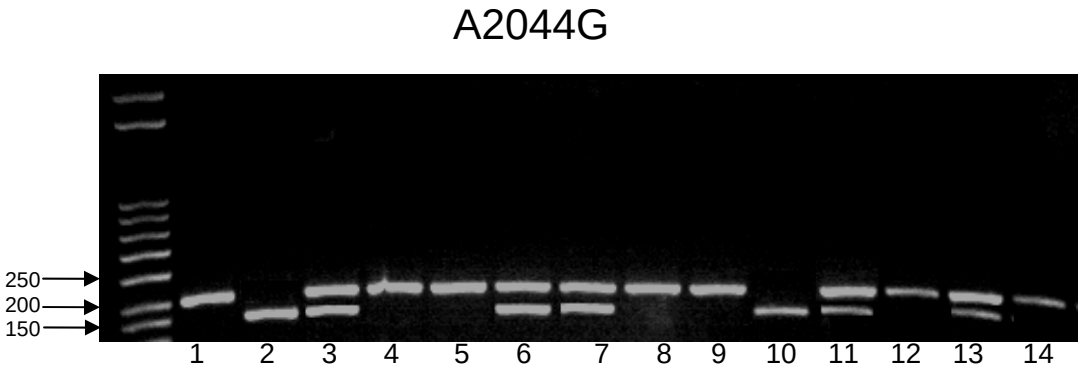


Figure 21: The exon 4 nonsynonymous polymorphism A2044G on agarose gel after digestion by *Nla*IV enzyme. Lanes 1, 4, 5, 8, 9, 12, and 14 showing a single band at 210 bp representing the AA genotype. Lanes 2 and 10 represent GG genotype with single band at 178 bp. The heterozygous genotype AG is seen in lanes 3, 6, 7, 11, and 13 with two bands at 210 and 178 bp.

Table 13: A2044 G polymorphism and its association with asthma, serum IgE levels, family history and severity of asthma

Exon 4 nonsynonymous polymorphism A2044 G					
Phenotype	**Subjects**	**Allele 1**	**Allele 2**	**Allele Frequency Difference (p value)**	**Risk for allele 2 (p value)**
Asthma	Controls Cases	0.73 0.53	0.27 0.47	0.0012	0.004
Serum IgE levels	Low IgE (<150 IU/mL) High IgE (>150 IU/mL)	0.67 0.49	0.33 0.51	0.03	0.009
Family History	Negative Positive	0.54 0.53	0.46 0.47	0.80	0.80
Severity of Asthma	Mild Severe	0.7 0.42	0.3 0.58	0.0005	0.007

4.3.6. 3' UTR polymorphism A2525G

The PCR-RFLP analysis revealed a 390 bp fragment in case of a GG genotype. Two fragments 253 and 157 bp long were seen with the AA genotype. All three fragments were seen on the gel in case of a heterozygous (Figure 22).

This polymorphism had no correlation with the presence of asthma (p = 0.17), family history of asthma (p= 0.12) or the severity of asthma (p= 0.76). A weaker association was seen with serum IgE levels (0.04). The allele frequencies in comparison with each phenotype, the allele frequency difference and the risk for allele 2 are given in Table 14.

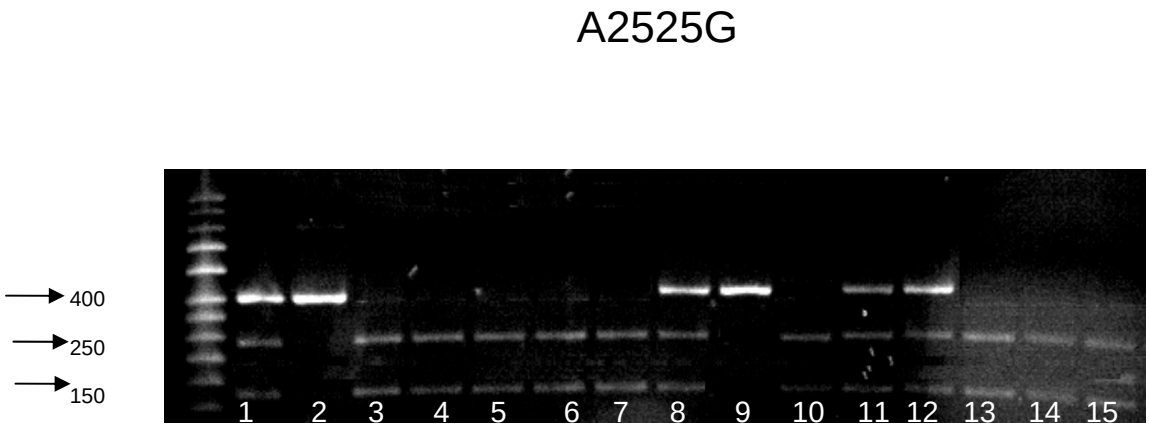


Figure 22: The 3' UTR polymorphism A2525G on agarose gel after digestion by *Nhe*I enzyme. Lanes 1, 8, 11 and 12 represent the heterozygous genotype AG with three bands at 390, 253 and 137 bp. Lanes 2 and 9 show a single band at 390 bp representing GG genotype. The AA genotype is seen in lanes 3, 4, 5, 6, 7, 10 13, 14, and 15 with two bands at 253 and 137 bp.

Table 14: A2525G polymorphism and its association with asthma, serum IgE levels, family history and severity of asthma

3' UTR polymorphism A2525G					
Phenotype	**Subjects**	**Allele 1**	**Allele 2**	**Allele Frequency Difference (p value)**	**Risk for allele 2 (p value)**
Asthma	Controls Cases	0.70 0.60	0.30 0.40	0.10	0.17
Serum IgE levels	Low IgE (<150 IU/mL) High IgE (>150IU/mL)	0.70 0.57	0.30 0.43	0.13	0.04
Family History	Negative Positive	0.52 0.65	0.48 0.35	0.11	0.12
Severity of Asthma	Severe Mild	0.60 0.60	0.40 0.40	0.98	0.76

5. DISCUSSION

Asthma is an inflammatory airways disease characterized by bronchial hyperresponsiveness and airway obstruction. In allergic asthma there is BHR to a variety of specific and non-specific stimuli, chronic pulmonary eosinophilia, elevated serum IgE levels and excessive airway mucus production [163].

Since no data was available on the spectrum of asthma and the precipitating factors of asthma for Pakistani population we emphasized on the clinical appraisal in our sample population. The randomly selected asthmatic patients visiting an asthma clinic for the follow up of their treatment were recruited for this study. When classified on the basis of their symptoms most of the patients belonged to the moderate intermittent to severe persistent class. A considerable number of patients reported a positive family history of asthma and/ or any other allergic condition. Seasonal exacerbations in their symptoms were high particularly in spring/winter though not many patients reported an allergy to pollens. Considering the environmental conditions in Lahore we stressed on environmental trigger factors in taking their history and exposure to dust and smoke turned out to be the major contributing factors. Not many patients reported pollen and exposure to other plants as triggering factors.

IgE has a well established role in the pathogenesis of asthma and has been associated with the severity of asthma and other allergic disorders like atopic dermatitis and allergic rhinitis. Serum total IgE levels were assessed in patients to

ascertain their atopy status and severity of the disease. As expected the levels were directly proportional to the severity of asthma and significantly higher in asthmatics as compared to the controls. Serum IgE levels were also used for association with the polymorphisms studied.

Hypothalamic pituitary adrenal axis was studied in these patients to see a possible suppression of the axis by inhaled corticosteroids. All of our patients reported taking inhaled steroids for the treatment of their symptoms, while none of them reported taking oral steroids. Oral steroid intake among asthma patients belonging to the lower socioeconomic status is quite common in Pakistani population. They are very cheap, easily available and most often used by quacks and other alternative medicine practitioners hence increasing our suspicion of oral steroid intake in these patients despite a negative history. No significant difference in the cortisol and ACTH levels was observed between patients and controls.

One reason could be that the HPA axis is under influence of many factors including cytokine levels, stress and exogenous steroids that it is difficult to interpret the results on the basis of ICS only. Recently there has been a lot of stress on the methods used for the determination of HPA axis but so far none has been established as reliable in the diagnosis and this could be the other reason for no significant difference [164, 165]. There are several tests measuring basal and dynamic cortisol and ACTH levels and different combinations have been explored

in previous studies. In our study we used a combination of cortisol and ACTH levels in the early morning samples.

IL-13 plays a pivotal role in the regulation of IgE synthesis, especially in those with allergic asthma [166-168]. Several polymorphisms in the IL-13 gene, located in chromosome 5q31±33, have recently been described and associated with the development of asthma. Atopy traits, such as elevated total serum IgE levels and eosinophilia are also associated with this disease and may predict the development of symptomatic asthma. IL- 13 is expressed in asthmatic airways and has an important role in the TH2 mediated allergic response and is therefore an excellent biologic candidate gene for the development or expression of diseases with atopy components such as asthma.

In the present study six polymorphisms of IL-13 gene were studied out of which four are in the 5' promoter region C-1512A, T-1112C, A-646G and C-469T, one polymorphism in exon 4 A2044G and one polymorphism from the 3' UTR A2525G. Two of these polymorphisms T-1112C and A2044G (Arg to Gln) have previously been studied in detail and have found to be associated with the development of asthma and high serum IgE levels respectively. In addition, SNP T-1112C appears to promote increased binding of nuclear proteins to the promoter region [19], whereas the amino acid change resulting from SNP 2044 could affect the interaction of IL-13 with IL-13R∝1 [21, 22].

Another promoter polymorphism C-1512A was studied by Graves et al and found to be in close linkage disequilibrium with A2044G [20]. We have included this polymorphism in our study to find out its association with asthma and/or related traits. In our study we have included two more promoter polymorphisms A-646G and C-469T, which have never been studied in asthma context before, to see their potential role in the development of asthma.

The 5' promoter region houses many important regulatory features and thus the polymorphisms in this region are suspected to have important effects on the development of asthma and related traits. This region codes for many transcription factors implicated in the regulation of T-cell gene transcription following engagement of antigen receptors [169]. T-cell regulatory factors, which are also present in this region, are responsible for controlling T cell development.

Another polymorphism from the 3' UTR region A2525G was selected for this study to see its regulatory effect on asthma since it is in close proximity with the polymorphism 2044. This polymorphism was discovered by Graves et al and they found it to be in close linkage disequilibrium with 2044 [20].

These six SNPs encompassed the entire IL-13 gene so that the contribution of genetic variation could be detected. Consistent with other reports that have evaluated IL-13 polymorphisms, we observed a significant association of several polymorphisms in IL-13 with various asthma phenotypes.

The asthma and atopy phenotypes studied in this population are the presence of asthma, and serum IgE levels. In addition to these phenotypes we have sought to identify a relationship between these polymorphisms and the family history of asthma and the severity of asthma as defined by their history and physical examination.

The promoter polymorphism T-1112C was found to be significantly associated with both the presence of asthma (p=0.0008) and the severity of asthma (p=0.003). A weaker association was found with serum IgE levels (p=0.01). Majority of previously conducted studies showed an association of this polymorphism with the presence of asthma and not serum IgE levels. Its association with serum IgE levels has only been demonstrated in two other populations, a German [118] and a Korean [119]. This polymorphism has also been associated with other diseases like Type 1 diabetes [126], schistosomiasis [141], respiratory syncitial virus [136] and latex allergy [138]. Its association with the severity of asthma points out at its possible role in the development of asthma. Functional studies are needed in patients on the basis of severity of asthma to further validate these results.

Nonsynonymous polymorphism 2044 was strongly associated with serum IgE levels (p=0.009), the presence of asthma (p=0.004) and severity of asthma (p=0.007) while no association was found with the family history of asthma in our population. Most of the studies associate this polymorphism with high IgE levels

but association with asthma [128]; eosinphilia [129] and atopy [133] have also been found in isolated studies. However, there are studies with conflicting reports from some populations in which they found no association with these traits. No correlation between the polymorphism 2044 and serum IgE levels was found in Mexican [130], and Costa Rican population [117]. This polymorphism has also been demonstrated in other diseases like Type 1 diabetes [137], onchocerciasis [134] atopic dermatitis [133] and juvenile idiopathic arthritis [124]. Its association with asthma and its severity is an important finding as it has previously been demonstrated in an isolated study.

The two new promoter polymorphisms A-646G and C-469T had a very low frequency in our population and thus could not be analyzed further for association with phenotypes.

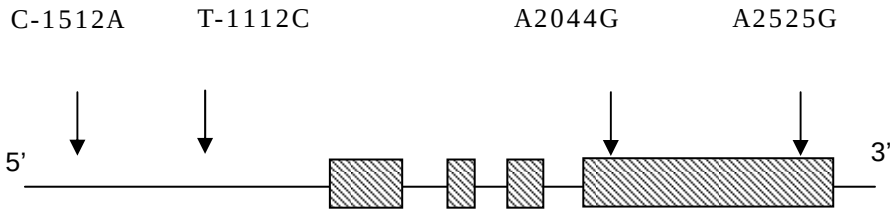
The other promoter polymorphism C-1512A which was previously found to be in linkage disequilibrium with A2044G, has been shown to be associated with asthma ($p=0.001$) and weakly with its severity ($p=0.02$) and family history of asthma ($p=0.03$). The functional role of this polymorphism in the development of asthma has not yet been explored. A functional study on this polymorphism will reveal its possible role and importance in the development of asthma.

The 3' UTR polymorphism A2525G has been found to be weakly associated with serum IgE levels ($p=0.04$). However, it showed no association with any other

phenotype in our population. This polymorphism was previously described to be in linkage disequilibrium with the polymorphism A2044G.

A comparison between results from this association study and other previously studied populations is given in Figure 23.

Inclusion of severity of asthma for association with the identified polymorphisms was another new aspect of this study. Leung et al., in 2001 conducted a study on hospital based cohort reporting an association between the exon 4 polymorphism and atopy but not asthma [116]. They concluded that the association between the said polymorphism and asthma could be weakened by the fact that most of their patients were mild to moderate asthmatics. It is therefore important to classify the patients into groups on the basis of their severity and then comparing the genotypes with different levels of severity. This point is supported by Weir et al., studying B2 adrenergic receptor haplotypes, showed that the severity of asthma in their recruited cohort significantly affected results on the association studies for asthma predisposition genotypes [170].



		C-1512A	T-1112C	A2044 G	A2525G
Asthma	*	++	++++	+++	-
	#	-	+++	+	++
IgE	*	-	++	+++	++
	#	-	+	+++	-
Severity of asthma	*	+	+++	+++	-
	#	-	-	-	-
Family History of asthma	*	+	-	-	-
	#	-	-	-	-
Atopy	*	-	-	-	-
	#	-	-	++	+
Other conditions	#		Schistosomiasis, RSV infection, COPD, T1D, latex allergy	Onchocerciasis, T1 Diabetes, Juvenile Rheumatoid arthritis	

Figure 23: Schematic diagram of IL-13 gene and location of four polymorphisms studied showing a comparison of association with different phenotypes in this population and other previously studied populations.

+: association -: no association *: Results from this study, #: results from previous studies

6. CONCLUSIONS

Our study population mainly comprised of moderate intermittent to severe persistent asthmatics. Exposure to dust and smoke turned out to be the major precipitating factors in local population. Their serum IgE levels were high in asthmatics and increased with the severity of asthma. They were significantly raised in patients with a history of allergy as compared to non allergic patients. No correlation could be found with the family history of asthma. Adrenal functions were normal in asthmatics as compared to the control population. This was probably due to multiple factors influencing their HPA axis including stress, cytokines and inhaled corticosteroids.

The promoter polymorphism T-1112C and the polymorphism A2044G showed strong associations with both the presence of asthma and serum total IgE levels, not seen in other previously studied populations. Their role in asthma development was further strengthened by their association with the severity of asthma. The other promoter polymorphism C-1512A had an association with the presence and severity of asthma. This was the only polymorphism that showed an association with the family history of asthma. The 3'UTR polymorphism A2525G showed weak association with serum IgE levels only.

k. APPENDICES

Appendix I: Patient Consent Form

Department Of Physiology and Cell Biology
University Of Health Sciences, Lahore

Consent by Subject to Participate In the Study

Polymorphisms of Interleukin 13 (IL13) in Local Asthmatic Population

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I _____S/O, W/O, D/O _____

voluntarily consent to participate in this research study described to me in detail and understand that the study is designed to add to medical knowledge and may of no direct benefit to myself. I acknowledge that the purpose of experiment, risks and discomforts involved and the nature and purpose of the procedure have been explained to me by Dr. Afia Hasnain. I have had the opportunity to ask questions about the experiment.

_____ _____
Name of Subject Signature of subject

I confirm that I have explained to the subject the nature and purpose of the procedures.

Dr Afia Hasnain _____
Name of Interviewer Signature of Interviewer

Prof. Dr. M Nawaz _____
Name of Supervisor Signature of Supervisor

Appendix II: Questionnaire

Asthma Questionnaire [171]

PATIENT #			
NAME			
AGE			
HOSPITAL			
ADRESS			
CONTACT No.			
☐ Height:		☐ Weight:	☐ BMI:
☐ Married		☐ Unmarried	☐ No of Children ____
1.	Frequency	☐ Continual ☐ Daily ☐ > twice weekly ☐ < twice weekly	
2.	Physical Activity	☐ Limited ☐ exacerbations effect activity ☐ no effect	
3.	Exacerbations	☐ Frequent ☐ > twice weekly ☐ Brief, may last for hours or days	
4.	Night time symptoms	☐ Frequent ☐ > once weekly ☐ > twice weekly ☐ < twice monthly	
5.	PEF	☐ < 60% ☐ > 60- <80 % ☐ > 80 %	
6.	Family History	☐ Yes Specify;_____ ☐ No	

7.	Asthma trigger factors	☐ Dust ☐ Smoke ☐ Animal dander ☐ Pollen ☐ Physical exercise ☐ Anxiety or stress ☐ Acute respiratory infection ☐ Food items ☐ Seasonal variation ☐ Aspirin
8.	Symptoms in response to allergens	

	Near cats, dogs or horses, near feathers, including pillows, quilts or duvets, or in a dusty part of the house; do you:	Near trees, grass or flowers, or when there is a lot of pollen about; do you:
start to cough?	☐	☐
start to wheeze?	☐	☐
Get a feeling of chest tightness?	☐	☐
Start to feel short of breath?	☐	☐
Get a runny, stuffy nose or sneeze?	☐	☐
Get itchy or watering eyes?	☐	☐
	☐	☐

9. Seasonal exacerbations in symptoms			☐ Yes ☐ No	
If yes, which season	☐ Winter	☐ spring	☐ summer	☐ autumn
10. Currently taking any medicines (including inhalers, aerosols or tablets) for asthma?				

Appendix III: Classification of asthma severity

Classification of Asthma Severity on the basis of history and physical examination [142]

Severity	Symptoms	Nighttime symptoms	Lung function
Severe Persistent	Continual symptoms Limited physical activity Frequent exacerbations	Frequent	FEV1 or PEF ≤ 60% predicted
Moderate Persistent	Daily symptoms Daily use of inhaled short-acting beta2-agonist Exacerbations affect activity Exacerbations ≥ 2 times a week; may last days	>1 time a week	FEV1 or PEF >60% –<80% predicted

Mild Persistent	Symptoms >2 times a week but <1 time a day Exacerbations may affect activity	>2 times a month	FEV1 or PEF ≥ 80% predicted
Mild Intermittent	Symptoms ≤ 2 times a week Asymptomatic and normal PEF between exacerbations Exacerbations brief (from a few hours to a few days); intensity may vary	≤ 2 times a month	FEV1 or PEF ≥ 80% predicted

l. REFERENCES

1. National Heart Lung and Blood Institute. Global initiative for asthma. Global strategy for asthma management and prevention. Dec 2007. NHLI/WHO Workshop report.

2. Gibson GJ. Lung Function And Bronchial Hyperresponsiveness: Physiological Aspects. In: Clark TJH, Godfrey S, Lee TH, Thomson NC, ed. Asthma. London: Arnold; 2000: pp. 32-59.

3. Elias JA, Zhu Z, Chupp G, Homer RJ. Airway remodeling in asthma. J. Clin. Invest. 1999; 104: 1001-1006.

4. Busse WW, Lemanske RF. Advances in Immunology: Asthma. N. Engl. J. Med. 2001; 344: 350-362.

5. Corry DB, Kheradmand F. Induction and regulation of the IgE response. Nature. 1999; 402: B18-B23.

6. World Health Organization. Fact sheet on asthma. Fact sheet no. 307, May 2008.
   http://www.who.int/mediacentre/factsheets/fs307/en/index.html

7. Matthew M, Denise F, Shaun H, Richard B. Global burden of asthma. A report developed for the global initiative for asthma (GINA). 2004.

8. Burrows B, Martinez FD, Halonen M, Barbee RA, Cline MG. Association of asthma with serum IgE levels and skin-test reactivity to allergens. N. Engl. J. Med. 1989; 320: 271-277.

9. Kobayashi Y, Kondo N, Shinoda S, Agata H, Fukutomi O, Orii T. Predictive values of cord blood IgE and cord blood lymphocyte responses to food antigens in allergic disorders during infancy. J. Allergy. Clin. Immunol. 1994; 94: 907-916.

10. Platts-Mills TA. The role of immunoglobulin E in allergy and asthma. Am. J. Respir. Crit. Care Med. 2001; 16: 1-5.

11. Howarth PH, Durham SR, Kay AB, Holgate ST. The relationship between mast cell-mediator release and bronchial reactivity in allergic asthma. J. Allergy Clin. Immunol. 1987; 80: 703-711.

12. Buske-Kirschbaum A, von Auer K, Krieger S, Weis S, Rauh W, Hellhammer D. Blunted cortisol responses to psychosocial stress in asthmatic children: a general feature of atopic disease. Psychosom. Med. 2003; 65: 806-810
13. Russel G. Commentary: Symptomatic adrenal insufficiency during inhaled corticosteroid treatment. Arch. Dis. Child. 2001; 85: 333–334.

14. Bisgaard H, Pedersen S. Safety of treatment. Eur. Respir. J. 1996; 21: 28–34.

15. Sizonenko P. Effects of inhaled or nasal glucocortioids on adrenal function and growth. J. Pediatr. Endocrinol. Metab. 2002; 15: 5–26.

16. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD. IL-13: central mediator of allergic asthma. Science. 1998; 282: 2258–2261.

17. Zurawski G, deVries J. Interleukin-13, an interleukin-4 like cytokine that acts on monocytes and B cells but not T-cells. Immunol. Today. 1994; 15: 19–26.

18. Wjst M, Fischer G, Immervoll T, Jung M, Saar K, Rueschendorf F, Reis A, Ulbrecht M, Gomolka M, Weiss EH, Jaeger L, Nickel R, Richter K, Kjellman NI, Griese M, von Berg A, Gappa M, Riedel F, Boehle M, van Koningsbruggen S, Schoberth P, Szczepanski R, Dorsch W, Silbermann M, Wichmann HE. A genome-wide search for linkage to asthma. Genomics. 1999; 58: 1-8.

19. van der Pouw Kraan TC, van Veen A, Boeije LC, van Tuyl SA, de Groot ER, Stapel SO, Bakker A, Verweij CL, Aarden LA, van der Zee JS. An IL-13 promoter polymorphism associated with increased risk of allergic asthma. Genes Immun.1999; 1: 61–65.

20. Graves PE, Kabesch M, Halonen M, Holberg CJ, Baldini M, Fritzsch C Weiland SK, Erickson RP, von Mutius E, Martinez FD.A cluster of seven tightly linked polymorphisms in the IL13 gene is associated with total serum IgE levels in three populations of white children. J. Allergy. Clin. Immunol. 2000; 105: 506–513.

21. Heinzmann A, Mao XQ, Akaiwa M, Kreomer RT, Gao PS, Ohshima K, Umeshita R, Abe Y, Braun S, Yamashita T, Roberts MH, Sugimoto R, Arima K, Arinobu Y, Yu B, Kruse S, Enomoto T, Dake Y, Kawai M, Shimazu S, Sasaki S, Adra CN, Kitaichi M, Inoue H, Yamauchi K, Tomichi N, Kurimoto F, Hamasaki N, Hopkin JM, Izuhara K, Shirakawa T, Deichmann KA. Genetic variants of IL13 signaling and human asthma and atopy. Hum. Mol. Genet. 2000; 9: 549–559.

22. Celedon JC, Soto-Quiros ME, Palmer LJ, Senter J, Mosley J, Silverman EK, Weiss ST. Lack of association between a polymorphism in the interleukin- 13 gene and total serum immunoglobulin E level among nuclear families in Costa Rica. Clin. Exp. Allergy 2002; 32: 387–390.

23. Holgate ST. Genetic and environmental interaction in allergy and asthma. J Allergy Clin Immunol.1999; 104: 1139-1146.

24. Holt PG, Macaubas C, Stumbles PA, Sly PD. The role of allergy in the development of asthma. Nature. 1999; 402: B12-B17.

25. Warner JO, Warner JA. Allergy and Asthma. In: Clark TJH, Godfrey S, Lee TH, Thomson NC, ed. Asthma. London: Arnold, 2000: 444-456.

26. McConnell W, Holgate ST. The Definition Of Asthma: Its Relationship To Other Chronic Obstructive Lung Diseases. In: Clark TJH, Godfrey S, Lee TH, Thomson NC, ed. Asthma. London: Arnold; 2000: pp.1-31.

27. Holgate ST, Lackie PM, Davies DE, Roche WR, Walls AF. The bronchial epithelium as a key regulator of airway inflammation and airway remodeling in asthma. Clin. Exp. Allergy. 1999; 2: 90-95.

28. Busse W, Elias J, Sheppard D, Banks-Schlegel S. Airway remodeling and repair. Am. J. Respir. Crit. Care Med. 1999; 160: 1035-1042.

29. Martinez FD, Holt PG. Role of microbial burden in etiology of allergy and asthma. Lancet. 1999; 354: 12-15.

30. Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang, Zhang Y, Elias JA. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. J. Clin. Invest. 1999; 103: 779-788.

31. Mendis S, Fukino K, Cameron A, Laing R, Filipe A Jr, Khatib O, Leowski J, Ewen M. The availability and affordability of selected essential medicines for chronic diseases in six low- and middle-income countries. Bull. World Health Organ. 2007; 85: 279-88.

32. Jamalvi SW, Raza SJ, Naz F, Shamim S, Jamalvi SM. Management of acute asthma in children using metered dose inhaler and small volume nebulizer. J. Pak. Med. Assoc. 2006; 56: 595-599.

33. Kuehni CE, Strippoli MP, Low N, Silverman M. Asthma in young south Asian women living in the United Kingdom: the importance of early life. Clin. Exp. Allergy. 2007; 37: 47-53.

34. Hussain SF, Omar MD, Arif H, Sameeruddin SR, Zubairi AB, Khan JA.Trends in hospital-based management of acute asthma from a teaching hospital in South Asia. Int. J. Clin. Pract. 2005; 59: 912-916.

35. Hussain SF, Zahid S, Khan JA, Haqqee R. Asthma management by general practitioners in Pakistan. Int. J. Tuberc. Lung Dis. 2004; 8: 414-417.

36. Shahzad K, Akhtar S, Mahmud S. Prevalence and determinants of asthma in adult male leather tannery workers in Karachi, Pakistan: a cross sectional study. BMC Public Health. 2006; 6: 292.

37. Johansson SG, Bennich HH, Berg T. The clinical significance of IgE. Prog. Clin. Immunol. 1972; 1: 157-181.

38. Sears MR, Burrows B, Flannery EM, Herbison GP, Hewitt CJ, Holdaway MD. Relation between airway responsiveness and serum IgE in children with asthma and in apparently normal children. N. Engl. J. Med. 1991; 325: 1067-1071.

39. Halonen M, Stern D, Taussig LM, Wright A, Ray CG, Martinez FD. The predictive relationship between serum IgE levels at birth and subsequent incidences of lower respiratory illnesses and eczema in infants. Am. Rev. Respir. Dis. 1992; 146: 866-870.

40. Hopp RJ, Bewtra AK, Nair NM, Townley RG. Specificity and sensitivity of methacholine inhalation challenge in normal and asthmatic children. J Allergy Clin. Immunol. 1984; 74: 154-158.

41. Burrows B, Lebowitz MD, Barbee RA, Cline MG. Findings before diagnoses of asthma among the elderly in a longitudinal study of a general population sample. J. Allergy Clin. Immunol. 1991; 88: 870-877.

42. Wiesch DG, Meyers DA, Bleecker ER. Genetics of asthma. J. Allergy Clin. Immunol. 1999; 104: 895-901.

43. Kay A B, Barata L, Meng Q, Durham S R, Ying S. Eosinophils and eosinophil-associated cytokines in allergic inflammation. Int. Arch. Allergy Immunol. 1997; 113: 196-199.

44. Umetsu DT, DeKruyff RH. Th1 and Th2 CD4+ cells in the pathogenesis of allergic diseases. Proc. Soc. Exp. Biol. Med. 1997; 215: 11-20.

45. Ishizaka K, Ishizaka T. Physicochemical properties of reaginic antibody. Association of reaginic activity with an immunoglobulin other than γA- or γG-globulin. J. Allergy. 1996; 37: 169-185.

46. Johansson SG, Bennich H. Immunological studies of an atypical (myeloma) immunoglobulin. Immunology. 1967; 13: 381-394.

47. King CL, Poindexter RW, Ragunathan J. Frequency analysis of IgE-secreting B lymphocytes in persons with normal or elevated serum IgE levels. J. Immunol. 1991; 146: 1478-1483.

48. Bos JD, Van Leent EJ, Sillevis SJ. The millennium criteria for the diagnosis of atopic dermatitis. Exp. Dermatol. 1998; 7: 132-138.

49. Novak N, Bieber T, Leung DYM. Immune mechanisms leading to atopic dermatitis. J. Allergy Clin. Immunol. 2003; 112: 128-129.

50. Gould HJ, Sutton BJ, Beavil AJ, Beavil RL. The biology of IgE and the basis of allergic disease. Annu. Rev. Immunol. 2003; 21: 579-628.

51. Miller WL, Tyrrell JB. Adrenal Disease. In: Felig P, Baxter JD, Frohman LA, eds. Endocrinology and Metabolism. New York: McGraw Hill; 1995: pp. 573–580.

52. Chrousos GP. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. N. Engl. J. Med. 1995; 332: 1351–62.

53. Orth DV, Kovacs WJ. The Adrenal Cortex. In: Wilson JD, Foster DW, Kronenberg HM, Larsen PR, eds. William's Textbook of Endocrinology. Philadelphia: WB Saunders Company; 1998: pp. 517–86.

54. Knutsson U, Dahlgren J, Marcus C, Rosberg S, Brönnegård M, Stierna P, Albertsson-Wikland K. Circadian cortisol rhythms in healthy boys and girls: relationship with age, growth, body composition, and pubertal development. J. Clin. Endocrinol. Metab. 1997: 82: 536–40.

55. Buske-Kirschbaum A, Jobst S, Wustmans A, Kirschbaum C, Rauh W, Hellhammer DH. Attenuated free cortisol response to psychosocial stress in children with atopic dermatitis. Psychosom. Med. 1997; 59: 419 –426.

56. Sternberg EM, Hill JM, Chroussos GP, Kamilaris T, Listwak SJ, Gold PW, Wilder RL. Inflammatory mediator-induced hypothalamic-pituitary adrenal adrenal axis activation is defective in streptococcal cell wall arthritis susceptible Lewis rats. Proc. Natl. Acad. Sci. USA 1989; 86: 2374 –2378.

57. Elenkov IJ, Chrousos GP. Stress hormones, Th1/Th2 patterns, pro/anti-inflammatory cytokines and susceptibility to disease. TEM 1999; 10: 359–368.
58. Akira S, Hirano T, Taga T, Kishimoto T. Biology of multifunctional cytokines: IL 6 and related molecules (IL 1 and TNF). FASEB J. 1990; 4: 2860-2867.

59. Hesse DG, Tracey KJ, Fong Y, Manogue KR, Palladino MA Jr, Cerami A, Shires GT, Lowry SF. Cytokine appearance in human endotoxemia and primate bacteremia. Surg. Gynecol. Obstet. 1988; 166: 147-153

60. van Deventer SJH, Buller HR, ten Cate JW, Aarden LA, Hack CE, Sturk A. Experimental endotoxemia in humans: analysis of cytokine release and coagulation, fibrinolytic, and complement pathways. Blood. 1990; 76: 2520-2526.

61. Boumpas DT, Chrousos GP, Wilder RL, Cupps TR, Balow JE. Glucocorticoid therapy for immune-mediated diseases: basic and clinical correlates. Ann. Intern. Med. 1993; 119: 1198-1208.

62. Hirano T, Akira S, Taga T, Kishimoto T. Biological and clinical aspects of interleukin 6. Immunol. Today. 1990; 11: 443-449.

63. Imura H, Fukata J, Mori T. Cytokines and endocrine functions: an interaction between the immune and neuroendocrine systems. Clin. Endocrinol. 1991; 35: 107-115.

64. Bernardini R, Kamilaris TC, Calogero AE, Johnson EO, Gomez MT, Gold PW, Chrousos GP. Interactions between tumor necrosis factor-a, hypothalamic corticotropin-releasing hormone, and adrenocorticotropin secretion in the rat. Endocrinology. 1990; 126: 2876-2881.

65. Sapolsky R, Rivier C, Yamamoto G, Plotsky P, Vale W. Interleukin-1 stimulates the secretion of hypothalamic corticotropin-releasing factor. Science. 1987; 238: 522-4.

66. Naitoh Y, Fukata J, Tominaga T, Nakai Y, Tamai S, Mori K, Imura H. Interleukin-6 stimulates the secretion of adrenocorticotropic hormone in conscious, free-moving rats. Biochem. Biophys. Res. Commun. 1988; 155: 1459-1463.

67. Perlstein RS, Mougey EH, Jackson WE, Neta R. Interleukin-1 and interleukin-6 act synergistically to stimulate the release of adrenocorticotropic hormone in vivo. Lymphokine Cytokine Res. 1991; 10: 141-146.

68. Perlstein RS, Whitnall MH, Abrams JS, Mougey EH, Neta R. Synergistic roles of interleukin-6, interleukin-1, and tumor necrosis factor in adrenocorticotropin response to bacterial lipopolysaccharide in vivo. Endocrinol. 1993; 132: 946-52

69. Perera BJC. Efficacy and cost effectiveness of inhaled steroids in asthma in a developing country. Arch. Dis. Child. 1995; 72: 312–316.

70. Calpin C, Macarthur C, Stephens D, Feldman W, Parkin PC. Effectiveness of prophylactic inhaled steroids in childhood asthma: a systematic review of the literature. J. Allergy Clin. Immunol. 1997; 100: 452–457.

71. Price J. The role of inhaled corticosteroids in children with asthma. Arch. Dis. Child. 2000; 82: 1–4.

72. Bisgaard H. Use of inhaled corticosteroids in pediatric asthma. Pediatr. Pulmonol. Suppl. 1997; 15: 27–33.

73. Wagener JS. Inhaled steroids in children: risks versus rewards. J. Pediatr. 1998; 132: 381–383.

74. Lipworth BJ. Adrenal suppression with inhaled corticosteroids. Ann. Allergy Asthma Immunol. 2001; 87: 359–361.

75. Dluhy RG. Clinical relevance of inhaled corticosteroids and HPA axis suppression. J. Allergy Clin. Immunol. 1998; 101: 447–450.

76. Todd GRG, Acerini CL, Ross-Russel R, Zahra S, Warner JT, McCance D. Survey of adrenal crisis associated with inhaled corticosteroids in the United Kingdom. Arch. Dis. Child. 2002; 87: 457–461.

77. Patel L, Wales JK, Kibirige MS, Massarano AA, Couriel JM, Clayton PE. Symptomatic adrenal insufficiency during inhaled corticosteroid treatment. Arch. Dis. Child 2001; 85: 330–334.

78. Marsh DG, Neely JD, Breazeale DR, Ghosh B, Freidhoff LR, Ehrlich-Kautzky E, Schou C, Krishnaswamy G, Beaty TH. Linkage analysis of IL4 and other chromosome 5q31.1 markers and total serum immunoglobulin E concentrations. Science 1994; 264: 1152-115

79. Meyers DA, Postma DS, Panhuysen CI, Xu J, Amelung PJ, Levitt RC Bleecker ER. Evidence for a locus regulating total serum IgE levels mapping to chromosome 5. Genomics 1994; 23: 464-470.

80. Postma DS, Bleecker ER, Amelung PJ, Holroyd KJ, Xu J, Panhuysen CI, Meyers DA, Levitt RC. Genetic susceptibility to asthma-bronchial hyperresponsiveness co inherited with a major gene for atopy. N. Engl. J. Med.1995; 333: 894-900.

81. Noguchi E, Shibasaki M, Arinami T, Takeda K, Maki T, Miyamoto T, Kawashima T, Kobayashi K, Hamaguchi H. Evidence for linkage between asthma/atopy in childhood and chromosome 5q31- q33 in a Japanese population. Am. J. Respir. Crit. Care Med. 1997; 156: 1390-1393.

82. Doull IJ, Lawrence S, Watson M, Begishvili T, Beasley RW, Lampe FHolgate T, Morton NE. Allelic association of gene markers on chromosomes 5q and 11q with atopy and bronchial hyperresponsiveness. Am J Respir Crit Care Med 1996; 153: 1280-1284.

83. CSGA. A genome-wide search for asthma susceptibility loci in ethnically diverse populations. The Collaborative Study on the Genetics of Asthma (CSGA). Nat. Genet. 1997; 15: 389-392.

84. Ober C, Cox NJ, Abney M, Di Rienzo A, Lander ES, Changyaleket B, Gidley H, Kurtz B, Lee J, Nance M, Pettersson A, Prescott J, Richardson A, Schlenker E, Summerhill E, Willadsen S, Parry R. Genome-wide search for asthma susceptibility loci in a founder population. Hum. Mol. Genet.1998; 7: 1393-1398.

85. Kamitani A, Wong ZY, Dickson P, van Herwerden L, Raven J, Forbes AB, Abramson MJ, Walters EH, Harrap SB. Absence of genetic linkage of chromosome 5q31 with asthma and atopy in the general population. Thorax. 1997; 52: 816-817.

86. Laitinen T, Kauppi P, Ignatius J, Ruotsalainen T, Daly MJ, Kääriäinen H, Kruglyak L, Laitinen H, de la Chapelle A, Lander ES, Laitinen LA, Kere J.Genetic control of serum IgE levels and asthma: linkage and linkage disequilibrium studies in an isolated population. Hum. Mol. Genet. 1997; 6: 2069- 2076.

87. Mansur AH, Christie G, Turner A, Bishop DT, Markham AF, Helms P Morrissson JF. Lack of linkage between chromosome 5q23-33 markers and IgE/bronchial hyperreactivity in 67 Scottish families. Clin. Exp. Allergy. 2000; 30: 954-961.

88. Ulbrecht M, Eisenhut T, Bönisch J, Kruse R, Wjst M, Heinrich J, Wichmann HE, Weiss EH, Albert ED. High serum IgE concentrations:

association with HLA-DR and markers on chromosome 5q31 and chromosome 11q13. J. Allergy Clin. Immunol. 1997; 99: 828-836.

89. Blumenthal MN, Wang Z, Weber JL, Rich SS. Absence of linkage between 5q markers and serum IgE levels in four large atopic families. Clin. Exp. Allergy. 1996; 26: 892-896.

90. Cookson WOCM, Sharp PA, Faux JA, Hopkin JM. Linkage between immunoglobulin E responses underlying asthma and rhinitis and chromosome 11q. Lancet. 1989; 1: 1292-1295.

91. Collee JM, ten Kate LP, de Vries HG, Kliphuis JW, Bouman K, Scheffer H, Gerritsen J. Allele sharing on chromosome 11q13 in sibs with asthma and atopy. Lancet. 1993; 342: 936-936.

92. Folster Holst R, Moises HW, Yang L, Fritsch W, Weissenbach J, Christophers E. Linkage between atopy and the IgE high-affinity receptor gene at 11q13 in atopic dermatitis families. Hum. Genet. 1998; 102: 236-239.

93. Shirakawa T, Hashimoto T, Furuyama J, Takeshita T, Morimoto K. Linkage between severe atopy and chromosome 11q13 in Japanese families. Clin. Genet. 1994; 46: 228-232

94. van Herwerden L, Harrap SB, Wong ZY, Abramson MJ, Kutin JJ, Forbes AB, Raven J, Lanigan A, Walters EH. Linkage of high-affinity IgE receptor gene with bronchial hyperreactivity, even in absence of atopy. Lancet. 1995; 346: 1262-1265.

95. Lympany P, Welsh K, MacCochrane G, Kemeny DM, Lee TH. Genetic analysis using DNA polymorphism of the linkage between chromosome 11q13 and atopy and bronchial hyperresponsiveness to methacholine. J. Allergy Clin. Immunol. 1992; 89: 619-628.

96. Rich SS, Roitman-Johnson B, Greenberg B, Roberts S, Blumenthal MN. Genetic analysis of atopy in three large kindreds: no evidence of linkage to D11S97. Clin. Exp. Allergy. 1992; 22: 1070-1076.

97. Hizawa N, Yamaguchi E, Ohe M, Itoh A, Furuya A, Ohnuma N, Kawakami Y. Lack of linkage between atopy and locus 11q13. Clin. Exp. Allergy. 1992; 22:1065-1069.

98. Coleman R, Trembath RC, Harper JI. Chromosome 11q13 and atopy underlying atopic eczema. Lancet. 1993; 341:1121-1122.

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

100. Martinati LC, Trabetti E, Casartelli A, Boner AL, Pignatti PF. Affected sib-pair and mutation analyses of the high affinity IgE receptor beta chain locus in Italian families with atopic asthmatic children. Am. J. Respir. Crit. Care Med. 1996; 153: 1682-1685.

101. Amelung PJ, Postma DS, Xu J, Meyers DA, Bleecker ER. Exclusion of chromosome 11q and the FCERIB gene as aetiological factors in allergy and asthma in a population of Dutch asthmatic families. Clin. Exp. Allergy. 1998; 28: 397- 403.

102. Barnes KC, Neely JD, Duffy DL, Freidhoff LR, Breazeale DR, Schou C, Naidu RP, Levett PN, Renault B, Kucherlapati R, Iozzino S, Ehrlich E, Beaty TH, Marsh DG. Linkage of asthma and total serum IgE concentration to markers on chromosome 12q: evidence from Afro-Caribbean and Caucasian populations. Genomics. 1996; 37: 41-50.

103. Nickel R, Wahn U, Hizawa N, Maestri N, Duffy DL, Barnes KC, Beyer K, Forster J, Bergmann R, Zepp F, Wahn V, Marsh DG. Evidence for linkage of chromosome 12q15-q24.1 markers to high total serum IgE concentrations in children of the German Multicenter Allergy Study. Genomics. 1997; 46:159-162.

104. Daniels SE, Bhattacharrya S, James A, Leaves NI, Young A, Hill MR, Faux JA, Ryan GF, le Söuef PN, Lathrop GM, Musk AW, Cookson WO. A genome-wide search for quantitative trait loci underlying asthma. Nature. 1996; 383: 247-250.

105. Meyers DA, Postma DS, Stine OC, Koppelman GH, Ampleford EJ, Jongepier H, Howard TD, Bleecker ER. Genome screen for asthma and bronchial hyperresponsiveness: interactions with passive smoke exposure. J Allergy Clin Immunol. 2005 Jun; 115:1169-75.

106. Kurz T, Hoffjan S, Hayes MG, Schneider D, Nicolae R, Heinzmann A, Jerkic SP, Parry R, Cox NJ, Deichmann KA, Ober C. Fine mapping and positional candidate studies on chromosome 5p13 identify multiple asthma susceptibility loci. J Allergy Clin Immunol. 2006 Aug; 118: 396-402

107. Zurawski SM, Vega F, Juyghe B, Zurawski G. Receptors for interleukin-13 and interleukin- 4 are complex and share a novel component that functions in signal transduction. EMBO J. 1993; 12: 2663–2670.

108. Akbari O, Stock P, Meyer E, Kronenberg M, Sidobre S, Nakayama T, Taniguchi M, Grusby MJ, DeKruyff RH, Umetsu DT. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. Nat. Med. 2003; 9: 582–588.

109. Schmid-Grendelmeier P, Altznauer F, Fischer B, Bizer C, Straumann A, Menz G, Blaser K, Wüthrich B, Simon HU. Eosinophils express functional IL-13 in eosinophilic inflammatory diseases. J. Immunol. 2002; 169: 1021–1027.

110. Hilton DJ, Zhang J-G, Metcalf D, Alexander WS, Nicols NA, Wilson TA. Cloning and characterization of a binding subunit of the interleukin 13 receptor that is also a component of interleukin 4 receptor. Proc. Natl. Acad. Sci. USA. 1996; 93: 497–501.

111. Aman MJ, Tayebi N, Obii NI, Puri RK, Modi WS, Leonard WJ. cDNA cloning and characterization of the human interleukin-13 receptor alpha chain. J. Biol. Chem. 1996; 271:29265–29270.

112. Caput D, Laurent P, Kaghad M, Lelias JM, Lefort S, Vita N, Ferrara P. Cloning and characterization of specific interleukin (IL)-13 binding protein and structurally related to the IL-5 receptor a chain. J. Biol. Chem. 1996; 271: 16921–16926.

113. Wills-Karp M, Chiaramonte M. Interleukin-13 in asthma. Curr. Opin. Pulm. Med. 2003; 9: 21–27.

114. Grünig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, Sheppard D, Mohrs M, Donaldson DD, Locksley RM, Corry DB. Requirement for IL-13 independently of IL-4 in experimental asthma. Science. 1998; 282: 2261–2263.

115. Howard TD, Whittaker PA, Zaiman AL, Koppelman GH, Xu J, Hanley MT, Meyers DA, Postma DS, Bleecker ER. Identification and Association of Polymorphisms in the Interleukin-13 Gene with Asthma and Atopy in a Dutch Population Am. J. Respir. Cell Mol. Biol. 2001; 25: 377-84.

116. Leung TF, Tang NLS, Chan IHS, Li AM, Ha G and Lam CWK. A polymorphism in the coding region of interleukin-13 gene is associated with atopy but not asthma in Chinese children. Clin. Exp. Allergy. 2001; 31: 1515-1521.

117. Celedon JC, Soto-Quiros ME, Palmer LJ, Senter J, Mosley J, Silverman EK, Weiss ST. Lack of association between a polymorphism in the interleukin-13 gene and total serum immunoglobulin E level among nuclear families in Costa Rica. Clin. Exp. Allergy. 2002; 32: 387-90.

118. Liu X, Beaty TH, Deindl P, Huang SK, Lau S, Sommerfeld C, Fallin MD, Kao WH, Wahn U, Nickel R. Associations between specific serum IgE response and 6 variants within the genes IL4, IL13, and IL4RA in German children: the German Multicenter Atopy Study. J. Allergy Clin. Immunol. 2004; 113: 489-495

119. Kim HB, Lee YC, Lee SY, Jung J, Jin HS, Kim JH, Kim BS, Kang MJ, Jang SO, Kim J, Kimm K, Shin ES, Lee SG, Hong SJ. Gene-gene interaction between IL-13 and IL-13Ralpha1 is associated with total IgE in Korean children with atopic asthma. J. Hum. Genet. 2006; 51:1055-1062.

120. Kabesch M, Schedel M, Carr D, Woitsch B, Fritzsch C, Weiland SK, von Mutius E. IL-4/IL-13 pathway genetics strongly influence serum IgE levels and childhood asthma. J. Allergy Clin. Immunol. 2006;117: 269-74

121. Moissidis I, Chinoy B, Yanamandra K, Napper D, Thurmon T, Bocchini J, Bahna SL. Association of IL-13, RANTES, and leukotriene C4 synthase gene promoter polymorphisms with asthma and/or atopy in African Americans. Genet. Med. 2005:406-410.

122. Howard TD, Koppelman GH, Xu J, Zheng SL, Postma DS, Meyers DA, Bleecker ER.Gene-gene interaction in asthma: IL4RA and IL13 in a Dutch population with asthma. Am. J. Hum. Genet. 2002; 70: 230-236.

123. Homma S, Sakamoto T, Hegab AE, Saitoh W, Nomura A, Ishii Y, Morishima Y, Iizuka T, Kiwamoto T, Matsuno Y, Massoud HH, Massoud HM, Hassanein KM, Sekizawa K. Association of phosphodiesterase 4D gene polymorphisms with chronic obstructive pulmonary disease: relationship to interleukin 13 gene polymorphism. Int. J. Mol. Med. 2006; 18: 933-939

124. Heinzmann A, Jerkic SP, Ganter K, Kurz T, Blattmann S, Schuchmann L, Gerhold K, Berner R, Deichmann KA. Association study of the IL13 variant Arg110Gln in atopic diseases and juvenile idiopathic arthritis. J. Allergy Clin. Immunol. 2003; 112: 735-9.

125. Wang M, Xing ZM, Lu C, Ma YX, Yu DL, Yan Z, Wang SW, Yu LS.A common IL-13 Arg130Gln single nucleotide polymorphism among

Chinese atopy patients with allergic rhinitis. Hum. Genet. 2003; 113: 387-90

126. Bugawan TL, Mirel DB, Valdes AM, Panelo A, Pozzilli P, Erlich HA. Association and interaction of the IL4R, IL4, and IL13 loci with type 1 diabetes among Filipinos. Am. J. Hum. Genet. 2003; 72: 1505-14.

127. Arima K, Umeshita-Suyama R, Sakata Y, Akaiwa M, Mao XQ, Enomoto T, Dake Y, Shimazu S, Yamashita T, Sugawara N, Brodeur S, Geha R, Puri RK, Sayegh MH, Adra CN, Hamasaki N, Hopkin JM, Shirakawa T, Izuhara K. Upregulation of IL-13 concentration in vivo by the IL13 variant associated with bronchial asthma. J. Allergy Clin. Immunol. 2002; 109: 980–987.

128. Chen W, Ericksen MB, Levin LS, Khurana HGK. Functional effect of the R110Q IL13 genetic variant alone and in combination with IL4RA genetic variants. J. Allergy Clin. Immunol. 2004; 114: 553-60.

129. Hunninghake GM, Soto-Quirós ME, Avila L, Su J, Murphy A, Demeo DL, Ly NP, Liang C, Sylvia JS, Klanderman BJ, Lange C, Raby BA, Silverman EK, Celedón JC. Polymorphisms in IL13, total IgE, eosinophilia, and asthma exacerbations in childhood. J. Allergy Clin. Immunol. 2007; 120: 84-90.

130. Lopez KI, Martínez SE, Moguel MC, Romero LT, Figueroa CS, Pacheco GV, Ibarra B, Corona JS. Genetic diversity of the IL-4, IL-4 receptor and IL-13 loci in mestizos in the general population and in patients with asthma from three subpopulations in Mexico. Int. J. Immunogenet. 2007; 34: 27-33.

131. Xi D, Pan S, Cui T, Wu J. Association between IL-13 gene polymorphism and asthma in Han nationality in Hubei Chinese population. J. Huazhong Univ. Sci. Technolog. Med. Sci. 2004; 24: 219-22

132. Hoffjan S, Ostrovnaja I, Nicolae D, Newman DL, Nicolae R, Gangnon R, Steiner L, Walker K, Reynolds R, Greene D, Mirel D, Gern JE, Lemanske RF Jr, Ober C. Genetic variation in immunoregulatory pathways and atopic phenotypes in infancy. J. Allergy Clin. Immunol. 2004 Mar; 113: 511-8.

133. Tsunemi Y, Saeki H, Nakamura K, Sekiya T, Hirai K, Kakinuma T, Fujita H, Asano N, Tanida Y, Wakugawa M, Torii H, Tamaki K. Interleukin-13 gene polymorphism G4257A is associated with atopic dermatitis in Japanese patients. J. Dermatol. Sci. 2002; 30: 100-7.

134. Hoerauf A, Kruse S, Brattig NW, Heinzmann A, Mueller-Myhsok B, Deichmann KA. The variant Arg110Gln of human IL-13 is associated with an immunologically hyper-reactive form of onchocerciasis (sowda). Microbes Infect. 2002; 4: 37-42.

135. DeMeo DL, Lange C, Silverman EK, Senter JM, Drazen JM, Barth MJ, Laird N, Weiss ST. Univariate and multivariate family-based association analysis of the IL-13 ARG130GLN polymorphism in the Childhood Asthma Management Program. Genet. Epidemiol. 2002 Nov; 23: 335-48.

136. Puthothu B, Krueger M, Forster J, Heinzmann A. Association between severe respiratory syncytial virus infection and IL13/IL4 haplotypes. J. Infect. Dis. 2006; 193: 438-41.

137. Maier LM, Chapman J, Howson JM, Clayton DG, Pask R, Strachan DP, McArdle WL, Twells RC, Todd JA. No evidence of association or interaction between the IL4RA, IL4, and IL13 genes in type 1 diabetes. Am. J. Hum. Genet. 2005 Mar; 76: 517-21.

138. Brown RH, Hamilton RG, Mintz M, Jedlicka AE, Scott AL, Kleeberger SR. Genetic predisposition to latex allergy: role of interleukin 13 and interleukin 18. Anesthesiology. 2005; 102: 496-502.

139. Schwartzbaum J, Ahlbom A, Malmer B, Lönn S, Brookes AJ, Doss H, Debinski W, Henriksson R, Feychting M. Polymorphisms associated with asthma are inversely related to glioblastoma multiforme. Cancer Res. 2005 Jul; 65: 6459-65.

140. Kouriba B, Chevillard C, Bream JH, Argiro L, Dessein H, Arnaud V, Sangare L, Dabo A, Beavogui AH, Arama C, Traoré HA, Doumbo O, Dessein A. Analysis of the 5q31-q33 locus shows an association between IL13-1055C/T IL-13-591A/G polymorphisms and Schistosoma haematobium infections. J. Immunol. 2005; 174: 6274-6281.

141. Ohashi J, Naka I, Patarapotikul J, Hananantachai H, Looareesuwan S, Tokunaga K. A single-nucleotide substitution from C to T at position -1055 in the IL-13 promoter is associated with protection from severe malaria in Thailand. Genes Immun. 2003 Oct; 4: 528-31.

142. van der Pouw Kraan TC, Küçükaycan M, Bakker AM, Baggen JM, van der Zee JS, Dentener MA, Wouters EF, Verweij CL.Chronic obstructive pulmonary disease is associated with the -1055 IL-13 promoter polymorphism.Genes Immun. 2002 Nov; 3: 436-9.

143. Sun HP, Chen JQ, Guo XR, Chen RH. The relationship between IL-13 gene polymorphism and the levels of serum IL-13 and serum eosinophil

cation protein in asthmatic children. Zhonghua Yi Xue Yi Chuan Xue Za Zhi. 2003 Dec; 20: 547-8.

144. Guidelines for the diagnosis and management of asthma; Expert panel report 3; National Institutes of Health; National Heart, Lung and Blood Institute.2007; NIH publications.

145. Johansson SG. Raised levels of a new immunoglobulin class (IgND) in asthma. Lancet. 1967; 2: 951-3.

146. Zetterstrom O, Johansson SG. IgE concentrations measured by PRIST in serum of healthy adults and in patients with respiratory allergy. A diagnostic approach. Allergy. 1981; 36: 537-47.

147. Wuthrich B. Serum IgE in atopic dermatitis: relationship to severity of cutaneous involvement and course of disease as well as coexistence of atopic respiratory diseases. Clin. Allergy. 1978; 8: 241-8.

148. Radermecker M, Bekhti A, Poncelet E, Salmon J. Serum IgE levels in protozoal and helminthic infections. Int. Arch. Allergy Appl. Immunol. 1974; 47: 285-95.

149. Savanat T, Thammapalerd N, Jaroonvesma N, Bunnag D. Total serum IgE level in patients with amoebic liver abscess and other parasitic infections. Southeast Asian J. Trop. Med. Public Health. 1977; 8: 419-454.

150. Kreiger DT. Rhythms of ACTH and corticosteroid secretion in health and disease and their experimental modification. J. Steroid Biochem.1975; 6: 785-791.

151. Chernow B, Alexander R, Smallridge RC, Thompson WR, Cook D, Beardsley D, Fink MP, Lake R, Fletcher JR. Hormonal responses to graded surgical stress. Arch. Intern. Med. 1987; 147: 1273-1278.

152. Migeon CJ, Lanes RL: Adrenal cortex: hypo- and hyperfunction. IN: Lifshitz F ed: Pediatric Endocrinology, A Clinical Guide, second edition. Marcel Dekker, Inc., New York, 1990, pp. 333-352.

153. Crapo L. Cushing's syndrome: A review of diagnostic tests. Metabolism. 1997; 28: 955-977.

154. Lee PDK, Winter RJ, Green OC: Virilizing adrenocortical tumors in childhood. Eight cases and a review of the literature. Pediatrics. 1985; 76: 437-444.

155. Drucker S, New MI. Disorders of adrenal steroidogenesis. Pediatr. Clin. North Am. 1987; 34: 1055-1066.

156. Leisti S, Ahonen P, Perheentupa J. The diagnosis and staging of hypocortisolism in progressing autoimmune adrenalitis. Pediatr. Res. 1983; 17: 861- 867.

157. Alsever RN, Gotlin RW. Handbook of Endocrine Tests in Adults and Children, second edition. Year Book Medical Publishers, Inc., Chicago, 1978.

158. Odell WD, Horton R, Pandian MR, Wong J: The Use of ACTH and Cortisol Assays in the Diagnosis of Endocrine Disorders. Nichols Institute Publication, 1989.

159. Gold EM. The Cushing's syndromes: Changing views of diagnosis and treatment. Ann. Intern. Med. 1979; 90: 829.

160. Plasma Cortisol, RIA for Physicians, Edited by J.C. Travis, 1:8, Scientific Newsletter, Inc. 1976.

161. Krieger DT. Physiopathology of Cushing's disease, Endocrine Review. 1983; 4: 22- 43.

162. Krieger DT, Liotta AS, Suda T, Goodgold A, Condon E. Human plasma immunoreactive lipotropin and adrenocorticotropin in normal subjects and in patients with pituitary-adrenal disease, J. Clin. Endocrinol. Metab. 1979; 48: 566-571.

163. Barnes PJ. Pathophysiology of Asthma. In: Barnes PJ, Rodger IW, Thomson NC Editors. Asthma, Basic Mechanisms and Clinical Management. Academic Press, London. 1998; pp. 487–506.

164. Zollner EW. Hypothalamic-pituitary-adrenal axis suppression in asthmatic children on inhaled corticosteroids (Part 1) – which test should be used? Pediatr. Allergy Immunol. 2007; 18: 401–409

165. Zollner EW. Hypothalamic-pituitary-adrenal axis suppression in asthmatic children on inhaled corticosteroids (Part 2) – the risk as determined by gold standard adrenal function tests: A systematic review. Pediatr. Allergy Immunol. 2007; 18: 469–474

166. Romagnani S. The role of lymphocytes in allergic disease. J. Allergy Clin. Immunol. 2000; 105: 399-408.

167. Punnonen J, Aversa G, Cocks BG, McKenzie AN, Menon S, Zurawski G, de Waal Malefyt R, de Vries JE. Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. Proc. Natl. Acad. Sci. USA. 1993; 90: 3730-3734.

168. Van der Pouw Kraan TC, Van der Zee JS, Boeije LC, De Groot ER, Stapel SO, Aarden LA. The role of IL-13 in IgE synthesis by allergic asthma patients. Clin. Exp. Immunol. 1998; 111:129-135.

169. Muegge K, Durum SK. Cytokines and transcription factors. Cytokine. 1990; 2: 1-8.

170. Weir TD, Mallek N, Sandford AJ, Bai TR, Awadh N, Fitzgerald JM, Cockcroft D, James A, Liggett SB, Paré PD. β2-adrenergic receptor haplotypes in mild, moderate and fatal/near fatal asthma. Am. J. Respir. Crit. Care Med. 1998; 158: 787-791.

171. International Union against Tuberculosis and Lung Diseases (IUATLD) questionnaire, Community Respiratory Health Survey (ECRHS), and International Study of Asthma and Allergies in Childhood (ISAAC). Questionnaires: a major instrument for respiratory epidemiology. European Respiratory Monograph. 2003; 23:1–25.