Libyan International Medical University

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Immunoglobulin Isotypes and Complement Components (C3, C4) as a Biomarkers Correlated to Wheezy Children: a Case-Control Study

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Abbreviations

IgE: Immunoglobulin E

IgA: Immunoglobulin A

IgG: Immunoglobulin G

IgM: Immunoglobulin M

C3: Complement 3

C4: Complement 4

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

CBC: Complete blood count

ANOVA: Analysis of Variance

Sig: Significant

In-sig: Insignificant

H0: null hypothesis

n=: number

e=: effected

Abstract

Wheeze chest is a common problem among preschool children, and represents a common disorder characterized by airways obstruction. Almost 30% of children manifest wheeze chest symptoms before the age of three and 50% before the age of six. Their parents report at least one attack within this period.

The major aims were to assess the correlation between the immunoglobulin (IgA, IgM, IgG, and IgE), complement, and the level of eosinophils and development of wheeze chest. And also correlate the level of IgE with the numbers of the attacks per year, age, family history, and eosinophilic count.

A total number of a randomized (n=72) cases were involved in this study, divided into 2 groups, (n=52) patients with recurrent wheezy chest attacks and (n=21) controls. Five milliliters of blood sample were equally collected from all participants.

All patients' patents/relatives were present while withdrawing the blood samples and all of them were informed that this study was for the purpose of a medical research and to fulfill a requirement of under-graduation project, and all signed for approval.

Three out of twelve variables have been found to be significant according to the correlation part in this study. Number of attacks strongly correlated with IgE with a P-value of (P=0.001), as well as the manifestation with a P-value of (P=0.002), while age was weakly correlated with IgE with a P-value of (P=0.005). The other 9 variables in this study were found to be insignificant, correlating with IgE.

The two groups this test was used to determine are the controls (n=21) and the effected individuals (e=52). All thirteen variables were included in the test showing that all are insignificant, except for IgE ($p<0.001$) highly significant, number of attacks ($p<0.001$) highly significant, manifestation ($p<0.001$) highly significant, family history ($p<0.001$) highly significant and IgM ($p=0.059$) significant. Measuring IgE, IgM, and IgA in both control and effected children, approved only for IgE only, while disapproved for IgM, IgA, IgG, C3, C4 and eosinophil since there was no significant differences between patients and controls groups.

Chapter one

Introduction and Literature Review

1.1. Background on wheezing

Wheeze chest is a common problem among preschool children, and represents a common disorder characterized by airways obstruction. Almost 30% of children manifest wheeze chest symptoms before the age of three and 50% before the age of six. Their parents report at least one attack within this period (El-Asheer et al. 2016).Wheeze chest is common throughout early life and childhood. However, it is very rare in neonatal period. (El-Gamaland El-Sayed,2011) ??

Recurrent attacks of wheeze chest have a significant morbidity and have been estimated that about one third of school-age children manifest the symptom during the first 5 years of life. In young children, wheezing is associated with a poor quality of life. (et al. Eur J Pediatr 2015)

When wheezing occurs for the first time, it appears to be triggered by some sort of viral infection. In this condition, the patient will be diagnosed with bronchiolitis if a respiratory distress takes place. Evident of respiratory syncytial virus (RSV) infection will make the diagnosis firmer. Wheezing can be classified into two main categories: unremitting and episodic wheeze. Unremitting wheezing is defined as children with distinct episodes of wheezing with a wide range of intermittent symptoms including wheezing at night or in response to exercise, laughter and cold air. Atopy is hardly ever associated with episodic wheezing and it rarely progresses to asthma later on in life. However, in the very first years of life atopic sensitization is often associated with preschool children who are suffering from unremitting wheezing. Environmental air pollution and prenatal exposure to tobacco smoke are considered to be environmental exposures affecting the growth of the airways. They are also considered to be associated with decreased postnatal lung function and with unremitting preschool wheezing. (Frey and von Mutius 2009)

1.2. Pathophysiology of wheezing

Asthma is a chronic disease that involves inflammation of the pulmonary airways and bronchial hyper-responses that results in the clinical expression of a lower airway obstruction that usually is reversible. Bronchial hyperresponsiveness is documented by lowered bronchial airflow after bronchoprovocation with methacholine or histamine. Many other factors that provoke airway obstruction include exercise, cold air, viral upper respiratory infection, respiratory allergens and cigarette smoke. Bronchial provocation with allergen induces a prompt early phase immunoglobulin

E (IgE,)- mediated decrease in bronchial airflow (forced expiratory volume in 1 second), followed in many patients by a late-phase IgE- mediated reaction with a decrease in bronchial airflow, for 4-8 hours. Moreover, bronchial asthma is well known by the presence of increased numbers of neutrophils, eosinophils, lymphocytes, and plasma cells in the bronchial tissues which it also involves bronchial secretions, and mucus. Initially, there is enrollment of leukocytes from the bloodstream to the airway by activated T-lymphocytes and CD. The activated T-lymphocytes also direct the release of inflammatory mediators from eosinophils, mast cells, and lymphocytes. Furthermore, the subclass 2 helper T-lymphocytes subset of activated T-lymphocytes produces interleukin (IL)-4, IL-5, and IL-13. IL-4 in conjunction with IL-13 signals the switch from IgM to IgE antibodies. (Web.a.ebscohost.com, 2019)

1.3. Etiology

The macroscopic picture of bronchial asthmatic patients displays lung hyperinflation, smooth muscle hypertrophy, lamina reticularis thickening, mucosal edema, epithelial cell sloughing, cilia cell disruption, and mucus gland hypersecretion. (Web.a.ebscohost.com, 2019)

Considering inflammation to be an etiological part of the disease, airways inflammation, involving T lymphocytes, activated eosinophils, and mast cells is an established feature of asthma, and has been the key target for treatment as it plays a crucial rule. Moreover, congenital conditions should not be missed. Most wheezing in infants is caused by either inflammation or anatomic abnormalities. (Louis et al. 2000)

1.4. The role of allergy and development of asthma

As a matter of contribution, immunologic and molecular influences contribute to the infant's propensity to wheeze. Basically, comparing children and adults to infants, tend to have higher levels of lymphocytes and neutrophils, rather than eosinophils and mast cells in bronchoalveolar lavage fluid. A wide range of inflammatory mediators have also been implicated in the wheezing infant such as histamine and leukotrienes. Fetal and early postnatal in which the structure and function of the lung are affected by factors including fetal nutrition and fetal and neonatal exposure to maternal smoking may also occur. (El-Gamal and El-Sayed 2011)

When the immune system is inappropriately stimulated, for example by environmental aeroallergens, it will result in a chronic inflammatory disease under the term of bronchial asthma. Bronchial asthma can be literally characterized by, reversible airway obstruction, bronchial hyperreactivity and mucus overproduction. Correspondingly, many epidemiological and genetic

studies have dealt with its origin. In fact, hundreds of genome-wide linkage analyses and association studies have identified several chromosomal regions harboring asthma susceptibility genes like chromosome 2q and 5q, in which it made bronchial asthma the most common disease of childhood during the last decades. (Bierbaum and Heinzmann 2007)

Forty percent of wheeze-chest children almost 80% of them with more troublesome asthma, continue to have symptoms in mid-adult life, which explains the increase of asthma in children thus it makes it a serious implication for adults. (Jain, Vinod Bhat, and Acharya 2010)

During childhood, wheezing is considered to be a heterogeneous condition. Some children will manifest asthmatic symptoms that occur in the respiratory system for the first time in early life, while others will only have a transient early wheezing. Many studies have been investigating and supporting the hypothesis of the immune system having major mechanisms that might contribute wheezing, suggesting an immunoglobulin E-mediated hypersensitivity type of mechanism. Researches have implied two proposals, whether an extended activation of T-helper 2-like immune response after bronchiolitis or a higher IgE levels in young children with persistent wheezing. (Lopez et al. 2002)

1.5. Risk factors associated with development of asthma

Considering risk factors of developing asthma in childhood with history of recurrent attacks of wheezing, it has been documented that the main risks can be classified under, phenotypes of asthma, breastfeeding, lung function, family structure, socio-economic status, allergic sensitization. It has been showed that, there is a strong association between wheeze chest and lower level of immunoglobulin IgG, IgM, and IgA, meaning that the more immunoglobulins drop down, the more the chance for babies to develop wheeze attachs, and increase the level of complement (C3 &C4). Moreover, it has been demonstrated that there is an increase in eosinophil counts among wheeze chest patients. (Subbarao, Mandhane and Sears, 2019)

1.6. Immunoglobulins

Immunoglobulins (Igs), also known as antibodies, are proteins present in the milk and colostrum of all lactating species. All Ig classes bind antigens and, in addition, exhibit one or more effector functions. In effect, the antibodies function as flexible adaptors linking various parts of the cellular and humoral immune system. While one part of an antibody binds to antigen, other parts interact with other elements. (Immunoglobulins : Structure and Function Immunoglobulins : Structure and Function n.d.)

1.6.1 Immunoglobulin A (IgA)

There has been some evidence that highly suggests allergic sensitization are being promoted by immunoglobulin A deficiency. Its Mechanism could be unexplainable and is not well understood yet, IgA may be believed to be competitively binding to allergens in that other immunologically active factors will be prevented from encountering the allergen. consequently, allergen-specific immune activation could be promoted deficiency of allergen-specific immunoglobulin A, which could lead to clinical allergy and allergic sensitization in inclined individuals. (Possin et al. 2010)

Major key roles are being well played in the immune protection by a major serum immunoglobulin known as immunoglobulin A along with the predominant antibody class in the external secretions that bathe mucosal surfaces. In order for IgA to be produced, the body needs a considerable amount of energy to do so. Immunoglobulin A Considered to be the second most important immunoglobulin following IgG, which present at concentrations of 2 – 3 mg/ml comparing with 12 mg/ml of IgG. Considerably, serum IgA is known for its fast metabolization rate, almost five times faster than IgG, making more sense for the previously mentioned immunoglobulins to have almost the rate of production. Bodily antigenic materials are various, although its main component is believed to be immunoglobulin A, which is being locally synthesized in all mucosal surfaces of the body. The gut-associated lymphoid (GALT) is considered to be a part of the mucosal immune system, which is highly specialized and does a very wide independent systemic immune system function. Generally, mucosal immune system shares common characteristics, mostly all parts of it, but when it comes to the lymphoid structures, it might vary from tissue to tissue. (Woof and Ken 2006)

1.6.2. Immunoglobulin A and wheeze chest

In correlation of IgA with wheeze chest children, it is established that infants who had suffered from upper or lower respiratory infections before the acute bronchiolitis, IgA was significantly higher than in infants without previous respiratory infections, which gives the hypothesis of IgA involvement with children who suffer from wheeze chest. (Carlsen et al., 2019)

1.6.3. Immunoglobulin G (IgG)

Almost 75% of the body's immunoglobulins are believed to be IgG, which is the simplest antibody class in humans. Four polypeptide chains formed by glycoproteins are what is making these molecules. They consist of two identical copies of each of two kinds of polypeptide chain: heavy (H) gamma "which it might be one of four subclasses" and light (L). These two chains must be linked together by disulfide bonds and noncovalent forces making the form of L-H-H-L. Immunoglobulin

G amino acid will differ from its individual IgG molecule, but within the molecule itself, the two L chains and the two H chains are typically identical to each other. In the upper airway, immunoglobulin A, secretory IgA and immunoglobulin g are the major components of respiratory secretions. Meanwhile, IgG mainly provides a primary protection against systemic and local infections but in the lower respiratory tract. (Immunoglobulin, Antibody, and Painter 2008)

1.6.4. Immunoglobulin G and wheeze chest

Deficiencies of IgG limited to one or more subclasses have been recognized in children with recurrent infections and one small study found evidence of IgG subclass deficiency in non-allergic children with chronic chest symptoms. As other forms of immunodeficiency have been associated with atopic diseases, a study under the name of IgG subclass deficiency in asthma studied IgG subclass concentration and have found low concentrations of IgG among 28 asthmatics children, disagreeing with the hypothesis of the correlation between IgG and asthma. (Loftus et al. 1988)

1.6.5. Immunoglobulin M (IgM)

Considerable amount of almost 15% of immunoglobulin M (IgM) has been detected with individuals in their adolescence. It has been known that IgM is one of the immunoglobulins that is formed in the very beginning of the immune response, but throughout time, it is increasingly being replaced by immunoglobulin G (IgG) which means the presence of specific antibodies of immunoglobulin M (IgM) class are diagnostic of any chronic or recent infection. During the second trimester, IgM is recognizable as it is being the first immunoglobulin to be found during this stage and also as it develops a well immunological competence. In case of presence of a viral infection in newborns, immunoglobulin M (IgM) is known for its role defeating these particular viruses, so the presence of IgM antibodies would be explanatory since it does not cross placenta from mother to baby and this explains the intrauterine viral infection. In spite of the fact that IgM has a low count in the airway secretions, IgM has a major role in mucosal defense, and it is capable of agglutinating bacteria and activating the complement cascade. Individuals with IgM deficiency appear to have a specific defect in B lymphocyte maturation, but the B lymphocytes are capable of secreting other antibody isotypes, meaning that Isolated IgM deficiency is not associated with recurrent respiratory infections. (Paolo Casali. 2008)

1.6.6. Immunoglobulin IgM and wheeze chest

Since IgM has a major role in mucosal defense, it is believed that the presence of high levels of IgM might have a role developing symptoms that associate with bronchial asthma and may also be associated with the elevated immunoglobulins. (Paolo Casali. 2008)

1.6.7. Immunoglobulin E (IgE)

In case of allergen exposure, normally the immune system produces a protein known as immunoglobulin E IgE which is also known for its major role in allergic reactions. Only if produced, immunoglobulin E IgE will bind on the allergy cells and waits for the proper allergen to return. Allergy symptoms will be caused in a way of the allergen and the IgE bound to the allergy cells in which they become activated to cause such reaction. A significantly strong association has been proven between asthma and total IgE or specific IgE. Several studies found a close correlation between serum IgE levels and self-reported asthma. Alternatively, it was found that allergic rhinitis to be independent of serum IgE concentration but associated with cutaneous reactivity to common seasonal aeroallergens in most individuals tested, and by that it is clearly understood that asthma is almost always associated with some type of IgE-related reaction and therefore has an allergic basis. (Possin et al. 2010)

In similarity with other immunoglobulins, immunoglobulin E (IgE) will always be released into the circulation in a response to an antigenic stimulus by plasma and B cells. Immunoglobulin E is most commonly associated with allergic disease and believed to mediate an exaggerated or maladaptive immune response to antigens. The typical immediate hypersensitivity reaction results from re-exposure of the host to particular antigen Once antigen-specific IgE has been produced. (Kelly and Grayson 2016)

The determination of allergen specific immunoglobulin E antibodies and total immunoglobulin E serum concentrations is the laboratory diagnosis of type I hypersensitivity reaction. Physiologic changes are noticeable in the first decade of life since serum concentration of total immunoglobulin E varies throughout the lifespan. Considering the defined reference intervals of total immunoglobulin E, almost 5% of healthy children may have serum total immunoglobulin E concentrations above the age-specific reference range, while 10% of children with clinical signs of hypersensitivity may have serum total immunoglobulin E concentrations within the age-specific reference range. (Topić et al. 2011)

1.6.8. Immunoglobulin E and wheeze chest

Screening of sensitized individuals based on serum total immunoglobulin E concentrations might prove inadequately reliable in some cases, as the concentration of circulating immunoglobulin E antibodies might not always correlate with local tissue immunoglobulin E synthesis. (Topić et al. 2011)

Population studies have shown an association between prevalence of asthma or bronchial hyperresponsiveness and total serum immunoglobulin E (IgE) levels, meaning that IgE levels increase among asthma or bronchial hyperresponsiveness. (Sunyer et al. 1996b)

1.7. The complement system

The complement system refers to a series of more than 20 proteins, circulating in the blood and tissue fluids and previously discovered by Jules Bordet as a heat-labile component of normal plasma that causes the opsonization and killing of bacteria. Almost all of proteins are normally inactive, but the activation of one protein enzymatically cleaves and activates the next protein in the cascade happens in response to the recognition of molecular components of microorganisms they become sequentially activated in an enzyme cascade. (Gani n.d.)

1.7.1. Complement 3 (C3)

Generally speaking of Complement C3, it is consisted of an α-chain and a β-chain that are connected by cysteine bridges. With a concentration of 1.3 mg/ml, C3 is considered to be the most abundant protein of the complement system in plasma. However, it is the central component of the human complement system, C3 in its native form is inactive. A crucial step in the complement activation cascade has to happen in order for the complement to be ready is the cleavage of C3 into C3b and C3a, which can be initiated by one or more of the three distinct pathways, called alternative, classical and lectin complement pathways.(Dinasarapu et al. 2012)

Given the importance of C3 in the immune response, it is one of the sources of the biologically active fragments that induce pro-inflammatory responses and mark the particles for clearance by cell lysis or phagocytosis and for activation and control of adaptive immunity.C3 split products issued from in vivo sequential proteolytic cleavages result in complement amplification. Moreover, complement 3 is synthesized mainly by hepatocytes and also by cells at extrahepatic sites including macrophages, monocytes, endothelial cells, fibroblasts, dendritic cells, and smooth muscle cells. (Ghannam 2019)

1.7.2. Complement 4 (C4)

As a part of the complement system, complement C4 activation occurs by the C4 splitting down into C4b and a small peptide also known as C4a. The small peptide, C4a diffuses away and acts as a chemotactic factor and an inflammatory paracrine. Meanwhile, C4b acts as an opsonin and remains bound to the complex at the surface of the pathogen which activates the next step of the process. (Randox Laboratories, 2019)

According to a recent study done in Benghazi, Libya under the name of complement components as inflammatory markers in childhood asthma, it was established that complement C4 level for patients with asthma was no different from the controls. (Journal et al. 2016)

1.7.3. Complements and wheeze chest

Some studies have reported increased plasma complement levels (C3 and C4) since the complement system plays an important role in immune responses of the host. The role of the complement system in asthma has been suggested, possibly through initiation and/or amplification of the inflammatory response in the airways through the complement activation cascade. Accordingly, C3 was more noticeable in many studies, whereas others have demonstrated no significant changes. (Fattah et al. 2010)

1.8. Eosinophils

Eosinophils are major effector cells in the immune system. approximately 8μm in diameter and are identified by their bi-lobed nucleus, pink staining with eosin and characteristic cytoplasmic granules (Figure1). Eosinophils have a beneficial role in host defense against nematodes and other parasitic infections and are active participants in many immune responses. However, eosinophils can also be damaging as part of the inflammatory process of allergic disease. (Buckland and London n.d.)

In a theoretical point of view, healthy individuals tend to have normal levels of almost every substance in the body. Speaking of which, eosinophils are present in the circulation in low numbers and are rarely found in the lung, being mostly confined to the tissues surrounding the gut. Closely to neutrophils, eosinophils are terminally end-differentiated cells programmed to undergo apoptosis in the absence of viability enhancing stimuli. Moreover, eosinophils have a limited life-span in the circulation of 8 to 20 hours, which is extended to 3–4 days in tissues.

However, asthma is recognized as a heterogeneous condition, eosinophilic asthma is a recently described phenotype of the disease characterized by increased blood or sputum eosinophils whose numbers correlate with disease severity. By the meaning of documentation, eosinophilic

involvement in inflammatory conditions affecting the skin, gastrointestinal tract, and upper and lower airways. (Rosenberg 2016)

1.8.1. Eosinophils and development of wheeze chest and allergy

An increased number of eosinophils in the tissues and/or blood representing the so-called eosinophilia. Atopic dermatitis may produce a more significant eosinophilia if affecting a large part of the body and if associated with significant atopy. While allergic rhinitis and asthma often produce a mild eosinophilia. (Kovalszki et al. 2016)

High levels of eosinophils could cause inflammation in the airways, affecting the sinuses and the lower airways as well as nasal passages. Generally, inflammation and other symptoms of asthma become more severe, as the level of eosinophils increases. (Adithya Cattamanchi, 2019)

1.9. Aims of the Study

Given the findings for the role of immunoglobulins, complement (C3 &C4), and eosinophils in wheeze chest, the major aims were to assess the correlation between the immunoglobulins (IgA, IgM, IgG, and IgE), complements, and the level of eosinophils and development of wheeze chest. Moreover, correlation of the level of IgE with, IgA, IgM, IgG, C3, C4, the numbers of the attacks per year, age, family history, and eosinophilic count.

1.10. Hypothesis

The hypothesis to be tested is that IgE level and Eosinophils are elevated while the other immunoglobulins and complement proteins are decreased in in children with wheezy chest compared with the control group.

Chapter Two

Materials and Methods

2.0. Materials

2.1. Type of study, Samples collection and Ethical approval

This study is a case control one since there are two categories involved, cases referred as patients and controls to compare the results to. University's committee ethical approval was obtained for this study on the 10th Feb 2019. Part of the participants were randomly involved in this study from Benghazi pediatric hospital, since they were hospitalized in the center. The hospital's ethical approval was obtained on the 30th March 2019. Meanwhile, the rest of the patients and controls have visited Libyan international medical university in order to withdraw and run all blood tests at the university's Lab. All patients' patents/relatives were present while withdrawing the blood samples and all of them were informed that this study was for the purpose of a medical research and to fulfill a requirement of under-graduation project, and all signed for approval. A total number of a randomized 72 cases were involved in this study, divided into 52 patients with recurrent wheezy chest attacks and 21 controls. Five milliliters of blood sample were equally collected from all participants.

2.2. Materials and Equipment

EDTA tubes 3ml (MD)

Clot activator tubes 5ml (Xinle)

Eppendorf tubes 1.5 ml

Distilled water

Syringes 5 ml

MICROLIT pipette and tips 1.0 ml

MICROLIT pipette and tips 200 µ

MICROLIT pipette and tips 100 µ

MICROLIT pipette and tips 5 µ

ELISA microplates (96-well)

EMP ELISA

Multichannel pipette

Incubator

Radial immunodiffusion plate IgA RID (Mod 01.06 ver. 2.1)

Radial immunodiffusion plate IgG RID (Mod 01.06 ver. 2.1)

Radial immunodiffusion plate IgM RID (Mod 01.06 ver. 2.1)

Radial immunodiffusion plate C3 RID (Mod 01.06 ver. 2.1)

Radial immunodiffusion plate C4 RID (Mod 01.06 ver. 2.1)

EUROLINE inhalation middle east (IgE) kit (DP3117-1601 E)

EUROLINE plot strips

EUROIMMUN total IgE kit (EV 3840-9601 E)

Microscopic slides 3 x 1 inches

GIEMSA stain 0.5 liters

Absolute alcohol 0.5 liters

Microscope oil 1ml

CELLTAC for CBC tests

HERMLE Centrifuge

ROCKING MIXER Shaker

2.3. Samples numbering

All samples were kept at the lab's fridge. Numbering the samples in this study started from 101 until 173. As for the EDTA tubes, numbering was for example 101 B, 102 B, 103 B … etc. While numbering the clot factor tubes was 101 A, 102 A, 103 A … etc. Serum and stained slides were ordinary numbered as 101, 102, 103, ….. 173.

2.4. Methods

2.4.1. Determination of total IgE concentration

The aim of this experiment was to determine the total IgE concentration, 100µl of patients' diluted samples, calibrators, positive and negative controls were all transferred into the microplates wells and incubated for 30 minutes at room temperature (25° C). washing was done manually using wash buffer prepared previously repeated 3 times after each step. Transferring 100 µl of both conjugate and substrate followed the mentioned procedures separately with 30 minutes incubation period for the conjugate and 15 minutes for the substrate intermediated by washing step. Lastly, a 100 µl of stop solution were pipetted into the wells making all samples ready for measurement at 450 nm wavelength within 30 minutes of adding the stop solution.

2.4.2. Determination of middle east IgE

Blot strips have been placed in incubation channels and were filled with 1.0 m of prepared working buffer. On a rocking shaker, samples were incubated for 5 minutes. Fluid was aspirated off and undiluted 400 µ of patients' samples were added to the strips to be incubated for 60 minutes on the rocking shaker. A three times washing was done after awhile followed by adding 1.0 m of conjugate with 60 minutes of incubation on the rocking shaker. Washing and additional substrate were done followed by 10 minutes of incubation on the rocking shaker. Lastly, stopping solution was added in which it was distilled water and all strips were being placed on formatted papers designed for this test to be measured with the software program.

2.4.3. Determination of IgA, IgG, IgM, C3 and C4

The examined proteins all went under the same procedure using the same principle, differing only in the incubation period. Materials were required for conducting these tests are micropipette to 5 µ and slide ruler. Working plates are removed from their envelops and kept at room temperature for few minutes. Five µ of undiluted sample were being placed in each well and left until completely adsorbed. IgA, IgG, C3 and C4 were all incubated for 72 hours at the lab's fridge, therefor, IgM was incubated for 96 hours (figure).

2.4.4. Determination of complete blood count (CBC)

Two CC of patients' blood sample were put in EDTA tubes to be tested for CBC and to make blood films for eosinophilic counting. Complete blood count tests were done by the 3 parts CELLTAC (figure).

2.4.4. Determination of eosinophils

In order to count eosinophils, blood films had to be done by the procedure of adding a blood drop into a slide, fixing it with absolute alcohol and then staining it with GIEMSA stain. Furthermore, after complete dryness of the slide, a drop of oil is added to the slide and put under the microscope to be read with high power lens x100. The strategy of counting eosinophils under the microscope was as easy as viewing 100 WBCs and exclude the number of eosinophils out of the total number.

2.5. Statistical Analysis

Research is basically what know as a problem or a systematic investigation that include, research development, evaluation and testing, designed to develop generalizable knowledge. This is a broad field that may include biomedical research, health services research and epidemiological studies.

(Institute of Medicine (US) Committee on Health Research and the Privacy of Health Information: The HIPAA Privacy Rule; 2009)

Over the last decades, medical journals have published a host of papers that statistics played a crucial role in all of them. This helped with research, planning and decision-making in the health sciences. (Ocaña-Riola 2016)

In this study, children with wheezy chest attacks are going to be involved, measuring mainly the level of IgE and correlate with other variables that be also measured. A total number of a randomized (n=73 cases) were involved in this study divided into (52 patients) with recurrent wheezy chest attacks and (n=21 controls) with no significant signs or symptoms of such a disease. Clinical and Laboratory data were statistically analyzed using a computer program (Minitab 13 for Windows).

2.5.1. Statistical tests:

The following tests were all used to statistically analyze the data: descriptive statistics, statistical inference including correlation, one-way ANOVA and T-test.

Chapter Three

Results

3.0. Descriptive statistics

3.1. Graphical representation

Meanwhile analyzing the data using Minitab, it was shown a number of 48 male cases (which represents 65.8% of the total number) and a number of 25 female cases (which represents 34.2% of the total number). These numbers make up the total number of all cases in this study (figure 9.). Patients with recurrent wheezy attacks were 52. Thirty-four were males (which represents 65.4% of the patients' number) and 18 were females (which represents 34.6% of the patients' number) (figure 10.).

According to the age group that was required for this study, 6 years old children were the oldest to accept and younger than 1-year old children were the youngest to accept as well. Using histogram, ages were ranging from months to 6 years showing a majority "highest number" for the age 3 mainly and the age 5 closely, along the other age groups with a minority "lowest number" for the age of 6 (figure 11). To further have a better look into the patients' status, a histogram chart was done only on the patients' number making the majority "highest number" for the ages of 1 and 5 years old mainly and the age of 3 closely along the other age groups with a minority "lowest number" for the age of 6 as well (figure 12).

Considering the residence or area of living to be an influence for respiratory complaints, all cases were asked about their residence whether they live in an urban or a rural area. Although a high number of the cases were urban residents, a number of 4 cases were rural areas residents (which represents 5.5% of the total number) while 69 cases of this study were urban residents (which represents 94.5% of the total number) (Figure 13).

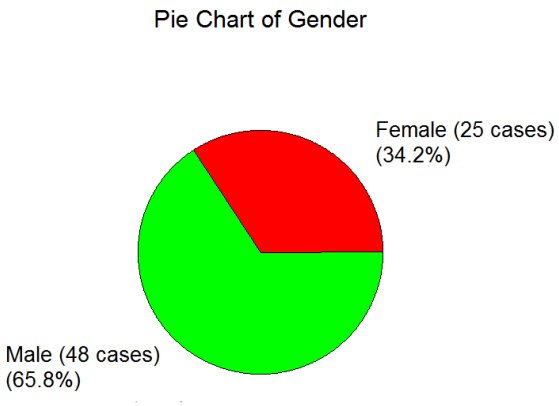

Figure 9: The total number of all children were 73. The number of female cases were 25, while the total number of male cases were 48. That means the male cases represent 65.8% of all patients and the female case represent 34.2% of the total number.

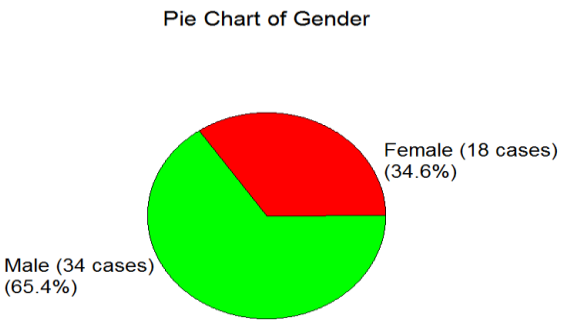

Figure 10: The total number of Patients with recurrent wheezy attacks were 52. The number of females were 18 (which represents 34.6% of the patients' number) and thirty-four were males (which represents 65.4% of the patients' number)

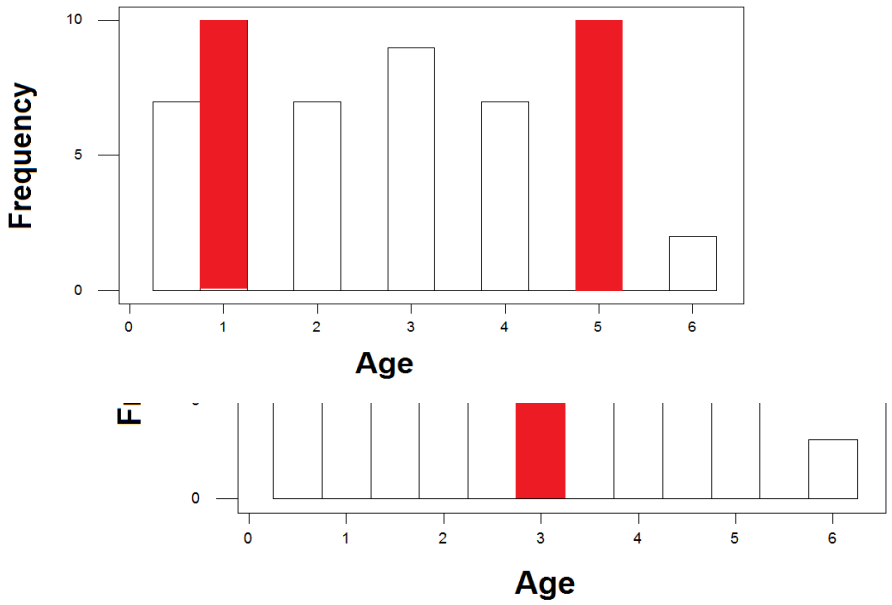

Figure 11: ages were ranging from months to 6 years showing a majority "highest number" for the age 3 mainly and the age 5 closely, along the other age groups with a minority "lowest number" for the age of 6.

Figure 12: highest number for the ages of 1 and 5 years old mainly and the age of 3 closely along the other age groups with a minority "lowest number" for the age of 6 as well.

Figure 13: a number of 4 cases were rural areas residents (which represents 5.5% of the total number) while 69 cases of this study were urban residents (which represents 94.5% of the total number)

3.2. Estimation of IgE

A total number of 73 cases divided into 52 patients and 21 controls in this study were all tested for total IgE, shown that only 29 patients had elevated levels of IgE while the others are having a normal range (figure 14).

3.3. Estimation of IgA

A total number of 73 cases divided into 52 patients and 21 controls in this study were all tested for total IgA, shown that only 17 cases had elevated levels of IgA while the others are having a normal range (figure 15).

3.4. Estimation of IgG

A total number of 73 cases divided into 52 patients and 21 controls in this study were all tested for total IgG, shown that 30 cases had elevated levels of IgG while the others are having a normal range (figure 16).

3.5. Estimation of IgM

A total number of 73 cases divided into 52 patients and 21 controls in this study were all tested for total IgM, shown that only 12 cases had elevated levels of IgM while the others are having a normal range. (figure 17).

3.6. Estimation of C3

A total number of 73 cases divided into 52 patients and 21 controls in this study were all tested for total C3, shown that 50 cases had elevated levels of C3 while the others are having a normal range. (figure 18).

3.7. Estimation of C4

A total number of 73 cases divided into 52 patients and 21 controls in this study were all tested for total C4, shown that 39 cases had elevated levels of C4 while the others are having a normal range. (figure 19).

3.8. Estimation of Eosinophils

A total number of 73 cases divided into 52 patients and 21 controls in this study were all tested for total Eosinophils, shown that only 4 cases had elevated levels of eosinophils while the others are having a normal range. (figure 20).

The total amount of the IgE in the all cases (patients and control)

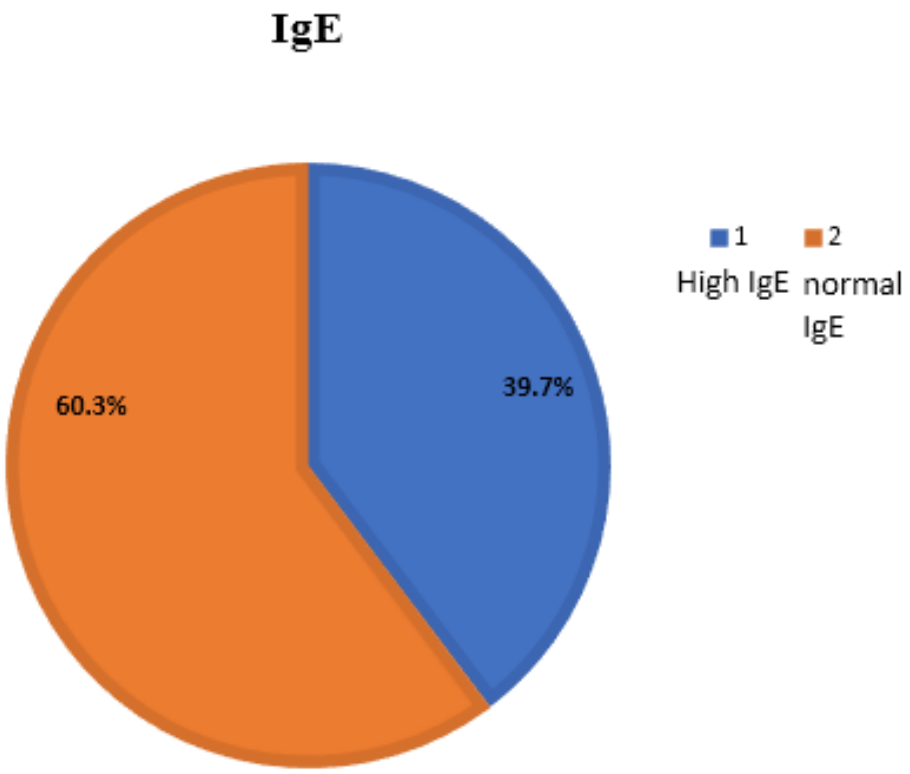

Figure 14: This figure has shown that, only 29 patients had elevated levels of IgE while the others are having a normal range. (39.7% of the all cases is positive for IgE).

The total amount of the IgA in the all cases (patients and control)

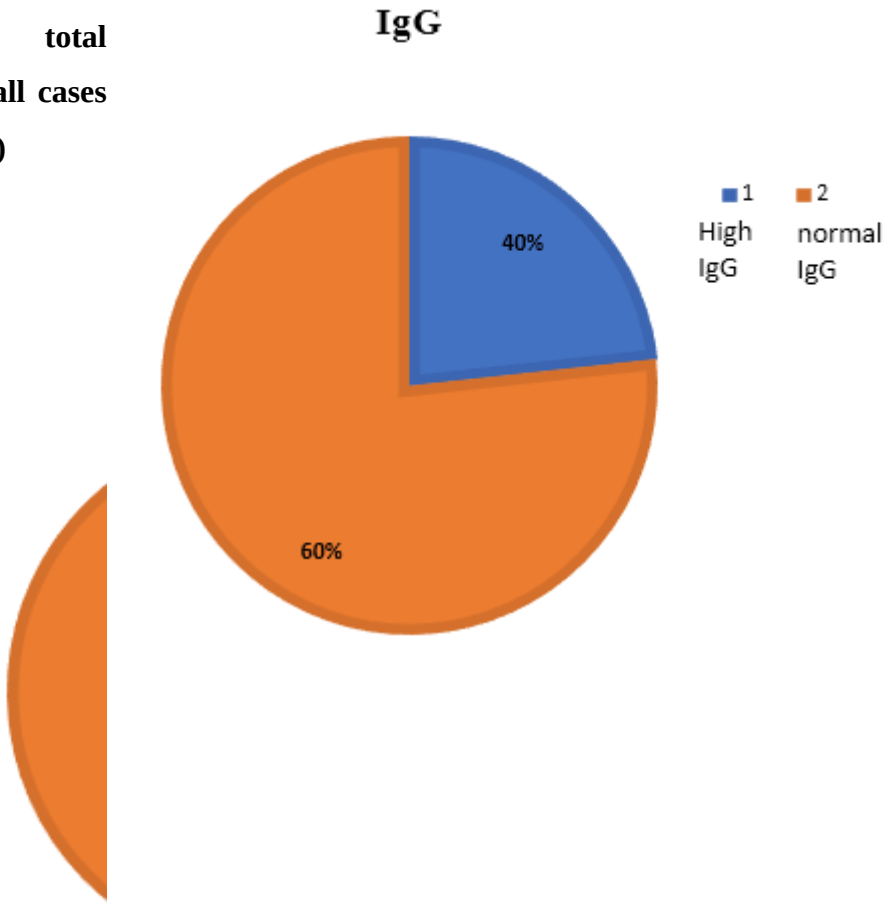

Figure 15: This figure has shown that, only 17 patients had elevated levels of IgA while the others are having a normal range. (23.2% of the all cases is positive for IgA)

The total amount of the IgG in the all cases (patients and control)

Figure 16: This figure has shown that, only 30 patients had elevated levels of IgG while the others are having a normal range. (40.01% of the all cases is positive for IgG)

The total amount of the IgM in the all cases (patients and control)

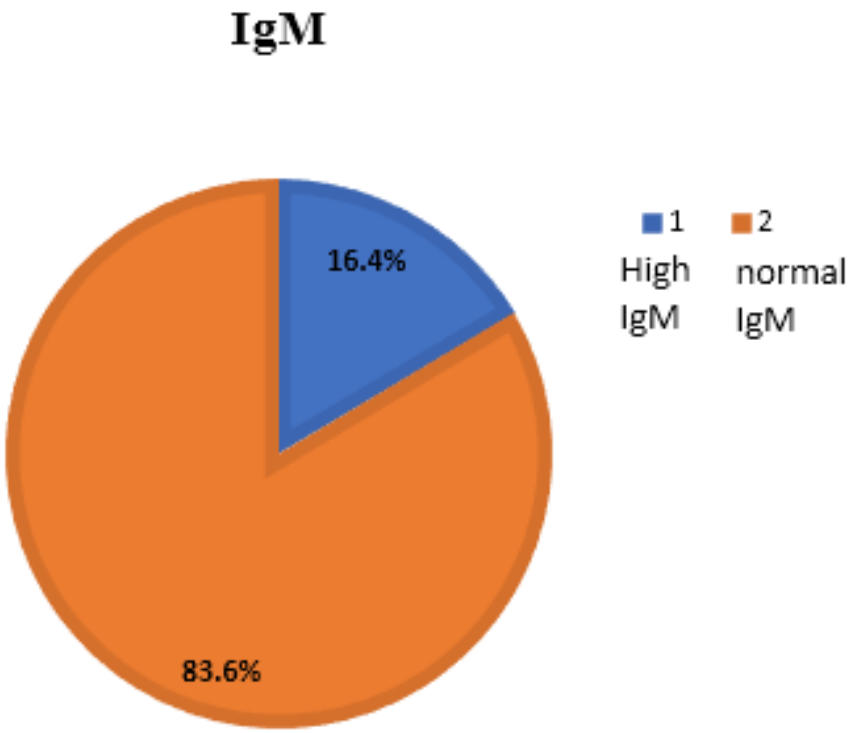

Figure 17: This figure has shown that, only 12 patients had elevated levels of IgM while the others are having a normal range. (16.4% of the all cases is positive for IgM)

The total amount of the C3 in the all cases (patients and control)

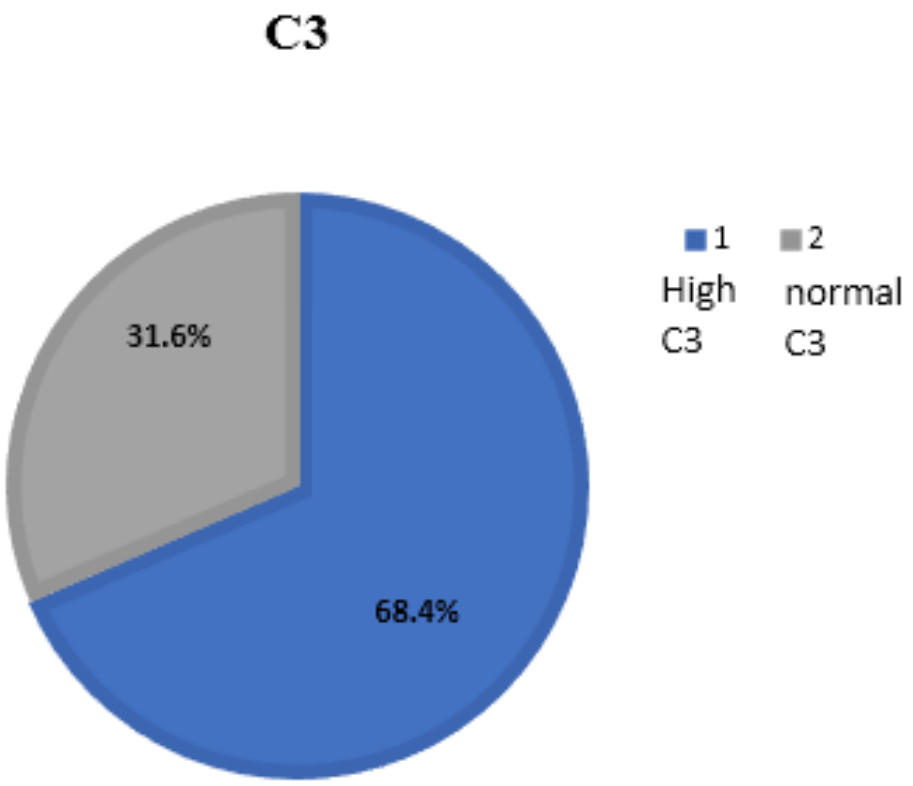

Figure 18: This figure has shown that, 50 patients had elevated levels of C3 while the others are having a normal range. (68.4% of the all cases is positive for C3)

The total amount of the C4 in the all cases (patients and control)

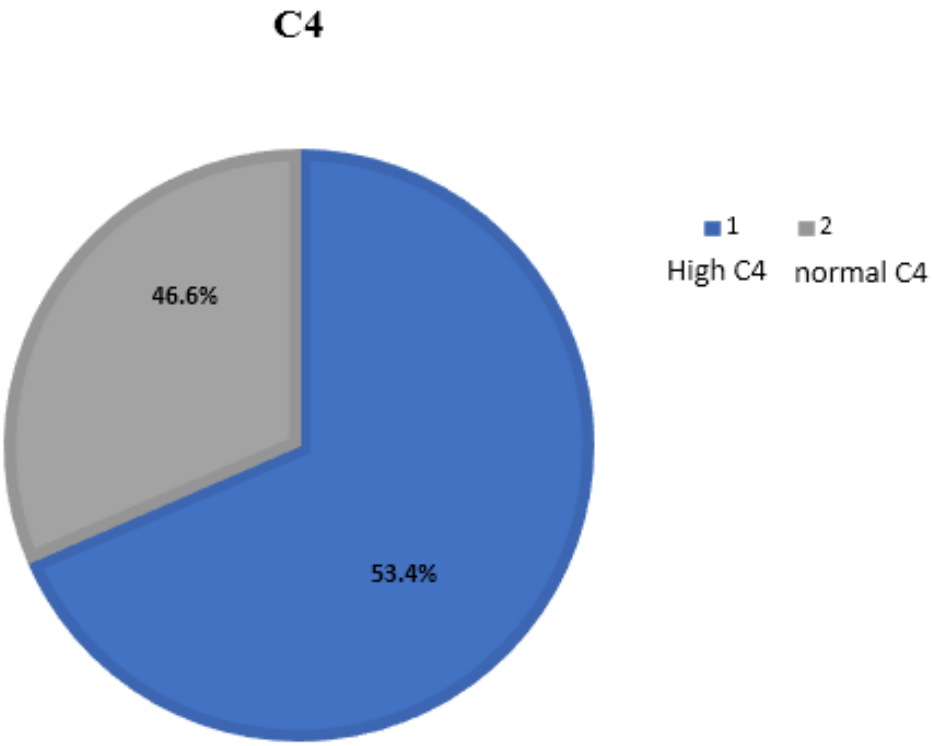

Figure 19: This figure has shown that, 39 patients had elevated levels of C3 while the others are having a normal range. (53.4% of the all cases is positive for C3)

The total amount of the eosinophils in the all cases (patients and control)

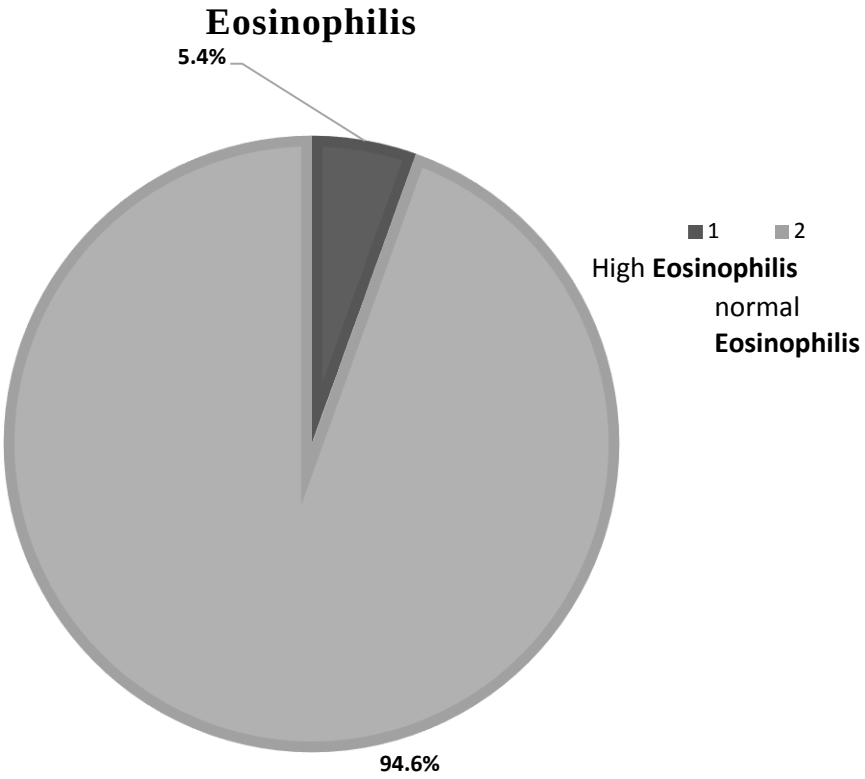

Figure 20: This figure has shown that, only 4 patients had elevated levels of eosinophils while the others are having a normal range. (5.4% of the all cases is positive for eosinophils)

3.9. Type of Allergen

As a part of this study, testing the type of the allergen to those who had elevated IgE was mandatory and a bar-chart was done to show a representative figure for the number of cases

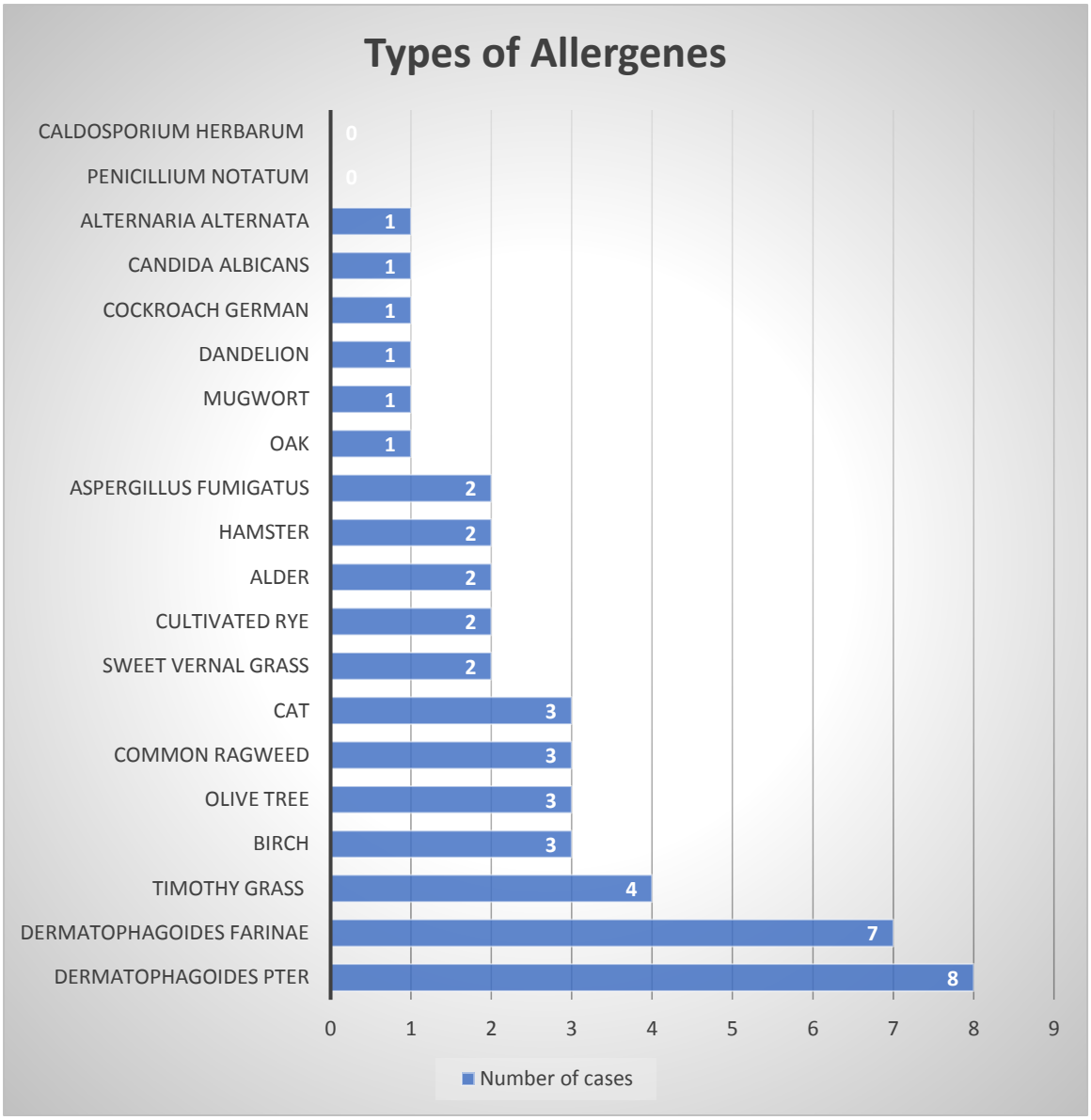

Figure 21: All IgE positive were screen for inhalation middle east (IgE) showing individuals not only allergic for one allergen, but many. Most of the cases shared Dermatophagoides Pter allergen and so the list goes descending in the number with 2 allergens were not found cladosporiumherbarum and penicillium notatum.

3.10. Statistical inference

3.10.1. Correlation

Correlation analysis is used to study the type and strength of a relationship between two variables, meaning that correlation and regression analysis are applied to data to define and quantify the relationship between two variables(Table1).

Three out of twelve variables have been found to be significant according to the correlation part in this study. Number of attacks strongly correlated with IgE with a P-value of (P=0.001), as well as the manifestation with a P-value of (P=0.002), while age was weakly correlated with IgE with a P-value of (P=0.005). Furthermore, the other 9 variables in this study were found to be insignificant, correlating with IgE.

Table 1: The correlation of IgE with most effective variables.

IgE		IgA	IgG	IgM	C3	C4	Gender
	P-value	0.471	0.505	0.917	0.590	0.463	0.403
	Comment	In-sig	In-sig	In-sig	In-sig	In-sig	In-sig
		Age	Residence	Number of attacks	Manifestation	Family history	Eosinophilic count
	P-value	0.005	0.874	0.001	0.002	0.463	0.094
	Comment	Sig	In-sig	Sig	Sig	In-sig	In-sig

3.10.2. One-way ANOVA

3.10.2.1. One-way ANOVA: IgE versus number of attacks

On applying one-way classification analysis of variance (ANOVA), the null hypothesis (Ho) says that there are no differences of IgE according to the variability of number of attacks factor. The result of this test gave P-value (P=0.011) which means that the Ho should be rejected and it concludes that the variability of IgE based on the number of attacks is **significant** and this indicates that at least one level of attacks differs compared to the other levels.

Table 2: This test designed to show the significant differences of IgE according to the number of attacks.

IgE vs number of attacks	P - value	Comment
	0.011	**Significant**

To have an idea about the significant difference among the number of attacks level, the multiple comparison tests via Tukey test was applied and gave the following results (Table 3):

Table 3: All of the number of attacks levels differences in IgE are not significant, except for control (no attacks) and (levels 3 and 4), also (levels 2 and 4). (0 for controls, 1 one attack per year, 2 two attacks per year, 3 three attacks per year, 4 more than three attacks per year).

Table 3		Number of Attacks				
		0	1	2	3	4
Number of Attacks	0		0.188	0.185	0.032 **Significant**	0.001 **Significant**
	1			0.620	0.855	0.233
	2				0.323	0.017 **Significant**
	3					0.170
	4					

3.10.2.2. One-way ANOVA: IgE versus manifestation

Table 4: This test designed to show the significant differences of IgE according to the manifestation.

IgE vs manifestation	P - value	Comment
	0.006	**Highly Significant**

On applying ANOVA, the Ho says that there are no differences of IgE according to the variability of manifestation. The result of this test gave P-value (P=0.006) which means that the Ho should be

rejected and it concludes the variability of IgE is based on the manifestation is **Highly significant** and this indicates that at least one level of manifestation differs compared to the other levels.

To have an idea about the significant difference among the manifestation level, the multiple comparison tests via Tukey test was applied and gave the following results (table 5):

Table 5		Manifestation		
		0	2	3
Manifestation	0		0.017 **Significant**	0.003 **Significant**
	2			0.079
	3			

Table 5: All of the manifestation levels differences in IgE are not significant, except for control (no attacks) and (levels 2 and 3). (0 for controls, 1 dermatitis, 2 allergic rhinitis, 3 both).

3.10.2.3. One-way ANOVA: IgE versus family history

IgE vs family history	P - value	Comment
	0.019	**Significant**

Table 6: This test designed to show the significant differences of IgE according to the family history.

On applying ANOVA, the Ho says that there are no differences of IgE according to the variability of family history. The result of this test gave P-value (P=0.019) which means that the Ho should be rejected and it concludes the variability of IgE is based on the family history is **significant** and this indicates that at least one level of family history differs compared to the other levels.

To have an idea about the significant difference among the family history level, the multiple comparison tests via Tukey test was applied and gave the following results (Table 7.):

Table 7		Family History				
		0	1	2	3	4
Family History	0		0.001 **Significant**	0.036 **Significant**	0.110	0.552
	1			0.469	0.074	0.084
	2				0.418	0.295
	3					0.624
	4					

Table 7: All of the Manifestation levels differences in IgE are not significant, except for control (no attacks) and (levels 1 and 2). (0 for controls, 1 no family history, 2 third line, 3 second line, 4 first line).

3.10.3 T-test

This test is a type of inferential statistic used to determine if there is a significant difference between the means of two groups, in this study the two groups are the controls and effected individuals (table 8).

Factor	P-value
IgE	0.000
IgA	0.190
IgG	0.990
IgM	0.059
C3	0.443
C4	0.218
Gender	0.919
Age	0.406
Residence	0.862
No. of attacks	0.000
Manifestation	0.000
Family history	0.000

Eosinophilic count	0.095

Table 8: as mentioned previously, the two groups this test was used to determine are the controls (n=21) and the effected individuals (e=52). All thirteen variables were included in the test showing that all are insignificant, except for IgE ($p<0.001$) highly significant, number of attacks ($p<0.001$) highly significant, manifestation ($p<0.001$) highly significant, family history ($p<0.001$) highly significant and IgM ($p=0.059$) significant.

Chapter Four

Discussion

Almost 30% of children known for their wheezing symptoms, which was a very important area to focus the lights on, specially that these symptoms happen to occur before their third birthdays. Accordingly, these facts were documented by many population-based birth cohort studies while all participants were in a respiratory infection status. Immunologic and molecular influences are not as good in comparison to older children or even adults, by which, infants are prone to wheeze due to anatomic factors related to the lung and chest wall. Immunocompetent and immunodeficient infants may or may not develop immunologic derangements that cause wheezing, which it was originally led by viral infections. Anatomic causes of wheeze may be extrinsic or intrinsic to the airway. Development of asthma in persistent wheezers is possible, however, not every wheeze is indicative of asthma. Testing for allergy in these infants is worthwhile and can be of significant value in avoidable allergens. (Lopez et al. 2002)

Almost 30% of children manifest wheeze chest symptoms before the age of three and 50% before the age of six. Their parents report at least one attack within this period (El-Asheer et al. 2016).

Wheeze chest is common throughout early life and childhood. However, it is very rare in neonatal period (El-Gamaland El-Sayed,2011).

Recurrent attacks of wheeze chest have a significant morbidity and have been estimated that about one third of school-age children manifest the symptom during the first 5 years of life. In young children, wheezing is associated with a poor quality of life (Dávila, I et al. 2015).

Some type of IgE-related reaction is known to be usually associated with asthma and therefore has an allergic basis. In contrast, various epidemiologic studies have shown a highly significant relationship between asthma and interaction to various allergens as were demonstrated by skin tests or the specificity of IgE in the serum presence.(Platts-Mills 2001)

In the present study, total number of participants was (n = 73) divided into patients and controls, represented by 34.2% females (n = 25) and 65.8% males (n = 48). According to the patients group, 34.6% were females (n= 18) and 65.4% were males (n = 34). Ages varied from months to 6 years, showing a majority “highest number” for the age 3 mainly and the age 5 closely, along the other age groups with a minority “lowest number” for the age of 6. Moreover, residence was one of the

variables of this study, and it has shown that almost all participants were urban residents with a percentage of 94.5% (n = 69) and only 5.5% (n = 4) were rural residents.

Regarding the immunoglobulins levels, IgE concentrations were high in 29 patients while relatively normal ranges to the rest of the patients and a totally normal concentrations to all controls. IgA levels were high only in 17 cases of the total number of this study, while the rest were had normal levels. IgG levels were elevated in 30 cases in this study while the rest were had normal levels. IgM was the second least to be noticed elevated in all cases with a number of only 12 cases, while the rest were had normal levels. Meanwhile, C3 was the highest levels to be noticed with a number of 50 cases been positive for C3. Complement 4 levels were high in 39 cases in this study while the rest were had normal levels. The first least to be noticed elevated in all cases with a number of only 4 patients was eosinophilic count.

There are few studies investigating the relationship between recurrent wheezy chest and the prevalence of immunoglobulins in general and IgE in specific. In this study, the aim was to estimate all immunoglobulins, complements, eosinophils and specific allergens in infants and children younger than 6 years of age with recurrent wheeze.

Regarding specific allergens, 20 different types were evaluated in patients who had elevated levels of IgE, and the findings were that all patients were not only allergic to 1 type but unlikely to many. Most definitely the majority of the cases shared DermatophagoidesPter (8 patients) and Dermatophagoidesfariaeni (7 patients) allergens and so the list goes descending in the number with 2 allergens were not found, cladosporiumherbarum and penicillium notatum (figure 21). Eight patients had DermatophagoidesPter allergen in their serum making it the highest one to be present in all cases, this finding supports the reports of Ella et al in which their study included 132 cases, DermatophagoidesPter and Dermatophagoidesfariaeni allergens were found in 48 cases making them the commonest allergens. (Ella et al. 2015)

Moreover, IgE levels were correlated to all other variables, including: IgA, IgG, IgM, C3, C4, family history, manifestation and age. Many other researches hypothesized that other immunoglobins may correlate significantly with IgE, however, this study disagrees, since the correlation was insignificant for all variables. The only significant ones are age, number of attacks and manifestation. Respectively, IgA, IgG and IgM correlated insignificantly with IgE (P=0.471) (P=0.505) (P=0.917) to support these findings, reports by Gu et al shown that the serum IgG, IgA and IgM levels showed

no correlation to serum IgE level, meaning that immunoglobins are considerably independent from each other.(Gu 2002)

On the other hand, findings by Liphaus BL et al suggest that in the 69 allergic patients, 39 had high IgE levels, those were significantly correlated with IgA levels with a p-value of (P=0.003). Meanwhile, there was no correlation obser9/9/*ved between IgE and IgM or IgG. (Liphaus et al. 2012)

Regarding the complement system, C3 and C4 levels were correlated to IgE and it has been found that the correlation was insignificant for both of them C3 (P=0.590) C4 (P=0.463). Despite the results of C4, the previously mentioned findings by Liphaus BL et al found that IgE correlated insignificantly with C3 as well, therefore, it correlated significantly with C4 with a P-value of (P <0.03), meaning that somehow when IgE levels elevate, C4 levels elevate as well. (Liphaus et al. 2012)

Blood eosinophilia and total serum IgE level have been proposed as screening tests for allergies. Regarding the heterogeneous disorder allergic rhinitis, it is characterized by one or more symptoms including sneezing, itching, nasal congestion, and rhinorrhea. The key to diagnosis of allergic rhinitis is awareness of signs and symptoms. IgE antibody tests to detect specific allergens are the standard method used today;in addition, diagnosis must be confirmed with a positive history and demonstration that the symptoms are the result of IgE-mediated inflammation.(Skoner 2001)

According to El-Asheer et al findings, eosinophilic count correlated significantly with IgE, by which, these findings contradict with this present study findings, since eosinophilic count correlated insignificantly with IgE (P<0.095).(El-Asheer et al. 2016)

According to the number of attacks variable, first it was correlated with IgE and shown a significant correlation (P=0.001). And for a further statistical testing, the significant differences of IgE according to the number of attacks test was done via one-way ANOVA, to represent an even confirmative result that the variability of IgE based on the number of attacks is significant (P=0.011). Moreover, comparing the patients group to the controls using T-test, a highly significant result was found with a p-value of (p<0.001), which means that values of IgE of patients are significantly high comparing to the healthy controls. In contrast, Peat et al study included 3 groups of early atopics, late atopics and nonatopics. The nonatopic group had a mean serum IgE level of 45.9 IU/ml, which was significantly lower than in the other two atopic groups. This difference between groups was significant at the (P <0.00l) (Peat et al. 1996).

According to the manifestation variable, it was correlated with IgE and shown a significant correlation (P=0.002). And for a further statistical testing, one-way ANOVA was done, to represent an even confirmative result that the variability of IgE based on manifestation is highly significant (P=0.006). Comparing the patients group to the controls using T-test, a highly significant result was found with a p-value of ($p<0.001$), which means that values of IgE of patients are significantly high comparing to the healthy controls. Relatively, findings by J. Sunyer et al found out that the difference between patients with current wheezing symptoms and IgE levels (in the terms of allergic rhinitis only) was significant with a p-value of ($p<0.01$) in which it supports this study's findings. (Sunyer et al. 1996a)

According to the family history variable, it was correlated with IgE and shown an insignificant correlation (P=0.463). For a further statistical testing, one-way ANOVA was done, to represent an even confirmative result that the variability of IgE based on family history is significant (P=0.006) and it was unlikely different than correlation. Comparing the patients group to the controls using T-test, a highly significant result was found with a p-value of ($p>0.001$), which means that values of IgE of patients are significantly high comparing to the healthy controls. Moreover, a study by Dávila I, et al showed that 2 groups were involved, patients with and without a family history and patients with a family history. When patients with and without a family history of asthma were analyzed separately, a significant correlation was only detected in the former ($P <0.001$) in patients with a family history vs P=0.412 in patients with no family history. By this, we can establish that this study and the mentioned one both agree on family history being a significant variable when compared patients to the healthy controls. (Dávila et al. 2015)

In this study, it has been found that IgE levels were significantly higher in infants and children with recurrent wheeze in patients group than those of healthy controls ($P>0.0001$). These findings are in agreement with El-Asheer et al as they also found significantly high IgE levels in patient groups with closely the same P-value. (El-Asheer et al. 2016)

In addition, IgA, IgG and IgM were found to be insignificant in comparison between patients and healthy individual, IgA (P=0.190), IgG (P=0.990), IgM (P=0.059) and by these findings suggesting that other immunoglobulins do not differ in the 2 groups (i, e. both patients and controls might have high/low levels and not demanding on whether the case is affected or healthy). The present study's findings come in contradiction with F.I.E. Najam et al, as the study shown a significant elevation of

IgG in patients as compared to controls (P<0.005), therefore, they have also shown a significant elevation of IgE in patients as compared to controls (P< 0.001), IgA and IgM were within the normal ranges, and there were no significant differences were between controls and patients, in which both of these results come in agreement with the present study findings. (Najam, Giasuddin, and S 1999)

According to the complements (C3 and C4), both of them were found insignificant in this study C3 (P=0.443) C4 (P=0.218), meaning both patients and controls might have high/low levels and not demanding on whether the case is affected or healthy. These findings are supported by F.I.E. Najam et al study on Complement Components in Childhood Asthma, in which it has been found that C4 was no different when compared patients to control(P>0.05). Therefore, C3 was found to be significantly high in patients group (P<0.001) by which, this finding disagrees with this study's findings. (Article 2005)

Chapter Five

Conclusion and Recommendations

5.1 Conclusion

This study aimed to assess the correlation between the immunoglobulins (IgA, IgM, IgG, and IgE), complements, the level of eosinophils and development of wheeze chest. All participants were in age of months to 6-year-old divided into 2 groups, patients and controls. Moreover, the study correlated the level of IgE with, IgA, IgG, IgM, manifestation, the numbers of the attacks per year, age, family history, and eosinophilic count. In contrast, insignificant correlation was found for all variables, except for manifestation, the numbers of the attacks and family history, which were found significant to IgE. Measuring IgE, IgM, and IgA in both control and effected children, approved only for IgE only, while disapproved for IgM, IgA, IgG, C3, C4 and eosinophil since there was no significant differences between patients and controls groups.

Furthermore, all IgE positive patients were tested for almost 20 specific allergens, given the result of Dermatophagoides Pter and Dermatophagoides fairnae to be the most abundant allergens among these patients.

Statistically, all of the following tests were used to analyze the data: graphical representation, correlation, one-way ANOVA and T-test

5.2 Recommendations

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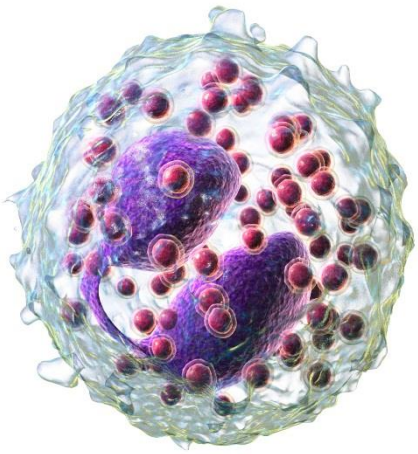

Figure 1: Eosinophil

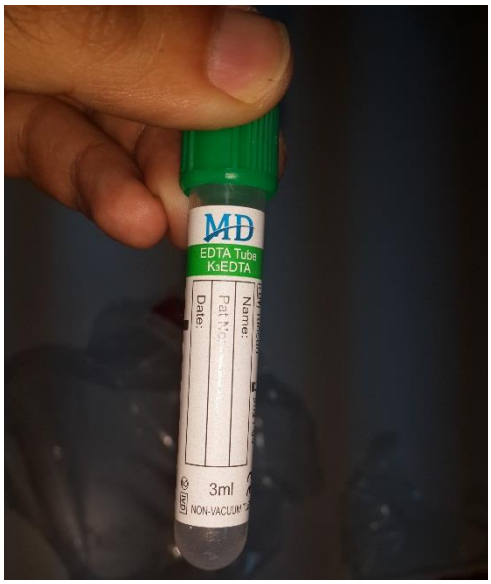

Figure 2: EDTA tube

Figure 3: MICROLIT pipette and tips 1.0 ml

Figure 4: MICROLIT pipette and tips 5 µ

Figure 5: Radial immunodiffusion plate IgA, M, G, C3 and C4 RIDs

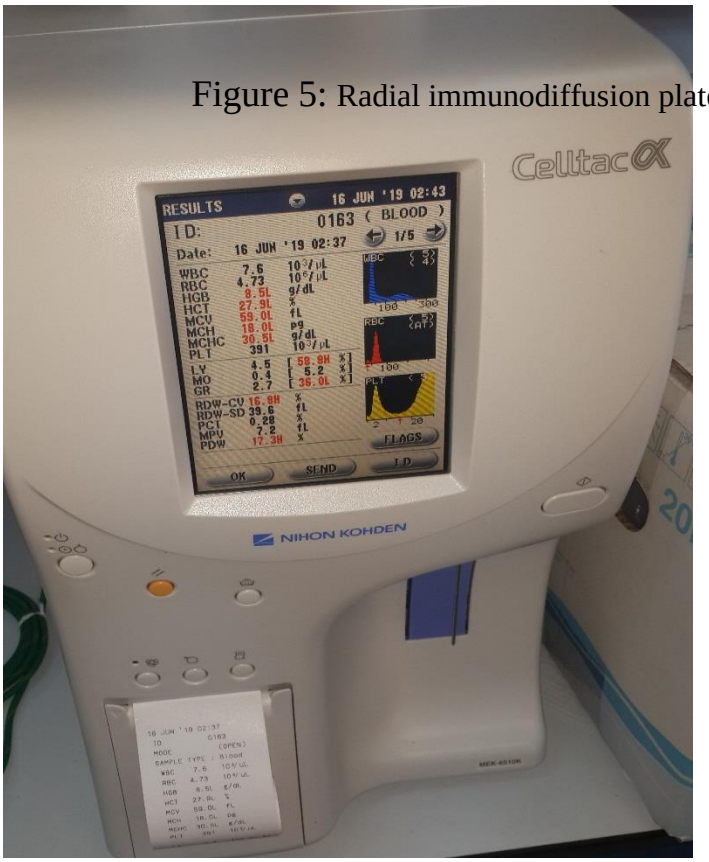

Figure 6: CELLTAC for CBC tests

Figure 7: HERMLE Centrifuge

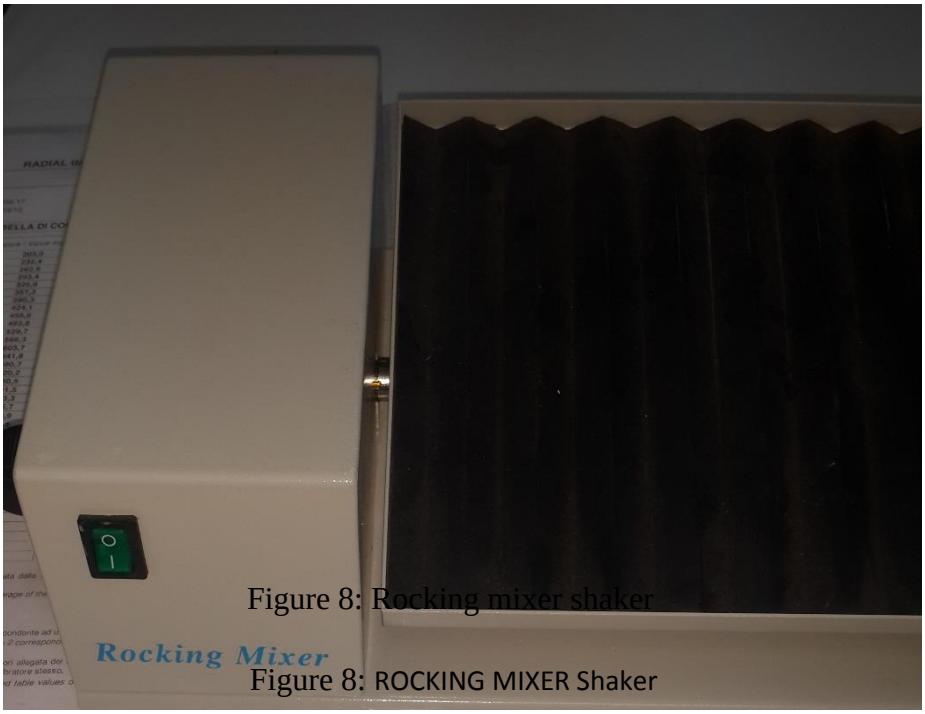

Figure 8: Rocking mixer shaker

Figure 8: ROCKING MIXER Shaker

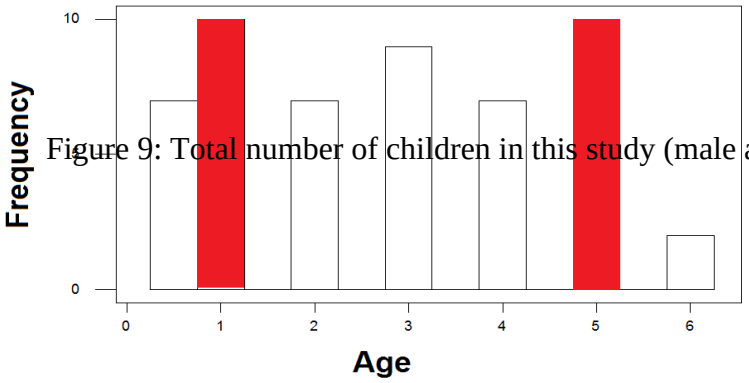

Figure 9: Total number of children in this study (male and female)

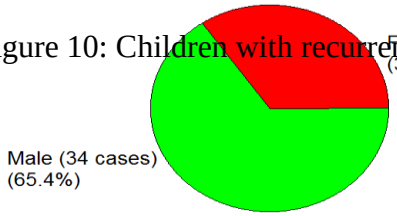

Figure 10: Children with recurrent wheezy attacks

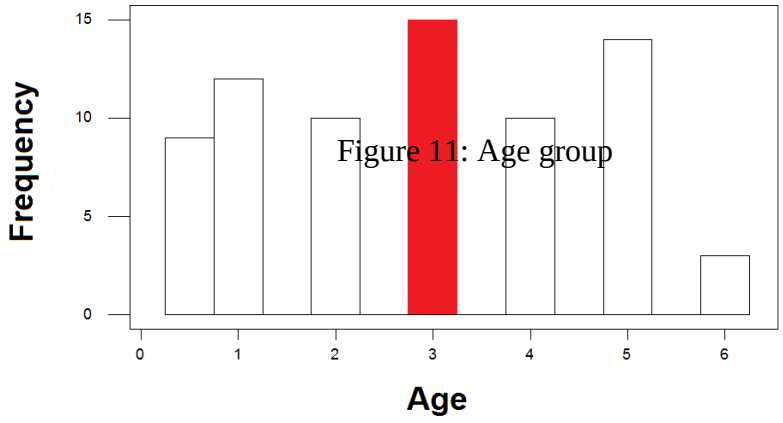

Figure 11: Age group

Figure 11: Age group

Figure 12: Highest age group

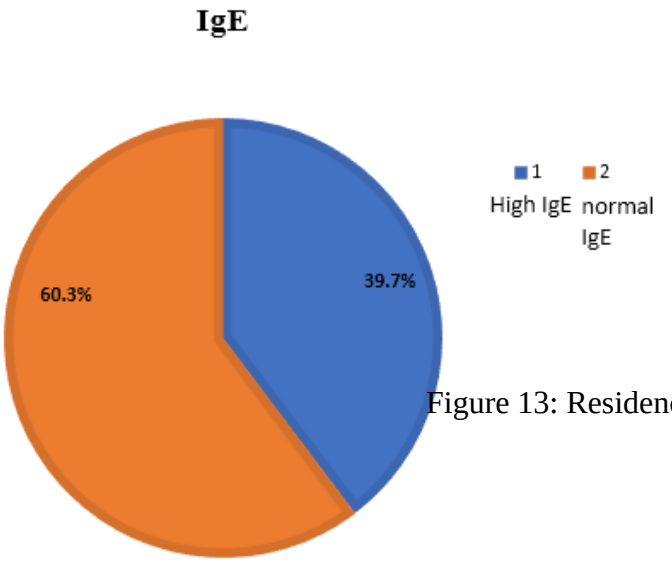

Figure 13: Residence

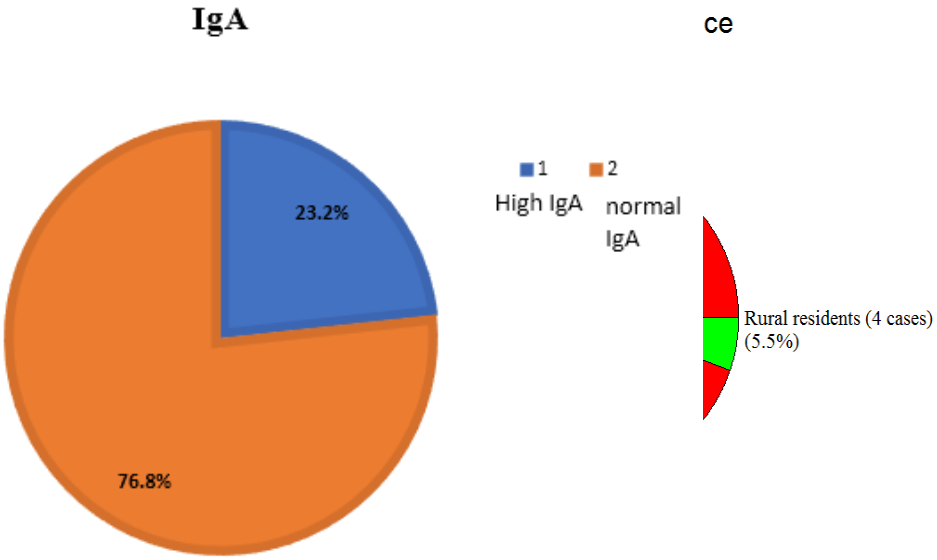

Figure 14: The total amount of the IgE in the all cases (patients and control)

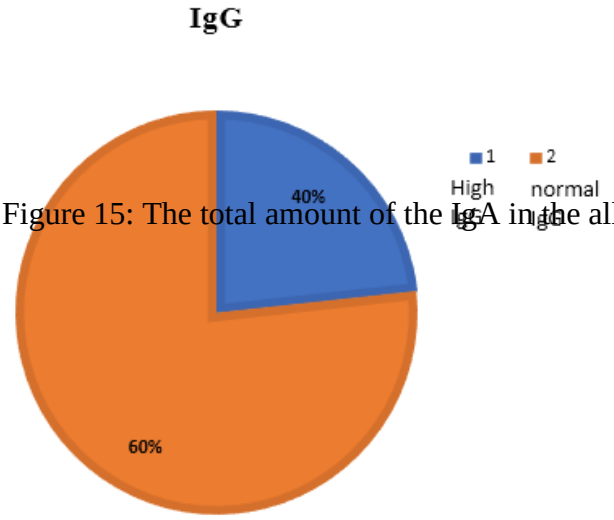

Figure 15: The total amount of the IgA in the all cases (patients and control)

Figure 16: The total amount of the IgG in the all cases (patients and control)

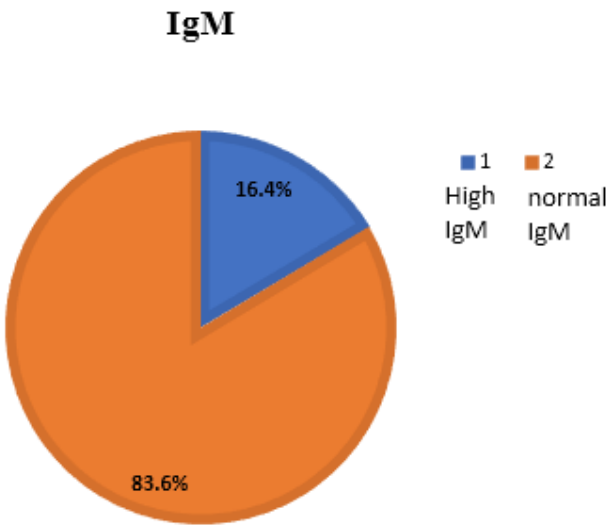

Figure 17: The total amount of the IgM in the all cases (patients and control)

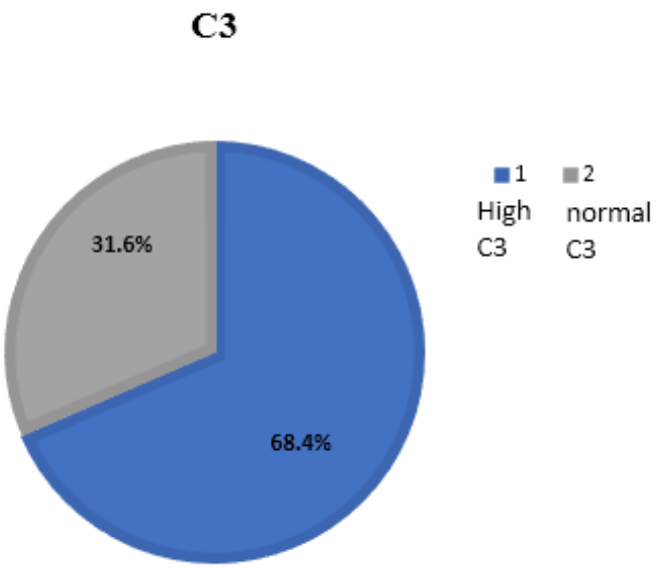

Figure 18: The total amount of C3 in the all cases (patients and control)

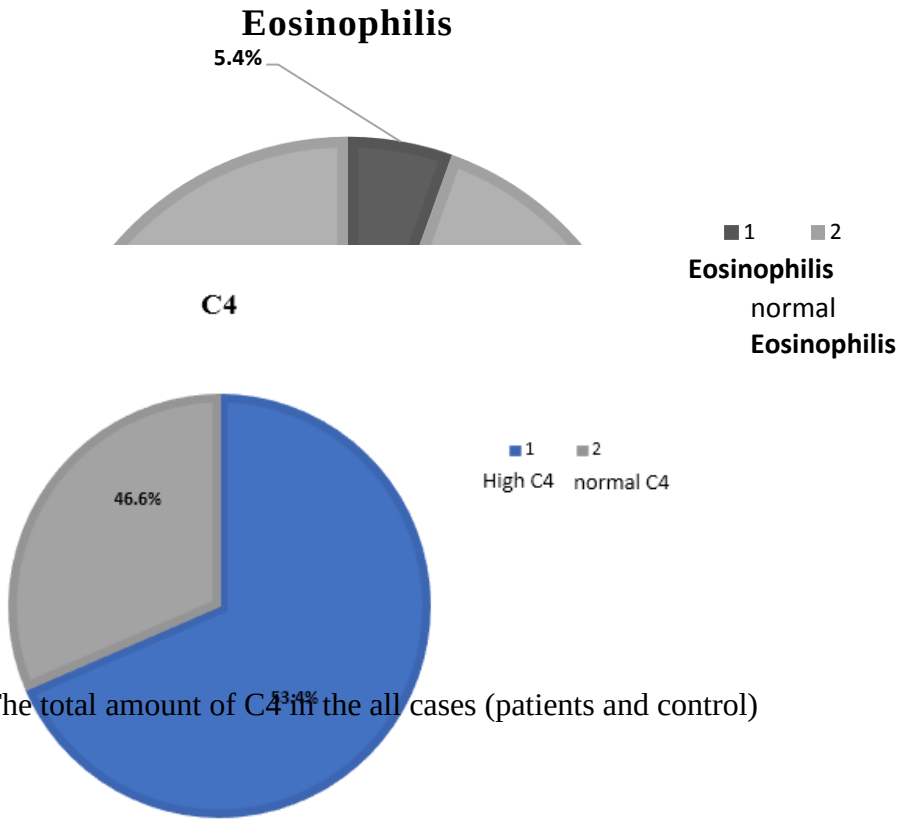

Figure 19: The total amount of C4 in the all cases (patients and control)

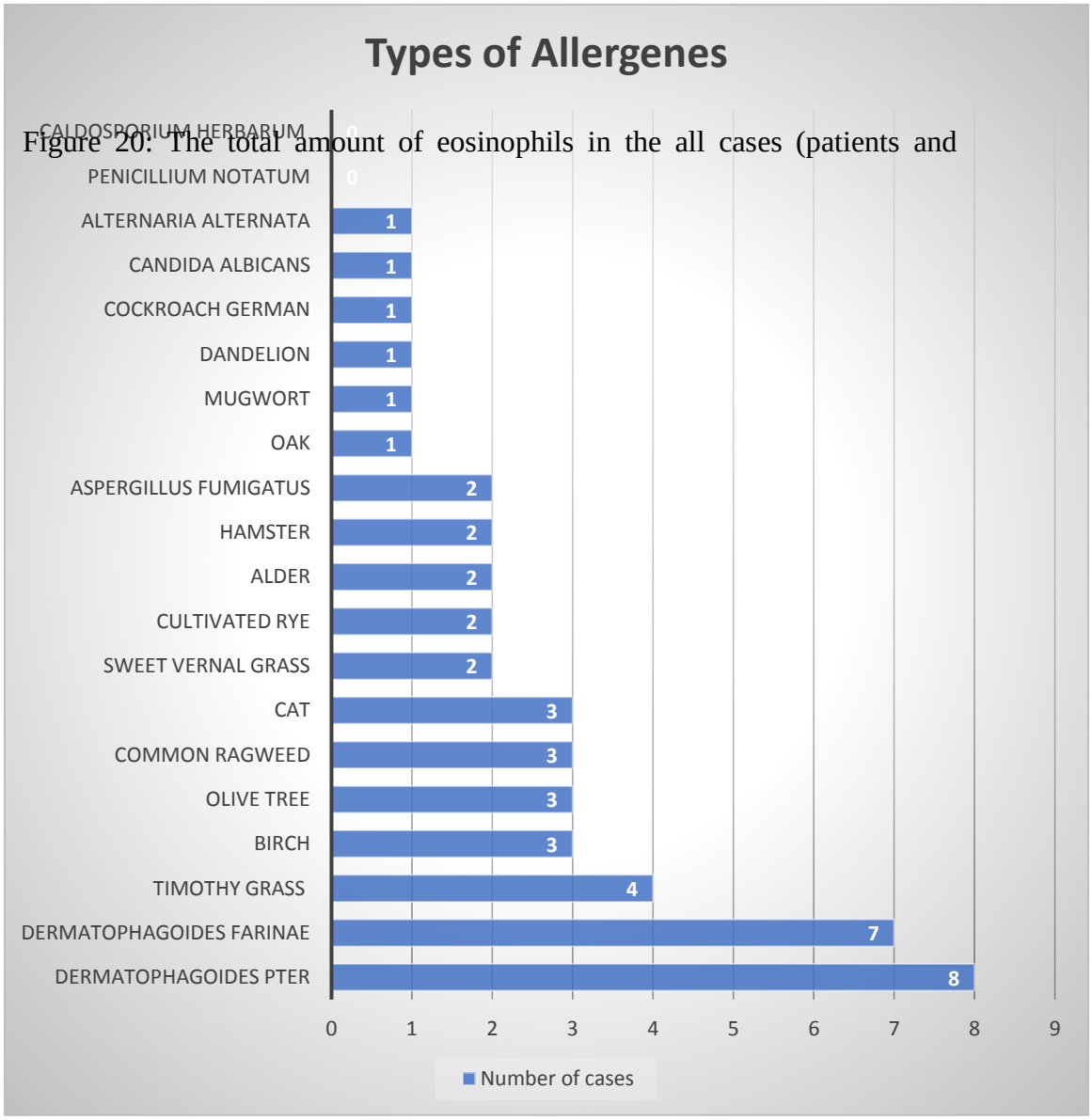

Figure 20: The total amount of eosinophils in the all cases (patients and

Figure 21: Type of allergen