Detection of Novel Single Nucleotide Polymorphism in Tyrosine Kinase Gene among Atopic Allergic Individuals in Pakistani Population.

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By

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A thesis submitted in partial fulfillment of the requirements for the Degree of

Master of Philosophy
In
Microbiology

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2017

Dedication

Dedicated To my Loving Parents for providing will to aspire and for giving me

courage to fight against odds.

Declaration

The material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Mehreen

Certificate

This thesis submitted by **Mehreen** is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University Islamabad, Pakistan: as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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Dated:

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List of Acronym/abbreviations (alphabetically)

AHR	Hyperresponsiveness
ASO	Antisense oligonucleotide
BAL	Broncho alveolar lavage
BCR	B cell receptor
bFGF	Basic fibroblast growth factor
BMI	Body mass index
CCL11	CC chemokine ligand 11
CCL2	CC chemokine ligand 2
CCL3	CC chemokine ligand 3
CCR2	CC chemokine receptor type 2
CRTH2	Chemoattractant receptor homologous molecule expressed on Th2 cells
CXCL8	CXC chemokine ligand 8
DCs	Dendritic cells
EBC	Exhaled breath condensate
FcRI/FcRII	Ig Fc receptor
FcεRI	IgE receptor
FoxP3	Fork head box-P3
GM-CSF	Granulocyte macrophages colony stimulating factor
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon gamma
ILCs	Innate lymphoidal cells
ILs	Interleukins
ITAM	Immunoreceptor tyrosine based activation motif
MCP-1	Monocyte chemotactic protein 1
MIP-1α	Macrophage inflammatory protein 1 alpha
MMP	Matrix mettalo proteinases
PAF	Platelet activating factor
PGE2	Prostaglandin-E2
RA	Rheumatoid arthritis
RANTES	Regulated on activation, normal T-cell expresses and secreted

SH-2	Src homology domain
SLE	Severe lupus erythematous
SNPs	Single nucleotide polymorphisms
Syk	Spleen tyrosine kinase
TGF-β	Tumor growth factor beta
Th-2	T helper type 2 cells
TIMP	Tissue Inhibitor of metalloproteinase
TNF-α	Tumor necrosis factor alpha
TNF-β	Tumor necrosis factor beta
Treg	Regulatory T-cells
TSLP	Thymic stromal lymphopoietin
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VLA-4	Very late actigen-4

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Mehreen

Abstract

"Detection of Novel Single Nucleotide Polymorphism in Tyrosine Kinase Gene among Atopic Allergic Individuals in Pakistani Population"

Allergic asthma and allergic rhinitis are airway allergic diseases caused by allergen sensitization. According to WHO, asthma and allergic rhinitis affect about 235 million peoples. As these allergies are multigene disorder, there is involvement of various genes in the pathogenesis of these allergies. One of the important signaling kinase is spleen tyrosine kinase (*Syk*) gene. It was selected as this kinase play important role in allergen mediated activation of receptors on the immune cells leading to degranulation and release of various cytokines and chemokines. This study was carried out to determine the involvement of *Syk* gene polymorphism in the air way allergies. For this purpose, 228 patients' blood samples were collected from National Institute of Health and Federal Government Hospital Islamabad. While screened blood samples are collected from Blood donor society of Red Crescent Society Islamabad, Pakistan. DNA Extraction was done by using phenol/chloroform method while PCR was used for specific exonic regions. Further analysis for genetic variation by SSCP was done. The comorbid disease was prevalent. Males were more effected (52%) and family history was a risk factor. This atopy was significantly associated with smoke (p=0.003), exercise (p= 0.022) weather changes p=0.018. Various socio-demographic risk factors like adult age group and lower socioeconomic status were found to be associated with disease manifestation. In the exon15, variant observed were not associated to the disease. In exon 15' peak disturbance is needed to verify for possible association of *Syk* gene and its variant with the disease outcome. There is need to study this gene in larger samples size to confirm its role conclusively in asthma and allergic rhinitis among local population.

Chapter 1: Introduction

Chapter 1 Introduction

1.1: Allergy:

Allergy is a state in which the body immunologically reacts to an innocuous particle from environment that poses no threat to it (Niggemann and Beyer, 2016). Such particles do not provoke immunological response in every person. These agents are termed as allergens, and their source could be air, animals, food, dust, bugs and drugs. The reaction to allergen is mediated by the production of specific antibodies, which activate the tissue resident mast cells to produce histamines and other chemical mediators to destroy it. As a result, body shows symptoms of allergy, which may be moderate to severe depending on allergen exposure and reaction against it (Grabbe and Schwarz, 1998). These types of responses are termed as hypersensitivity type 1 reactions in an individual and known commonly as allergic response. Hypersensitivity type 1 reactions are specialized immunological responses made against allergen through T helper type 2 (Th-2) cells whose activities preferentially produces IgE antibodies (Wawrzyniak *et al.*, 2016). Allergies can be of various types depending on type of causative allergen and site of exposure (Janeway *et al.*, 1997; Lilja and Wickman, 1998).

The most common allergies are respiratory tract allergies, which are predominately asthma and rhinitis due to immense exposure of airways to air containing numerous types of allergens like dust mites (Bartlett *et al.*, 2016), molds spores, grass pollen (Zhang *et al.*, 2016), environmental irritants like smoke, fibers, cold air *etc* (Suzuki *et al.*, 2016; Rönmark *et al.*, 2016). Other allergies could be against food like peanut, egg, soybean or wheat (Sicherer *et al.*, 2016). Insect bite can have venom which also act as allergen (Singh, 2013). Some drug also induce allergy such as penicillin and sulfa drugs. Sometime allergy can be life threatening due to exaggerated allergic response in short period called as anaphylaxis shock (Muraro *et al.*, 2017). Allergy can be checked in susceptible person by introducing various allergen extracts to skin either by prick, injection or in form of patch. If allergen causes allergy, it will appear as bump, wheals, redness, itching. Also, blood test can be performed to analyze the amount of IgE antibody, which is an indicator for allergy (Pepys, 1973).

1.2: Atopic Allergies:

The word atopy was introduced by Coca and Cooke in 1923, native of Greek that is ἀτοπία means "Placeless-ness" (Ring, 1991; Turner *et al.*, 2016). This term is used to show two characteristics of the allergy. Firstly, the allergy is IgE mediated and secondly, it is genetically predisposed. The atopic allergies in the family can increase the chance for the next generation to become allergic due to genetic predisposition (Mihrshahi *et al.*, 2007). These responses are initiated in the body after allergen exposure during early childhood or in later stages of life. Child with single allergic parent may have moderate chance of allergic disease while the one having both parents allergic have higher chance. It is observed that allergies are interrelated and one type can lead to other types of allergies. This stepwise leading of one type of allergy into other is called atopic march. In atopic march progression of eczema into asthma is observed in 30% cases of allergy while the 80% of allergic asthma also possesses concurrent allergic rhinitis (Bousquet *et al.*, 2008; Brożek *et al.*, 2010).

1.3: Allergic asthma and Allergic rhinitis:

Allergic asthma is characterized by presence of IgE, causing inflammation of lower airways which results hypersensitivity to allergens entered upon respiration. It starts with sensitization and leads into inflammation, bronchospasm, hyper-responsiveness and remodeling of airway lining (Al-Sahab *et al.*, 2011; Papaiwannou *et al.*, 2014; Jiang *et al.*, 2014; Storms *et al.*, 2015). The eosinophils are major player of inflammation in this case. The symptoms can be severe coughing, sound of wheezing, tightness of chest, mucus secretion and trouble in breathing. The condition is reversible as it is caused by allergen induced inflammation. The incidence rate of asthma worldwide is 235 million cases according to world health Organization (WHO) report (2013) (Raedler *et al.*, 2015; Rantala *et al.*, 2015).

Rhinitis means "nasal inflammation" that causes symptoms like rhinorrhea, sneezing, watery and itching eyes, and excess mucus secretions in throat. It may occur throughout the year due to presence of allergen or irritant constantly in environment, or it may be

seasonal due to specific allergen and it is termed as hay-fever. Rhinitis as an allergy occurs more commonly as in UK, where 60 million allergic rhinitis were reported (Fasano, 2010; Okubo *et al.*, 2014).

Asthma and rhinitis are IgE mediated responses localized in the airways. They are interrelated according to many studies due to atopy and site of inflammation (Nielsen *et al.*, 2002). It has been observed that half of the allergic-rhinitis individuals have concurrent asthma, while majority of asthmatic patients are also affected with allergic-rhinitis (Njue *et al.*, 2016). According to the European Community Respiratory Health Survey (ECRHS), allergic rhinitis and asthma both are characterized by the onset of bronchospasm as a result of sensitization to variety of allergens. Another study shows interaction due to specific allergen reaction through nasal mucosa resulting in bronchial hyper-responsiveness (Giavina-Bianchi *et al.*, 2016). Similar studies in adults and children shows direct bronchial symptoms with or without effecting upper-airways. It has been observed that treatment for allergic rhinitis relief bronchial responses showing upper airway impact on lower airways. Likewise, it has been observed that asthma specific allergens induce upper airways responses in allergic-rhinitis patients. Lower airways inflammation is observed in allergic rhinitis patients even in absence of asthma likewise in case of asthma upper airways hypersensitivity exist even in absence of allergic rhinitis (Takemura *et al.*, 2016). These associations of airways show combined airways disease phenomenon in which both physiology and immunology play important role in their correlation (www.aafa.org/page/rhinitis-nasal-allergy-hayfever.aspx) (Fasano, 2010).

1.4: Pathophysiology:

In allergic asthma and rhinitis pathology, various similarities and differences occurred. The ciliated epithelial membrane covering the upper and lower airway lining and ability to secrete mucus in both linings is a prominent similarity between two. While the difference lies in the presence of frequent blood vessels in nose and smooth muscles in bronchi. In allergic asthma and allergic rhinitis, the allergic response initiated with development of sensitization after exposure to specific allergen (Bergeron and Hamid,

2006). Sensitization means development of IgE antibodies, as allergen enters the airways and captured by dendritic cells, these cells activate the T-cells to divides into Th2 cells, which in turn activates B cell for class switching of residence Ig-M antibody to allergen specific IgE antibody. After sensitization exposure to allergen causes early response in atopic individual, in which allergen cross link two cells (mast cells) by binding to IgE antibodies on surface of Fc receptors (Giavina-Bianchi *et al.*, 2016). This cross linkage activates the cell by down signaling resulting in release of various chemicals and chemokines like histamines, which causes airflow obstruction by smooth muscle constriction in allergic asthma individuals while in allergic rhinitis patient the engorgement of vessels causes runny nose and congestion. Later exposure causes late-phase responses that continues the allergic inflammation by activated Th2 cells and eosinophils causes further immune cell recruitment at site of inflammation worsening the disease condition. Early phase responses are similar in allergic asthma and allergic rhinitis patients while late phase responses differ as occur after long term inflammation and contrasts in remodeling of membranes (Bourdin *et al.*, 2009). In allergic asthmatic patient, the epithelial disruption and narrowing of passage occur, while in allergic rhinitis both phenomena are comparatively low as compared to allergic asthma (Riedler *et al.*, 2001). The pattern of immune cells (mast, basophil, dendritic), mediators (histamine, leukotriene, chemokines) and interleukins (IL4, IL5, IL13) involved in inflammation of both atopic conditions are similar (Almqvist *et al.*, 2007; Proud and Leigh, 2011)

1.5: Allergens:

The most abundantly occurring allergies are related to airways and food. Allergic asthma and allergic rhinitis has greatly influenced the world and recent studies have shown some similarities among these two, one being sharing common allergens. Asthma is chronic airway inflammation caused by interplay of environmental and genetic factors (Sarnowski *et al.*, 2016; Chen *et al.*, 2016). Allergic rhinitis is nasal allergy provoked by certain environmental allergens and stimuli. Variable triggers are responsible for the onset of bronchial hypersensitivity in asthma and in rhinitis case. These are environmental stimuli that on contact irritate and results in inflamed airways. They are

not same for all patients they may vary. Pollen (grass, ragweed, mulberry) are abundantly present in spring season affecting the people. Allergic rhinitis occurring in spring called hay fever, asthma also get worse in this season (Wisniewski *et al.*, 2016). Dust mites, and cockroaches' residues and excretion present indoor also causes allergies especially their common sources are carpets and bedsheets. Fungal spores are present both indoor/outdoor in damp places and causes symptoms of both allergic rhinitis and asthma that upon severity affect the lungs. Proteins in hair and dander of common pets (dogs, cats) are also known to worsen the asthma condition and causes allergic rhinitis upon exposure which may last for many days (Ownby and Johnson, 2016; Apfelbacher *et al.*, 2016).

Indirect or direct smoke exposure, heartburn/acid reflux, stress and strong emotions, allergic reaction to food or sulfites, exercise, food preservatives, medication used for cardiovascular disease such as aspirin or beta blockers can also trigger the allergic inflammation reactions. In many studies, attempted to develop link between asthma and urbanization, which still in-conclusive (Laidlaw and Boyce, 2016).

In a study, dust mites, parthenium leaves and cockroaches were found to be major allergens for both conditions. Among animal dander, the cat fur was majorly involved than other animal hair (Mishra *et al.*, 2016).

1.6: Risk factors:

Various risk factors are associated with the initiation, development and pathogenesis of allergic asthma as well as allergic rhinitis. While studying the risk factors association among co-morbid cases of rhinitis and asthma, it was seen that the risk factor pattern was same as observed in allergic asthma case alone. The common risk factor is atopy, which make the person easy target for the allergy development (Program *et al.*, 2011; Rönmark *et al.*, 2016).

Genetic predisposition plays important role for developing asthma and rhinitis disease. Allergic asthma has the higher chance of occurrence in individuals with positive family history. Several studies carried on monozygotic twins showed strong association of genetic linkage with allergic asthma, allergic rhinitis, allergic eczema and food allergy.

Childhood susceptibility to allergic hyper-responsiveness and family history of allergy make the individual easy target of asthma in the later life (Barrett, 2008; Giavina-Bianchi *et al.*, 2016).

Age is a very important risk factor in atopy but there are inconsistent reports in the literature on the association of age with asthma development. Some work cite asthma to be more common in children than adults (Krauskopf *et al.*, 2013). Recent evidence shows, that the allergic rhinitis decreases with age. Whereas some studies report, no specific correlation between age and onset of asthma (Yao *et al.*, 2011). It seems that gender also plays an important role in asthma development along with age as in early childhood boys are more likely to develop asthma than girls while in adulthood the facts are inverse (Almqvist *et al.*, 2008). The allergic rhinitis is seen to be frequently present in young adults (20-44 years), with majority cases of males as compared to females. Sensitivity check against histamines shows high number of male patients were sensitive to histamine as compared to females. Allergic rhinitis prevails in all ages while asthma more frequently occurred in children (Bordignon and Burastero, 2006; Cazzoletti *et al.*, 2015).

Early sensitization is strongly associated with onset of allergic rhinitis and moderately to allergic asthma. Airways hypersensitivity upon allergen exposure shows one's ability to be able to develop allergic disease. Dust, mold, pet dander, toxic chemicals are the common allergens. Allergens can worsen the already developed asthma or rhinitis symptoms. Domestic animal dander is the risk factor for asthma wheezing (Pegas *et al.*, 2011; Garcia-Larsen *et al.*, 2016).

Tobacco smoke either direct or indirect is related to onset of asthma. Smoke effect the lungs and thus increase the possibility of asthma development. Smoking during pregnancy make the fetus easy target of allergens. After birth making fetus susceptible for various allergies more commonly asthma and rhinitis in early childhood. Early childhood exposure to tobacco smoke increase the risk for allergy development (Thacher *et al.*, 2014; Feleszko *et al.*, 2014).

Asthma risk is also found aggravated by pollution in the air. Asthma majorly prevail in urban population as compared to rural areas. In children who were brought up/frequent visitor to the farm environment were resistant to develop allergies or asthma. It was attributed to repeated exposure to bacteria in the environment, which decreased the exacerbation susceptibility by suppressing Th2 responses (Depner *et al.*, 2016). Other particles like ozone, smog, gases and fume exposure during occupation irritate the airways and causes allergic rhinitis and asthma.

In many studies performed in obese children, the level of increased IgE shows link of increased weight with allergies. Body mass index (BMI) was also found to be linked with allergies, as inflammatory responses due to increase in weight also made these obese persons vulnerable to asthma and allergies (Silverberg, 2016). The western children from upper economic strata were found to more likely develop asthma exacerbations as compared to other airways allergies (Weinmayr *et al.*, 2015). Obesity is linked with asthma onset in both male and female. Obese females with waist greater to 88cm are at higher risk for asthma as compared to males. Difference in risk may the hormones in female (Brumpton *et al.*, 2013; Ilmarinen *et al.*, 2015).

Early childhood viral infection causes airway exacerbation and leads to onset of asthma when individual is adult. These infections can affect the lung functioning, thus worsening the asthma condition during cold when infections are frequent (Link, 2014). Childhood rhinovirus infections are more commonly associated with the asthma onset later in life. Many studies with rhinovirus infections shows 80% to 85% of association with asthma development (Teach *et al.*, 2015).

As these two conditions (asthma and rhinitis) are also atopic in nature most cases, there is also involvement of genetic factors.

1.7: Genes involved in atopic allergies:

Initial linkage study on genetic association for asthma was conducted on the base of locating genetic markers on genome and linked genes to it whether responsible for disease or not, however it did not provide any direct impact of these genes on asthma. Linkage studies provide association of larger chromosomal region with variety of genes

involved in asthma. Atopic allergies are found to be associated with the chromosomal region 5q 31–32, which have genes like *CD14*, *IL13*, *IL4* and *SPINK5* that participate in T cell activity and skin morphogenesis. Recent Genome wide association study focused on the development of link between SNPs spanning regions in whole genome and their association with a particular disease. This study identified variety of candidate genes involved in asthma and allergies. According to another study that was carried out in UK by GABRIEL (Moffatt *et al.*, 2010) and EVE (Program *et al.*, 2011) consortium, 6 genes were found to be common in asthma patients which are *TSLP*, *IL33*, *IL1RL1*, *IL18*, *HLA-DQ*, *ORMDL3* and *SMAD3*. While some other genes were also reported to be involved in multiple aspects of asthma pathogenesis, those are *RORA*, *ORMDL3*, *SLC22A5*, *IL2RB* and *IL13*. These genes were found to be associated with mechanisms or pathways involved in prognosis of asthma and allergies, like inflammation, T-cell activation, mast cell/ basophil cell degranulation *etc*. As allergic asthma and allergic rhinitis are heterogeneous diseases, that's why no single gene with its direct impact on diseases has yet been observed (Meltzer *et al.*, 2005; Yi *et al.*, 2014; Norman, 2014; Thomsen, 2015). All these attempts over the years has enabled researchers to proof involvement of various genes of receptors, cells and mediators participating in the pathways. In the recent years, therapeutic studies on asthma, rhinitis and inflammatory pathways have shown signaling molecules like spleen tyrosine kinase an intracellular enzyme as vital player, Inhibition of which causes improvement in asthma severity. However, no study has been carried on the role of Syk kinase and its genetic variation on the disease pathogenesis. There is need to understand not only the role but effect of *Syk* gene mutations/SNPS on the Syk signaling hence leading to asthma outcome.

Aim and Objectives

Aim and Objectives:

The aim of this study was to evaluate any possible mutations or SNPs in *Syk* gene and the comorbidity in allergic asthmatic patients with rhinitis from Pakistani patients visiting allergy center Islamabad.

The objectives related to the study were:

1. Collection data using questionnaire to investigate the prevalence, possible triggers and symptoms of asthma disease alone and along with allergy like rhinitis.
2. Patient's blood sampling from Federal Government Hospital (FGH) for asthmatic patients and allergy center Islamabad for atopic patients. For controls blood from healthy individuals taken from FGH lab, Pakistan Red Crescent Society, Islamabad.
3. Use of PCR and vertical gel electrophoresis for detection of possible variants in various exons of *Syk* gene.
4. Statistical analysis of the information received through questionnaire for finding significant association between allergic asthma and allergic rhinitis by SPSS software.

Chapter 2: Review of Literature

Review of Literature:

Allergies are increasing (worldwide (Simons, 2009; Anandan *et al.*, 2010; Uphoff *et al.*, 2015). Among these who are suffering from allergies are 40% adults and 50% children, these ailments are asthma, allergic rhinitis, eczema and drug reactions (Matasar and Neugut, 2003). For more than half century these diseases are continuously striking the industrial countries of the world, most prominently the western world (Pawankar *et al.*, 2011). The allergic asthma (Alshabanat *et al.*, 2015; Dannemiller *et al.*, 2016) and allergic rhinitis are in abundance in comparison to other allergies with prevalence rate of 18% and 20% respectively (Jackson *et al.*, 2013; Wheatley and Togias, 2015). Dermatitis has prevalence of about 20% (Thomsen, 2014). About 6% of world population has allergy to one food type (Kronenberg, 2012; Sicherer, 2014). Allergic shocks occur sometime in life about 2% effected people in which 0.3 % is fatal (Patterson, 2009). It is one of the leading chronic diseases of present world ranking 3rd in number among children.

European countries have high asthma and allergy prevalence around the world, in America 1 asthma case occurs in each 12 adults and 11 children (Sigurs *et al.*, 2005; Kim and Mazza, 2011; Gilchrist and Lenney, 2012). In Canada this prevalence is 10% (Kovesi, 2012). In USA $18 billion is annual medical consumption on asthma (Jiang *et al.*, 2014; Vos *et al.*, 2015).

The prevalence among rest of the continents including Middle East countries is low as compared to European world (El-Sharif, 2002; Gilchrist and Lenney, 2012). In a comparative study for prevalence of allergic diseases among Middle East countries, asthma being abundantly present in Saudi Arabia population mostly affecting children. The percentage of allergic rhinitis was found higher in Morocco, Lebanon, Qatar and Pakistan. Skin allergies are dominating in Qatar and Pakistani population. (Al Ghobain *et al.*, 2012; Hasnain *et al.*, 2016).

Various studies at different times in different regions of Pakistan showed higher prevalence of allergic disease. In a study at Karachi in 2007, high prevalence of allergies

was observed. Among all these allergies, the percentage of allergic rhinitis was highest of all while asthma was secondary to nasal problems (Noori *et al.*, 2007). In another study of school children in Karachi indicated high prevalence of rhinitis as compared to asthma (Hasnain *et al.*, 2009). A study conducted in the same year in Islamabad showed allergic rhinitis being dominating among other allergies. The episodes of wheezing along with rhinitis, however, was comparatively low (Yusuf, 2012).

Another study in 2007 at Bahawalpur (Ashraf et.al, 2015), was carried out for general medical practices for asthma treatment and similar study for risk-factor association (Amber *et al.*, 2010) showed that asthma is a becoming serious problem of concern to be focused for better interventions (Ashraf, 2015; Kamran *et al.*, 2015). In survey based study at Karachi (2016), 44% of the patients have comorbid asthma and rhinitis (Naveed *et al.*, 2016).

A work on children during 2005-2006 in pediatric department of ISRA university hospital showed that the asthma is observed in infants with age of 12 months to 8 years, where it was predominately affecting males. All these infants were atopic in nature (Majeed *et al.*, 2008).

In southern Punjab study on prevalence of asthma showed female population with age 20-60 years were frequent target of disease as compared to male of same age (Khan *et al.*, 2016). According to a survey report in Europe, allergic rhinitis and asthma in adults, asthma manifestations occurred in 50% of the allergic rhinitis cases (Ramsdale *et al.*, 1985; Demoly *et al.*, 2006). While 80% of the asthma cases also reported rhinitis like symptoms (Casale and Dykewicz, 2004). Both allergies exist at same time in many cases irrespective of age group and share common risk factors as atopy. Childhood exposure to allergens like dust-mites was noted to increase bronchial exacerbation in atopic individuals (Ciprandi *et al.*, 2013). In follow up cases of European survey, further association between allergic asthma and allergic rhinitis was proved (Leynaert *et al.*, 2000; Cruz *et al.*, 2007; Brożek *et al.*, 2010). In a study on patients with allergic rhinitis, nasal allergens exposure showed increase in airway hyper-responsiveness, sputum

eosinophils, eosinophilic cationic protein (Bonay *et al.*, 2006; Shaaban *et al*, 2007) vascular cell adhesion molecule-1, E-selectin, and eosinophilic inflammation in bronchial mucosa. Similar studies on allergic rhinitis patients without having asthma induces in patients' nasal lamina propria increased eosinophils, IL-5 expression in epithelium and eotaxin-positive cells (Braunstahl *et al.*, 2000; Fasano, 2010). Allergic asthma and allergic rhinitis both share epidemiological, clinical, immunological and physiological aspects of pathology (Slavin, 2000; Giavina-Bianchi *et al.*, 2016).

The similarities and differences in response of inflammation by both of these diseases depends upon the structural similarities and differences of nose and tracheal linings. On the basis of these perspectives, both of these allergies are being studied as united airway disease. Within both airways, the epithelium act as a barrier where different innate and adaptive reactions are orchestrated in case of airways allergies (Giavina-Bianchi *et al.*, 2016). Some similarities in upper lining among both airways are the presence of ciliated epithelium and columnar cells along with goblet cells for secretion of mucus. While difference lie in lower layer as the nasal submucosa is rich with large vessel linings and attached to bone, while lower airways are characterized by the presence of smooth muscles and bronchi attached to cartilage. Nasal epithelium act as strong anatomical barrier and withstand long term inflammation as compared to bronchial lining due to presence of tight junctions, peptidases and a large antioxidant apparatus, also the lymphoid apparatus of nasal mucosa is well developed with better regeneration abilities.

Allergic rhinitis is multifactorial chronic inflammation of nasal mucosa with common symptoms of sneezing, rhinorrhea, pruritus, nose congestion. While asthma is a long term polygenic disorder of lower respiratory tract, characterized by variable and reversible episodes of air flow. The airways in both conditions become hypersensitive to allergen, and develop Th2 mediated inflammatory responses (Polosa *et al.*, 2000; Shaida *et al.*, 2001; Braunstahl *et al.*, 2001; Okubo *et al.*, 2014).

On secondary exposures allergen binds the already produced antibodies and activates immune cells (mast cells) to release various mediators like histamines, leukotrienes,

cytokines, chemokines (Kim and Mazza, 2011). The responses shown against these chemicals are termed as early or late phase. In early phase histamines irritate the sensory nerves as a result sneezing and mucus secretion (rhinorrhea) with in half hour of exposure. In late phase leukotrienes mainly released by eosinophil cells play important role in nasal congestion occurred due to swelling of vessels, plasma leakage and interstitial edema. The mucosal lining swells due to the interplay of chemical mediators, such as histamine, PAF, prostaglandin D2 and particularly leukotriene. After 6-7 hours of allergen exposure late phase responses can be observed. Chronic lesions as a result of long term inflammation are also observed in rhinitis cases (MEZAWA, 1988; Ichikawa *et al.,* 1991; Okubo and Okuda, 1998).

Characteristic phenotype of allergic asthma is associated with high blood eosinophil count along with increased amount of total IgE. According to some researchers' asthma is a systemic respiratory disease as involved both upper and lower airways in case of comorbidity along with other atopic allergies like allergic rhinitis. In early phase reaction the interleukin 5, 9 and 13 are released by T-cells, promotes the increase of IgE and eosinophil count in airways. After formation IgE binds to immune cell and causes degranulation to release histamine, cysteinyl leukotriene, cytokines and chemokines for inflammation of airways characterized by edema, bronchospasm and mucus hypersecretion. Late phase responses are carried out by same mediators upon further exposure to allergen (Del Prete *et al.,* 1993; Rotmagnani, 1998; Galli *et al.,* 2005; Okubo *et al.,* 2014; Ferreira *et al.,* 2014).

The cellular inflammation frame work in both diseases is dependent on eosinophil, mast-cell, and CD4+ T-cell infiltration. Mediators in both cases are similar cysteinyl leukotrienes, interleukin [IL]-4, [IL]-5, [IL]-13, chemokine, and eotaxin (KleinJan *et al.,* 1999; Nag *et al.,* 2002). Inflammation at start after allergen exposure is carried out by histamines in both of the diseases but the chronic results are different due anatomical difference lies between upper and lower airways. The collagen deposition in basement membrane of epithelium causes narrowing the airflow passage in case of asthma. The

deposition of collagen in case of allergic rhinitis is comparatively low (Bousquet *et al.*, 2004).

The pathological responses in allergic asthma and allergic rhinitis are divided mainly into inflammation and remodeling. During inflammation key players starting the response are interleukins along with other factors aiding the process. These interleukins produced by basophils and mast cells, with their prominent role are IL-4 /IL-13, IL-5, IL-9, known for IgE switching, controlling mast cells and for eosinophil recruitment respectively (Shahid *et al.*, 2002) [IL]- 3, IL-6 IL-10, IL-12, {IL}-18, [IL]-2, Interferon (IFN)-γ, tumor necrosis factor (TNF)-α, TNF-β and granulocyte macrophage colony stimulating factor (GM-CSF) are seen to release by these effectors (Ronchetti *et al.*, 2001).

Bronchoalveolar lavage (BAL) in asthma patient is also observed in which tryptase appear to be the indicator of mast cell degranulation. Chymase poses direct threat by provoking tissue damage cascade (Pearlman, 1999; Bousquet *et al.*, 2000; Barnes, 2002). Lipid mediators are released immediate after their production including prostaglandin and leukotrienes (Nakae, 2007).

In exhaled breath condensate (EBC) test of asthma and rhinitis, patients show low pH as compared to controls indicating the inflammation. Patients of asthma have high level of TNFα (Brunetti *et al.*, 2006; Matsunaga *et al.*, 2006) TNFα is of prime importance in establishing airway hyper-responsiveness by increasing number of adhesion factors in nasal and bronchial lining (Nakae, 2007).

Other cytokines produced by mast cells include CXCL 8, tumor growth factor (TGF)-β, basic fibroblast growth factor [bFGF] (Bradding *et al.*, 2006), CC-chemokine ligand-3 or macrophage inflammatory protein (MIP-1α), CCL2 or monocyte chemotactic protein (MCP-1) (Stone *et al.*, 2010), and CCL11 or eotaxin-1, which are involved in cell migration (Teran, 2000) Basophils show immense production of IL4 and also causes various receptor to be expressed on T-cells which are involved in hypo sensitization such as CCR2, CCR3, Ig Fc-receptors (FcRI and FcRII) and CRTH2 (high affinity receptor

for PGD2). The cytokines released here are important in the later phase responses (Bochner and Schleimer, 2001).

Later phase organization consists of eosinophils (as marker of chronic situation) neutrophils, T cells, macrophages, dendritic cells (DCs), endothelial cells and epithelial cells affecting the membrane anatomy (Elias, 2000).

Eosinophil recruitment in late response is vital step, which is maintained by IL5 and eotaxin,that results in long term inflammation critical for airway remodeling, hyper-responsiveness and epithelial shedding. Peroxidases released by the eosinophils are involved in vasodilation, smooth muscle constriction, edema, mucus secretion and also activate dendritic cells. While TGFβ causes airway remodeling and thickened the submucosa of airway lining. Proteases along with eosinophilic-neurotoxin and major basic proteins cause tissue damage in air ways (Giavina-Bianchi *et al.*, 2016). Eosinophilic cationic protein is elevated in asthma sputum samples and is capable of pore formation in membranes (Zimmerman *et al.*, 2000; Hamid and Minshall 2000; Koh *et al.*, 2007).

Asthma linked morbidities are due to the presence of increased eosinophil count in sputum, nasal mucosa and all over the respiratory tract showing that allergic asthma and allergic rhinitis shows similarities in pathology (Giavina-Bianchi *et al.*, 2016). Patients challenged by different allergen for observing effector cell responses show the same pattern of degranulation in both allergic conditions (Kämpe *et al.*, 2011).

Neutrophil aggravate ongoing processes by releasing cytokines like TGF-β, CXCL8, proteases as elastase, matrix metalloproteinase 9, lysozyme, reactive oxygen species and nitric oxide (NO). Elastase being diverse in nature participating at different points along with its primary action on elastin, it also effects collagen. Moreover, it activates matrix metalloproteases, and other immune cells. It interferes with epithelial and endothelial membrane functions. It hampers the ciliary motion by lying a mucus layer on them and increase the cell damage by apoptosis. CXCL8 is usually observed in elevation in severe

asthmatic individuals and is related to chemotaxis of neutrophils (Shute *et al.*, 1997; Sampson, 2000; Gibson *et al.*, 2001).

The increasing evidences on endotype of united airways disease is involvement of type 2 innate cells in the inflammatory diseases along with Th2 adaptive cell. In allergic asthma and allergic rhinitis, various type 2 cytokines are released by type 2 cells that bind the Fc receptor of granulocytes causes variety of pro-inflammatory processes resulting in smooth muscle constriction, increase blood flow to vasculatures and immune cell attraction. Type 2 cytokines are IL-4, IL-5, IL-9 and IL-13 involved in tissue eosinophilia and mast cell hyperplasia, mucus production by goblet cells and airway hyper-responsiveness (AHR), which is a hallmark of allergic asthma (Kapsenberg *et al.*, 1999; Bonsignore *et al.*, 2015; Kabata *et al.*, 2015; Agache and Akdis, 2016).

Th1/Th17 inflammatory cells and non-allergic mechanisms such as environmental factors, psycho-social stress, and activation of metabolic pathways, resident cells in the remodeled phenotype or epithelial barrier dysfunction further modulate the profile of type 2-driven inflammation. In addition, type 2-driven inflammation is characterized by a high cellular plasticity that enable the cells to adapt to a specific inflammatory milieu. Several sub-endotypes might exist within the type 2 immune response complex endotype such as the IL-5-high, IL-13-high or IgE-high endotype7 and their preponderance differs between allergic diseases (Kapsenberg *et al.*, 1999; Bonsignore *et al.*, 2015; Kabata *et al.*, 2015; Agache and Akdis, 2016).

Different course of animal experimentations have shown that even in absence of Th2 cells, innate cells can cause inflammation by IL25, IL33 and TSLP as detected in nasal lavage of rhinitis patients. Genome wide association studies showed ILC2 involvement in allergies (Matsushita *et al.*, 2015; Klose and Artis 2016).

Macrophages are antigen presenting cells and can block inflammation by releasing PGE2 and IL-10 acting as inhibitor. But during provocative responses against allergen (Chanez *et al.*, 1996; Barnes, 2002).

The macrophages are thought to cease the inhibitors in patients with lung exacerbation (Gosset *et al.*, 1999; Rosenwasser, 2001). Activated macrophages produce IL-1, IL-6, CCL2, CXCL8, CCL3, and IFN-γ. TNF-α, along with other players causes pathology (Vissers *et al.*, 2005). Differentiation of macrophages by IL6 (Diehl and Rincón, 2002) proceeds in inflammatory milieu by releasing free radicals, bioactive lipids, nitrates, nitrogen oxide, directly affecting bronchi. Moreover, in chronic allergic responses defected effector T-cell known as exhausted T-cells are formed (Thepen *et al.*, 1989; Wherry, 2011).

Dendritic cells participate in initiation and maintenance leading to persistence response. Allergy arises as dendritic cells present allergen to naïve T cells and causes Th2 proliferation. As a result, immune mediated responsiveness starts at site of action. As in case of asthma DCs are key player of maintaining long term inflammation with increased expression of high affinity Fc receptor (TUNON-DE-LARA *et al.*, 1996) also active proliferation of allergen specific T-cells and FoxP3+ T-reg cells (Penna *et al.*, 2002; Hammad *et al.*, 2003).

The recruitment of immune cell is possible at site of inflammation after migration through the endothelial lining of vascular system. The early -phase cytokines and chemokines upregulate adhesion molecules such as, E-selectin, P-selectin, vascular adhesion molecule (VCAM)-1 and intercellular adhesion molecules (ICAM)-1. This systemic recruitment of leukocytes is hall mark of later chronic reactions (Pober *et al.*, 1987; Bochner *et al.*, 1995). Greater number of VCAM-1 and ICAM-1 are required for immune cells migration which are observed in IgE mediated responses (Howarth, 1992; Manolitsas *et al.*, 1994). A late activation antIgEn (VLA-) 4, appears in late-phase during inflammation on leukocytes in case of chronic inflammation.

Epithelial cells are first line of defense in respiratory tract, which also play critical role in atopic diseases. BAL fluid from atopic asthma patients show elevated number of CCL22 and CCL17 (Panina-Bordignon *et al.*, 2001; Howarth *et al.*, 2004) although these are expressed in non-asthmatic controls but at low level (Sekiya *et al.*, 2000). Moreover,

another chemokine CCL11 along with its receptor CCR3 is also upregulated in patients with such lower airway pathology (Ying *et al.*, 1997). Bronchial epithelial cells of atopic are also observed with presence of various cytokines in abundance such as ICAM-1 (CD-54), CXCL8, GM-CSF and IL-6 (Marini *et al.*, 1992). Increased number of CXCL8 are observed as marker of severity as absent in acute allergic asthma patients and healthy subject. IL-17 upregulates CXCL1, CXCL6 and CXCL8 (Prause *et al.*, 2003).

In the lower airways remodeling related to allergic asthma pathology is well studied. The disease stress causes damage to airways in which structural (Vignola *et al.*, 2003) are made at epithelial, lamina propria and submucosa of the patients. Further these changes are aided with inflammation caused by the various factors involved (Fahy *et al.*, 2000).

In various studies on patients, base membrane diameter increases in a complex manner by immunoglobulins by deposition of collagen I and III, tenascin and fibronectin (Roche *et al.*, 1989; Jeffery *et al.*, 1989; Chetta *et al*,. 1997; Vignola *et al.*, 2003). The interstitial matrix and fibers below basement membrane undergone catalytic based digestion forming disturbed network in patients (Carroll *et al.*, 2000; Vignola *et al.*, 2003)

Blood vessels are also affected in patients in number with increased permeability and diapedesis especially in fatal asthma cases (Carroll *et al.*, 1997; Bousquet *et al.*, 2000). In animal model, smooth muscles become abnormal by swelling up and enlargement (Salmon *et a.*, 1999; Elias *et al.*, 2003). Effect on gland in asthma may include goblet cell hyperplasia, mucous gland hypertrophy and increased mucus production (Fahy *et al.*, 2001; Benayoun *et al.*, 2003).

Upper airways during allergic rhinitis show inflammatory cascade like in asthma condition while remodeling is different may be due to structural differences and differential cytokine expression in asthma, or it may be origin dependent involving embryological genetic factors leading to differential pattern (Bousquet *et al.*, 2003; Sobol *et al.*, 2003). Promoting factors for increased blood vessel in nasal allergies have been observed such as platelet derived vascular endothelial cell growth factor (VEGF) (Bousquet *et al.*, 2004; Salib and Howarth, 2004). While clear picture about the

phenomenon can't be established (Abrams *et al.*, 1999). Changes being observed in nasal vasculatures are dilation, complement protein attachment, and arteriolar wall thinning (Salib and Howarth, 2003). Many molecules have been found with change in expression in asthma, such molecules can act as biomarker (Coste *et al.*, 1998). Tewfik *et al* elaborated nitric oxide involvement in fibroblast activation in nasal allergy and also their increased level as biomarker of allergic state (Tewfik *et al.*, 2003).

IL6 member Oncostatin M is also present in increased number in nasal allergy patient (Kang *et al.*, 2005). Heme oxygenase enzyme confer protection against oxidation mediated cell injury (Elhini *et al.*), as it is elevated in the tissues of patients, and was proposed as therapeutic target in future (Elhini *et al.*, 2006). Matrix metallo proteinases (MMP9) are enzymes responsible for extracellular matrix changes (Atkinson and Senior, 2003) but MMP9 elevation was observed in nasal mucosa of patients (Van Toorenenbergen *et al.*, 1999) while MMP9 along with eosinophilic cationic protein is increased in late response after nasal allergen provocation. Perennial allergic rhinitis patients also were also seen to have high ratio of TIMP-1 and TIMP-2 mRNA (Shaida *et al.*, 2001; Ponikau *et al.*, 2003).

Beside these cytokines, chemokines and mediator, there is need to study the molecules and receptors involved in the initiation of allergy response are important. In case of Ig E mediated asthma and rhinitis, FcεRI along with it downward signaling molecules, adaptors and transcription factors play important role. As they modulate the early as well as the late response, their expression, regulation as well as genetics is important. Many studies including genome wide recent work highlight the importance of various genes along with their genetic variation in allergy pathogenesis and other outcomes. A number of these gene are *IL33, IL1RL1, IL18, HLA-DQ, ORMDL3, SMAD3, RORA, SLC22A5, IL2RB, IL13* (Moffatt *et al.*, 2010; Nakanishi *et al.*, 2013), *ADAM33, CD14, IL4, IL4R* (de Faria *et al.*, 2008), *TNF, ADRB2, MS4A2* (alias FCER1B) and *TSLP* (Bunyavanich *et al.*, 2011). Beside the signaling partners like Lyn and Fyn, ZAP and Syk kinases are also important in generating various pathways leading to release of cytokines and mediator (Ulanova *et al.*, 2005).

ZAP and Syk family are tyrosine kinases, which are cytoplasmic enzymes that are involved in protein phosphorylation in a stream of signal transduction downward of FcεRI receptor (Sanderson *et al.*, 2009; Mócsai *et al.*, 2010). These enzymes are specific for transferring the phosphate group to the tyrosine amino acids in protein. Both of these are structurally similar but functionally different members.

Syk is 72kDa intracellular kinase without any transmembrane region and is encoded by *Syk* gene. Syk consist of three domains two SH2 domain at N-terminal and one kinase domain at C-terminal. Both domain at N-terminal are linked by inter domain-A while the two termini N-terminal and C-terminal are combined by inter domain-B (Siraganian *et al.*, 2002). Syk is involved in diverse biological functions that's why it is not only present in hematopoietic cells but also found in epithelial, fibroblast and osteoclast cells (Mócsai *et al.*, 2010). Syk can be activated by phosphorylation of tyrosine residues in ITAM-dependent or ITAM-independent manner. In ITAM-dependent manner immune-receptor, tyrosine-base activation motif (ITAM) present in the transmembrane part of the receptor is being phosphorylated by Src family kinases upon crosslinking of receptors. *Syk* gene binds these phosphorylated ITAM motifs by the help of SH2 domains and activated (Underhill and Goodridge, 2007; Fuller *et al.*, 2007). While in ITAM-independent process the inter-domain A and B of Syk contains tyrosine residues which are the sites for auto-phosphorylation and are involved in Syk mediated signaling (Tsang *et al.*, 2008)

Syk is involved in adaptive immune responses by activation and development of B lymphocyte, signaling through Fc receptors (FcRs) and B cell receptor (BCR) (Cheng *et al.*, 1995; Crowley *et al.*, 1997; Geahlen, 2009). It also participate in the innate responses by signaling through Dectin-1 receptor in pathogen recognition event (Rogers *et al.*, 2005). Diversity of *Syk* is due to its involvement in other biological functions. *Syk* deficient fetuses present a defect in lymphatic vascular development (Turner *et al.*, 1995). Osteoclast functioning and development requires *Syk* regulated signaling, while GPVI, C-type lection CLEC2, and α2bβ3 integrin receptors of platelet requires *Syk* mediated signaling (Poole *et al.*, 1997; Zou *et al.*, 2007; Boylan *et al.*, 2008).

Syk mediates LPS-stimulated production of RANTES and IL-1 in nasal fibroblast (Yamada *et al.*, 2001). Epithelial linked Syk is found to be important mediator of downstream signaling during airways inflammatory reactions (Coopman *et al.*, 2000; Ulanova *et al.*, 2005)

Syk inhibition in various animal model shows improvement in disease condition due to blockage of downstream signaling. Such studies provide an insight to novel therapeutic methodologies by proving its involvement in diseases like immune thrombocytopenic purpura, rheumatoid arthritis, systemic lupus erythematous, allergic rhinitis and asthma (Matsubara *et al.*, 2006; Pine *et al.*, 2007)

In various studies OVA-sensitized or Ova-induced asthma animals were pre-treated with R406 and other inhibitors against *Syk* that inhibits the development of airway inflammatory responses, eosinophilic recruitment, remodeling of bronchial lining by goblet cells and airway hyper-responsiveness (Braselmann *et al.*, 2006; Bahjat *et al.*, 2008). Likewise, R788 inhibitors show improvement in systemic lupus erythematous (SLE) symptoms by reducing kidney infiltrates (Bahjat *et al.*, 2008) and also reducing antibody mediated severity in rats. Two human based studies with *Syk* inhibitor R788 shows symptoms to be rectify in patients with active RA (Weinblatt *et al.*, 2010). Similarly, R406 show improvement patients suffering from ITP (Podolanczuk *et al.*, 2009).

According to study conducted by (Yamamoto *et al.*, 2003) by using *Syk* inhibitor BAY61-3606 also shows inhibition of edema, constriction and inflammation of bronchioles asthma rat model. Moreover, pre-exposure of anti-sense oligonucleotides (ASO) aerosols against *Syk* inhibited inflammation, lung eosinophilia and tracheal contraction (Stenton *et al.*, 2002). In first clinical trial with *Syk* inhibitor R112 improved patient response with allergic rhinitis and asthma (Matsubara *et al.*, 2006). These observations lead toward the novel therapeutic strategies for the allergic and asthmatic patients.

In different studies it has been observed that genetic changes in *Syk* gene that can lead to suppression or overexpression of protein, which is involved in different pathological disorders. Most of evidences have shown their involvement in various types of cancers. For example, Copeman *et al* observed *Syk* transcripts in different tissues i.e. epithelium, mammary gland tissue as well as in non-invasive breast cell lines. Defect at genetic level is proved by absence or reduction of Syk in invasive breast cell carcinoma. It is also observed that during breast cancer development, hypermethylation of *Syk* locus occurs leading to the loss of Syk protein expression. This type of defect is observed in highly aggressive and metastatic human mammary carcinoma (coopman *et al.*, 2000). In a review on human colon carcinoma, suppression in *Syk* gene expression is observed (Krisenko *et al.*, 2015).

It has been observed that increased Syk level is associated with hyper response in disease manifestation. In case of ovarian tumors lower expression of Syk is observed in low malignant grades while hyper expressive Syk causes high grade malignancy. (Prinos *et al.*, 2011). Likewise, in case of rheumatoid arthritis *Syk* gene expressed excessively that can be observed in wide variety of immune cells involved in the disease pathogenesis (Shrivastava *et al.*, 2015) In case of small cell lung cancer early tumor formation occur due to presence of highly expressed *Syk* gene. The expression of Syk is exaggerated in epithelial lining as compared to normal expression present in healthy individuals as reviewed by (Krisenko *et al.*, 2015). In case of lymphomas elevated Syk expression level is observed which causes immense B cell mediated responses and their expression is necessary for survival of diseased cell. In case of follicular lymphomas B cell receptor engagement activates hyperactive responses due to the presence of constitutively active Syk protein in such cell. These responses are not observed in non-malignant B cell (Leseux *et al.*, 2006; Irish *et al.*, 2006).

In immune response the proteins in pathological cascades are activated at time of requirement by interaction of various proteins. While in malignant conditions the immune cells show continuous presence of proteins like Syk protein. This type of unusual presence of *Syk* is involved in chronic signaling causes, hyper-responsiveness to B cell

dependent signaling. In diffuse large B-cell lymphoma pathology phosphorylated *Syk* is involved in chronic signaling (Cheng *et al.*, 2011). Chronic lymphocytic leukemia progression depends on presence of *Syk* signaling (Buchner *et al.*, 2009). In Mantle cell lymphoma constitutive Syk expression causes the presence of increased amount of proteins in tumor cells and are also observed in cell lines (Bertoni *et al.*, 2006). In B cell acute lymphocytic leukemia also shows active *Syk* involvement consitutively expressed in the cells showing B cell receptor independent responses (Perova *et al.*, 2014).

The proteins are expression of gene transcripts any change during their transcription can lead to defected protein or change in protein expression. In marginal zone lymphomas modulation in *Syk* gene transcription has observed. (Ruiz-Ballesteros *et al.*, 2005).

In case of breast cancers various polymorphisms in *Syk* gene are present, which are associated with tumor progression. These variation causes altered protein response in patients and observed to be a prominent risk factor in breast carcinogenesis. Various Pakistani studies show such polymorphisms with in *Syk* gene associated with breast cancer. Within the kinase domains of *Syk* gene, about twelve variants are found in both intronic and exonic regions. These novel mutations show increased risk for cancer prognosis (shakeel *et al*, 2012). The mutations and variants present in cancer cases causes decreased transcript level in malignant cells as compared to normal cells. Such decreased level is to avoid tumor suppression role of *Syk* (Inayat *et al.*, 2012).The suppression can be due to production of mutated products of gene that fails to act normally. It has found that single nucleotide variants in 5`UTR region can lead to defected Syk proteins, which decreases resistance against cancer progression (Kanwal *et al.*, 2012).

These studies have shown involvement of *Syk* gene in inflammatory processes and also in asthma which makes it center of interest for further studies. The suppression and overexpression in *Syk* gene in various cancer studies have been observed which is caused due to variations at genetic level. As inflammation related studies have proved overexpression of *Syk* gene in case of asthma this lead to an idea that may be there is some type of variation present like in case of cancers, which causes hyper responsiveness

and altered expressions of protein. As previously there is no step has taken to study, the involvement of *Syk* at genetic level with in atopic allergies or asthma makes it a good point to be focused for research studies.

Chapter 3: Material and Methods

3.1: Study Design and Sample size:

This study was case-control study, carried out to analyse presence of mutations and polymorphism in *Syk* gene among asthma patients and healthy individuals. Asthma patients visiting different allergy centres were included in this study. Total 228 asthma patients examined and diagnosed clinically by physicians were included in this study while 205 healthy individuals participated in the study. Demographic and clinical data was assessed by using designed questionnaire which included gender, age groups, living conditions, family history and smoking profile along with other clinical features (Appendix I).

3.2: Blood samples collection from patients and controls:

Blood samples were collected after taking the written consent of asthma patients as well as controls (Appendix II). Asthmatic patients included in this study were one visiting National Institute of Health Islamabad (NIH) and Federal Government Hospital, Islamabad. While control samples were taken from general population.

By using 5 cc sterile syringes, 3-4 ml peripheral blood was taken from asthma patients as well as controls and immediately transferred to EDTA vials. These samples were kept in ice box to maintain 4°C and then brought to Medical Microbiology and Immunology Laboratory at Department of Microbiology, Quaid-i-Azam University (Islamabad) for analysis of *Syk* gene polymorphisms.

3.2.1: Inclusion and Exclusion Criteria:

All patients with allergic asthma and comorbid along with allergic rhinitis were included in the study. While for controls blood samples of healthy individuals were collected. Patients above 13 year of age were included in this study. Whereas children below age of thirteen years were excluded. Patients having any metabolic disorder, microbial infections or other allergies were also excluded from the study.

3.3: Phenol chloroform method for DNA extraction from blood samples:

In two consecutive days, Genomic DNA of patients and controls were extracted from blood samples by using "Phenol chloroform method". For extraction, various chemicals were used as each has a particular function. A complete list of chemicals used for stocks and working solutions preparation in DNA extraction is given in Appendix III, IV and V.

Day 1:

In 1.5ml eppendorf, 750µl blood was taken from patients as well control samples. A total of 500µl Solution A was then added in the eppendorf and these tubes were inverted for 15 min. These tubes were subjected to centrifugation at 13,000 rpm for 5 min to form the pellet. After centrifugation, supernatant was discarded carefully, so that pellet remained intact. Solution A, 500µl was again added to the pellet and with the help of vortex this pellet was broken and dissolved in the solution. Again centrifugation at 10,000 rpm was performed for 10 min and after that supernatant was discarded. A 400µl Solution B was then added in the pellet followed by 15µl SDS (20%). Finally, 4µl of Proteinase K was added and for the digestion of proteins the tubes were placed overnight in incubator at 37°C.

Day 2:

Solution C and D were prepared by taking equal amount of solutions in falcon tube. For the layer formation, these solutions were left at room temperature for 1 hour. After formation of layers, solution C+D 500µl was added in these overnight incubated tubes. Tubes were centrifuged at 10,000 rpm for 13 minutes. Supernatant was shifted in the new eppendorf carefully by not disturbing pellets. Equal amount to supernatant, chloroform-isoamyl alcohol (24:1) was added in the new tubes and were placed in centrifuge at 10,000 rpm for 10 min. After centrifugation, again supernatant was picked and transferred to the new eppendorf. Later, Sodium acetate and isopropyl Alcohol (chilled) was added in the tube in 55µl and 500µl amount respectively. Tubes were inverted

several time for mixing purpose and then centrifuged at 10,000 rpm for 4 minutes. In order to obtain DNA pellet, supernatant after centrifugation was discarded carefully. For washing of the DNA pellet, 70% ethanol (500µl) was added in the tubes and centrifugation was done at 10,000 rpm for 5 min. After washing, supernatant was discarded and the DNA pellets were allowed to air dry at room temperature. When the samples got dried, 100µl Tris EDTA buffer (1X) was added and the samples were preserved at -20°C.

3.4: Genotypic analysis of *Syk* gene among study population:

For detection of variations in *Syk* gene, Polymerase Chain reaction (PCR) and single stranded conformational polymorphism analysis (SSCP) was done. Variants that were detected by these techniques were sent for commercial sequencing by Macrogen (Korea) for the analysis of mutations/polymorphisms (SNPs).

3.4.1: Polymerase Chain reaction for amplification of *Syk* gene exons:

Gene specific primers were designed (Primer3) and used for the amplification of *Syk* gene's exons via PCR technique. Genomic DNA was quantified before amplification by using gel electrophoresis. In PCR amplification, various conditions like annealing temperature, primer concentrations and thermocycler conditions were tested and optimum conditions were assessed. A wide range of temperature 48 to 64 ^{0}C was examined for the optimization of exons 15 and 15' of *Syk* gene. Likewise, different concentrations (from 0.1µl to 0.5µl) of primer were analysed. Thermocycler conditions were also checked for the amplification purpose. All the PCR reagents and thermocycler conditions that were optimized are mentioned in Table 3.1-3.3 respectively. After optimizing all the conditions, amplification of these exons was performed by using patients and controls DNA.

3.4.2: Visualization of DNA and PCR products via Horizontal Gel Electrophoresis:

Horizontal gel electrophoresis was used for the quantitative analysis of extracted DNA and for qualitative analysis of amplified PCR products of both patients and controls. For DNA visualization, 1% agarose gel and for PCR products 2% agarose was prepared. For DNA- 0.5g agarose while for PCR products- 1.0 g agarose was weighted and mixed with 50ml of Tris Borate EDTA buffer (1X). The mixture was then microwaved for 2 min in order to form a clear gel. This solution was left at room temperature and gel casting tray was assemble. Before pouring of gel into pre-assembled apparatus, 2-3µl ethidium bromide was added for staining and gel visualization. The gel was then carefully transferred to the casting tray and left for 30 min for proper solidification. After solidification, combs were removed and the gel was placed in the electrophoretic chamber containing 400ml of TBE buffer (1X). For loading of the DNA, loading dye (2-3µl) was mixed well with DNA (2µl) and loaded carefully in the wells. While for PCR products, 4µl of amplified products were directly loaded in the wells. Voltage of electrophoretic apparatus was 90V for both DNA and PCR products. Electrophoresis was performed for 25 min for DNA while PCR products time was 40 min. Quantitative visualization of DNA and qualitative visualization of amplified products was done by using UV-trans illuminator. List of ingredients for TBE and loading dye are given in appendix VI.

3.4.3: Analysis of amplified products by SSCP on 8% PAGE:

SSCP is a technique, which analyses varied banding pattern and mobility shift among study population. After PCR, SSCP analysis was performed by using 8% poly acrylamide gel electrophoresis.

In order to perform 8% PAGE, apparatus includes 2 glass plates with varying length, 3 spacers, a comb and multiple clamps was used. Plates were first rinsed by 70% ethanol and air dried. After air drying, these plates were assembled in such a way that the larger rectangular plate was separated by the smaller one by the help of spacers between the three sides of the plates leaving the upper side opened. Leakage was avoided from the sides of the plates by using clamps that tightened the plate assembly. After assembling

the apparatus, 8% polyacrylamide gel was prepared. For the preparation of 8% PAGE, chemicals that were used for the stock and working solution preparation are mentioned in Table3.4 and Appendix VII.

After gel preparation, the gel was transferred to the assembled apparatus instantly. Pouring was done carefully in order to avoid bubble formation followed by comb insertion between the gel containing glass plates. The gel was then allowed to solidify for 1 hour. After gel solidification, the clamps were removed along with the lower spacer so that gel was dipped in TBE buffer. The gel plates were then placed vertically in the electrophoretic column by the help of clamps while the combs were removed in such a way that the wells were not disturbed. Electrophoretic column consist of a larger horizontal tank and a small vertical tank. Both the tanks were filled with TBE buffer (1X) in an appropriate level so that wells were completely dipped.

After assembly and gel solidification, amplified products were denatured at 95°C for 8 min. For denaturation purpose, form-amide solution (5µl) was added in 10µl PCR products and then placed in the thermocycler for denaturation. After denaturation step, these products were immediately transferred onto the ice and after 10 min these were loaded in the wells after mixing with 5µl loading dye. The electrophoresis was performed for 3-5 hours at 120V.

For visualization purpose, gel was first stained with ethidium bromide staining solution (100mg/ml). The gel was dipped in staining solution for 5 min and visualized under UV-trans illuminator. After visualization of banding pattern, variants were selected on the basis of band shift. These variants were then again amplified and then sent for sequencing after purification.

3.4.4: Purification of PCR products:

After detection of variants by SSCP analysis, selected variants were amplified again and were subjected to purification before sequencing. Purification was done by multiple steps. In amplified products equal amount of binding buffer was added followed by the

addition of 10µl sodium acetate (3M) with the indication of colour change. Binding of the DNA was indicated by yellow colour while orange colour indicated inverse. As the band length of amplified exon were 415bp (exon 15') and 444bp (exon 15), for this length 1:2 of isopropanol (100%) was added and mixed well. Afterward, it was transferred to purification-column and centrifugation was done for 40-50sec followed by discarding of supernatant. After discarding of supernatant, for washing of the purification-column wash buffer was added and centrifugation of 50 sec was done. Supernatant was discarded again and the column was then positioned on the collection tube. Centrifugation was again carried out in order to remove remaining wash buffer. In a new Eppendorf vacant column was shifted followed by the addition of 40µl elution buffer and centrifugation. After the final centrifugation step, column was disposed, while the purified DNA was collected and stored at -20°C.

3.4.5: Sequencing of selected variants:

Sequencing was carried out for those samples that were selected on the basis of band shift on 8%PAGE. For sequencing, these selected samples were again amplified. Apart from amplification, purification was also performed. For the sequencing purpose, purified PCR samples (25µl) were labelled properly. By using paraffin, the samples tubes were wrapped tightly and shifted to ice or -20°C overnight. After that, purified products were sent to Macrogen, Korea for sequencing.

3.5: Statistical analysis:

A number of statistical tools like Microsoft Excel, SPSS 16.0, and online calculators were used for the analysis of demographic as well as clinical data. By using Microsoft excel, percentages and mean of different variables were evaluated. Data was arranged in the form of graphs and tables by using Microsoft excel and word. SPSS 16.0 and online Chi-square calculator was used to assess association between demographic and clinical variables.

Table No.3.1: Primer used for the amplification of various exons of *Syk* Gene

S.no	Exons	Forward Primer	Reverse Primer	Band size
1.	Exon15	TCAAGCTCCCGATCTCAAGT	TGGAATAGACTGAGGGCACA	415bps
2.	Exon15`	TGACCCAACTGACTCCAACA	CCCCACCGTTGTTTGTTTAG	444bps

Table No. 3.2: Reaction mixture for the amplification of *Syk* gene exons and optimized quantities

S.no.	Reagents	Volume (µl)	Final Volume (10µl)
1.	Master mix (2X) (Thermo Scientific)	5	9
2.	Forward Primer (e-oligos)	0.3	
3.	Reverse Primer (e-oligos)	0.3	
4.	PCR Water (Thermo Scientific)	3.4	
5.	Extracted DNA	1	1

Table 3.3: Various temperatures with their duration optimized for *Syk* gene exon amplification.

S.no.	Stages		Temperature °C	Time	No. of Cycles
1.	Initial Denaturation		95°C	5 minutes	1X
2.	Denaturation		95°C	45 second	35 X
3.	Annealing	Exon 15`	60°C	45 seconds	
		Exon 15	55°C		
4.	Extension		72°C	45 seconds	
5.	Final Extension		72°C	7 minutes	1X

Table No. 3.4: Composition of 8% Poly acrylamide gel (PAGE)

S.no.	Reagents	Amount	Final volume
1.	30% Arcyl-Bisacrylamide solution	13.5mL	**50 mL**
2.	10X-TBE	5mL	
3.	10% Ammonium per sulphate	350µl	
4.	TEMED	25µl	
5	Distilled water (to adjust total volume upto 50mL)	31.125mL	

Chapter 4: Results

4. RESULTS:

4.1. Description of study population:

This study was a case-control study based on candidate gene approach. The major aim of this study was detection of genetic variations in *Syk* gene among allergic asthmatic/allergic rhinitis individual. This study was conducted after ethical approval by ethical committee of Quaid-i-Azam University. For this study, 228 blood samples of asthma patients were enrolled from National Institute of health and Federal Government hospital, Islamabad. In case of controls, a total of 206 blood samples of healthy individuals were collected from various institutes from general population. Blood samples from both patients and controls were taken along with written consent and filled questionnaire.

4.2. Clinical and Demographic characteristics of study population:

The questionnaire designed to collect data about disease history in relation with various risk factors (atopy, gender, age, tobacco smoke, viral infections), allergens (pollen, dust, air, smoke, cool air, animal dander) and possible genetic predisposition for atopy. Various parameters of study are then compared with one another and their percent relations. The significance of association among variables to allergies was analyzed for the data by carrying out statistical analysis using SPSS software.

Males and females were more or less equal in number in the study. The number of male patients was higher than the male in general population (controls) as compared to females. A total number of patients were 228, in which more than half about 119 were males and 109 were females. Among 206 controls, 164 were males and 41 females. The study population was categorized into 4 age groups, in which first group ranging from 14-30 years which comprised of 102 patients and 153 controls. The second group had 68 patients with 40 controls which had age range from 31-45 years. The third group contained 47 patients with 12 controls having age range from 46-60 years. The last number of patients was in age group above 60 years. Thus, the younger patients with age

14-30 years was the major affected group with allergies, however, least number belonged to above 60 years. When patients and controls were grouped on basis of marital status as married, unmarried and widowed, numbers of patients/controls in these groups were 153/79, 62/126, and 13/0 respectively (Table 4.1).

Socioeconomic status of the patients and control was divided into 3 classes on the basis of income level, which were lower class, middle class and higher class. Majority of patients (104) belonged to lower class with least number were from higher class (38). The number of controls mostly had middle class income; lower numbers belonged to higher income bracket (Figure 4.1). Asthmatic patients were living in the urban areas mostly as compared to rural setting. Another factor studied was work environment, as exposure due to environmental condition effect the asthma outcome. A high percentage of male patients were outdoor workers, it was 56% followed by office worker 39%, students 20% and unemployed 4%. While in case of females, a very few number was working outdoor which were 6 in number and 17 were students. Majority of the females were placed in indoor environment as they were residing at their homes (Table.4.2).

Table 4.1: Patients and controls' gender, age and marital status in percentage.

S.no	Characteristics		Patient %	Control %
1.	**Gender**	1) Male	52%	80%
		2) Female	48%	20%
2.	**Age groups**	1) 14-30 years	45%	75%
		2) 31-45 years	30%	20%
		3) 46-60 years	20%	5%
		4) 60+ years	5%	0%
3	**Marital Status**	1)Married	67%	39%
		2) Un Married	27%	61%
		3) Widowed	6%	0%

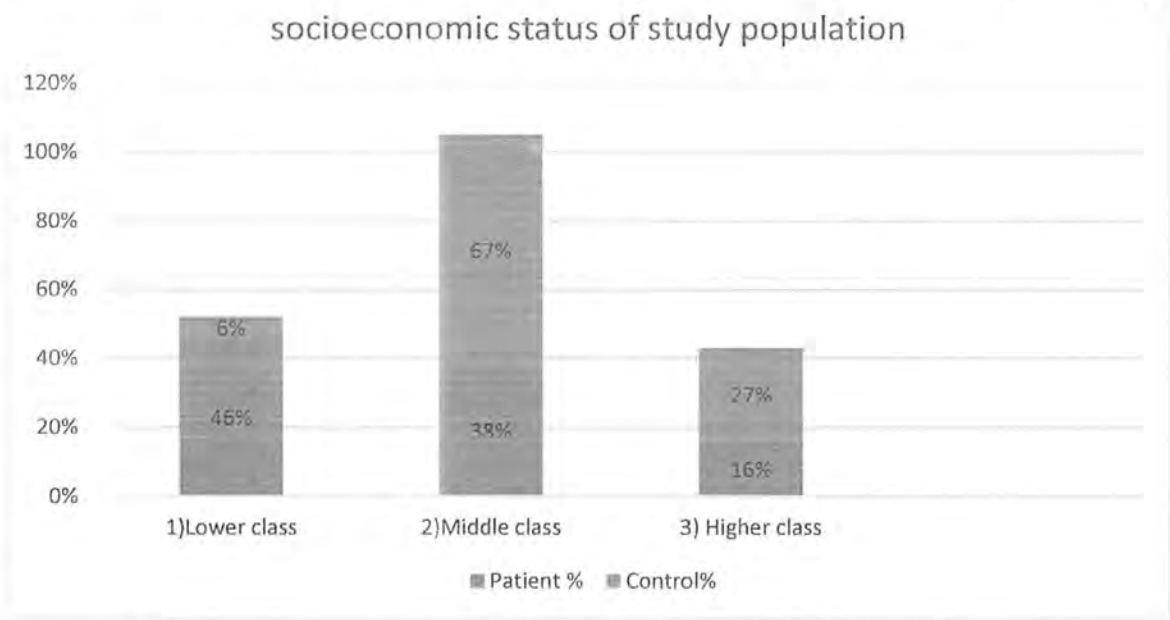

Figure 4.1. Socioeconomic status among patients and control

Table 4.2: Distribution of patients according to living and work environment.

S.no	Characteristics			Percentage (%)
1.	**Living Environment**		Urban	65%
			Rural	35%
2.	**work environment**	**Women**	1)Home maker	83%
			2)Outdoor worker	6%
			3) Student	11%
		Men	1)Office worker	33%
			2)Outdoor worker	47%
			3)Unemployed	3%
			4) Student	17%

The major study population belongs to Punjab region (105) among patients and Punjabi being the dominant ethnic group with 127 individuals in total. The patients from capital and adjoining area were 60 in number followed by patients from Khyber Pakhtunkhwa belonging to Pashto ethnicity. Other patients were from various regions of Pakistan which included Azad Jammu Kashmir, Sindh, Baluchistan and others with descending percentages. Among other ethnic group Kashmiri, Sindhi, Baluchi and Saraiki were more prominent (Table 4.3).

In patients' and controls, a high number were non-smoker. A very few patients were smoker while moderate number of controls were smoker. While few patients with previous history of smoking were also present with 7% of total. Caffeine consumption was divided into low, medium and high level on the basis of daily consumption of tea, coffee and energy drinks. A higher number of patients (94) were taking less caffeine, while medium caffeine consumers were 57 and a higher caffeine taker were 48 patients. Some patients had no consumption of caffeine at all. Most controls had low caffeine uptake or no caffeine uptake, while individuals of medium and high caffeine uptake were 39 and 23 (Figure 4.2-4.3)

The symptoms of the disease are commonly related to the airways manifestations. These are upper respiratory problems (URP), lower respiratory problem (LRP), or combination of two as LRP/URP. Majority cases observed had both type of symptoms at a time hence termed as LRP/URP. About 135 patients had both type of symptoms, while least common were URP only in 18 patients out of total while moderate level of LRP was observed (75 patients).

Symptoms appear distinctly in different patients, hence showing varied pattern of disease symptoms. These variations are due to disease condition, day/night difference and seasonal changes. The patients with symptoms throughout year were 119 patients while rest of 109 patients had seasonal symptoms. The episodic cases were 92 followed by continual cases which were 136 patients. Day and night dependent differences were also present where severe conditions occurring at night among 139 patients. Daytime

complication cases were 57 in number and least number of 32 cases had early morning complications (Table 4.4)

Table 4.3: Distribution of patients according to region and ethnic groups

S.no	Characteristics		Patient n	Percentage (%)
1.	**Regions**	1.Punjab	105	46
		2.Federal	60	26
		3.Khyber Pakhtunkhwa	41	18
		4.AzadJammuKashmir	10	4
		5.Sindh	4	2
		6.Baluchistan	2	1
		7.Other	6	3
2.	**Ethnic groups**	1.Punjabi	127	55.7
		2.Pashtun	50	21.9
		3.Saraiki	10	4.4
		4.Urdu	10	4.4
		5.Kashmiri	8	3.5
		6.Baluchi	1	0.4
		7.Sindhi	2	0.88
		8.Others	20	8.8

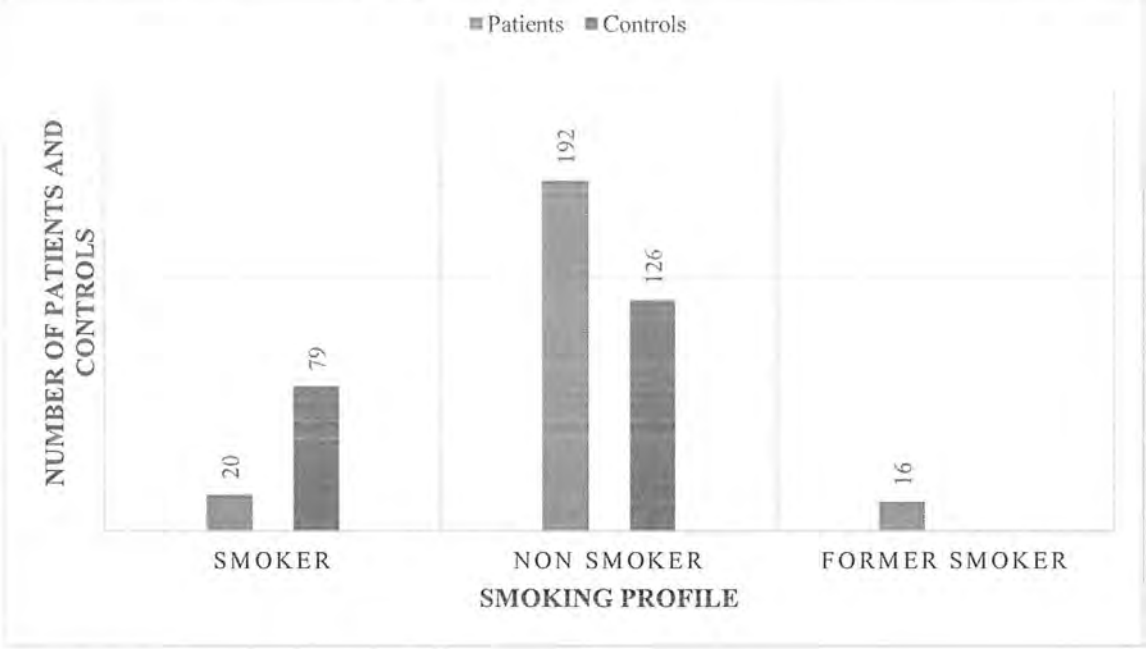

Figure 4.2: Distribution of patients according to smoke history.

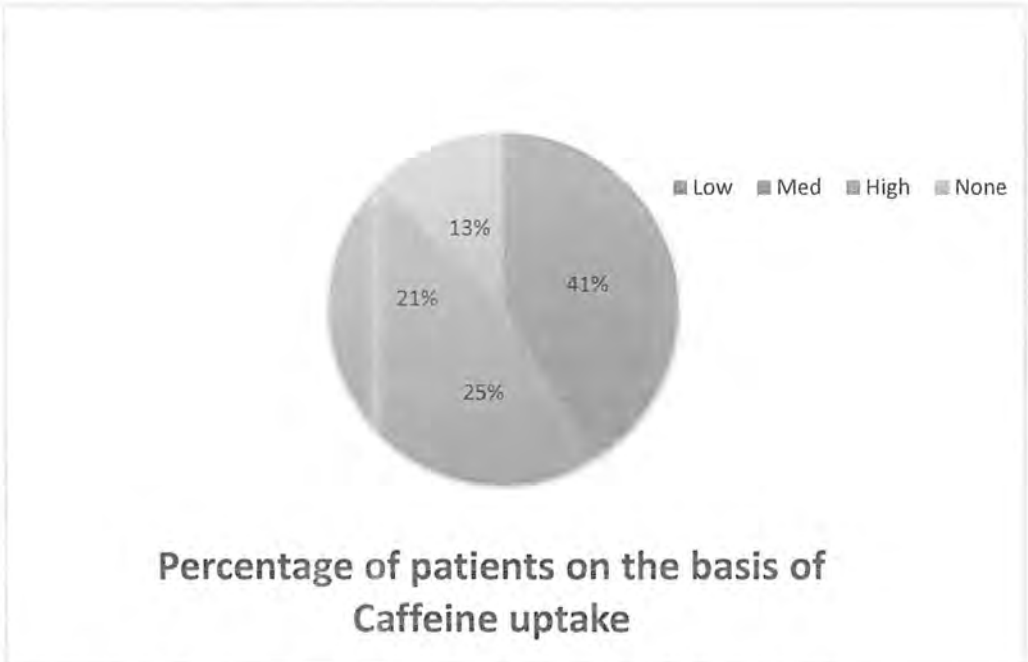

Figure 4.3: Distribution of patients according to caffeine uptake

Table 4.4: Common symptoms and their pattern of occurring in patients

S.no.	Features			Patient (n)	Percentage
1.	Symptoms		LRP	75	33%
			LRP/URP	135	59%
			URP	18	8%
2.	Pattern of symptom	On the basis of Seasonal trends	Seasonal	109	48%
			Perineal	119	52%
		On the basis of symptom occurrence	Continual	136	60%
			Episodic	92	40%
		According to day/night Variation	Nocturnal	139	61%
			Diurnal	57	25%
			Early Morning	32	14%

Season related complication cases were found to be occurring mostly in the spring season among 76 patients. Patients effected by spring/winter which is more than one season were 56 patients. Some 65 case had symptoms throughout the year. The winter related disease variations were observed in 21 patients (Figure 4.4)

On the basis of comorbid conditions, patients were divided into two groups. In one group the allergic patients suffering only from asthma, which were 110 in number. In the second group, patients who had asthma along with rhinitis were placed which were 118 patients (Table 4.5).

Various allergens are responsible for provocation of allergic responses. In this study, the studied allergens responsible for the disease symptoms development were diverse in nature. The pollens and dust were major triggers affecting majority of cases which were 202 in patients. Other triggers were food (141 cases), weather changes (110 cases), cool air (123 cases), smoke or perfume (181 cases), exercise (96 cases) and molds (34 cases) as shown in Figure 4.5.

Associations of asthma with family history was also assessed, among all patients enrolled 131 patients had family history of allergies. Majority of the patients enrolled were only clinically assessed and diagnosed by allergist, these patients were not tested by skin prick test only 82 patients had skin prick test. Among studied individuals, only 81 patients were vaccinated for allergy suppression while majority 147 never had vaccine against allergic responses (Table.4.6)

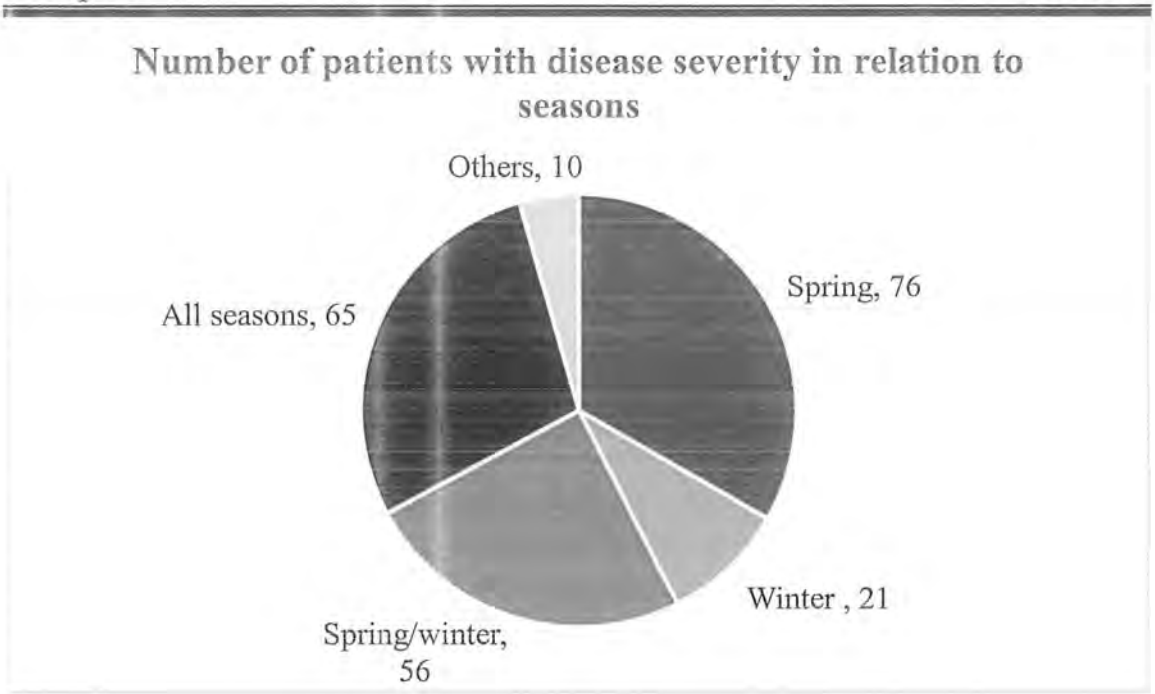

Figure 4.4: Patients with seasonal variation of disease

Table 4.5: Distribution of patients on the basis of types of atopic allergy occurrence

Occurence of Disease	# of Patients	Percentage (%)
Atopic Asthma	110	48
Asthma+Rhinitis	118	52

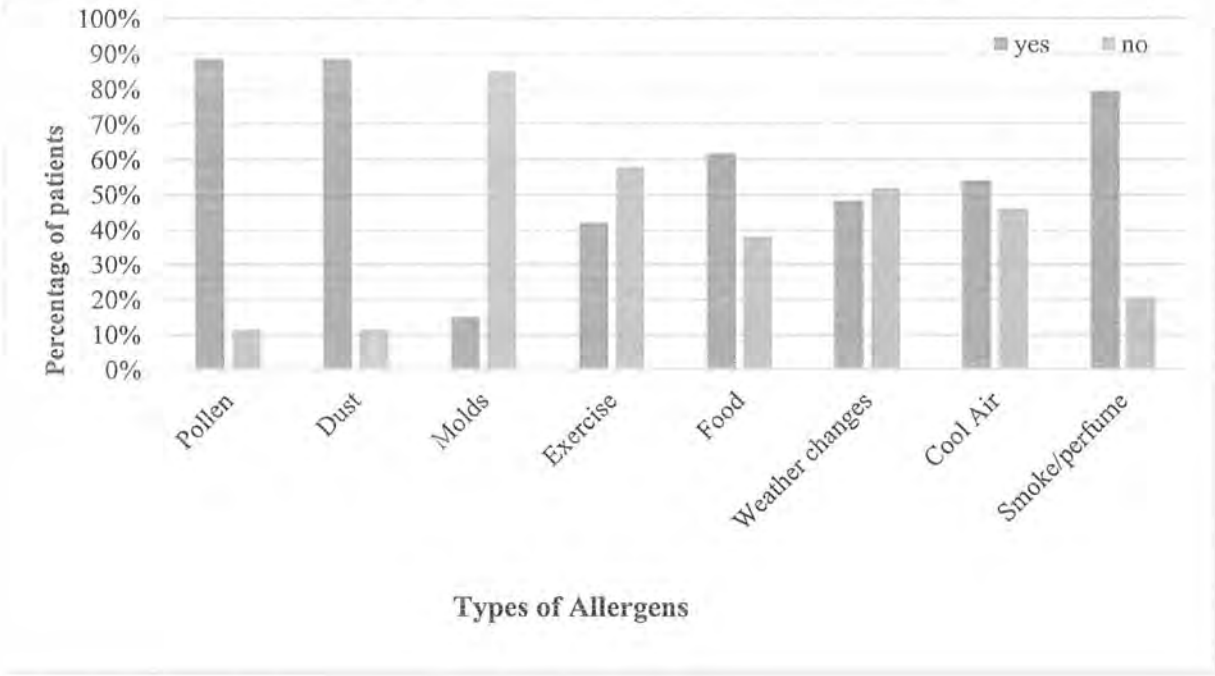

Figure 4.5: Distribution of patients on the basis of association with different allergen

Table 4.6: Association of asthma in patients with family history, skin prick test and vaccination

S.no.	Characteristics		Number of patients	Percentage (%)
1.	Family History	Positive	131	57
		Negative	97	43
2.	Skin Prick Test	Yes	82	36
		No	146	64
3.	Vaccination	Yes	81	36
		No	147	64

Statistical analysis was done by using SPSS (version. 20). Significant associations between variables were also calculated, a value of ≤ 0.05 indicates a significant association. There was significant association of family history with allergic symptoms as *p*-value obtained was 0.016. Family history of atopy was significantly associated with various allergens like smoke, weather change and exercise. Smoking history does not associate with family history it may act as independent variable. Family history was found strongly associated with LRP or LRP/URP disease symptoms while URP is not associated with family history (Table 4.7).

4.3. Detection of novel mutation in *Syk* gene:

This study was aimed to detect the novel mutations or variations among atopic allergic individuals in Pakistani populations. To carry out this genetic level study, DNA was extracted from study population shown in Figure (4.6-4.7). Further amplified products were obtained by PCR for the exon 15 and exon 15` as shown in Figure 4.8-4.9. Possible allelic differences were observed by running amplified products of exon15 and exon15` on vertical acrylamide gel. Band with possible shifts were observed carefully by SSCP (Figure 4.10-4.11-4.12-4.13) and visualized by BIORAD Gel Documentation System. Selected variants observed with shifts on gel were sent for sequencing to Macrogen Korea.

After sequencing, these results were interpreted by Bioedit software version (7.2.6). In exon 15, the variants 93650015 T>A were detected as shown in dna electropherograms (Figure 4.14). These variations were observed in control samples which are normal healthy individuals.

In exon 15`, poly A region was observed a few bases away from start site that causes noisy peaks away from the homopolymer (Figure 4.15). Four type of substitutions were observed which were 93650818 A>C, 93650830 G>A, 93650832 G>A and 93650839 G>A (Figure 4.16) in the same sequence. These are novel variations as no such reported variants are available in any of data base like Ensemble, NCBI or others. Complementary strand of exon 15` was sequenced for resolving noise due to homopolymer and for the

confirmation of variations observed in the preceding sequence. The complementary strand shows no such variation also no peak disturbance due to poly A as shown in Figure 4.17.

Table 4.7: Association of symptoms, allergens and smoking profile with Family history of allergies.

Variables		Family History		P-Value
		No	Yes	
Symptoms	LRP	41	34	0.010*
	LRP/URP	47	88	0.004*
	URP	9	9	0.505
Allergens	Pollen	86	116	0.979
	Dust	86	116	0.979
	Smoke	68	113	0.003*
	Exercise	32	63	0.022*
	Food	54	87	0.09
	Weather changes	38	72	0.018*
	Cool air	53	70	0.857
Smoking profile	Smokers	7	13	0.475
	Non Smokers	82	109	0.788
	Former smokers	8	8	0.532

Genomic DNA of Patients:

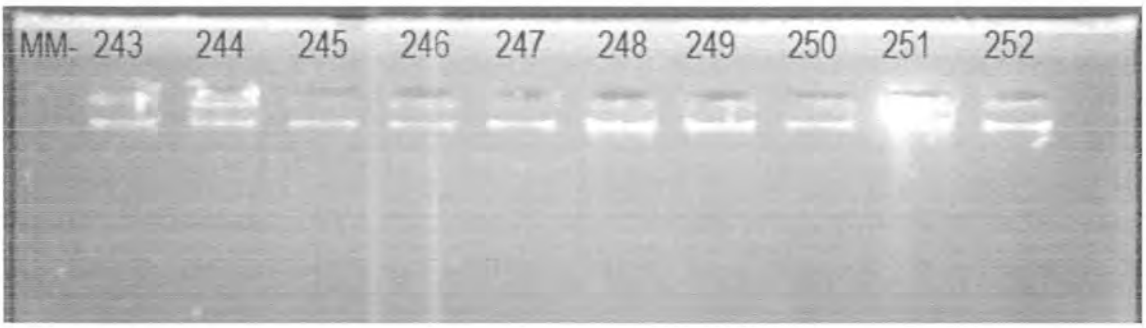

Figure.4.6: 1% agarose gel for DNA extracted from allergic patients (MM 243-252).

Genomic DNA of Controls:

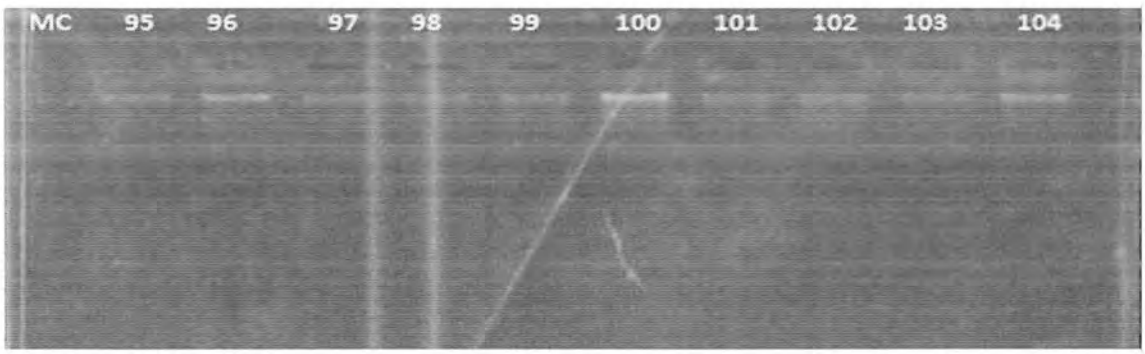

Figure.4.7:1% agarose gel of DNA extracted from Controls (MC95-104)

Exon 15:

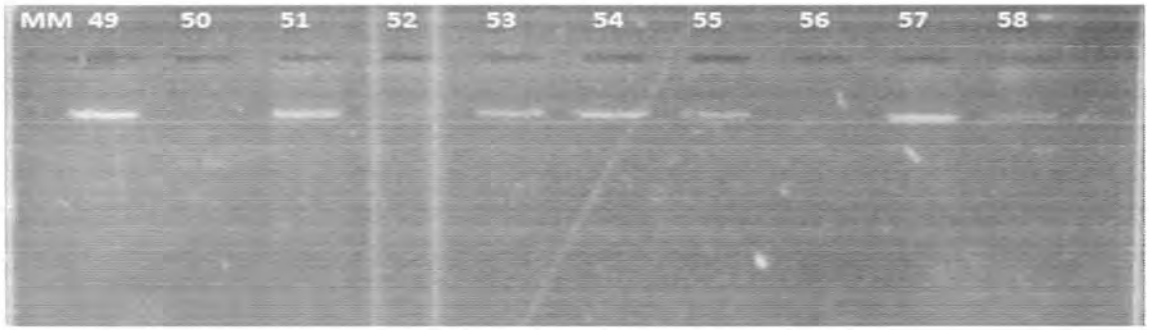

Figure.4.8: Amplicons of exon15 starting from lane 1(MM 49, 51-55, 57-58) showing amplicon having band size of 444bp.

Exon 15`:

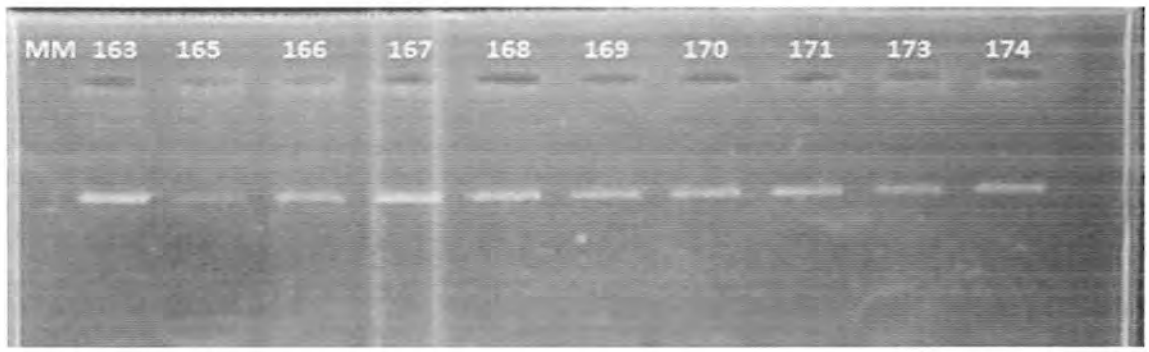

Figure.4.9: Amplification of exon15` starting from lane 1 (MM163, 165-171, 173-174) showing amplicons having band size of 415bp.

SSCP of Exon 15:

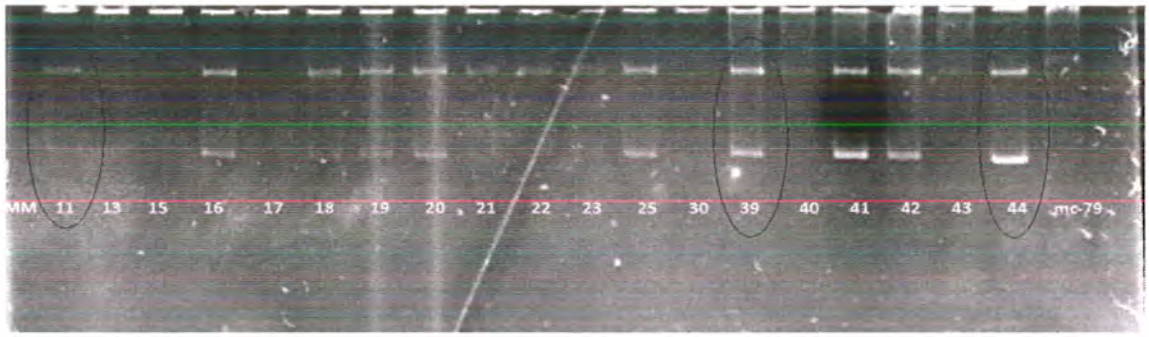

Figure.4.10: 8% acrylamide showing denatured amplicons of exon 15. Lane 1-19 shows allergic patients that includes MM 11,13,15,16,17,18,19,20,21,22,23,25,30,39,40,41,42,43 and 44. The last lane shows control sample MC-79.

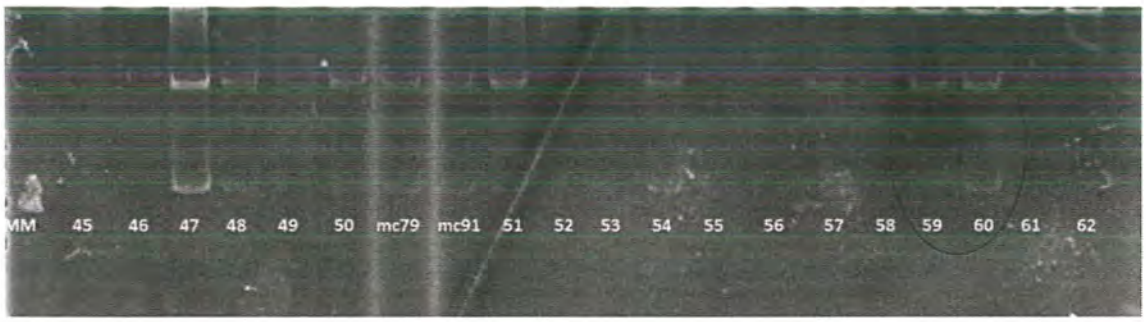

Figure.4.11:8% acrylamide showing denatured amplicons of exon 15. Lane shows PCR products of allergic patients that includes MM 45,46,47,48,49,50 from lane 1-6 and lane 9-20 contains MM-51,52,53,54,55,56,57,58,59,60,61 and 62. Control samples in lane 7-8 (MC-79 and MC-91.

SSCP of Exon 15`:

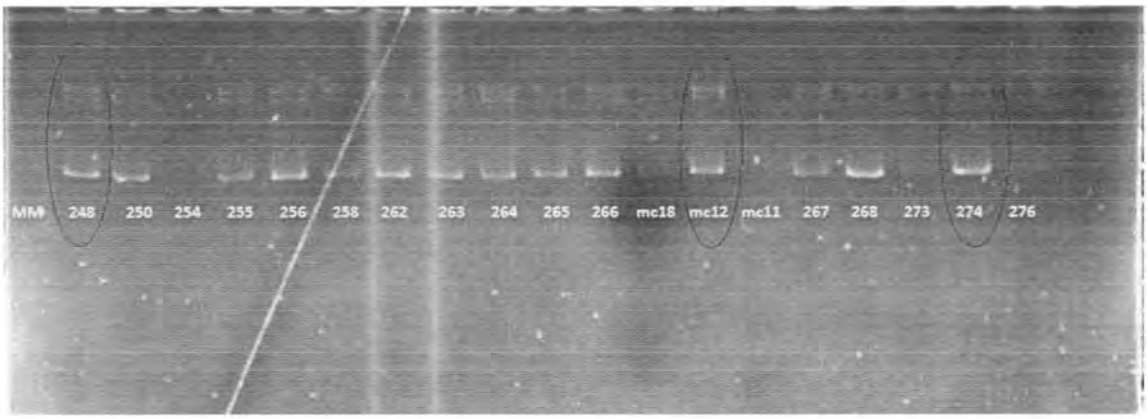

Figure.4.12: 8% acrylamide showing denatured amplicons of exon 15`. Patients amplified products Lane 1-11 (MM 248, 250, 254, 255, 256, 258, 262, 263, 264, 265, 266) and lane 16-20 (MM 267,268,273,276), control samples lane 12, 13, 14 (MC-18, 12, 11). Variations observed in three lanes in sample MM-248, MM-274 and MC-12.

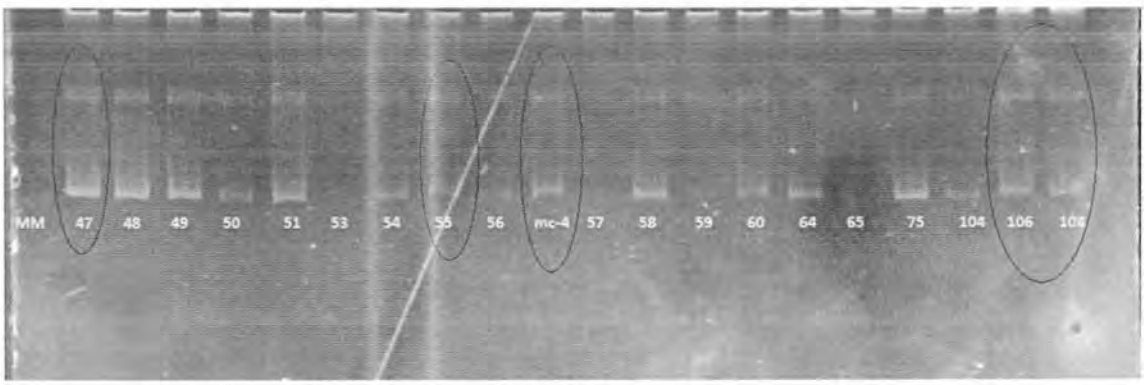

Figure.4.13: 8% acrylamide showing denatured amplicons of exon 15`. Lane 1-9 (MM 46-108) and in lane 10 control amplicon (MC-4), variations observed in lane 1,8,10,19 and 20 with sample number MM-47,55,106,108 and MC-4

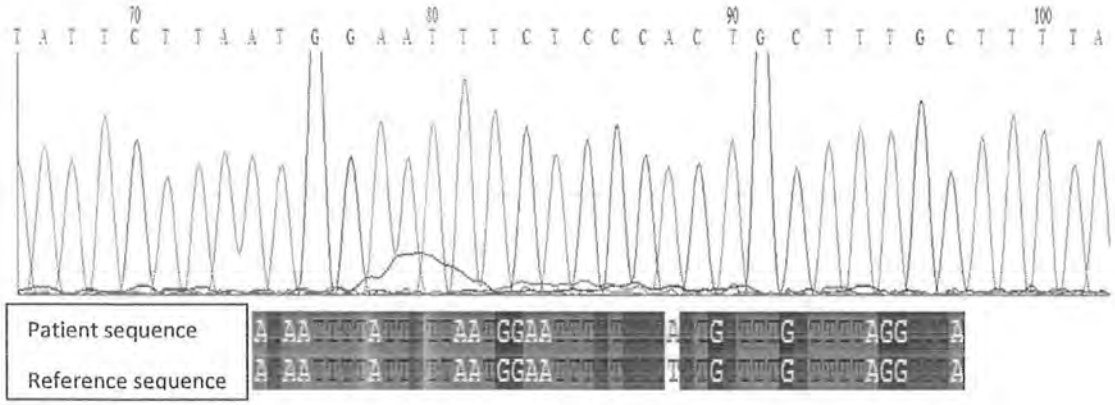

Figure 4.14: Exon-15 variant in control samples

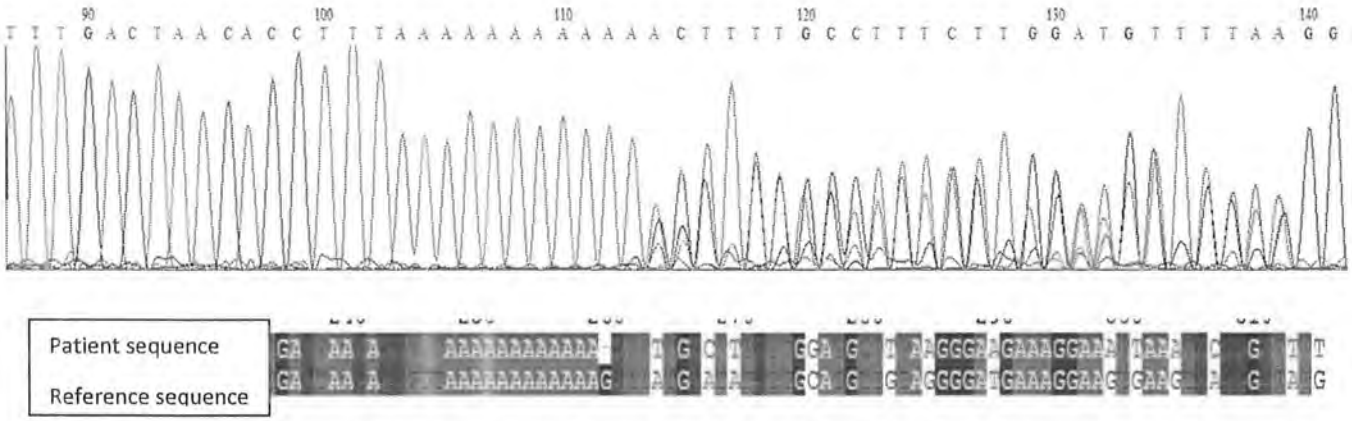

Figure 4.15: Exon 15` Poly-A region and disturbed peaks in patient's sample.

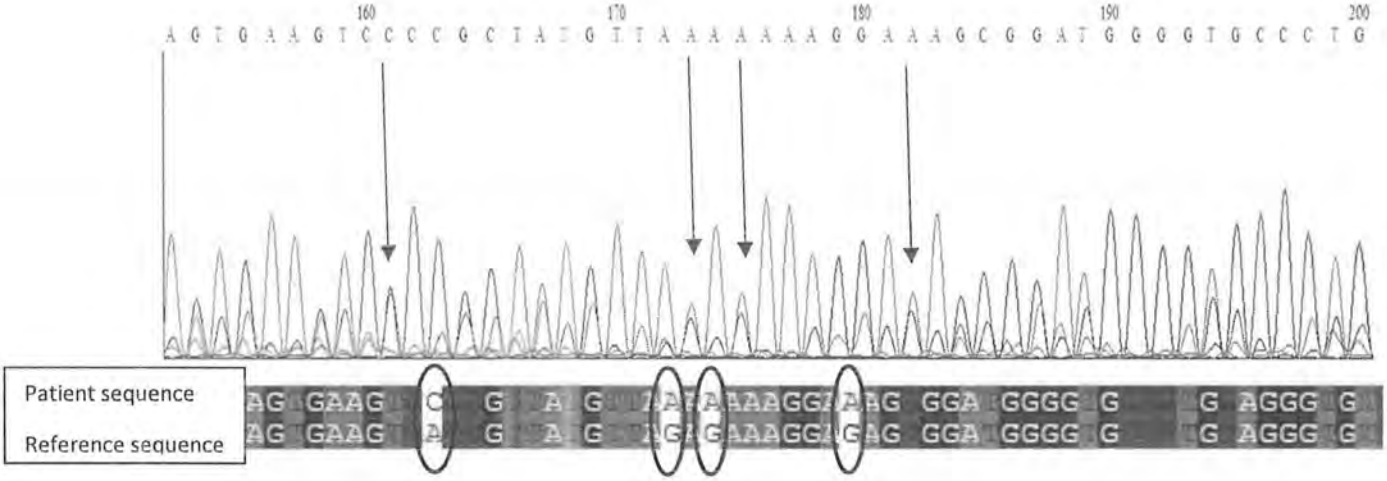

Figure 4.16: Exon 15` Poly-A region and disturbed peaks in patient's sample.

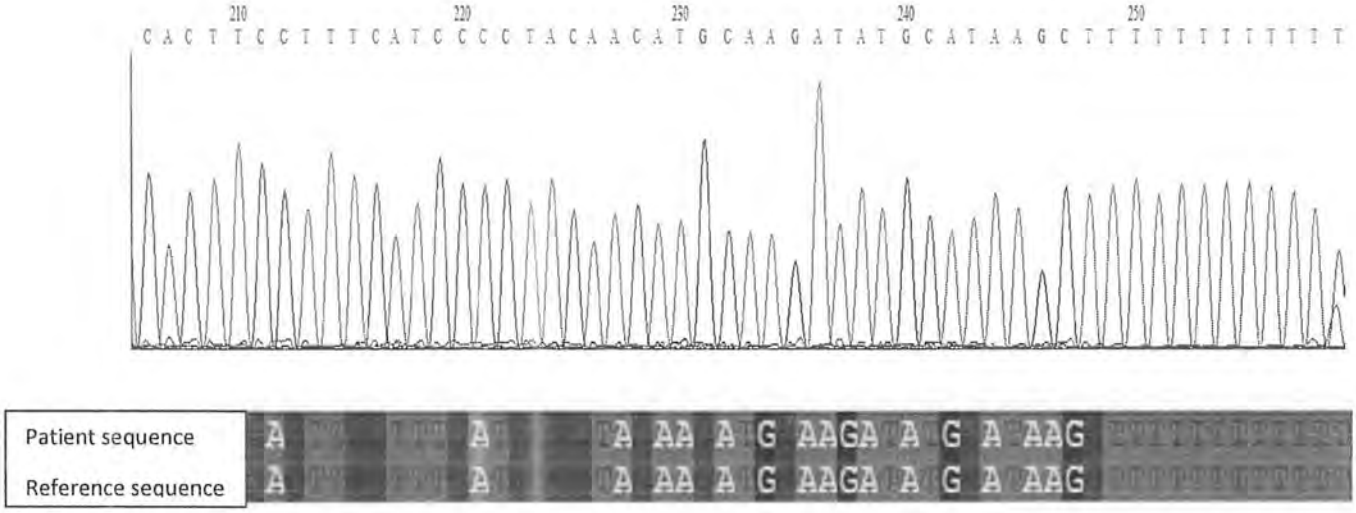

Figure 4.17: Complementary strand of Exon 15`showing normal peaks without noise and no variations in sequence.

Chapter 5: Discussion

Allergic diseases related to airways, allergic asthma and allergic rhinitis are on rise around the world affecting large populations. According to WHO report 2013, asthma and allergic rhinitis affected about 235 million peoples (CDC report 2010; WHO 2013). Such a high burden of these diseases is because of various risk factors including age, gender, family history and socioeconomic status (Rönmark *et al.,* 2016). Similarities between asthma and allergic rhinitis were observed on basis of clinical, demographic, sensitization and pathophysiological responses (Slavin, 2008). Allergen are mainly responsible for this burden, most common of which are, dust, pollen and animal dander (Mishra *et al.,* 2016). Various genes have been found to be associated with allergic rhinitis and allergic asthma that increases risk for their initiation and development (Ferreira *et al.,* 2014). Rhinitis and asthma occur independently as well as a comorbid state (Burte *et al.,* 2015). Rhinitis act as independent risk factor for asthma development (Njue *et al.,* 2016).

Various clinical and demographic factors associated with the disease were evaluated in present study. These factors also play important role along with genetic factors for the allergic disease progression. The focus of the study was mainly on determining the association of *Syk* gene novel mutations/SNPs with allergic asthmatic and allergic rhinitis in patients. Syk is an important signaling protein involved in receptor mediated responses (Mocsai *et al.,* 2010). It is an important therapeutic target due to its involvement in inflammatory responses and also promotes various cancers (Geahlen, 2014). Advancement in cancer studies on *Syk* gene, due to its involvement and atypical responses in the malignant cells shows genetic variations (Faryal *et al.,* 2016). These variations are the cause of various pathologies and cancer prognosis. But no such studies have been reported in case of asthma and allergies.

According to data of the study, male were in higher number than female. However, the observed ratio is in accordance with previous Pakistani studies. In these studies, the number of male patients was also double the number of female patients (Ahmad *et al.,* 2011; Noman *et al.,* 2016). Such higher ratio of 68.75% male patients was also observed in another study by Khan and Ajmal, (2016). A study by Abramson *et al.,* (2016) also

observed same percentage of females (51.9%). In another study, however, females (11%) were in higher ratio than males (8.1%) (Wang *et al.*, 2016). The difference in male to female ratio among various studies may be due different geographical area and change in study method.

There is some work on age related change in immune responses and their effect on asthma and allergy (Mathur, 2010). Therefore, prevalence of these diseases with increasing age group also changes (Yao *et al.*, 2011). According to present study, allergies was higher in 14-30 years age group among patients, while above 60 years of age few patients were present. Other study also shows same common age group among patients (20-60 years) with few patients above age of 60 years (Khan and Ajmal, 2016). Naveed *et al.*, (2016) showed in their study that 25% patients belonged to 18-30 years of age group. But in other study on prevalence of allergies, a lower ratio of allergic asthma and allergic rhinitis present in age group less than 60 years (Wüthrich *et al.*, 2013).These studies results are in line with the current study which showed that allergies are not common in individuals belonging to age greater than 60years.

Most of the present study population was resident of the urban areas of Pakistan. In other study on Pakistani patients also had allergies in urban residents (57%), which is the same pattern of prevalence in relation to urban and rural setting (Noman *et al.*, 2016). The urbanization is found to play an important role for disease prevalence worldwide, which is growing with industrialization in developing countries (Gaviola *et al.*, 2015). This proves urbanization as an important factor of disease prevalence in Pakistan as it's a developing country.

In present work, some ethnic groups were observed to be relatively higher such as Punjabi as compared to other ethnic group. Second high group was Pathan ethnic as compared to other groups observed in this study. In other study, more than half of allergy cases were also reported from Punjab region (51%) compared with Khyber Pakhtunkhwa 23.4% cases (Ahmad *et al.*, 2007). In North America, association of asthma with ancestry was seen through genome wide association studies, which showed that it contributes in

complex asthma genetic makeup (Program *et al.*, 2016). Such high ratio of one ethnic group and genetic linkage of disease indicate ethnicity as an important factor which contribute in inflammatory asthma disease.

In this study, most of the patients were from socioeconomic class with low income touching poverty line, in these patients there was severe disease manifestation. Same trend with regard to socioeconomic status was also seen in work reported by Khan and Ajmal, (2016). In a comparative study on impact of socioeconomic status with asthma and allergies, families with economically less stable were found to be more prone to disease (Beck *et al.*, 2013). These studies clearly indicate the impact of socioeconomic status as that low income class with increased the chances of asthma.

Direct smoking and indirect smoking affects equally the lungs in disease development. While indirect/passive smoking has observed to have high impact on asthma than direct smoking (Khan and Ajmal, 2016). Asthma being an airway disease can also get worse by smoking as it irritates the airways (CDC, 2013). In current study, data was collected regarding smoking history of patients and was found that majority never smoked in their life, while only few were former smokers. Same finding were observed in other study, where 126 asthma participants were nonsmoker and only 74 smokers (Noman *et al.*, 2016). Another study showed that higher ratio of patients with asthma were nonsmoker (87.5%) with remaining percentage of smoker on regular and occasional base (Naveed *et al.*, 2016). In another report infrequent current smoking is observed. The ratio of current smoker, former smoker and no smoking at all was observed 26.3%, 26.3% and 42.2% respectively (Abramson *et al.*, 2016). Heaney *et al.*, (2010) also found that difficult cases of asthma were few but had smoker profile among patients.

Occurrence of asthma symptoms was recorded in present work which appeared to be severe at one instance of day as compared to other instance around clock. Among these, disease condition becomes more sever at nighttime in maximum number of patients. Least symptoms in early morning were present while diurnal complications were observed to be present moderate number of patients. In other study, 37.1% patients

showed severe asthma exacerbation and shortness of breath in night, which caused impairment in sleep (Noman *et al.*, 2016). The evening time complications in a study was recorded to be more prevalent in asthmatics with difficult breathing, continuous cough and chest tightness (Alavoine *et al.*, 2015). The night time worsening in inflammatory allergic responses is due to variation in biological functions with circadian clock (Nakao *et al.*, 2015). This shows evening time plays an important role in worsening of already developed allergic asthma and allergic rhinitis condition.

The season changes bring various climatic changes along with new vegetation. In spring season pollens are on rise and affects allergic patients differently by initiating symptoms or worsen the condition in particular season (Bergman, 2016). Among them spring season affect most of the allergic patients while some are affected by both winter and spring. Some patients show no response on weather change. In a study, seasonal variations was observed with perennial symptoms in 30% patients, 35% become worse in spring while 22.9% were affected due to winters (Noman *et al.*, 2016). During the mid of season, the trees are full grown with high production rate of pollens in the air, which poses threat to allergic patients being sensitized to aeroallergens.

Children are more prone to seasonal allergic attacks (Ito *et al.*, 2015). In a study in Europe by Canova *et al.*, (2013), the reports of allergic attacks were common in spring and may be in summer as compared to winter due to high grass pollen concentration in environment. In other study grass/pollen rise caused more affected cases in winter with 37% patients, while in spring, autumn, and summer it was observed to be 30%,18% and15% respectively (Mahboub *et al.*, 2014). The difference in various studies could be due to geographical and vegetation variations that may impact the residential population differently.

In this study, two types of allergic conditions were observed, one was allergic asthma and other was mix cases of allergic asthma and allergic rhinitis as comorbid condition. The prevalence of allergic asthma along with comorbid rhinitis patients was high as compared to alone allergic asthma patients. In other study, mix cases of asthma/rhinitis

were low in comparison to singly occurring allergic rhinitis and allergic asthma (Ahmad *et al.*, 2011). Noori *et al.*, (2007), also reported mix cases of asthma/rhinitis with low frequency (8%). In another study, higher number of allergic rhinitis (97.9%) followed by 53.4% comorbid cases with presence of seasonal asthma (Zhang and Zhang, 2014). These findings suggest that with the passage of time the comorbid allergic conditions are increasing.

Various atmospheric agents are responsible for allergic rhinitis and allergic asthma. These include dust, pollen, smoke, strong fragrance or perfumes and insect repellent (Manohar *et al* 2014). According to this study, among the local Pakistani population is more prone to pollen and dust for sensitization leading to inflammation. After these, food of various type provokes allergic responses, followed by weather changes, cold wind, smoke, intense fragrance, exercise and fungus. These findings are in accordance with another study where pollen and dust was major triggers among allergens in local population. Paper mulberry pollens were prominent followed by thresher raw, cotton and foods (Ahmad *et al.*, 2011). Another allergen based study in different region has shown that among poly sensitized patients with dust were 59% followed by grass/pollen 44% as top allergens for airways inflammation (Mahboub *et al.*, 2014). These allergen profiles among different studies have shown that pollen and dust is the prime cause of allergic rhinitis and allergic asthma.

Family history plays an important role in allergic diseases. Patients with parental allergic diseases are more prone toward asthma (Fuertes *et al.*, 2015). According to recent findings, one of the important risk factor for asthma and allergic rhinitis is positive family history of these diseases (Rönmark *et al.*, 2016). In this present study, patients with family history were in majority having allergic diseases running in their families, like asthma and rhinitis. Family history was found to be significantly higher in asthmatics (Noori *et al.*, 2007) , also another work recently also found 68.75% patients having positive history for allergies among siblings and elders of family showing the strong family history linkage with these diseases (Naveed *et al.*, 2016). In another recent study, family history was evaluated by observing paternal and maternal impact on allergy onset

in later life. Maternal asthma and atopy were observed to be 4.6% and 13.7% respectively. While paternal asthma and atopy was observed 5.8% and 9.9% case respectively (Abramson *et al.*, 2016). In a population based study strong family history increased the risk of rhinitis and asthma (Rönmark *et al.*, 2016). These studies show that family history plays crucial role in allergic onset in offspring with allergic parents.

To dissect the role of *Syk* gene and its variation on asthma pathogenesis, *Syk* gene variant were detected by SSCP and sequencing. Several variants were observed in these exons. Variants in exon 15 were not linked to asthma and rhinitis onset because only control samples show variations, not observed in any of the patient's sample. These could be natural variations, that may play role towards or against any other disease. In case of exon 15`, both genders had variants among patient's sample. The prominent age group was 14-30 years in exon15`. Those individuals were resident of urban area and belonged to Punjab ethnicity. The exon15` showed variants in patients from lower middle class. The variations were mostly observed in patients with mix disease condition (asthma/rhinitis), predominately those patients had both LRP and URP with positive family history for atopy. Sequencing results visualized on electropherogram shows peak disturbance due to presence of poly-A region. In order to sought this problem sequencing using complementary strand was carried out. The results show no association of this exon with allergic asthma and rhinitis. Exon 15` is an important region of the *Syk* gene as it lies in coding region of kinase domain at 3` end. Any substitution in this coding region effects the gene and protein kinase domain might get lost. Due to such vital part of *Syk* gene, peak disturbance in exon 15` after poly-A region is needed to be further verified by designing short multiple primers or via anchored primer technique. No such work has been carried out earlier, therefore, no comparison can draw, but there is possibility to identify new SNPs, such polymorphism can be studied in future to understand their role in initiation, progression, severity and endotypes of these allergies. Hence, such knowledge can be used to develop better prognostic, diagnostic and treatment strategies.

Conclusion

Conclusion:

This case control study was carried out, in which *Syk* gene polymorphism was assessed for association with allergic asthma and allergic rhinitis development. Various factors are found to be associated with disease condition.

- The age (14-30 years) was found to have impact in prevalence of both allergic asthma and allergic rhinitis.
- Male were more prominent than females.
- Majority of study population was residing in urban settings with low income level and ethnicity was Punjabi
- Smokers are also found to be affected at lower frequency with allergic asthma and rhinitis along with former smoking history in few cases.
- Most prominent condition in the study was comorbid allergic asthma and allergic rhinitis.
- Disease symptoms appeared most frequently at night time.
- Seasonal variations also affected allergic patients, the most prominent season for airways allergy attack was observed to be the spring season.
- Family history is found to be strongly associated risk factor for allergic onset.
- The exon 15 and exon 15' of *Syk* gene had no variants in allergic asthma and rhinitis patients.

Future Prospects

Future prospects:

- Exon 15` needed to be further verified by designing short multiple primers or via anchored primer technique.
- Further studies on the *Syk* gene involvement in allergic population with increased sample size to authenticate the association.
- Need for investigation of genetic association of allergic asthma and allergic rhinitis with other candidate genes among Pakistani population.
- Studies should be focused to evaluate family risk on genetic level, and genetic basis of ethnicity.
- The comorbidity of allergic asthma with other type of allergies is needed to observe possible risk factors for disease onset.
- In order to develop therapies against asthma circadian rhythm observed in allergic diseases is need to evaluate more elaborately.
- There is need of public education on possible risk factors involved and preventive strategies from allergic hypersensitivities.
- National survey program to evaluate the overall disease burden in Pakistan and annual economic impact.

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Appendix

Table. 7.1: stock solutions for DNA extraction:

S.no.	Solution	Chemicals	Amount	Distill H_2O (mL)	Final Volume (mL)
1.	Tris HCL (1 M)	Tris HCL	14.532 mg	120	150
2.	EDTA (0.5M) pH-8.8	EDTA	73	450	500
		NaOH	Few drops for pH adjustment		
3.	Sodium dodecyl sulphate (20%)	SDS	20 g	50	100
4.	Ethanol (70%)	Absolute ethanol	70 mL	30	100
5.	Sodium acetate	CH3COONa	9.84 g	25	40
6.	T.E Buffer	EDTA (0.2M)	10 mL	70	100
		TrisHCl (1M)	20mL		

Table.7.2: Preparation of Bromophenol Blue

S.no.	Chemical	Amount(g)	Distill water (mL)	Final volume (mL)
1.	Bromophenol blue	0.05	20	50
2.	Sucrose	8.0		

Table.7.3: working solutions for DNA extraction

S.no	Solutions	Chemicals	Amount (g/mL)	Water (ml)	Final volume (mL)
1	Solution A	Sucrose (0.32M)	27.63	150	250
		Tris (10mM	0.303 g		
		MgCl2 (5mM)	0.254 g		
		Triton X 100	2.5mL		
2	Solution B	Tris (10 mM)	0.364 g	200	300
		NaCl (400mM)	7.02 g		
		EDTA	0.1752 g		
3	Solution C	Tris HCL (10mM)	0.605 g		500
		Phenol	500 mL		
4	Solution D	Chloroform	20 mL		500
		Iso-amyl Alcohol	480 mL		

Table.7.4: 10X-TBE for Gel electrophoresis:

S.no.	Chemicals	Amount (g)	Distill Water (mL)	Final Volume (mL)
1.	Tris Base	27	220	250
2.	EDTA	2.325		
3.	Boric acid	14.5		

Table.7.5: Acryl-Bisacrylamide 30% solution for 8% PAGE

S.no.	Chemicals	Amount (g)
1	Acryl Amide (Serva)	145 g
2	Bisacrylamide	5g
Dissolve in 250 mL of water and then raise the final volume up to 500mL		

Table.7.6: Ammonium per Sulphate 10% solution

S.no.	Chemicals	Amount
1.	Ammonium per sulphate	5g
2.	Distill water	45mL

Table.7.7. Showing Patients socioeconomic background.

S.no	Socio economic status	Patient %	Control %
1.	Lower class	46	6
2.	Middle class	38	67
3.	Higher class	16	27

Table.7.8. Patients with seasonal variation of disease.

S.no	Seasonal Variation	Patients	Percentages
1	Spring	76	33%
2	Winter	21	9%
3	Spring/Winter	56	25%
4	All seasons	65	29%
5	Others	10	4%

Table 7.9: Patients association with different allergen.

S.no	Different allergens that trigger symptoms	Patient *n*	Percentage %
1.	Pollen	202	89
2.	Dust	202	89
3.	Molds	34	15
4.	Exercise	96	42
5.	Food	141	62
6.	Weather changes	110	48
7.	Cool Air	123	54
8.	Smoke/perfume	181	79

Table.7.10: Caffeine uptake and smoking history in Patients and control.

S.no	Characteristics		Patient *n (%)*	Control *n (%)*
1.	**Smoking history**	1.Non smoker	192 (84%)	126 (61%)
		2.Smoker	20 (9%)	79 (39%)
		3.Former smoker	16 (7%)	0 (0%)
2.	**Caffeine uptake levels**	1.Low	94 (41%)	73 (36%)
		2.Medium	57 (25%)	39 (19%)
		3.High	48 (21%)	23 (11%)
		4.No	29 (13%)	70 (34%)

Appendix I: Consent form for Patients:

"ریسرچ میں رضامندی کا فارم"

(*Syk* Gene Polymorphism Analysis In Local Allergic Asthmatic Patient)

شرکت کنندہ:

تاریخ پیدائش:

MRنمبر:

محقق: ڈاکٹر رانی فریال

محکمہ خرد بینی حیاتیات، قائد اعظم یونیورسٹی

+92-51-8003456

تعلیم کا مقصد:

اس مطالعہ کا بنیادی مقصد الرجک دمہ کی شدت کا تعلق سک جین کے ساتھ معلوم کرنے کے لئے ہے ۔ میں نے جائزہ لینا ہے کہ سک جین مقامی الرجک دمہ کے مریضوں میں پائی جاتی ہے یا نہیں۔ یہ جائزہ دمہ کی شدت کی وجہ معلوم کرنے میں اہم کردار ادا کرے گا ۔ اس بیماری کو پیدا کرنے والے جینز کی نشاندہی کرنے سے ہی ہمیں اس بات کا علم ہوگا کہ ہم کس طرح اس بیماری سے بچنے کے لیے نئے طریقہ علاج بنا سکتے ہیں۔ اور ان کو لاگو کر کے بیماری سے نتجات حاصل کر سکتے ہیں۔

طریقہ کار:

1. اس مطالعے میں 1.5 ملی لٹر خون آپ سے لیا جائے گا
2. اور چند سوالات آپ کی طبی تاریخ اور دمہ کی خاندانی تاریخ کے بارے میں کیے جائیں گے۔ یہ چند منٹ لے گا

ممکنہ خطرات

- تربیت یافتہ عملہ آپ کے بازو سے خون کا نمونہ لے گا۔ بازو سے خون لینے کی وجہ سے تھوڑا سا درد ہو گا اور بازو پر ہلکا سا نشان پڑ سکتا ہے۔یہ نشان چند ہی دنوں میں ختم ہو جائے گا۔

ممکنہ فوائد

- سٹڈی کے دوران اگر ہمیں آپ کے خون کے نمونے میں کوئی خاص بات نظر آئی تو تحقیق کرنے والے عملے کا کوئی فرد آپ سے رابطہ کرے گا۔
- اس سے آپ کو علاج میں رہنمائی ملے گی۔ اور ہمارے موجودہ علم میں اضافہ ہو گا۔

رازداری

فراہم کردہ معلومات کو راز رکھا جائے گا ۔ پرنسپل تفتیش کار کے سوائے کسی کو بھی رسائی نہ ہوگی ۔ آپ کا نام اور شناخت کسی بھی وقت ظاہر نہیں کیے جائیں گے۔آپ کی دسے کئی جانچ پڑتال کے نتائج صرف آپ کے علاج اور مستقبل میں اس بیماری سے بچنے کیلیے کی جانے والی ریسرچ میں استعمال کیے جائیں گے۔ آپ کے خون کے نمونوں پر آپ کے نام کی بجائے کوڈ نمبر لگایا جائے گا اور ان کوڈ نمبروں کی فائل کو محفوظ رکھا جائے گا جس تک صرف تحقیق کرنے والے افراد کو ہی رسائی حاصل ہو گی۔آپ کے نمونوں سے حاصل شدہ DNAاور خلیوں کو کوڈ نمبر لگا کر غیر معینہ مدت کیلئے محفوظ کر لیا جائے گا تاکہ اس سے نئے جینز دریافت کیا جا سکے اور اس سٹڈی کو جاری رکھا جا سکے۔

دستیاب ذرائع کی معلومات

اگر کوئی مزید سوالات ہوں تو آپ پرنسپل تفتیش کار (ڈاکٹر رانی فریال [Dr.Rani faryal])، خرد بینی حیاتیات(department of Microbiology) ، درج ذیل فون نمبر 90643008-051 پر قائد اعظم یونیورسٹی کے شعبہ سے رابطہ کر سکتے ہیں ۔

اجازت نامہ

اس تحقیقی مطالعہ میں شرکت کے لیے رضاکارانہ طور پر میں نے اس فارم کو پڑھا ہے اور سمجھ لیا ہے ۔ اور میں رضاکارانہ طور پر حصہ لینے کے لئے تیار ہوں اور سمجھتا / سمجھتی ہوں کہ کسی کو بھی اس سٹڈی سے انفرادی مالی فائدہ نہیں ہو گا۔

شرکت کنندہ کا نام: _____

شریک کے دستخط یا انگوٹھا _____

پرنسپل تفتیش کار کے دستخط _____

رضا مندی کا فارم حاصل کرنے کے شخص کے دستخط _____

Dateتاریخ: _____

Appendix II: Questionnaire for Patients data.

QUAID-I-AZAM UNIVERSITY
(Department of Microbiology)

Islamabad

Thank you for your willingness to participate. You were selected by a scientific sampling procedure, and your cooperation is very important to the source of the study. This is a questionnaire you are asked to fill out. Please answer the questions as frankly and accurately as possible. ALL INFORMATION IN THIS STUDY WILL BE KEPT CONFIDENTIAL AND USED FOR MEDICAL RESEARCH ONLY. Your personal physician will be informed about the test results if you desires. The questions can be answered by checking the best answer or by filling in a blank with a number or word.

Name:_____. DOB_____. Age: _____ gender: • Male / • Female, Province/City:_____.

Marital status: • Single • Married • Separated • Divorced • Widowed

Education: • K – 12 • Some College • Degree Obtained

Main reasons for the visit: _____

(□CHECK THOSE BOXES WHICH RELATE TO YOUR SITUATION)

1. Are you bothered by any of the following symptoms?

Upper Respiratory Problems	**Lower Respiratory Problems**	**Skin Problems**
□Nasal congestion	□ Asthma	□ Acne
□Runny nose	□ Wheezing	□ Hives or swelling episodes
□Itchy nose	□ Chest tightness	□ Eczema
□Red or itchy eyes	□ Shortness of breath	□ Frequent boils
□Sinus pressure or pain	□ Frequent or constant cough	□ Pimply rashes
□Poor sense of smell	□ Pneumonias	□ Blistery rashes

□ Frequent ear infections	□ Frequent bronchitis	□Itchiness in general
□Frequent colds	□ Frequent croup	□ Itching skin rash
□ Hoarse voice		□ Dry skin
□ Frequent sinus infections		

Others: _____

These symptoms started _____ years ago (or _____ months ago), at the age of _____

2. Patterns of symptoms:

· Perennial	· Seasonal	· Both
· continual	· episodic	· Both
· Diurnal variation	· nocturnal	· On Awakening in early morning

3. Check the months during which you have symptoms:

Month	None	Mild	Severe	Month	None	Mild	Severe
January				July			
February				August			
March				September			
April				October			
May				November			
June				December			

4. Recurring or current symptoms:

Symptoms	None	Mild	Severe	Symptoms	None	Mild	Severe
Plugged nose				Sneezing			
Swollen eyes				Coughing			
Mouth-breathing				Nasal itching			
Sinus pressure/headache				Wheezing			
Runny nose				Eye itching			
Loss of sense of smell				Shortness of breath			
Post-nasal drainage				Skin itching or eczema			
Ear plugging				Red eyes			
Hives or swelling episodes				Watery eyes			

Others:_____

5. Are the symptoms triggered by any of these?

□ pollen □ animals □ dust □ mold □ smoke or scents □ weather changes □ foods

6. Symptoms are improved by travel:	***7. Things you notice make the symptoms worse:***	
□To a dryer climate	□House cleaning	□Rainy weather
□To the mountains	□Being outdoor	□Smoke
□To the beach	□Cool air	□ Perfumes
□Out of state	□Moldy or damp areas	□ Exercise
Where?	□Clear weather	□Getting up in the morning
	□Food **(mention if any)**	□Soap powders
	□Colds or flu	□Hair sprays

8. Tobacco exposure: I never smoked (skip other questions):

□I currently smoke	Cigarettes #pks./day :_____
□Every day smoker	Pipe - #/day :_____
□Smoke some days only	Cigars - #/day :_____
□Former smoker	Smokeless tobacco #/day:_____
□year quit	# of years total :_____

N/A because patient is child_____

Does anyone smoke in the home? □ Yes □ No

9. Environment:

· Rural, · Urban

Home setting:	***Pets:***	***Flooring:***	***Heating/Air Conditioning:***
· Older house · Newer house · Farm · Manufactured home · Apartment	□Indoors □ Dogs □ Cats □Other _____	□Carpet □ Hardwood □ Tile □ Cemented floor	□ Central heat □Space heater □ Fireplace □No heating □ Central air conditioning □ No air conditioning

What kind of trees are on your property, if known?

10. DEMOGRAPHICS

· Child / Student · Employed · Retired · Unemployed · Disabled

Occupation: _____ Income Level:_____

Birthplace: _____Ethnic group:_____

11. WORK ENVIRONMENT:

· Homemaker · Office worker · Outdoor worker