University of Kerbala

College of Science

Department of Biology

Study the Association of Respiratory Syncytial Virus and Rhinovirus Infection with some cytokines and rs6749704 polymorphism in Pediatric Asthma

A Dissertation

Submitted to the Council of the College of Science / University of Kerbala in Partial Fulfilment of the Requirements for the Degree of Doctorate of Philosophy in Biology

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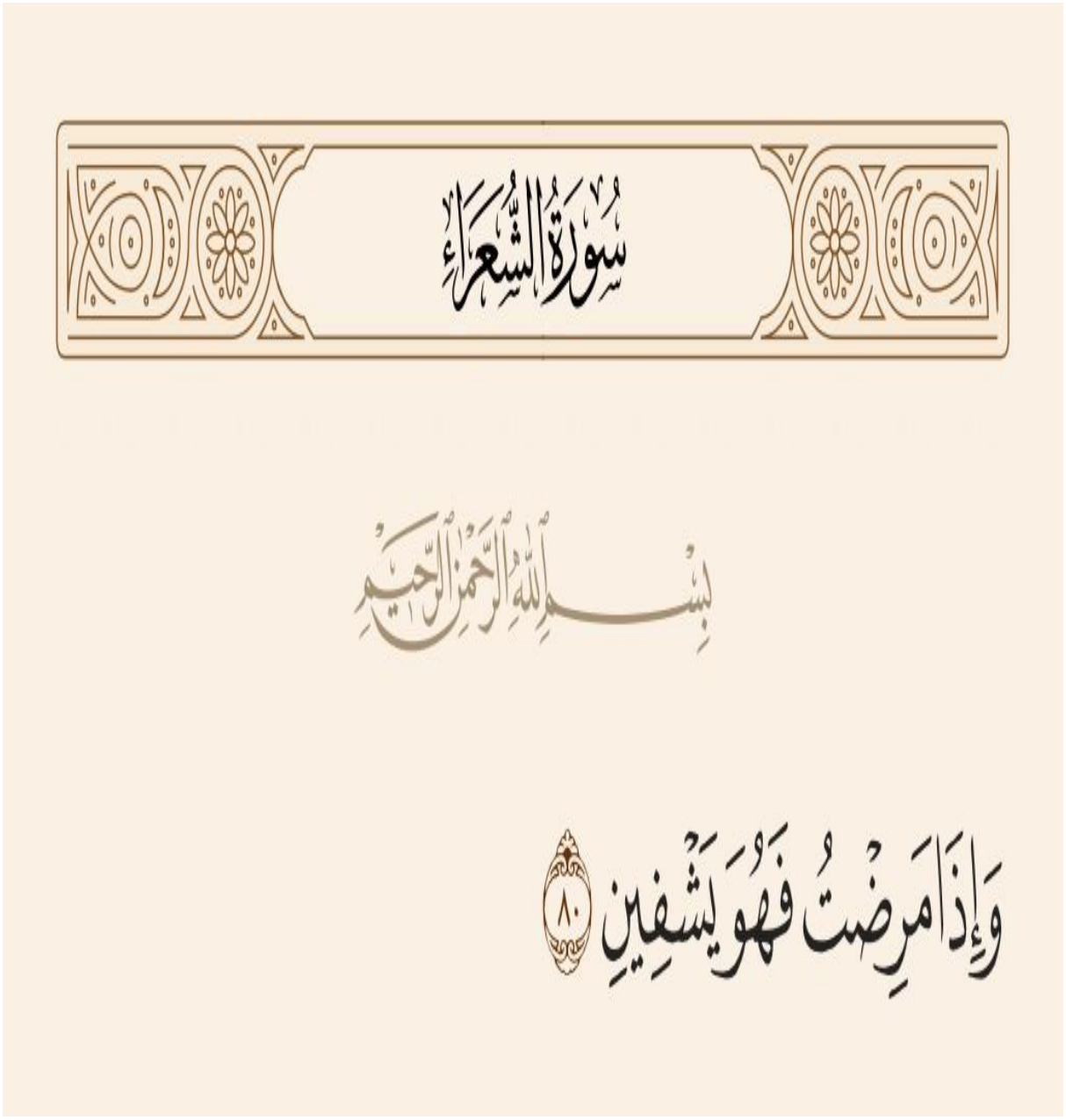

صدق اهلل العلي العظيم

[سورة الشعراء)08( ]

Dedication

To My dear family especially my father and mother My dear brothers and my sisters

My dear wife

To all My dear teachers who had a role in getting me to this point.

Mohammed O.Hamad

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List of Abbreviations

Code	Words
AA	Atopic Asthma
AHR	Airway Hyper-Responsiveness
ABPA	Allergic bronchopulmonary aspergillosis
AE	Asthma Exacerbation
APC	Antigen Presenting Cells
ASMC	Airway Smooth Muscle cells
AFLP	Amplified fragment length polymorphism
AR	Allergic Rhinitis
BPA	Bronchopulmonary aspergillosis
BP	Base Pair
BA	Bronchial asthma
BMI	Body Mass Index
CD4	Cluster of differentiation 40
CD28	Cluster of differentiation 28
CD80	Cluster of differentiation 80
CD86	Cluster of differentiation 86
CD154	Cluster of differentiation 154
CX3CR1	C-X3-C motif chemokine receptor 1
CSR	Class switch recombination
CBC	Complete blood count
CD40L	Clusters for differentiation 40 ligand
CDC	Centres for Disease Control and Prevention
CLB	Cell Lysis Buffer
Chr.	Chromosome
CWD	Column Wash Solution
CCL20	Cysteine-cysteine motif chemokine ligand 20
CXCL10	C-X-C motif chemokine ligand 10
CysLT1R	Cysteinyl Leukotrienes Receptor
CSR	Class switch recombination
DNA	Deoxyribonucleic acid
DCs	Dendritic Cells
DHPLC	Denaturing high performance liquid chromatography
ECRHS	European Community Respiratory Health Survey
ELISA	Enzyme Linked Immuno Sorbent Assay
EDTA	Ethylene Di amine Tetra Acetic Acid
EC	Eosinophil Count
FcεRI	Fc epsilon Receptor I

FEV1	**Forced Expiratory Volume**
GM-CSF	**Granulocyte Macrophage-colony Stimulating Factor**
GINA	**Global Initiative for Asthma**
GRCh38	**Genome Reference Consortinum38**
GBD2019	**Global Burden of Disease 2019**
HRV	**Human Rhinovirus**
HDM	**House dust mites**
HRP	**Horse Radish Peroxidase**
HRM	**High resolution melting**
HWE	**Hardy Weinberg Equilibrium**
HCSIF	**Human Cytokine Synthesis Inhibitory Factor**
ICSs	**Inhaled Corticosteroids**
IRES	**Internal Ribosomal Entry Sites**
IP10	**Interferon γ -induced protein 10**
IL3	**Inter leukine-3**
IL5	**Inter leukine-10**
IL13	**Inter leukine-13**
ILC2	**Innate lymphoid cells 2**
IgE	**Immunoglobulin E**
JAK	**Janus kinase**
LRT	**Lower Respiratory Tract**
LPS	**Lipopolysaccharide**
LABA	**Long-acting Beta2-agonists**
MS	**Multiple Sclerosis**
MHCII	**Major histocompatibility complex class II**
MALDI-TOF	**Matrix-assisted laser desorption/ionization time-of-flight**
OR	**Odds Ratio**
O.D	**Optical Density**
OPRFs	**Overlapped open reading frames**
PCR-RFLP	**Polymerase Chain Reaction-Restriction fragment length polymorphism**
P14	**Patch 14**
PEF	**Peak Expiratory Flow**
PK	**Proteinase K**
Pg	**Picogram**
PCR	**Polymerase Chain Reaction**
RFLP	**Restriction Fragment Length Polymorphism**
RPM	**Round per minutes**
RSV	**Respiratory Syncytial virus**
RA	**Rheumatoid Arthritis**
RDRP	**RNA-dependent RNA polymerase**
RANTES	**Regulated upon activation, normal Tcell expressed& secreted**

RNP	**Ribonucleoproteins**
RPM	**Round per minutes**
sIAM1	**Soluble Intracellular Adhesion Molecule-1**
SA	**Saudi Arabia**
SCYA20	**Small Inducible Cytokine A20**
SLE	**Systemic Lupus Erythematosus**
SPSS	**Specific Software Statistical Package for the Social Sciences**
SABA	**Short-acting beta-2-agonist**
SICB	**Small-inducible cytokine B10**
SNPs	**Single Nucleotide Polymorphisms**
SCs	**Systemic Corticosteroids**
SSCP	**Single-strand conformation polymorphism SSCP**
Th2 cell	**T-helper 2 cell**
Treg	**T regulatory**
TBE	**Tris-boric acid–EDTA**
TGF-β	**Transforming Growth Factor Beta**
TLR	**Toll Like Receptor**
TSIgE	**Total serum Immunoglobulin E**
US	**United States**
µl	**Microliter**
UAE	**United Arab Emirates**
URI	**Upper respiratory tract infections**
UV	**Ultra-Violet**
VPg	**Viral Protein Genome**
WHO	**Word Health Organization**

List of appendices

Summary

Immunological and molecular study was performed on asthmatic children infected with RSV and HRV to detection the association between Respiratory Syncytial virus (RSV) and Human Rhinovirus (HRV).infections and genetic polymorphism (rs6749704) in *CCL20* , and subsequent alteration in immune response with disease and severity.

The present study is a case-control study conducted on 100 in / and outpatient's distributed between 50 atopic asthma (35 male and 15 female) and 50 non atopic (31 male and 19 female) who attended the consulting clinic for respiratory diseases and asthma in Kerbela Teaching Hospital for Children during the time period extending from February to June 2022 in Kerbela province. Their ages were between 1-6 years. Fifty healthy children (32 male and 18 female) with the same age and sex to the patients, were selected from the local community were considered as the control group. The characteristics and clinical data were collected from consultants, asthmatic children and /or their parents through a questionnaire. In addition, a questionnaire was made to collect the data from healthy control and /or their parents.

Sera of patients' and healthy control' blood which were used to qualitative determination of Respiratory Syncytial virus -IgM, Human Rhinovirus -IgM and measure concentrations total serum immunoglobulin E (TSIgE), CCL20, CXCL10, IL-10 and TGF-β for all samples by Enzyme Linked Immuno Sorbent Assay (ELISA) technique. Whilst, the second part of the blood was placed in EDTA tubes to determine complete and differential blood count including percentage of (eosinophil and neutrophils count) by using blood film and (Sysmex XN-350 five differential automated hematology analyzers, and for the genomic DNA extraction. The DNA extraction was used to detect *CCL20* (rs6749704) single nucleotide polymorphism (SNP) by Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR- RFLP).

In the present data, a total of 24 child with asthma infected with RSV and HRV, distribution 10 as RSV and 14 as HRV.

The obtained result showed there was a highly significant association between TSIgE concentration in atopic asthmatic patients and mean rank (275.77)IU/mL while in non-atopic was (36.17) IU/mL compared to healthy control(11.153) IU/mL. Regarding, The mean rank of percentage eosinophil and neutrophil in atopic patients were (6.2), (27.87) and mean rank of non-atopic were (2.1), (52.34), there was a highly significant association between atopic, non-atopic asthmatic patients and healthy control(*P*=0.0001).Whereas in healthy control were (1.7) (48.42) (*P*=0.0001). Further there was a highly significant association between increasing CCL20 serum concentration in asthmatic patients compared to healthy control, and the mean rank of CCL20 in atopic patients was (52.2 pg/ml), non-atopic patients (35.7pg/ml) and mean rank of healthy control was (23.9pg/ml). Additionally, eosinophil and neutrophil count and TSIgE level were higher in the moderate compared to mild asthma in the atopic and non-atopic($P = < 0.05$).

The results of the current study showed significant differences in the distribution of the CC genotype of *CCL-20* (-786T>C) between the atopic, non-atopic asthmatic children and control group (*P*=0.000). Therefore, the CC genotype could be considered a risk factor for developing atopic and non-atopic asthma in Iraqi children. Genotypes analysis by using Hardy-Weinberg distribution showed significant differences between observed and expected patient numbers for *CCL-20* (-786T>C) in the non-atopic patients but not in the atopic group. In conclusion, a high incidence of asthma occurred between males compared to females children and suggested that *CCL20* (-786T>C) promoter polymorphism may contribute to the predisposition of asthma and there was a significant association among genotype of this gene and level of CCL20 and development of the disease in children infected with RSV and HRV.

A family history of asthma is considered a risk factor. CCL - 20 and CXCL10 are significantly associated with the development of asthma in children infected with RSV and HRV.

An increased risk of asthma was associated with the CC genotype of *CCL-20* (-786T > C) that was revealed as an etiological with the risk by their association with high CCL-20 level , high percentage eosinophil and neutrophil count , and distribution in the atopic and non-atopic respectively in asthmatic children more than in the control group.

Chapter One
Introduction
and
Literature review

1. 1. Introduction

Asthma is an airway inflammation disease characterized by variation airflow limitation and signs of respiratory, also causing Airway Hyper-Responsiveness(AHR), reversible blockage, mucus over-production, eosinophil and Th2 infiltration and modification of airway wall with a prevalence ranging 1-21% globally (Assis *et al*.,2019). It has affected an estimated over260 million people in 2019, according to estimates of the Global Burden of diseases (GBD) 2019 (Vos *et al*., 2020). Whilst in Iraq was roughly15.8% among children below 5 years old (Salem *et al*., 2002).The known signs of respiratory in children with asthma include cough, wheezing, shortness of breath, and chest tightness. Worsening of signs comes as a result of the presence of risk factors and comorbidities. Delay in diagnosing and managing comorbidities can ultimately lead to poor asthma management, a decline in pulmonary function, and an increased risk of unpredictable exacerbation and hospitalization (Reddel *et al*., 2022). Wheezing and asthma are affected by viral infections in children, wheezing diseases in infants are typically viral in origin, and children who suffer more severe wheezing attack are more likely to have asthma flare-ups frequently and to acquire the disease later in childhood (Mikhail and Grayson,2019).

Syncytial virus (RSV) and Human Rhinovirus (HRV)can cause severe lower respiratory tract infections, especially in the infants hospitalization. The determinants of the outcome of RSV and HRV infections are not fully known, but both viral and host factors play a part, in moderate-to-severe persistent asthmatics, viral infection was associated with increased CCL2, CXCL10, sICAM1, CCL4, CCL5, CCL20 and CCL24, however virus positive sample with mild intermittent asthma showed no important increase in protein expression

(Song *et al*., 2014). The immune responses towards virus infection is important for the viral control and clearance is crucial, however, if not properly regulated, it may lead to immunopathology (Roy Wong and Perlman, 2022). Immunity of lung to asthmatic children having RSV and HRV starts with the attraction of immune system cells into the lungs, the regulation of the inflammatory process is primarily governed by chemokines and cytokines, which are diminutive proteins synthesized in reaction to the innate immune system's recognition of viral or infectious agents (Nuriev and Johansson, 2019).

Chemokines are regarded as chemo-attractants for immune cells, changes in the chemokine profile result in substantial dysregulation of immune responses, inadequate or misdirected immunity that cause increased viral replication and viral defect to lung tissue, airway inflammatory response of asthma is known to be related with release of a number of inflammatory mediators and cytokines, and the later play pivotal role in organization of immune response. However, both pro-inflammatory and anti-inflammatory cytokines are affected by hereditary factors (Luu Quoc *et al*., 2022; Sulfiana and Iswanti, 2022). In fact, the clinical signs of asthma perhaps commutates a disequilibrium in pro-and anti-inflammatory cytokines concentrations (Oliveira *et al*., 2023).

TGF-β is a cytokine that dominates proliferation, cellular differentiation and other functions in most cells, also a basic mediator that contributes to pro-inflammatory responses and fibrotic tissue modification within the lung in asthmatic children. It is a multiple functional cytokine that has been related to the pathogenesis of sub-epithelial fibrosis and airway wall modification in bronchial asthma, IL-10 is immune-regulatory cytokines produced by Treg cells and monocyte, IL-10 and TGF-β are

contributed in the arrangement of Th1 and Th2 responses in both non atopic and atopic asthmatic children patients below respective immunotherapy, and asthmatic children who perhaps trouble in the function of Treg (Hori, 2021). On the other hand, CXCL10 also known as IP-10 or SICB10 is an 8.7 kDa protein that in humans is encoded by the CXCL10 gene. CXCL10 is a small cytokine belonging to the CXC chemokine family, it is a chemokine related to dendritic cells (DCs) conscripting during viral infection, which can organize sneak of effector T-cells follow upto the lungs(Goritzka *et al.*,2015;Elemam *et al*., 2022).

C-C motif ligand 20 (CCL-20) is a small cytokine from the CC chemokine family, a powerful chemotactic for leukocytes especially lymphocytes, and weakly attracts neutrophils, further, contributes to the composition and functionality of mucosal lymphoid tissues by chemo-attraction of DCs and lymphocytes against the epithelial cells ambient these tissues. It takes away its effects on its cells by linking and stimulating the chemokine receptor CCR6, (CCL20 are pivotal role in the function of the innate immune system).They also conscript innate immune effectors out of the circulation and into the tissue where, in cooperation with other chemo-attractants, they guide these cells to every site of tissue injury. Chemokine plays an important role for the positioning of innate immune sentinels in peripheral tissue, then immediately after innate immune activation, directs these stimulated cells to drain lymph node in order to start and establish an adaptive immune response (Sokol *et al*., 2015). SNPs in the human genome are the most significant and fundamental type of variation in the genome.

It is responsible for genetic changes that generate vulnerability to the majority of autoimmune illnesses such as relationship between asthma susceptibility in various ethnic groupings and SNPs in the genes for

chemokines and their receptors(Jackson *et al*., 2018; Micheal and Grayson., 2019). SNPs in *CCL20* are associated with other diseases in children and adults, the genetic variations in *CCL20* (-786 T>C) are thought to play a role in the expression of inflammation, as well as they relate to other serious diseases such as RA, multiple sclerosis and ischemic heart disease ,further, SNP was utilized as a genetic marker in the risk factors of diseases(El Sharkawi *et al*., 2019). The current study concentrates on CCL20 and CXCL10 as there is scanty studies on them across the world and no available information for them in Iraqi pediatric population. Greater understanding of relationship among RSV and HRV infections, atopic inflammation and asthma, as well as to an important difference in development of atopic and non-atopic asthma, with several proposed mechanisms explaining association among viral infections, development of asthma and asthma, exacerbations. Understanding these complex association is important for developing asthma prevention strategies and targeted asthma therapies.

Therefore this study aims to elucidate effects of RSV and HRV infection on levels of these former chemokines and subsequent alterations in immune response with disease course, and severity in both atopic and non-atopic asthma. This is achieved through the following objectives:

1. Immunological detection by ELISA for both RSV and HRV by specific IgM in patients and control group.
2. Measurement of TSIgE, CCL20, CXCL10, TGF-β and IL-10 concentration in patients and control group by using ELISA.
3. Correlation of viral infections and CCL20, CXCL10 levels with atopic and non-atopic asthma by using ELISA.
4. Detection of CCL20 (rs6749704) gene polymorphism by PCR-RFLP.

1. 2. Literature Review

1. 2. 1. Asthma Overview

Asthma is the most common chronic lung condition disease in children in which the airways narrow and become inflamed, it is a heterogeneous, multifactorial disease with variable and mostly reversible airway obstruction due to spasms and secretions in the bronchi always resulting from an allergic reaction or hypersensitivity and then lead to difficulty in breathing, rhonchus, wheezing, cough, chest tightness, the severity of these signs different from asthmatic children patients to another patient and time to time, particularly early the morning or late at night (Asher and Pearce, 2014; Grose, 2018). Symptoms episodes are generally related to widespread, but variable, airflow obstruction within the lungs which is usually reflected either spontaneously or with suitable asthma treatment such as bronchodilator (Hizawa, 2023).

It appears that there is an association to a Th1-Th2 unbalance which is affected by decreased and changed microbial exposure, excess obesity, epithelial microbiome variable and nutritional factors, although the hereditary aspects of disease have been broadly investigated and many candidate genes diagnosed epigenetic and environmental factors seems to play a basic portion in phenotype expressiveness (Luo *et al.*, 2022). Asthma is a long-term condition and universally health complex problem, chronic inflammatory illness of the LRT and non-communicable diseases affected people,virtually 300 million individuals of all ages, ethnic groups and countries during the world (Majellano *et al*., 2019). Asthma is still poorly controlled although a broad array of update treatment available for using, also 5-10% of asthmatic patients

have a weak response to Inhaled Corticosteroids (ICSs) and stay on elevated doses of (SCs) (Heffler *et al*., 2019; Corren *et al*., 2022).

1. 2. 2. Classification

Asthma has been divided into extrinsic (atopic) and intrinsic (non-atopic) asthma, both types cause the same symptoms, the difference between them, including the causes and triggers asthma symptoms, the treatments are similar for each type, although the prevention strategies differ, atopic asthma is deemed as the most prevalent form of asthma in childhood (Froidure *et al*., 2016). It always starts after various triggers predispose, its characteristics by eosinophilic airway inflammation binding to the positive IgE antibodies, as apparent by serology or skin compunction testing. On the other hand, non-allergic asthma in children is advanced with age and a family members history of atopic sensitization which is absent. Intrinsic (non-allergic) asthma caused via viral infections, cold, humidity, chemicals, smoking and perfume smells, which forms a percentage of 10-40% out of asthmatic patients (Chau-Etchepare *et al*, 2019).

Atopic asthma has a genetic correlation to increase IgE and eosinophil production, also children patients have mast cell linking IgE molecules that are found in their airways (Froidure *et al*. , 2016). Furthermore, if one parent is atopic, the recurrent child's risk for asthma is 25%, maternal asthma results in early asthma development, also paternal asthma affects asthma development later in life with declined risk of it development over time, in comparison, when both parents are asthmatics, child's risk for asthma development is 50%, increasing over time, moreover, number of asthmatic siblings seems to affect the child asthma risk (Paaso *et al*., 2013). On the basis of frequency and severity

of clinical signs, asthma has been classified into eosinophilic and noneosinophilic (neutrophilic and paucigranulocytic) depending on etiology and underlying inflammation (Yamasaki *et al*., 2022). Asthma has been divided according to (GINA) into intermittent, mild persistent, moderated persistent, and severe persistent. WHO classified severe asthma into untreated severe asthma, difficult treat asthma and treatment resistant severe asthma (Bai *et al.*, 2007). Some children who identified with asthma find it improves or disappears completely as they get older, this is known as childhood asthma which influence around 1.1 million children in UK, bear in mind, though, that it can return later in life, especially if it's moderate or severe rather than mild (Padem and Saltoun, 2019).

Individuals with this illness may categorized as allergic mediated by IgE, atopic asthma is produced by allergens like pollen, pets, dust mites and 80% of people with it have a related condition like hay fever, eczema or food allergies, whereas non-atopic asthma doesn't link to an allergy stimulators like pollen, dust, pets and less common than allergic asthma, and causes of it are not well understood. It develops later in life and can be more severe and caused by viral URTI such as RSV and HRV, occupational, aspirin, diclofance exacerbated respiratory disease, exercise induced, cough variant asthma (Padem and Saltoun, 2019).

1. 2. 3. Epidemiology of Asthma

1. 2. 3. 1. Prevalence and Incidence

Asthma is a global health problem that affects about 300 million individuals of all ages, ethnic groups and countries currently suffers from it in worldwide, according to WHO estimates, approximately 250,000 people die prematurely each year from asthma (D'Amato *et*

al., 2016). It has been estimated that more than 25 million children and adults in the US have it , and epidemiological studies have shown that asthma is less prevalent more in men than women, and it is more prevalent and severity among children, especially males versus females through various ages (Fuseini and Newcomb., 2017).

In the study of Saglan *et al*., (2022) showed that the prevalence of asthma was less in females than in males. The prevalence has been increasing at an alarming rate and has more than doubled in the last decade, in addition it is prevalence is estimated to be 3-38% in children and 2-12% in adults (Cavkaytar and Sekerel, 2014). It is varies significantly in different regions of the world, for example, in the US over 9 million children have been diagnosed with asthma, of which 75% have active illness and it has different prevalence among ethnic groups, which is more prevalent (11%) among African American of all ages and African American children (17%). However, a few studies that fulfilled these criteria have delineated asthma trends among children and adults, the spread incidence of asthma has been increased both locally and global with a many of risk factors being linked to it (Amoah *et al*., 2012).

Asthmatic patients usually were males in the US compared to the girls. This case in early age was increasing among these children, with a higher spread in preschool and school-age children in comparison more than 10 years of age , further, the percentage of annual asthma spread is less in adults (7.9%) than children (8.1%) (Akinbami *et al*., 2016; Pate *et al*., 2021). Different countries have prevalence of asthma reported in the range of 2.4-18.4%, such as in a Swedish study conducted in 2008, the prevalence of asthma was found to be 11.8% (Rönmark *et al*.,2016). The prevalence of asthma was 11.0% (95% CI: 8.9–13.2; males 10.3%,

females 11.4%, urban 13.0% and rural 8.9%. The study of Wang *et al*., (2012) showed that prevalence of asthma in China was found to be 3.15 times higher in participants with a history of allergic diseases in the mother than in non-allergic mothers, whilst, there are a limited epidemiology studies on the asthma prevalence and incidence in Iraq. In the UAE, the prevalence was 13% out of the study included 3200 children, frequency of wheezing was higher in males than in females. In a study conducted with the ECRHS survey in Saudi Arabia in 2016, the frequency of wheezing with in12 months was 18.2% and the difference between men and women was not significant while in Kuwait was 11.9 (Ziyab, 2017; Al Ghobain *et al*., 2018). The prevalence of asthma at 5-6 years of age, an important increase is observed in cumulative incidence of wheezing and recurrent wheezing up to 3 years of age followed by stabilization, full-term pregnancy and the minimization of respiratory infections at an early age could reduce the prevalence of asthma at the age 6 years (Alfonsoa *et al*., 2020).

1. 2. 3. 2. Effect Sex and Age on Asthmatic Patients

Asthma is the most common chronic condition in children and adults in western countries, affecting 1 in 7 children, whilst 1 in 12 adults, most children who develop symptoms of asthma are <5 years of age, the disease is a frequently of misdiagnosed or not suspected in infants and toddlers. In addition, the prevalence of asthma is different in males and females throughout their lifespan, additionally boys in particular are more likely to develop asthma than girls, this pattern is reversed after puberty, this indicates to sex-specific factors, such as fluctuations in hormone levels, could play a role in x-disease's pathogenesis. Asthma is higher prevalence and incidence in boys than girls seen before puberty. Many studies referred that 30% of the asthma patients are before 14

years old, In addition, children with group aged 1-9 years especially 6-9 had a higher risk ratios for asthma (Lin *et al*., 2017; Naeem and Silveyra, 2019).

1. 2. 4. Risk Factors of Asthma

Asthma is a condition that is likely caused by gene-environment interactions, therefore development of childhood asthma is complex with a strong interaction of genetic, epigenetic, and environmental factors, it is critical how the immune system of a child responds to these influences and whether effective strategies for a balanced and healthy immune maturation can be assured., pregnancy and early childhood are particularly susceptible for exogenous influences due to developing nature of a child's immune system, in contrast, endogenous influences such as family history and the genetic background are immutable, Prenatal influences such as mother's nutrition, smoking and infections affect complex interplay of innate and adaptive immune regulation as well as peri and postnatal influences including mode of delivery (Mims, 2015; Krusche *et al* ., 2019).

1. 2. 4. 1. Hereditary

Asthma has a genetic ranging from 35-95%, as well as hereditary studies have diagnose hundreds of genetic differences related to an increased risk of asthma in children, furthermore epigenetic variants in the way the genetic code is translated have also been linked to the advance of asthma, further many genes are correlated to many allergic features, including TSIgE, atopy and allergic diseases. Asthma and atopic phenotypes are caused by hereditary changes in a common immune regulation pathway and stimulated Th2 cytokine secretion by epithelial cell cytokine (Ober and Yao, 2011; Han *et al*., 2017). If

someone has a parent with asthma, one will acquire asthma 3-6 times more than another someone without parent with asthma (American Lung Association, 2019).

2. 4. 2. Asthma and Allergen

Allergy is defined as disease following a response by the immune system to an otherwise innocuous antigen, while allergens are antigens that elicit hypersensitivity or allergic reactions (Zhang and Ailin , 2015). An antigen is any molecule that can bind specifically to an antibody or T-cell receptor, the name arises from the ability to generate antibodies. Allergens can enter the body via the skin, airways or gastrointestinal tract (Janeway *et al*., 2005). Asthma is an atopic illness, and it is the most common kind of asthma particularly in children allergens exposure leads to the development of allergic inflammation, allergy is the main element that causes asthma predisposition (Der Sarkissian, 2019; Miteva *et al*., 2023).

In predisposed individuals, initial exposure of professional antigen-presenting cells to allergen causes the activation of allergen-specific Th2 cells and IgE synthesis, known as allergic sensitization. Subsequently exposures to allergen lead to the infiltration of inflammatory cells causing early allergic responses and late allergic responses. Early- phase reaction is an IgE-mediated and it can occur within minutes of allergen exposure (Li *et al*., 2022). Ninety percent of children with childhood asthma have a topic, compared with about 50% of adults with asthma and signs of atopic asthma looked after inhaling allergens like pollen, dust mites, mold and it generally worsens after exercising in cold air, inhaling smoke, dust and pollutants a strong odor, exercising in cold air and humidity (Han *et al*., 2017).

1.2. 4. 3. Passive Smoking

Passive smoking is an influencing factor in advancing asthma, also active smoking has shown to be a risk factor for developing atopic asthma, demonstrating that there was a dose-response correlation between the effect of smoking exposure and the hazard of updated onset asthma. Also, several studies such as Den Dekker *et al*.,(2015) illustrated a clear correlation among smoking and an increased risk of advancing asthma in children, and study Alfonso *et al*., (2020) have also showed that potent association between parental and maternal smoking and advancement of asthma in children depending on predispose cigarette smoke which has associated with early childhood asthma signs. In addition, childhood exposure to passive smoke has been shown to be correlated with an excess risk of advancing asthma as an adults. Beside to the influence of exposure to prenatal or postnatal passive smoke was recorded at 21-85%, which excess risk of occur wheezing and asthma in children, in addition to the frequency of asthma at 3-6 years of age and connected with smoking exposure a clear hazard factors childhood (Boskabady *et al*., 2022).

1. 2. 4. 4. Changes in Weather

Asthma correlates with various weather changes that cause its aggravation, such as air quality (high humidity), air temperature, irritants, allergens and atmospheric pressure. There are more asthma allergens in the air after storms, dry winter air, breathing in cold may be challenging, on chilly days, breathing by the nose assists in warming and humidifying the air before it enters the lungs cold (D'Amato *et al*., 2018). In addition to the cold , dry air which is a consequent of asthma stimulator that can lead to severe symptoms, especially in those who

take part in winter sport, also sand storms were stimulate asthma sign. Moreover, windy and wet weather which induce mold growth and pushes pollen and mold into the air can aggravate asthma sign (D'Amato *et al*., 2018).

1.2. 4. 5. Psychological Factors

Asthma is an autonomic disease that is associated with stress and includes excess sadness and anxiety, stress is an emotional and physical state that can contribute to the initial of asthma. It can also exacerbate flare-ups in individuals who already have an asthma identification (Nasiri Kalmarz*et al*., 2022).

Psychological factors, particularly chronic stress, can influence the effectiveness of asthma, and asthma activity in children with parental stress levels, psychological stress perhaps exacerbate asthma sign, because there is a potent association between asthma and psychological problems, unessential increase pressure and/or exercise should stay away, due to it is thought that excess the inflammatory response of the respiratory tract in response to allergens and triggers. The resulting changed neuroimmune responses perhaps influence the expression of immune-mediated problems like asthma also, enhance an individual's sensitivity to several environmental factors that perhaps participate to asthma aggravation hazard (Teijeiro and Gómez, 2021).

Previous studies like Lovell *et al*., (2011) indicated that cortisol, which is a by-product of the sympathetic nervous system, namely the hypothalamus pituitary cortical axis, further has a detrimental effect on the function of the immune system, the drop in cortisol and excess serotonin concentrations, can relieve stress with better sleep and can assist with worsen breathing.

1. 2. 4. 6. The Diet System

Breast-feeding in the infants protects against the advancement of atopic disease at progress age, particularly in children with allergic . Genetic studies revealed that infants who are fed cow's milk or soybeans are subject to an occurrence of wheezing in early childhood is more consequently greater contract to those fed breast milk(Adeyeye *et al*, 2019). Diet is a key source of allergen predisposition in asthmatic children, some data reported that dietary habits in western states such as the excess use of foodstuffs ready-made, reduction antioxidants (from fresh vegetables and fruits) participate to the present excess. For allergic and asthmatic illness, found allergen foods that can provoke asthma attack such as nuts, peanuts, shellfish, fish, eggs and dairy products. Some people get wheezy after eating specific foods, such as eggs, cow's milk, soy, fish, shrimp, shellfish, salads and fresh fruits (Adeyeye *et al*, 2019).

1.2. 4. 7. Body Mass Index (BMI)

The study conducted by revealed liÖhman Magnusson *et al*.,(2015) a strong correlation among BMI and asthmatic children whether females and males, and it is refers to that elevated BMI through the first 4 years does not excess the danger of asthma at school age between children who have advanced a normal weight by age of 7 years. Though, elevated BMI at age of 7 years is connected with an excess hazard of asthma and sensitization to inhalation allergens, not just are obese young individuals more likely to have asthma, but they perhaps also be more likely to have serious asthma, participating to a potential demand for health care and effective asthma medication (Oudjedi *et al.*,2020). Obesity is the most common asthma co-morbidity, exacerbation

respiratory signs and weak control (Kopel *et al.*,2017). Moreover, Kochuieva *et al*., (2021) illustrated that excessing weight at age of 1 year was participating with a reduced risk of asthma and best lung function at age 6-8 years. Obesity is extremely known in severe asthmatics, there have been leastwise two differentiated obese asthma phenotypes, one an early onset, allergic phenotype and the other a late onset, female dominated, non-atopic phenotype, moreover, the fraction of the children with the decreased height is excessing monotonically and crucial in the groups of excessing severities, coincidently, they are with decreased height is excessed importantly in groups of excessing bronchial asthma severities (Tashiro and Shore, 2019; Fainardi *et al*., 2022).

1.2 .4 .8. Infections

Infectious conditions perhaps lead to damage the immune system, which exacerbation asthma which is linked with inflammation or infection of the airways, airway hyper-reaction and mucosal production. Respiratory viral infections cause 80-85%, of severe asthma exacerbations in children. with HRV being most commonly detected just as RV-A and RV-C associated with wheezing illness in early childhood. Notably, these viruses are more often correlated with exacerbations of asthma compared to RV-B (Kloepfer *et al*., 2017).

The RV-B might slightly the risk of exacerbation asthma symptoms in children with greater severity. Moreover, children with asthma exacerbations have higher total IgG1 titers to HRV compared with the control (Iwasaki *et al*., 2014*;* Esquivel *et al*.,2017). On the other hand, RV-C might be more strongly correlated with more severe exacerbations, which may necessitate hospitalization (Annamalay *et*

al.,2017; Altman *et al*.,2020). However, exacerbations of frequent wheezing and severe asthma in early childhood are often due to other respiratory viruses (influenza, SV and parainfluenza, acting alone or in combination. For example, a study of 175 children in age of 2-15 years with asthma exacerbations showed that HRV was most frequently detected 73%, followed by influenza A 27% and RSV 7.7%. In this study, RSV appears to drive asthma exacerbation in infants without a pre-existing history of atopy, while HRV is more likely to lead for asthma in children who have already developed an allergic phenotype (Duenas, 2016;Mikhail and Grayson, 2019). Respiratory viruses interact with host and environmental factors to increase risk of wheezing illnesses in infants and children result to increase risk of exacerbations in asthmatic children. In addition, high exposure to allergens in children with allergic asthma lead to increases the risk for virus-induced exacerbations. Children with established asthma , allergy and genetic factors can increase risk of virus-induced wheezing illnesses and asthma exacerbations, also allergy and viral respiratory tract infections synergistically increase the risk acute asthma exacerbations (Annamalay *et al*., 2017). Infections with RV increase the frequency and quantity of *S. pneumoniae*, *M. catarrhalis* and *H.* influenzae which detected in airway secretions.Stress and depression have been associated with acute wheezing disease predominantly viral in children, mechanism of this association was unknown but was not due to enhanced Th2 responses or impaired antiviral responses(Kloepfer *et al*., 2017; Altman *et al*., 2020).

1. 2. 5. Human Rhinoviruses (HRVs)

Human Rhinoviruses are tiny, non-enveloped viruses and members of the recently expanded Enterovirus genus that back to the family Picornaviridae, positive-sense RNA genome, with a single-stranded. as

shown in Figures 1 and 2.Further RV-A and RV-B were first discovered by isolation on monkey kidney cells in 1950s ,whilst in 1953,Winston Price, from the Johns Hopkins University could isolated the first rhinovirus from nasal passage samples in human (Jiang *et al*., 2021).

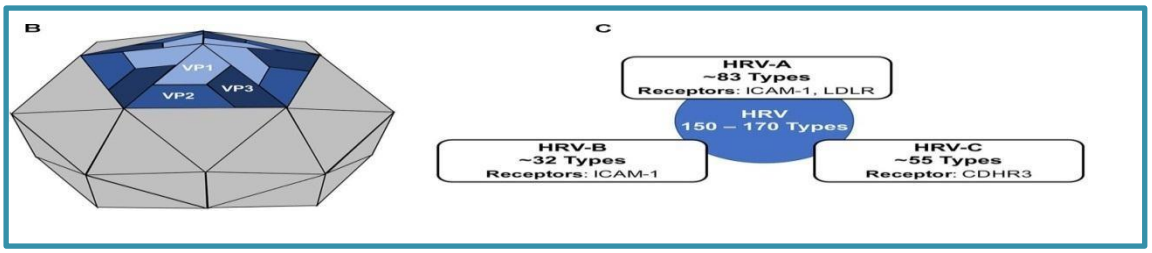

Figure1. 1. Illustration of the HRV adapted from (Kusel *et al*., 2006).

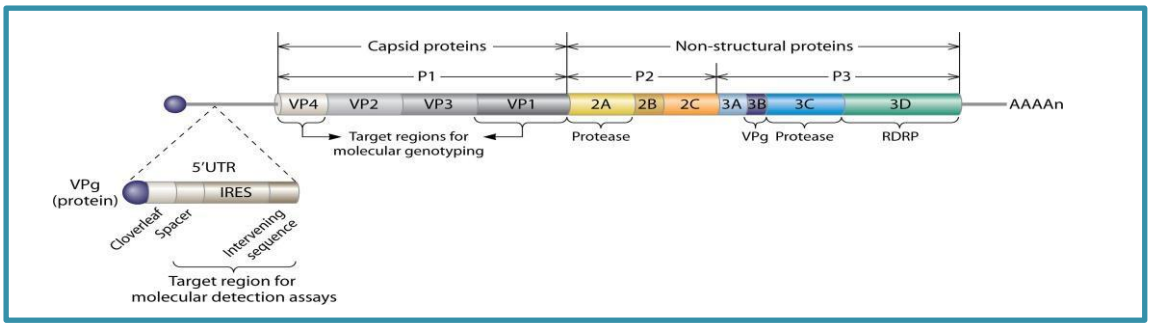

Figure1. 2. Genomic structure of HRV. A 7.2 kb single-stranded, positive-sense RNA virus called HRV has five un-translated sections, a single ORF, a tiny VPg, and one ORF. While the P1 protein is digested to produce the HRV capsid, the P2 and P3 proteins are used to make VPg, protease& RDRP or IRES (Kusel *et al*.,2006).

Its structure and organization are typical for picornaviruses, infectious non-enveloped virions of roughly 30 nm in diameter, consist of an icosahedral protein shell (capsid) (composed of a capsid that contains four viral proteins, VP1, VP2, VP3 and VP4)that surrounds and protects the genome (single-stranded positive sense RNA genomes of between 7200 and 8500 nucleotides in length. At the 5' end of the genome is a virus-encoded protein and, as in mammalian mRNA, there is a 3' poly-A tail. Structural proteins are encoded in the 5' region of the genome and non-structural at the 3' end).To date, there are currently more than 150 known serotypes of HRV, which differ based on proteins on their

surface and the receptors they bind at the surface of epithelial cells in the respiratory tract, the strains that bind to (ICAM)-1 belong to the so-called “major group HRVs” while the “minor group HRVs”, including ~10 strains, bind to the low-density lipoprotein receptor, one striking characteristics of HRVs is the ability to replicate rapidly and demonstrate high mutation rates, resulting in distinct genetic diversity, since most HRVs replicate best at 33–35°C, it was long thought that infections were limited to the upper airways where the temperature of the mucosal surface is relatively low.

The primary route of entry for human rhinoviruses is the upper respiratory tract (mouth and nose). Rhinovirus A and B use "major" ICAM-1 (Inter-Cellular Adhesion Molecule 1), also known as CD54 (Cluster of Differentiation 54), on respiratory epithelial cells, as receptors to bind to. Some subgroups under A and B uses the "minor" LDL receptor instead, Rhinovirus C uses cadherin-related family member 3 (CDHR3) to mediate cellular entry. As the virus replicates and spreads, infected cells release distress signals known as chemokines and cytokines (which in turn activate inflammatory mediators).Infection occurs rapidly, with the virus adhering to surface receptors within 15 minutes of entering the respiratory tract. There are 171 rhinovirus (RV) genotypes recognized and classified into three main phylogenetic species depending on their sequence structures: HRV-A, B and C(83,32 and 56 types) respectively (Kusel *et al*. , 2006; Warner *et al*. , 2019).

In addition, It is the most common cause of upper respiratory tract infection(URI) which include nearly 171 rhinovirus (RV)genotypes recognized and divided to RV-A(83 types), RV-B(32 types), and RV-C (56 types). It can be detected year-round, however, the incidence of rhinovirus is higher in the autumn and winter, with most infections

occurring between September and April. Furthermore humidity may influence rhinovirus seasonality. Young children (<5 years old) experience a high rate of infection which can be detected in community surveillance studies of children up to 34% of the year (Pan *et al.* , 2018).

In an effort to identify the etiology of the common cold, while RV-C genotypes, which are not cultivable using ordinary culture methods, have been identified decades after following the rise of molecular techniques. In addition to common immunological detection including antigen testing , and viral culture, seeking for a molecular detection methods are very important in applying for viral identification screening which has revealed the quite unexpected existence of a further species of human rhinoviruses (Pan *et al.* , 2018). HRV-Cs have been shown to be highly prevalent with frequent detection of this species in association with bronchiolitis, other lower respiratory tract disease and asthma exacerbations (Jiang *et al.* , 2021).

1. 2. 6. Respiratory Syncytial Viruses(RSVs)

Respiratory Syncytial Viruses are enveloped viruses with a negative-sense (−) a single-stranded RNA virus genome returning to the order Mononegavirales, family Pneumoviridae, genus Orthopneumovirus, Its name is derived from the large cells known as syncytia that form when infected cells fuse (Rima *et al*., 2017). RSV was identified in 1955 when researchers isolated a virus from a population of chimpanzees with respiratory illness, in 1957, this same virus was identified by Robert M. Chanock in children with respiratory illness studies of human antibodies in infants and children revealed that the infection was common in early life, the virus was later renamed human respiratory syncytial virus (HRSV). HRSV is a medium-sized (~150 nm). Further

many particles are spherical, filamentous species have also been identified. The genome rests within a helical nucleocapsid and is surrounded by matrix protein and an envelope containing viral glycoproteins. Its genome is linear and approximately 15,000 nucleotides in length and contained 10 genes, encoding seven structural and four non-structural proteins (Rima *et al*., 2017). Two viral glycoproteins, designated G (large glycoprotein) and F (fusion glycoprotein), are involved in virus-host cell attachment and cell fusion as shown in Figure1.3 and Figure1.4. Annexin II has identified as a potential RSV receptor on airway epithelial cells, while L-selectin/ CD62L and CX3CR1 appear to be the potential receptors for G-protein on leukocytes and immune-effector cells(Taleb *et al*., 2018). Nowadays, RSV diagnosis largely relies on nucleic acid/PCR-based tests(Wu,2020).

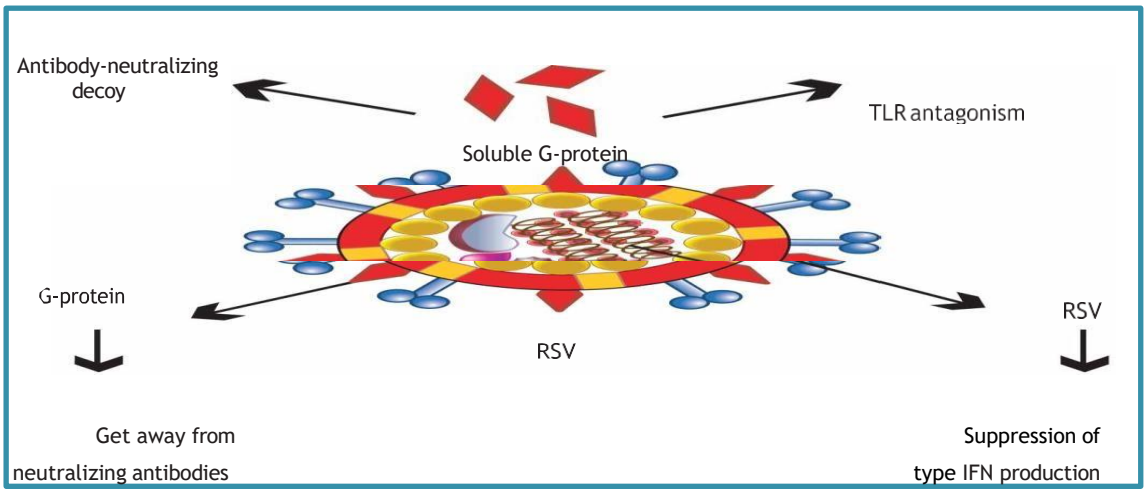

Figure1. 3. Illustration of the RSV adapted from (Ogra, 2004).

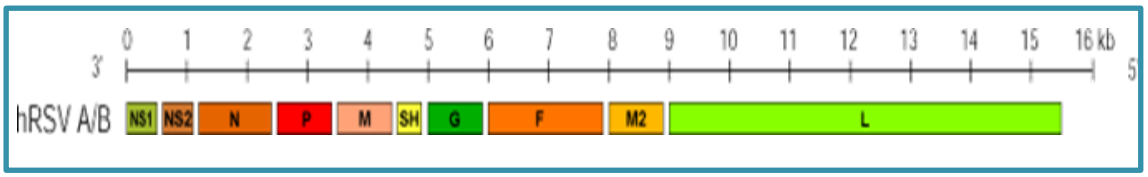

Figure1. 4. Genomic structureof the RSV adapted from (Ogra, 2004).

Strictly speaking, the high sensitivity of these tests may complicate clinical interpretation, as the presence of small amounts of viral targets may not necessarily prove their pathogenesis role. However,

prospective case - control studies in asymptomatic and symptomatic children have shown that a positive RSV test results is always of clinical relevance, independent of viral quantity (Jansen *et al*., 2014). RSV has a major impact on the infant mortality by viral infection in the US and worldwide, further the RSV virions are either spherical particles of 100–350 nm in diameter or long filaments up to 10 µm and 60–200 nm in diameter. The RSV virion comprises an RNA synthesis (RNP) complex packaged in a lipid envelope derived from the host cell membrane. The RNA synthesis RNP consists of four proteins essential for the RSV RNA synthesis: the nucleoprotein (N), the large polymerase protein (L), the phosphoprotein (P), and the processivity factor M2-1(Jansen *et al*., 2014). The RSV envelope contains three membrane proteins: the glycoprotein (G), the fusion protein (F), and the small hydrophobic protein (SH). The matrix protein (M) lays between the RNP and the envelope, acts as the cushion. RSV encodes ten sequential viral genes (NS1–NS2–N–P–M–SH–G–F– M2–L), and each gene is flanked by conserved gene start (GS) and gene end (GE) sequences, this genes encoding for 11 proteins (Jansen *et al*., 2014).

Each gene encodes an mRNA with the 5 methylated cap and 3 polyA tail to be translated into a single corresponding protein, except the M2 gene, which has two slightly overlapped (ORFs) encoding two proteins: M2-1 and M2-2. Two extragenic regions are at the genome ends, a 344-nt leader (Le) and a 5 155-nt trailer (Tr). Studies on immune response in primary RSV infection have demonstrated that infants and children produce fewer antibodies (IgM, IgG, IgA and secretory IgA) to both glycoprotein F and glycoprotein G(Soto *et al*., 2020; Ascough *et al*., 2022). The virion of RSV consists of enveloped lipid with an asymmetrical spherical shape, 150 to 300 nm in measurement, other

morphology of virion that are 60-100 nm and up to 10 nm in length, filament-like, could be seen in both infected cultures and preparation of the virus, the viral envelope is a lipid bilayer obtained from the host plasmatic layer (Gonnin *et al*., 2023).

1. 2. 7. Pathogenesis of Asthma

1. 2. 7.1. Asthma Exacerbation

Severe asthma aggravation is progressive excesses in asthma symptoms, including shortness of breath, wheezing and coughing, furthermore, it is an acute or sub-acute episode of airflow obstruction occurring on a background of chronic airway inflammation and hyper responsiveness it. It is events that require urgent action on the part of the patient and physician to prevent a serious outcome, such as hospitalization or death (Reddel *et al*., 2015). There are many factors can contribute to asthma aggravation including exposure to allergens, infections, underuse of asthma control medication and pollutants. In most cases, there is multiple contributing factors and this is especially true for severe exacerbations.Viral infections are of special importance because it contribute to 90% of exacerbations, especially during the fall and spring in temperate climates, when viral respiratory tract infections are most common, most episodes of asthma exacerbation in preschool aged children involve symptoms of airway obstruction and wheezing associated with a cold and cough, that is caused by the viral infection, firstly HRV as shown in Figure 1.5. In addition, HRV is according to the National Institute of Mental Health and Prevention (2007), play a major role in seasonal peaks of exacerbations that coincide with the return of children to schools after summer and spring breaks. There exists a plethora of respiratory viruses that have the potential to cause

wheezing illnesses, HRVs and RSV are the most closely related to aggravation asthma in children (Heymann *et al*., 2004).

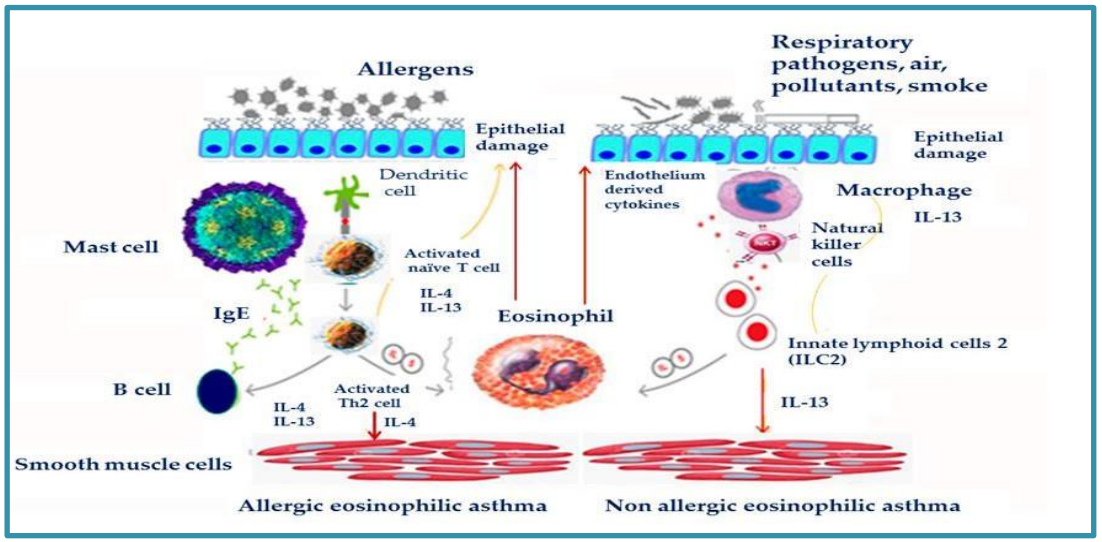

Figure1.5. Pathogenesis of asthmatic acute exacerbations(License, 2019).

Esquivel *et al*.,(2017) refers to Asthma exacerbations results from a variety of risk factors including RVs which can cause a spectrum of illnesses ranging from asymptomatic infections to severe lower respiratory tract illnesses, this is also true for children with asthma, and most RV infections in children with asthma do not cause exacerbations.

1. 2. 7. 2. Asthma Severity

The degree of asthma in each person is different, and it may change over time. The severity of asthma varies from person to person and might fluctuate over time, especially in youngsters. According to NAEPP/EPR3 Guidelines, the severity of asthma was categorized based on the frequency of symptoms(daytime, night-time, and exertional) (Urbano, 2008) as indicated in Table1.1. Stratification of asthma severity is an integral part of asthma management linking appropriate treatment to establish control asthma. Precision assessment of severe asthma is crucial for monitoring the health of people with this disease (Majellano, 2019).

Table1.1. Assessment of children's asthma severity adapted from (Urbano, 2008)

Competent of severity		Classification of asthma severity			
		Intermittent	Persistent		
			Mild	Moderate	Severe
Day time symptoms		≤2 days/week	>2 days/week but not daily	Daily	Throughout the day
Night time awakenings	Age 0-4 years	0	1-2/month	3-4/month	>1/week
	Age ≥ 5 years	≤2/month	3-4/month	>1/week but not nightly	Often 7/week
	SABA use for symptoms	≤2 days/week	>2 days/week but not daily	Daily	Several times daily
	Interference with normal activity	None	Minor limitation	Some limitation	Extreme limitation
Lung function (≥5 years)	FEV1% predicted	>80	>80	60-80	<60
	FEV1/FVC	>0.85	>0.8	0.75-0.8	<0.75

Different endotypes of asthma may be influenced by genetic variants to varying degrees, and there may be a genetic component to illness severity(Thomsen *et al*., 2010; Slager *et al*., 2012).

1. 2. 8. Immune Response of Asthma

Children with asthma have a reduced lung function in concert with impaired immune responses and altered immune cell subsets however, little is known regarding the effects of asthma on immune responses in children (Hosseini *et al*., 2021). Non-atopic asthmatics show no sensitization and rather neutrophilic inflammation, while atopic asthma is characterized by sensitization to specific allergens, high IgE levels, and eosinophilia, childhood allergic asthma has been characterized by a Th cell type 2-shifted endotype and decreased innate immunity gene expression, further, Tregs play a critical role in the development of it,

possible a reduced number of Tregs in children with allergic airway disease, in addition, some studies have shown increased or decreased number of Tregs at different ages in asthmatic children (Lee *et al*.,2007; Raedler *et al*., 2015; Schröder, 2017).

Besides Tregs quantity, impaired function of Tregs is important for asthma development , distinctly, non-allergic asthma showed increased pro-inflammatory IL-1β / IL-17-shifted neutrophilic inflammation and insufficient suppression of IL5, IL-13 and IFN-γ by Tregs (Raedler *et al*., 2015; Zhang, *et al*., 2022).

It has been suggested that Th1and Th17 high inflammation have a role in development of childhood asthma is complex with a strong interaction of genetic, epigenetic and environmental factors (Krusche *et al*., 2019). Allergen exposure results in the activation of numerous cells of the immune system such as NK cells, neutrophils, eosinophils , structural cells such as epithelial cells that may contribute to an altered immune response in childhood asthma, DCs and Th2 lymphocytes, DCs in the airway epithelium and sub mucosa detect inhaled allergens as shown in Figure 1.6 (Holgate, 2012).

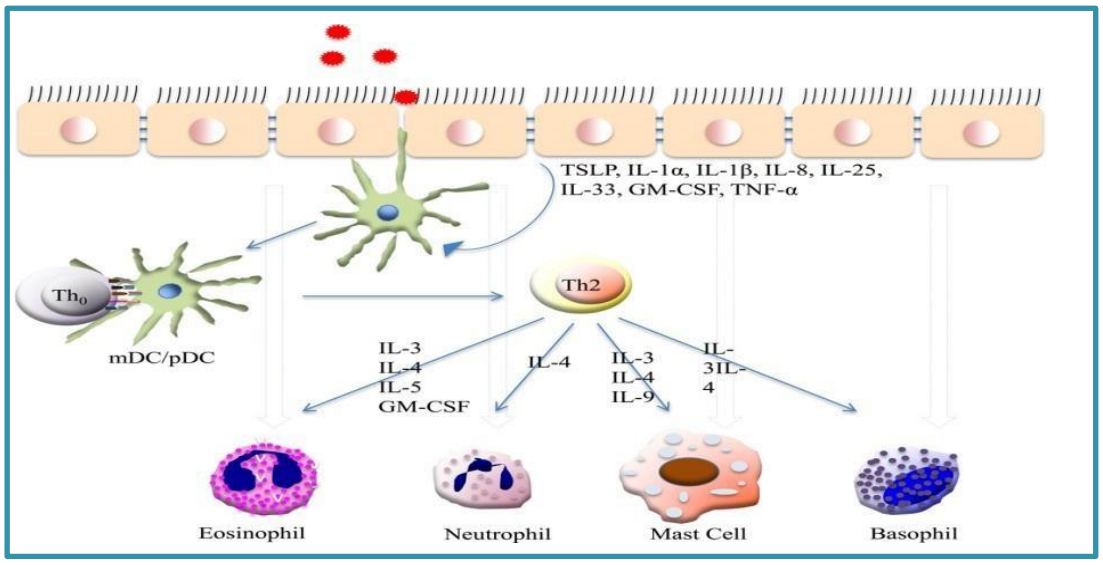

Figure 1. 6. Effect of DCs on immune cells cytokines adapted from (Gaurav and Agrawal, 2013).

It is then migrate to secondary lymphatic systems where they process and present antigens via MHC II to T and B lymphocytes, leading to proliferation of Th1 or Th2 and B-lymphocytes produce IgE, which binds to high affinity FcεRI on basophils and mast cells in response to allergen presentation by airway DCs, T-helper lymphocytes of adaptive immune system control many aspects of disease during secretion of IL-4, IL-5, IL-13, IL-17 and IL-22 and these are counter balanced by cytokines produced by Treg cells like IL-17 (Lambrecht and Hammad, 2019). Whereas, role of type 2 innate lymphoid cells (ILC2) in childhood asthma is less clear, studies have demonstrated that ILCs have a key role in development of virus induced asthma exacerbations, ILC2 secrete IL9, IL13 and IL5 in response to IL-25 and IL-33stimulation (Jia *et al*.,2016; Hershenson, 2021).

1. 2. 8 .1. Role of T helper (Th2) cytokines in asthma

Concept of Th2-cell-mediated immunity to allergens driving asthma pathogenesis in humans has dominated thinking over the last 30 years and, importantly, has pushed forward treatments with biological substances that target Th2 cell cytokines (León and Ballesteros-Tato, 2023). It has been found that besides Th2cells, other innate immune cells like mast cells, ILC2s and basophils can produce Th2-cell-associated cytokines in asthma, and the terminology gradually shifted from Th2 cell high asthma to Type-2-high asthma. It has been found that besides Th2 cells, other innate immune cells like mast cells, ILC2s and basophils can produce Th2-cell-associated cytokines in asthma, and the terminology gradually shifted from Th2 cell high asthma to Type-2-high asthma. It is currently estimated that only about half of asthma patients have evidence of Type 2 immunity in their airways (Fahy, 2009). Correlation between a Th1/Th2 cytokines imbalance and 25-

hydroxy-vitamin D (vit D) level in early pulmonary disease (Tang *et al*.,2023). In childhood, type-2-high asthma is frequently caused by atopic sensitization and exposure to inhaled allergens, driven by allergen-specific CD4+Th2 cells, and demonstrated by presence of allergen-specific IgE in serum(Karagiannis *et al*., 2015). The production of Th2-cell-associated cytokines by CD4+T cells is under genetic and epigenetic control, a polymorphism in a gene locus at position5q31, which contains IL4, IL13 and RAD50 genes, has been associated with asthma and atopy in multiple studies, this locus is also hypo-methylated in (PBMCs) and contains enhancer histone modifications (H3K4me2)in the memory Th2 cells of asthma patients (Demenais *et al*., 2018).

The T helper 2 type cytokines, including IL4, IL-5 and IL-13 are thought to drive pathology in asthmatic patients and play a role in driving many of the hallmarks of atopic inflammation. IL-4 is important for atopic sensitization and IgE production, and IL-5 is crucial for eosinophil survival, IL-13 has pleiotropic effects in the lungs, including a central role in development of airway hyper responsiveness and tissue remodelling as shown in Figure1.7. (Lloyd and Hessel, 2010; Finkelman *et al*., 2010).

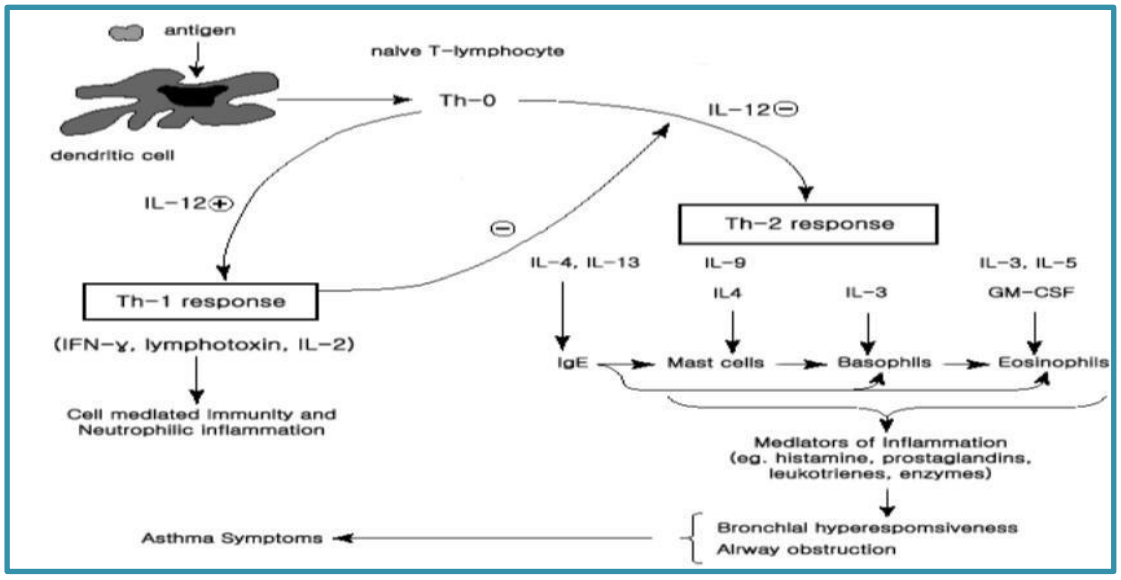

Figure1. 7. Immunopathogenesis of asthma adapted of (National Asthma, 2002).

IL-13 induces features of the atopic response by a complex array of actions on resident airway cells instead of through traditional effector pathways involving eosinophils and IgE-mediated events, also it is effect on asthma include the induction of goblet cell increased mucus, metaplasia, secretion, airway hyper reactivity and potent activators of inflammatory responses, and fibrosis (Gour and Wills-Karp, 2015). The IL-5 is critical molecular switch for the development, migration, recruiting of eosinophils to lung during atopic inflammation, IL-5 exerts its biological actions via stimulation of the IL-5 receptor expressed by eosinophils and a lesser extent, basophils (Possa *et al*., 2013).

1. 2. 8. 2. Role of Immunoglobulin E (IgE) in Asthma

Discovery of IgE begun in the 1960s. It has direct role in the pathogenesis of bronchial asthma and its connection with skin reactivity to allergen exposition, at that time, researchers worked on the discovery of a protein, called regain in the hopes of providing an explanation for the immunological concepts that explain asthma-associated type I hypersensitivity symptoms and anaphylactic attacks. However, the extremely low amounts of IgE in human blood made the entire process very difficult. among every type of antibody (Laffleur *et al*., 2017).

IgE is the most potent antibody and can induce severe inflammatory reactions even in minute doses. IgE response can remain for years even without allergen stimulation, this may be due to long-lived plasma cells that produce IgE (Colas *et al*., 2022). IgE play a significant role in atopic patients they are coupled to IgE-specific receptors on mast cells and basophils and are specific for antigens such pollens and house dust mites, which trigger the release of intermediates (arachidonic acid metabolites histamine) and IL-4, IL-5 and TNF, which are crucial for

the initial and final phases of allergic reaction and related eosinophil infiltration in the airway (Johansson and Stockholm, 2016). IgE production is primarily controlled by IL-4,IL-13 and the immediate interaction that occurs among T and B lymphocytes as shown in Figure 1.8.

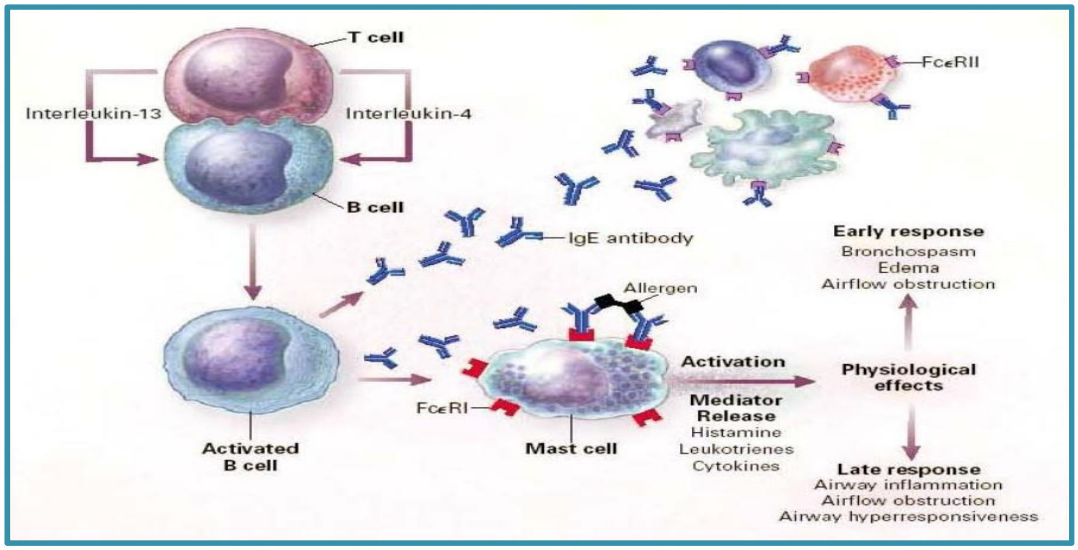

Figure 1. 8. Interactions between CD4Tand Bcells and IgE synthesis adapted from (Busse and Lemanske, 2001).

Via bonds among T-lymphocytes' cell surface molecules, in particular (CD28 and CD154) and B-lymphocytes' outer membrane receptors (CD40 and CD80 or CD86) (Novosad et al., 2020).The maturation of B lymphocytes into active plasmatic cells, isotype switching and the creation of particular Ig class are all processes that are started by the interaction of these two stimuli, commonly known as the immunological synapse . It's interesting to note that B-lymphocytes are capable of receiving both signals by IL-13 and IL-4 as well as T-lymphocytes alone (Rabe *et al*., 2018). IgE levels have been reported to decrease in relation to anti-IL-13 (Corren *et al*., 2013). A significant increase of total IgE levels occurs in patients with different diseases including allergic asthma, atopic eczema, intermittent rhinitis and persistent rhinitis bound to perennial allergens. IgE levels culminate

about 4-6 weeks after pollen season, high levels are measured in patients suffering from allergic bronchopulmonary aspergillosis (ABPA) (serum levels can exceed 1000 kIU/L), and further in most eosinophilic syndromes because increase IL4 , IL-5and IL13 (Stone *et al*.,2010). IgE is synthesis either by class-switch recombination from IgM in germinal centre B cells or through sequential switch from IgM to IgG1 and then from IgG1to IgE which may occur outside of germinal centres. Although, it is thought that IgE memory of allergens originally sits in IgG,it was shown human blood or tonsil B-cells undergo CSR to IgE upon CD40 ligation and activation IL-4 or IL-13produced by Th2 cells and ILC2 (Spits *et al*. , 2013). The complexes of IgE molecule with allergen bind to the FcεRI on surface of the projections of dendritic cells (that penetrate from sub epithelial spaces up to the airway lumen). The presence of FcεRI on surface of dendritic cells is very likely strengthening the processes of antigen presentation to naïve T-helper lymphocytes with their subsequent maturation towards the Th2 subset, up to1000 times, resulting in significant reduction of triggering threshold for the allergic reaction, already in this early phases (Matucci *et al*., 2018) as shown in Figure 1.9.

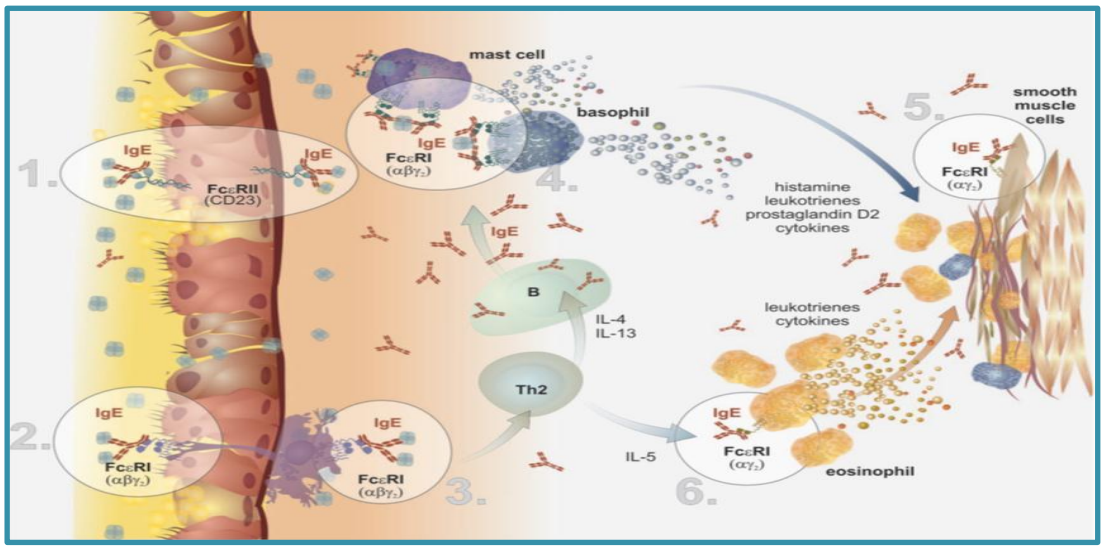

Figure1. 9. Roles of IgE in asthma pathology adapted from (Pelaia *et al.* , 2017).

1. 2. 8. 3. Role of Eosinophil in Asthma

Eosinophils are granulocytes in blood, produced in the bone marrow with other white blood cells. Their percentage about 1-3% of whole white blood cells, they have been implicated in the pathogenesis of asthma, generally, eosinophilic phenotype is associated with a good response to corticosteroids and to Th2 targeted therapy, such as anti-IL-5 treatments (Haldar *et al*., 2009). Eosinophilic asthma has been associated with allergic sensitization and a Th2-dominant inflammatory response. It plays multiple functions and is an important component of allergic and asthmatic type-2 immune responses, IL-13, and chemokines including eotaxins, and the adhesion molecules P-selectin and vascular cell adhesion molecule-1, other chemo-attractants for eosinophils include complement product C5a and the lipid mediators platelet-activating factor and leukotriene B4 as shown in Figure 10.

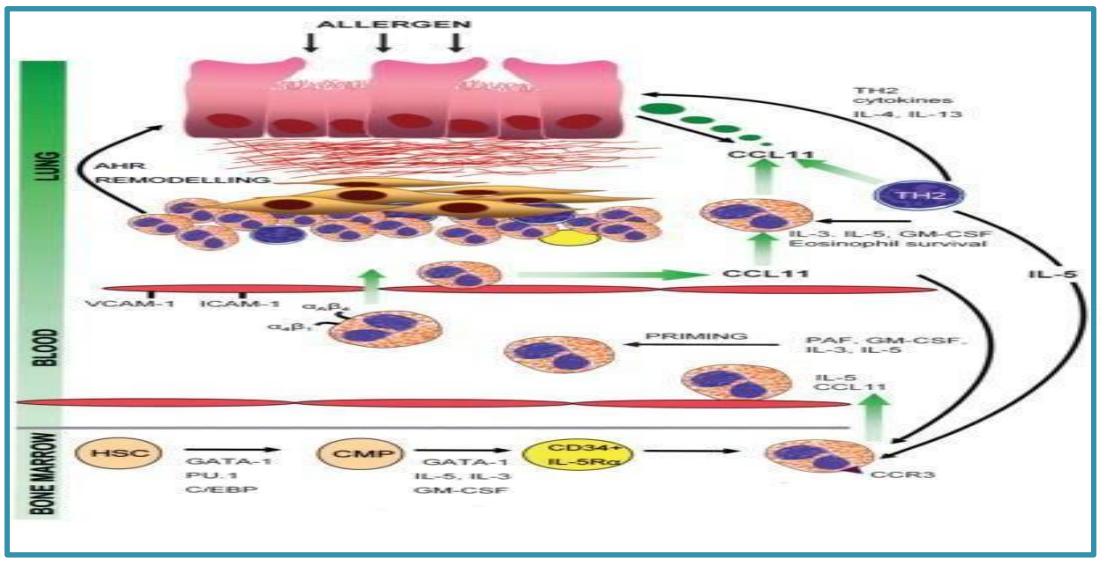

Figure1.10. Eosinophil development and trafficking adapted from (Foster *et al*., 2003).

Other chemoattractants for eosinophils include complement product C5a and the lipid mediators platelet-activating factor and leukotriene B4 in addition to IL13,eotaxins,P-selectin,and vascular cell adhesion molecule

-1.It performs a variety of duties and is an essential component of type 2 allergy and asthmatic immune responses(Zietkowski *et al*., 2008).

1. 2. 9. The cytokines and chemokines

Cytokines are a broad category of small proteins(~5-25 kD) synthesized by cells, and that influence the behaviour of other cells, their function is accomplished via specific cytokine receptors on the cells that they affect. They are also important mediators that control immune and inflammatory responses through intricate networks and act as biomarkers for many diseases (Liu *et al*., 2021). The first biologically active chemokines were discovered in the late 1980s and early 1990s, based on the presence of leukocyte chemo-attractant activity present in the culture supernatants of human mononuclear leukocytes stimulated by pro-inflammatory agents such as bacterial endotoxin and cytokines, which demonstrated potent chemotactic activity for monocytic leukocytes, they are small chemo-attractant proteins, that stimulate migration and activation of cells,especially lymphocytes and phagocytic cells, they are the most direct and immediate cell factors that selectively induce the migration of specific inflammatory cells by binding with different receptors (Chen *et al*.,2018). They are classified into four groups (CC, CX, CX3C and CXC) depending on the presence and position of first cysteine residues(Palomino and Marti, 2015).

It is a group of low molecular weight 8-10 KDa polypeptides that are designated for their targeted cell chemotaxis. There are four conserved cysteines in the protein structure of chemokine molecules, further they are key drivers of anti-viral inflammatory response, such as RSV and HRV infection, many chemokines are produced at high levels through the bacterial and viral infection, for example CCL20, in addition to the

synergistic effect of TNF-α and interferon-γ, which can induce CXCL10 expression in airway epithelial cells, and specific cells are recruited by several unique chemokine/chemokine receptor interactions (Schneider *et al*.,2013; Song *et al*., 2014). CXCL10 regulate the infiltration of effector T-cells into the lungs and they are all produced during RSV infection in mice and humans (McNamara *et al*., 2005).

1. 2. 9. 1. Interleukin - 10 (IL-10) and Transforming Growth Factor Beta (TGF-β)

Interleukin-10 protein is a homodimer, each of its subunits is178 amino acid long, also known as human cytokine synthesis inhibitory factor (CSIF), gene encoding human IL-10 which is located on chromosome 1 covering a total of 5.1 kb pairs comprising five exons. It is a cytokine-mediated interactions among inflammatory cells and contribute to pathogenesis of allergic asthma , including inhibitory for B and T cells, IgE production, mast cell proliferation, and they induce apoptosis of eosinophils (Minshawi *et al*. , 2020).

In addition, IL-10 is deemed as a key regulators of immune response that has been suggested for treatment of allergy and asthma because of its immunosuppressive and anti-inflammatory properties, which is synthesized by activated CD4and CD8 T lymphocytes, activated monocytes, mast cells, and inhibits synthesis a range of cytokines produced by Th1 that leads to polarization of immune response to the Th2-profile (Li *et al*., 2011). In humans, its signals is sent through a receptor complex consisting of two IL-10 receptor-1 and two IL-10 receptor-2 proteins, it is humoral factors involved in the suppressive function of many effector cells, Treg cells, and disease processes, allowing the clearance of infection while minimizing damage to the host, and its levels are inversely correlated with disease incidence and

severity and play critical roles in maintaining immune homeostasis (Mosser and Zhang, 2008). A combination of TGF-β and IL-10, no single cytokine, is required to suppress B cell activation induced by (TLR) stimulation (Komai *et al*., 2018).

TGF-β is a cytokine that controls proliferation, cellular differentiation, and other functions in most cells, also a major mediator involved in pro-inflammatory responses and fibrotic tissue remodelling within asthmatic lung, it is a multifunctional cytokine which has been linked to the pathogenesis of sub-epithelial fibrosis and airway wall remodelling in bronchial asthma (Cheng *et al*. , 2022). Allowing the clearance of infection while minimizing damage to the host, myeloid and lymphoid lineages secrete IL-10 in response to different stimuli (Meng *et al*., 2019). Includes CD4 and CD8 T cells, B cells, mast cells, eosinophils, NK cells, neutrophils, macrophages, and monocytes (Howes *et al*. , 2014). Recently, local macrophages like microglia and cardiac macrophages were added to list of IL-10 producer cells(Yang *et al*., 2020). Changes in blood concentrations of IL10 and TGF produced by activated T-lymphocyte before and after prednisolone therapy with clinical improvement demonstrate the role of soluble IL10 and TGF on activity and regulation of asthma. They are a powerful inhibitor of monocyte/macrophage function, causing many pro-inflammatory cytokines to be produced as well as less IL-10 being produced by the alveolar macrophages of people with atopic asthma (Chung, 2022).

1. 2. 9. 2. C-C motif ligand 20(CCL20) and C-X-C motif ligand 10 (CXCL10) Chemokines

Chemokines play an important role for the recruitment and migration of inflammatory cells by binding with different receptors as shown in Table 1.2. In addition, they can induce various types of inflammatory

cells which would release a variety of inflammatory factors, that cause the immediate phase reaction of airway inflammation mediated by IgE and induce chronic persistence airway inflammation with eosinophils and Th2 (Erle and Sheppard, 2014). The CC family contains ~28 types of chemokines with chemotaxis effects on almost all inflammatory cells except neutrophils. The CXC chemokine family contains more than 15 types of chemokines, which possess potent effects on the recruitment of neutrophils and monocytes. Studies have shown that a variety of cells are involved in expression and secretion of chemokines, including macrophages, monocytes, eosinophils, basophils, neutrophils, mast cells, DCs and lymphocytes (Mitchell and O'Byrne , 2017;Capucetti, *et al*.,2020).Chemokines in airway epithelial cells and its role in the pathogenesis of airway inflammation in asthma as shown in Table1.2.

Table1.2.Airway epithelium chemokine expression that is associated with asthma adapted from (Gao *et al*., 2015).

Name of Chemokine	Other Name	complete name	Receptors
CC family			
CCL2	MCP-1	Monocyte chemotactic protein-1	CCR2, 10
CCL3	MIP-1α	Macrophage inflammatory protein-1α	CCR1, 3, 5
CCL4	MIP-1β	Inflammatory of Macrophage protein-1β	CCR5,8
CCL5	RANTES	controlled after stimulation normal T-cell generated and released	CCR1, 3, 5
CCL11	Eotaxin-1	Eotaxin-1	CCR3, 5
CCL13	MCP-4	Monocyte chemotactic protein-4	CCR2, 3
CCL17	TARC	Thymus activation regulated chemokine	CCR4
CCL20	**MIP-3α**	**Macrophage inflammatory protein-3α**	**CCR6**
CCL22	MDC	Macrophage-derived chemokine	CCR4
CXC family			
CXCL1	GRO-α	Growth-regulated oncogene-α	CXCR1, 2
CXCL5	ENA-78	Epithelial-derived neutrophil-activating peptide 78	CXCR2
CXCL8	IL-8	Interleukin-8	CXCR1, 2
CXCL10	**IP-10**	**Interferon-inducible protein-10**	**CXCR3, 4**
Non CC or CXC family			
TSLP	No	Thymic stromal lymphopoietin	TSLPR
IL-33	No	Interleukine-33	ST2 ,IL-33R

It has previously been demonstrated that TNF-α stimulates expression of C-C motif chemokine ligands CCL2, CCL4, CCL5, CCL11 and CCL20. Further, airway epithelial cells release CCL5 and CCL20 upon stimulation by IL-1β (Liu *et al*., 2018). Other inflammatory cytokines can induce the expression of chemokines, for examples,CCL11 and CCL20 are produced following IL-4 or IL-13stimulation (Bao *et al*., 2022). Further, it has been demonstrated that clusterin induces production of CCL20 by regulating the oxidative stress environment in airway epithelial cells from mice studies (Hong *et al*., 2016). HRV, RSV and bacterial infection induces airway epithelial cells to secret high levels of CXCL10 and CCL20 in human nasal mucosa and gland epithelial cells (Song *et al.,* 2014).

Additionally, Janus kinase (JAK) pathway and the synergistic effect of TNF-α and interferon-γ can induce expression of CXCL10 in airway epithelial cells (Michi *et al*., 2020). Based on these observations, these associated signaling molecules have also been used as potential targets of anti-inflammatory treatment. It is commonly stated that inhibition of JAK pathway in the airway epithelium may provide an alternative anti-inflammatory approach to glucocorticosteroid -resistant asthma in vitro. CXCL10 is an important chemokine secreted by the airway epithelium that functions as a biomarker for virus-induced asthma. Long-acting beta 2 (β2) agonists (LABAs) are frequently used as inhaled medication for asthma(Chien *et al*., 2012; Fenwick *et al*., 2015).

1. 2. 10. Diagnosis of Asthma in Children

A diagnosis of asthma should be suspected in patients with recurrent cough, wheeze,chest tightness and dyspnea, also it is diagnosed through medical history, family history, physical exam and should be confirmed

by using test that measures airflow in and out of the lungs (spirometry preferred). Spirometry testing measures the flow and volume of air blown out after a child takes a very deep breath and then forcefully exhales, other tests may be recommended to ensure that other conditions are not the cause of a child's wheezing or coughing. This may include a chest X-ray and allergy testing or immune problems in skin or blood, which recommended to identify triggers of asthma (Quirt *et al*., 2018).

Further the possibility of a first manifestation in infants or toddlers makes diagnostic determination difficult in this age group because congenital disorders and a wide range of differential diagnostic possibilities have to be taken into account and may need to investigated along with perinatal aspects, a special feature in this age group is that many children suffer from asthma-like symptoms (Quirt *et al*., 2018). Several factors, including a detailed medical history, family history, a physical exam (your nose, throat and upper airways) are applied for diagnosis of asthma. Use a stethoscope to listen to your breathing. Further, spirometry is the main test doctors use to diagnose asthma in children 5 years or older(Gregory *et al*.,2019).

Eosinophilic asthma can occur in atopic and non-atopic patients but the pathways for eosinophil recruitment are quite distinct measurement of eosinophils in blood for the evaluation and management of allergic diseases, asthma severity and a relevant factor in the pathophysiology of the disease as shown in Figure1.6. Further, blood eosinophils are known to be an indirect marker of airway inflammation in asthma, additionally, atopy is an hereditary disease with a frequency of Th2 cells and it predisposes to asthma, a Th2 reaction can activate not only immediate IgE in sensitized individuals, but also viruses, contaminants and

allergens such as (HDM) and molds can cause both innate and adaptive immune reactions of type 2 even in the absence of strong IgE antibodies (Caminati *et al*., 2018; Nieto-Fontarigo *et al*., 2019).

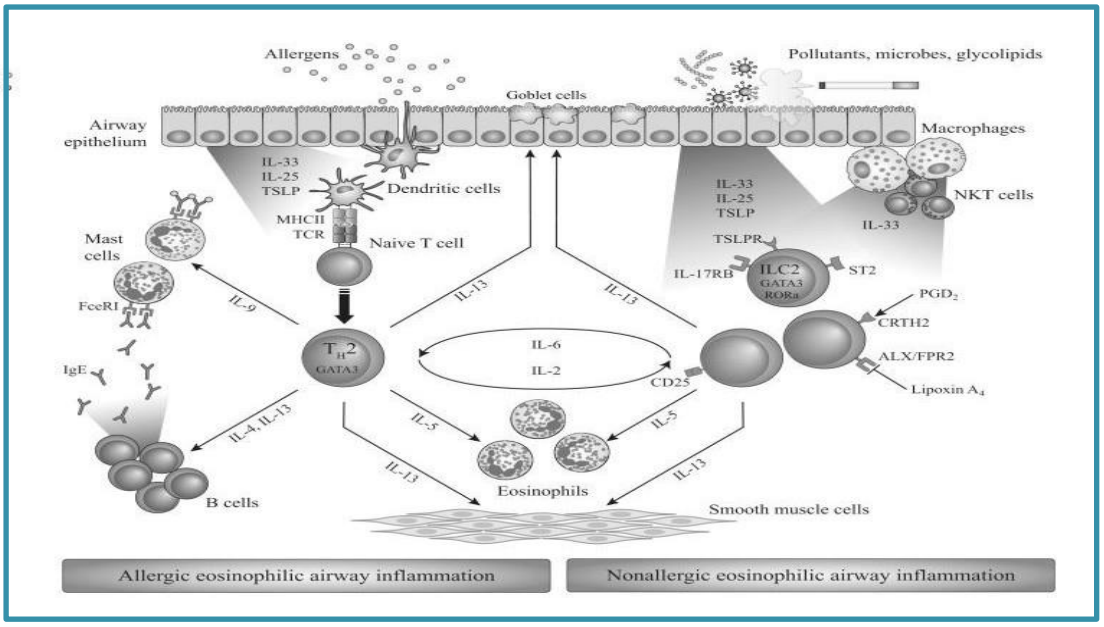

Figure1.11.Atopic and non atopic eosinophilic pathways in asthma adapted from (Brusselle *et al* .,2013).

When asthma is persistent and uncontrolled despite treatment, blood eosinophil levels can reliably predict eosinophilic asthma in those people (Wang *et al*., 2021). Moreover, blood eosinophil counts could add predictive value to the global initiative for asthma control-based risk assessment, TSIgE concentration was predictive in asthma, and it perhaps is utilized to discriminate between non-asthmatic and asthmatic peoples in conjunction with other biomarkers, TSIgE antibodies test to measure sensitisation to inhalant allergens is a useful diagnostic indicator for the presence of asthma in children (Matucci *et al*.,2018).

1. 2. 11. Treatment of Asthma

The aim of asthma treatment is to obtain clinical control and reduce future risks to the patient, initial treatment after diagnosis to achieve the best possible results, a maintenance therapy with a controller should be

initiated as quickly as possible after the diagnosis of asthma (Fitzpatrick *et al*.,2016). For pre-school children with intermittent symptoms, the initial therapy consists of an inhaled short-acting beta-2agonist(SABA), regular follow-up should occur in a period of 2-3 months to optimize the treatment strategy. Gold standard in asthma therapy is still a low-dose ICS as a controller together with an on-demand (SABA), a considerable number of asthmatic children remain symptomatic despite treatment with ICSs, resulting in significant morbidity and reduced quality of life (Duse *et al*., 2022).

To mitigate the adverse effects of drugs, especially the restrictions on body growth resulting from the use of ICS, which is of utmost significance in pre-schoolers, anti-viral measures encompass methods that augment resistance to various RVs by administering interferons or other immune-stimulatory agents. This holds particular significance for individuals with severe asthma who exhibit a concomitant allergic inclination prior to undergoing systemic steroid therapy.

Further, biological material taken by asthmatic children, like as proteins and microorganisms, may aid in fostering the growth of strong mucosal immune systems that can fend off viral infection. Two new therapeutic approaches has been suggested firstly, identify a new class of probiotics selected to promote resistance to viral illnesses and secondly, develop strategies to inhibit pathogenic bacteria that synergize with viruses and add to illness severity (Teach *et al*.,2015).

1.2.12. Single nucleotide polymorphisms(SNPs) in Asthma

The polymorphism is defined by Cavalli-Sforza and Bodmer,(1981), and the most common type of genetic variation among people in the DNA sequence, which includes differences in genotypes ranging from a

single nucleotide site to large nucleotide sequences visible at a chromosomal level, each SNP represents a difference in a single DNA building block, such as a SNP may replace the nucleotide cytosine (C) with the nucleotide thymine (T) in a certain stretch of DNA, or a G nucleotide present at a specific location in a genome may be replaced by an A as shown in Figure 1.12 (Monga *et al*., 2017).

Single nucleotide polymorphisms (SNPs)help predict an individual's response to certain drugs, vaccine and it can establish ancestries and in identify genes involved in complex diseases such as heart disease, diabetes, cancer, and they are responsible for genetic effects that produce susceptibility to most autoimmune diseases (Chiarella *et al* ., 2023). SNP detection are polymorphic allele-directed specific analysis such as allele specific PCR, RFLP and AFLP, further melting curve analysis, which is combined with the real-time PCR techniques using hydrolysis probes, hybridization probes, or double-stranded DNA-binding fluorescent dyes (Matsuda, 2017).

In addition, multiplex probe amplification (probe-based PCR) is used for SNPs genotyping and pathogen nucleic acid detection. PCR-RFLP is a method for genotyping SNPs,allows rapid detection of point mutations after the genomic sequences are amplified by PCR (Matsuda, 2017).

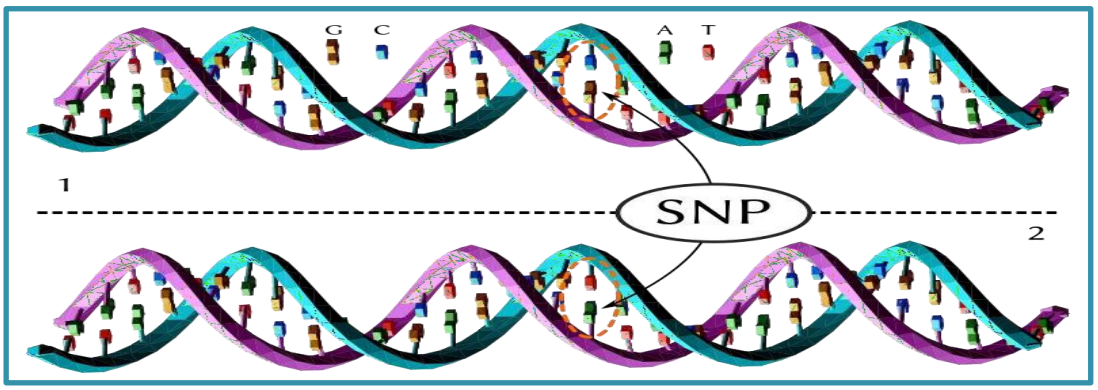

Figure1. 12. Single nucleotide polymorphisms (SNPs) adapted from (Monga *et al.* , 2017).

1. 2. 12. 1. Asthma-Associated Genes

Over a 100 different genes that have been associated with asthma and the list is still growing, in general, asthma susceptibility genes fall mainly into four categories genes relating to functioning of the immune system (innate immunity and immunoregulation), genes relating with epithelial and mucosal biology (immunity) and function, genes relating with lung function, airway remodelling and disease severity and associated with TH2-cell differentiation and effector functions (Bossé and Hudson, 2007; Loxham and Davies, 2017).

1.2.12.2.Single Nucleotide Polymorphisms of Chemokine Genes

Researchers' interest in the impact of chemokine gene polymorphisms on gene expressions and disease led to the explored the cytokine, chemokine, and cytokine gene polymorphism associations with asthma risk (Chiarella *et al*., 2023).The majority of polymorphisms are SNPs found in the enhancer, promoter,or other regulatory sequences of chemokine and cytokine genes (Raeiszadeh Jahromi *et al*., 2015). Numerous research have examined the connection between its gene variations and the chance of developing asthma (Ranjbar *et al*., 2022).

1. 2. 12. 3. The C-C motif ligand 20 (CCL20) Polymorphism

The C-C motif ligand 20 is a four-exon gene localized to chromosome 2q33- q372 and encodes a 95-amino acid polypeptide. CCL20 is a small chemokine about 8 kDa encoded by the gene SCYA20 located on chr.2 as shown in Figure1.13(Nelson *et al*., 2001).

A number of polymorphisms in the *CCL20* promoter region are exhibited to be related to cytokine serum levels. Most of the studies confirmed that CCL20 was associated with different inflammatory

diseases and autoimmune diseases such as MS (Jafarzadeh *et al*., 2014; El Sharkawi *et al*., 2019). Further, this variation is linked to levels of CCL20 and its CCR6 receptor are elevated in many autoimmune diseases which help in the recruitment of Th17 to site of inflammation (El Sharkawi *et al*., 2019). Also this variant is associated with reduced pulmonary capacities (Daneshmandi *et al*., 2012). Previous molecular research demonstrated that the CCR6-CCL20 is a key modulator of a number of inflammatory disorders (Liu *et al*.,2018; Ellinghaus *et al*., 2016).

Burke *et al*., (2015) showed that CCL20 expression is increased in pancreatic islets from obese mice, and that inflammation strongly and rapidly promotes CCL20 levels in rat and human pancreatic β cells. Population genetic studies point towards the significant involvement of CCR6-CCL20 axis in RA research suggests that the CCR6-CCL20 axis plays a substantial role in RA(Kochi *et al*., 2010).

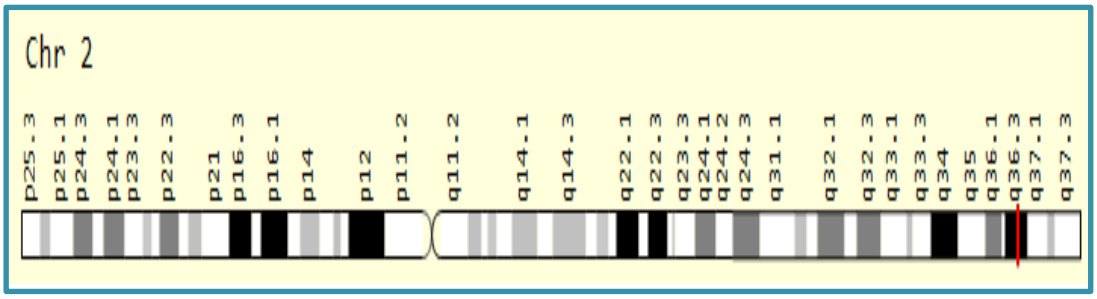

Figure1.13. Cytogenetic location,Chr.2 p14. molecular location of *CCL20* adapted from (NCBI, 2023).

Chapter Two

Materials and Methods

2. Material and Methods

2.1. Subjects and Study Design

2.1.1. Subjects

Consecutive 100 patients children asthmatic were diagnosed in the present study to have asthma based on clinical confirmed by physian , radiologist and laboratory finding, who attended at out- patient of the asthma clinic at Karbala Teaching Hospital for Children in Karbala city for the period extending from February 2022 to June 2022. All children should meet the criteria of the American Thoracic Society for asthma (Chung *et al*., 2014). In the current study, asthmatic children were divided into two groups according to the type of asthma: atopic and non-atopic. Their ages were ranged between 1-6 years old. Fifty children were grouped as the healthy control groups with the same ages and sex of the patients who randomly selected from the local community.

2.1.2. Inclusion and Exclusion Criteria

2.1.2.1. Inclusion criteria: Children were identified with asthma (atopic and non atopic) either newly diagnosed or on controller therapy and all met the criteria of the American Thoracic Society for asthma.

2.1.2.2. Exclusion criteria: Patients whose age is less than one year or those with other chronic diseases, in addition to other inflammatory conditions, autoimmunological, immunosuppressive diseases and Coronavirus disease 2019 (COVID-19) or infections other than respiratory tract infection.

2.1.3. Study Design

This is a case-control study which involved 100 asthmatic children who

were divided into two groups according to the type of asthma including atopic (No.50) and non-atopic (No.50) distributed in (66 male and 34female) and 50 non-asthmatic children (33 male and 17 female). The healthy controls children (non-asthmatic) were in the same age and sex of asthmatic children. A study design that is enrolled in this study was shown in Figure 2.1.

2.2. Ethical and Scientific Approval

Un approval for this study protocol was given by Karbala Health Directorate's ethical committee. Study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients verbal and analytical approval before sample was taken. Health measures and safety was given during sampling.

2.3. Patients Data Collection

The Demographic and clinical data were collected through patients and /or their relatives through a questionnaire.

2.3.1. Self-Reporting Questionnaires: The questionnaires were planned to scan the children at the Kerbala Teaching Hospital for Children and the Study Committee for data collection from children with asthma and non-asthmatic children and/or their parents, taking into account international and local criteria (Appendix I and Appendix II).

Types of treatment: Two basic types of treatment were prescribed by the physicians which were montelukast and inhaled corticosteroids.

Asthma Severity: The degree of asthma severity were identified based on the international standards diagnosed in the NAEPP/EPR 3 Guidelines (Urbano, 2008) by the specialist pediatrician.

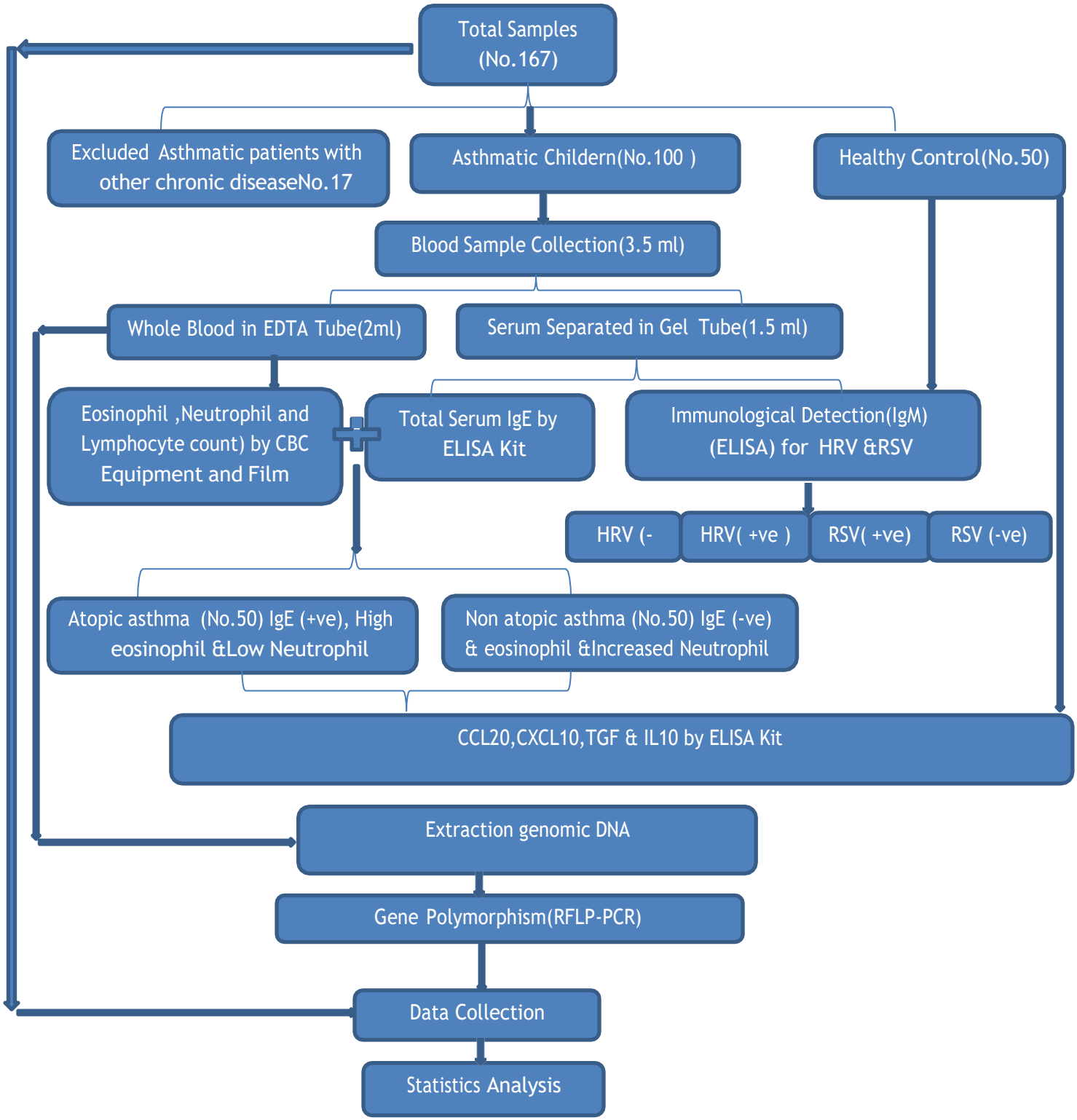

Figure 2.1. A flow chart illustrating the study design and methods.

2.4. Materials

2.4.1. Equipment and Instruments

In the present study, the following equipment and instruments were used (Tables 2.1 and 2.2).

Table2.1.Equipment with its manufacturing companies and country of origin

Equipment	Manufacturing Company	Country
Autoclave	Labtech	Korea
Bench Centrifuge	Hettich – Cleaver	Germany
Cooling centrifuge(universal 320	Hettich	Korea
Deep Freezer	Stuart	U.K
Digital camera	Sony	Japan
ELISA Devices (washer and reader)	Biokit ELx800	U.S.A
Gel Electrophoresis	Biometral	Germany
Hematology analyzer	Sysmex XN 350	Japan
Laptop	Dell	U.S.A
Incubator	Bender	Germany
Microscope	Olympus	Japan
Microwave Oven	Samsung	Korea
Nano drop	Thermo	U.S.A
Printer	Canon Inc.	China
Refrigerator	Concord	Lebanon
Sensitive balance	Sartorius	Germany
Thermal and gradient thermal	Cleaver Scientific	U.K

cyclers		
U.V Trans illuminator	Biometral	Germany
U.V PCR cabinet	Labconco	U.S.A
Vortex	Thermolyne	
Water bath	Gallen	kamp
Water distillator	Lab Tech	korea

Table 2. 2. Instruments with its country of origin

Instruments	Country
Cylinders (250,500 ml)	Germany
Racks	
Cold medical box	China
Disposal syringes (3 and 5 ml)	
EDTA tube (anticoagulant tubes)	
Eppendorf tube (0.5 ml and 1.5 ml)	
Filter paper	
Filter Tips for PCR (100 µl and 200 µl)	
Flasks (different size)	
Gel and Clot Activator Tube	
Gloves	
Slide holder	
Slides	
Tips (Yellow and Blue)	
Micropipettes (different size)	Japan
PCR StripTubes Sterile (DNase/RNase)free	USA

2.4.2. Chemicals and Biological Materials

Table 2.3 Chemicals and biological materials with their manufacturing companies and country of origin

Chemicals and biological materials	Manufacturing Company	Country
Agarose	Himedia	India
Deionized water	Bioneer	Korea
Distal water	Labtech	
Ethanol (96%)	BDH	UK
Ethidium bromide		
Ethylene diamine tetra acetic acid (EDTA)	Himedia	India
Leishman stain	AFCO	Jordan
Proteinase K	Promega	U.S.A
Tri-Borate EDTA Buffer(TBE buffer)10X	BDH	UK

2.4.3. ELISA Kit

Table 2.4. ELISA kits used in the present study

ELISA kit	Manufacturing Company	Country
Human serum IL-10,TGF β,CCL20,CXCL10, HRV IgM and RSV IgM	Sunlong	China
Total serum IgE (Diagnostic)	AccuBind	U.S.A

2.4.3.1. ELISA Kit Content of Total Serum IgE

Table 2.5. ELISA kit for detection of the human total serum IgE (LOT NO. 25K1D1) and (CAT NO. 2525-300A).

Components	Format
1. Antibody-coated micro plate wells	12×8
2. Calibrator A 0.0 IU/ml (IgE,Human)	1 ml ×1
3. Calibrator B 5.0 IU/ml (IgE, Human)	1 ml ×1
4. Calibrator C 25 IU/ml (IgE, human)	1 ml ×1
5. Calibrator D 50 IU/ml (IgE, human)	1 ml ×1
6. Calibrator E 150 IU/ml (IgE , human)	1 ml ×1
7. Calibrator F 400 IU/ml (IgE, human)	1 ml ×1
8. Enzyme conjugate horse radish peroxidase (HRP)	13 ml ×1
9. Wash buffer	20 ml ×1
10. Substrate Solution A	7 ml ×1
11. Substrate Solution B	7 ml×1
12. Stop solution (1N HCl)	8 ml×1
13. Plate Sealer	2 pics

2.4.3.2. ELISA Kit Content for detection Serum IL-10 ,CCL20 and TGF-β and CXCL10

Table2.6.ELISA kit for detection of serum IL-10,TGF-β,CCL20 and CXCL10 kit CXCL10kit(LOT NO. 20220922)

Components	Format	Storage
1. User manual	1	R.T.

2. Sealed bags		1	R.T.
3. Closure plate membrane		2	R.T.
4. Micro ELISA striplate		12×8	2-8°C
5. Standard	IL-10 and CCL20: 135 pg / ml	0.5ml×1vial	2-8°C
	TGF-β and CXCL10:270pg/ml		
6. Standard diluent		1.5ml×1vial	2-8°C
7. Sample diluent		6 ml×1 vial	2-8°C
8. HRP - Conjugate reagent		6 ml×1 vial	2-8°C
9. Chromogen solution A		6 ml×1 vial	2-8°C
10. Chromogen solution B		6 ml×1 vial	2-8°C
11. Stop solution		6 ml×1 vial	2-8°C
12.Concentrated Wash Solution (30X)		20ml×1 vial	2-8°C

2.4.3.3. ELISA kit Contents of Serum HRV-IgM and HRSV-IgM

Table 2. 7. ELISA kit for detection of HRV-IgM and HRSV-IgM (LOT NO. 20220426

Components	Format	Storage
1. User Manual	1	R.T.
2. Closure plate membrane standard	2	R.T.
3. Sealed bags	1	R.T.

4.Concentrated HRP conjugate reagent	6 ml×1 vial	2-8°C
5. Negative Control	0.5 ml×1 vial	2-8°C
6. Positive Control	0.5 ml×1 vial	2-8°C
7. Sample diluent	6 ml×1 vial	2-8°C
8.Chromogen Solution A	6ml×1 vial	2-8°C
9.Chromogen Solution B	6ml×1 vial	2-8°C
12. Stop Solution	6 ml×1 vial	2-8°C
10. Concentrated Wash Solution(30X)	20 ml×1 vial	2-8°C
11. Micro ELISA plate	12×8	2-8°C

2.4.4. Deoxyribonucleic Acid (DNA) Extraction Kit

Table 2. 8. Human genomic of deoxyribonucleic acid (DNA) extraction kit
Cat No.FABGK100 and Lot No.CC310122622

DNA extraction kit contents	Volume	Manufacturing Company	Country
RBC Lysis Buffer	135ml	Favorgene	Taiwan
FATG Buffer	30ml		
FABG Buffer	40ml		
W1Buffer	45 ml		
Wash buffer*(Concentrate)	25 ml		
Elution Buffer	30ml		
FABG Mini Column	100pcs		
Collection tubes	200 pcs		

User Manual	1		

2.4.5. Polymerase Chain Reaction Kit

Polymerase Chain Reaction kit contents of gene amplification is demonstrated in Table 2-8.

Table 2.9. Reagents equipment for PCR

Reagent and Equipment of PCR	Volume	Company	Country
GoTaq® G2 Green Master Mixes	2 × 1.25 ml	Promega	U.S.A
Nuclease-free water	2 × 1.25 ml		
Bromophenol blue loading solution	3 × 1 ml		
CCL20 forward primer	10.9 nmol	Mcrogen	South Korea
CCL20 reverse primer	13.5 nmol		
100 and 50 bp of ladder	0.5 ml	Solgent	Korea

2.4.6. Restriction Enzyme Kit

Table 2.10. Restriction enzyme kit

Restriction enzyme kits contents	Volume	Company	Country
Rsa I	2000 unit	ThermoFisher	U.S.A.
Buffer Tango of Rsa I	1ml of 10 x		

2.5. Methods

2.5.1. Body Mass Index (BMI)

Children's BMI is calculated by dividing a person's height in meters squared by their weight in kilograms (Centres for Disease Control and Prevention, 2019).

2.5.2. Samples Collection and Processing

Blood in volume 3.5 ml of venous was withdrawn from each participant which was obtained by disinfecting antecubital fossa with 70% ethanol and then made vein puncture by disposal 5ml syringes after applying a tourniquet. Blood then was divided into EDTA tubes and gel tubes according to further management of the sample. Volume of 3.5 ml of blood was dispensed into 2 ml to EDTA tube for the haematological and molecular tests in order to investigate gene polymorphism for the CCL20 gene. Further 1.5 ml of blood was put into gel tube and allowed to clot then serum was separated by centrifugation at 3000 round per minutes (RPM) for 15 minutes. After that, serum was transferred to four newly labeled Eppendrof tubes and stored in deep freeze (-20°C) to be used for immunological assays, including ELISA analysis for TSIgE, CXCL10, CCL20, TGFB and IL-10 and IgM for both HRV and RSV.

2.5.3. Complete Blood Count

The blood samples were taken from patients which place in EDTA tubes and shaken before being quickly inspected in a Sysmex XN-350 five differential automated hematology analyzer (Sysmex, Japan) to count white blood cells.

2.5.4. Peripheral Blood Smear

Blood smear was prepared according to (Dacie and Lewis, 2017).

2.5.5. Immunological Diagnosis by Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA Kits was applied to the *in vitro* qualitative and quantitative determination of human serum (HRV IgM, RSV IgM as shown in Appendix IV) and (TSIgE ,CCL20, CXCL10, IL-10 and TGF-β) for patients and control group respectively.

2.5.5.1. Measurement The Level of The Total Serum IgE

Serum was analysed to determine the total IgE concentration by Awareness Technology automated immunoassay analyzer (BioTek, USA) using AccuBind EUROIMMUN total IgE ELISA kit.

2.5.5.1.1. The Principle of The Test (Indirect ELISA)

The test kit contains microtiter wells coated with polyclonal antibodies against human IgE. In the beginning, serum from patient samples were incubated in the wells. IgE found in the sample would bind to the antibodies. After that a second incubation was carried out using an enzyme-labelled anti-human IgE. The color reaction would be catalyzed by the addition of a substrate. Then stop solution would stop the reactions. Finally, the determination of the IgE concentration was measured by means of the calibration curve at a wavelength of 450 nm.

2.5.5.1.2. Preparation of The Reagents

All reagents left to room temperature (25℃) before use.

1. Substrate Reagent: the contents of the vial labeled solution (A) Poured into the vial labeled solution (B) and placed the yellow cap on the mixed reagent for easy identification. And stored at 4℃.
2. Wash Buffer: Diluted the contents of wash concentrate to1000 ml with distilled water in a suitable storage container. The diluted buffer was stored at 4℃ for up to 60 days. 20 ml of concentrated wash buffer was diluted into 980 ml of wash buffer with distilled water. If crystals have formed in the concentrated, the buffer is warmed with water bath and mixed gently until the crystals have completely dissolved. And the solution cooled to room temperature (25℃) before used.

2. 5. 5. 1. 3. Procedure of The Test

1. Sample incubation: 25 µl of serum sample and calibrator was transferred to micro plate wells and 100 µl of biotin reagent was added into each microtiter plate wells and left for 30 min at room temperature. to mix the contents the micro plate was gently swirled for 20-30 sec. and covered by sealed with closure plate membrane. Then, the contents of the micro plate was discard by decantation or aspiration,and bloted the plate with absorbent filter paper.

2. Washing: automatic wash was conducted 2-3 times with 300µl of wash buffer.

3. Enzyme Conjugate incubation: 100µl of enzyme conjugate was added into each micro plate wells, and left without shake the plate after enzyme conjugate addition, and covered by sealed with closure plate membrane and allowed to incubate for 30 min at room temperature.

4. Washing:Automatic wash was conducted 2-3 times with 350µl of wash buffer.

5. Substrate incubation: 100 µl of chromogen/substrate solution which were added into each micro plate wells, and left without shake the plate after substrate addition and left 15 min at room temperature.

6. Stopping: 50 µl of stop solution was added into each micro plate wells and gently mix for 15-20 seconds.

7. photometric measurement was done at 450 nm within 30 min of adding stop solution.

2.5.5.1.4. Interpretation of The Result

1. The average absorbance value 450 nm was calculated for each set of reference standards, and samples.

2. A standard curve was constructed by plotting the mean absorbance obtained for each reference standard against its concentration in IU/mL.on graph paper, with absorbance on the vertical Y-axis and concentration on the horizontal or X-axis.

3. The mean absorbance value was used for each sample, determine the corresponding concentration of IgE in IU/mL from the standard curve.

2. 5. 5. 2.Measurement of The Chemokines and Cytokines Concentrations

2. 5. 5. 2.1. Measurement of The Total Serum CCL20, CXCL-10, IL-10 and TGF-β

Total CCL20, CXCL10, IL-10 and TGF-β concentration was measured by Awareness Technology automated immunoassay analyzer (BioTek, USA) using Sunlong ELISA kit as shown in Appendix III.

2. 5. 5. 2.1.1. Principle of The test

This test is used the Sandwich-ELISA principle, in which the microtiter plate wells were pre-coated with an antibody specific to human CCL-20,CXCL-10, IL-10 and TGF-β. Standards or samples were added to the microtiter wells and combined with the specific antibody. After that a biotinylated detection antibody specific for Human (CCL-20,CXCL-10, IL-10 and TGF-β) and Avidin-Horse Radish Peroxidase (HRP) conjugate were added successively to each microtiter well and incubated. after that, free components were washed away and substrate solution was added to each well and incubated. Finally the enzyme-substrate reaction was terminated by the addition of a stop solution and the color would turn from blue to yellow. IL-10 concentration was measured by means of the standard curve at a wavelength of 450 nm.

2. 5. 5. 2. 1. 2. Preparation of The Reagents

1. Dilution of Standards as shown in appendix IV.
2. Substrate Reagent: sensitive to light and contaminants.
3. Wash Buffer: 30 ml of concentrated wash buffer was diluted into 850 ml of wash buffer with distilled water. The buffer was warmed 37°C with water bath and mixed gently until the crystals were completely dissolved in case crystals was formed in the concentrated. The solution cooled to room temperature before used.

2. 5. 5. 2. 1. 3. Procedure of The Test

1. Sample incubation: Fifty microliter of standard or sample (10 µl serum + 40µl sample diluent buffer) was added to each well, samples loaded onto the bottom without touching the well wall. The contents were mixed well with

gentle shaking and incubated for 30 min at 37°C, after sealed with closure plate membrane.

2. Washing:carefully peel off closure plate membrane,aspirate and refill with the wash solution. Discard the wash solution after resting for 30 sec. Repeat the washing procedure for 5 times with 350 µl of wash buffer.

3. Conjugate incubation: 50µl HRP conjugate was added and incubated for 30 min at 37°C, then aspirated and washed five times with 350 µl of wash buffer.

4. Substrate incubation: Fifty microliter of substrate A reagent was added firstly, then 50µl of substrate B reagent was added secondly to each well , mix with gently shaking and incubate for 15 min at 37°C.

5. Stopping: In order to terminate the reaction, 50 µl stop solution was added to each well. The color in the well would change from blue to yellow and read absorbance (O.D.) at 450 nm using a microtiter plate reader within 15 min of adding the stop solution. The OD value of the blank control well was set as zero.

2. 5. 5. 2. 1. 4. Interpretation of The Results

The curve was plotted on log-log graph paper, with standard concentration on x-axis and OD values on y-axis.

2. 5. 6. Molecular procedure steps

2. 5. 6. 1. Extraction of Human Genomic Deoxyribonucleic Acid (DNA)

The genomic DNA was extracted from the the nucleated cells of study groups under the aseptic condition according to manufacturer instructions of

FavorPrep™ Blood/ Genomic DNA Extraction Mini Kit, Taiwan.Cat No. FABGK100 and Lot.NoCC310122622.

2. 5. 6. 1. 1. Deoxyribonucleic Acid Extraction Procedure

1. The whole blood sample was mixed thoroughly for at least 10 minutes at room temperature.
2. Two hundred µl of whole blood was added to a 1.5 ml micropcentrifuge tube (not provided).
3. Added 30 µl Proteinase K (PK) (10mg / ml not provided) Solution to the sample, and briefly mixed then incubate for 15 min at 60°C .
4. Two hundred µl of Cell Lysis Buffer (FABG) was added to the tube, then caped and mixed by vortex for at least 10 seconds.
5. The processed sample was incubated at 70°C water bath for 15 min.to lyse the sample, during incubation, invert the sample every 3 min.
6. Absolute ethanol(200 µl) (96~100%) added to the clear lysate and immediately mixed by vortex for 10 seconds, and then precipitates were broken by pipetting.
7. A FABG column Placed to a collection tube then transferred the sample mixture (including any precipitate) carefully to FABG column.

Then centrifuged at speed 14000 rpm for 1 min and discarded the collection tube then placed FABG column to a new collection tube.

8. Immediately, 400 µl W1 Buffer was added to the FABG column and centrifuged for 30 sec. at speed 1400rpm. then discarded the flow-through and placed the FABG column back to the collection tube.

Note. Make sure that ethanol has been added into W1 buffer when first open.

9. 600µl wash buffer(Preparation of wash buffer by adding ethanol(96-100% and volume of ethanol 100ml)) was added to the FABG column and centrifuged for 30 sec. at speed 1400rpm then discarded the flow-through and placed the FABG column back to the collection tube .

10. The column was centrifuged for an additional 3 min to dry. Important Step:This step will avoid the residual liquid to inhibit subsequent enzymatic reactions .

11. The dry FABG column was Placed to a new 1.5 ml microcenterifuge tube

12. A Standard volume (100 µl) of preheated elution buffer was added to the membrane center of the FABG column.

18. The FABG column was incubated for 10 min at 37°C in an incubator.

Important Step

20. Eluted the DNA by Centrifugation for 1 minute at full speed 14000 rpm.

15. DNA extract was stored at 4 °C or -20°C .

13. one hundred µl of nuclease-free water was added to the column and centrifuged for 1 minute at maximum speed.

14. The ReliaPrep™ Binding column was discarded, and the elute stored in -20°C until used.

2. 5. 6. 1. 2. Detection of DNA Extraction Efficiency

Agarose gel electrophoresis 1 % was adopted to confirm the presence and integrity of the extracted DNA (Gaikwad, 2016).

2.5.6.1.3. Detection of DNA Extraction Efficiency,Concentration and Purity

The extracted genomic DNA was checked by using Nano drop spectrophotometer (THERMO. USA), which check and measures the purity of DNA through reading the absorbance at (260 /280 nm) as follows

1. After opening up the Nano drop software, the appropriate application was chosen (Nucleic acid, DNA).
2. A dry chem- wipe was taken and the measurement pedestals were cleaned several times. Then carefully 1µl of ddH_2O was pipetted onto the surface of the lower measurement pedestal.
3. The sampling arm was lowered and OK was clicked to initialized the nano drop, then the pedestals was cleaned off and 1µl of the appropriate. Blanking solution was added as black solution which is the same elution buffer of DNA samples.
4. The pedestals were cleaned and 1µl of DNA sample was pipetted for measurement.
5. The purity of DNA was determined by reading the absorbance in Nano drop spectrophotometer at 260 nm and 280 nm, so the DNA 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. A ratio of ~1.8 is generally accepted as “pure” for DNA, 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.

2.5.6.2. Polymerase Chain Reaction(PCR)Preparation

2.5.6.2.1. Preparation of The Primer Suspension

The primers were prepared according to manufacturer instructions to form a stock solution with a concentration of 100 pmol/µl by dissolving the lyophilized primers with deionized distilled water. Then the working solution was prepared by dissolved 10 µl of 100 pmol/µl with 90 µl of deionized distilled water to form 100 µl of 10 pmol/µl.

Table 2.11.The specific primers and their sequences

Primer	Sequences	Product Size (bp)	Region of SNP	Reference
CCL-20 [-786T>C (rs6749704)]	F: 5-TTTGACATTTGCTGTGCTGAC-3	214	227813076-227813183 (107 bp)	Jafarzadeh *et al.*, 2014)
	R: 5-GGCTCAAACCTCAGCTTCAC-3			

2.5.6.2.2. Preparation of Tris-boric acid–EDTA(TBE) Buffer Solution

All TBE powder 1X dissolved in 1000 ml.

2. 5. 6 .2. 3. Polymerase Chain Reaction (PCR) Protocol

The polymorphism of the CCL-20 gene (AppendixVI) was identified by restriction fragment length polymorphism PCR(RFLP-PCR method. RFLP detect SNPs by use of restriction enzymes. Restriction endonucleases are enzymes that cut DNA into short pieces. Each restriction enzyme targets differ

in the nucleotide sequence of DNA strand and therefore cuts at different sites(MeSH, 2019).

2. 5. 6. 2. 4. Polymerase Chain Reaction (PCR) Master Mix Preparation

Master mix of PCR was prepared according to Promega protocol(Go Taq®Promega Green Master Mix) as shown in Table 2.12. The addition of PCR master mix components was done in a UV PCR cabinet to prevent contaminations by nucleic acid. All PCR components were assembled in PCR tube, and mixed by a refrigerated microcentrifuge at 50 rpm for 10 sec. PCR program consists of 30 cycle for chemokine gene primer.

Table 2.12. Protocol of PCR mixture

Component		Volume in a 25 µl reaction
Go Taq®Promega Green Master Mix 2x		10 µl
Template DNA		2 µl
Primers	Reverse (10 pmol/µl)	2 µl
	Forward (10 pmol/µl)	2 µl
Nuclease Free water		4 µl
Total		20

2. 5. 6. 2. 5. Thermocycling Conditions

The PCR thermocycler conditions for each gene were done by using convention PCR thermal cycler system. The running conditions according to detected by Jafarzadeh *et al.*, (2014) as shown in Table 2.13.

Table 2. 13. PCR Thermo Cycling Condition for CCL20 Gene Detection

Step	Temperature	Time	Number of cycle
Initial Denaturation	94 ˚C	5 Minutes	
Denaturation	94 ˚C	30 Seconds	30 cycles
Annealing	58 ˚C	30 Seconds	
Extension	72 ˚C	30 Seconds	
Final Extension	72 ˚C	5 Minutes	
Hold	4 ˚C	∞	

2. 5. 6. 2. 6. Restriction Enzyme Preparation

Rsa I, ThermoFisher (Scientific)

This enzyme prepared according to ThermoFisher protocol, it recognizes a sequence the GT AC of CCL20(-786C>T) gene and cuts best at 37 ˚C in Tango buffer. After PCR cycles were finished. Ten units of enzyme were added to 10 µl of CCL20 PCR product with 1-2 µl of enzyme buffer and 18 µl nuclease-free water.Tubes were then incubated for 1-16 hours at 37 ˚C.

2. 5. 6. 2. 7. Gel electrophoresis

A. Preparation of Gel Electrophoresis

Agarose gel was prepared in a concentration of 1.5-2 % for DNA profiling according to Su *et al*., (2008). Agarose gel was prepared through the following steps:

1.Dissolving 2gm of agarose powder in 100 ml of TBE buffer (90 ml D.W. were added to 10 ml TBE buffer 10X, the final concentration was 1X).

2. Then the mixture was placed in a microwave for 1 minute at 90 ˚ C, then allowed to cool to 50 ˚C, and 4 µl ethidium bromide (5%) was added.

3. The agarose-ethidium bromide solution was poured into the gel tray of electrophoresis apparatus containing the combs, and the two ends of the gel tray were sealed with combs fixed.

4. The agarose was allowed to solidify at room temperature for 20 min.

5. The combs and the seals were removed gently from the tray.

6. The combs were made wells that used for loading DNA samples.

7. Five microliters of amplified PCR product were loaded to the agarose gel wells followed by DNA marker (ladder) to one of the wells.

8. The gel tray was fixed in electrophoresis chamber and 1X TBE buffer was added to the chamber until the surface of the gel was covered. Then the electric current was set on 70 volt for 75-80 min.

B. Agarose Gel Documentations

The amplified PCR products and restriction enzyme products of CCL-20 were separated in 1.5% agarose gel and visualized with UV light, using the gel documentation system.

1. Agarose Gel Electrophoresis Documentations

The amplified PCR products were separated in 1.5% agarose gel and visualized with UV light, using the gel documentation system. The positive results of PCR products were distinguished when the DNA band base pairs of sample was equal to the target product size for (CCL-20) 214 bp. Finally, the

gel was photographed, using Cleaver Gel Documentation System by a digital camera.

2. Agarose Gel Electrophoresis for Restriction Enzyme Products

The result of CCL20 restriction enzyme products was distinguished when the DNA band of the sample separated into two bands with a length of 129 bp and 85 bp for wiled alleles type while only one band with a length of 214 bp in the mutant alleles type. The positive results were distinguished when the DNA band base pairs of sample was equal to the target product size for *CCL20*. Finally, the gel was photographed, using Cleaver Gel Documentation System by a digital camera

2. 6. Statistical Analysis

For statistical analysis, data were entered into the SPSS version 20 for Windows program (GraphPad Software, San Diego, California, USA).The results were expressed using mean ±SD. ANOVA was used to compare different means. Statistical significance was defined as a *P*-value <0.05, and extreme significance as a *P*-value <0.001 . The relationship between the levels of CCL20, CXCL10, IL-10, and TGF-B and the percentage eosinophil count and total serum IgE levels was also explained using the Pearson correlation.

Genotypes of *CCL20* -786T>C were shown as percentage frequencies, and one-tailed Fisher's exact probability (P) and Kingdom by anova calculator was used to determine whether there was any significant changes in the distributions of these variables among asthmatic patients and controls. Additionally ,the odds ratio(OR) was estimated to define the association between a genotype with the disease. Allele frequencies of genes were calculated by direct gene counting methods.

2.6.1. Hardy Weinberg Equilibrium (HWE)

In genome-wide searches for markers with empirically confirmed genotypes, testing for HWE is frequently employed as a quality control filter. HWE is a crucial quality assurance step in population-based genetic association studies, which have been shown to be effective in identifying genes linked to a wide range of complex human disorders with significant public health implications. In the past, statistical tests for HWE were a crucial tool for identifying genotyping errors, and they are still crucial for the quality assurance of next-generation sequence data and permit evaluation of miss in gness. In addition, can indicate inbreeding, population stratification, and even problems in genotyping (Namipashaki *et al*., 2015; Graffelman *et al*., 2017).

Chapter Three

Results and Discussion

3. Results and Discussion

3 .1. Distribution of The Asthmatic Patients and Healthy Control Group

Hundred and fifty samples were collected in the current study, which included 100 asthmatic patients divided to 50 atopic and 50 non-atopic who have suffered from virus(suspected) infection and had flu, cough and fever and attended to the asthma clinic in Kerbela pediatric hospital, beside to 50 healthy control revealed the following results:

3. 1. 1. Asthma Distribution by Age and Sex

The two groups of asthmatic patients in the present study were divided into 5 categories according to their age ranging between 1-6 years old distributed into(1-2, 2.1-3,3.1-4, 4.1-5,and 5.1-6 years) as shown in Table 3.1.

Table 3. 1. Distribution of asthmatic patients depending on age and sex

Age(years)	Males No.	%	Females No.	%	Total No.	%
1-2	9	56.3	7	43.8	16	16.0
2.1-3	11	55.0	9	45.0	20	20.0
3.1- 4	10	90.9	1	9.1	11	11.0
4.1-5	7	77.8	2	22.2	9	9.0
5.1- 6	29	65.9	15	34.1	44	44.0
Total	66	66	34	34	100	100

The highest frequency of asthmatic children age was in the group 5.1-6 years in both sexs followed by 2.1-3 years which were 44.0%&20.0 %, respectively and the lower frequency was 9.0 % at 4.1-5 years as shown in Figure 3.1.

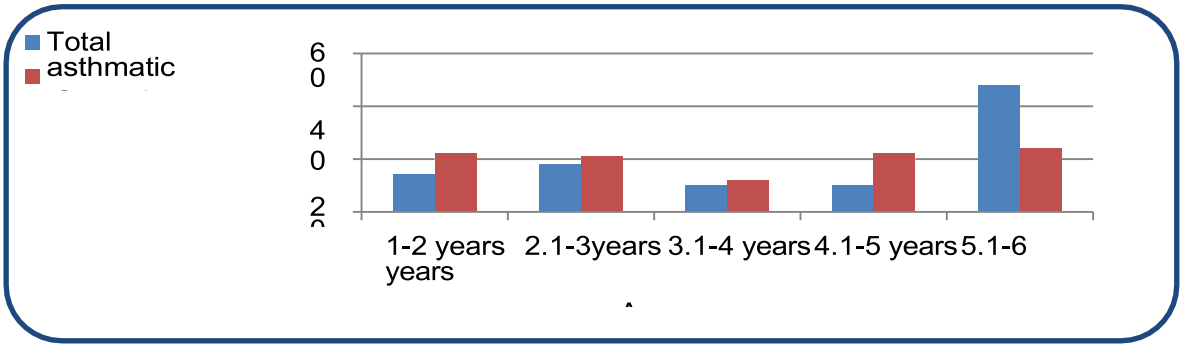

Figure 3. 1. Age distribution of total asthmatic children.

Abood *et al*., (2023) mention that 43% and 30% of asthmatic children patients belonged to the age group of <5years and 5-10years, respectively. This result is in agreement with frequency of asthma in Shanghai, China, and identified related risk factors for asthma in children at the age of 3-6 (Ren *et al*., 2022). In addition, Rahimian *et al*., (2021) found that the frequency of asthma in Iranian children was lower in comparison to other region in the world (6%), and in boys and girls were 9% and 8%, respectively. The current data clarified that asthma was more frequent in 5.1- 6 years (44.0%) and (40.0%) in atopic and non-atopic asthmatic children, respectively (as shown in Figures 3.2 and 3.3). The result showed that there was no significant difference in age distribution between asthmatic patients and healthy control as presented in Table 3.2.

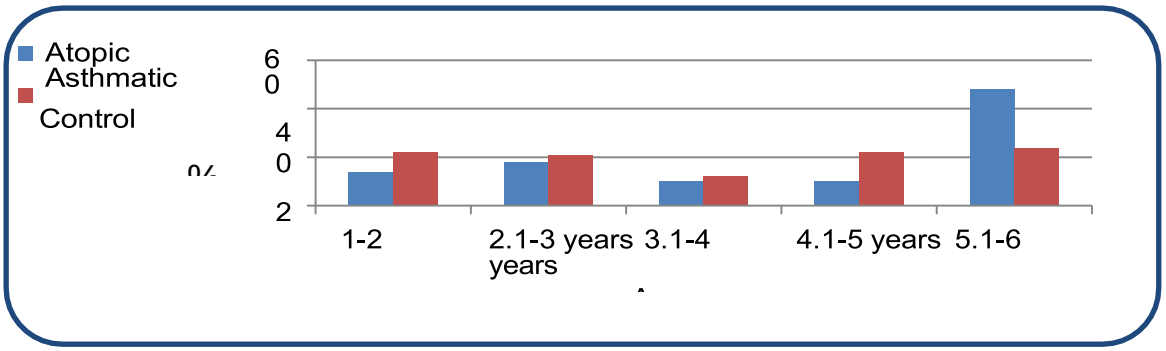

Figure 3. 2. Age distribution of atopic asthmatic children

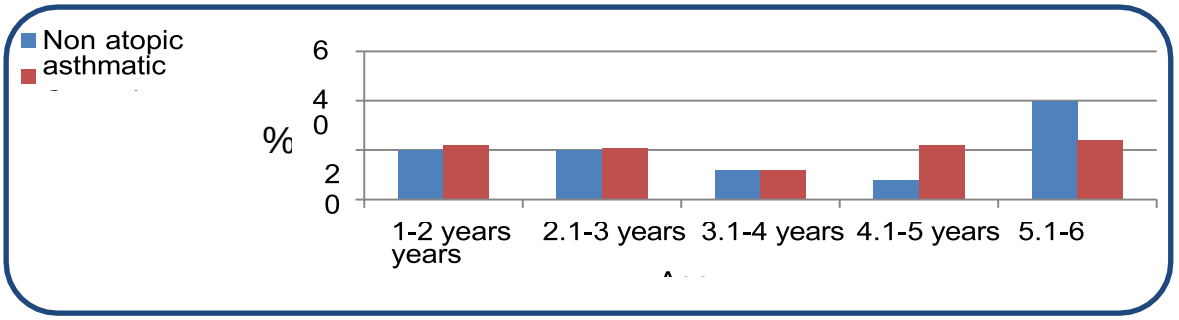

Figure 3. 3. Age distribution of non atopic asthmatic children.

This group had a higher risk percentage for asthma than did those in other age groups. The possible explanation for this result is that children were typically higher sensitive to allergens and viruses because their bodies are still

developing,further decrease in age lead to the children become more effective to exposure to respiratory disease like asthma.Moreover, frequency of bronchial asthma in children asthmatic patients may be due to the inadequate treatment or healthcare of asthma, increase in passive smoking or exposure to air pollutions and increase pulmonary diseases or microbial infections in airways in some population (Ren *et al*., 2022).

Table 3. 2. Difference in mean age between asthmatic and control group.

	Healthy control	Cases (Asthma)		P.value
		Atopic	Non atopic	
Age (years)	1- 6	1- 6	1- 6	0.3
Mean`	4.0002	4.4504	4.1800	
Standard Deviation (SD)	1.55302	1.66115	1.73429	
Standard Error (SE)	0.21963	0.23492	0.24527	
No.	50	50	50	

(P<0.05), Non significant, No.Number,by one way ANOVA test

This result showed there was no significant difference in age distribution among asthmatic patients and control as shown in Table 3.2.The present results showed that a frequency of asthma in male, with a percentage of 66.0%, whereas the percentage was 34.0% in female in the group of asthmatic children Table 3.3. Furthermore, the percentage of males /females in the groups of atopic and non atopic asthmatic patients were 70.0/30.0,62.0/38.0, respectively, while 64.0/36.0 for the control group as shown in Table 3.3. Significant differences were found among sexes between the patient group and the control group (P=0.01). Further , male in a group of children were associated with both atopic and nonatopic asthma. The obtained data are in agreement with Naeem and Silveyra, (2019**)** who found that males suffer more often from asthma than females, and these may be because physical activity in males comparison to females. The study of Trivedi and Patel (2020) revealed that of 109 children with asthma, the percentage of males and females was 2.6:1with a significant male predominance. It is clear that the male children are at increased risk for asthma and this is probably related to

narrower airways and increased airway tone which predisposes them to enhanced airflow limitation in response to a variety of stimuli especially HRV and RSV infections. This difference disappears after the age of 10 years when airway diameter/length percentage is the same in both sexes, because of changes in thoracic size that occurs in puberty in males but not in females(Ripoll *et al*., 2020). Furthermore, the present result may be because males more effective than females and in Iraq the males have more contact with environmental pollution than females, in addition the questionnaires in the present study confirmed the male has more exposure to dust than female.

Table 3. 3. Difference in sexes between asthmatic patients and control group.

Asthma types	Total No.	Sex	Asthmatic patients		P.value		
			No.	%			
Atopic	50	Male	35	70.0	0.242**	0.32**	0.01*
		Female	15	30.0			
Nonatopic	50	Male	31	62.0	0.150**		
		Female	19	38.0			
Healthy children	50	Male	32	64.0	0.174**		
		Female	18	36.0			

(P<0.05),No: Number, **: Non significant difference between male and female in atopic, non atopic patients and healthy children,*.Significant difference between atopic, non atopic patients and healthy children, by on way ANOVA test

3.1. 2. Body Mass Index (BMI) Distribution

Regarding BMI, the mean of it were 15.03, 15.6 and 17.07 in atopic, non atopic and healthy control as shown in Table 3.4.

Table 3. 4. Difference in BMI between the asthmatic patient and control groups.

	Asthmatic Patients No.100 Mean± S. D		Healthy controls No.50 Mean ±S. D	P.value
	Atopic No.50	Non Atopic No.50		
Weight	18.25± 6.041	17.11±5.572	19.74± 5.196	0.26
Hight	108.74±16.112	102.88±15.907	113.92 ±15.514	
BMI	15.03 ±1.535	15.64±2.933	17.7± 1.640	

BMI of children was calculated according to the web site-Centres for Disease Control &Prevention(Centres for Disease Control and Prevention,2019),by one wayANOVA test.

In addition a significant difference was not found between asthmatic patients and healthy control in weight, height, and BMI. In contrary a study conducted by Lin *et al*., (2017) which found that the first ages in obese children had a higher risk percentage for asthma in comparison with other age groups. These differences might be due to ethnicity differences. The relationship between excess weight gain and asthma in childhood remains inadequately defined, previous studies mentioned the relation between asthma and body weight gain (Bruske *et al*., 2014; Han *et al*., 2019). For example, Ekstrom *et al*., (2017) illustrated that persistent asthma no consistent association was observed among males whereas among females associated with high BMI throughout childhood. The correlation between asthma and obesity may be related to the potency of ICS use in paediatric patients with moderate-severe asthma and affects BMI trajectory (Han *et al*., 2019). On the other hand previous results showed, that asthmatic children were shorter than healthy (Protudjer *et al*., 2015). It might be possible to attribute this to any dose of systemic corticosteroids daily suppressing the growth percentage for as long as the treatment is maintained (Loke *et al*., 2015). BMI is a risk factor associated with atopic and non atopic asthma (Jallab and Hasan, 2018).

3. 1. 3. Resident Distribution

Most of the children in the current study lived in urban areas than rural as presented in Figures 3.4, 3.5, and 3.6 (P=0.000). The lower prevalence in rural may be due to beneficial effects of exposure to farm environments and less exposure to air pollution. Whilst pollen in farms in rular may be exacerbate asthma in children but in less compared to other causes to trigger asthma in urban.

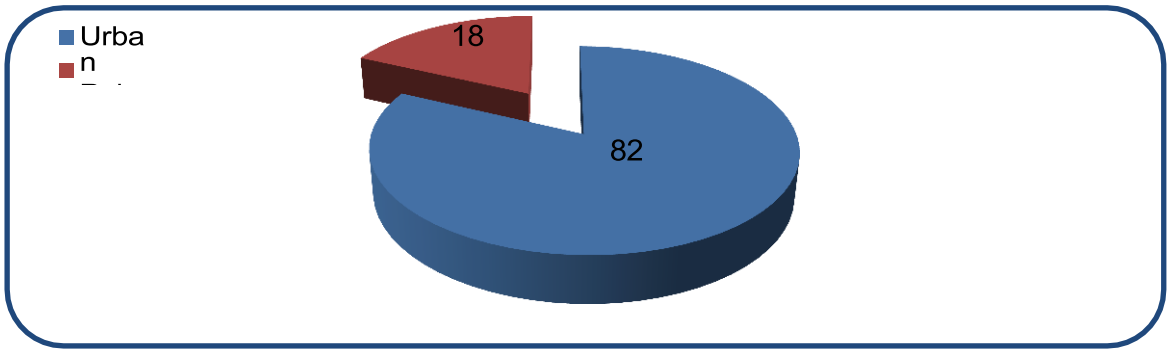

Figure 3.4. Resident distribution of total asthmatic children

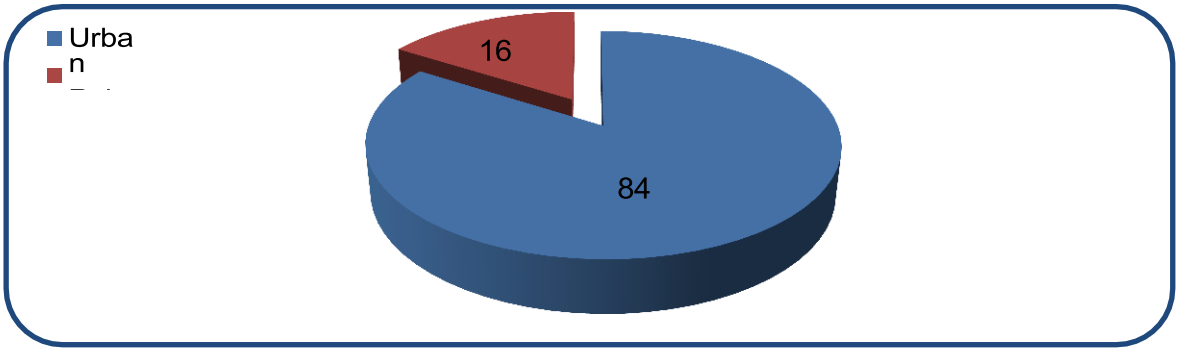

Figure 3. 5. Resident distribution of Atopic asthmatic children

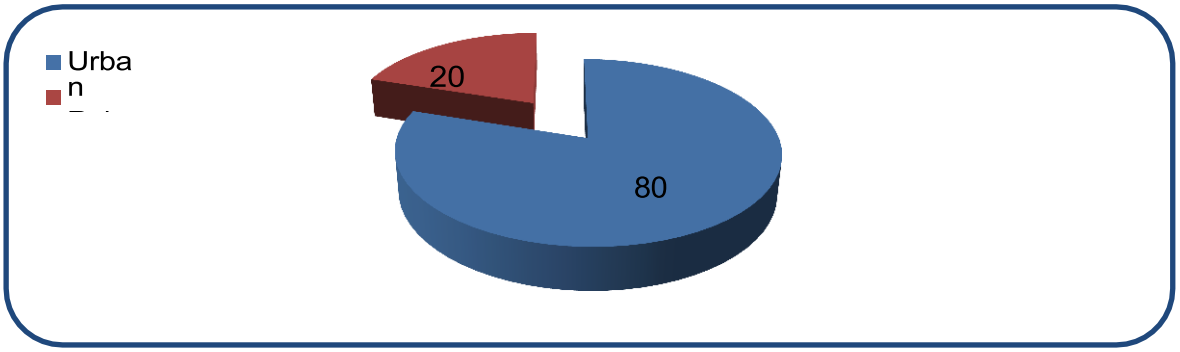

Figure 3. 6. Resident distribution of non atopic asthmatic children

The current results support the evidence of a difference in childhood asthma frequency between urban and rural locations, and that once a child has asthma, certain crowded urban exposures may aggravate the disease (Grant and Wood, 2022). The study of Lama *et al*., (2013) mentioned that 56 (80%) of asthmatic children lived in urban whilst in rural were14(20%). Rennie *et al*., (2020) showed that atopic and nonatopic asthma phenotypes were vary by specific housing and other environmental conditions such as virus and bacterial infection.

3. 2. Major Characteristics of Asthmatic Children

3. 2. 1. Severity of Asthma

The present study included 33,15 and 2 atopic asthmatic children with mild, moderate and severe asthma respectively, whereas non atopic children was 31,15, and 4, respectively as shown in Figurs 3.7and 3.8. Children with asthma age from 1-10 years, and significant correlations between higher levels of HRV or RSV-specific antibodies at age one and atopic in children up to 10 years (Amat *et al*., 2018).

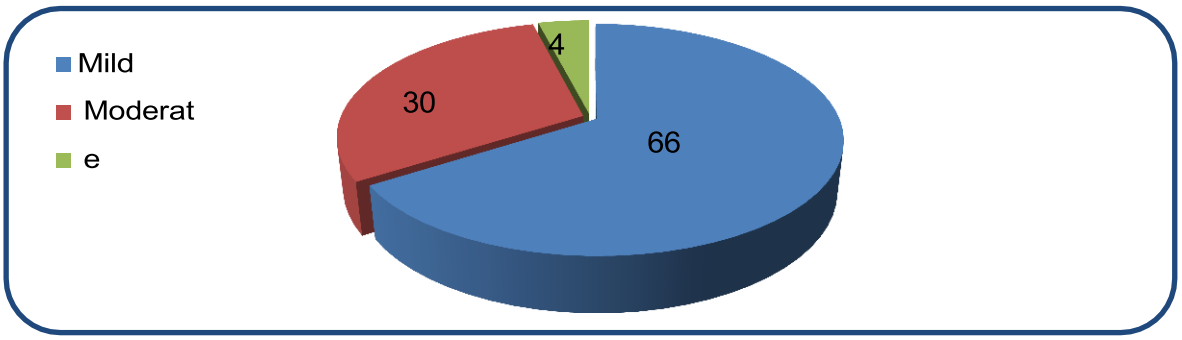

Figure 3. 7. Asthma severity distribution of atopic asthmatic children

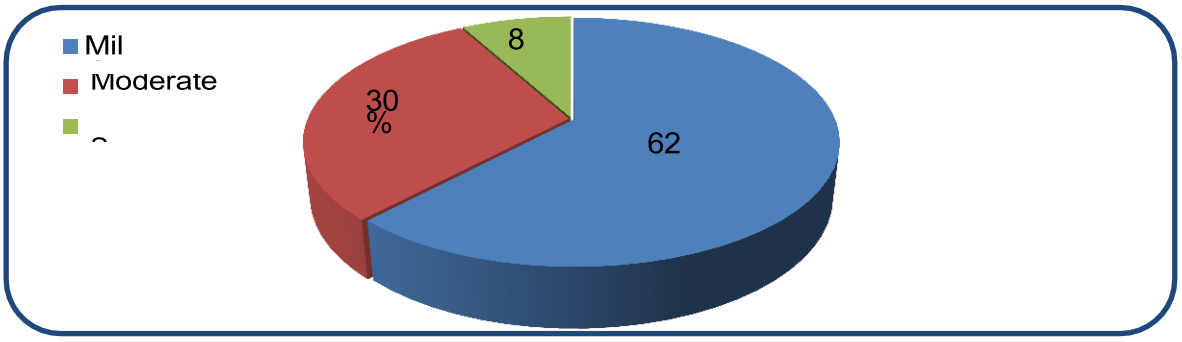

Figure 3. 8. Asthma severity distribution of nonatopic asthmatic children

It can be seen in which study that there was a significant frequency of recurrent bronchial obstruction in 3 years after the initial episode irrespective of its severity or the type of virus involved. Allergic sensitization is possible to be a certain risk factor for asthma severity in the longer term in infants and children, especially those with RSV-hRV co-infection, severity in their

study were mild 27(38%), moderate 29(40%) and severe16 (22%). The current results roughly are in agreement to a study introduced by Abood *et al*., (2023) who showed that the asthmatic children patients 59% mild versus 41% were moderate.In addition Hinks *et al.* (2015) who found moderate asthma more than mild and severe asthma. These differences might be due to ethnicity differences.

3. 2. 2. Type of Treatment

3. 2. 2. 1. Treatment of Atopic Asthmatic Children

In the current results 17 of atopic asthmatic children received regular treatment, with either inhaled corticosteroids (ICSs) or montelukast(5 took ICSs and 12 took montelukast, also 3 took (ICS and beclomethazone) and 2 took (ICS and fluticasone) whereas 28 of them were newly diagnosed as shown in Table 3.5 and Figure 3.9.

Table 3. 5. Types of Treatment for the Atopic Asthmatic Children.

Type of treatment	No.of Patients	%
Montelukast	12	24
ICS	5	10
ICS &Beclomethazone	3	6
ICS &Fluticasone	2	4
No treatment	28	56

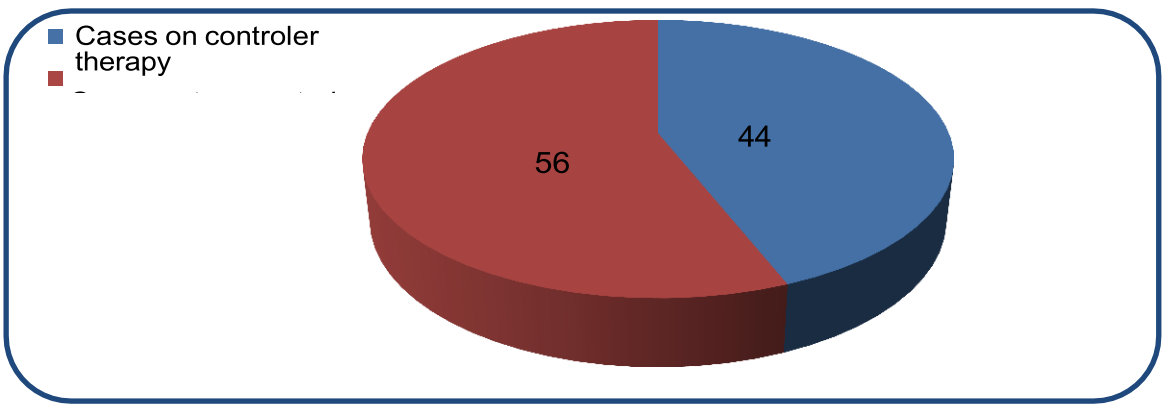

Figure 3. 9. Treatment distribution in the atopic asthmatic children.

Guilbert *et al*., (2014) showed that severe asthma in children was characterized by sustained signs despite treatment with high doses of ICS. Most asthmatic children in the current study were taking montelukast treatment compared to other drugs due to its effectivity and fewer side effects. In addition, Abood *et al*., (2023) revealed that 75% of asthmatic children were receiving montelukast and 25% were taking ICS, and 69% were well controlled versus 31% were not well controlled.

3. 2. 2. 2. Treatment of Non Atopic Asthmatic Children

In the non atopic children group, 29 of them were received regular treatment with either montelukast, ICS, beclomethazone or fluticasone, 19 child took montelukast, 5 took beclomethazone,3 took ICSs and 2 took fluticasone), whereas 21of them were newly diagnosed as shown in Table 3.6 and Figure 3.10.

Table 3. 6. Type of treatment for the non atopic asthmatic children

Type of treatment	No.of Patients	%
Montelukast	19	38
ICS	3	6
Beclomethazone	5	10
Fluticasone	2	4
No treatment	21	42

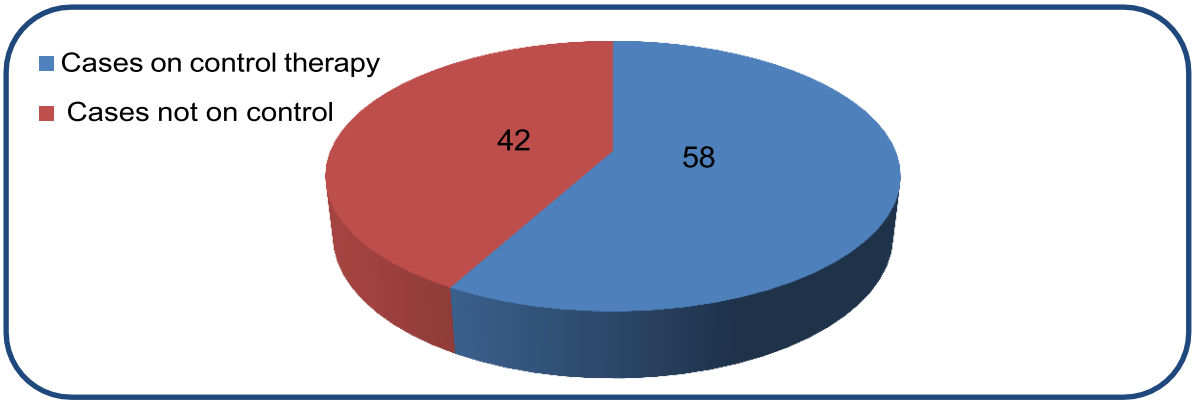

Figure 3. 10. Treatment distribution in the non atopic asthmatic children.

Sawanyawisuth *et al*., (2020) showed that ICS significantly reduced hospital admission in asthma exacerbation in children. It may be used alone for mild-to moderate asthma exacerbation and combination with SC for moderate-to-severe asthma exacerbation. Asthma severity is the intrinsic intensity of disease, and assessment is generally most accurate in patients who are not receiving controller therapy (Andrew *et al*., 2016).

3. 2. 3. Family History of Asthma, Allergic Diseases, and Passive smoking

The current study showed that there was a association between a family history of asthma diseases and asthma in children as presented in Table 3.7.The present results indicated that 45 asthmatic children distributed between 22 atopic child and 23 nonatopic child with a family history of asthma, (17and 3), (4 and 5), (4,1) and (1, 4) of atopic and nonatopic with a family history of allergic disease (asthma and allergic rhinitis), allergic rhinitis, (asthma and eczema) and (eczema, asthma and allergic rhinitis), respectively. On the other hand, only (2 and 2) from non atopic with a family history of (asthma, allergic rhinitis and allergic conjunctivitis) and (asthma, allergic rhinitis, eczema and allergic conjunctivitis), respectively. Two of atopic children and10 non- atopic without a family history of asthma / allergic diseases. Additionally 10 non- atopic without a family history of asthma / allergic diseases were noticed. Support to the results, recent asthma family history study confirmed that asthma was strongly associated with family history of asthma and deemed as a risk factor (Hameed *et al*., 2019). The reasons for this result could be attributed to epigenetic variations in how the genetic code is translated and have also been shown to have a role in the development of asthma in children. A percentage of 88.0% of asthmatic children were associated with a family history of asthma and allergic diseases.

Table 3. 7. Demographic of clinical characteristics of the studied groups

Clinical characteristics		Atopic		Non Atopic		P.value
		Frequency No.	%	Frequency No.	%	
Asthma (Family)	Yes	22	44	23	46	0.04 *
	No	28	56	27	54	
Asthma and allergic rhinitis (Family)	Yes	17	34	3	6	0.24
	No	33	66	47	94	
Asthma and allergic rhinitis (patient)	Yes	5	10	2	4	0.45
	No	45	90	48	96	
Allergic rhinitis (Family)	Yes	4	8	5	10	0.43
	No	46	92	45	90	
Allergic rhinitis (patient)	Yes	13	26	15	30	0.25
	No	37	74	35	70	
Allergic conjunctivitis (patient)	Yes	1	2	3	6	0.46
	No	49	98	47	94	
Allergic rhinitis and allergic conjunctivitis (patient)	Yes	9	18	11	22	0.33
	No	41	82	39	78	
Eczema, allergic rhinitis and allergic conjunctivitis (patient)	Yes	2	4	4	8	0.46
	No	48	96	46	92	
Eczma, asthma and allergic rhinitis (patient)	Yes	7	14	3	6	0.35
	No	43	86	47	94	
Asthma and eczema (Family)	Yes	4	8	1	2	0.45
	No	46	92	49	98	
Eczema , asthma and allergic rhinitis (Family)	Yes	1	2	4	8	0.45
	No	49	98	46	92	
Asthma ,allergic rhinitis and allergic conjunctivitis (Family)	Yes	0	0	2	4	0.48
	No	50	100	48	96	
Asthma,allergic rhinitis,eczema &allergic conjunctivitis (Family)	Yes	0	0	2	4	0.48
	No	50	100	48	96	
No history of asthma/allergic diseases (Family)	Yes	2	4	10	20	0.38
	No	48	96	40	80	
No history of asthma/allergic diseases (patient)	Yes	13	26	12	24	0.29
	No	37	74	38	76	
Smoke (family)	Yes	31	62	33	66	0.17
	No	19	38	17	34	

(P<0.05), * : Significant difference, No.: Number,by on way ANOVA test

In addition, co-infection RSV- HRVwere correlated with the risk of sensitization to aeroallergens at 3 years(*P*=0.02)(Amat *et al*., 2017). On the other hand, atopic and non atopic asthmatic children who were exposed to passive smoking in the present study were 31(62.0%), 33 (66%), whereas 19

(38.0%), 17(34%) were non exposure, respectively, there with non significant association (*P*=0.17). Further, in the control 8(16.0%) exposure to passive smoking compared with 42 (84.0%) as non exposure. He *et al*., (2020) showed a strong link between parental smoking in the first 2 years and the prevalence of wheeze, asthma, and nocturnal cough. It seems possible that the family history of passive smoking is considered to be the main risk factor for the development of asthma, beside the effect of parental smoking on intensifying their asthmatic children status which was shown by increasing drug used for their management, frequnency and severity of recurrent symptom (Boskabady *et al.* , 2022). These differences may be due to exposure duration,long passive smoking duration which increase risk of asthma onset ,heavy passive smoking or light,or past passive smoking.

3. 3. Detection of HRV and RSVin asthmatic and control children

A total of 150 blood samples including 100 samples of suspected viral HRV and RSV infected asthmatic patients and 50 samples of the control were used in ELISA. The current results showed that 10 and 1 samples (20% and 2.0%), in the atopic were a positive HRV and RSv respectively . However in non atopic were 4 and 9 (8.0% and 18.0%) respectively, were considered as a positive result (+ve IgM). On the contrary, (-ve IgM) was obtained in the control for this test as illustrated in Table 3.8.

Table3.8.Identification of HRV&RSV in asthmatic children&control(IgM positive)by ELISA

Comparison groups		No.	No. HRV	%	No. RSV	%	Total	%	P-value
Asthmatic patients	Atopic	50	10	20	1	2	11	22	0.434
	Non-atopic	50	4	8	9	18	13	26	0.234
Control		50	0	0	0	0	0	0	
Total		150	14	9.33	10	6.7	24	16	
P-value		0.250			0.362				

(P<0.05), Non significant, by on way ANOVA test, No: Number

It is widely accepted that HRV is the most crucial trigger of asthma exacerbations and this virus has a large diversity about 170 genottypes,very effective replication, a tendency to create Th2 a biased inflammatory environment and an association with specific risk genes in people predisposed to asthma development (Jartti *et al*., 2020). Whilst, Al-Janabi *et al*.,(2020) showed that 50 children with asthma aged 2-15 years enrolled distributed as35(70%) were males and15(30%) were females, and 40 children with 24(60%) of them were males and 16(40%) were females. In the same study, no significant difference was detected between the cases and the control regarding age and sex, and this is in agreement with the present results as shown in Tables 3.9 and 3.10.

Table 3.9. Association of age groups with HRV and RSV infection(IgM positive) in the asthmatics and control group

Comparison groups		HRV				P-value	RSV				Total	P-value
		+veIgM		-veIgM			+veIgM		-veIgM			
		No.	%	No.	%		No	%	No.	%		
Atopic (Age group)	1-2 years	2	4	5	10	0.090	1	2	6	12	7	0.447
	2.1-3 years	3	6	6	12	0.205	0	0	9	18	9	0.500
	3.1-4 years	1	2	4	8	0.344	0	0	5	10	5	0.500
	4.1-5 years	0	0	5	10	0.500	0	0	5	10	5	0.500
	5.1-6 years	4	8	20	40	0.442	0	0	24	48	24	0.500
Nonatopic(Age group)	1-2 years	1	2	9	16	0.430	4	8	6	12	10	0.295
	2.1-3 years	0	0	10	20	0.0500	3	6	7	14	10	0.366
	3.1-4 years	1	2	5	10	0.374	1	2	5	10	6	0.437
	4.1-5 years	0	0	4	8	0.500	1	2	3	6	4	0.395
	5.1-6 years	2	4	18	36	0.430	0	0	20	40	20	0.500
											100	
Non-asthmatics		0	0	50	100		0	0	50	100		

(P<0.05), Non significant, No: number, by on way ANOVA test

Moreover,the same study found that 5 out of 22 (22.7%) children were from (2-5 years) age group, 11 out of 21 (52.4%) children were from(>5-10 years) age and 4 out of 7 children were from(>10-15 years) age were infected by RSV as IgM positive. In general, RV is a risk factor for later atopic asthma, whereas RSV is more likely associated with later nonatopic asthma.

The study conducted by Kim *et al*., (2018) declared that HRV, RSV and others were associated with 80-85% of asthma exacerbation in children.

Table3.10.Association of the sex with HRV and RSV infection in the asthmatics and control

Comparison groups		Total No.	No. +ve IgM HRV				P.value	No. +ve IgM RSV						P.value
			Male	%	Female	%		Male	%	Female	%	Total	%	
Asthmatic patients	Atopic	50	8	16	2	4	0.090	1	2	0	0	11	22	0.323
	Non-atopic	50	3	6	1	2	0.126	7	14	2	4	13	26	
Total of patients		100	11	11	3	3		8	8	2	4	24	24	
Control		50	0	0	0	0		0	0	0	0	0	0	
Total of patients &control		150	14			9.3		10			6.7	24	16	
P-value			0.272					0.410						

(P<0.05), Non significant,by on way ANOVA test, **No.Number**

Seven patients out of the 50 (14%) atopic asthmatic children with acute HRV infection lived in the urban area and 3(6%) were living in a rural area, whereas in case RSV was 1(2%) patients and (0), respectively. On the other hand, in the non atopic 3(6%) pateints with acute HRV infection were living in the urban area and 1 (2%) patients were in rural areas. Further, For RSV were 10(20%) and 2(4%) patients were living the urban and rural areas, respectively and the residence had no association with acute RSV infection in asthmatic children. There were no significant associations between the passive smoking with acute HRV infection in asthmatic patients as presented in Table 3.11. Al-Janabi *et al*.,(2013) showed that 14 out of the 20(70%) asthmatic children with acute RSV infection lived in urban area, whereas (30%) of patients in rural. The same study concluded that residence and maternal smoking had no association with acute RSV infection. Out of the 226 children with RSV infections, 18(8.0 %) had coinfections with RV, whereas RV was detected in 31(14%) of 226 control children (Karppinen *et al*., 2016).These results are somewhat similar to those obtained by Shameran and Al-Mola, (2014) and Ali *et al*., (2014) in Iraq found RSV infection rates were 24% and 20%, respectively.

Table 3. 11.Association of residence and passive smoking with RV and RSV infection in asthmatics and non asthmatic children

Comparison groups			HRV				P-value	RSV					P-value
			+ve IgM		-ve IgM			+ve IgM		-ve IgM			
			No.	%	No.	%		No.	%	No.	%	Total	
Atopic	Residence	Urban	7	17.1	34	82.9	0.031	1	2.4	40	97.6	41	0.0001
		Rular	3	33.3	6	66.7		0	0	9	100	9	
	Passive smoking	+ve	8	25.8	23	74.2	0.041	1	3.2	30	96.8	31	0.04
		-ve	2	10.5	17	89.5		0	0	19	100	19	
Non-atopic	Residence	Urban	3	9.1	30	90.9	0.01	5	15.1	28	84.8	33	0.02
		Rular	1	5.9	16	94.1		4	23.5	13	76.5	17	
	Passive smoking	+ve	4	12.1	29	87.9	0.14*	6	18.2	27	81.8	33	0.032
		-ve	0	0	17	100		3	17.6	14	82.4	17	

Significant difference between groups (P<0.05), *except passive smoking in (+ve IgM and-ve IgM) HRV in non atopic was non significant , No:Nnumber, by one way ANOVA test

3. 4. Immunological Study

3.4.1.Relationship of SerumCCL20 Level and Immunological Parameters

3. 4.1.1. Relationship of Serum CCL20 Level with Percentage Eosinophil and Neutrophile Count in The Atopic and Non Atopic Asthmatic Children

The current results of the CCL-20 were found to be a positive linear correlation with percentage of Eosinophil Count (EC) (*P*=0.001). Whereas,a negative correlation with percentage of Neutrophil Count (NC) with a significant difference (*P*=0.0001) as shown in Figures 3.11. and 3.12. The present results also demonstrated that the serum levels of CCL20 in both newly diagnosed and previously diagnosed patients were significantly higher than in the healthy group.

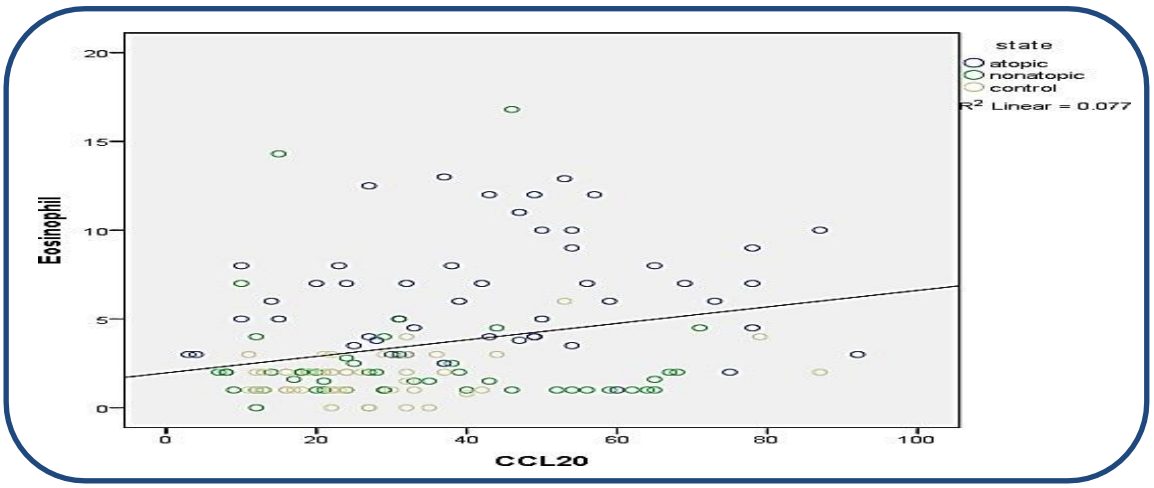

Figure 3. 11. Scatterplot % of eosinophil count according to serum CCL-20 pg/ml. Pearson correlation coefficient: r = +0.278 (weak linear correlation) (*P* = 0.001).

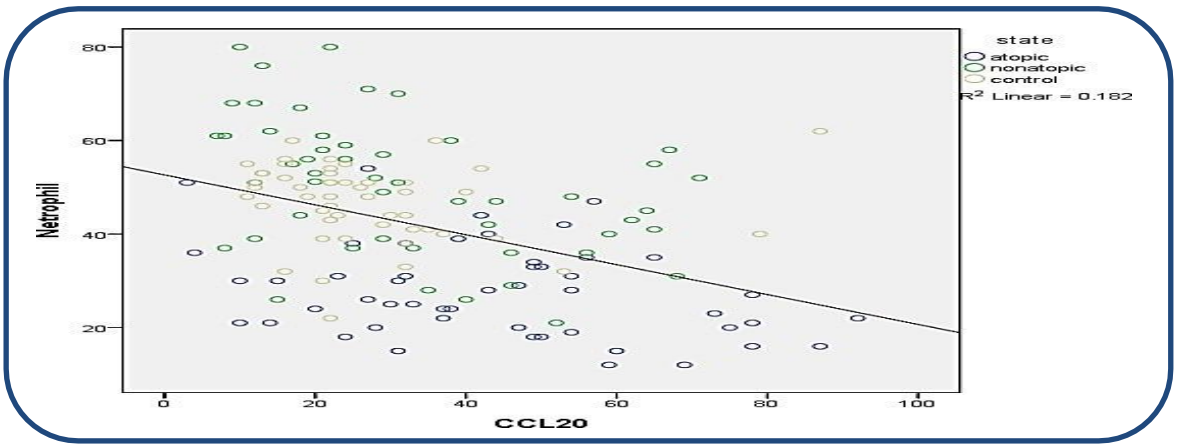

Figure 3. 12. Scatterplot % of neutrophil according to serum CCL-20 pg/ml. Pearson correlation coefficient: r = -0.427 (medium linear correlation) (*P* = 0.0001).

The stimulated airway epithelium by allergens increases the production of the CCL-20 chemokine, which attracts DCs to the tissue, from mediators that contribute to the innate immune response in asthma include C-C motif chemokine ligand 20 (CCL20)(Soleha *et al*., 2021). As mentioned before, CCL20 plays an important role in migration of Th17 cells to inflamed tissues (Zhang *et al*. 2016). After the Th17cells' entrance into the lesion areas, they secrete many inflammatory cytokines, causing the creation of a cytokine cascade by inflammatory cells. This cytokine environment promotes the inflammatory process and stimulates the production of CCL20, including the trafficking of more Th17 cells to the inflammatory lesion. These explanations provide a potential interpretation for the continuous presence of Th17 in the lesions through appositive feedback loop by CCL20.

Therefore, CCL20 may participate in both induction / initiation and maintenance phases of disease.

In addition, Lewis *et al*.,(2019) found that HRV and RSV load was associated with protein abundance of CCL20 and CXCL10 that together attract neutrophils and monocytes to the airways, but not with IL-4, IL-13, CCL5 or CCL24, cytokines that promote blood eosinophil chemotaxis and allergic airways disease. In study of Spencer *et al*., (2009) showed that along with responding to immune signals, eosinophils were a source of cytokines, chemokines and growth factors such as IL-10,CXCL10,CCL20 and TGFβ. Furthermore, Lewis *et al.* , (2018) showed that increased abundance of airway cytokines and CCL20 following viral infection in asthmatic children. The IL-1β-induced CCL20 expression and protein release were increased in ASMCs from moderate compared with mild asthmatic children patients and healthy control (Faiz *et al* ., 2018).

In mild persistent asthmatic children increased eosinophile ,In addation, viral infection was associated with increased CXCL10 and CCL20 (Lewis *et al*., 2018). On the other hand, Ikawa *et al*., (2021) showed that CCL20 level in systemic sclerosis patients was significantly higher than in healthy controls. Moreover, Trivedi and Pate (2020) illustrate that AEC increased significantly (P<0.0001) with increasing severity of asthma especially through found HRV and RSV and increased CCL20 levels, and of the total 109 patients with asthma, 44(40.4%) had intermittent asthma, 30(27.5%)mild persistent, 25(22.9%) moderate persistent and 10(9.2%) severe persistent asthma, and increased AEC was found in 61(56 %) patients. In a previous study, Kamil *et al.*,(2022) illustrated that the serum level of CCL20 was a highly significant increase in rheumatoid arthritis (RA) patients in comparison with control. In addition, levels of CCL20 and its CCR6 receptor are elevated in many autoimmune diseases, which aids in

attracting Th17 cells to the site of inflammation(El Sharkawi *et al*., 2019). Furthermore, Jafarzadeh *et al.,* (2014) showed that mean serum levels of CCL20 in both newly diagnosed and previously diagnosed patients were significantly higher in comparsion to the healthy(*P*=≤0.05and *P*=0.001 respectively). The same results showed higher levels of CCL20 in patients who play an important role in the pathogenesis of (MS). Nakagome *et al*.,(2018) showed that eosinophils contribute to the development of asthma exacerbation by virus infection-related CXCL10,CCL20 and interaction with other cells such as neutrophils, also eosinophils correlated to CCL20 and CXCL10 level in have been considered important proinflammatory and epithelial-damaging cells in children with asthma having virus infection.

3.4.1.2. Relationship between Serum CCL20 Level with TSIgE in The Atopic and Non Atopic Asthmatic Children

The results of serum CCL20 were found to have a positive linear correlation with TSIgE (*P*=0.024) as shown in Figure 3.13. The present study shows the elevated tsIgE (≥100 IU/ml) in the atopic asthmatic children with HRV and RSV infection,whereas in the non atopic with the same viruses were <100.

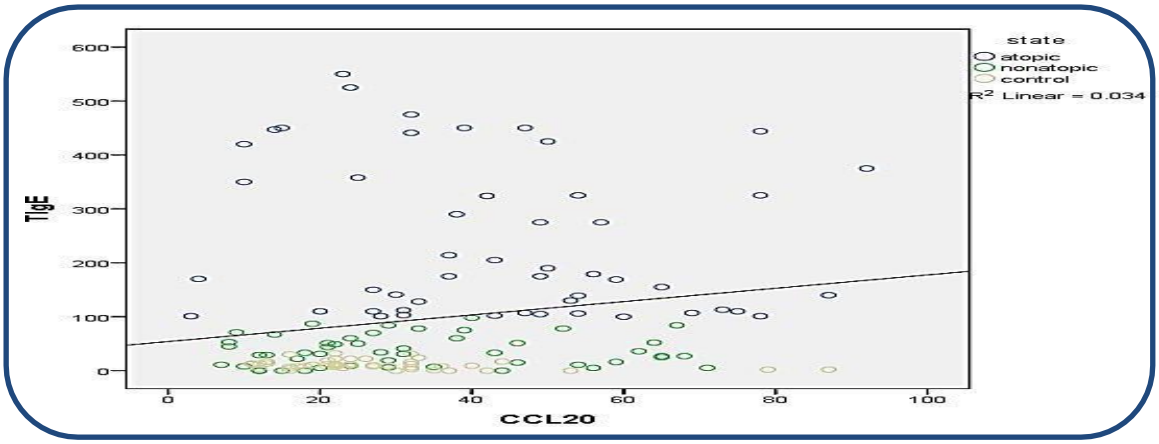

Figure 3. 13. Scatterplot to TSIgE according to serum CCL-20 pg / ml. Pearson correlation coefficient: r = +0.185(weak linear correlation)(*P*=0.024).

Lama *et al*.,(2013) indicate that the mean TSIgE level was> 100IU/ml in the asthmatic patients,whereas in the control group was<100 IU/ml.

In addition, Abood *et al*., (2023) showed that highly significant differences (*P*<0.001) in TSIgE between asthmatic patients and healthy controls. CCL20 and CXCL10 were important mediators of the immune response to HRV and RSV in asthmatic patients, also it promote chronic inflammatory states (Kallal *et al*., 2010). It is thought to be that CCL20 is produced following IL-4 or IL-13 stimulation. Furthermore, CCL5 and CCL20 are released by airway epithelial cells upon stimulation by IL-1β (Liu *et al*.,2018).

3. 4. 2. Distribution of Eosinophil,Neutrophile count and TSIgE in The Atopic and Non Atopic Asthmatic Children and Control Group

These results revealed that EC and TsIgE increased in the atopic children, on the contrary , EC and TsIgE were not elevated in the non atopic children. as shown in Table 3.12.

Table3. 12. Percentage of Eosinophil,Neutrophil count and TSIgE among asthmatic patients and control

Parameter	Atopic Asthmatic patients No.50	Non Atopic Asthmatic patients No.50	Healthy controls No.=50	P-value
%Eosinophil count(EC)	6.2±2.5	2.1±0.43	1.7 ±0.81	0.000*
%Neutrophil count (NC)	27.87±12.7	52.34±21.7	36.42±17.8	
TSIgE (IU/mL)	275.77±12.95	36.17±13.6	11.153±6.92	

(P<0.001),EC:Eosinophil count,TSIgE:Total Serum ImmunoglobulineE, *highly Significant by on way ANOVA .

But may be lymphocytes were high due to virus infection. It showed a significant difference in the EC and TSIgE level between atopic and non atopic patients and control, whilst non atopic characterized an increased percentage of neutrophil (*P*=0.000).These results were in congruence with study indicating that the elevation eosinophil counts may be a valuable indicator of asthma diseases (de Groot *et al*., 2015). Beside, the present result agree with result of Gerday *et al*., (2022) who showed that non-atopic patients displayed greater blood neutrophil (median 57% and mean TSIgE

levels were 42 IU/mL, *P*=0.01),however atopic patients were more eosinophilic (median 2.8% and mean TSIgE levels were 214 IU/ml, (*P*=< 0.001). Additionally, Ahmad Al Obaidi *et al*.,(2008) found that the mean tsIgE level was 554 IU/ml and 69IU/ml in patients and control groups, respectively. The current results are presented in Table 3.12 which indicates that TSIgE levels in different age groups were significantly higher in atopic and non atopic asthmatic patients compared to the control group (*P*=0.000). The results of the current study are consistent with results of Hameed *et at*., (2023) who found a high significant difference (*P*=< 0.001) in the levels of TSIgE in the asthmatic patients in comparison to the healthy group. In addition, Satwani *et al*.(2009) showed that eosinophilia along with raised serum TSIgE level is deemed to be a significant allergic marker in asthmatic children having HRV and RSV. Moreover, Joshi *et al*.,(2020) illustrated that the elevation level of TSIgE may increase in severity of atopic asthma.

3.4.3. Relationship between Asthma Severity and Immunological Parameter in The Atopic and Non Atopic Asthmatic Patients

Patients having mild asthma were more than patients with moderate and severe asthma. These data were in congruence with data obtained by Saad *et al.,* (2018). This study showed a high elevation leveled CCL20 and CXCL10 in most mild, moderate and severe,with significant differences among patients compared to the control($P \leq 0.05$)as shown in Table 3.13. In addition, TSIgE was higher in the moderate compared to the mild asthma as in Tables 3.13 and 3.14.This result agreed with the mean serum levels of CCL20 in (MS) patients which were significantly higher than those in the control group (P<0.001) Jafarzadeh *et al*., 2014). The CCL20 is produced by a variety of cells such as endothelial cells, neutrophil, NK cells, Th17cells, Bcells, Langerhan's cells, dendritic cells and macrophages in response to stimulators like IL-1α, IL-β, IL-6, IL-17,IL-21, IFN-γ and TNF-α (Lee *et al*., 2013).

Table3.13.Relationship between asthma severity and immunological parameter in the Atopic asthmatic children

Parameters (pg/ml)		Severity of Asthma				
		Atopic / mean± S.D			Control(50)	P.value
		Mild (No.33)	Moderate(No.15)	Severe (No.2)		
CCL20 levels		43.545±22.1	50.625±32.1	62.54±32.1	23.9±10.1	0.018*
CXCL10 levels		44.667±21.13	46.687±23.14	79.1 ±39.12	29.6±9.02	0.036*
TGF-β levels		40.545±19.9	43.25±17.3	24.5±44.2	43.7±19.01	0.015*
IL-10 levels		27.45±11.3	38.75±31.4	8.3±2.3	51.4±23.1	0.041*
%Eosinophil count		6.4±3.1	5.9±2.2	7.0±3.1	1.8±0.71	0.021*
% Neutrophil count		29.54±11.2	28.0±13.2	23.5±19.8	41.4±12.2	0.004*
TSIgE IU/mL	1-2 years	173.6 ±73.1	170±78.2	0	7.1±2.1	0.285**
	2.1-3 years	238.9±100.1	0	0	11.4±3.1	0.002*
	3.1-4 years	243.75±160.3	450±211.3	0	9.5±2.1	0.001*
	4.1-5 years	203.75±89.6	0	0	17.0±8.1	0.005*
	5.1-6 years	213.3±120.1	340.58±140.3	393±131.7	21.3±7.8	0.038*

(P<0.05),*: Significant difference between mild, moderate and severe in atopic patients and control regarding parameters,: Non significant difference, by One way ANOVA test.**

Further, EC and TSIgE were higher in atopic patients, whilst in the non atopic were not increased and were normal in control group. CCL20 has affected the immune cells and its dysregulation contributes to autoimmune and inflammatory conditions (SLE and RA)(Koga *et al*. , 2016). This result is in agreement with the findings of Faiz *et al*., (2018) mentioned that, CCL20 expression level was increased in ASMCs from moderate compared to mild asthmatic patients and control group. Beside that, the result of this study has indicated a non significant difference in the mean level of IL-10 in the atopic,while a significant in the non atopic patients. It is clear that IL-10 was decreased in the serum of the atopic and non atopic patients compared to the control group (*P*=0.000). Moreover the present result showed a significant difference (P=0.05, P=0.07) in the serum level of TGF-B and CXCL-10 in the atopic asthmatic patients compared to healthy control. On the reverse in the non atopic was not significant(*P*=0.002, *P*=0.0001) respectively. Releasing of CXCL10 was induced by IFN-γ in great quantities by bronchial epithelial cells. Therefore CXCL10 release was specific to acute virus-induced asthma

(Kato *et al*., 2015). Moreover, Huoman *et al*., (2021) mentioned that children with asthma having CXCL10 levels were consistently elevated at 1 and /or 8 years, whereas asthma later in life was preceded by increased CXCL10 levels after birth. In the present study, significant difference was found in the mean level of serum IL-10 in the atopic group. Similarly, a significant difference was revealed in the non atopic patients. It is clear that IL-10 was significantly lower in the serum of the atopic and non atopic patients compared to the control group as shown in Tables3.13 and 3.14.These results are consistence with the results of Jahromi, *et al*., (2014) who found that IL-10 was evaluated in the asthmatic patients compared to the healthy group. A study was conducted by Al-Ameri, (2013) revealed that the level of IL10 was significantly lower in asthmatics reflecting the poor regulatory T helper cell function in this group compared with the control.

Table 3.14.Relationship between asthma severity and immunological parameter in Non Atopic asthmatic children

Parameters (pg/ml)		Mild (No.31)	Moderate (No.15)	Severe (No.4)	Control(50)	P.value
CCL20 levels		33.16±16.3	34.8±14.2	39.0±20.12	23.9±10.1	0.002*
CXCL10 levels		35.87±20.15	38.3±15.61	41.8±21.98	29.6±9.02	0.001*
TGF-β levels		27.8±11.2	26.2±20.1	27.5±18.2	43.7±19.01	0.005*
IL-10 levels		19.4±10.4	21.2±9.11	19.5±12.5	51.4±23.1	0.038*
% Eosinophil count		1.8±0.4	2.7±1.5	1.6±0.7	1.8±0.71	0.004*
% Neutrophil count		50.3±21.11	49±28.3	44.7±25.11	41.4±12.2	0.0001*
* TSIgE * IU/mL : (	1-2 years	30.775±17.2	0	0	7.1±2.1	0.123**
	2.1-3 years	48.125±23.1	53±30.1	0	11.4±3.1	0.126**
	3.1-4 years	32.9±13.2	0	45.285±.03	9.5±2.1	0.228**
	4.1-5 years	39.35±11.2	0	0	17.0±8.1	0.023*
	5.1-6 years	49±23.1	17.6±9.2	36±.01	21.3±7.8	0.123**

(P<0.05), *: Significant difference between mild, moderate and severe in non atopic patients and control regarding parameters. **: Non Significant difference in TSIgE between ages groups, by One way ANOVA test.

The present results indicated that a significant difference(*P*=0.01, *P*=0.04) in Serum level TGF-β and CXCL10 in the atopic asthmatic patients compared to the control, moreover level TGF-β and CXCL10 in the non atopic was significant (*P*=0.005, *P*=0.001) respectively. It is clear that TGF- β decreased

in the serum of the atopic and non atopic patients and the current results are compatible with the results of Mohamed *et al*., (2012) who showed that a decrease the mean levels of TGF- β in the atopic, non atopic and control.

3.4.5.Comparison of Immunological Parameter between Patients with no Controller Therapy and Patients with Controller Therapy

A high TSIgE level was found in both the atopic asthmatic children on controller therapy and non on controller therapy. In addition, these results revealed that the level of serum CCL-20 in asthmatic children on controller therapy was lower than the level in patients with not controller (37.4±12.6 pg/ml and 48.3 ±20.3 pg/ml in the atopic, in addition, in the non atopic was (32.2±41.2 and 39.75±22.6 pg/ml, respectively) as shown in Table 3.15.

Table3.15.Comparison of Immunological Parameter between Patients with no Controller Therapy and patients with Controller therapy

Parameters pg /ml		Asthmatic Patients No.100						p-value
		Atopic No.50 / mean± S.D			Non atopic No.50 / mean± S.D			
		Not on controller therapy No.28	On controller therapy No.22	p-value	Not on controller therapy No.20	On controller therapy No.30	control	
CCL20 levels		48.3±20.3	37.4±12.6	0.08**	39.75±22.6	32.2±41.2	23.9±10.1	0.05**
CXCL10 levels		44.9±43.8	35.4±36.2	0.07**	31.3±18.6	24.6±51.2	29.6±9.02	0.07**
TGF-β levels		31.3±17.3	38.8±33.2	0.06**	30.3±61.3	34.4±25.6	43.7±19.01	0.07**
IL10 levels		22.0±31.8	14.6±42.3	0.12**	24.4±16.2	18.5±33.5	51.4±23.1	0.08**
%Eosinophil count		6.1±2.1	6.3±1.9	0.01*	2.4±1.2	2.2±1.21	1.8±0.71	0.13**
% Neutrophil count		27.2±13.4	28.1±14.2	0.08**	50.34±31.2	47.6±21.4	41.4±12.2	0.05**
TSIgE IU/mL	1- 2 years	162±48.4	190±121.3	0.01*	27.8±15.4	16.3±11.1	7.1±2.1	0.01*
	2.1-3 years	225±120.3	210.8±140.2	0.05**	51±23.4	47.9±25.4	11.4±3.1	0.16**
	3.1-4 years	149±114.2	245±151.5	0.02*	26.2±11.2	0	9.5±2.1	0.02*
	4.1-5 years	252.4±98.2	0	0.15**	45±21.11	50.7±32.3	17.0±8.1	0.50**
	5.1-6 years	247.9±143.2	297.5±104.3	0.50**	38.1±21.4	41±25.3	21.3±7.8	0.03*

(P<0.05),:Non significant difference between not on controller therapy and on controller therapy for atopic and non atopic patients and control regarding parameters,*: Significant difference. by One way ANOVA test.**

The present results showed non significant difference in CCL20 CXCL10,TGF- β and IL-10 level (*P*= ≥0.05) in the atopic and non atopic asthmatic children.This result may be due to the event of montelukast can

regulate the T helper1(Th1) / Th2 balance, block CCL-20 mRNA expression, and improve the airway inflammation caused by acute bronchial asthma (BA) and the clinical symptoms and lung function (Qu *et al*., 2018). On the other hand, a significant difference was found in eosinophil count (EC)percentage ($P \leq 0.05$) and non significant of neutrophils percentage in atopic, while in the non atopic there was no significant difference ($P=0.1$) in EC and non significant difference for neutrophils (between asthmatic children who received treatment and those not on treatment. This is in agreement with the results obtained by Kopriva *et al*., (2004) who found that there were no significant changes in eosinophil density, or eosinophil activation following treatment with montelukast. Despite several earlier research mentioning the role of montelukast in lowering blood eosinophil levels (Yamakawa *et al*., 2010), However, another study contradicts them, stating that eosinophil density or activation changes do not appear to be the source of the short-term clinical response to montelukast (Friesen *et al*., 2009). Further, the use of a single EC as a criterion for eligibility for specific therapeutics is insufficient to identify all patients with the potential to benefit(Mathur *et al*., 2016).

3.4.5.Relationship of Immunological Parameters with HRVand RSV According to Severity in The Atopic and Non Atopic Children

The present result showed that level of CCL20 and CXCL10 in severe was (77.5 and 79.7) pg/ml compeared to moderate was (69.6 and 73.6) pg/ml in atopic asthmatic children with HRV and no significant difference ($P= 0.3$, $P=0.2$) as shown in Table 3.16. A high total serum TSIgE level is found in both asthmatic children with RSV and HRV.Whereas, in the non atopic(mild, moderate and severe) with HRV was a significant difference in CCL-20 and CXCL10 levels ($P=0.02$, $P=0.04$), further, a significant difference for CXCL10($P=0.03$) was found in the patients with RSV and no significant difference for CCL20 ($P=0.2$) as shown in Table 3.17.

Table 3. 16. Association of the immunological parameters with HRVand RSV according to severity in atopic children

Parameters (pg/ml) mean± S.D		HRV			P-value	RSV		
		Severity of asthma				Severity of asthma		
		Mild (No.=7)	Moderate (No.=1)	Severe (No.=2)		Mild (No.=0)	Moderate (No.=1)	Severe (No.=0)
CCL20 levels		75.3±11.2	69.6	77.5±17.1	0.341**	0	152	0
CXCL10 levels		81.1±20.1	73.9	79.7±12.2	0.201**	0	49	0
IL-10 levels		28.3±5.5	17.6	9.3±1.2	0.036*	0	13.5	0
TGF-β levels		20.9±4.32	31.5	25.2±7.1	0.251**	0	64	0
%EC		6.4±0.8	6.4	6.4±2.4	0.302**	0	6	0
% Neutrophil		18.9±9.2	19	27±10.1	0.071**	0	30	0
TSIgE IU/mL	1-2 years	211.3±40.3	231	0	0	0	0	0
	2.1-3 years	131±20.1	0	0	0	0	0	0
	3.1-4 years	116±23.1	0	0	0	0	0	0
	4.1-5 years	0	0	0	0	0	450	0
	5.1-6 years	282±25.1	0	233±20.01	0.011*	0	0	0

(P<0.05),:Non significant difference between mild,moderate and severe in atopic patients regarding parameters. *Significant difference. by One way ANOVA test.**

HRV-C has been associated with asthma exacerbation (AE),HRV is the most predominant viral infection responsible for pediatric AE in Taiwan,HRV-C is responsible for more of these exacerbations than HRV-A or HRV-B (Su *et al*., 2020).

Table3.17.Association of immunological parameters with HRVand RSVaccording according to severity in the non atopic children

Parameters (pg/ml) mean± S.D		HRV			p-value	RSV			p-value
		Severity of asthma				Severity of asthma			
		Mild (No.=1)	Moderate (No.=1)	Severe (No.=2)		Mild (No.=4)	Moderate (No.=3)	Severe (No.=2)	
CCL20 levels		51.5	60.5	81.0±21	0.02*	68.8±12	57±13.2	91 ±22.1	0.21**
CXCL10 levels		55.3	48.7	79.0±13	0.04*	62.9±21	55.7±15.4	83 ±15.2	0.035*
IL-10 levels		13.5	17.1	11.3±4.1	0.000*	22.2±5.6	21.0±1.2	9 ±0.87	0.02*
TGF-B levels		24.0	21.2	17.2±2.1	0.32**	24.1±11.2	31.1±10.2	17±3.2	0.002*
%EC		4.3	1.7	1.5±1.2	0.02*	1.8±0.2	2.1±0.3	1±0.5	0.041*
% Neutrophil		44.5	60.5	67.0±14.1	0.1**	47.2±21.1	45.3±12.1	66±21.5	0.022*
TSIgE IU/mL	1-2 years	0	0	23±1.1	0.000*	40.7±13.3	32.0±7.1	5±0.43	0.000*
	2.1-3 years	0	0	0	0.000*	51.5±20.1	36.0±20.1	7±0.31	0.011*
	3.1-4 years	0	0	19±2.3	0.000*	21.0±0.89	0	0	0.000*
	4.1-5 years	0	0	0	0.000*	0	27.0±12.3	0	0.012*
	5.1-6 years	39	16	0	0.01*	0	0	0	0.000*

(P<0.05),*:Significant difference,: Non significant difference between mild, moderate and severe regarding parameters in non atopic patients infected with HRV and RSV, by One way ANOVA test.**

In addition, RSV-mediated pathogenic pathways in predispose and induce airway dysfunction and non-allergic asthma development and exacerbation especially in infants and children (Manti and Piedimonte, 2022).

3. 6. PCR Based Detection of SNPs

3. 6. 1. Extraction of Human Genomic DNA

Deoxyribonucleic Acid (DNA) was extracted as described in lane 2.5.6.1.1. quality and integrity were checked by agarose gel electrophoresis as in appendix VII and Nano drop before performing PCR.

3. 6. 2. *CCL-20* (-786T>C) Gene Amplification

The results were a single clear band with a molecular size of 214 bp. The size of the amplicon was determined by comparing with DNA ladder 50-1500 bp, as illustrated in Figure 3.14 and 100-1000 bp, as shown in Figure 3.15.

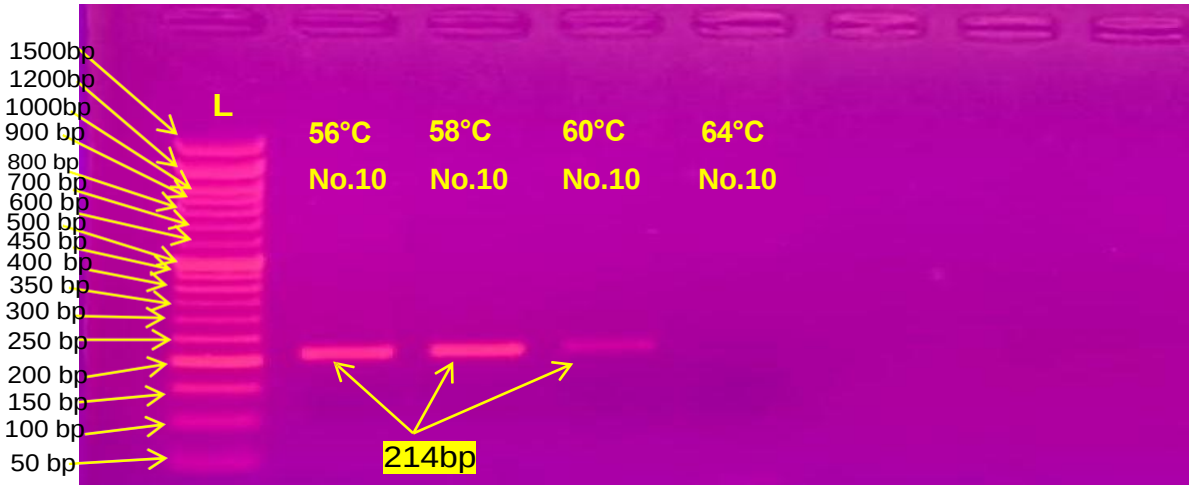

Figure 3. 14. Gel electrophoresis to PCR product of *CCL-20* (214bp) with DNA ladder 50bp(50-1500pb) on agarose gel 2% in 70 volt, 80 min.No:Number

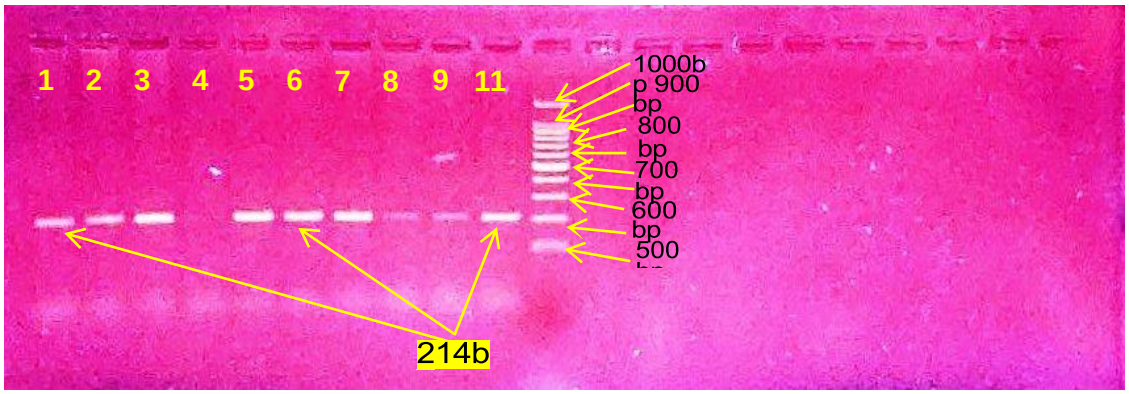

Figure 3. 15. Gel electrophoresis to PCR product of *CCL-20* (214bp)with DNA ladder (L) 100 bp (100-1000bp) on agarose gel 1.5% in70 volt, 70 min.

3. 6. 2. 1. Genotype of *CCL-20* (-786T>C)

CCL20 (T-786 C) PCR product was digested with RsaI,thermofisher,USA. The sequence of the restriction was 5'G T↓A C 3'. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) product is recognized on gel electrophoresis, thus the amplified PCR product of *CCL20* covers rs6749704 with a molecular size of 214bp. RsaI, a restriction enzyme has merely a restriction site on this region, consequently, the fragment was digested into 3 different visible fragments with 214, 129 and 85 bp, of a heterozygotic form (T/C). On the other hand, in the homozygotic form, a 214 bp fragment (without any digestion) (C/C) or 129 and 85 bp as a result of digesting both alleles (T/T) in the case which were then observed. The digested products were electrophoresed on 1.5 % agarose gel as a presented in Figure 3.16. Genetic polymorphism of CCL-20 (-786T>C) in the atopic and non atopic asthmatic children was observed with three genotypes (TT, TC and CC) as shown in Tables 3.18 and 3.19, which illustrated the distribution of genotypes of CCL-20 in the asthmatic patients and healthy control as shown in Figures 3.16. and 3.17, respectively.

The homozygous genotype (CC) was recorded at a high frequency in the atopic asthmatic children (44.0%) compared to the control group (12%) with a significant difference (P=0.000). Additionally, the frequency of genotype (TT) was (14.0%) and (56%) in the patients and control groups, respectively, revealed significant differences between the groups. However, the frequency of heterozygote genotype (TC) was (42.0%) and (32.0%) in the patients and the control group, respectively but no significant differences were found among them. The T and C alleles frequency were 35 (44.0%), and 65 (56.0%) in the patients, while in the control group were (72.0%) and (28.0%), respectively, and significant differences were shown between T and C in each group, as illustrated in Table 3.18 (P=0.0001).

Table 3. 18. The statistical evaluations of *CCL-20* SNP between the atopic patients and control groups

CCL-20 (786T>C)		Cases N.	%	control No.	%	Total	%	O.R	P-value	(95%CI)
TT		7	14	28	56	35	35	0.1279	0.0001	0.0483 - 0.3390
TC		21	42	16	32	37	37	1.5388	0.301	0.6794 - 3.4855
CC		22	44	6	12	28	28	5.761	0.0001	2.0787 - 15.9710
Total		50	100	50	100	100	100			
Allele	**T**	35	44	72	72	107	53.5	0.209	0.0001	0.1150 -0.3814
	C	65	56	28	28	93	46.5	4.775	0.0001	2.6221 - 8.6973
Total		100	100	100	100	200	100			

Odds ratio with 95%confidential interval of atopic asthma in comparison with control with designated genotype.Fisher's exact test

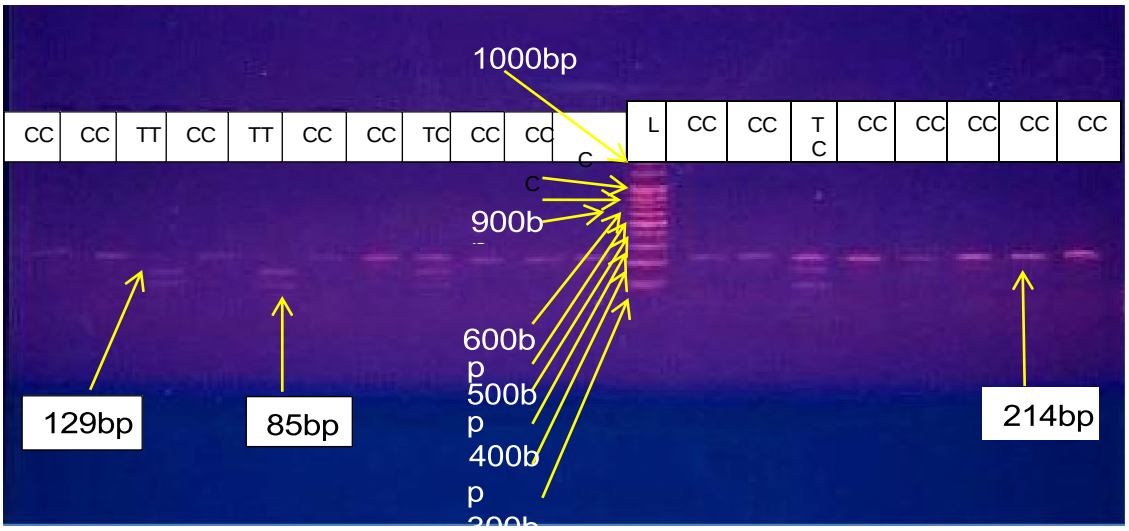

Figure 3.16.Gel electrophoresis of PCR-RFLP products for the patients group ,which illustrated genotype of *CCL-20* SNPon agarose 1.5% in 70 volts, 75 min and results were detected by UV-documentation system. The restriction process showed three types of genotype, in the case of a heterozygotic form (T/C), (214, 129 and 85 bp).While in the homozygotic form CC (214 bp), or two 129 and 85 bp digesting both alleles (T/T). L:(Ladder), refers to DNA molecular marker 100–1000bp.

The analysis revealed that the allele C was a risk allele and the genotype CC was a risk genotype. The current results showed that *CCL20* (-786T>C) polymorphism was significantly associated with the asthma disease at the Iraqi children patients. It is found that C allele and CC genotype in the asthmatic children patients were higher than in the control group, with a significant association. In contrast, the "T" allele and TT genotype have a rather

preventive role. This indicates that the "T" allele may be protective. The *CCL20* TT genotypes may have a protective role in asthma pathogenesis in Iraqi children patients. Polymorphisms of SNP (rs6749704)of *CCL20* relation with susceptibility to Rheumatoid Arthritis (RA),CCL20 affects immune cells in a variety of ways, and it dysregulation contributes to autoimmune and inflammatory condition(Kamil *et al*., 2022). CCL20 and the receptor (CCR6) altered Thelper (TH17) cells (El Sharkawi *et al*., 2019). In the same study, the analysis of logistic regression for *CCL20* (rs6749704) revealed that the CC genotype was significantly more frequent in (RA) patients (*P*= 0.000,OR=22.52, CI 95% (4.76–205.87). Additionally, the C allele was significantly more frequent in (RA) patients in comparison to the control group (70.83% versus 16.67%, *P* = 0.000, OR=12. 14, CI 95% (5.57–27.36). Thus the CC genotype and C allele and higher serum level of the chemokine CCL20 were associated with RA disease in Iraqi patients. On the other hand, in the non atopic asthmatic children, the frequency of homozygous genotype CC was significantly higher in the asthmatic children (60.0%) compared to the control group (12.0%) (*P*=0.000).

Table 3. 19. The statistical evaluations of *CCL-20* SNP between the non atopic patients and control groups.

***CCL-20* (786T>C)**		**Cases No.**	**%**	**Controls No.**	**%**	**Total**	**%**	**O.R**	**P-value**	**(95%CI)**
TT		9	18	28	56	37	37	0.172	0.0002	0.0693 - 0.4295
TC		11	22	16	32	27	27	0.599	0.2622	0.2449 - 1.4667
CC		30	60	6	12	36	36	11.00	0.0001	3.9524- 30.6144
Total		50	100	50	100	100	100			
Allele	**T**	29	29	72	72	101	50.5	0.151	0.0001	0.0815 - 0.2802
	C	71	71	28	28	99	49.5	6.619	0.0001	3.5693 - 12.2760
Total		100	100	100	100	200	100			

Odds ratio with95% confidential interval of atopic asthma in comparison with control with designated genotype. Fisher's exact test

Similarly, a significant difference in the genotype TT frequency was shown between the patients (18.0%) and control (56%) groups (*P*=0.0002).

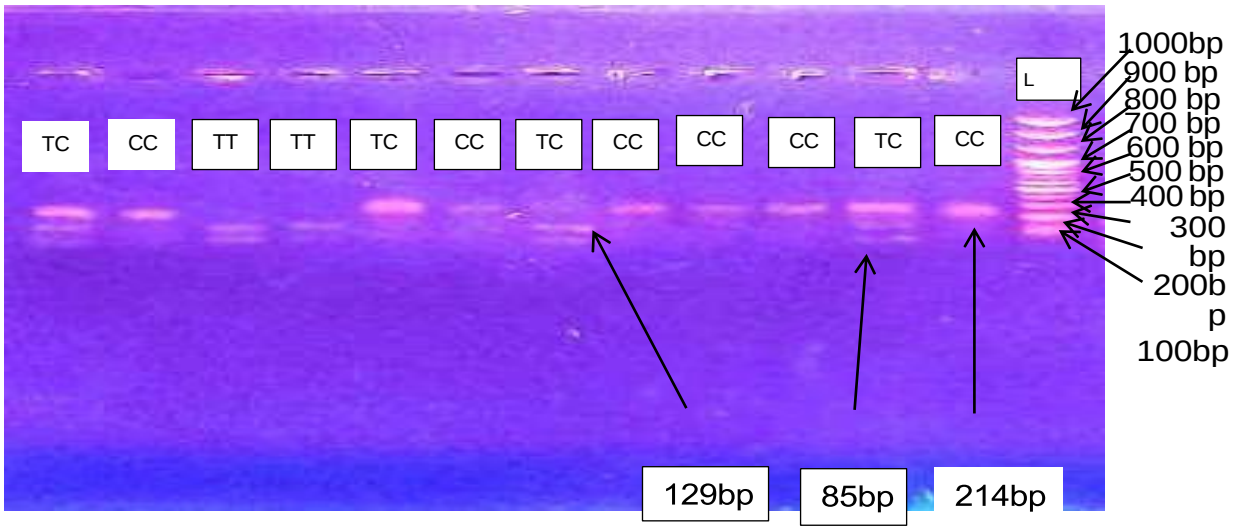

Figure 3. 17. Gel electrophoresis of PCR-RFLP product for the control, which illustrated genotype of *CCL-20* SNP on agarose1.5%in70volts,75min,and results were detected by UV documentation system. The restriction process showed three types of genotype, in the case of a heterozygotic form (T/C), (214, 129, and 85 bp). While in homozygotic form CC (214 bp), or two129and 85bp digesting both alleles(T/T).L:Ladder,DNA molecular marker100-1000bp.

Moreover, the present study revealed that the frequency of allele C was (71.0%) in the patients compared to the control (28.0%) group, whereas the allele T was (29.0%) in the patients in compared to the control (72.0%) group with a significant difference (*P*=0.000), as illustrated in Table 3.19. In contrast, a significant difference was not found regarding heterozygote genotype TC frequency between the patients group(22.0%) and control group (32.0%) (*P*=0.2622). From the literature, a study by Jafar zadah *et al*., (2014) on *CCL20* rs6749704 in Multiple Sclerosis (MS) patients showed that the genetic variantion at rs6749704 did not significantly differ between the (MS) and healthy groups. In contrast to the other MS patterns, Secondary Progression Multiple Sclerosis (SPMS) patients had a much lower frequency of the TC genotype. Additionally, the frequency of TC genotype in the patients with (SPMS) was lower than in the control group, with a borderline significance. These data indicate that rs6749704 may have a relationship with some patterns of MS disease. the rs6749704 SNP T>C in *CCL20* on Egyptian patient with (MS) autoimmune disease revealed that the TC genotype was

significantly more prevalent in (MS) patients (*P*=0.01,OR=2.6 (95% CI1.4–4.8) (El sharkawi *et al* ., 2019). Furthermore, C allele was significantly more frequent in the (MS)patients than in the control(27.7% versus15.8%,*P*=0.006, OR=2.0 95% CI (1.2–3.4) which was agreed with the current study under allelic model and disagree under genotyping model. The results of *CCL-20* SNPs for the atopic and non atopic asthmatic children and control group are in congruence with the expected Hardy-Weinberg equilibrium (according to the website OEGE-Online Encyclopaedia for Genetic Epidemiology Studies) as shown in Tables 3.20 and 3.21.

Table 3. 20. Expected frequencies for genotypes of the *CCL-20* SNP in the atopic patients using Hardy-Weinberg equilibrium

Gene Position	Genotype	Observed asthmatic patients No.(%)	Expected asthmatic patients No.(%)	p-value	Observed healthy control No.(%)	Expected healthy control No.(%)	p-value
***CCL-20* (786T>C)**	**TT**	7 (14%)	6.12(12.25%)	0.58	28(37.6%)	25.92(53.29%)	0.34
	TC	21(42%)	22.75 (45.5%)		16 (47.1%)	20.16(39.42%)	
	CC	22 (44%)	21.13 (42.25%)		6 (15.3%)	3.92 (7.29%)	

Analysis of the HardyWeinberg equilibrium of CCL-20(-786T> C)revealed that the asthmatic patient and the control group were in agreement with the equilibrium, and no significant differences and significant variations between the observed and expected genotypes frequencies were observed for the atopic and non atopic asthmatic children respectively(*P*=0.5, *P*=0.001).

Table 3. 21. Expected frequencies for genotypes *CCL-20*SNP in the non atopic patients using Hardy-Weinberg equilibrium

Gene Position	Genotype	Observed asthmatic patients No.(%)	Expected asthmatic patients No.(%)	p-value	Observed healthy control No.(%)	Expected healthy control No.(%)	p-value
***CCL-20* (786T>C)**	**TT**	9(18%)	4.21 (8.41%)	0.001	28 (37.6%)	25.9 (53.29%)	0.23
	CT	11 (22%)	20.5 (41.18%)		16 (47.1%)	20.2 (39.42%)	
	CC	30 (60%)	25.2 (50.41%)		6 (15.3%)	3.9 (7.29%)	

3. 6.2. 2. Correlation Asthma Severity and *CCL-20*(-786T>C)Genotypes in Asthmatic Children

By comparing of genotype frequencies, a significant association of genotypes *CCL-20* with mild, moderate and severe asthma was found as shown in Table 3.22.

Table 3. 22. Correlation *CCL-20* (786T>C)genotypes and asthma severity in the atopic asthmatic group

CCL-20 (786T>C) genotypes	Asthma Severity			
	Mild 33(66%)	Moderate15(30%)	Severe2(4%)	P.value
TT	5 (15.1%)	2(13.3%)	0(0)	0.0219
TC	15(45.5%)	5(33.3 %)	1(50%)	
CC	13 (39.4%)	8 (53.3%)	1 (50%)	
T Allele	25 (37.9%)	9(30.0%)	1(25%)	
C Allele	41 (62.1%)	21(70.0%)	3(75%)	

Significant difference (P<0.05). Statistics Kingdom by ANOVA calculator

The current data also revealed , that significant differences in the prevalence of the allele T and allele C for *CCL-20* in the patients with mild forms of the disease in comparison to that in individuals with moderate and severe asthma were observed continuously as presented in Table 3.22.

Since the (-786T>C) transition is located in the promoter region of *CCL-20*, it could affect the expression of the gene and, hence, influence the serum level of CCL-20. It should be kept in mind that the association with asthma was found for the genotype CC of *CCL-20*, it could be assumed that this variant of the gene was expressed at a higher level in comparison with that of TT and TC genotype caused increased production of CCL-20 and in turn increase of eosinophil count. In addition, in the non atopic a negative significant association of genotypes *CCL-20* with mild , moderate and severe asthma were found as shown in Table 3.23. Furthermore, a non significant prevalence of the allele T and allele C of *CCL-20* in the patients with mild and moderate forms of the disease in comparison with that in individuals with severe asthma.

Table 3. 23. Correlation *CCL-20*(-786T>C)genotypes and asthma severity in non atopic asthmatic

***CCL-20* (786T>C) genotypes**	**Asthma Severity**			
	Mild 31(62%)	**Moderate15(30%)**	**Sever4(8%)**	**p-value**
TT	6(19.35%)	3(20.0%)	1(25.0%)	0.07
TC	5 (33.3%)	4 (26.7%)	1(25.0%)	
CC	20(60.6%)	8(53.3%)	2(50.0%)	
T Allele	17 (27.4%)	10(33.3%)	3(37.5%)	
C Allele	45(72.6%)	20(66.7%)	5(62.5%)	

Non significant difference (*P* >0.05). Statistics Kingdom by ANOVA calculator

To the best of our knowledge, the present study may be the first time to investigate the association of rs6749704 of CCL20 gene with the asthma disease in Iraqi children.

Chapter Four

Conclusion and Recommedations

4. 1. Conclusion

1. High CCL-20 and CXCL10 whereas low IL10 and TGFB level was associated with severe asthma and not welled control in children.
2. The role of TSIgE and (CCL-20 , CXCL10, IL10 and TGFB) cytokines are significant in the pathogenesis of asthma in Iraqi children.
3. In the asthmatic children infected by HRV and RSV, the eosinophile count and TSIgE are associated with increased atopic asthma severity.
4. Asthma in children has been associated with the site-specific CCL-20 (-786T > C genotype in Iraqi asthmatic children.
5. The CC genotype of *CCL-20* (- 786T > C) in the promoter sequences may result in the altered CCL- 20 production that leads to altered inflammatory responses by increased Eosinophil Count , TSIgE and hence, contributes to the pathogenesis of asthma.
6. In asthmatic children infected by HRV and RSV, the CC genotype in *CCL-20* (-786T>C) could be an important risk factor for asthma in Iraqi children.
7. In the asthmatic children infected with HRV and RSV, the TT genotypes in *CCL20* (rs6749704) polymorphism could be protective genotypes for asthma in children.

4.2. Recommendations

1. Suggestion of HRV and RSV - specific IgE in an allergy panel test in Iraq.
2. Assessment of HRV and RSV infection in asthmatic children for better management of the disease.
2. Performing SNP in *CCL-20* (-786T>C) to children with severe asthma and autoimmune diseases is also a possible area of study.
3. Another study is needed to detect the difference in response to biological asthma therapy (Anti-CCL-20) according to polymorphism in *CCL-20* (-786T>C).
4. Other studies are required to take the association between other SNPs of the *CCL20* (rs6749704) and asthma disease.
5. the CCL20 SNPs genotyping is an important aspect of research; a re-evaluation is required but on the basis of DNA-sequencing. This will definitely help in giving better results.

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Appendix

Appendix I: Asthmatic patients questionnaires

ASTHMA QUESTIONNAIRE

File Number: . . . . . .Date : . . . . . .

Patient Name: . . . . . phone number . . . . . .Age.....year

Gender male female Address : . . . . . .

Weight : . . . . . kg Height: ..... cm

Treatment :

a- Montelukast : daily dose 4mg 5mg 10mg

b- I C S : daily dose . . . . . c- Fluticasone d- Beclomethasone

Asthma Severity:

a- Mild b- Moderate c- Severe

History : Personal

Eczema	Yes	NO	shortness of breath	Yes	NO
Allergic Rhinitis (AR)	Yes	NO	wheezing	Yes	NO
Allergic conjunctivitis	Yes	No	cough	Yes	NO

Drug allergy : Yes NO Type : . . Food allergy : Yes Type : . . NO

Family

Eczema	Yes	NO	Drug allergy : Yes	NO	Type :
Asthma	Yes	NO	Food allergy : Yes	NO	Type :
Allergic Rhinitis (AR)	Yes	NO	Allergic conjunctivitis		
Animal in the house	Yes	NO			

Cat Dog Chicken Birds cow sheep

Smoker in the house Yes NO

Trigger for Asthma

- Viral infection	Yes	NO	- Cold air	Yes	NO
- Exercise	Yes	NO	- Dust	Yes	NO
- Play	Yes	NO	- Fumes	Yes	NO

Parameter: % eosinophil count…….

Total serum IgE ……

Appendix II: Healthy controls questionnaires

Healthy controls QUESTIONNAIRE

Date : . . . . . .

Participant Name: . . . . . Age.....year

Gender male ☐ female ☐ Address : . . . . . .

Weight : . . . . . kg Height: .....cm

History : Personal (hx.)

Allergic diseases Yes ☐ NO ☐

Other diseases Yes ☐ No ☐

Family (hx.)

Allergic diseases Yes ☐ NO ☐

Other diseases Yes ☐ No ☐

Parameter:

Absolute eosinophil count…….

Total serum IgE ……

Appendix III: Contents of ELISA Kit

Appendix IV: Procedure

Detection of Serum Human (RSV-IgM and RV-IgM)

The ELISA Kits was applied to the *in vitro* qualitative determination of human serum RSV and RV IgM for patients and control group, by using Sunlong ELISA kit (LOT NO. 20220426).

The Principle of The Test

The ELISA used was based on the qualitative enzyme immunoassay technique. The microtiter wells provided in this kit has been pre-coated with an antigen specific to RSV-IgM and HRV-IgM. At the beginning, serum from patient samples were incubated in the wells. RSV-IgM and HRV-IgM found in the sample would bind to the specific antigen. After that a second incubation was carried out using an enzyme-labelled anti-human RSV- IgM or HRV IgM. The color reaction would be catalyzed by the addition of a substrate. Then stop solution would stop the reactions. The (OD) is measured by photometric at a wavelength of 450nm. Finally, The qualitative determination of human (RSV-IgM and RV-IgM) was detected by comparing with the CUTOFF value.

Preparation of Reagents

All reagents are left to the room's temperature (25℃) before used.

1. In the microtiter wells, two wells left as negative control, two wells as positive control and one empty well as blank control. (blank control well dont added samples and HRP-Conjugate reagent).

2. Adding samples: Added 50 ul of Negative and positive control to the negative and positive control wells respectively. For sample wells,40µl sample dilution buffer and 10µl sample are added in it. Samples loaded onto the bottom without touching the well wall. Mixed with gentle shaking.

3. Incubation: Incubated 30min at 37℃ after sealed with closure plate membrane.

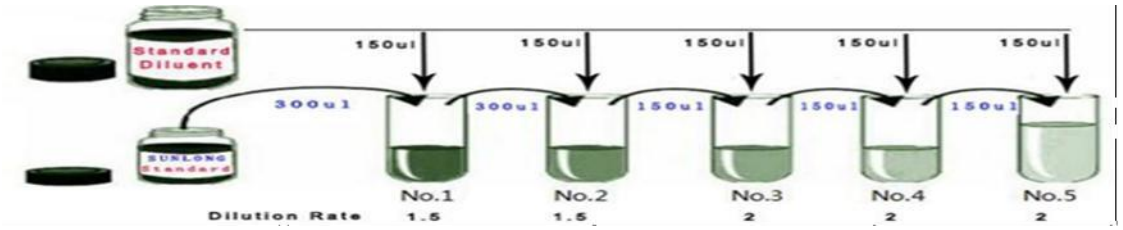
4. Dilution: Diluted the concentrated washing buffer with distilled water.
5. Washing: Closure plate membrane removed carefully, aspirated and refilled with the wash solution. Then Discarded the wash solution after resting for 30 sec, and repeated the washing 5 times with 350µl of wash buffer.
6. Added 50 µl HRP-conjugate reagent to each well except the blank well.
7. Incubation: As described in Step 3.
8. Washing: As described in Step 5.
9. Coloring: 50µl chromogen solution A and 50µl chromogen Solution B Were added to each well, mixed with gently shaking and incubated for15 minutes, and avoid light during coloring.
10. Termination: 50 µl of stop solution was added into each micro plate wells and gently mix for 15-20 seconds.
11. Photometric measurement was done at 450 nm within 15 min of adding stop solution.

Determine The Result

The average value of positive control ≥1.00; the average value of negative control ≤ 0.10. Calculated the critical value (CUT OFF): critical value = the average value of negative control + 0.15. The samples of Human (RSV-IgM and RV-IgM) were considered negative when the OD value < CUT OFF, whilst it considered positive when the OD value ≥ CUT OFF.

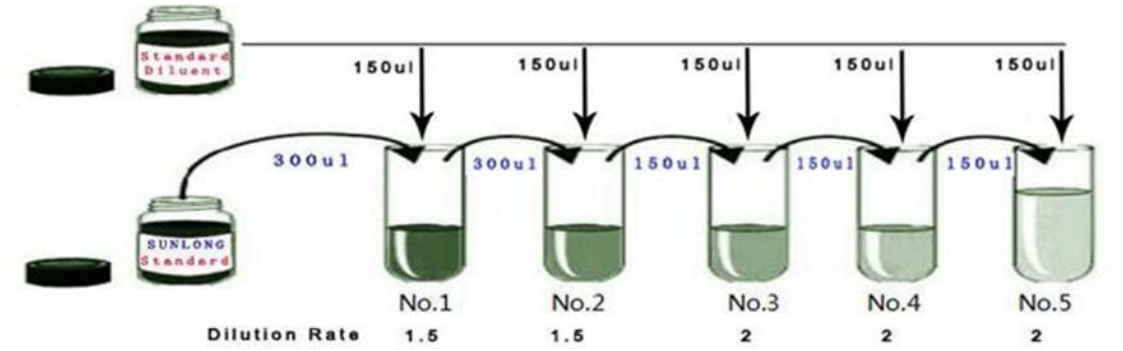
AppendixV: Dilution of Standards

For CCL20 and IL-10



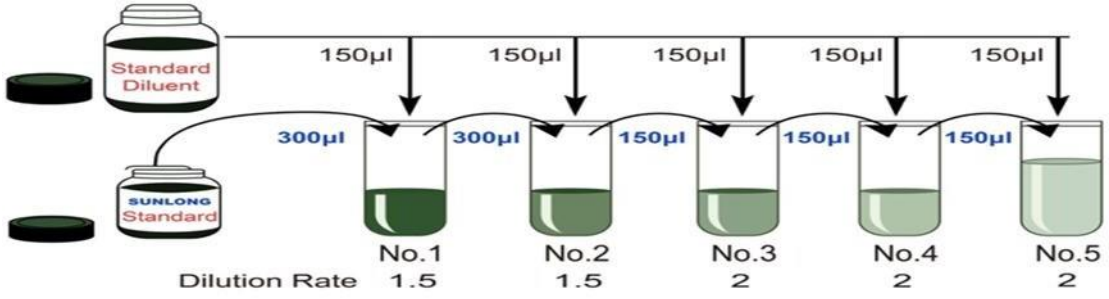
135pg/ml	90pg/ml	60pg/ml	30pg/ml	15pg/ml	7.5pg/ml

For CXCL-10



360pg/ml	240pg/ml	160pg/ml	80pg/ml	40pg/ml	20pg/ml

For TGF-β



270 pg/ml	180 pg/ml	120 pg/ml	60 pg/ml	30 pg/ml	15 pg/m

Appendix VI:*CCL20* [(Homo sapines)(Human)]

CCL20 (-786T>C) rs6749704

Region of SNP (rs 6749704): 227813076-227813183 (107 bp)

Region of *CCL20*: 227813842-227817556 (3714 bp)

Homo sapiens chromosome 2, GRCh38.p14 Primary Assembly

TAGGTTTTCTTTCCTCAACAATTCTGAGGCTCTATATTGAGTTATATTAG ACATCATCATG

GAGAGTTAAAGGTAGGTAAGGATTATTTTCTGAACTGCAATATTGA

Appendix VII: Gel Electrophoresis of Genomic DNA

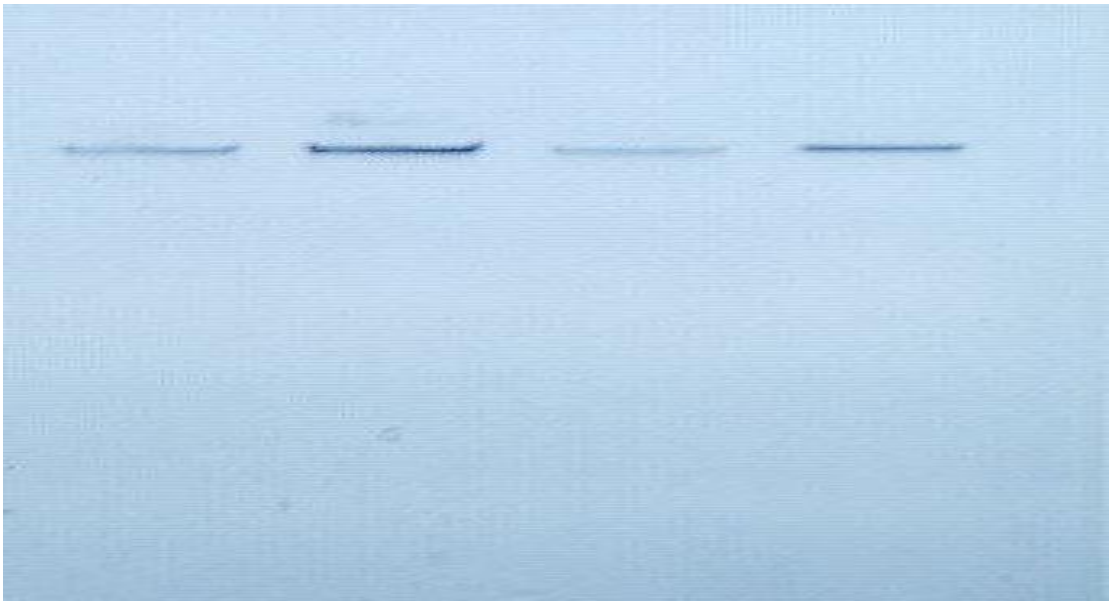


Figure 7.1. Evaluation of DNA extract quality and integrity .1 % agarose gel electrophoresis of genomic DNA in 75 volt and 30 min.

الخلاصة

تم إجراء دراسة مناعية وجزيئية للأطفال المصابين بالربو والخمج بالفايروس التنفسي المخلوي والفايروس الانفي لتحديد الارتباط بين العدوى الفايروسية والتغاير الجيني rs6749704 للجين المشفر للمحرك الخلوي CCL20 ، والتغيير اللاحق في الاستجابة المناعية للمرض وشدته.

الدراسة الحالية هي مقارنة بين المرضى والاصحاء حيث شملت ١٠٠ طفل مصاب بالربو منهم الراقدين وكثير منهم مراجعين ٥٠ منهم ربو تحسسي (٣٥ من الذكور و ١٥ أناث) و ٥٠ ربو ليس تحسسي (٣١ من الذكور و ١٩ أناث) تم جمع عينات دم المرضى المشاركين في هذه الدراسة من عيادة الربو في مستشفى كربلاء التعليمي للأطفال في الفترة الممتدة من شباط إلى حزيران ٢٠٢٢. تراوحت أعمارهم بين سنة وست سنوات. شملت مجموعة الاصحاء ٥٠ طفلًا سليمًا (٣٢ من الذكور و ١٨ من الاناث) من نفس أعمار وجنس المرضى تم اختيارهم بشكل عشوائي من المجتمع ، تم جمع البيانات الديموغرافية والسريرية من الأطباء الاستشاريين والمرضى و / أو والديهم من خلال الاستبيان . بالإضافة إلى ذلك ، تم الحصول على البيانات من مجموعة الاطفال الاصحاء و / أو والديهم خلال استبيان خاص بهم.

تم استخدام المصل المفصول من دم المرضى والاصحاء للتحديد النوعي للغلوبين المناعي نوع M الخاص بالفايروسين قيد الدراسة، ومستويات الغلوبين الكلي نوع E والمحركات المناعية CCL20 و CXCL10 و IL-10 وTGF-β باستخدام تقنية الامتزاز المناعي المرتبط بالإنزيم. بينما وضع الجزء الثاني من الدم في انابيب حاوية مادة مانعة للتخثر لتحديد عدد خلايا الدم الكامل والتفريقي، وبضمنها النسبة المئوية للخلايا الحامضية والمتعادلة باستخدام صورة الدم وجهاز تحليل الدم التفريقي الآلي، فضلا عن استخراج الحمض النووي من خلايا الدم. استخدم الحمض النووي المستخرج للكشف عن التغاير الجيني (rs6749704) في الجين المشفر للمحرك الخلوي CCL20 في الموقع (-786T>C) بواسطة استخدام تقنية تفاعل البلمرة المتسلسل- تعدد اشكال اطوال قطع التقييد.

تم تحديد ٢٤ طفل مصاب بالربو والخمج بالفايروس التنفسي المخلوي والفايروس الأنفي في الدراسة الحالية ،١٠كانوا مصابين بالتنفسي المخلوي و١٤منها كانت بالأنفي.

أظهرت النتائج الحالية وجود علاقة معنوية عالية بين تركيز الغلوبين الكلي نوع E في مرضى الربو التحسسي (٢٧٥.٧٧) وحدة عالمية / مل ومرضى الربو غيرالتحسسي (٣٦.١٧) وحدة عالمية / مل مقارنة بالأصحاء (١١.١٥٣) وحدة عالمية / مل. فيما يتعلق بالنسبة المئوية لعدد الخلايا الحامضية والمتعادلة كانت بمرضى الربو التحسسي (٦.٢) و (٢٧.٨٧) في حين كانت بمرضى الربو غير التحسسي(٢.١) و (٥٢.٣٤) بينما كانت بالأصحاء (١.٧) و (٤٨.٤٢) وكانت هناك علاقة معنوية عالية (P =0.0001). كذلك أظهرت النتائج الحالية وجود علاقة ذات دلالة إحصائية بين تركيز CCL20 (٥٢.٢) بيكوغرام/مل في مصل الدم لدى مرضى الربو التحسسي ومرضى الربو غير التحسسي(٣٥.٧) بيكوغرام/مل مقارنة مع الاصحاء(٢٣.٩) بيكوغرام/مل.ايضا كانت نسبة تلك الخلايا وتركيز الغلوبين الكلي نوع E عالي في الحالات المتوسطة قياسا بالخفيفة لمرضى الربو التحسسي وغير التحسسي (P = < 0.05)

أظهرت نتائج الدراسة الحالية فروقًا ذات دلالة إحصائية في توزيع النمط الوراثي CC لـ -*CCL* *20* (-786T> C) بين الأطفال المصابين بالربو التحسسي وغير التحسسي والاصحاء (P=0.0001). لذلك يمكن اعتبار النمط الوراثي CC عامل خطر لتطوير نسبة احتمالات الإصابة بالربو التحسسي واللاتحسسي لدى الاطفال العراقيين. أظهر تحليل الأنماط الوراثية باستخدام توزيع هاردي وينبرج وجود فروق ذات دلالة إحصائية بين أعداد المرضى الذين لوحظوا والمتوقعون بالنسبة لجين الكيموكاين ٢٠ في الموقع T-786C بالنسبة لمرضى الربو غير التحسسي ، بينما على العكس في التحسسي.

في الختام ، كانت نسبة عالية من الإصابة بالربو بين الذكور مقارنة الإناث لدى الأطفال، اذ أن تعدد الأشكال في المحفز في المنطقة الجينية (-786T> C) *CCL20* قد يساهم في الاستعداد للربو، وكان هناك ارتباط معنوي بين النمط الجيني لهذا الجين ومستوى الكيموكاين وتطور المرض عند الأطفال المصابين بفيروس التنفسي المخلوي والفايروس الانفي. يعتبر التاريخ العائلي للإصابة بالربو أحد عوامل الخطورة. يرتبط CCL-20 و CXCL10 بتطور الربو عند الأطفال المصابين بفيروس الانف البشري والمخلوي التنفسي. كذلك ارتبطت زيادة خطر الإصابة بالربو لدى الاطفال بالنمط الجيني CC لـ -*CCL* *20* (-786T>C) ،اذ تم الكشف عن ارتباطه بمستوى CCL-20 المرتفع ، وارتفاع نسبة عدد الحمضات والعدلات قي مرضى الربو التحسسي وغير التحسسي ، وتوزيعه لديهم أكثر مقارنة بالاصحاء.

جـــامعـــــة كربلاء

كليــة العلــوم

قســم علـوم الحيـاة

دراسة العلاقة الترابطية بين الخمج بالفايروس التنفسي المخلوي والفايروس الأنفي والتغاير الجيني في rs6749704 مع بعض المحركات الخلوية في الربو عند الاطفال

اطروحة مقدمة الى مجلس كلية العلوم / جامعة كربلاء

وهي جزء من متطلبات نيل درجة الدكتوراه في علوم الحياة

كتبت بواســـــطة

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بكلوريوس علوم حياة / جامعة الكوفة ٢٠٠١

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