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Immunomolecular study of IFNγ, IL-4 , IL-10 and FOXP3 In Asthmatic Patients

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Certification

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Introduction and Literature Review

1.1 Introduction

Airway allergy is a worldwide health problem, an estimated 300 million persons worldwide have asthma and about 400 million persons suffer from allergic rhinitis , the prevalence of both diseases is markedly increasing (Droste *etal.,* 2014) . Allergic reactions occur to normally harmless environmental substances known as allergen ; these reactions are acquired , predictable and rapid (Sicherer and Leung, 2007) . Strictly , allergy is one of four forms of hypersensitivity and is called type I or (immediate) hypersensitivity, its characterized by excessive activation of certain white blood cells called mast cells and basophiles by a type of antibodies known as IgE , resulting in an extreme inflammatory response (Burke and Maibach, 2010) . Common allergic reactions include eczema, hives, hay fever , asthma attacks , food allergies and reaction to venom of insect sting such as wasps and bees (Winther *etal.*, 2014). Interferons are proteins made and released by the cells of the most vertebrates in response to the presence of pathogens such as viruses , bacteria, parasites or tumor cells and they allow communication between cells to trigger the protective defenses of the immune system that eradicate pathogen or tumor cells (Marsh *etal.*, 2013) .

IFNs belong to the large class of glycoproteins known as cytokines . Although they are named after their ability to "interfere" with viral replication within host cells, IFNs have other functions ; they activate immune cells such as natural killer cells and macrophages , they

increase recognition of infection or tumor cells by up-regulating antigen presentation to T lymphocytes and they increase the ability of uninfected host cells to resist new infection by virus (Marsh *etal.,* 2013) . Certain host symptoms such as aching muscles and fever are related to the production of IFNs during infection, there are several single nucleotide polymorphisms (SNPs) in gene coding for IFNγ have been reported has a specific association with number of different diseases (Meltzer, 2012) .

Also there are many different factors that play important role in mechanism of asthma as IgE which is released after Ag sensitization and finally leading to degranulation of basophiles and mast cells ; IL-4 which is responsible for survival and maintenance of basophiles and immunomodulation of immune response from IgM towards IgE ; IL-10 has contradictory effect in case of asthma , and Treg cells has immunoregulatory effect (Munaser *etal.,* 2011) .There is also a specific relationship between nasal carriage of Staphylococcus aureus and allergic rhinitis so that in this study shade light on a relationship between Staphylococcal nasal carriage with asthma exacerbation (Woo-Tung *etal.,* 2013) . The polymorphism in IFNγ play a crucial role in many immune – and non – immune – mediated disorder including asthma , as many other cytokine do have similar roles . The present study aimes at the following ;

Aim of the study

Analyzing the allelic distribution of Interferon Gamma gene 874 A/T Single Nucleotide polymorphism in asthmatic patients and to study the relationship of the allele with markers of allergy (Total IgE, Specific IgE (allergen assay) , IL-4, IL-10, FOXP3, Eosinophil count, Nasal Staphylococcal infection) .

Objectives are

1 – Using of ASO – PCR for detection of IFNγ 874 A/T polymorphism .

2 – Using of RT-PCR for detection of IL-10 and FOXP3 gene expression .

3 – ELISA for titration of (IL-4 , IgE and IFN γ) .

4 – Revealing possible associations between the for mentioned variables

1.2 Literatures Review

1.2.1 Hypersensitivity

Excessive or inappropriate immune response sometimes leads to host tissue damage resulting from prolonged or repeated antigen exposure (Sicherer and Leung, 2007) . These reactions, called hypersensitivity reactions , cause tissue injury by the release of chemical substances that attract and activate cells and molecules resulting in inflammation (Ngoc *etal.,* 2005). These reactions are classified into four hypersensitivity types depending upon the mechanism (s) that underlie the tissue damage the first three types involve antigen – antibody reactions while the fourth is antibody independent, involving cell- mediated immune response, only type I also called (immediate hyper sensitivity) is rapid , occurring within minutes of exposure to an antigen , and always involve IgE mediated degranulation of basophils or mast cells (Marsh *etal.,* 2013) . .

Type II reaction is initiated by the binding of an antibody to cell membrane or to the extracellular matrix (Portnoy and Amado ,2006). Type III hypersensitivity reactions involve the interaction of antibodies with soluble molecules to make soluble antigen – antibody complexes that become deposited in tissues (Cline and Burrows ,1989).

Type IV hypersensitivity reactions are those in which cells of the immune system directly attack host cells in the absence of antibody, these reactions include contact dermatitis , delayed type hypersensitivity and occasionally cytotoxic T lymphocyte responses (Burke *etal.,* 2010).

1.2.1.1 Type I Hypersensitivity

Commonly called allergic or immediate hypersensitivity reactions (Kay 2005) . Type I responses occur within minutes to hours of antigen exposure (Sicherer and Leung , 2007) . Some individuals develop IgE antibodies in response to relatively harmless environmental antigens or allergens (Mensinga *etal.,* 2013) . IgE molecules readily bind to Fc receptors (FcR or CD23) on the surface of mast cells and basophile, unlike other FcR , FcR bind antigen – free immunoglobulin IgE , and the IgE – CD23 complexes function as antigen – specific cell surface receptors (Winther *etal.,* 2014) . Cross linking of surface – bound IgE molecules generates intracellular signals via CD23 , leading to mast cell or basophiles degranulation and release of vasoactive amines (e.g histamine) and other inflammatory mediators that cause vascular endothelial cell junctions to loosen (vasodialation) and increase vascular permeability , resulting in fluid accumulation in the tissues (edema) (Rich *etal.,* 2008) . Histamine also induces smooth muscle contraction in arterial and arteriole walls (vasoconstriction) to accelerate fluid distribution from the central trunk of the body into peripheral tissues (Meltzer, 2012) . .

Because mast cells accumulate in respiratory passages , intestinal walls and the skin , type I reactions are often most pronounced in these tissues (Kerkhof *etal.,* 2003) . Sites affected are typically those where the initiating antigen is most often encountered , antigens that enter the body by inhalation localize primarily to the nasopharyngeal and bronchial tissues where smooth muscle contraction and vasodilatation increase mucous production and constriction of respiratory passage , in

combination , these responses can produce the severe and potentially fatal disorder known as asthma (Wen *etal.*, 2003).

1.2.1.1.1 Asthma

(Asthma ,"Panting") is one of the few chronic diseases in the developed world that is increasing in prevalence, despite better understanding of its pathogenesis and improved treatments (Borish *etal.*, 2004). It is one of the most common disorders encountered in clinical medicine in both children and adults , it affects approximately 5% of the adult population in the western world and its reported incidence is increasing dramatically in many developed nations. The cost of the disease is substantial, and the market for the pharmaceutical industry is estimated at $5.5 billion /year (Munasir *etal.*, 2011) .

Asthma is a very complex and difficult term to define in simple manner, currently it is considered to be a group of different disorders characterized by three major features, 1 - intermittent and reversible airway obstruction leading to recurrent episodes of wheezing, breathlessness, chest tightness and cough, 2- Broncho hyper responsiveness (BHR) which is defined as an increased sensitivity to Broncho-constrictors such as histamine and cholinergic agonists and, 3 - airway inflammation (Tliba and Amrani ,.2008) .

Pathophysiology of asthma is still poorly understood and its cause remains unknown, many scientific reports suggest that, it involves the activation of many inflammatory cells like mast cells, macrophages / monocytes, eosinophils, Treg cells ,T-helper type-2 lymphocytes (Th2), dendritic cells, basophils, neutrophils and platelets, it is now increasingly recognized that structural cells may also be important

sources of mediators in asthma (Schmid-Ott *etal.,* 2001) . Air-way epithelial cells, smooth muscle cells, endothelial cells and fibroblasts are all capable of synthesizing and releasing inflammatory mediators (Chong *etal.,* 2006) . To date, greater than 50 different mediators have been implicated in asthma (Yoshizawa *etal.,* 2002) . It is now clear that cytokines play a critical role in orchestrating, perpetuating and amplifying the inflammatory response in asthma that is evident in the asthmatic airways (Pestka *etal.,* 2004) . They considered as novel mediator of chronic obstructive pulmonary disease (COPD), rheumatoid arthritis, inflammatory bowel disease and allergic inflammation (Sheffer and Taggart ,.1993) . Although insight into the pathophysiology of asthma has increased substantially over recent years, a number of issues remain to be further clarified. The occurrence of asthma has increased significantly in 2015, approximately 300 million persons world wide have asthma and about 400 million persons suffer from allergic rhinitis and its caused 350,000 deaths anualy (Cui *etal.,* 2015) .

1.2.1.1.2 Signs and symptoms : -

Asthma is characterized by recurrent episodes of wheezing , shortness of breath , chest tightness and coughing . Sputum may by produced from the lung by coughing, but is often hard to bring up (Sapigni *etal.,* 2014) . During recovery from an attack , it may appear pus – like due to high levels of WBC (Eosinophils) (Toma *etal.,* 2005) . Symptoms are usually worse at night and in the early morning or in response to exercise or cold air (Cox ,.2011) . Some people with asthma rarely experience symptoms , usually in response to triggers , whereas other may have marked and persistent symptoms, associated

condition ; number of other health conditions occur more frequently in those with asthma including gastroesophageal reflex disease (GERD) , rhinosinusitis , and obstructive sleep apnea (Staikuniene and Sakalauskas ,.2003) psychological disorders are also more common with anxiety disorders occurring in between 16-52 % and mood disorders in 14-41 % (American Thoracic Society, 1995) . However its not known if asthma causes psychological problems or if psychological problems lead to asthma (Emerson *etal.,* 2000) .

1.2.1.1.3 Causes of Asthma

Asthma is caused by a combination of complex and incompletely understood environmental and genetic interactions, these factors influence both its severity and its responsiveness to treatment (Grammatikos ,.2008) .

a - Environmental causes

Many environmental factors have been associated with asthma 's development and exacerbation including allergens , air pollution and other environmental chemicals (Wosinska-Becler *etal.,* 2004).

Smoking during pregnancy and after delivery is associated with greater risk of asthma – like symptoms, low air quality from factors such as traffic pollution or high ozone level has been associated with both asthma development and increased asthma severity (LaGrutta *etal.,* 2005) . Exposure to indoor volatile organic compounds may be trigger for asthma ; formaldehyde exposure for example , has appositive association (De Castro *etal.,* 2003) . Also, phthalates in certain types of pace are associated with asthma in children and adults (Meager, 2006) .There is an association between acetaminophen (paracetamol) use and

asthma (Hussein *etal.,* 2006) . The minority of evidence does not however support a causal role. A 2014 review found that the association disappeared when respiratory infections were taken into account (Sapigni *etal.,* 2014) .Paracetamol used by mother during pregnancy is also associated with an increased risk (Yu *etal.,* 2006) .

Asthma is associated with exposure to indoor allergens, common indoor allergens include : dust ,mites , cockroaches , animal dander and mold (Staikuniene and Sakalauskas, 2003) Efforts to decrease dust mites have been found to be ineffective (Riechelmann *etal.,* 2015) . Certain viral respiratory infections , such as respiratory syncytial virus and rhino virus may increase the risk of developing asthma when acquired as young children (Miller *etal.,* 2009).

b. Genetics

Family history is a risk factor for asthma ,with many different genes being implicated (Sengler *etal.,* 2002) . If one identical twin is affected ,the probability of the other having the disease is approximately 25% (Gamero *etal.,* 2007). By the end of 2005, 25 genes had been associated with asthma in six or more separate populations ,including GST M1, IL-10, CTLA-4, SPINK5, LTC4S, IL4R and ADAM33 , among others (Ono and Abelson , 2005) Many of these genes are related to the immune system or modulating inflammation, even among this list of genes supported by highly replicated studies, results have not been consistent among all populations tested (Romagnani, 2004) . In 2006 over 100genes were

associated with asthma in one genetic association study alone (Johansson and Lundahl , 2001) .

The risk of allergic sensitization and the development of allergies varies with age ,with young children mostly at risk (Ohly, 2006) Several studies have shown that IgE levels are highest in childhood and fall rapidly between the ages of 10 and 30 years(Kawai *etal.,* 2001) .

The peak prevalence of hay fever is highest in children and young adults and the incidence of asthma is highest in children under 10 (Yunginger *etal.,* 2000) . Over all, boys have a higher risk of developing allergies than girls (Novak and bieber, 2003) . Although for some diseases, namely asthma in young adults, females are more likely to be affected these differences between the sexes tend to decrease in adulthood (Kim *etal.,* 2010). Ethnicity may play role in some allergies , however, racial factors have been difficult to separate from environmental influences and changes due to migration (Hoffmann *etal.,* 2002). It has been suggested that different genetic loci are responsible for asthma, to be specific, in people of European, Hispanic, Asian and African origins (Maher *etal.,* 2007) .

c. Hygiene hypothesis :-

Allergic diseases are caused by inappropriate immunological responses to harmless antigens driven by a Th2-mediated immune response (Garn and Renz , 2007) . Many bacteria and viruses elicit a Th1-mediated immune response , which down-regulates the Th2 responses (Woo *etal.,* 2013) . The first proposed mechanism of action of the hygiene hypothesis was that insufficient stimulation of the Th1 arm of the immune system leads to an overactive Th2 arm, which in

turn leads to allergic disease (Shirai and Leung , 2003). It has been proposed that the reduced exposure to bacteria and viruses is due, in part, to increased cleanliness and decreased family size in modern societies (Yang *etal.,* 2005). Exposure to bacterial endotoxin in early childhood may prevent the development of asthma, but exposure at an older age may provoke bronchoconstriction (Bachert *etal.,* 2012). Evidence supporting the hygiene hypothesis includes lower rates of asthma on farms and in households with peants (Garn and Renz , 2007) . Use of antibiotics in early life has been linked to the development of asthma (Meager, 2009) . Also delivery via caesarean section is associated with an increased risk (estimated at 20-80%) of asthma –this increased risk is attributed to the lack of healthy bacterial colonization that the newborn would have a acquired from passage through the birth canal (Pestka *etal.,* 2004) . In other words individuals living in too sterile an environment are not exposed to enough pathogens to keep the immune system busy (Sicherer, 2007) . Since our bodies evolved to deal with ascertain level of such pathogens, when they are not exposed to this level, the immune system will attack harmless antigens and thus normally benign microbial objects-like pollen) will trigger an immune response (Ohly, 2006) . The hygiene hypothesis has been extensively investigated by immunologist and epidemiologists and has become an important theoretical frame work for the study of allergic disorders (Wuthrich and Schmid-Grendelmeier, 2003) .

Epidemiological data support the hygiene hypothesis, studies have shown that various immunological and autoimmune diseases are much less common in the developing world than the industrialized world and that immigrants to the industrialized world from the developing

world increasingly develop immunological disorder in relation to the length of time since arrival in the Industrialized world (Grimbaldeston *etal.,* 2006) .

1.2.1.1.4 Pathophysiology of asthma :-

A - **Acute response :**

In the early stages of allergy , type I hypersensitivity reaction against an allergen encountered for the first time and presented by a professional antigen – presenting cell causes a response in a type of immune cell called Th-2 lymphocyte , which belongs to a subset of T cells that produce a cytokine called IL – 4 these Th2 cells interact with other lymphocytes called B cells , whose role is production of antibodies coupled with signals provided by IL-4 this interaction stimulates the B-cell to begin production of large amount of a particular type of antibody known as IgE (Akpinarli *etal.,* 2002) . Secreted IgE circulates in the blood and binds to an IgE specific receptor (called Fc $\varepsilon_{\text{ }}$RI) on the surface of other kinds of immune cells called mast cells and basophils, which are both involved in the acute inflammatory response , the IgE coated cells, at this stage, are sensitized to the allergen (Kawai *etal.,* 2013) .

If later exposure to the same allergen occurs , the allergen, can bind to the IgE molecules held on the surface of the mast cells or basophills, cross-linking of the IgE and Fc receptors occurs when more than one IgE –receptor complex interacts with the same allergenic molecule, and activates the sensitized cells (Elizabeth *etal.,* 2010). Activated mast cells and basophiles undergo a process called degranulation during which they release histamine and other

inflammatory chemical mediators (cytokines , interleukins , leukotrienes and prostaglandin) from their granules into the surrounding tissue causing several systemic effects , such as vasodilation , mucous secretion, nerve stimulation and smooth muscle contraction (Karaglannidis *etal.*, 2004) . This results in rhinorrhea , itchiness , dyspnea and anaphylaxis , depending on the individual , allergen and mode of introduction ,the symptoms can be system – wide (classical anaphylaxis) , or localized to particular body systems so that asthma is localized to the respiratory system and eczema is localized to the dermis (Wuthrich and Schmid-Grendelmeier, 2003) . .

B - Late - phase response

After chemical mediator of the acute response subside, late phase responses can often occur (Yunginger *etal.*, 2000) . This is due to the migration of other leukocytes such as neutrophils , lymphocytes , eosinophils and macrophages to the initial site and the reaction is usually seen 2-24 hours after the original reaction (Simon *etal.*, 2004).

Cytokines from mast cells may play a role in the persistence of long – term effects (Williams and Galli , 2000) . Late phase responses seen in the asthma are slightly different from those seen in other allergic responses , although they are still caused by release of mediators from eosinophils and are still Dependent on activity of Th2 cells (Kaliner , 2012) .

1.2.1.1.5 Diagnosis :

Effective management of allergic diseases relies on the ability to make an accurate diagnosis (Cox, 2011) . Allergy testing can help confirm or rule out allergies (Droste *etal.*, 2014) . Correct diagnosis , counseling and avoidance advice based on valid allergy test result reduces the incidence of symptoms and need for medication and improves quality of life (Portony and Amado, 2006). To assess the presence of allergen – specific IgE antibodies , two different methods can be used;

a- skin prick test, b - allergen assay, booth methods are recommended and may be with similar diagnostic value (Bener *etal.*, 2002).

Allergy undergoes dynamic changes overtime (Grimbaldeston *etal.*, 2006) . Regular allergy testing of relevant allergens provides information on if and how patient management can be changed , in order to improve health and quality of life (Lieberman and Sicherer, 2011) .

Annual testing is often the practice for determining whether allergy to milk , egg , soy and wheat have been outgrown , and the testing interval is extended to 2-3 years for allergy to peanut , tree nuts , fish and crustacean shellfish(Sicherer and Sampson, 2010) .

Results of followup testing can guide decision – making regarding whether and when it is safe to introduce or re-introduce allergenic food into the diet (Kay , 2005) .

a - Skin prick testing

Skin testing is also known as " puncture testing " and "prick testing " due to the series of tiny punctures or pricks made into the

patient's skin (Bener *etal.,* 2002) . Small amounts of suspected allergens and / or their extracts e.g (pollen, grass, mite proteins, peanut extract) are introduced to sites on the skin marked with pen or dye (the ink / dye should be carefully selected lest it cause an allergic response itself) (Garn and Renz, 2007) . A small plastic or metal device is used to puncture or prick the skin (Munaser *etal.,* 2011) . Sometimes , the allergens are injected intradermally into the patient's skin , with a needle and syringe common are as for testing include the inside Forearm and the Back (Williams and Galli, 2000) .

If the patient is allergic to the substance, then a visible inflammatory reaction will usually occur within 30 minutes (Lieberman *etal.,* 2011) . This response will range from slight reddening of the skin to a full-blown hive (called " wheal and flare") in more sensitive patients similar to mosquito bite (Sicherer and Sampson, 2010) . Interpretation of the results of the skin prick test is normally done by allergists on a scale of severity, with +/- meaning borderline reactivity , and 4+ being a large reaction (Grammatikos, 2008) . Increasingly , allergists are measuring and recording the diameter of the wheal and flare reaction (Muller, 2004) . Interpretation by well-trained allergists is often guided by relevant literature (Bernstein *etal.,* 2008) . Some patients may believe they have determined their own allergic sensitivity From observation, but a skin test has been shown to be much better than patient observation to detect allergy (Kay, 2005) . If a serious life-threatening anaphylactic reaction has brought a patient in for evaluation , some allergists will prefer an initial blood test prior to performing the skin prick test (Platanias, 2005) . Skin tests may not be an option if the patient has widespread skin disease or has

taken anti histamines in the last several days (La Grutta *etal.,* 2005) .

*** Patch testing**

Is a method used to determine if a specific substance causes allergic inflammation of the skin (Broll, 2009) . It tests for delayed reactions . (Ono and Abelson, 2005) . It's used to help ascertain the cause of skin contact allergy or contact dermatitis (Beltrani and Boguneiwicz ,. 2003) . Adhesive patches, usually treated with number of common allergic chemicals or skin sensitizers, are applied to the back, the skin is then examined for possible local reactions at least twice, usually at 48 hours after application of the patch, and again two or three days later (Ohly, 2006) .

b – Blood testing (Allergen assay)

An allergy blood test is quick and simple, and can be ordered by a licensed health care provider, unlike skin-prick testing, a blood test can be performed irrespective of age, skin condition, medication , symptom, disease activity and pregnancy (Chang *etal.,* 2006) . Adults and children at any age can take an allergy blood test , for babies and very young children, a single needle stick for allergy blood testing is often more gentle than several skin tests (Toma *etal.,* 2005) .

Multiple a allergens can be detected with single blood sample and its very safe since the person is not exposed to any allergens during the testing procedure (Rich *etal.,* 2008) . The test measures the concentration of specific IgE antibodies in the blood (Bettiol *etal.,* 2006) . Quantitative IgE test results increase the possibility of ranking how different substances may affect symptoms (Winther *etal.,* 2014).

A general rule of thumb is that the higher IgE antibody value, the greater the like hood of symptoms (Rich *etal.*, 2008) . Allergens found at low levels that today do not result in symptoms can nevertheless help predict future symptom development (Lai *etal.*, 2005) . The quantitative result can help determine what a patient is allergic to , help predict and follow the disease development , estimate the risk of a severe reaction and explain cross reactivity (Feher *etal.*, 2010) . Allow total IgE level is not adequate to rule out sensitization to commonly inhaled allergens, statistical methods , such as Roc curves , Predictive value calculations and likehood ratios have been used to examine the relationship of various testing methods to each other (Feher *etal.*, 2010) . These methods have shown the patient , with high total IgE have a high probability of allergic sensitization , but further investigation with allergy test for specific IgE antibodies for a carefully chosen of allergens is of often warranted (Kerkhof *etal.*, 2003) . .

1.2.1.1.6 Medication

A-chemotherapy

Several antagonistic drugs are used to block the action of allergic mediators or to prevent activation of cells and degranulation processes , these include antihistamines , glucocrticoides ,epinephrine (adrenaline), theophylline and commonly sodium, Antileukotriene agents , such as montelukast (singulair) or zafirlukast (Accolatate), are FDA approved for treatment of allergic diseases, anti cholinergic, decongestants ,mast cell stabilizers, and other compounds thought to impair eosinophil chemotaxis, are also commonly used, these drugs help to alleviate the symptom of allergy, and are imperative in the recovery of acute

anaphylaxis , but play little role in chronic treatment of allergic disorders (Anderson, 2005) .

B - Immunotherapy

Desensitization or hyposensitization is a status in which the person is gradually vaccinated with progressively larger doses of the allergen in question, this can either reduce the severity or eliminate hypersensitivity altogether (Cullinan *etal.,* 2004) . It relies on the progressive skewing of IgG antibody production , to block excessive IgE production seen in atopy (Gotzsche and Johansen, 2008) . In a sense , the person builds up immunity to increasing amounts of the allergen in question (Jacquemin *etal.,* 2009) . Studies have demonstrated the long – term efficacy and the preventive effect of immunotherapy in reducing the development of new allergy (Krieger *etal.,* 2005). Meta – analyses have also confirmed efficacy of the treatment in allergic rhinitis in children and in asthma (Bernstein *etal.,* 2008) .

A review by the Mayo-clinic in Rochester, confirmed the safety and efficacy of allergen immunotherapy for allergic rhinitis and conjunctivitis , allergic forms of asthma and stinging insect based on numerous well – designed scientific studies (Ono and Abelson , 2005).

In addition , national and international guidelines confirm the clinical efficacy of injection immunotherapy in rhinitis and asthma , as well as the safety (Krieger *etal.*,2005) .

A second form of immunotherapy involves the intravenous injection of monoclonal anti – IgE antibodies ,these bind to free and B-cell associated IgE , signaling their destruction they do not bind to IgE already bound to the Fc receptor on basophils and mast cells , as this

would stimulate the allergic inflammatory response (Bakolis *etal.,* 2015) .

The first agent of this class is omalizumab while this from of immunotherapy is very effective in treating several types of atopy , it should not be used in treating the majority of people with food allergies (Holt and Thomas, 2005) .

A third type, sublingual immunotherapy , is an orally administrated therapy that take advantage of oral immune tolerance to non – pathogenic antigens such as food and resident bacteria this therapy currently account for 40 percent of allergy treatment in Europe, in the united states , sublingual immunotherapy is gaining support among traditional allergists and is endorsed by doctors treating allergy (Bakolis *etal.,* 2015) .

Allergy shot treatment is the closest thing to a " cure " for allergic symptoms , this therapy requires a long – term commitment (Luczynska *etal.,* 2006) .

On this basis, our review will concentrate on a better understanding of the exact functional role of each T reg cells , IgE and different cytokines as (IL-4 , IL-10 , IFNγ) in the sensitization process and relationship between gamma interferon polymorphism on these parameters and on the severity of altered airway behavior and from the other hand discus the relationship between Staphylococcal nasal infection and asthma exacerbation .

1.2.2 Cytokines

Cytokines are small, extracellular signaling proteins usually less than 80 KD in size and many are glycosylated. These mediators play an important role in the co-ordination and persistence of inflammation in asthma, although the precise role of each cytokine remains to be determined (Renauld , 2001) . Cytokines are regulatory peptides so can be produced by virtually every nucleated cell type in the body and they have pleiotropic regulatory effects on hematopoietic and many other cell types that participate in host defense and repair processes (Lafaille , 2010) . Cytokines often possess overlapping biological activities, exert different effects at different concentrations, can synergize or antagonize the effects of other cytokines and regulated in a complex manner and function via cytokine cascade (Chang *etal.,* 2006) . Cytokines and their receptors exhibit very high affinity for each other. Because of this high affinity, picomolar concentrations of cytokines can mediate a spectrum of biological effect, they affect closely adjacent cells, and therefore function in a predominantly paracrine fashion, they may also act at a distance (endocrine) and have effects on the cell of origin (autocrine) (Yoshimura *etal.,* 2007) . The studied Items INF-γ , IL-4, IL-10 , Foxp3, total and specific IgE .

1.2.2.1 Interferon (IFN)

Three classes of human IFN have been characterized on the basis of antigenic, biological and biochemical properties (Pestka , 2007).

IFNα and IFNβ are acid stable proteins synthesized by leukocytes and Fibroblasts , respectively that have been treated with virus or

synthetic polynucleotides (Meager , 2009). The IFNα gene family consists of twelve or more members where as IFNβ is encoded by single gene (Lajoie-Kadoch *etal.*, 2009). The structures of IFNα and IFNβ proteins are similar and infect , are recognized by the same receptor (Sicherer *etal.*, 2007). The genes for IFNα and IFNβ do not contain introns and share 30-95 % sequence homology and both have been localized to the short arm of chromosome 9 (Ngoc *etal.*, 2005) .

1.2.2 .1.1 Gamma interferon IFNγ

Is a dimerized soluble cytokine that is the only member of the type II class of interferons (Platanias , 2005). The existence of this interferon , was described by E.F. Wheelock as a product of human leukocytes stimulated with phytoaoemagglutinin and by others as a product of antigen – stimulated lymphocytes (Maher *etal.*, 2007).

IFNγ is not homologues to other interferons and is encoded by a single gene that contains three introns Located on the Long arm of chromosome 12 qu 14 (Gamero *etal.*, 2007) .

a. IFNγ Functions

IFN γ or type II interferon, is a cytokine that is critical for innate and adaptive immunity against viral , some bacterial and protozoal infections (Meager , 2006) . IFN γ is an important activator of macrophages and inducer of class II major histocompatibility complex (MHC) molecule expression (Miller *etal.*, 2009) . Aberrant IFNγ expression is associated with a number of auto inflammatory and autoimmune disease (Zhaojun *etal.* , 2006) . The importance of IFNγ in

the immune system stems in part from its ability to inhibit viral replication directly, and most important from its immunostimulatory and immunomodulatory effects (Nurse *etal.,* 2012) . IFN γ is produced predominantly by natural killer and natural killer T cells as part of the innate immune response and by CD4 Th1 and CD 8 cytotoxic T lymohocyte (CTL) effector T cells once antigen – specific immunity develops (Hussein *etal.,* 2009) But later studies showed that myeloid cells, dendritic cells and macrophages in particular , also secrete IFNγ that is likely important for cell self a activation during the onset of the infection (Pravica *etal.,* 2000) . Also , IFNγ the only type II interferon , and its serologically distinct from type I : it's acid – labile , while the type I variants are acid stable (Lai *etal.,* 2005) .

b. Receptor binding :-

Cellular responses to IFNγ are activated through its interaction with a heterodimeric receptor consisting of interferon gamma receptor 1 (IFNGR1) and interferon gamma receptor 2 (IFNGR2) (Feher *etal.,* 2010) . IFNγ binding to the receptor activates the JAK-STAT path way (Keslacy *etal.,* 2007) . IFNγ also binds to the glycosaminoglycan heparan sulfate (HS) at the cell surface . However , in contrast to many other heparan sulfate binding proteins where binding promotes biological activity , the binding of IFNγ to Hs inhibit its biological activity (Pravica *etal.,* 2000) .

c. Biological activity

IFNγ has antiviral , immunoregulatory and antitumor properties it alters transcription in up to 30 genes producing a variety of physiological and cellular responses (Hussein *etal.,* 2009) , among the effects are :

1 - Promoting NK cell activity .

2 - Increasing antigen presentation and lysosome activity of macrophage

3- Activity inducible nitric oxide syntheses iNos .

4 - Induce the production of IgG2 and IgG3 from activated plasma cells.

5 - Promoting Th1 differentiation by upregulating the transcription factor T-bet , ultimately leading to cellular immunity : cytotoxic CD8 + T cells and macrophage activity – while suppressing Th2 differentiation , which would cause a humoral (antibody) response.

6 - Cause normal cells to increase expression of class I MHC molecules as well as class II MHC on antigen – presenting cells to be specific , through induction of antigen processing genes including subunits of the immune proteasome (MECL1,LMP2, LMP7) as well as TAP in addition possibly to the direct upregulation of MHC heavy chains and B2 – micro globulin itself.

7 - Promotes adhesion and binding required for leukocyte migration.

8 - Induce the expression of intrinsic defense factors – for example, with respect to retroviruses , relevant genes include TRIM5α and Tethrin representing directly antiviral effects.

IFNγ is the primary cytokine that defines Th1 cells ; Th1 cells secrete IFNγ , which in turn cause more undifferentiated CD4+ cells (Th0) to differentiate into Th1 cells , representing a positive feedback

loop while suppressing Th2 cells differentiation (Grewe *etal.,* 2014).

d. The relationship between IF Nγ and Asthma :-

Airway inflammation plays a crucial role in the pathogenesis of bronchial asthma (Burrows *etal.,* 2013). T-Lymphocytes are involved in the inflammatory / immune response in asthma causing tissue infiltration and damage , these cells recognize and respond directly to allergens and by releasing several cytokines , may orchestrate the inflammatory response (Kowalski *etal.,* 2011). Activated CD4 + T cell have been detected in the peripheral blood , bronchoalveolar lavage and bronchial mucosa of patients with asthma (Tomassen *etal.*, 2013).

Human CD4+ T cell can be divided into two distinct subpopulations based on cytokine production profile : T helper cell type I (Th1) cells which produce large amounts of Interleukin (IL-2) and gamma interferon (IFNγ) and Th2 cells which release iL-4 and iL-5 (but little or no IFNγ and IL-2) (Grewe *etal.,* 2014) .CD4+ Th2 like cells have been detected in the airways of atopic , asthmatic patients and have been shown to accumulate during the Late – phase skin reaction to allergen in a topic individuals (Asher *etal.,* 2004).

Results from in vitro studies of the cytokine production of blood derived , antigen specific CD4+ T cell clones and lines have suggested that Th2-like cells are preferentially induced following the stimulation by allergen of lymphocytes from atopic donors , despite the considerable progress toward defining the characteristics of the inflammatory process in allergic asthma, the mechanism underlying the persistence of inflammation in the airways of asthmatic patients are still poorly under stood (Baldacci *etal.,* 2001) .

Apoptosis plays a critical role in the resolution of inflammation (Trautmann *etal.*, 2000) . Stimulation of activated T cells through the T cell receptor /CD3 complex triggers the apoptosis of these cells (Shirai *etal.*, 2003) . The interaction between Fas and Fas ligand is the main pathway of activation – induced apoptosis of mature T cells (Renauld, 2001). Activated T cells have recently been reported to be resistant to Fas mediated apoptosis in asthmatics , suggesting that the defect in apoptosis may be involved in the pathogenesis of asthma (Sengler *etal.*, 2002) .

IFNγ play an important role in regulating the proliferation and apoptosis of T – lymphocytes (Tang *etal.*, 2013).

Lymphocytes from mice with disrupted genes for IFNγ or IFNγ receptor-binding chain (IFNγ-R1) display hyper proliferation in response to mitogen and alloantigen (Zhaojun *etal.*, 2006).

IFNγ upregulates the expression of Fas protein on T- cell surface, furthermore , we have previously shown that activated Th2 clones that do not normally express detectable level of Fas L on their surface do so in the presence of IFNγ (Shirai *etal.*, 2003). Many researchers found that the defect in IFNγ production that characterizes a Th2 response was responsible for a decrease in the apoptosis of allergen –specific T cells and thus , for their persistence in allergic airway inflammation, also A Fas-blocking monoclonal antibody prevent the inhibitory effect of IFNγ on allergen – induced proliferation (Gamero *etal.*, 2007) . There are several SNPs in Gene coding for IFNγ have reportedly been associated with Systemic Lupus Erythematosus (Kim et al 2010) , Hepatitis C infection (Tomassen *etal.*, 2013) so the researchers found there is no association between treatment response and

polymorphism among studied patients, other study proved that IFNγ SNPs increase the susceptibility to cervical carcinomas among women (Lai *etal.,* 2005 and Chong *etal.,* 2006) , also, IFN γ SNPs increase susceptibility to pneumonia induced sepsis due to change in Th1 cytokines production might induce shift toward Th2 dominant phenotype (Feher *etal.,* 2010) , while, in case of T.B. infection The IFN-γ +874 A allele found to be associated with increased susceptibility to tuberculosis and individuals with AA genotypes at risk for tuberculosis infection, whereas the T allele has a protective role against TB and individuals carrying TT genotypes have prophylaxis against TB infection (Al- Zubadi, 2013).

1.2.3 Regulatory T cell :-

The regulatory T cells (Tregs) , formerly Known as suppressor T cells are a subpopulation of T cells which modulate the immune system , maintain tolerance to self antigens , and a brogate autoimmune disease (Grewe *etal.,* 2014) . These cells generally suppress or down regulate induction and proliferation of effector T cells (Zhaogun *etal.,* 2006). Additional regulatory T cells Known as T reg 17 cells have recently been identified (Yoshimura *etal.,* 2007) . Mouse models have suggested that modulation of Tregs can treat autoimmune disease and cancer , and facilitate organ transplantation (Matsumoto *etal.,* 2009) .

1.2.3.1 Regulatory T cell populations :-

T reg cells are component of the immune system that suppress immune responses of other cells , this is an important " self – check " built into the immune system to prevent excessive reactions (Bassuny *etal.,* 2003).

Regulatory T cells come in many forms with the most well-understood being those that express CD4 , CD25 and Foxp3 (CD4 + CD25 +) regulatory T cells, these " T regs " are different from helper T cells (La Grutta *et al.,* 2005) . Another regulatory T cell subset is T reg 17 cells (Piccirillo *etal.,* 2002) . Regulatory T cells are involved in shutting down immune responses after they have successfully eliminated invading organisms and also in preventing autoimmunity cells (Piccirillo *etal.,* 2002). Additional regulatory T cells populations include Tr1, Th3 , CD8 + CD28 – and Qa-1 restricted T cells (Schmid-Ott *etal.,* 2001). The contribution of these populations to self tolerance and immune homeostasis is less well defined (Kagoshima *etal.,* 2001). Foxp3 can be used as a good marker for CD4 + CD25+ T cells (Zhaojun *etal.,* 2006).

1.2.3.2 T regs Functions :-

The immune system must be able to discriminate between self and non self , when self and non – self discrimination fails, the immune system destroys cells and tissues of the body and as a result causes autoimmune diseases (Bassuny *etal.,* 2003) . Regulatory T cells actively suppress activation of the immune system and prevent pathological self – reactivity i.e auto – immune disease (Ray *etal.,* 2010). The critical role of regulatory T cells play within the immune system is evidenced

by the sever autoimmune syndrome that result from a genetic deficiency in regulatory T cells (IPEX) syndrome (Hamad and Lambrecht, 2006).

The molecular mechanism by which T reg cells exert their suppressor / regulatory activity has not been definitively characterized and is the subject of intense research in vitro experiments have given mixed results regarding the requirement of cell – to – cell contact with the cell being suppressed (Wu *etal.*, 2008) . The immunosuppressive cytokines TGFβ and interleukin – 10 (IL- 10) have also been implicated in T reg cell function (Wen *etal.*, 2003).

1.2.3.3 T reg cell and diseases including asthma :-

An important question in the field of immunology is how the immunosuppressive activity of T reg cells is modulated during the course of an ongoing immune response while the immunosuppressive function of T reg cells prevent the development of autoimmune disease , it is not desirable during immune responses to infectious microorganism (Williams and Rudensky, 2007) . The current hypothesis suggest that , upon encounter with infectious microorganisms , the activity of T reg cells may be down regulated either directly or indirectly by other cells to facilitate elimination of the infection (Medzhitov, 2007) .

Th2 lymphocytes play an important role in the initiation , progression and persistence of allergic disease including asthma (Johansson and Lundahl , 2001) . However , little is known about immunoregulatory mechanisms that determine susceptibility to , severity of or persistence of asthma , disturbance in Th1 Th2 balance , although having furthered the present understanding of immunoregulation in asthma but not adequately explain many (pre) clinical observations

(Romagnani , 2004). In recent years, the general knowledge regarding the regulation of infectious , autoimmune disease , asthma and allergen immunotherapy by T reg cells has rapidly increased (Sweilam *etal.*, 2008) . Many different T reg subsets have been described , including CD8+ T reg cells , natural killer cells and several different CD4+ T reg cell subsets (Walker *etal.*, 2003) . There are two major and well described CD4+ T reg cell subsets naturally occurring CD 25+ T reg cells and adaptive T reg cells that are postulated to prevent immune response against self antigens and adaptive immune responses respectively (Sweilam *etal.*, 2008) . The adaptive T reg cells are further sub divided into T reg1 and Th3 that mediate suppression exclusively via the cytokines IL-10 and TGFβ respectively (Emmanuel *etal.*, 2006) .

The discovery of the mutual inhibitory effects of Th1 and Th2 cells prompted the postulate that an imbalance between these two arms of the immune response would underlie Th1 mediated autoimmune disease as well as Th2 mediated allergic disease, including asthma (Gemou – Engesaaeth, 2008) .

First, Th1 cells don't always appears beneficial in mouse models of allergic asthma , and were found to contribute to or to exacerbate , disease manifestations (Hori *etal.*, 2003 and Wu *etal.*, 2008). This is in accordance with the observed appearance of Th1 cells or the cytokine IFNγ, not only in chronic a topic dermatitis and asthma ,but also during allergic sensitization (Meadus, 2003) . Secondly, Th2 - skewed parasitic helminth infection didn't appear to be associated with increased manifestations of allergy and asthma but, on, the contrary, appeared to protect against these disease (Smith *etal.*, 2001). Finally , epidemiological data from the 1960s onwards have shown parallel rise

in the prevalence of Th2 mediated allergic disease , including asthma and Th 1 mediated (auto) immune disease, such as type 1 diabetes , multiple sclerosis and crohns disease where these data don't exclude a mutual inhibitory effect of Th1 and Th2 responses (Medzhitov , 2007) . A more powerful control mechanism is likely to be at play at present , naturally T reg cells (n T reg and a Treg) respectively are taking the center stage as the crucial immunoregulatory cells that are capable of suppressing Th1 and Th2 mediated adaptive immune responses in a cell contact - dependent fashion directly or by acting on APCs, they also appear to offer exciting new views of the initiation and progression of a asthma and other allergic disease (Medzitov , 2007) .

1.2.3.4 Natural Treg :-

CD4+ CD25+ are generated in the thymus and reside in the blood and other peripheral lymphoid tissues at a frequency of 5-10% of all CD4 cells and in the bone narrow ≥ 20% both in mice and human (Lee *etal.,* 2002) . Passive transfer of n T reg cells has been shown to suppress autoimmune diabetes , inflammatory bowel disease and transplant rejection in rodent (Hara *etal.,* 2001) . n T reg cell express normal α, β, TCR repertoire also a high TCR affinity for self peptide that might be encounterd in the periphery , which may explain the constitutive expression of the activation marker (CD25) however the target autoantigens recognized by n Treg cells are at present unknown (Piccirillo *etal.,* 2002 and Torrego *etal.,* 2004) . n Treg cells can be generated in the periphery from conventional CD4 T cells upon exposure to pathogen – derived antigens also low dose antigenic peptide via prolonged subcutaneous infusion the cytokine TGF β or glucocorticoids (Wills – Karp, 1999) .Studies performed so far with

human CD4 + CD25+ n Treg cells without exception, confirmed data obtained in mouse studies (Umland *etal.*, 2002) . Human CD4+ CD25+ n Treg cells show poor proliferative responses upon stimulation with anti CD3 even in the presence of IL-2 , suppress proliferative responses of conventional CD4 CD25 Tcells and produce low level of immunosuppressive cytokines IL-10 and TGF-β while the suppression at least in vitro cannot be overcome by addition of neutralizing antibodies to these cytokines or their respective cell surface receptors (Richards *etal.*, 2000) . The role of IL-10 or TGF-β in vitro may be indirect since n Treg cells were found to aid the generation of a Treg cells that mediate suppression by these cytokines (John *etal.*, 1998) .

1.2.3.5 Foxp3 and natural T reg cells :-

Expression of specific transcription factors have been shown to be crucial to the differentiation of Th cells subsets from naïve unpolarized T cells (Zhaojun *etal.*, 2006) . Notably , T bet and GATA-3 are crucial to Th1 and Th2 cytokine expression, respectively (Moore *etal.*, 2001and Wan and Flavell, 2007).

Recently the transcription factor foxp3 was shown be selectively expressed by n Terg (Hawrylowicz and Garra, 2005) . The foxp3 mRNA level in resting n Treg cells is about 100 fold higher than in resting and activated conventional CD4+ T cells , therefore represent more specific marker for n Terg cells than cell surface molecules currently used such as CD25 CTLA - 4 and GITR (Borish *etal.*, 1996) . Data demonstrate that Foxp3 is a master regulatory gene for development and function of n Treg cells and confirms its role

in the suppression of autoimmune diseases and this also firmly demonstrated in human by Yang *etal.*, (2005).

Foxp3 is essential to n Treg cell function may open novel therapeutic strategies aimed at the induction of Foxp3 to convert conventional CD4 + T cells into n Treg cells with the ultimate goal to reverse aberrant Th2-mediated allergic asthmatic response (Lim *etal.*, 1998 and Zhaojun *etal.*, 2006) .

1.2.3.6 Adaptive T – regulatory cells :-

The a Treg cells are generated in the periphery from naïve T cells after encountering antigens presented by tolerogenic Dcs which are functionally distinct from immunogenic Dcs that promote Th1 or Th2 cells generation (Akdis *etal.*, 2004) . As already indicated , a Treg cells are subdivided into Tr1 and Th3 that mediate suppression via the cytokines IL-10 and TGFβ respectively (Arzu *etal.*, 2012).

The suppressive action of these cytokines is predominantly directed at inhibiting the expression of MHCII and costimulatory molecules by APCs (Heaton *etal.*, 2005) .

TGF-β inhibit proliferation and cytokines secretion by resting but not activated T cells (Arzu *etal.*, 2012) and IL-10 may sensitize activated T – cells to the suppressive action of TGF- β by enhancing TGF- β receptor type II expression on these cells (Borish *etal.*, 2004).

TGF – β has been shown to induce the expression of immunosuppressive IL-10 by T cells) (thus IL-10 and TGF–β may have synergistic , suppressive effects on T – cells proliferation and immuno-stimulatory cytokine secretion (Emmanuel , 2006).

1.2.3.7 Role of dendritic cells in generating adaptive Treg cells :-

Dcs play a crucial role in the generation of all adaptive T cell sub sets , including a T reg cells (Stampfli *etal.*, 1999 and Broll, 2009) .

Immature Dcs in peripheral tissues acquire pathogens and or antigens from their vicinity through phagocytosis , endocytosis and pinocytosis and mature upon recognition of so – called "danger signals" (Oh *etal.*, 2002 and Hamzaoui *etal.*, 2011) .

These signals are derived either directly from encountered pathogens / antigens or from endogenous mediators that are present or generated in the local microenvironment (Wuthrich and Schmid-Grendelmeier, 2003) .

Microbial pathogens contain ligands (pathogen associated molecular pattern PAMP) that are recognized through so – called " pattern recognition receptors " PPRs of which the family of Toll – like receptors (TLRs) and C – type Lectin receptors (CLRs) . The maturation of Dcs is characterized by high levels of expression of MHCII and costimulatory molecules such as , B7-1 , B7-2 and CD40 and by proinflammatory cytokine secretion (Yu *etal.*, 2006) . TLRs are not only involved in DC maturation , but also in the abrogation of T reg cell mediated suppression . Thus the combined action may enable development of full – blown immune responses (Sudha *etal.*, 2013) .

However , TLRs are also instrumental to the induction of tolerogenic DCs , indicating that the codes instructing development of immunogenic or tolerogenic DCs are very complex (Corrigan *etal.*, 1991and Schmid-Ott *etal.*,2001) .

Tolerogenic Dcs generally are semi-mature cells with increased expression of BMHCII and B7 – 2 but low levels of expression of

CD40 and absent production of the proinflammatory cytokines IL-6 and TNFα (Lajoie-Kadoch *etal.*, 2009).

Repetitive stimulation of T- cells with immature , antigen presenting Dc has been shown to generate IL-10 producing Tr1 cells (Platanias, 2005). The mechanisms by which so called tolerogenic DCs induce the development of a Treg cells is not completely understood (Hawrylowicz *etal.*, 2002) . The induction of Tr1 and Th3 cells is related to the production of IL-10 or TGFβ by (semi – immature) Dcs respectively, many pathogens including viruses , parasites , fungi and bacteria so called regulatory – type PAMPs have been shown to induce the production of either one or both of these cytokines by DCs and to facilitate induction of a Treg cells in this way (Oh *etal.*, 2002). The instruction of DCs by microbial ligands leading to immunogenic or tolerogenic responses may be an important link to the hygiene hypothesis of allergic diseases including asthma (Garn and Renz, 2007).

More ever , this knowledge may be of use in the development of novel therapeutics that target Dcs .

1.2.3.8 Treg cells and asthma :-

Therapeutically Treg cells may interfere with the development of allergic diseases and asthma at different stages , such as allergic sensitization , progression to allergic inflammation , airway remodeling and airway hyperesponsiveness (AHR) , and persistence of disease manifestations (Sicherer and Leung , 2007) .

Sensitization to environmental allergens is known to occur typically in early childhood or even before birth , but subsequent progression to

persistent atopic asthma , which may not manifest itself for several years , is restricted to only a subset of atopics (Asher *etal.*, 2004) .

This has led to the hypothesis that the induction of a down – regulatory Th1 responses , supposedly by microbial stimulation , during the first years of life prevents allergic sensitization and progression to atopic asthma (Kam *etal.*, 1993). Irrespective to this hypothesis , Treg cells may also be instrumental in preventing allergic sensitization and progression to asthma (Maloy and Powrie, 2001) . More research into the mechanisms by which n Treg cells promote the development of tolerance to harmless environmental allergens during intrauterine and neonatal life is needed (Weiner, 2001) . It may lead to novel strategies for primary prevention of allergic diseases including asthma (Sakaguchi *etal.*, 2001). n Treg cells from atopic donors suppressed proliferation and IL-5 production of autologous CD4+CD25- Tcells in particular during the pollen season (Zhaojun *etal.*, 2006) . Although further studies are needed , observations suggest that there may be an inverse correlation between n Treg cell activity and clinical manifestations of allergic diseases, if so , it needs to be determined whether clinical disease manifestations are the cause or the consequence of decreased n Treg activity (Jonuleit *etal.*, 2000) . The suppression by CD4+CD25+ Tcells was partially depend on cell contact and not due to increased IL-10 or TGF- β production , supporting the conclusion that these cells were indeed n Treg cells however , involvement of the immunosuppressive cytokines cannot be completely excluded since no blocking studies were carried out (Sundstedt *etal.*, 2003) . Studies so for indicate the potential role of n Treg cells in the spontaneous reversal of asthma and other allergic diseases and further

studies may shed new light on this intriguing phenomenon (Barrat *etal.*, 2002) .

Despite the limited number of studies , it can be concluded that n Treg and a T reg cells are important in the prevention of allergic sensitization as well as progression to established allergic diseases , including asthma , in established disease they function to reduce severity and to prevent exacerbation (Vieira *etal.*, 2004) .

Although these studies need to be expanded and confirmed by others , Treg cells may offer new possibilities for the monitoring of diseases and development of novel therapeutic strategies (Belkaid *etal.*, 2002) .

1.2.3.9 T reg cells and hygiene hypothesis :-

Inverse relationship between the extent of exposure to microbial agents during early childhood and the prevalence of Th2 mediated allergic diseases, however , the supposed underlying mechanism , that Th1 reactions as induced by microbial agents would suppress development of allergen – induced Th2 – reactions , appear untenable (Grewe *etal.*, 2014) .

The new concept, therefore, assumes that the rise in allergic and autoimmune diseases is the result of a decrease in immunoregulatory control of Th1 as well as Th2 reactions , probably due to decreased exposure to microbial agents (Novak and Bieber ,. 2003).

In many of the mouse infection models , in vivo depletion or transfer studies have shown that the pathogen – induced T – reg cells inhibit specific T cell responses and inflammatory immunopathology and prevent complete pathogen elimination from the host (Ramsdell,

2003). The Treg cell dependent pathogen survival is not necessarily harmful to the host , because as demonstrated for Leishmania major , it leads to persistent immunity against re – infection , while complete eradication leads to loss of immunity thus T reg cell – induced persistence of low number of pathogens in the body would result in persistently elevated levels of T reg cell activity (Hara etal., 2001) .

This in turn may help to prevent the development of untoward allergic and autoimmune reactions by so – called by stander suppression , since the suppressor effector function of n Treg cells and suppression by IL-10 derived from Tr1 cells are antigen non specific (Sudha *etal.*, 2013). In other words , T reg cell including pathogens may elevate the level of T cell regulation and so prevent sensitization to environmental allergens, in addition to various pathogens , the intestinal flora may be a persistent cause of increased T reg cell surveillance in the body (Hara *etal.*, 2001) . This is clearly indicated by the observation that murine CD4 + T cells isolated from enteral lamina properia , mesenteric lymph nodes or peripheral blood did not react to an extract of the sonicated luminal content of colon and caecum , while vigorous proliferation was seen upon depletion of the CD4+CD25+Tcells (Zhaojun *etal.*, 2006).

Regarding the protective activity of probiotic lactic acid bacteria against development of allergic diseases and observations that children with higher counts of lactic acid bacteria in their feces had a lower prevalence of allergic diseases (Ritz *etal.*, 2002) . .

1.2.4 Interleukin 10 (IL-10) :-

IL-10 is long chain-α helical structure having molecular weight 34-40 KD (Emmanuel ,. 2006) . In humans Th0, Th1, and Th2-like CD4+ T cell clones, cytotoxic T cells, activated monocytes and peripheral blood T cells including CD4+ and CD8+ T cells , Tr1 , macrophages, keratinocytes and many tumor cells have the capacity to produce IL-10 (Lai *etal.*, 2005) . IL-10, previously known as cytokine synthesis inhibitor factor (CSIF) was originally identified as a product of Th2 clones that suppressed the production of cytokines by Th1 clones responding to stimulation of antigen (Takanashi *etal.*, 1999) . Mast cells also have the capacity to produce IL-10. Constitutive IL-10 secretion occurs in the healthy lung with the major source being the alveolar macrophage; however, the circulating monocyte elaborates more IL-10 than the alveolar macrophage (Sudha *etal.*, 2013).

1.2.4 .1 Role of IL-10 in asthma:-

IL-10 is a pleotropic cytokine that has the potential to downregulate both Th1 and Th2- driven inflammatory processes (Schuster *etal.*, 2000) . It is of interest that IL-10 might also have a beneficial effect on airway remodeling, as it has been shown to reduce collagen type-I synthesis and vascular smooth muscle proliferation (Arzu *etal.*, 2012). The precise functional role of IL-10; how-ever appears to be somewhat unclear (Zeibecoglu *etal.*, 2000) . Whether IL-10 expression is changed in asthma is uncertain, as in some studies reduced and in others increased bronchoalveolar lavage fluid levels were found (Sudha *etal.*, 2013). IL-10, administered exogenously at the time of secondary antigen presentation, reduces

antigen-induced airway eosinophilia in animal models (Borish *etal.*, 2004) , where as airway eosinophilia and total serum IgE levels are increased in sensitized IL-10 knockout mice (Droste *etal.*, 2014).

However, the effects of IL-10 on airway hyperresponsiveness are somewhat more contradictory (Kerkhof *etal.*, 2003) . In some studies, endogenous production of IL-10 was shown to dampen airway responsiveness (Hamzaoui *etal.*, 2000), where as others have shown that IL-10 enhances the allergen induced increase in airway responsiveness, despite the reduction in eosinophil recruitment (Jason *etal.*, 2001) .

It behaves like a potent anti-inflammatory and immunosuppressive molecule inhibiting the production and release of Il-2 and IFN γ and a range of proinflammatory cytokines such as TNF α, IL-1, IL-6 and IL-12 (Keslacy *etal.*, 2007) .

Functions of IL-10 include promotion of B-cell survival , growth and differentiation , suppression of inflammatory cytokine synthesis by monocytes and macrophages and the down – regulation of T cell responses both directly and through effects on DC (Boyce and Austen, 2005) . IL-10 has also been implicated in the generation and maintenance of regulatory Tcells , fur the emphasizing its important role in immune regulation (Hawrylowicz and Garra, 2005) .

IL-10 was found to suppress IgE production by cells Based on its ability to suppress cell – mediated and antibody mediated responses (Lim *etal.*, 1998) . IL-10 is now considered to be a major immunosuppressive cytokine with apotential as a therapy for various inflammatory diseases (Wilson *etal.*, 2009) . Subsequent studies showed that IL-10 also can down-regulate Th2 clones and their production of IL-

4 and IL-5, in addition, IL-10 expresses a wide variety of effects on other immune cells, including stimulation of B cell differentiation and Ig secretion (Von Mutius, 2009).

The true biological effects of IL-10 have been difficult to delineate because the activities of this molecule on immune responsiveness vary considerably (Kim *etal.,* 2010).

IL-10 has been suggested as a potential therapy of allergic inflammation and asthma (Wang *etal.,* 2010). To define the role of IL-10 in controlling the development of allergic inflammation and AHR, we used an established mouse model of eosinophilic airway inflammation and allergen-driven alterations in airway function, Here, we describe that IL-10- deficient mice, sensitized and challenged to ovalbumin (OVA), fail to develop AHR despite a significant eosinophilic airway inflammatory response (Li *etal.,* 2003).

Only after reconstitution with IL-10 could changes in airway responsiveness be detected, these data imply a major role for IL-10 in the regulation of airway function downstream of the inflammatory cascade (Johnston *etal.,* 2007). IL-10 is able to inhibit the cytokine synthesis by both Th1 and Th2 human cell clones (Woodruff *etal.,* 2001). In contrast, IL-2 is a growth and activating factor for Th1and Th2 cells (Lim *etal.,* 1998). The relationship and balance between IL-2 and IL-10 play a crucial role in operating cellular complex network in asthma and remain to be elucidated (Kips, 2001) . Several studies have suggested a role for Th2 like subset of CD4+ T lymphocytes contributes to eosinophil influx and activation by demonstration of mRNA for IL-3, IL-4, IL-5, GMCSF, but not Th1 cytokines such as IFN-γ except IL-2 in

BAL fluid of atopic asthma compared with normal control subjects (Chong *etal.*, 2006).

1.2.5 Interleukin 4 (IL-4) :-

1.2.5.1 Synthesis and release :-

IL-4 is a short chain α-helical bundle having molecular weight 18 KD (Akpinarli *etal.*, 2002) . The major cellular sources of IL-4 are thymocytes, mature T-cells, mast cells, basophils and CD4+ Th2 cells (Zhaojun *etal.*, 2006) . IL-4 is thought to be an upstream cytokine that regulates allergic inflammation by promoting Th2 cell differentiation and IgE synthesis (Akpinarli *etal.*, 2002).

IL-4 synthesis can be induced by stimulation of the antigen receptors on T-lymphocytes and by IgE Fc receptor cross linking in mast cells and basophils (Woo-Tung *etal.*, 2013) . IL-4 was originally identified as a B-cell growth factor, which drives the optimal stimulation of B-cells by antigen (Wong *etal.*, 2001) . Interestingly, corticosteroids enhance the capacity to induce IL-4 synthesis from CD4+ T-cells (Bailey *etal.*, 2002). In addition, IL-4 stimulates the expression by B cells of major histocompatibility complex (MHC) Class-II molecules, B7, CD40, surface IgM and low affinity receptor, resulting in enhance antigen presenting capacity of B-cells (Badthorn *etal.*, 2011) .

1.2.5.2 Role of IL-4 in asthma :-

IL-4 demonstrates a broad range of biological activities, in general terms: it can be described as the main cytokine involved in the pathogenesis of allergic disorders (Akpinarli *etal.,* 2002).

Additional effects that seem of particular importance for asthma include stimulation of mucus producing cells and fibroblast, thus also implicating IL-4 in the pathogenesis of airway remodeling (Arzu *etal.,* 2012) .

Another potentially important activity of IL-4 in allergic inflammation is its ability to induce the expression of vascular cell adhesion molecule-1 on endothelial cells this will produced enhanced adhesiveness of the endothelium for T-cells, eosinophils, basophils and monocytes, which are characteristics of allergic reactions (Akbari *etal.,* 2001) . Because of these properties IL-4 has long been considered as a potential target in allergies and asthma, numerous *in vivo* studies highlighted its role in IgE production (Faith *etal.,* 2003) . However, it soon became clear that the role of IL-4 in IgE production could not be extended to airway hyperreactivity, which remains the hallmark of asthma (Wilson *etal.,* 2009).

Over expression of IL-4 in lungs leads to a lymphocytic and eosinophilic inflammation, but without airway hyperreactivity (Kawai *etal.,* 2001).

Shalev *etal.,* (2011) , reported that, inhalation of recombinant human IL-4 induced airway eosinophilia and caused some degree of bronchial hyperresponsiveness in atopic asthmatics (Winther *etal.,* 2014). Thus, an essential effector role for IL-4 in experimental asthma has been difficult to show, although IL-4 appears to play an important

role in Th2 cell development and recruitment to the airways (Hoves etal., 2006) . The local production of IL-4 in the bronchial mucosa by multiple cell types enhances Th2 expansion (Cope *etal.,* 2011) . In addition to the pre-established allergen-specific Th2 T cells resident in the bronchial mucosa of allergic patients, possible sources of IL-4 include NK1.1 cells (both T and non-T) and mast cells (Schmid-Ott *etal.,* 2001) . IL-4 production by mast

cells is favored if they are sensitized, via FceRI, by preformed IgE specific for the allergen (Kawai *etal.,* 2001). IL-4 supports the differentiation and expansion of allergen- specific Th2 cells, which then provide 2 signals, IL-4 and CD40L, that drive IgE production by B cells (Laouini *etal.,* 2003) .

1.2.6 IgE and Asthma :-

IgE is set apart from the other immunoglobulin isotypes by its very low plasma levels and short half-life (Woo-Tung *etal.,* 2013).

In addition to triggering immediate-hypersensitivity reactions and late-phase responses, there is accumulating evidence that preformed IgE can augment humoral and cellular immune responses to allergens (Tomassen *etal.,* 2013). IgE blockers have recently been developed and show some promise in the treatment of allergic diseases(Xin-Yang *etal.,* 2015). The production of IgE is tightly regulated and involves a complex network of cellular and molecular signals (Kawai *etal.,* 2013).

As the millennium draws to a close, it is clear that IgE production represents only one feature of a larger specific immune response orchestrated by the Th2 subset of CD4+ Th cells. Th2 cells are critical in maintaining both the state of chronic and relapsing eosinophil-

predominant inflammation and the acute hypersensitivity responses characteristic of the atopic diseases (Deo *etal.*, 2010) .

1.2.6.1 IgE functions in allergic responses (immediate hypersensitivity)

Asthma, allergic rhinitis, and atopic dermatitis are almost invariably accompanied by elevated levels of IgE (Elizabeth *etal.*, 2010).

Genetic analyses of families have shown that bronchial hyperresponsiveness (BHR) and IgE levels are linked (Hatachi *etal.*, 2003). Thus, in clinical practice, allergen-specific IgE (as demonstrated by skin testing or in vitro assays) is generally believed to be inextricably connected to the induction of allergic airway symptoms, and is used as a guide for environmental modification and immunotherapy (Saraiva and O´Garra, 2010) . The interaction of IgE with antigen is known to lead to a variety of immunological sequelae (Winther *etal.*, 2014) . Cross-linking of IgE bound to mast cells by FceR I triggers the release of preformed vasoactive mediators, synthesis of prostaglandins and leukotrienes, and the production of cytokines (Nomura *etal.*, 2003) .

In the bronchial mucosa, these mediators of immediate-hypersensitivity reactions rapidly induce mucosal edema, mucous production, smooth muscle constriction, and eventually elicit an inflammatory infiltrate (Monetini *etal.*, 2004) . IgE-induced mast cell degranulation in vivo is often followed by a late-phase reaction (LPR), a second wave of hypersensitivity responses occurring many hours after the acute reaction and dependent upon eosinophils. In asthmatics, this manifests as a second wave of decreased airflow 4–8 hours after the initial allergen contact (Ellis *etal.*, 2012).

1.2.6.2 Regulation of IgE production:

2 signals are required for the isotype switch.

The strong association of IgE with the atopic diseases, and its documented role in immediate hypersensitivity reactions in the airway, has driven efforts to define the cellular and molecular interactions that regulate its production (Ellis *etal.,* 2012). The immunoglobulin heavy chain gene locus (IgH) present in the genome of all naive IgM+ B cells, contains all the genetic information required to synthesize each of the immunoglobulin isotypes (Joo *etal.,* 2012). In the case of IgE, it is now clear that two distinct signals, both of which can be provided by Th cells, are required to drive this process of isotype switching (Tanaka *etal.,* 2012).

a. The first signal can be provided by the cytokines IL-4 or IL-13; stimulates transcription at the Ce gene locus, which contains the exons encoding the constant-region domains of the IgE e-heavy chain (Bettiol *etal.,* 2006) . A second signal, delivered by the interaction of CD40L on the surface of T cells with CD40 on the B-cell membrane, activates the genetic rearrangement (called deletional switch recombination) that brings into proximity all of the elements of a functional e-heavy chain (Yu and Wang , 2011).

IL-4 and IL-13 are the only cytokines that can support IgE production by cultured B cells (Yu and Wang, 2011). IL-4 is produced primarily by Th2 cells, but is also derived from NK cells, mast cells, and basophils(Tang – Feldman *etal.,* 2011) . IL-13 is produced by activated Th2 cells, as well as by Th0 cells, mast cells, and dendritic cells, the receptor for each cytokine is multimeric, IL-4 and IL-13 receptors share the IL - 4R α chain, the high affinity IL-13 receptor contains the IL-4R

α chain and an IL-13 binding chain (IL-13Rα1 or IL-13Rα2), whereas the IL-4 receptor is composed of IL-4Rα and the common cytokine receptor γ chain (Ozdemir *etal.*, 2008) . Although the two cytokines exert many of the same influences on target cells, they differ in several important respects.

Because T cells do not express IL-13 receptors, exogenous IL-13 does not promote Th2 responses or suppress Th1 differentiation in antigen-stimulated cultures in the same way as IL-4 (Frossard and Eigenmann, 2008).

b. CD40/CD40L interaction: The second signal for IgE isotype switches recombination.

CD40L, which is absent on resting T cells, appears on their surface after T-cell receptor (TCR) activation by antigen/MHC complex (Torres – Aguilar *etal.*, 2010). Binding of the newly expressed CD40L with CD40 on B cells provides the second signal for the induction of deletional switch recombination to IgE (Pacciani *etal.*, 2010) . A number of observations have pointed to a critical role for CD40/CD40L interactions in isotype switching to IgE(Cai *etal.*, 2012). IgE production by IL-4–stimulated B cells in culture requires the presence of T cells (Shah *etal.*,2012). Anti-CD40 antibodies, which directly activate CD40L, can completely substitute for this T-cell help (Liu *etal.*, 2011). Soluble human CD40 inhibits CD40/CD40L interaction, and blocks IL-4–driven IgE synthesis in such cultures (Pacciani *etal.*, 2010).

1.2.7 Eosinophils and allergic asthma

Eosinophils are circulating granulocytes produced in the bone marrow along with other white blood cells and travel at relatively low levels in the bloodstream, making up 1–3% of white blood cells and these are the major cell types that can be recruited to sites of immunological or inflammatory responses (Brightling, 2001). The effector function of eosinophils is related to their release of toxic granule proteins, reactive oxygen species (ROS), cytokines, and lipid mediators (Busse, 2001) . Although eosinophils has been traditionally considered as cytotoxic effectors cells, new insights into molecular pathways allowed a better understanding of the immunomodulatory functions of these cells (Das *etal.,* 2006) . More recently, eosinophils have also been demonstrated to participate in host defense against respiratory viruses (Esnault *etal.,* 2012). With the capacity of the eosinophils to store preformed cytokines, chemokines, and growth factors available for immediate use, they play multiple roles favoring the initiation and maintenance of immune responses in inflammation, besides maintaining epithelial barrier function, affecting tissue remodeling, and bridging innate and adaptive immunity (Fanat *etal.,* 2009). The local accumulation of eosinophils is involved in the pathogenesis of allergic diseases such as asthma (Fattouh and Jordana, 2008). Allergic asthma is associated with eosinophilic inflammation in the airways (Aihara *etal.,* 2003). The proinflammatory mediators derived by eosinophil are major contributors to inflammation in asthma, including airway epithelial cell damage and loss, airway dysfunction of cholinergic nerve receptors, airway hyperresponsiveness, mucus hypersecretion, and airway remodeling, characterized by fibrosis and collagen deposition (Aihara

etal., 2004). Currently, the term "eosinophilic asthma" has been used to characterize an asthma phenotype with prevalence of eosinophils in the bronchial airways, and this phenotype can be identified by peripheral eosinophil count (Busse, 2001).

Materials and Methods

2.1 Samples collection

2.1.1 Patients

In this case control study, a total of 100 patients with asthma who have referred to Hilla Allergy Specialized Center after they were suspected to have asthma according to their clinical manifestations , radiological changes, skin test and confirmed by allergen assay . Venous blood samples and nasal swap were collected from 100 patients suffering from asthma.

The age of the patients are ranging from (10 - 75) years old including (40 males) and (60 females) .

2.1.2 Control

Venous blood samples were taken from apparently healthy persons not suffering from any respiratory problems and have – ve family history to asthma , include 100 persons equal 50 males and 50 females with age range approximately matched to that of patients all control group confirmed diagnosed by radiological X – ray and allergen assay who give – ve results .

2.2 Materials

2.2.1 Laboratory Instruments and equipments .

All the instruments and equipments were required in this study showed in the table (2-1).

Table (2-1) laboratory instruments and equipments

No.	Instruments	Company	Country
1.	Autoclave	Hirayama	Japan
2	Distillator	FineTech	Korea
3.	E-graph –UV (Gel documentation)	ATTO	Korea
4.	ELISA instrument system	BioTek	USA
5.	Gas burner	GFL	Germany
6.	Gel electrophoresis	Cleaver	USA
7.	Glass EDTA tubes 10 ml	AFCO	Jordan
8.	Glass gel tubes 10ml.	AFCO	Jordan
9.	Glass slides, flasks and Beakers	Hirschman	Germany
10.	Incubator	Memmert	Germany
11.	Light microscope	Olympus	Japan
12	Micro centrifuge	Beckman	Germany
13.	Oven	Memmert	Germany
14.	PCR Thermocycler apparatus	Bioneer	Korea
15.	Platinum wire loop	Himedia	India

16.	Refrigerator	Concord	Lebanon
17.	Screw capped tube 10 ml	AFCO	Jordan
18.	Sensitive electron balance	Sartorius	Germany
19.	Water bath	Kottermann	Germany
20	Electrophoresis	Bionner	Korea
21	Exispin vortex centrefuge	Bionner	Korea
22	Miniopticon Real Time PCR	Bio – Rad	USA
23	Vortex	CYAN	Belgium
24	Nnodrop	Thermo Scientific	UK
25	High speed cold centrifuge	Eppendrof	Germany
26	Digital Camera	Sumsung	Korea
27	Biological safety cabinet	Labogene	Denmark
28	Centrifuge	Gemmy	Taiwan
29	Medical injection Syringes	MEDECO	UAE
30	Microcentrifuge tubes 1.5ml	BIO BASIC	Canada
31	Micropipettes (different volumes)+ tips	BIO BASIC	Canada
32	Screw capped tubes 30 ml.	Hirschman	Germany
33	Minineph analyzer	MININEPH	England
34	Spectrophotometer	Optima sp3000	Japan
35	Vitek	bioMérieux	France

2.2.2 Chemicals and biological materials

2.2.2.1 Chemical materials: Chemical materials are listed in table (2-2)

Table (2-2) Chemical Materials

N0.	Chemicals	Company/country
1.	Glycerol	B.D.H , England
2.	99% , 95% and 70% alcohol (Ethanol)	Flukachemika Switzerland
3.	Cedar Oil	Labort / India
4.	Tracking dye	Geneaid
5.	Tris-EDTA Buffer solution(TE)	Promega-USA
6.	Tris Borate-EDTA –Buffer solution(TBE)10x	Bio basic-Canada
7.	Ethidium Bromide	Bio basic-Canada
8	Neutralizing Buffer(phosphate Buffer)	Merck, Germany
9.	Chloroform	Labort / India
10.	DEPC water	Bioneer / Korea
11	EDTA	Sigma – Aldrich / USA
12	Ethanol 100%	Labort / India
13	Isopropanol	Labort / India
14	RNase free water	Bioneer / Korea
15	Free nuclease water	Bioneer / Korea

2.2.3. Commercial kits:

The commercial kits were used in the study are listed in table(2-4)

Table (2-4) Commercial kits used in the study

No.	Type of kits	Company/country
1-	Genomic DNA extraction kit from fresh blood	Geneaid-Taiwan
2-	PCR preMix 20 µl reaction	Bioneer-Korea
3-	DNA ladder 100-1500bp	Promega-USA
4-	Primers of INF-ɣ gene polymorphism , primers of internal control, primers of FoxP3 and primers of IL-10	Bioneer-Korea
5-	ELISA kits to evaluate the concentrations of IFN-ɣ , IL-4 and total IgE	Peprotech-UK

a- Genomic DNA extraction kit from fresh blood :

Table (2-5) Genomic DNA extraction kit

Materials	Company
1- RBC Lyses Buffer 2- GB Buffer 3- RNase A(10mg/ml) 4- Absolute Ethanol 5- GD column and 2ml collection tube 6- Washing Buffer (W1) 7- Washing Buffer (W2) 8- Elution Buffer	Genaid

b- PCR preMix 20 µl reaction (Bioneer)

Table (2-6) PCR preMix contents

Components	20 µl reaction
Top DNA polymerase(Taq)	1 U
Each: dNTPs (dATP, dGTP, dCTP, dTTP)	250µM
Tris-Hcl(pH 9.0)	10 mM
Kcl	30 mM
Mgcl2	1.5 mM
Stabilizer and tracking dye	

c – Kit for allergen assay

(Quantitative determination of allergen – specific IgE in human serum)

Table (2-7) allergen assay kit

Components	**Company/ country**
1-Anti IgE enzyme conjugate	**DR.FOOKE / Germany**
2- Concentrated washing buffer (50x)	
3-Substrate buffer	
4-Substrate tablets	
5-Stop solution(1 N NaOH)	

d- DNA ladder 100-1500bp (Promega)

Table (2-8) DNA ladder components

Materials

1-Ladder consist of 11 double-stranded DNA with size (100-1500bp).
2-Loading dye has a composition :
[15% Ficoll, 0.03% bromophenol blue, 0.03% xylene cyanol, 0.4% orange G, 10mM Tris-HCl (pH 7.5)50mm EDTA].

e. ASO -PCR Kits

Table (2-9) ASO -PCR Kits

No.	Kit	Company	Country
1	AccuPrep®Genomic DNA extraction kit	Bioneer	Korea
	Binding buffer (GC)		
	Proteinase K 25 mg		
	W1 buffer		
	Wash buffer		
	Elution buffer		
	Binding column		
	Collection 2ml tube		
2	**AccuPower™ PCR PreMix**	**Bioneer**	**Korea**

	Taq DNA polymerase dNTPs (dATP, dCTP, dGTP, dTTP) Tris-HCl pH 9.0 KCl		
	$MgCl_2$		
	Stabilizer and Tracking dye		

f. Real-Time PCR Kits

Table (2-10) Real-Time PCR Kits

No.	**Kit**	**Company**	**Country**
1	Total RNA Extraction Kit AccuZol™	Bioneer	Korea
	Trizol reagent 100ml		
2	DNase I enzyme kit	Promega	USA
	Dnase I enzyme 10x buffer DEPC water Stop reaction		
3	AccuPower® RocketScript™ RT PreMix 96 plate	Bioneer	Korea
	RocketScript Reverse Transcriptase (200 u) X Reaction Buffer (1 x 5) DTT (0.25 mM)		

	(dNTP (250) µM each		
	RNase Inhibitor (1 u)		
4	AccuPower ® Greenstar ™ qPCR PreMix 96 plate	Bioneer	Korea
	SYBER Green fluorescence		
	Taq DNA polymerase		
	dNTPs (dATP, dCTP, dGTP, dTTP)		
	DEPC water		

g. ELISA kits to evaluate the concentrations of IFN-ɣ and IL-4 and Total IgE

Table (2-11) ELISA kits for titration of the concentrations of IFN-ɣ and IL-4 and Total IgE

Components	Description	volume
Antibody-coated Microplate	Capture antibody-coated microplate for IFN-ɣ , IL-4 and IgE (12x 8-well strips)	2 plates for each item
Detection Antibody solution	Biotinylated detection Antibody.	22ml
standard	Lyophilized standard. Use after reconstitution with 1X diluent. Reconstituted standard may be stored at<-20oC for up to 2 weeks. Avoid multiple freeze-thaw cycles.	2 vials
Avidin-HRP Solution	HRP-conjugated Avidin.	23ml

5X Diluent(PEK5XDIL01)	For dilution of standards and samples. Use after diluted to 1X concentration.	36ml
20X Wash Buffer (PEK20XWB01)	For microplate washing. Use after diluted to 1X concentration.	50ml
ABTS Solution (PEKABTS01)	Substrate solution for color development.	22ml
Plate Sealer	Adhesive strips	6 ea

2.2.4. Primers

a. ASO PCR IFN-ɣ gene polymorphism 874 A/T primers that used in this study were designed by (Pravica *etal.*, 2000).

Table (2-12) ASO - PCR primers

Primer	**Sequence**
Common primer	**5'-TCAACAAAGCTGATACTCCA-3'**
T allele primer	**5'-TTC TTA CAA CAC AAA ATC AAA TCT-3'**
A allele primer	**5'-TTC TTA CAA CAC AAA ATC AAA TCA-3'**

b. Real-time PCR primers were designed in this study by using NCBI- Gene Bank data base and Primer 3 design online, supported from (Bioneer, Korea) company.

Table (2-13) Real-time PCR primers

Primer	**Sequence**	
GAPDH	**F**	**ATGGGAGTTGCTGTTGAAGTCA**
	R	**CCGAGGGCCCACTAAAGG**
FoxP3	**F**	**TGTGCTAGGGCGGTATGAGA**
	R	**GCTGGGGTGCAACTATGGG**
IL-10	**F**	**ACATCAAGGCGCATGTGAAC**
	R	**ACGGCCTTGCTCTTGTTTTC**

2.3.3 Phosphate buffer solution:

A weight of 80 gm of NaCl, 0.34gm of KH_2PO_4, and 1.12gm of K_2HPO_4 were all dissolved in 1000 ml of D.W. The pH was adjusted to 7.3, then the solution was sterilized in autoclave (Rutella *et al*., 2006).

2.3.4 Agarose gel

Agarose gel was prepared by dissolving 2 gm of agarose powder in 100ml of 1X TBE buffer (pH 8) in boiling water bath, allowed to cool to 50^{o}C and 1µl of ethidium bromide at the concentration of 0.5mg/ml was added (Shalev *etal.*, 2011) .

2.4 Methods:

2.4.1 Collection of clinical specimens :

2.4.1.1 Collection of Blood

Up to 3ml of blood that used for serum separation collected in non-heparinized gel tubes and 2ml of blood collected in heparinized tubes for DNA and RNA extraction was drawn according to (Lim *etal.*, 2012) .

2.5 Genotyping assay

2.5.1 ASO PCR

ASO-PCR technique was performed for detection interferon gamma gene polymorphism in the blood of asthmatic patients samples as well as in healthy blood samples as control group. methods were carried out according to method described by (Parasons *etal.*, 2012) as following steps:

2.5.1.1 Genomic DNA Extraction

Genomic DNA was extracted from patients blood samples as well as from blood of healthy control samples by using AccuPrep®Genomic DNA extraction kit (Bioneer. Korea), and done according to company instructions as following steps:

1. A 200µl frozen blood sample was transferred to sterile 1.5ml microcentrifuge tube, and then added 20µl of proteinase K and mixed by vortex and incubated at 60℃ for 10 minutes.
2. After that, 200µl of Binding buffer (GC buffer) was added to all tubes samples and mixed well by vortex to achieve maximum lysis efficiency, and then all tubes were incubated at 60℃ for 10 minutes.

3. A 100µl of isopropanol was added to mixture and mixed well by pipetting, and then the lysate was carefully transferred into (GD) Binding filter column that fitted in a 2 ml collection tube, and then closed the tubes and centrifuged at 8000 rpm for 1 minute.
4. The throughout samples lysates were discarded in disposal bottle, and then 500µl Washing buffer 1 (W1) was added to each Binding filter column, and centrifuged at 8000 rpm for 1 minute.
5. The throughout washing buffer 1 was discarded in disposal bottle, and then 500µl Washing buffer 2 (W2) was added to each Binding filter column, and centrifuged at 8000 rpm for 1 minute.
6. The throughout washing buffer 2 was discarded in disposal bottle, and then the tubes were centrifuged once more at 12000 rpm for 1 minute to remove isopropanol completely.
7. After that, GD Binding filter column that containing genomic DNA was transferred to sterile 1.5ml microcentrifuge tube, and then 50µl of Elution buffer was added and left stand the tubes for 3 minutes at room temperature until the buffer is completely absorbed into the binding filter for elution the extracted DNA.
8. Finally, all tubes were centrifuged at 8000 rpm for 1 minute to elute DNA, and store at -20℃freezer until used.

2.5.1.2 Genomic DNA Profile

The extracted blood genomic DNA was checked by using Nanodrop spectrophotometer (THERMO. USA), which measured DNA concentration (ng/µL) and check the DNA purity by reading the absorbance at (260 /280 nm) as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).
2. A dry chem-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipette 2µl of free nuclease water onto the surface of the lower measurement pedestals for blank the system.

2.5.1.3 ASO PCR master mix preparation

PCR master mix was prepared by using (AccuPower PCR PreMix Kit) and this master mix done according to company instructions as following table:

Table (2-14) ASO PCR master mix

PCR Master mix	**Volume**
DNA template	5µl
Common primer (10 pmol)	1µl
T allele primer (10 pmol)	1µl
A allele primer (10 pmol)	1µl
PCR water	12µl
Total volume	20µl

After that, these PCR master mix component that mentioned above placed in standard AccuPower PCR PreMix Kit that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl2,stabilizer, and tracking dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler (Mygene. Korea) .

2.5.1.4 PCR Thermocycler Conditions

PCR thermocycler conditions were done for each gene independent as following tables:

Table (2-15) PCR technique steps

PCR step	Temp.	Time	repeat
Initial denaturation	95°C	5min.	1
Denaturation	95°C	30 sec.	40cycle
Annealing	52°C	30 sec.	
Extension	72°C	30 sec.	
Final extension	72°C	5min	1
Hold	4°C	Forever	-

2.5.1.5 PCR product analysis

The PCR products were analyzed by agarose gel electrophoresis following steps:

1- 2% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C.

2- Then 0.5µ of ethidium bromide stain were added into agarose gel solution.

3- Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and 10ul of (100bp Ladder) in one well.

4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for 1hour.

5- PCR products were visualized by using UV Transilluminator.

2.5.2 Quantitative Reverse Transcription Real-Time PCR

Quantitative Reveres Transcription Real-Time PCR technique was performed for estimation of relative gene expression analysis. This technique was done according to method described by manufactured company as following :

2.5.2.1 Total RNA extraction (For IL-10 and FOXP3 genes)

Total RNA were extracted from blood by using (Total RNA extraction kit Accuzol (TM), Bioneer, Korea) and done according to company instructions as following steps:

1- 200µL of frozen blood was homogenized by using micropestle in 1 ml of Trizol® reagent.

2- 200µl chloroform was added to each tube and shaken vigorously for 15 seconds.

3- The mixture was incubated on ice for 5 minutes. Then centrifuged at 12000 rpm, 4C°, for 15 minutes.

5- Supernatant was transferred into a new eppendorf tube, and 500µl isopropanol was added. Then, mixture mixed by inverting the tube 4-5 times and incubated at 4C° for 10 minutes. Then, centrifuged at 12,000 rpm , 4C° for 10 minutes.

6- Supernatant was discarded, and 1ml 80% Ethanol was added and mixed by vortex again. Then, centrifuge at 12000 rpm, 4C° for 5 minutes.

7- The supernatant was discarded and the RNA pellet was left to air to dry.

8- 50µl DEPC water was added to each sample to dissolve the RNA pellet, Then, the extracted RNA sample was kept at -20.

2.5.2.2 Estimation of RNA yield

The extracted total RNA was assessed and measurement by Nanodrop spectrophotometer (THERMO. USA), There are two quality controls were performed on extracted RNA. First is to determine the quantity of RNA (ng/µL), the second is the purity of RNA by reading the absorbance in spectrophotometer at 260 nm and 280 nm in same Nanodrop machine as follow:

1- After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, RNA).

2- A dry Chem-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipet 1µl of ddH2O onto the surface of the lower measurement pedestal.

3- The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of the appropriate blanking solution was added as black solution which is same elution buffer of RNA samples.

4- After that, the pedestals are cleaned and pipet 1µl of RNA sample for measurement.

5- The purity of RNA, also determined by reading the absorbance in Nanodrop spectrophotometer at 260 nm and 280 nm, so the RNA has its absorption maximum at 260 nm and the ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

2.5.2.3 DNase I Treatment

The extracted RNA were treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using samples (DNase I enzyme kit) and done according to method described by promega company, USA instructions as follow :

Table (2-16) DNase I enzyme kit

Mix	**Volume**
Total RNA	**10** µl

100ng/ul	
DNase I enzyme	**1** µl
10X buffer	**4** µl
DEPC water	**5** µl
Total	**20** µl

After that, The mixture was incubated at 37C° for 30 minutes. Then, 1µl stop solution was added and incubated at 65C° for 10 minutes for inactivation of DNase enzyme action.

2.5.2.4 cDNA synthesis

DNase-I treatment total RNA samples were used in cDNA synthesis step by using AccuPower® RocktScript RT PreMix kit that provided from Bioneer company, Korea and done according to company instructions as following table:

Table (2-17) RT PreMix kit

RT master mix	**Volume**
Total RNA 100ng/ul	10ul
Random Hexamer primer	1ul
DEPC water	9ul
Total	20ul

This RT Pre-Mix was placed in AccuPower Rocket Script RT Pre-Mix tubes that contains lyophilized Reverse transcription enzyme . Then dissolved completely by vortex and briefly spinning down. The RNA converted into cDNA in thermocycler under the following thermocycler conditions:

Table (2-18) cDNA synthesis steps

Step	**Temperature**	**Time**
cDNA synthesis (RT step)	50 °C	1 hour
Heat inactivation	95 °C	5 minutes

2.5.2.5 Quantitative Real-Time PCR (qPCR) master mix preparation

qPCR master mix was prepared by using AccuPowerTM Green Star Real-Time PCR kit that dependant syber green dye detection of gene amplification in Real-Time PCR system and include the follow :

Table (2-19) qPCR master mix kit

qPCR master mix	**Volume**
cDNA template (100ng)	3µL
Forward primer(10pmol)	1 µL
Reverse primer (10pmol)	1 µL
DEPC water	15 µL
Total	20 µL

After that, these qPCR master mix component that mentioned above Accopwer Green star qPCR premix standard plate tubes that contain the syber green dye and other PCR amplification components, then the plate

mixed by Exispin vortex centrifuge for 3 minutes, than placed in Miniopticon Real-Time PCR system in the following thermocycler protocol in the following table :

Table (2-20) qPCR steps

qPCR step	**Temperature**	**Time**	**Repeat cycle**
Initial Denaturation	95 °C	5min	1
Denaturation	95 °C	20 sec	45
Annealing\Extention Detection(scan)	58 °C	30 sec	
Melting	60-95°C	0.5 sec	1

2.5.2.6 Data analysis of qRT-PCR

The data results of q RT-PCR for target and housekeeping gene were analyzed by the relative quantification gene expression levels (fold change) Livak method that described by (Ganguly *etal.*, 2012).

The relative quantification method, quantities obtained from q RT-PCR experiment must be normalized in such a way that the data become biologically meaningful. In this method, one of the experimental samples is the calibrator such as (Control samples) each of the normalized target values (CT values) is divided by the calibrator normalized target value to generate the relative expression levels. After

that, the ΔCT Method with a Reference Gene was used as following equations :

Table (2-21) equations for Data analysis of qRT-PCR

Gene	**Test(Treatment group)**	**Cal. (Control group)**
Target gene	CT (target, test)	CT (target, cal)
Reference gene	CT (ref, test)	CT (ref, cal)

First, normalize the CT of the reference (ref) gene to that of the target gene, for calibrator sample:

ΔCT (calibrator) = CT (ref, calibrator) – CT (target, calibrator)

Second, normalize the CT of the reference (ref) gene to that of the target gene, for the test sample:

ΔCT (Test) = CT (ref, test) – CT (target, test)

ΔΔCT= ΔCT (test) – ΔCT (calibrator)

Fold change = 2-ΔΔCT

Ratio (reference/target) = 2CT (reference) – CT (target)

So, the relative expression was divided by the expression value of a chosen calibrator for each expression ratio of test sample.

2.6 Serological examination (ELISA) : Titration of (Total IgE , IFN γ , IL-4)

2.6.1 Reagents preparations (manufactured instructions provided by Labscience co.) :-

Bring all reagents to room temperature (18 - 25) before use .

1 – Wash buffer :- Dilute 30 ml of concentrated wash buffer into 750 ml of distilled water . put unused solution back at 4 C° if crystals have formed in the concentrate , you can warm it with 40C water path (heating temperature should not exceed 50 C°) and mix it gently until

the crystals have completely dissolved , the solution should be cooled to room temperature before use .

2 - Standard :- prepare standard within 15 minutes before use , centrifuge at 10000 rpm for one minute and reconstitute the standard with 1.0 ml of reference standard and sample diluent . Tighten the lid , let it stand for 10 minutes and turn it upside down for several times , after it dissolves fully mix it thoroughly with pipette . This reconstitution produces a stock solution of 200ng/ml . Then make serial dilutions as needed (making serial dilution in the wells directly is not permitted).

The recommended concentrations are as follows : 200 , 100 , 50 , 25 , 12.5 , 6.25 , 3.13 , 0 ng/ml . If you want to make standard solution at the concetraion of 100ng/ml , you should take 0.5 ml standard at 200 ng/ml , and it to an EP tube with 0.5 ml reference standard and sample diluent , and mix it . Procedures to prepare the remained concentrations are all the same . The undiluted standard serves as the highest standard serves as the highest standard 200ng /ml the reference standard and sample diluent serve as the zero ng/ml (this serial dilution in case of total IgE), while in case of IL – 4 : the recommended concentrations are as follow : 2000, 1000 , 500, 250 125 62.5 . 31.25 , 0 ng/ml if you want to make standard solution at the concentration of 1000ng/ml , you should take 0.5 ml standard at 2000 ng/ml , and it to an EP tube with 0.5 ml reference standard and sample diluent , and mix it . procedures to prepare the remained concentrations are all the same . the undiluted standard serves as the highest standard serves as the highest standard 2000ng /ml the reference standard and sample diluent serve as the zero ng/ml, and in case of IFN the recommended concentrations are as

follows : 1000 , 500 , 250 , 125 , 62.5 , 31.25 , 15.63 , 0 ng/ml . if you want to make standard solution at the concetraion of 500 ng/ml , you should take 0.5 ml standard at 1000 ng/ml , and it to an EP tube with 0.5 ml reference standard and sample diluent , and mix it . Procedures to prepare the remained concentrations are all the same . the undiluted standard serves as the highest standard serves as the highest standard 1000ng /ml the reference standard and sample diluents serve as the zero ng/ml .

3 – Biotinylated detection Ab :- calculate the required amount before experiment (100µl / well) in actual preparation , you should prepare 100 – 200 µl more centrifuge the stock tube before use , dilute the concentrated biotinylated detection Ab to the working concentration using biotinylated detection Ab diluent .

4 – Concentrated HRP conjugate

Calculate the required amount before experiment 100 µl/ well in actual preparation , you should prepare 100 – 200 µL. Dilute the concentrated HRP conjugate to the working concentration using concentrated HRP

5 – substrate reagent :- as it is sensitive to light and contaminates, so you should not open the vial until you need it , the need dosage of the reagent can be aspirated with sterilized tips and the unused residual reagent should not be dumped back into the vial again >

6 – Washing procedure :

Automated washer by added 350 µl wash buffer into each well, the interval between injection and suction should be set about 60s

2.6.2 Assay for IFN γ , Total IgE , IL-4 measurement

Bring all reagents and samples to room temperature before use, centrifuge the sample again after thawing before the assay. All the reagents should be mixed thoroughly by gently swirling before pipetting, avoid foaming . It's recommended that all samples and standards be assayed in duplicate.

1. Add Sample: Add 100μL of Standard, Blank, and Samples per well. The blank well is added with Reference Standard & Sample diluent , solutions are added to the bottom of micro ELISA plate well, avoid inside wall touching and foaming as possible. Mix it gently. Cover the plate with sealer provided. Incubate for 90 minutes at 37 ℃ .
2. Biotinylated Detection Ab: Remove the liquid of each well, don't wash. Immediately add 100μL of Biotinylated Detection Ab working solution to each well. Cover with the Plate sealer. Gently tap the plate to ensure thorough mixing. Incubate for 1 hour at 37°C.
3. Wash: Aspirate each well and wash, repeating the process three times. Wash by filling each well with Wash Buffer (approximately 350μL) (a squirt bottle, multi-channel pipette, manifold dispenser or automated washer are needed). Complete removal of liquid at each step is essential. After the last wash, remove remained Wash Buffer by aspirating or decanting. Invert the plate and pat it against thick clean absorbent paper.
4. HRP Conjugate: Add 100μL of HRP Conjugate working solution to each well. Cover with the Plate sealer. Incubate for 30 minutes at 37 C°.
5. Wash :Repeat the wash process for five times as conducted in step 3.
6. Substrate: Add 90μL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for about 15minutes at 37 C°. Protect the

plate from light. The reaction time can be shortened or extended according to the actual color change, but not more than 30minutes.When apparent gradient appeared in standard wells, user should terminate the reaction.

7. Stop: Add 50μL of Stop Solution to each well. Then, the color turns to yellow immediately. The order to add stop solution should be the same as the substrate solution.

8. OD Measurement: Determine the optical density (OD value) of each well at once,using a micro-plate reader set to 450 nm. User should open the micro-plate reader in advance, preheat the instrument, and set the testing parameters.

9. After experiment, put all the unused reagents back into the refrigerator according to the specified storage temperature respectively until their expiry.

2.9 Statistics analysis

Data were translated into a computerized database structure . Statistical analysis were computer assisted using SSPS version 23 . Frequency distribution made at first then CT value , t test , correlation , OD ratio , Roc curve were obtained .

Conclusions and Recommendations

Conclusions

1. Statistically significant increasing in serum IgE, IL-4, IFNγ, FOXP3 and IL-10 in asthmatic patients compared to healthy controls .
2. The risk of having asthma was tested for the presence of T allele of IFNγ 874 gene polymorphism compared to its absence , the risk was increased by 80% but failed short of statistical significance .
3. The optimum cut-off value of serum IgE was ≥ 3.8 iu/ml , IFNγ ≥ 229.8 pg/ml and IL-4 ≥191 pg/ml, also the optimum cut-off value for FOXP3 fold change was ≥2.46 while IL-10 was ≥ 3.74
4. IFNγ 874 A/T (SNP) had no effect on FOXP3, IL-10 fold change , serum IgE, IFNγ and IL-4 concentrations .
5. Differences in measured parameters had no effect on the severity of asthma which measured clinically .
6. Disease duration, Treatment , age groups, residence and type of allergic response had no effect on measured parameters .
7. Gender had no effect on measured parameters except IFNγ was significant higher among male cases compared to females .
8. Vaccination status had statistically significant increasing in FOXP3 , IL-10 , IL-4 but not IgE and IFN γ.
9. There was no important or statistically significant between the three ordered categories of serum IgE with median FOXP3, IL-10 and IFNγ but there was significant gradual increasing of IL-4 .
10. There was no important or statistically significant between the three ordered categories of serum IFNγ with median IL-4 con., but the

median of FOXP3 and IL-10 was significantly higher in lowest IFNγ and lowest among those with highest category of serum IFNγ .

11. Median FOXP3 and IL-10 fold change was obviously higher among asthmatics with highest serum IL-4 category and lowest among those with lowest serum IL-4 categories .

Recommendations

1 – Study of the relationship between T allele of IFNγ gene polymorphism as predisposing factor to other diseases in Iraq.

2 – Studying the risk of having asthma for the presence of T allele of IFNγ 874 gene polymorphism in large population .

3 – Expansive study about the vaccination (immune therapy) to reach novel strategy for elimination of asthma .

4 – Study of another food and non – food allergens and determine its relationship with asthma .

Results and Discussion

Results :

The results presented in this chapter were based on the analysis of 100 Asthma cases and another 100 healthy controls. The age of cases group ranged between 7 and 80 years with a mean of 37.8 years +/- 15.9 years (standard deviation). The age of control group ranged between 14 and 42 years with a mean of 21.8 years +/- 5 years (standard deviation). An equal gender distribution was observed in healthy controls, while females were slightly more frequent in cases (59%) as shown in table (3-1) .

Table (3-1): Description of the study samples by age and gender.

	Study group			
	Healthy controls		Cases (Asthma)	
	N	%	N	%
Gender				
Female	50	50.0	59	59.0
Male	50	50.0	41	41.0
Total	100	100.0	100	100.0
Age (years)				
Range	(14 to 42)		(7 to 80)	
Mean	21.8		37.8	
SD	5		15.9	
SE	0.5		1.6	
N	100		100	

3.1 Case-control differences

As shown in table (3-2), the original (unadjusted) Ct values of the two tested markers were used in exploring case-control differences. The mean Ct value for Foxp3 among cases (28.81) was significantly lower than that of healthy controls (31.89). Similarly, the mean Ct value for IL10 among cases (28.59) was significantly lower than that of healthy controls (30.89), figure (3-1)

Adjustment of the resulting Ct values was done by subtracting the resulting Ct value of the tested marker from that of a control marker known as

"GAPDH" to remove the artefacts in measurements caused by extraneous factors unrelated to Asthma disease effect. The GADPH marker is known to be unaffected by Asthma disease, therefore it can be used as a reference marker to

elicit a possible increase or decrease in the tested marker activity. The resulting adjusted measure is called "ratio" which is measured on a log scale.

Table (3- 2): Case-control differences in FoxP3 and IL10 Ct values

	Study group		
	Healthy controls	**Cases (Asthma)**	**P**
Foxp3 Ct value			<0.001
Range	(30.32 to 33.61)	(28.01 to 30.53)	
Mean	31.89	28.81	
SD	0.67	0.64	
SE	0.067	0.064	
N	100	100	
IL10 Ct value			<0.001
Range	(29.36 to 32.86)	(27.12 to 29.78)	
Mean	30.85	28.59	
SD	0.72	0.59	
SE	0.072	0.059	
N	100	100	

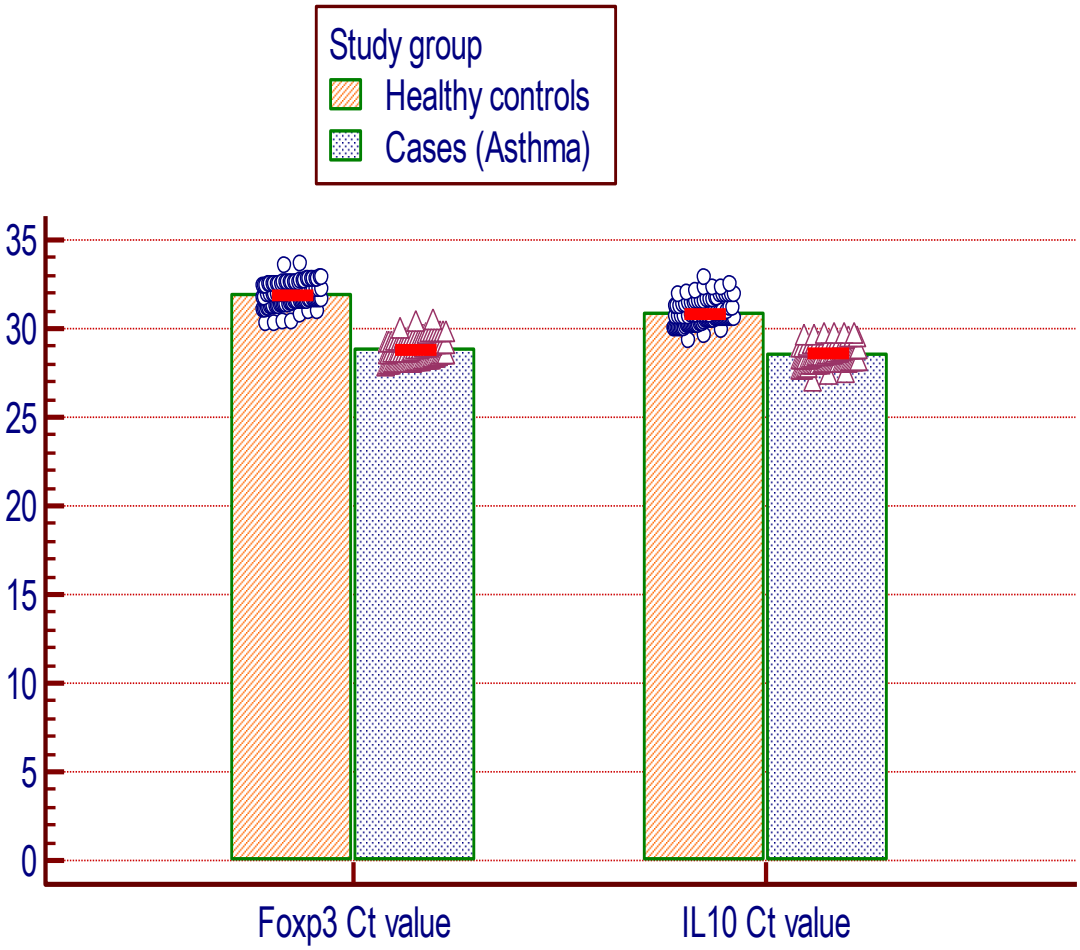


Figure (3-1) : Dot diagram with error bars showing the case-control difference in mean (with its 95% confidence interval) Foxp3 and IL10 Ct value.

As shown in table (3-3), the mean Foxp3 ratio among cases (2.83) was significantly higher than that of healthy controls (-0.25). Similarly, the mean Foxp3 ratio among cases (3.06) was significantly higher than that of healthy controls (0.79), figure (3-2).

The adjusted ratio measures of the two markers were finally presented as a "fold change" measurements. The fold change gives a clear estimate of the amount of marker activation since it is measured in ordinary units rather than log units associated with "ratio" values presented earlier. The fold change results from exponentiation of a base value of 2 to the ratio value. i.e. the fold change measure of a tested marker tells exactly how many folds the activity of the tested

marker is increased compared to a control marker (GADPH) known to be unaffected by the disease process.

Table (3-3): Case-control differences in FoxP3 and IL10 adjusted Ct values (results presented as ratio values).

	Study group		
	Healthy controls	**Cases (Asthma)**	**P**
Foxp3 ratio			<0.001
Range	(-2.37 to 2.79)	(0.79 to 5.78)	
Mean	-0.25	2.83	
SD	1.2	1.18	
SE	0.12	0.118	
N	100	100	
IL10 ratio			<0.001
Range	(-1.45 to 3.39)	(1.08 to 6.04)	
Mean	0.79	3.06	
SD	0.97	1.03	
SE	0.097	0.103	
N	100	100	

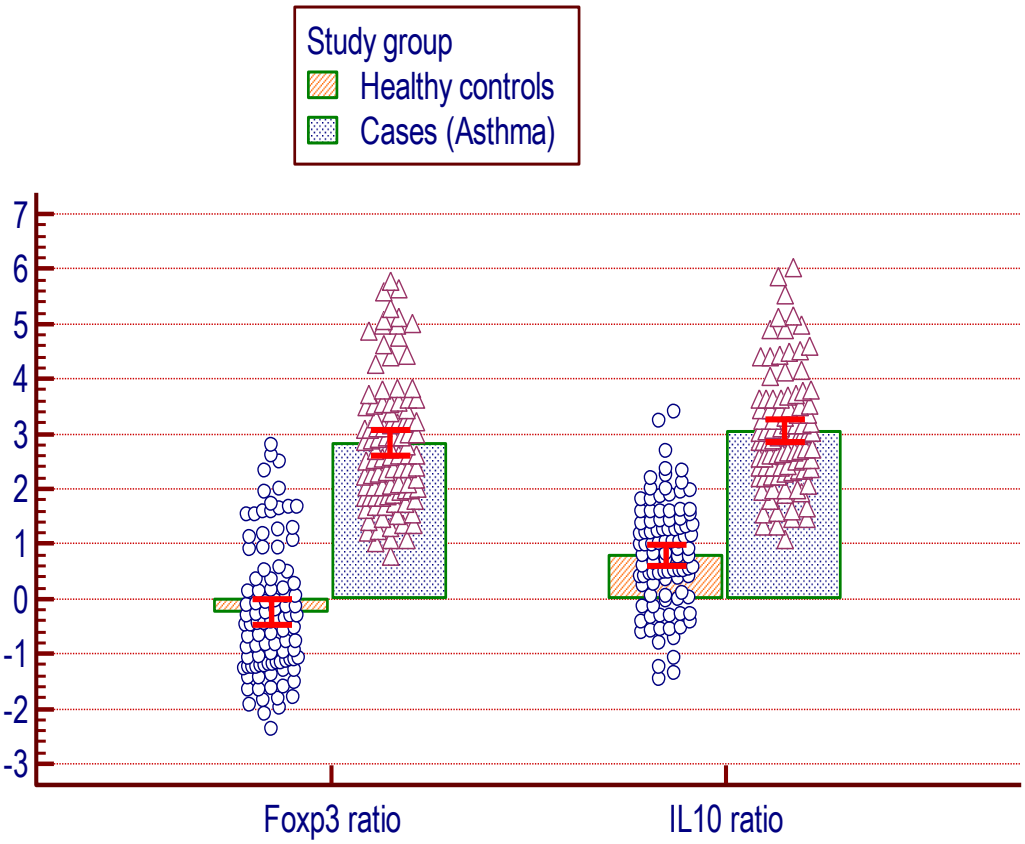


Figure (3-2) : Dot diagram with error bars showing the case-control difference in mean (with its 95% confidence interval) Foxp3 and IL10 ratio.

As shown in table (3-4), the median Foxp3 fold change among cases (6.21) was significantly higher than that of healthy controls (0.735), in our study we found statistically significant increasing of FOXP3 expression in asthmatic patients compared with healthy control and this agreed with Pelaia *etal.,* (2012) who found the same results. And this increasing may be resulted from taking of Glucocorticoides as found by Pelaia *etal.,* (2012) or may be as a result of the effect of immunotherapy in vaccinated asthmatic patients (discussed later in vaccination paragraph) as found by Luzina *etal.,* (2012) and this increasing in the FOXP3 is accompanied by significant increasing in the IFNγ which is characteristically induce the expression of FOXP3 i.e IFNγ has ability to convert CD4+CD25- Treg cells into CD4+CD25+FOXP3+ Treg cells (Zhaojun *etal.,* 2006) .

Table (3-4) : Case-control differences in FoxP3 and IL10 adjusted Ct values (results presented as fold change values).

	Study group		
	Healthy controls	**Cases (Asthma)**	**P**
Foxp3 fold change			<0.001
Range	(0.19 to 6.93)	(1.73 to 55.02)	
Median	0.735	6.21	
Inter-quartile range	(0.44 to 1.335)	(3.84 to 11.555)	
N	100	100	
Mean Rank=	54.2	146.9	
IL10 fold change			<0.001
Range	(0.37 to 10.5)	(2.12 to 65.89)	
Median	1.775	7.905	
Inter-quartile range	(1.05 to 2.685)	(5.2 to 12.09)	
N	100	100	
Mean Rank=	54.9	146.1	

Similarly, the median IL-10 fold change among cases (7.905) was significantly higher than that of healthy controls (1.775), figure(3-3), in this study also found significant increasing of IL-10 in asthmatic patients compared with control group and this agreed with other study by Schaller, (2010) who found increased number of IL-10 mRNA positive cells in BAL and higher IL-10 secretion by macrophages have been shown in asthmatics when compared

with controls , other study found that the number of IL-10 mRNA cells were slight increased in sputum samples of allergic asthmatics when compared with controls (Yu *etal.,* 2006) . Makela *etal.*,(2000) showed higher production of IL-10 in induced sputum samples of asthmatics during acute exacerbation while disagreed with many studies explained significant decreasing of IL-10 in asthmatic patients compared with control groups as Lee *etal.*, (2002) ; Hara *etal.*, (2001) and Borish *etal.*, (2004) .

IL-10 is a pleotropic cytokine that has the potential to down regulate both Th1 and Th2 driven inflammatory processes , whether IL-10 expression is changed in asthma is uncertain, in some studies increased and in other decreased so that after these conflicting results, the findings of this study did not reveal different IL-10 levels in asthmatics over controls also between mild , moderate and severe asthmatics . There was also no effect of IL-10 level on clinical severity and this agreed with Torrego *etal.*, (2004) .

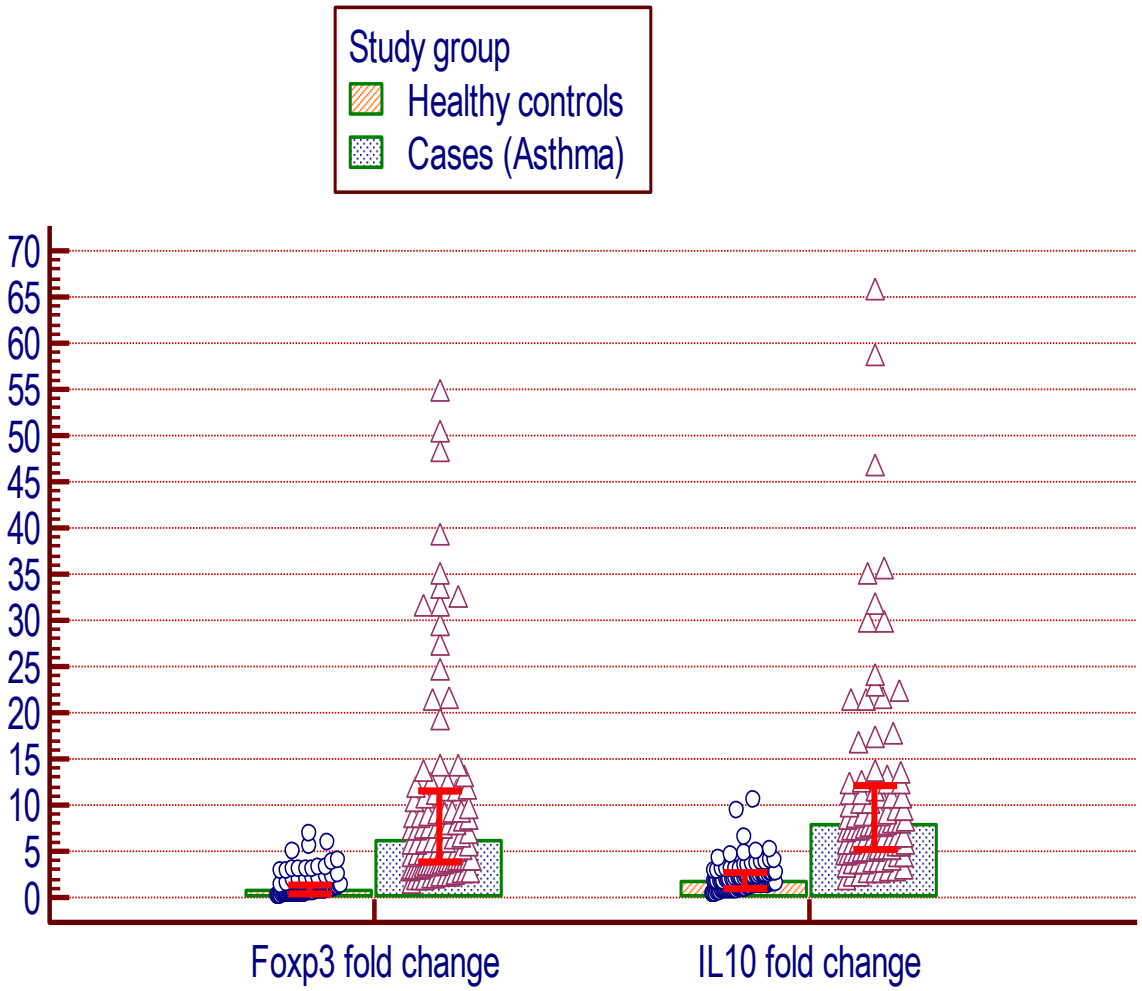


Figure (3-3) : Dot diagram with error bars showing the case-control difference in median (with its interquartile range) Foxp3 and IL10 fold change

Table (3-5) show the median serum IgE which was significantly higher among cases with Asthma (27 iu/ml) compared to healthy controls (1.65 iu/ml), figure (3-4), it was significantly higher in asthmatic patients than healthy control group and this result matching with Hussein *etal.*, (2006) and Kawai *etal.*, (2013) who reported a significant correlation between asthma and other allergic diseases with IgE level, Johansson and Lundahl, (2001) found that the total serum IgE in allergic conjunctivitis was higher than that of control, in addition, Staikuniene and Sakalauskas, (2003) have reported an elevation of serum IgE level by a more than 2 – fold in allergic skin patients . Cline and

Burrows, (1989) reported the highest total IgE levels were seen in the asthmatic patients at age group 8-14 years, however in the present study, no significant relationship of serum Total IgE levels with any age group was observed. Interestingly no association was found with either sex, though many studies have reported males to have raised IgE levels (Aceves and Broide, 2008).

Table (3-5) : Case-control differences in mean of selected serum measurements.

	Study group		
	Healthy controls	**Cases (Asthma)**	**P**
Serum IgE conc (in/ml)			<0.001
Range	(0.2 to 3.8)	(3.8 to 215.8)	
Median	1.65	27	
Inter-quartile range	(1.2 to 2)	(16.15 to 39.65)	
N	100	100	
Mean Rank=	50.5	150.5	
Serum IFN gamma conc (pg/ml)			<0.001
Range	(10 to 202)	(154.5 to 1402)	
Median	147	464	
Inter-quartile range	(119 to 163.25)	(347.25 to 692)	
N	100	100	
Mean Rank=	50.8	150.2	
Serum IL4 conc (pg/ml)			<0.001
Range	(10 to 78)	(304 to 2390)	
Median	33.5	644.5	
Inter-quartile range	(22 to 45)	(527 to 700)	
N	100	100	
Mean Rank=	50.5	150.5	

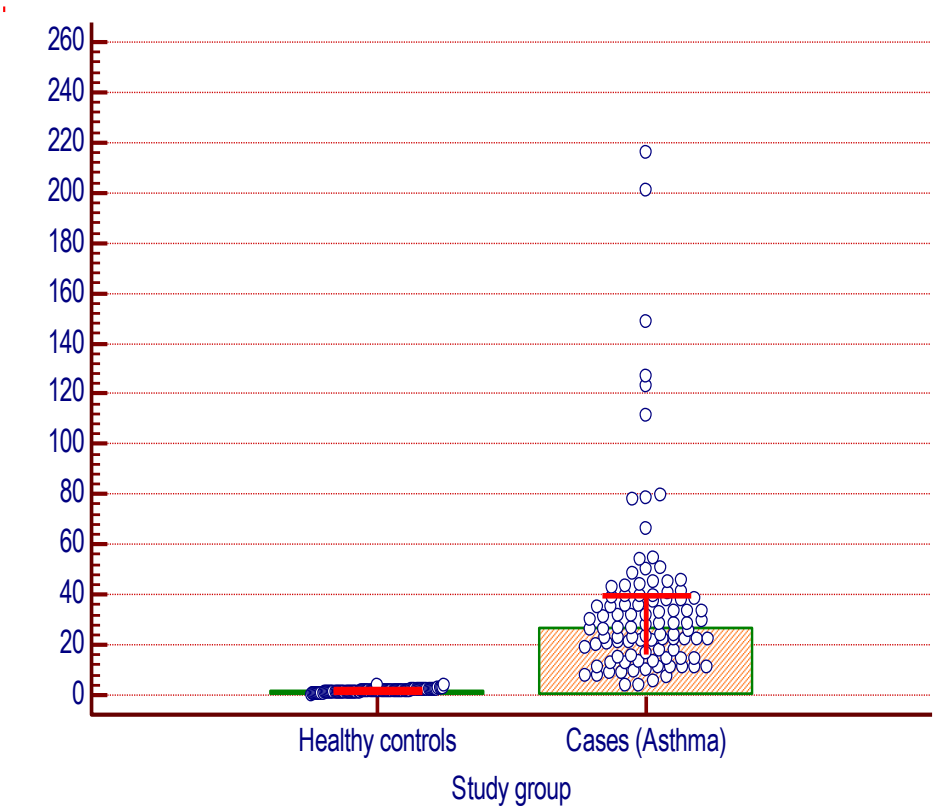


Figure (3-4) : Dot diagram with error bars showing the case-control difference in median (with its interquartile range) serum IgE.

Our findings have referred to significant increase in eosinophil count in asthmatic patients (580±1.76) compared with healthy control (180±1.41) (P 0.001) , eosinophil count /ml in mild asthmatic cases (340) , in moderate (485) while in severe (665) (P.001) also found eosinophil count / ml in case of asthmatic patients carrying AA was (645), in case of AT(522) and in case of TT was (402) (P.001) and this result agreed with Nurse *etal.,* (2012) who found polymorphism didn't cause significant increasing in eosinophil count in asthmatic patients . This study corresponding with Dewson *etal.,* (2001) and Sudha *etal.,* (2013) who found significant increasing in eosinophil count in asthmatic patients peripheral blood , other studies found significant increasing in eosinophil count in both allergic dermatitis and allergic conjunctivitis when compared with control group as found by Akuthota *etal.,* (2011) who found

statically differences in peripheral blood eosinophil count and this mismatch with Winther *etal.,* (2014) who found no statistically differences in eosinophil count in allergic diseases in general and this mismatch may be attributed to differences in environment , population or season in which these studies conducted (Lara *etal.,* 2010) . In clinical practices, the peripheral blood eosinophil counts are widely used to demonstrate the allergic etiology of disease , to monitor its clinical course and to address the choice of therapy (Simon *etal.*, 2001), therefore , peripheral blood eosinophil count can be useful for observing the association of host factors and environmental determinants as indicators of allergy prevalence (Ferreira *etal.,* 1998) , also there was significant correlation between peripheral blood eosinophil count and total IgE levels in asthmatic patients (P 0.001) and this consistent with A Japanese study conducted by Yoshizawa *etal.,* (2002) to evaluate the role of eosinophil in allergic diseases by correlating eosinophil count and IgE level and revealed a positive correlation between the number of peripheral blood eosinophils and IgE level and matched with Hussein *etal.,* (2011) who reported a positive correlation between the two parameters.

Eosinophils regulate the immune response through direct effects on T – cell activities . Both Th1 and Th2 cytokines generation by CD4+Tcells are also influenced by eosinophils . Eosinophils regulate the allergen – dependent Th2 pulmonary immune responses mediated by denderitic cells and T lymphocytes as well as suppress Th1 responses . And Eosinophils only release their granules proteins with cytotoxic , immunological and remodeling – promoting properties when they reach the target organ , in this case, the lungs showing that these cells have basically local effects on inflammation (Blease *etal.,* 2000) . Total Eosinophil counts reflect asthmatics activity and are useful for early detection of exacerbations , Schyder *etal.,* (2002) have demonstrated that there is strong association between Eosinophil count in peripheral blood and airway

hyperresponssiveness .

Clinically , the quantity of Eosinophil in the airways and sputum is directly related to the degree of airway hyperresponssiveness (Adamko *etal.,* 1999). The evidence supporting an important role for the Eosinophils in causing allergic disease has lead to a number of pharmaceutical companies developing specific anti IL-5 therapies and this gives a novel strategies for asthma and other allergic conditions therapy .

Similarly, the median serum IFN gamma was significantly higher among cases with Asthma (464 pg/ml) compared to healthy controls (147 pg/ml), figure (3 – 5) and this consistent with (Yoshimura *etal.,* 2007) who found significant increasing in IFNγ in asthmatic patients and disagreed with numerous researches which proved that IFNγ decrease in case of asthma . IFNγ known as Th1 cytokine which its releasing lead to suppression of Th2 cytokines which are chiefly responsible for allergic status including asthma, in this study this significant increasing of IFNγ in asthmatic patients may be due to several contributors; it may or may not be resulted from effect of IFNγ polymorphism as proved by (Pestka, 2007) , another opinion IFNγ do not produced by Th1 cells only , there are many different cells take part in this process , also, increasing of IFNγ may be due to presence of infections, or, increased IFNγ in case of polymorphism may be its non functional as proved by (Medzhitov, 2007) which result that the Th2 cytokines not suppressed by this IFNγ and remain raised .

However, Grewe *etal.,* 2014 stated that IFNγ, but not IL-4, is correlated with the clinical severity of atopic dermatitis , and this may be related to the capacity of IFN γ to enhance eosinophil viability and activate vascular endothelial molecules, which in turn increases infiltration by eosinophil and induces atopic dermatitis .

Asthma is a complex of different difficult events in which many different factors take part in this process not only mast , basophil cells and IgE but another unknown factors and cells so that, we cannot fully proved mechanisms

of asthma and the prophylaxis , prevention and treatment are not fully understood .

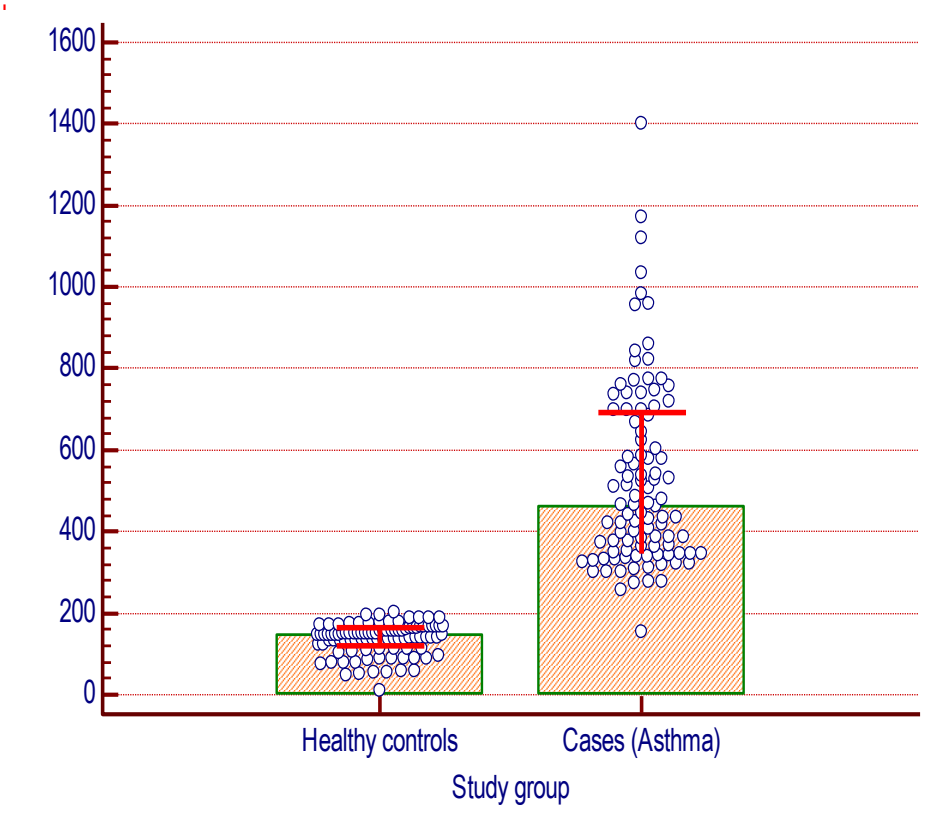


Figure (3-5) : Dot diagram with error bars showing the case-control difference in median (with its interquartile range) serum IFN gamma.

In addition, the median serum IL4 was significantly higher among cases with Asthma (464 pg/ml) compared to healthy controls (147 pg/ml), figure (3-6) , In this study we found statistically significant increasing in IL-4 titer in asthmatic patients and this agreed with (Schnyder *etal.*, 2002) who found the same result and this increasing can be explained that the IL-4 is necessary for

differentiation of naïve CD+ T cells within the Th2 subpopulation secreting IL-4 , IL-5 , IL-6 , IL-10 and IL-13 although IL-4 induce IgE synthesis and enables the immediate type of hypersensitivity reactions, IL-4 is critical in isotype switching from IgM to IgE which responsible for classic allergy and implicated in the pathophysiology of asthma , expression of VCAM-1 on endothelial cells and for inducing the differentiation of Th2 cells, production of IL-5 which is essential for the differentiation of eosinophils and transmigration across

endothelium and mucus secretion (Miyazaki *etal.,* 2006). IL-4 is of critical importance in the differentiation of Th2 cells and is therefore an "upstream" cytokine that is an attractive therapeutic target in the treatment of atopic diseases (Cuvelier and Patel, 2001). Excessive IL-4 production by Th2 cells has been associated with elevated IgE production and allergy (Cuvelier and Patel, 2001).

Our observed correlation between high titers of IgE and IL-4 in asthmatics is compatible with the hypothesis which explain that the maintenance of elevated IgE synthesis in these patients is at least partly IL-4 dependent (Ding *etal.*, 2013).

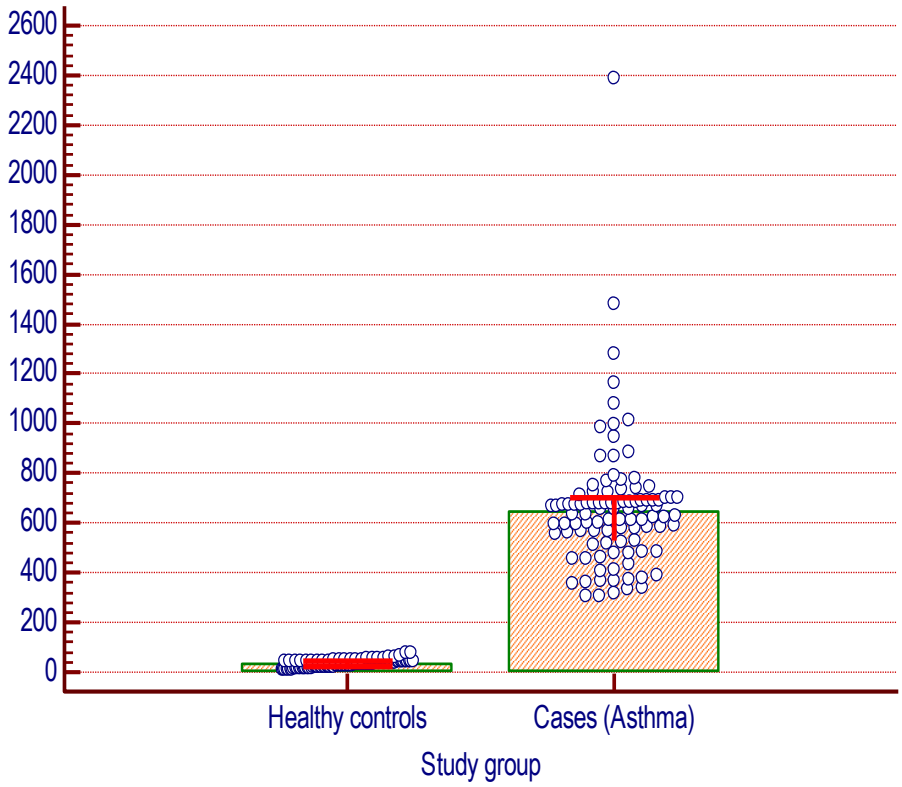


Figure (3-6) : Dot diagram with error bars showing the case-control difference in median (with its interquartile range) serum IL4.

3.2 IFN-gamma +874 gene Polymorphism and risk of Asthma

As shown in table (3-6), the AA genotype was the least frequent genotype among cases with Asthma, therefore, it would be used as the reference category for the other two genotypes to compare the risk of having Asthma because its (AA) wild gene. The risk of having Asthma was increased by 67% in the presence of TA gene compared to the reference genotype (AA). The observed increase in risk, however failed to reach the level of statistical significance, and this is may be due to small size of our population . Similarly, the risk of having Asthma is increased by 2 times in the presence of TT gene compared to the reference genotype (AA). The observed increase in risk also failed to reach the level of statistical significance also may be due to small studied population, figure (3-7) .

Table (3-6) : Case-control difference in IFN-gamma +874 gene Polymorphism.

	Study group						
	Healthy controls		Cases (Asthma)				95% Confidence
	N	%	N	%	P	OR	interval OR
IFN-gamma +874 gene Polymorphism							
AA	30	31.3	20	20.2	Reference		
TA	43	44.8	48	48.5	0.15[NS]	1.67	(0.83 to 3.37)
TT	23	24.0	31	31.3	0.08[NS]	2	(0.93 to 4.42)
Total	96	100.0	99	100.0			

P (Chi-square) = 0.18[NS]

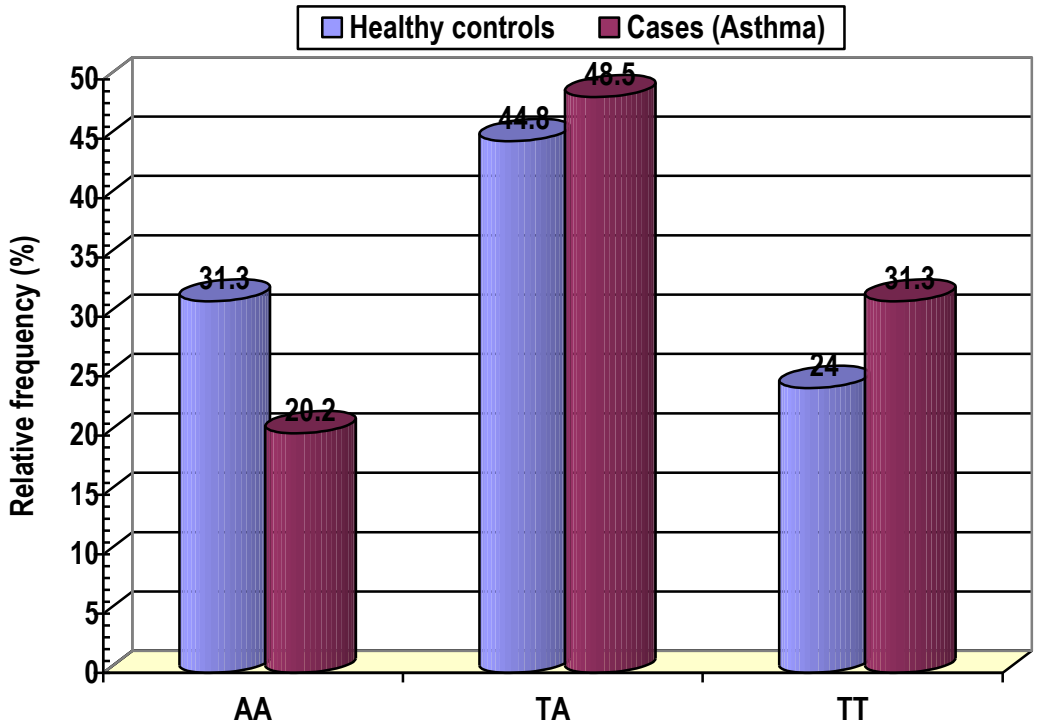


Figure (3-7) : bar chart showing the case-control differences in IFN-gamma +874 genotype frequencies.

The risk of having Asthma was tested for the presence of T allele of IFN-gamma +874 gene Polymorphism compared to its absence. It was increased by 80%, but failed short of statistical significance, table (3-7) and figure (3-8). In the current study we found that, the frequency of allele T 874 of INF- ɤ gene was greater (but not significant) in allergic patients than in control

subjects, this is consistent with the finding of other studies; Hussein *etal.,* (2011) who found the same results. Grewe *etal.,* (2014) stated that IFN-ɤ gene polymorphism is correlated with the allergic skin diseases (his study was conducted in allergic skin diseases only), they mentioned that the correlation may be related to the capacity of IFN-ɤ to enhance Eosinophil viability and activate vascular endothelial molecules, which in turn increases infiltration by eosinophils and induces allergic diseases.

More ever Hoffmann *etal.,* (2002) have conducted similar study on 329 normal volunteers and patients, they reported no association between allergic disease and IFN-ɤ alleles.

From our observations on T allele (AT,TT), the polymorphism may act as predisposing factor to severity of clinical sings in the patients suffering from "asthma " as within this study we found some cases carrying AT, or TT having severe clinical signs accompanied with huge titer of both IL-4 (1282 , 1480 , 2390) and IgE (127 , 200 , 215), refer to table 11, but its not reach to significant statistically because of small studied population . So that IFNγ 874 A/T (SNP) may be considered as predisposing factor to severity of clinical sings in asthma and there are many studies about IFNγ 874 A/T (SNP) and its relationship with other studies as follow : Hussein *etal.,* (2009) and Nicklas *etal.,* (2014) proved that IFNγ 874 A/T (SNP) contribute to susceptibility to atopic diseases by decreasing the amount of IFN γ. Other study from China reported a significant association of IFNγ 874 A/T (SNP) and severe acute respiratory syndrome (Chong *etal.,* 2006).

One study found a significant association between IFNγ 874 A/T (SNP) and Systemic Lupus Erythematosus suggesting that elevated IFN γ is associated with increased susceptibility to Systemic Lupus Erythematosus (Kim *etal.,* 2010).

Lai *etal.,* (2005) reported that IFNγ 874 A/T (SNP) is associated with individual susceptibility to Cervical carcinogenesis . Other study found a

significant association of TT genotype of IFNγ 874 A/T (SNP) and ischemic stroke in south Indian population (Rich *etal.,* 2008) .

Feher *etal.,* (2010) couldn't find any association between IFNγ 874 A/T (SNP) and Alzheimer disease .

Al-Zubadi, (2013) found in him study on T.B patients that the T allele has a protective role against T.B and individuals carrying TT genotype have a prophylaxis against T.B while AA individuals at risk for T.B infection .

This discrepancy could be due to differences in population and age groups; in other words, each analysis may identify the allele or haplotype responsible for the phenotype in that specific population .

From this study may concluded that the IFNγ 874 A/T (SNP) increase susceptibility to asthma and the identification of variants of IFNγ gene and their role in the development of atopic diseases and asthma provides a focus for the development of novel diagnostic and therapeutic strategies .

Table (3-7) : Case-control difference in relative frequency of T allele IFN-gamma +874 gene polymorphism

	Study group						
	Healthy controls		Cases (Asthma)				95% Confidence
	N	%	N	%	P	OR	interval OR
T allele (IFN-gamma +874 gene Polymorphism)							
Negative	30	31.3	20	20.2	Reference		
Positive	66	68.8	79	79.8	0.08[NS]	1.8	(0.93 to 3.45)
Total	96	100	99	100			

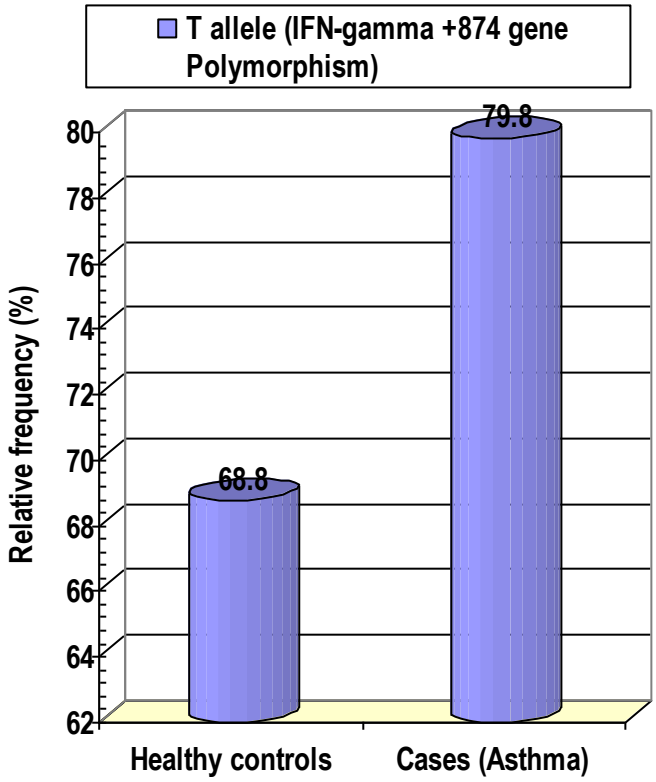


Figure (3-8) : bar chart showing the case-control differences in T allele (IFN-gamma +874 gene Polymorphism) frequency.

3.3 The Diagnostic values of the tested parameters in Asthma :-

The performance of adjusted Ct values (fold change) for Foxp3 and IL4 was compared to that serum IgE, IL4 and IFN gamma in the context of diagnosis of Asthma cases compared to healthy controls. Serum IgE and serum IL4 concentration were associated with a perfect test for diagnosis of Asthma (AUROC=1), followed closely by serum IFN gamma ceoncentration (AUROC = 0.997). The third rank in this list was occupied by Foxp3 and IL10 fold change (AUROC = 0.964 and 0.956 respectively), which provides an excellent test for differentiating between Asthma cases and healthy controls, table (3-8) and figure (3-9) .

Table (3-8) : The area under ROC curve (AUROC) for selected measurements when used as test to diagnosing Asthma cases differentiating them from healthy controls.

	AUROC	P
Serum IgE conc.	1.000	<0.001
Serum IL4 conc.	1.000	<0.001
Serum IFN gamma conc.	0.997	<0.001
Foxp3 fold change	0.964	<0.001
IL10 fold change	0.956	<0.001

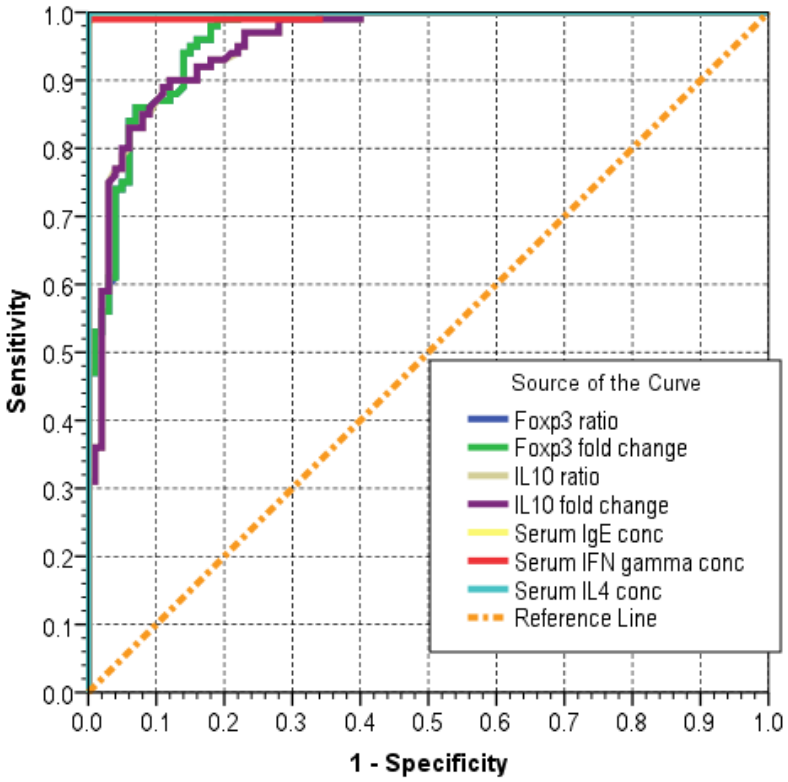
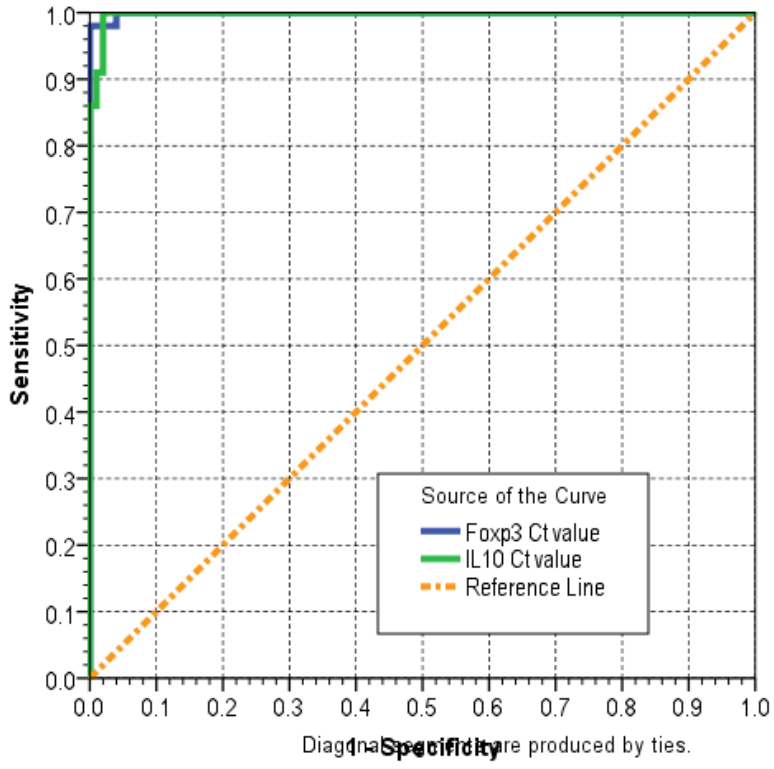


Figure (3-9) : ROC curve showing the test performance of selected quantitative measures in diagnosing Asthma cases differentiating them from healthy controls.



As shown in table (3-9), the optimum cut-off value for serum IgE was ≥3.8 iu/ml, that of IFN gamma was ≥229.8 pg/ml and that for serum IL4 was ≥191 pg/ml. A positive test at each of these optimum cut-off values associated with a perfect (or almost perfect) sensitivity, specificity and accuracy. A positive test (a person obtaining a serum value of ≥ the optimum cut-off value for any of these tests) would be 100% (or almost 100%) diagnostic for the presence of Asthma in any clinical context. A negative test on the other hand would exclude a possible diagnosis of Asthma with 100% confidence in any clinical context (under any pretest probability).

The optimum cut-off value for Foxp3 fold change was ≥2.46, which is associated with the most accurate test performance (90% accuracy). A positive test (obtaining a fold change ≥2.46 for a tested individual) at this optimum cut-off would establish a possible diagnosis of Asthma with 87% confidence level in a clinical context with equal odds of having Asthma (pretest probability =50%). The confidence level in a positive test is increased to 98.4% in a clinical context

were the disease is highly probable (pre-test probability=90%). A negative test on the other hand would exclude a possible diagnosis of Asthma with 99.2% confidence in a clinical context were Asthma is of low probability (pretest probability=10%). Raising the cut-off value of Foxp3 fold change to ≥6.98 results in a highly specific (100%) test. A positive test (obtaining a fold change 7 or more for a tested individual) at this cut-off value would establish a possible diagnosis of Asthma with 100% confidence in any clinical context. Lowering the cut-off value of Foxp3 fold change to ≥1.61 results in a highly sensitive (100%) test. A negative test (obtaining a fold change < 1.61 for a tested individual) would exclude a possible diagnosis of Asthma with 100% confidence in any clinical context, table (3-9) .

The optimum cut-off value for IL10 fold change was ≥3.74, which is associated with the most accurate test performance (89% accuracy). A positive test (obtaining a fold change ≥3.74 for a tested individual) at this optimum cut-off would establish a possible diagnosis of Asthma with 88.2% confidence level in a clinical context with equal odds of having Asthma (pretest probability =50%). The confidence level in a positive test is increased to 98.5% in a clinical context were the disease is highly probable (pre-test probability=90%). A negative test on the other hand would exclude a possible diagnosis of Asthma with 98.8% confidence in a clinical context were Asthma is of low probability (pretest probability=10%). Raising the cut-off value of Foxp3 fold change to ≥10.72 results in a highly specific (100%) test. A positive test (obtaining a fold change 10.72 or more for a tested individual) at this cut-off value would establish a possible diagnosis of Asthma with 100% confidence in any clinical context. Lowering the cut-off value of Foxp3 fold change to ≥2.09 results in a highly sensitive (100%) test. A negative test (obtaining a fold change <2.09 for a tested individual) would exclude a possible diagnosis of Asthma with 100% confidence in any clinical context.

Table (3-9) : Validity parameters for selected measurements when used as test to diagnosing Asthma cases differentiating them from healthy controls.

Positive if ≥ cut-off value	Sensitivity	Specificity	Accuracy	PPV at pre-test probability = 50%	PPV at pre-test probability = 90%	NPV at pre-test probability = 10%
Foxp3 fold change						
1.61 (Highest sensitivity)	100.0	78.0	89.0	82.0	97.6	100.0
2.46 (Optimum cut-off)	94.0	86.0	90.0	87.0	98.4	99.2
6.98 (Highest specificity)	47.0	100.0	73.5	100.0	100.0	94.4
IL10 fold change						
2.09 (Highest sensitivity)	100.0	60.0	80.0	71.4	95.7	100.0
3.74 (Optimum cut-off)	90.0	88.0	89.0	88.2	98.5	98.8
10.72 (Highest specificity)	31.0	100.0	65.5	100.0	100.0	92.9
Serum IgE conc						
3.8 (Optimum cut-off)	100.0	99.0	99.5	99.0	99.9	100.0
Serum IFN gamma conc						
229.8 (Optimum cut-off)	99.0	100.0	99.5	100.0	100.0	99.9
Serum IL4 conc						
191.0 (Optimum cut-off)	100.0	100.0	100.0	100.0	100.0	100.0

3.4 Factors associated with outcome measurements among cases group

Five outcome (dependent) variables were tested among cases with Asthma, namely: Foxp3 fold change, IL10 fold change, Serum IgE conc, Serum IFN gamma conc and Serum IL4 conc. The possible changes in average value of these measurements against selected explanatory variables were explored.

3.4.1 IFN-gamma +874 Polymorphism

As shown in table (3-10), there were no obvious or statistically significant differences in median Foxp3 fold change and IL10 fold change between the three gene morphologies of IFN-gamma +874. Also in the current study we didn't find significant correlation between IFNγ 874 A/T (SNP) and IgE level and this agreed with Ohly, (2006) who revealed that there was no significant association between the IFNγ 874 A/T (SNP) and IgE level in atopic German newborns, while disagreed with Hussein *etal.*, (2011) who reported significant

association between IFNγ 874 A/T (SNP) with IgE level in atopic patients . There were no obvious or statistically significant differences in median Serum IFN gamma conc and Serum IL4 conc between the three gene morphologies of IFN-gamma +874.

Table (3-10) : the difference in median of selected outcome measurements between the three genotypes IFN-gamma +874 gene Polymorphism among Asthma cases.

	IFN-gamma +874 Polymorphism			
	AA	**TA**	**TT**	**P**
Foxp3 fold change				0.39[NS]
Range	(2.11 to 55.02)	(2.07 to 35.13)	(1.73 to 50.56)	
Median	7.8	5.28	6.7	
Inter-quartile range	(3.25 to 13.085)	(3.62 to 9.205)	(4.59 to 13.33)	
N	20	48	31	
Mean Rank=	52.9	45.9	54.4	
IL10 fold change				0.22[NS]
Range	(2.55 to 65.89)	(2.12 to 35.13)	(2.81 to 58.78)	
Median	8.035	6.47	8.75	
Inter-quartile range	(5.55 to 12.715)	(4.855 to 11.095)	(5.91 to 13.33)	
N	20	48	31	
Mean Rank=	53.3	44.9	55.8	
Serum IgE conc				0.16[NS]
Range	(7.7 to 200.8)	(3.8 to 215.8)	(3.8 to 148.5)	
Median	35.3	24.65	24	
Inter-quartile range	(22.1 to 44.4)	(14.55 to 35.35)	(11.2 to 44.9)	
N	20	48	31	
Mean Rank=	60.7	46.2	49	
Serum IFN gamma conc				0.24[NS]
Range	(278.5 to 1402)	(154.5 to 1119)	(257.5 to 1173)	
Median	392.25	473	506.5	
Inter-quartile range	(340.25 to 535.25)	(350 to 700.25)	(377 to 740)	
N	20	48	31	
Mean Rank=	40.6	51.2	54.2	
Serum IL4 conc				0.38[NS]
Range	(305 to 1165)	(319 to 2390)	(304 to 1282)	
Median	619	640	672	
Inter-quartile range	(447.5 to 672)	(498.5 to 697)	(570 to 736)	
N	20	48	31	
Mean Rank=	43.9	49.2	55.2	

3.4.2 Disease severity

As shown in table (3-11), there were no obvious or statistically significant differences in median Foxp3 fold change, IL10 fold change, Serum IgE conc, Serum IFN gamma conc and Serum IL4 conc between the three clinical severity grades of Asthm. In addition, there were no obvious or statistically significant trend (linear correlation) between these measurements and disease duration.

Table (3-11) : the difference in median of selected outcome measurements between the three disease severity categories among Asthma cases.

	Disease severity			
	mild	**moderate**	**severe**	**P**
Foxp3 fold change				0.84[NS]
Range	(1.73 to 55.02)	(2.57 to 35.13)	(2.07 to 50.56)	
Median	6.19	9.825	6.15	
Inter-quartile range	(4.14 to 9.45)	(3.61 to 11.85)	(3.84 to 11.76)	
N	34	10	56	
Mean Rank=	48.9	55	50.7	
r=0.021 P=0.83[NS]				
IL10 fold change				0.66[NS]
Range	(2.8 to 65.89)	(2.55 to 35.13)	(2.12 to 58.78)	
Median	6.995	8.51	8.185	
Inter-quartile range	(5.35 to 9.32)	(5.33 to 21.66)	(4.855 to 12.47)	
N	34	10	56	
Mean Rank=	47.1	55.4	51.7	
r=0.064 P=0.52[NS]				
Serum IgE conc				0.86[NS]
Range	(7.7 to 200.8)	(10.2 to 54.2)	(3.8 to 215.8)	
Median	27.55	21.1	27.7	
Inter-quartile range	(20.3 to 38.7)	(12.7 to 38.2)	(17.7 to 40.05)	
N	34	10	56	
Mean Rank=	50.9	45.7	51.1	
r=0.012 P=0.9[NS]				
Serum IFN gamma conc				0.3[NS]
Range	(275.5 to 1402)	(303 to 700.5)	(154.5 to 1033)	
Median	528	489.5	433.25	
Inter-quartile range	(367 to 740)	(342 to 543)	(344.5 to 612.5)	
N	34	10	56	
Mean Rank=	56.8	46.8	47.4	
r=-0.142 P=0.16[NS]				
Serum IL4 conc				0.77[NS]
Range	(304 to 1480)	(319 to 1080)	(336 to 2390)	
Median	628.5	641.5	663	
Inter-quartile range	(482 to 679)	(584 to 699)	(562.5 to 712.5)	
N	34	10	56	
Mean Rank=	47.6	51	52.2	
r=0.07 P=0.49[NS]				

3.4.3 Disease duration

The duration of Asthma symptoms was classified into three categories: Short (<=1 year), Average (2-9 years) and prolonged (10+ years). There were no obvious or statistically significant differences in median Foxp3 fold change, IL10 fold change, Serum IgE conc, Serum IFN gamma conc and Serum IL4 conc between the three disease duration categories. In addition, there were no obvious or statistically significant trend (linear correlation) between these measurements and disease duration, table (3-12) .

Table (3-12) : the difference in median of selected outcome measurements between the three disease duration categories among Asthma cases .

	Duration of the disease (years)-categories			
	Short (<=1 year)	Average (2-9 years)	prolonged (10+ years)	P
Foxp3 fold change				0.85[NS]
Range	(1.73 to 55.02)	(2.07 to 35.13)	(2.11 to 48.41)	
Median	7.44	6.15	6.085	
Inter-quartile range	(4.32 to 12.52)	(3.63 to 10.375)	(4 to 11.79)	
N	22	40	38	
Mean Rank=	53.4	49	50.4	
r=-0.08 P=0.43[NS]				
IL10 fold change				0.98[NS]
Range	(2.12 to 65.89)	(2.99 to 35.13)	(2.8 to 58.78)	
Median	7.835	8.02	7.625	
Inter-quartile range	(3.98 to 13.05)	(5.23 to 10.96)	(5.33 to 11.71)	
N	22	40	38	
Mean Rank=	51.7	50.1	50.2	
r=-0.062 P=0.54[NS]				
Serum IgE conc				0.89[NS]
Range	(7 to 200.8)	(3.8 to 123.2)	(3.8 to 215.8)	
Median	26.25	24.65	29.35	
Inter-quartile range	(16.6 to 38.7)	(16.3 to 39.65)	(15.7 to 39.7)	
N	22	40	38	
Mean Rank=	48.8	49.8	52.2	
r=0.066 P=0.52[NS]				
Serum IFN gamma conc				0.28[NS]
Range	(257.5 to 1119)	(278.5 to 1402)	(154.5 to 1173)	
Median	433.25	568.75	429.5	
Inter-quartile range	(344.5 to 566)	(360 to 721)	(354.5 to 539)	
N	22	40	38	
Mean Rank=	46.2	56.2	47	
r=0.031 P=0.76[NS]				
Serum IL4 conc				0.97[NS]
Range	(373 to 1480)	(305 to 1080)	(304 to 2390)	
Median	625.5	656.5	663.5	
Inter-quartile range	(564 to 701)	(540.5 to 690)	(479 to 713)	
N	22	40	38	
Mean Rank=	49.8	50.1	51.4	
r=0.015 P=0.88[NS]				

3.4.4 Age

The age of Asthmatic group was classified into four categories. There were no obvious or statistically significant differences in median Foxp3 fold change, IL10 fold change, Serum IgE conc, Serum IFN gamma conc and Serum IL4 conc between the four age groups of Asthmatic cases. In addition, there were no obvious or statistically significant trend (linear correlation) between these measurements and disease duration, table (3-13) .

Table (3-13) : the difference in median of selected outcome measurements between the four age groups of Asthma cases.

	Age group (years)				
	<20	**20-34**	**35-49**	**50+**	**P**
Foxp3 fold change					0.12[NS]
Range	(2.4 to 13.33)	(2.07 to 50.56)	(1.73 to 48.41)	(2.11 to 55.02)	
Median	4.32	7.75	8.3	6.28	
Inter-quartile range	(3.25 to 6.15)	(4.15 to 14.38)	(4 to 19.38)	(3.76 to 9.19)	
N	13	27	34	26	
Mean Rank=	34.9	54.9	55.6	46.9	
r=0.02 P=0.84[NS]					
IL10 fold change					0.06[NS]
Range	(2.55 to 13.05)	(2.12 to 46.85)	(2.8 to 58.78)	(2.55 to 65.89)	
Median	5.35	8.15	10.65	7.37	
Inter-quartile range	(5.17 to 8.22)	(6.14 to 11.71)	(5.01 to 21.52)	(5.33 to 9.32)	
N	13	27	34	26	
Mean Rank=	36.3	54.2	58.1	43.8	
r=0.004 P=0.97[NS]					
Serum IgE conc					0.39[NS]
Range	(8 to 38.1)	(7.7 to 148.5)	(3.8 to 215.8)	(5.8 to 200.8)	
Median	27	22.7	31.65	28.6	
Inter-quartile range	(11.1 to 32.7)	(14.2 to 39.6)	(21.3 to 42.7)	(17.7 to 45.6)	
N	13	27	34	26	
Mean Rank=	42.1	45.6	54.6	54.4	
r=0.144 P=0.15[NS]					
Serum IFN gamma conc					0.77[NS]
Range	(275.5 to 1402)	(278.5 to 1119)	(257.5 to 822.5)	(154.5 to 1173)	
Median	525.5	480	440	449	
Inter-quartile range	(362 to 707)	(340.5 to 603)	(342 to 645)	(372 to 748)	
N	13	27	34	26	
Mean Rank=	52.8	50.1	46.8	54.6	
r=-0.03 P=0.77[NS]					
Serum IL4 conc					0.31[NS]
Range	(305 to 692)	(354 to 1480)	(304 to 1080)	(368 to 2390)	
Median	672	633	584.5	673.5	
Inter-quartile range	(598 to 679)	(577 to 722)	(458 to 713)	(584 to 768)	
N	13	27	34	26	
Mean Rank=	49.2	52.2	44	58	
r=0.008 P=0.94[NS]					

3.4.5 Gender

The Asthma cases gender had no obvious or statistically significant differences in median Foxp3 fold change, IL10 fold change, Serum IgE conc, and Serum IL4 conc. Only the median serum IFN gamma conc was significantly higher among male cases compared to females, table (3-14) and this corresponding with many studies proved that the IFN gamma significantly increased in females rather than males as found by La-Grutta *etal.,* 2005; Schmid-Ott *etal.*,2013 ; Trautmann *etal.,* 2000 .

Table (3-14) : the difference in median of selected outcome measurements between male and female gender of Asthma cases.

	Gender		
	Female	**Male**	**P**
Foxp3 fold change			0.28[NS]
Range	(1.73 to 50.56)	(2.11 to 55.02)	
Median	7.43	5.7	
Inter-quartile range	(4.14 to 11.79)	(3.61 to 9.38)	
N	59	41	
Mean Rank=	53.1	46.8	
IL10 fold change			0.24[NS]
Range	(2.55 to 58.78)	(2.12 to 65.89)	
Median	8.15	6.47	
Inter-quartile range	(5.46 to 12.47)	(4.63 to 10.36)	
N	59	41	
Mean Rank=	53.3	46.4	
Serum IgE conc			0.86[NS]
Range	(3.8 to 215.8)	(3.8 to 200.8)	
Median	26.3	27	
Inter-quartile range	(17.7 to 39.7)	(15.7 to 38.1)	
N	59	41	
Mean Rank=	50.9	49.9	
Serum IFN gamma conc			0.027
Range	(257.5 to 1033)	(154.5 to 1402)	
Median	424.5	536	
Inter-quartile range	(342 to 583.5)	(397.5 to 740)	
N	59	41	
Mean Rank=	45.2	58.2	
Serum IL4 conc			0.61[NS]
Range	(304 to 2390)	(305 to 1480)	
Median	624	669	
Inter-quartile range	(564 to 692)	(514 to 713)	
N	59	41	
Mean Rank=	49.3	52.3	

3.4.6 Vaccination status

As shown in table (3-15), there were no obvious or statistically significant differences in Serum IgE conc and Serum IFN gamma conc but median Foxp3 fold change, IL10 fold change and Serum IL4 conc had significant differences between vaccinated and non-vaccinated Asthma cases, as we found non significant decreasing of IgE which may be due to small population size, but from our observations there were an improvement of clinical signs of asthmatic patients and this may be explained that the immunotherapy modulate the immune response leading to produce IgG (IgG4) which appear immediately after allergen entry and neutralize it before its titter increasing and before its binding with IgE which found on mast and basophils . IgE titter remained raised, this agreed with McHugh *etal.,* (2013) who explained the causes by presence many different mechanisms to produce this immunoglobulin , a similar finding that of Nicklas, (2014) who found in British study on 375 asthmatic volunteers that both IgE and IgG4 were increased and proved the activity of immunotherapy and immunomodulation towards IgG4 and improvement of clinical signs . However , other studies question the role of total IgE as a useful indicator of allergic skin disease , Kaliner, (2012) found that 40% of allergic patients had normal total IgE levels . Wüthrich and Schmid-Grendelmeier, (2003) mentioned that the overlap IgE levels made it suggestive but not diagnostic of allergic diseases and explained it by the presence of non IgE mediated inflammatory mechanisms which may play a significant role in the mechanisms of allergic diseases . Nevertheless , IgE – mediated local release of mast cells in atopic areas could lead to acute exacerbations of atopic manifestations after allergen exposure, although this doesn't imply an obligatory role for IFNγ – IgE in pathogenisity of chronic atopic diseases .

The Significant increasing in IL-4 titter found in this study is coincided with previous result about immunotherapy because its responsible for allergic

response at first and secondly it causes immunomodulation towards IgG4 production and this consistent with Takai *etal.,* (2015) .

In case of FOXP3 and IL-10 there is a significant increasing in vaccinated asthmatic patients and after followed the patients data we found an improvement in healthy status (disease severity) and this agreed with Buckner, (2010) ; Sakaguchi *etal.,* (2010) and Miyao *etal.,* (2012) .

Foxp3 is a good marker for determination of Treg cells expression and there is an proved opinion that the number of IL-10 producing Treg cells potentially Tr1 cells may be related to the prevention of asthma exacerbations (Raynor *etal.,* 2012) . The beneficial effects of allergen immunotherapy likely to be mediated through the reduction of allergen induced inflammation and this mechanism till now unknown but one hypothesis that the T lymphocyte orchestrate the activation and differentiation of T – lymphocyte in relation to allergen immunotherapy both allergen specific hypo reactivity and a shift in the cytokine profile of reacting lymphocytes (Th2 to Th1) .

Most studies in the field of immunotherapy focused T reg cells (Tr1 and Th3) which are the most important cells that produce FOXP3 and IL-10 which are responsible for suppressive effect of immunotherapy and immunomodulation towards IgG (IgG4) production and positive feedback to generate Tr1 cells but till now the mechanism unknown . However, if the details of this immunological pathway will be elucidated , novel strategies to improve immunotherapy may become available not for better treatment of allergic diseases but also for efficient treatment of allergic asthma possibly with long – term improvement of disease.

Table (3-15) : the difference in median of selected outcome measurements between vaccinated and non-vaccinated Asthma cases.

	Vaccine		
	Negative	**Positive**	**P**
Foxp3 fold change			0.003
Range	(1.37 to 3.02)	(33.73 to 55.7)	
Median	2.19	18.23	
Inter-quartile range	(3.92 to 11.79)	(3.73 to 10.94)	
N	59	41	
Mean Rank=	50.2	50.9	
IL10 fold change			0.002
Range	(2.12 to 3.89)	(40.55 to 65.02)	
Median	2.94	30.31	
Inter-quartile range	(4.92 to 12.64)	(5.35 to 9.99)	
N	59	41	
Mean Rank=	51.8	48.6	
Serum IgE conc			0.4[NS]
Range	(3.8 to 215.8)	(3.8 to 127.2)	
Median	24	28.6	
Inter-quartile range	(14.3 to 39.7)	(21.9 to 38.2)	
N	59	41	
Mean Rank=	48.4	53.5	
Serum IFN gamma conc			0.65[NS]
Range	(257.5 to 1119)	(154.5 to 1402)	
Median	445.5	480	
Inter-quartile range	(344.5 to 622)	(348 to 700.5)	
N	59	41	
Mean Rank=	49.4	52.1	
Serum IL4 conc			0.001
Range	(319 to 410)	(1012 to 2390)	
Median	128	558	
Inter-quartile range	(515 to 703)	(564 to 684)	
N	59	41	
Mean Rank=	50.4	50.6	

3.4.7 Treatment status

As shown in table (3-16), although the median Foxp3 fold change, IL10 fold change and Serum IgE was slightly higher among treated asthma cases compared to untreated, the difference failed to reach the level of statistical significance. In addition, there were no obvious or statistically significant differences in median Serum IFN gamma conc and Serum IL4 conc between treated and untreated Asthma cases, as we observed at the duration of study and follow up of patients carts and data we found the effect of treatment through the times of drug consumption only and the sings return after withdrawal of drugs and this consistent with other studies which proved that as Karagiannidis *etal.,* (2004) who found the same results.

Table (3-16) : the difference in median of selected outcome measurements between treated and untreated Asthma cases

	Treatment		
	Negative	**Positive**	**P**
Foxp3 fold change			0.16[NS]
Range	(2.07 to 50.56)	(1.73 to 55.02)	
Median	6.005	6.83	
Inter-quartile range	(3.61 to 7.78)	(4 to 12.52)	
N	34	66	
Mean Rank=	44.8	53.4	
IL10 fold change			0.09[NS]
Range	(2.12 to 46.85)	(2.55 to 65.89)	
Median	6.455	8.22	
Inter-quartile range	(4.79 to 8.75)	(5.7 to 12.52)	
N	34	66	
Mean Rank=	43.5	54.1	
Serum IgE conc			0.12[NS]
Range	(5.8 to 123.2)	(3.8 to 215.8)	
Median	22.55	28.25	
Inter-quartile range	(12.6 to 38.7)	(20.8 to 39.7)	
N	34	66	
Mean Rank=	44.3	53.7	
Serum IFN gamma conc			0.09[NS]
Range	(276.5 to 1402)	(154.5 to 1173)	
Median	532	434.5	
Inter-quartile range	(384 to 748)	(345.5 to 587.5)	
N	34	66	
Mean Rank=	57.3	47	
Serum IL4 conc			0.9[NS]
Range	(304 to 1480)	(336 to 2390)	
Median	633	657	
Inter-quartile range	(568 to 693)	(524 to 713)	
N	34	66	
Mean Rank=	51	50.2	

3.4.8 Family history of Asthma

As shown in table (3-17), there were no obvious or statistically significant differences in median Foxp3 fold change, IL10 fold change, Serum IgE conc and Serum IL4 conc between Asthma cases with positive and negative family history. In addition, the median Serum IFN gamma conc was obviously higher among those with positive family history (533.25) compared to those with negative family history (419.25), but the difference observed failed short of statistical significance.

Table (3-17): the difference in median of selected outcome measurements between Asthma cases with positive and negative family history of the disease.

	Family history		
	Negative	**Positive**	**P**
Foxp3 fold change			0.56[NS]
Range	(1.73 to 55.02)	(2.07 to 50.56)	
Median	6.19	6.445	
Inter-quartile range	(3.68 to 10.23)	(4.03 to 12.305)	
N	56	44	
Mean Rank=	49	52.4	
IL10 fold change			0.93[NS]
Range	(2.12 to 65.89)	(2.8 to 58.78)	
Median	7.82	7.93	
Inter-quartile range	(5.305 to 11.14)	(5.09 to 12.495)	
N	56	44	
Mean Rank=	50.3	50.8	
Serum IgE conc			0.69[NS]
Range	(3.8 to 215.8)	(7 to 148.5)	
Median	28.25	26.25	
Inter-quartile range	(17.15 to 40.9)	(14.8 to 39.15)	
N	56	44	
Mean Rank=	51.5	49.2	
Serum IFN gamma conc			0.06[NS]
Range	(154.5 to 1402)	(278.5 to 1173)	
Median	419.25	533.25	
Inter-quartile range	(343.25 to 583.25)	(385.5 to 727.25)	
N	56	44	
Mean Rank=	45.7	56.7	
Serum IL4 conc			0.33[NS]
Range	(305 to 2390)	(304 to 1480)	
Median	613.5	662.5	
Inter-quartile range	(469.5 to 706)	(580 to 696.5)	
N	56	44	
Mean Rank=	48	53.7	

3.4.10 Type of allergic response

As shown in table 3(-19), there were no obvious or statistically significant differences in median Foxp3 fold change, IL10 fold change, Serum IgE conc, Serum IFN gamma conc and Serum IL4 conc between Asthma cases with delayed and rapid inflammatory response.

Table (3-19) : the difference in median of selected outcome measurements between Asthma cases with delayed and rapid response.

	Delayed response		
	rapid	**delayed**	
Foxp3 fold change			0.51[NS]
Range	(1.73 to 50.56)	(2.07 to 55.02)	
Median	6.23	6.19	
Inter-quartile range	(3.61 to 10.94)	(4.14 to 11.79)	
N	49	51	
Mean Rank=	48.5	52.4	
IL10 fold change			0.94[NS]
Range	(2.55 to 46.85)	(2.12 to 65.89)	
Median	7.31	8.15	
Inter-quartile range	(5.46 to 10.94)	(4.73 to 12.47)	
N	49	51	
Mean Rank=	50.3	50.7	
Serum IgE conc			0.17[NS]
Range	(3.8 to 79.8)	(3.8 to 215.8)	
Median	23.7	30.1	
Inter-quartile range	(14.2 to 38.1)	(19 to 41.4)	
N	49	51	
Mean Rank=	46.4	54.4	
Serum IFN gamma conc			0.11[NS]
Range	(275.5 to 1402)	(154.5 to 1119)	
Median	525.5	421.5	
Inter-quartile range	(367 to 707)	(343.5 to 622)	
N	49	51	
Mean Rank=	55.3	45.9	
Serum IL4 conc			0.14[NS]
Range	(304 to 1080)	(336 to 2390)	
Median	613	669	
Inter-quartile range	(482 to 684)	(570 to 724)	
N	49	51	
Mean Rank=	46.2	54.7	

3.4.11 Residence

As shown in table (3-20), there were no obvious or statistically significant differences in median Foxp3 fold change, IL10 fold change, Serum IgE conc, Serum IFN gamma conc and Serum IL4 conc between rural and urban Asthma cases.

Table (3-20) : the difference in median of selected outcome measurements between urban and rural Asthma cases.

	Urban Vs rural residence		
	rural	**urban**	**P**
Foxp3 fold change			0.19[NS]
Range	(2.07 to 55.02)	(1.73 to 35.13)	
Median	7.44	5.8	
Inter-quartile range	(4.21 to 10.65)	(3.48 to 11.73)	
N	50	50	
Mean Rank=	54.3	46.7	
IL10 fold change			0.9[NS]
Range	(2.12 to 65.89)	(2.55 to 35.13)	
Median	8.045	7.59	
Inter-quartile range	(4.73 to 12.47)	(5.35 to 10.94)	
N	50	50	
Mean Rank=	50.9	50.1	
Serum IgE conc			0.37[NS]
Range	(3.8 to 200.8)	(7.7 to 215.8)	
Median	29.35	26.25	
Inter-quartile range	(19 to 41.4)	(14.2 to 38.1)	
N	50	50	
Mean Rank=	53.1	47.9	
Serum IFN gamma conc			0.25[NS]
Range	(154.5 to 1119)	(275.5 to 1402)	
Median	423	528	
Inter-quartile range	(344.5 to 685)	(367 to 699)	
N	50	50	
Mean Rank=	47.1	53.9	
Serum IL4 conc			0.08[NS]
Range	(339 to 2390)	(304 to 1080)	
Median	671.5	623.5	
Inter-quartile range	(557 to 774)	(524 to 679)	
N	50	50	
Mean Rank=	55.6	45.4	

3.6 Association between the five selected outcome measurements among cases with Asthma

As shown in table (3-29), the serum IgE measurements among cases with Asthma were classified into three ordered categories based on quartile method. The median Foxp3 fold change, IL10 fold change, Serum IFN gamma conc showed no important or statistically significant differences between the ordered categories of serum IgE. In addition, there were no obvious linear correlation between serum IgE and each of the remaining tested measurements while there was significant gradual increasing of IL-4 with three ordered categories of the Serum IgE and this corresponding with Hooper, (2013) and this good indicator to agonist effect between IL-4 and IgE production .

As shown in table (3-30), the serum IFN gamma measurements among cases with Asthma were classified into three ordered categories based on quartile method. The median Serum IL4 conc showed no important or statistically significant differences between the ordered categories of serum IFN gamma. In addition, there were no obvious linear correlation between serum IFN gamma and serum IL4.

The median Foxp3 and IL10 fold change was significantly higher among asthmatics in the lowest category of serum IFN gamma and lowest among those in the highest category of serum IFN. In addition there was a statistically significant weak negative linear correlation between serum IFN gamma and each of FoxP3 and IL10 fold change, table (3-30) .

Although the median FoxP3 and IL10 fold change was obviously higher among asthmatics with highest serum IL4 category and lowest among those with lowest serum IL4 categories, the differences observed were not significant statistically. In addition, no significant linear correlation was observed between serum IL4 and each of the fold change measurements, table (3-31) .

Table (3-29) : the difference in median of selected outcome measurements between the ordered categories of serum IgE among Asthma cases.

	Serum IgE conc-cases group-quartiles			
	Lowest (first) quartile (<= 16.2)	**Average (inter-quartile range) 16.3 - 39.7**	**Highest (fourth) quartile (39.8+)**	**P**
Foxp3 fold change				0.13[NS]
Range	(2.13 to 21.66)	(1.73 to 48.41)	(2.46 to 55.02)	
Median	6.11	5.475	10.65	
Inter-quartile range	(4.15 to 8.82)	(3.63 to 9.22)	(3.73 to 27.51)	
N	25	50	25	
Mean Rank=	48.6	46.4	60.5	
r=0.174 P=0.08[NS]				
IL10 fold change				0.08[NS]
Range	(2.81 to 23.04)	(2.55 to 58.78)	(2.12 to 65.89)	
Median	8.15	6.395	10.43	
Inter-quartile range	(6.23 to 12.64)	(4.67 to 10.36)	(6.02 to 17.53)	
N	25	50	25	
Mean Rank=	53.6	44.3	59.8	
r=0.045 P=0.66[NS]				
Serum IFN gamma conc				0.07[NS]
Range	(276.5 to 842.5)	(154.5 to 1402)	(326 to 1173)	
Median	463.5	403.75	543	
Inter-quartile range	(342 to 587.5)	(337.5 to 700)	(434.5 to 668)	
N	25	50	25	
Mean Rank=	48	46	61.9	
r=0.151 P=0.13[NS]				
Serum IL4 conc				0.002
Range	(120 to 182)	(305 to 782)	(1282 to 2390)	
Median	98	413	672	
Inter-quartile range	(109 to 118)	(233 to 684.2)	(870 to 898)	
N	25	50	25	
Mean Rank=	48.2	46.9	60.1	

Table (3-30) : the difference in median of selected outcome measurements between the ordered categories of serum IFN gamma Asthma cases.

	Serum IFN gamma conc-cases group-quartiles			
	Lowest (first) quartile (<= 347.3)	**Average (inter-quartile range) 347.4 - 692.0**	**Highest (fourth) quartile (692.1+)**	**P**
Foxp3 fold change				0.003
Range	(2.13 to 48.41)	(2.11 to 55.02)	(1.73 to 9.38)	
Median	8.51	7.225	4.15	
Inter-quartile range	(5.9 to 14.38)	(3.48 to 12.52)	(3.63 to 6.54)	
N	25	50	25	
Mean Rank=	62.7	52.2	35	
r=-0.364 P<0.001				
IL10 fold change				0.008
Range	(2.8 to 58.78)	(2.55 to 65.89)	(2.12 to 10.36)	
Median	8.63	9.13	6.23	
Inter-quartile range	(6.47 to 21.52)	(5.22 to 13.33)	(4.67 to 7.89)	
N	25	50	25	
Mean Rank=	57.2	54.9	35	
r=-0.237 P=0.018				
Serum IL4 conc				0.28[NS]
Range	(319 to 747)	(336 to 2390)	(304 to 1480)	
Median	613	630.5	677	
Inter-quartile range	(530 to 684)	(515 to 701)	(601 to 742)	
N	25	50	25	
Mean Rank=	45.2	49.5	57.9	
r=0.149 P=0.14[NS]				

Table (3-31) : the difference in median of selected outcome measurements between the ordered categories of serum IL4 among Asthma cases.

	Serum IL4 conc-cases group-quartiles			
	Lowest (first) quartile (<= 527.0)	**Average (inter-quartile range) 527.1 - 700.0**	**Highest (fourth) quartile (700.1+)**	
Foxp3 fold change				0.19[NS]
Range	(2.35 to 32.61)	(2.11 to 50.56)	(1.73 to 55.02)	
Median	5.7	6.085	9.19	
Inter-quartile range	(4 to 9.22)	(3.73 to 9.45)	(4.15 to 27.51)	
N	25	50	25	
Mean Rank=	46.5	48	59.6	
r=0.086 P=0.4[NS]				
IL10 fold change				0.2[NS]
Range	(2.8 to 23.04)	(2.55 to 46.85)	(2.12 to 65.89)	
Median	7.75	7.73	9.67	
Inter-quartile range	(4.63 to 10.43)	(5.33 to 10.94)	(5.82 to 21.55)	
N	25	50	25	
Mean Rank=	45.4	48.6	59.3	
r=0.139 P=0.17[NS]				

الخلاصة

هدفت هذه الدراسة (دراسة حالة - سيطرة) بيان الدور المحتمل للتغايرات الوراثية في مورث (IFNγ) بالنسبة للتغاير في قاعدة نترو جينية واحدة هي T/A في الموقع +874 في الجزء الغير مشفر داخل الجين كونها عامل محتمل للإصابة بمرض الربو .

تم أجراء هذه الدراسة في محافظة بابل ، مركز الحلة التخصصي للحساسية والربو ومركز الرصافة التخصصي للحساسية والربو للفترة من شهر آذار / 2015 لغاية شهر أيلول / 2015 . تم جمع (200) عينة دم وقسمت الى مجموعتين : المجموعة الأولى تضمنت مائة عينة دم تم جمعها من مرضى الربو والمجموعة الثانية تتألف من مائة عينة دم تم جمعها من أشخاص سليمين ظاهريا . وجدت النتائج التالية : هناك زيادة مقبولة أحصائيا في المحتوى المصلي من (IgE) ، (IL-4) و (IFN γ) في مرضى الربو مقارنة مع مجموعة السيطرة الأصحاء . هناك زيادة مقبولة أحصائيا في التعبير الجيني لكل من (FoxP3) ، (IL-10) في مرضى الربو مقارنة مع مجموعة السيطرة الأصحاء . ان خطر حصول مرض الربو تم أختباره من حيث وجود الأليل (T) في حالة تغاير النيوكليوتيد الأحادي للأنترفيرون گاما في القاعدة A/T الموقع ٨٧٤ في مرضى الربو ، نسبة الخطر زادت عن 80 % ولكنها فشلت في الوصول لحالة القبول الإحصائي . ان قيمة حد العتبة للمحتوى المصلي من (IgE) كان أقل من 3.8 وحدة دولية / ملل وللأنترفيرون كاما اقل من 229.8 بيكو غرام / ملل والانترليوكين – 4 اقل من 191 بيكو غرام / ملل بالنسبة لنتائج تقنية ELISA . أما في حالة التعبير الجيني فوجد ان قيمة حد العتبة لمقدار التغيير في (FoxP3) أقل من 2.46 بينما هو اقل من 3.47 بالنسبة (IL-10) . الأختلافات في المعايير المقاسة ليس لها أي تاثير على شدة مرض الربو المقاسة سريريا . مدة المرض ، العمر ، الأقامة والعلاج المستعمل ليس لها أي تأثير على المعايير المقاسة ماعدا الجنس حيث وجد ان هناك زيادة معنوية واضحة في مستوى الكاما انترفيرون في الذكور مقارنة بالأناث . في حالة العلاج المناعي (اللقاح) نلاحظ بانه هناك زيادة معنوية مقبولة احصائيا في حالة التعبير الجيني لكل من (IL-10) و (FoxP3) و (IL-4) ولكن لاتوجد مثل هذه الزيادة في حال (IgE) ، (IFNγ) . بالنسبة لحالات الأصابة الأنفية بالمكورات العنقودية الذهبية هناك تأثير معنوي على مستويات (IL-4 , IFNγ , IgE) ولكن لايوجد تأثير معنوي على مستوى التعبير الجيني (IL-10 , FoxP3) في مرضى الربو مقارنة مع مرضى الربو الغير مصابين بهذه البكتريا . ومن هذا نستنتج أنه لاتوجد علاقة بين حالة تغاير النيوكليوتيد الأحادي للأنترفيرون گاما في القاعدة A/T الموقع ٨٧٤ مع مستويات (IL-4 , IFNγ , IgE , IL-10 , FoxP3) المقاسة في مرضى الربو .