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Association between Genetic Polymorphisms and *Chlamydia pneumoniae* in Asthmatic Children in Iraq

A Thesis

Submitted to the Council of the College of Medicine, University of Babylon, in a Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Science / Medical Microbiology.

By

Raghdah Maytham Hameed Mahdi

B.Sc. College of Health and Medical Technologies/ Middle Technical University of Baghdad (2014)

MSc. Microbiology \ College of Medicine University of Karbala (2019).

Supervised by

Professor

Dr. Huda H. Al-Hasnawy

Department of Microbiology

University of Babylon

Professor

Dr. Haidar Abdul Amir Najim Abood

Department of Pharmacology

University of Kerbala

2023 AD **1444 AH**

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

﴿وَمَا يُلَقَّاهَا إِلاَّ الَّذِينَ صَبَرُوا وَمَا يُلَقَّاهَا إِلاَّ ذُو حَظٍّ عَظِيمٍ﴾

صدق الله العلي العظيم

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Supervisors Certification

We certify that this thesis entitled "**Association between Genetic Polymorphisms and *Chlamydia pneumoniae* in Asthmatic Children in Iraq**" was prepared by "**Raghdah Maytham Hameed**" under our supervision at the College of Medicine, University of Babylon, as a partial requirement for the degree of Doctorate of Philosophy in Microbiology.

Professor

Dr. Huda H. Al-Hasnawy

Department of Microbiology

University of Babylon

Professor

Dr. Haidar Abdul Amir Najim Abood

Department of Pharmacology

University of Kerbala

In view of the available recommendation, we forward this thesis for debate by the examining committee

Professor

Dr. Hayam K. Al-Masudi

Head of Department of Microbiology

College of Medicine

University of Babylon

/ / 2023

Decision of Examination Committee

We, the examination committee, certify that we have read the thesis entitled (**Association between Genetic Polymorphisms and *Chlamydia pneumoniae* in Asthmatic Children in Iraq**) and have examined the student (**Raghdah Maytham Hameed**) in its contents, and that in our opinion it is accepted as a thesis for Degree of Doctorate of philosophy in Medical Microbiology with **Excellent** estimation.

Signature

Prof. Dr. Fatima Abdul Hussein Mejbel

University of Kufa / College of Science

(Chairman)

Signature

Prof. Dr. Maysa Salih AL-Shukri

University of Babylon / College of Medicine

(Member)

Signature

Prof. Dr. Lina Fadhil Hamza

University of Babylon / College of Pharmacy

(Member)

Signature

Prof. Dr. Ifad Kerim Al-shibly

University of Babylon / College of Medicine

(Member)

Signature

Assistant Prof. Dr. Rebee Mohsin Hasani

University of Babylon / College of Medicine

(Member)

Signature

Prof. Dr. Huda Hadi Al-Hasnawy

College of Medicine / University of Babylon

(Member and Supervisor)

Signature

Prof. Dr. Haidar Abdul Amir Najim Abood

College of Medicine / University of Karbala

(Member and Supervisor)

Approved for the college committee of graduate studies

Signature

Prof. Dr. Mohend Abbass Nori Al-Shalah

Dean of college of Medicine

University of Babylon

DEDICATION

To those who supported me morally….My family

To that who was the source of my strength....My instructor ***Ahmed Al-jabiri***

To that who died just before I finished my research.....My friend ***Mohanad Kadhim Al-Ibraheemi****. His determination and excellence will always inspire me.*

I give this humble effort ... with my sincere love for them.

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Summary

Asthma is a chronic inflammatory disorder of the airways of the lungs; considered a major global health problem, it affects an estimated 262 million people in 2019. A molecular and Immunological study was performed to detect the correlation between asthma and *Chlamydia pneumoniae* infection. Further, to determine the influences of genetic variants of the interleukin-4 (IL-4) C589T gene on total serum immunoglobulin E (IgE), serum IL-4 level, *Chlamydia pneumoniae* IgG and IgE levels, asthma severity, and asthma control.

This case-control study was performed on 174 subjects. After performing IgE tests, asthmatic patients with IgE < 100 IU/ml were excluded (41 patients) because this study was designed for IgE-mediated asthma, and 100 IU/ml is the cut-off value of a positive IgE level. This study involved 87 asthmatic children with high total IgE (57 male and 30 female) who attended the asthma clinic at Karbala Teaching Hospital for Children in the period extending from January 2022 to May 2022. Their ages ranged between one to sixteen years. The control groups included 87 children (48 male and 39 female) with age and sex-matched to the patients, randomly selected from the local community. A questionnaire collected the clinical and demographic data from patients and /or their parents. Blood was collected from each participant, notice that the sera were used to determine total serum IgE levels, Human *Chlamydia pneumoniae* IgG levels, and Human *Chlamydia pneumoniae* IgE levels for all samples and serum IL-4 for asthmatic children

only. At the same time, whole blood was used for DNA extraction. DNA extraction was used to detect SNPs in the IL-4 gene (IL-4 C-589T) by the restriction fragment length polymorphism PCR (PCR-RFLP) technique. The software SPSS version 21 was used to analyze data statistically.

The study showed a significant difference in the human *Chlamydia pneumoniae* IgG and IgE levels between asthmatic children and control (*p-value* <0.001 and 0.024, respectively). Further, in asthmatic children, there is a significant positive linear correlation between total serum IgE level and human *Chlamydia pneumoniae* IgE under age control (*p-value*=0.019). In the same line, there is a highly significant positive linear correlation between *Chlamydia pneumoniae* IgG and *Chlamydia pneumoniae* IgE in asthmatic children (*p-value* <0.001). In addition, the IL-4 level was higher in asthmatic patients with severe and not-well-controlled asthma compared to other asthma groups (*p-value* =0.007 and 0.004, respectively). Additionally, the study showed that the frequency of heterozygous IL-4 CT genotype was significant in patients (34.5%) compared to the control (17.24%). Therefore, the CT genotype could be considered a risk factor for developing asthma (odds ratio= 2.526, *p-value*= 0.01). Genotypes analysis using Hardy-Weinberg distribution showed no significant differences between observed and expected patient numbers for the IL-4 C589T gene. In asthmatic children, there were significant associations between high total serum IgE level, severe, and not-well-controlled asthma with CT genotype of IL-4 C589T polymorphism (*p-value* 0.02, <0.001, and 0.001, respectively). In contrast, high *Chlamydia pneumoniae* IgE levels were found to be associated with CC genotypes (*p-value*= 0.01).

In conclusion, children with asthma have more persistent *Chlamydia pneumoniae* infections than non-asthmatic children. The presence of *Chlamydia pneumoniae*-specific IgE may provide ongoing stimulation of allergic responses by *Chlamydia pneumoniae*. Drooping of *Chlamydia pneumoniae* IgE level in asthmatic adolescents who live in urban. High IL 4 level was associated with more severe asthma and not welled control in children. Asthma in children has been associated with the site-specific IL 4 C589T genotype in Iraqi asthmatic children. CT genotype of IL 4 C589T association with high more severe asthma and not welled control in children. CT genotype of IL 4 C589T association with high total IgE levels but not IL-4. IL-4 C589T polymorphism was not implicated in developing high *Chlamydia pneumoniae* IgE antibodies in asthmatic children.

List of contents

List of tables

List of figures

List of appendices

List of Abbreviations

Code	Words
ATP	Adenosine Triphosphate
AFLP	Amplified Fragment Length Polymorphism
APC	Antigen-Presenting Cells
ACT	Asthma Control Test
BslF I	Bacillus stearothermophilus FI
Bfgf	Basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
$x^{\wedge 2}$	Chi-Square
cLPS	Chlamydial Lipopolysaccharide
CI	Confidence intervals
DCs	Dendritic cells
EB	Elementary bodies
ELISA	Enzyme-linked immune assay
FEV1	Forced expiratory volume
HWE	Hardy Weinberg Equilibrium
Hsp60	Heat-Shock Protein 60
FcεRI	High-Affinity IgE Receptor
HEp-2	HL Cells and Epithelial Cell Lines
HRP	Horse Radish Peroxidase
IgE	Immunoglobulin E
ICS	Inhaled Corticosteroid
IFN-β	Interferon beta
IFN-γ	Interferon Gamma
IL-	Interleukin
IU	International Units
JAK	Janus kinase
MHC	Major Histocompatibility Complex
MMPs	Matrix Metalloproteinases
MIF	Micro-Immunofluorescence
µl	Micro Liliter
NO	Nitric Oxide
N	Number
OR	Odds Ratio

Omp	Outer Membrane Protein
pg	Pictogram
PCR	Polymerase Chain Reaction
PK	Proteinase K
POMP	Putative outer Membrane Protein
RFLP	Restriction Fragment Length Polymorphism
RB	Reticulate Bodies
RPM	Rounds per Minute
SABA	Short-acting β2 Agonist
STAT	Signal Transducer and Activator of Transcription
SNPs	Single Nucleotide Polymorphisms
SSPS	Specific Software Statistical Package for the Social Sciences
S.D	standard Deviation
GINA	The Global Initiative for Asthma
TWAR	Taiwan acute respiratory agent
Th	T-helper cell
TNF-α	Tumor Necrosis Factor α
T3S	Type III Secretion Apparatus

CHAPTER ONE

Introduction

and

Literature Review

1.1. Introduction:

Asthma is an inflammatory disorder in the airway, leading to airway obstruction, mucus hyper-production, hyper-responsiveness, and airway wall remodeling. Asthma is characterized by reversible airway obstruction and T-helper 2 (Th2) infiltration. Asthma typically presents a history of respiratory symptoms such as wheezing, cough, shortness of breath, and chest tightness and is characterized by underlying chronic airway inflammation. Asthma affected an estimated 262 million people in 2019, according to the Global Burden of Disease. In Iraq, the prevalence of asthma was 8.9% prevalence in older and 15.55% in younger children (Wu *et al.*, 2016, Asher *et al.*, 2017, Alavinezhad and Boskabady, 2018, Song *et al.*, 2022).

Asthma is a condition that is likely caused by complex interactions between multiple genetic and environmental influences. Atypical bacterial infections appear to play a role in asthma induction and exacerbation in children and adults. In addition, several gene polymorphisms have been associated with susceptibility to asthma and allergy (Bijanzadeh *et al.*, 2011, Webley and Hahn, 2017).

Chlamydia pneumoniae is an obligate intracellular bacterial pathogen and a respiratory pathogen in humans that occurs worldwide. *Chlamydia pneumoniae* is involved in the pathogenesis of multiple inflammatory diseases, including asthma (Gautam and Krawiec, 2022). Several studies indicate that bacteria can induce the differentiation of naïve T cells into Th2 or Th17 cells and elicit Th2 cytokine release. *Chlamydia pneumoniae* stimulates the production of IL-4 in peripheral blood mononuclear cells (Smith-Norowitz *et al.*, 2016).

IL-4 mediates essential pro-inflammatory functions in asthma, including differentiation of Th2 cells leading to cytokine release, induction of the IgE isotype switch, promotion of eosinophil transmigration across the endothelium, expression of vascular cell adhesion molecule-1, and mucus secretion (Steinke and Borish, 2001).

IgE is an antibody produced by B-cell after differentiation into plasma cells in lymph nodes. IgE is the most potent antibody classes and can trigger dramatic inflammatory reactions even when present in minute amounts. IgE plays a central role in the allergic response (Buc *et al.*, 2009, Laffleur *et al.*, 2017).

SNPs in the human genome are variations in the DNA sequence that can modify an individual's response to environmental exposure (Wang, 2005). SNPs are the most essential and basic form of variation in the genome. They are responsible for genetic effects that produce susceptibility to most autoimmune diseases, which may occur every 100 to 300 bases (Bell, 2002, Information, 2005).

Several studies suggested that the -589T allele in rs2243250 is associated with increased serum or plasma IL-4 levels and is linked to total serum IgE levels. IL-4 -589T allele was associated with probable asthma, rhinitis, and atopy in a cohort of infants at risk for allergic disease. In addition, the IL-4 -589T allele was associated with lower Forced expiratory volume (FEV1) values in a population of white subjects with asthma. These data suggest that the -589T polymorphism may influence asthma severity (Shirkani *et al.*, 2019).

Many previous studies mentioned the positive correlation between *Chlamydia pneumoniae* infection and asthma (Calmes *et al.*, 2021), but

the mechanism of action of this bacterium on asthmatic patients is still ununderstood.

On the other hand, many previous studies detected an association between IL-4 C589T polymorphism and asthma. Some previous studies showed that the T allele frequency for the C-589T IL-4 gene promoter in asthma patients was higher than for normal subjects (Jin and Zheng, 2021). In contrast, other studies showed no or weakly significant association between IL-4 589 C <T polymorphism and asthma (Hussein *et al.*, 2020). Thus, this work was performed to study the polymorphism in IL-4 among asthmatic patients in our local population. Further, to determine this polymorphism's impact on an asthmatic patient's characteristics.

The genetic variation may differ in a subject's response to environmental exposure. Unfortunately, no previous studies locally or internationally detected the influence of IL-4 polymorphism on the immune response to *Chlamydia pneumoniae* infection.

The Aim of Study:

This work aimed to investigate the possible correlation between IL-4 gene polymorphisms and characteristics of asthmatic children, such as severity and immunological biomarkers (IL-4 levels and total IgE levels) and specific *Chlamydia pneumoniae* IgE levels. This aim was achieved by the following objectives.

Objects:

- **Immunology:** Detection of *Chlamydia pneumoniae* by specific *Chlamydia pneumoniae* IgG and IgE antibody levels in patient's sera by ELISA. Detection of some immune markers (total IgE levels, IL-4 levels in patient's blood by ELISA.
- **Genetics:** Detection of IL-4 polymorphisms by PCR-RFLP

1.2. Literature Review:

1.2.1. Asthma Overview:

Asthma is a chronic, inflammatory disease of the air passages characterized by variable respiratory symptoms and variable airflow limitation. It is an obstructive pulmonary disorder with exacerbations characterized by symptoms of shortness of breath, chest tightness, cough, and wheezing (Maslan and Mims, 2014, Mims, 2015, Papi *et al.*, 2018). Asthma is the most common non-communicable, chronic disease in children and adults, characterized by variable airway obstruction, airway hyperresponsiveness, and airway inflammation. It is a common condition due to chronic inflammation of the lower respiratory tract (McCracken *et al.*, 2017, Papi *et al.*, 2018).

Asthma is a chronic disorder arising from poorly understood heterogenic gene-environment interactions. Asthma exacerbations are commonly initiated by upper respiratory tract infections and/or environmental allergens. However, other known factors increase the risk of patient exacerbation, such as cigarette smoking (Graham and Eid, 2015, Mims, 2015, Hernandez-Pacheco *et al.*, 2022).

Significantly, allergens or environmental exposure to toxic agents, such as pollutants, diesel exhaust, and detergents, may lead to asthma development by affecting the epithelial barrier. On the other hand, identifying novel genes for asthma suggests that many genes with minor effects rather than a few genes with substantial effects contribute to the development of asthma. These genetic effects may, in part, differ in a subject's response to environmental exposure, although some genes may also exert their influence independently of the environment (von Mutius, 2009, Boonpiyathad *et al.*, 2019).

1.2.2. Epidemiology of Asthma:

1.2.2.1. Prevalence and Incidence:

The clinicians were provided with an annually updated evidence-based asthma management and prevention strategy from the Global Initiative for Asthma (GINA) Strategy Report (Reddel *et al.*, 2021).

Asthma affected an estimated 262 million people in 2019, according to the Global Burden of Disease (Song *et al.*, 2022). Pediatric asthma is a worldwide public health problem. The Global Asthma Report 2022 detected that 1 in 10 children have asthma symptoms. The average annual asthma prevalence is higher in children (9.5%) than in adults (7.7%) (Loftus and Wise, 2016, Serebrisky and Wiznia, 2019, Network, 2022).

The observed adjusted prevalence of asthma in the Middle East is lower than the reported prevalence in North America and Europe, which ranges from 4.4% to 7.6% (Tarraf *et al.*, 2018). In Iraq, the prevalence of asthma was 8.9% prevalence in older and 15.55% in younger children (Alavinezhad and Boskabady, 2018). Further, the International Study of Asthma and Allergies in Childhood (ISAAC) recorded the prevalence of clinically diagnosed childhood asthma in Iraq as 16.3% in primary school children (Abood and Al-Zaubai, 2020).

1.2.2.2. Gender and Age:

In children, boys have an increased prevalence of asthma, while in adults, women have an increased prevalence and severity of asthma (Chowdhury *et al.*, 2021). ISAAC Phase III estimated asthma prevalence to be 10.6% in males and 7.9% in female adolescents (13-14 years old), 10.3% in males, and 8.5% in female children (6-7 years old) (Network, 2022).

An estimated 42% of adults reported asthma onset before age 16 with active asthma, including 14% starting at 5–9 years of age (Mirabelli *et al.*, 2013). In addition, children aged 7 to 9 years had a higher risk ratio for asthma than those in other age groups (Lin *et al.*, 2017). Another study showed that 30% of patients developed asthma before 14 years old (Hsu *et al.*, 2004).

1.2.2.3. Socioeconomic Status:

The most significant burden of childhood asthma was seen among low socioeconomic status young people, often concentrated in urban areas with high poverty rates. Asthma prevalence is generally greater in urban compared to rural populations (Milligan *et al.*, 2016, Lawson *et al.*, 2017, Rodriguez *et al.*, 2019).

Children who grow up in crowded urban neighbourhoods have higher asthma rates and experience more significant morbidity due to asthma. The differences in asthma prevalence in urban and rural areas may be because people have different lifestyles, cultures, and other environmental exposures and genetic backgrounds. Several environmental and lifestyle factors associated with the urban living were suspected of promoting the development of asthma, particularly in the first few years of life (Gern, 2010, Jie *et al.*, 2013).

Although rural children also encounter socio-demographic disparities that might be expected to worsen asthma. Whereas in rural areas, risk factors for asthma and asthma-like symptoms may show some differences when compared to urban areas. Thus, the prevalence of asthma in children was lower in rural than in urban areas (Valet *et al.*, 2011, Uğurlu *et al.*, 2014, Zhu *et al.*, 2015).

1.2.3. Diagnosis of Asthma in Children:

Asthma is diagnosed through a medical history, family history, physical exam, and a test that measures airflow in and out of the lungs. Spirometry testing measures the flow and volume of air blown out after taking a deep breath and then forcefully exhaling. The other tests to diagnose asthma include chest X-rays and skin or blood testing for allergies or immune problems (Sawicki *et al.*, 2021).

Serum IgE level was predictive in asthma, and it may be used to differentiate between asthmatic and non-asthmatic individuals in conjunction with other biomarkers. IgE antibodies test to measure sensitization to inhalant allergens is a useful diagnostic indicator for asthma in children (Shoormasti *et al.*, 2018, Sonntag *et al.*, 2019).

1.2.4. Asthma Severity:

Asthma severity is a vital independent risk factor for future exacerbations. As the severity of asthma increases, the exacerbation rates also become more frequent (Nakwan, 2021). According to the GINA report, asthma severity was classified as mild-moderate-severe asthma (Yılmaz *et al.*, 2022). Classifying the severity of asthmatic attacks based on the National Asthma Education and Prevention Program and the Global Initiative for Asthma guidelines was compared with physician assessment and benchmarked against asthma-related health care use. Guideline-based asthma severity symptom components were derived from patient-reported questionnaires. Prebronchodilator FEV measurements determined lung function levels; patients reported asthma-related medication and recent healthcare use (Miller *et al.*, 2005, Khajotia, 2008). Table 1-1 showed that the information enables the

pediatrician to select the right medication and determine the proper dose to keep the condition in check (Cloutier *et al.*, 2020).

Table (1-1): Assessment of Asthma severity in Children (Cloutier *et al.*, 2020)

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)	FEV_1% predicted	>80	>80	60-80	<60
	FEV_1/FVC	>0.85	>0.8	0.75-0.8	<0.75

Mild asthma is defined as well-controlled with as-needed inhaled corticosteroid (ICS)-formoterol, or with low dose ICS plus as-needed short-acting β2 agonist (SABA) (Yılmaz *et al.*, 2022). Previously, patients with mild asthma were divided into intermittent or persistent classes based on the frequency of symptoms and reliever medication usage (Shahidi and Fitzgerald, 2010). Today, the GINA report said that mild intermittent and persistent asthma are no longer distinguished. Both

are considered mild asthma because it has been stated that this ancient distinction is entirely arbitrary and not based on evidence (GINA, 2022).

People with moderate persistent asthma are not well controlled on low doses of ICSs. A combination of this drug and long-acting inhaled beta2 agonists provides improved control compared with doubling the maintenance dose of ICSs. Moderate asthma is associated with increased stored and secreted mucin. These findings suggest that acute degranulation of hyperplastic goblet cells may represent a mechanism for asthma exacerbations in mild and moderate asthma. Further, chronic degranulation of goblet cells may contribute to chronic airway narrowing in moderate asthma. The moderate persistent asthma symptoms: nighttime symptoms occur more than five times per month, the symptoms affect activity happening more than two times per week and may last for days, and lung function tests are 60% to 80% of predicted values based on age, sex, and height (O'Byrne and Parameswaran, 2006, Morosco, 2007).

Severe asthma is defined as asthma that requires treatment with high-dose ICSs plus a second controller to prevent it from becoming uncontrolled or which remains uncontrolled' despite this therapy. Children with severe asthma were at increased risk for adverse outcomes, including medication-related side effects and recurrent and life-threatening exacerbations that significantly impair their quality of life. The airway narrowing causes lung hyperinflation, ventilation-perfusion imbalance, and increased work of breathing that may lead to ventilatory muscle fatigue and respiratory failure, which is life-threatening. Diagnosing and treating severe asthma are time-consuming and require special experience (Papiris *et al.*, 2002, Fitzpatrick, 2016, Chung, 2018).

1.2.5. Treatment of Asthma:

Guidelines for asthma treatment have identified that the primary goal of management is achieving reasonable asthma control, thus reducing the risk of exacerbations. Phenotypic presentations in asthmatic children are various and might contribute to differential responses to asthma controller therapy. Mild to moderate asthma often responds to traditional medications, while severe asthma can be refractory to ICS, long-acting β-agonists, and leukotriene receptor antagonists. Further, children with more severe asthma are often unresponsive to recent efforts, and there remains a need for agents with properties that may achieve control in these patients (Darveaux and Busse, 2015, Berry and Busse, 2016, Fitzpatrick, 2016, Pongracic *et al.*, 2016).

Many children with asthma remain symptomatic despite treatment with ICSs, resulting in reduced quality of life and significant morbidity. Thus, the biological drugs are approved to treat patients with severe uncontrolled asthma, reduce the risk of exacerbation, maintain asthma symptom control, and reduce the need for systemic corticosteroids. This biological treatment includes omalizumab (anti-IgE), dupilumab (anti-IL-4/IL-13), and benralizumab, mepolizumab, reslizumab (anti-IL-5 pathways) (Vogelberg *et al.*, 2015, Busse, 2019, Calzetta *et al.*, 2021).

Many factors can play a role in asthma induction and exacerbation and response to treatment, such as genetic variation, ethnicity, and respiratory infections, such as viruses and atypical bacteria. Thus, treatment decisions on childhood asthma management should be critically made (Koo *et al.*, 2016, Leusink *et al.*, 2016, Webley and Hahn, 2017, Zhang *et al.*, 2017, Tesse *et al.*, 2018).

1.2.6. Asthma Control:

Determining the state of asthma control and identifying risk factors for poor asthma control is a crucial strategy for curbing the financial burden of the disease and its negative health impacts (Mulugeta *et al.*, 2022). The measurement of underlying asthma activity potentially by biomarkers to assess asthma control will lead to an improved assessment of the overall control of asthma (Fu *et al.*, 2014). Poor asthma control is associated with substantial impairment in quality of life due to the inverse relationship between the number of asthma control problems and quality of life (Chen *et al.*, 2007). Controlling asthma symptoms can be assessed by the Asthma Control Test (ACT). The ACT includes five items on a 20-point score from zero to six, referring to the previous four weeks; the lower the total score, the worse (Lindgren *et al.*, 2020).

Well-controlled asthma symptoms were defined as ACT scores≥ 20 and the absence of exacerbations in the previous six months (Lindgren *et al.*, 2020). In general, when asthma is well-controlled, there is no need for patients to modify their lifestyle to avoid unfavourable outdoor conditions (GINA, 2022). If asthmatic patients with well-controlled, a stepping-down treatment is a considerable choice. The step-down treatment for well-controlled mild asthma was relatively efficient in maintaining asthma control, reducing the risk of severe exacerbation, and stabilizing pulmonary function (Lam *et al.*, 2021).

Both children and adults with not well-controlled asthma had significantly lower quality of life. They were more likely to require an office or emergency department visit for asthma than patients with higher ACT scores (Guilbert *et al.*, 2011). Patients with not well-controlled asthma had ACT scores of less than 20 (Pavord *et al.*, 2017). Not well-

controlled asthma increases the risks of severe asthma exacerbations following pneumococcal pulmonary and upper respiratory infections (O'Byrne *et al.*, 2013). Patients with uncontrolled severe persistent asthma have more significant morbidity, greater use of health care resources, and more impairment in health-related quality of life compared to their peers with the well-controlled disease (Krings *et al.*, 2019). The failure in asthma control can be due to the complex interaction among different variables, such as the role of guidelines diffusion and implementation, disease-related factors, or patient-related factors (Braido, 2013).

1.2.7. Risk Factors of Asthma:

Asthma is a condition that is likely caused by complex interactions between multiple genetic and environmental influences (Hernandez-Pacheco *et al.*, 2022). Risk factors are associated with asthma mortality and morbidity, such as exposure to environmental triggers, low-income households, chronic stress, child psychological problems, parental stress, obesity, physical inactivity, and unhealthy diets (Oland *et al.*, 2017). Personal smoking and environmental air pollution have an inconsistent and likely generally small effect in causing asthma (Cockcroft, 2018). There was a significant relationship between cold temperatures and pediatric outpatient visits for asthma. The lower the temperatures, the higher the risk of asthma attacks among children (Xu *et al.*, 2018). Respiratory tract viruses have emerged as the most frequent triggers for exacerbations of asthma in children and adults (Van Meel *et al.*, 2018). Exercise is likely the second most common trigger of asthma (after viral respiratory infections); some of these activities provoke respiratory symptoms in the asthmatic child (Brennan Jr *et al.*, 2018).

Further, atypical bacterial infections play an important role in asthma induction and exacerbation in children and adults (Resiliac and Grayson, 2019). The role of bacterial infection in asthma is varied in that it may contribute to the initial development of the clinical onset of asthma or exacerbate established asthma (Darveaux and Lemanske, 2014). Bacterial organisms can increase inflammation and airway hyperresponsiveness in a patient with known asthma (Bozan *et al.*, 2018). *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* are responsible for chronic inflammation and have been implicated in the pathogenesis of asthma when the host immune system was unable to eradicate the bacteria (Calmes *et al.*, 2021).

Several gene polymorphisms have been associated with susceptibility to asthma and allergy (Shi *et al.*, 2022). Some genes may influence the development of asthma, while others modify asthma severity or the patient's response to therapy (Meyers *et al.*, 2014). In addition, a family history of asthma and allergic diseases are strong determinants of asthma. However, the magnitude of the effect varies according to the hereditary group, so some subtypes have a more robust genetic component (Paaso *et al.*, 2014). In families with one allergic parent, the child's risk of developing asthma was increased by asthma in a parent (Schoos *et al.*, 2020).

1.2.8. Bacterial Infection and Asthma:

Respiratory infections are thought to be a significant contributing factor to poor lung function and the onset of the disease (Mthembu *et al.*, 2021). Instead of protecting from asthma, several respiratory bacteria have been implicated in asthma pathogenesis (Sevin *et al.*, 2010). Epidemiological studies showed strong associations between asthma and

infection with atypical bacteria (Edwards *et al.*, 2012). Bacterial organisms are clinically relevant contributors to asthma exacerbations (McCauley *et al.*, 2019). Bacterial infections and colonization also have been associated with recurrent and exacerbation of wheezing (Darveaux and Lemanske, 2014). Asymptomatic bacterial colonization of the hypopharynx at one month was associated with a higher risk of developing persistent wheezing and asthma (Rahman Fink *et al.*, 2018).

A growing body of basic and clinical science implicates the atypical bacterial pathogens *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* as potentially essential factors in asthma. It appears that acute infections with *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* can cause asthma in some previously asymptomatic individuals; however, the quantitative role of these atypical bacteria as asthma initiators is unknown (Hahn, 2021, Liu *et al.*, 2021). On the other hand, data suggested that the changes induced by allergic disease and chronic airway inflammation impair immunity, predisposing those with asthma to develop a bacterial infection (Sevin *et al.*, 2010). Thus, the exact mechanism of *Chlamydia pneumoniae* in asthma is unclear and requires further study to detect it.

1.2.8.1. *Chlamydia pneumoniae*:

1.2.8.1.1. *Chlamydia pneumoniae* Overview:

Taxonomic analysis of the *Chlamydia pneumoniae* is Kingdom-Bacteria, Subkingdom-Negibacteria, Phylum-Chlamydiae, Class-Chlamydiae, Order-Chlamydiales, Family-Chlamydiaceae, Genus-Chlamydophila, and Species-*Chlamydophila pneumoniae* (System, 2012). Three chlamydial organisms are pathogenic to humans: *Chlamydophila pneumoniae*, *Chlamydia trachomatis*, and *Chlamydophila psittaci* (Oba *et*

al., 2020). *Chlamydia pneumoniae* is distinct from the other two chlamydial species that infect humans in the morphology of the elementary body and shares less than 10% of the DNA homology with those species (Kuo *et al.*, 1995).

Chlamydia pneumoniae is called Taiwan acute respiratory agent (TWAR) because two isolates were first isolated from respiratory infections a decade ago, TW-183 and AR-39 (Grayston *et al.*, 1986). The history of the TWAR organism began in Taiwan in 1965, when a field trial was underway testing an inactivated yolk sac-grow trachoma vaccine (Woolridge *et al.*, 1967). *Chlamydia pneumoniae* is an enigmatic animal and human pathogen. Molecular typing studies suggested that animal strains are ancestral to human strains and that *Chlamydia pneumoniae* crossed from animals to humans due to at least one relatively recent zoonotic event (Roulis *et al.*, 2013).

Chlamydia pneumoniae is an obligate intracellular bacterial pathogen and a respiratory pathogen in humans that occurs worldwide. It causes community-acquired pneumonia in children and adults and may be responsible for epidemics in enclosed populations. *Chlamydia pneumoniae* is challenging to diagnose due to its nonspecific clinical presentation and can only be isolated in tissue culture (Goldstein *et al.*, 2007, Gautam and Krawiec, 2022).

About 70% of the respiratory tract infections caused by *Chlamydia pneumoniae* are with mild symptoms or asymptomatic. Still, 30% of *Chlamydia pneumoniae* infections are responsible for more severe respiratory illnesses, such as community-acquired pneumonia with atypical symptoms. Further, *Chlamydia pneumoniae* infection accounts for 5% of pharyngitis, sinusitis, and bronchitis cases in both

immunocompetent and immunocompromised hosts (Gautam and Krawiec, 2020).

In children, *Chlamydia pneumoniae* infections seem to have a more critical role in causing respiratory tract disease; they have been frequented in children under five and are associated with wheezing. Further, *Chlamydia pneumoniae* plays a considerable role in community-acquired infection in children of all ages. Such conditions are more challenging when untreated with adequate antimicrobial agents (Principi *et al.*, 2001, Alves *et al.*, 2020).

1.2.8.1.2. Epidemiology of *Chlamydia pneumoniae*:

According to seroepidemiologic surveys, *Chlamydia pneumoniae* infection seems to be both endemic and epidemic. Such studies indicate that *Chlamydia pneumoniae* infection is widespread, with frequent reinfection during a lifetime. *Chlamydia pneumoniae* is recognized worldwide as a common cause of respiratory infections in children and adults. Over 60% of subjects with chronic bronchitis have specific *Chlamydia pneumoniae*. The incidence of *Chlamydia pneumoniae* pneumonia may be as high as 50% in children with community-acquired pneumonia. Serologic surveys detected rising *Chlamydia pneumoniae* antibody prevalence rates beginning in school-age children that reached 30–45% in adolescents (Blasi *et al.*, 1998, Hammerschlag, 2003, Choroszy-Krol *et al.*, 2013).

1.2.8.1.3. Clinical Manifestation of *Chlamydia pneumoniae*:

Most patients infected with *Chlamydia pneumoniae* remain asymptomatic, and the clinical presentations of it can vary widely. People with *Chlamydia pneumoniae* infection commonly present with cough,

malaise, headache, pharyngitis, and laryngitis. Furthermore, 60% of *Chlamydia pneumoniae* infection people have wheezing. *Chlamydia pneumoniae* can also cause lower respiratory tract infections like pneumonia and bronchitis (Sharma *et al.*, 2017, National Center for Immunization and Respiratory Diseases, 2021).

The incubation period of *Chlamydia pneumoniae* is approximately three to four weeks which is more than most respiratory bacterial infections (Oba *et al.*, 2020). Although most *Chlamydia pneumoniae* infections are asymptomatic or mild, severe complications can occur. These severe complications include asthma exacerbation, myocarditis, and encephalitis (National Center for Immunization and Respiratory Diseases, 2021).

Chlamydia pneumoniae is called an atypical organism because the clinical presentation is often less dramatic than illness caused by *Haemophilus influenzae* or *Streptococcus pneumoniae* (Jeffrey *et al.*, 2000).

1.2.8.1.4. Life Cycle of *Chlamydia pneumoniae*:

Chlamydia pneumoniae is a small gram-negative bacterium with a size range from 0.2 to 1 micrometer that undergoes several transformations during its growth. The life cycle of *Chlamydia pneumoniae* consists of two alternating forms: elementary bodies (EB) and reticulate bodies (RB). EBs are metabolically inactive but infectious, while the RBs are metabolically active but non-infectious (National Center for Immunization and Respiratory Diseases, 2021).

Respiratory secretions transmit *Chlamydia pneumoniae* from human to human (Oba *et al.*, 2020). After infection, the infectious EBs attach to the host cell by electrostatic binding and are taken into the cell by receptor-

mediated endocytosis (Hammerschlag *et al.*, 2015). The bacterial nucleoid is highly compacted in EBs due to the condensation of nuclear material by HctA and HctB, which is bacterial histone-like proteins (Brickman *et al.*, 1993). Then, EBs inject pre-packed T3SS effectors into the host cytoplasm as contact with the host is established, leading to the reorganization of actin and uptake of the EBs. Once inside the cell, the EBs remain within a membrane-lined phagosome, inhibiting phagosomal–lysosomal fusion. Thus, EBs inhabit a nonacidic vacuole dissociated from late endosomes and lysosomes, which help the bacteria avoid host immune pathways. Next, the EB (residing inside the inclusion) differentiates into RBs that undergo binary fission (Gitsels *et al.*, 2019).

The RBs rely on the host cell to synthesize adenosine triphosphate (ATP) (National Center for Immunization and Respiratory Diseases, 2021). Immediately produce early effectors by RBs, which prevent lysosomal degradation by modifying the inclusion membrane. Then, the inclusion starts traveling across microtubules towards the microtubule organizing center and away from the periphery. RBs hijack host cell metabolites when the inclusion reaches the nutrient-rich peri-Golgi region to support the growth of both its and the inclusion membrane, which is necessary to allow room for the expanding RBs. After 36 hours, the RBs reorganize themselves and condense to differentiate back into EBs (Gitsels *et al.*, 2019).

Host cell function is minimally disrupted despite the accumulation of 500 to 1000 infectious EBs in the inclusion. After 48 hours, the release may occur via exocytosis, cytolysis, or extrusion of the full inclusion, leaving the host cell intact. The EBs then exit the host cell and start a new infectious cycle. This strategy enables the organism to cause silent chronic infection (Figure 1-1) (Gitsels *et al.*, 2019).

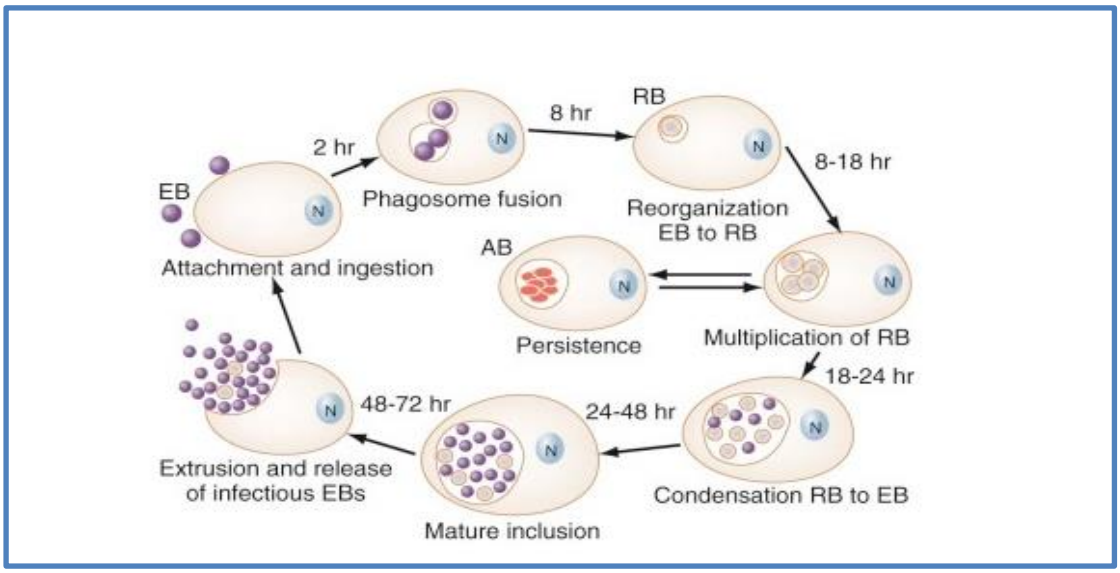

Figure (1-1): Life cycle of *Chlamydia pneumoniae* (Hammerschlag *et al.*, 2015)

1.2.8.1.5. Pathogenesis of *Chlamydia pneumoniae:*

Chlamydia pneumoniae can spread systemically in at least two ways: First, carried within recirculating lymphocytes, macrophages, and monocytes from the respiratory tract. Second, direct access to the bloodstream following a severe pulmonary infection and causing chlamydial bacteriaemia for a short interval. *Chlamydia pneumoniae* may infect bronchi, lungs, endothelial cells, macrophages, and monocytes (Gieffers *et al.*, 2004, Porritt and Crother, 2016).

When *Chlamydia pneumoniae* resides in vascular endothelial cells or alveolar macrophages in chronic infections, the bacteria and their structural components have easy access to circulation. *Chlamydia pneumoniae* infection induces the secretion of cytokines, including interleukin 8 (IL-8) and tumor necrosis factor α (TNF-α), and reactive oxygen species from alveolar macrophages and peripheral blood mononuclear cells. The continuous induction of cytokines by *Chlamydia pneumoniae* may lead to chronic inflammation of the vascular endothelium. The inflammation is caused because cytokines, such as

interferon-gamma (IFN-γ), have been demonstrated to restrict the growth of intracellular pathogens (Quinn and Gaydos, 1999, Gieffers *et al.*, 2004, Richard, 2018).

Furthermore, *Chlamydia pneumoniae* that induced epithelial cytokine liberation may contribute significantly to inflammatory airway diseases like bronchial asthma or chronic obstructive pulmonary disease. *Chlamydia pneumonia* causes lung parenchyma patchy consolidation as the exudate of neutrophils, fibrin, and RBCs gathers in infected areas (Krüll *et al.*, 2006, Jain *et al.*, 2022).

Because of the unique life cycle of *Chlamydia pneumoniae*, it occupies an intracellular niche that enables the bacterium to multiply and survive. Within the inclusion body, Chlamydia can genetically switch between a replicative and a persisting non-replicative state, linking the pathogen to acute and chronic diseases (Kern *et al.*, 2009). In addition, *Chlamydia pneumoniae* expresses a variety of potent virulence factors, including outer membrane protein–A (OmpA/B, Omp3, OmcB, POMP), chlamydial lipopolysaccharide (cLPS), heat-shock protein 60 (cHsp60/ GroEL-1), the chlamydial protease- or proteasome-like activity factor (CPAF), a type III secretion apparatus (T3S), peptidoglycans, and peptidoglycan-like structures (Krüll and Suttorp, 2007).

OmpA of the chlamydial EBs is the main component to protect chlamydia against the environment outside the host, attachment to host cells, and defense against the host's immune response. At the same time, OmcB might mediate chlamydial attachment and the initial invasion steps into target cells (Hackstadt, 1999, Krüll and Suttorp, 2007).

Chlamydia pneumoniae induces mononuclear phagocyte foam cell formation by cLPS. cLPS can be released from intracellular into inclusion

chlamydia to the inclusion membrane, host cell cytoplasm, surface, and surrounding infected cells. This release might impact the chlamydial infection pathogenesis and the host's immune disposition of infected cells. (Kalayoglu *et al.*, 2000, Netea *et al.*, 2002).

Chronic, persistent *Chlamydia Pneumoniae* infection can trigger the secretion of cHSP60 in the vessel wall resulting in the augmentation of inflammation (Richard, 2018). cHsp60/GroEL-1 can act as an extracellular agonist. It might be a key player in activating different intracellular signal transduction pathways with a prolonged and profound proinflammatory phenotype expression in treated cells (Bulut *et al.*, 2002). Moreover, the GroEL protein is an effective inducer to proliferate human vascular smooth muscle cells and hyperinflammatory response stimulation in animals (Sasu *et al.*, 2001).

Bacterial T3SS engaged with a eukaryotic host and caused conformational changes in pump action, which underpin effector injection. T3SS mediates the delivery of bacterial effector proteins into eukaryotic cytosol, membranes, and nuclei. Chlamydia uses a T3S system throughout the developmental cycle to transport several effector proteins across the inclusion and host cell plasma membrane (da Cunha *et al.*, 2014, Diepold and Wagner, 2014, Mueller *et al.*, 2014, Nans *et al.*, 2015).

1.2.8.1.6. Role of *Chlamydia pneumoniae* in Asthma*:*

Chlamydia pneumoniae has been implicated in asthma pathogenesis and chronic inflammation responsibility when the host immune system fails to bacteria eradication. Failure of *Chlamydia pneumoniae* eradication can lead to chronic infection, where *Chlamydia pneumoniae* enters a latency state in which it does not multiply and is dormant but is viable. *Chlamydia pneumoniae* also seems to be able to impair the

infected cells' apoptosis leading to inducing ciliostasis in the bronchi (Calmes *et al.*, 2021).

During the latency state, *Chlamydia pneumoniae* continues to synthesize stress protein, a cHsp60/GroEL-1. This protein can elicit an intense host inflammatory response at its production sites and appears involved in scarring processes and tissue injury (von, 2002).

Chlamydia pneumoniae contributes to airway remodeling by inducing the production of Interferon beta (IFN-β), IL-6, and Matrix metalloproteinases (MMPs) that can promote smooth muscle cell proliferation. Infection with *Chlamydia pneumoniae* also led to an increase of immunoglobulin E (IgE), IL-4, and IFN-γ and induced the secretion of TNFα and IL-8 (Johnston and Martin, 2005, Smith-Norowitz *et al.*, 2020).

Early-life chlamydial lung infection results in long-lasting alterations in hematopoietic cells, modulates immune responses, alters lung structure and function, and increases the severity of allergic airway disease in later life (Horvat *et al.*, 2010, Starkey *et al.*, 2012). Further, chlamydia-infected non-immune mammalian cells produce proinflammatory chemokines, growth factors, and other cellular modulators (Stephens, 2003).

Moreover, repeated infections with *Chlamydia pneumoniae* may induce Th1-type and Th2-dominant cytokine responses in the airways of neonatal mice. More significantly, this infection elicits pathogen-specific IgE production, which differentially affects the development of key features of allergic airway disease and asthma phenotype in the adult. These are asthma hallmarks and further confirm the role of chlamydial

infection in the initiation and pathology of asthma, at least in mice (Horvat *et al.*, 2007, Kaiko *et al.*, 2008, Patel and Webley, 2013).

Notably, previous data suggest a link between *Chlamydia pneumoniae* and asthma exacerbation. In mice with chronic and recurrent *Chlamydia pneumoniae* infection, an increase in the thickness of the subepithelial basement membrane suggestive of airway remodeling was observed (Chen *et al.*, 2009). The airway remodeling contributes to the thickening of airway walls, airway narrowing, sub-phenotypes of irreversible airflow obstruction, airway edema, bronchial hyperresponsiveness, and mucus hypersecretion. Airway remodeling is associated with poor clinical outcomes and increased disease severity among asthmatic patients(Bergeron *et al.*, 2010, Shifren *et al.*, 2012).

Chlamydia pneumoniae has also been found to cause ciliostasis in bronchial epithelial cells *in vitro* and completely abort ciliary motion within 48 hours. A decrease in the ciliary activity of ciliated bronchial cells produced by *Chlamydia pneumoniae* can contribute to both initiation and pathogenesis of respiratory infections induced by this pathogen. This effect of *Chlamydia pneumoniae* on the ciliary activity of ciliated bronchial epithelial cells is a specific property of *Chlamydia pneumoniae*. Ciliary dysfunction is closely related to asthma severity since ciliary dysfunction is associated with a progressive decline in lung function over time (Blasi *et al.*, 2002, Thomas *et al.*, 2010, Joskova *et al.*, 2020).

Chlamydia pneumoniae infection of human smooth muscle cells *in vitro* increased the production of IL-6, basic fibroblast growth factor (bFGF), and induced IFN-β production. These data provide a mechanism by which chlamydial infection of smooth muscle cells elicits a cytokine

response that may modulate inflammation and tissue remodeling during chronic asthma (Clements *et al.*, 2000, Rödel *et al.*, 2001).

Several studies indicate that bacteria can induce the differentiation of naïve T cells into Th2 or Th17 cells and elicit Th2 cytokine release. *Chlamydia pneumoniae* stimulates IL-4 production in peripheral blood mononuclear cells (Smith-Norowitz *et al.*, 2016).

Despite, *Chlamydia pneumoniae* entering into a state of quiescence with intermittent periods of replication, but is still able to produce HSPs and antigenic variation. Interestingly, the release of these effector proteins into the host cell cytoplasm is caused by the chlamydial T3S apparatus. This secretion system appears to remain fully functional during chronic infection (Kern *et al.*, 2009).

The previous meta-analysis detected the possibility of some undetected antigens responsible for generating a cryptic IgE response in some asthma patients with *Chlamydia pneumoniae* (Hahn, 2021). Four chlamydial antigens induced *Chlamydia pneumoniae*-specific IgE responses in serum (LPS, Crp A, HSP 60, and putative outer membrane protein (POMP)). The presence of *Chlamydia pneumoniae*-specific IgE antibodies in stable asthmatics (without acute airway infection) with *Chlamydia pneumoniae* provides further evidence for ongoing stimulation of allergic responses by *Chlamydia pneumoniae* (Smith-Norowitz *et al.*, 2020, Calmes *et al.*, 2021).

In some patients with *Chlamydia pneumoniae* infection, the production of specific *Chlamydia pneumoniae* IgE antibody may be an underlying mechanism leading to ongoing or worsening asthma symptoms (Smith-Norowitz *et al.*, 2020). The specific IgE generated and sustained by *Chlamydia pneumoniae* chronic infection may be one of

several mechanisms that contribute to asthma pathogenesis (Hahn *et al.*, 2012).

Since IgE antibodies play a central role in allergic inflammation, producing chlamydia-specific IgE may significantly exacerbate chronic, allergic airway diseases. Thus, chlamydia has a direct effect on asthma pathogenesis which, unlike most aeroallergens that a patient can avoid, the chlamydial organisms reside in the lower airways. They are continuously secreting bacterial antigens (Patel *et al.*, 2012).

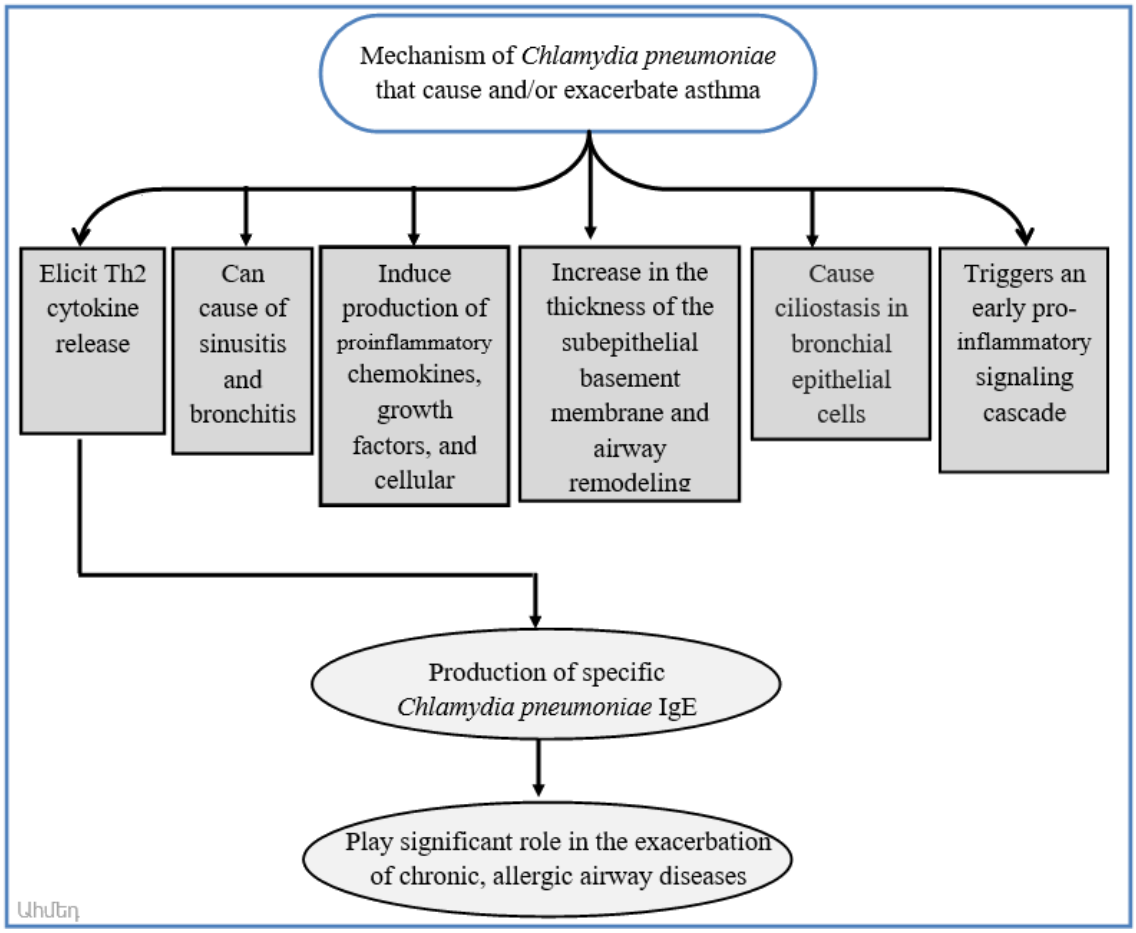

Figure (1-2): Role of *Chlamydia pneumoniae* in asthma (original figure)

1.2.8.1.7. Diagnosis of *Chlamydia pneumoniae:*

Diagnostic assays for *Chlamydia pneumoniae* are much needed for improved patient care, a better understanding of the pathogen

epidemiology, and appropriate use of antimicrobial therapy (Puolakkainen, 2013).

The specific laboratory diagnosis of *Chlamydia pneumoniae* infection can be made by isolating the organism from nasopharyngeal or throat swabs, pleural fluid, or sputa (phlegm), if present. The nasopharynx is the optimal site for the isolation of the organism (Hammerschlag *et al.*, 2015).

Culture is highly specific but has a long turnaround time, is technically expensive and demanding, and its sensitivity is highly dependent on transport conditions (Laupland and Valiquette, 2013). *Chlamydia pneumoniae* cannot be propagated in cell-free media; thus, this pathogen requires in a tissue culture. *Chlamydia pneumoniae* grows readily in cell lines derived from respiratory tract tissue, specifically HL cells and epithelial cell lines (HEp-2) (Campbell and Kuo, 2009).

Serological methods for diagnosing of *Chlamydia pneumoniae* infection vary widely (Villegas *et al.*, 2010). Chlamydia-specific IgM, IgG, and IgA antibodies were detected with an enzyme-linked immune assay (ELISA) and a micro-immunofluorescence (MIF) assay with *Chlamydia pneumoniae* elementary bodies. The MIF is specific for *Chlamydia pneumoniae* and can distinguish between recent and past infections (Miyashita *et al.*, 2008, Rahman and Kaltenboeck, 2019).

Antigen detection tests, such as ELISA and direct fluorescent antibody assay, and molecular detection methods, such as PCR, may provide a rapid diagnosis without requiring stringent transport conditions (Smolejová *et al.*, 2023). Still, diagnosis by the nasopharyngeal specimen, culture, serum antibody titers, or PCR is usually delayed concerning the

onset of symptoms, antibiotic treatment, or disease resolution (Burillo and Bouza, 2010).

1.2.8.1.8. Treatment of *Chlamydia pneumoniae:*

Newer macrolides, including clarithromycin and azithromycin, have *in vitro* activity against *Chlamydia pneumoniae* (Karnak and Beder, 2002). Results of two pediatric multicenter pneumonia treatment studies found that 10-day courses of clarithromycin and erythromycin and five days of azithromycin suspension were equally productive, eradicating the bacterium in 79–86% of the children (Gautam and Krawiec, 2022).

Quinolones (levofloxacin and moxifloxacin) also have some activity but are less effective than macrolides or tetracyclines (Karnak and Beder, 2002). Clarithromycin therapy improves lung function in patients infected with *Chlamydia pneumoniae* (Kraft *et al.*, 2002).

Ambroxol may be a supporting drug in respiratory infections caused by *Chlamydia pneumoniae* (Kókai *et al.*, 2021). Ambroxol works to thin down and break up phlegm and treats respiratory diseases by clearing congestion (Melisa Puckey, 2022).

1.2.9. Pathogenesis of Asthma:

1.2.9.1. Immune Response:

Asthma is primarily an inflammatory disorder of the airways associated with Th2 cell-dependent promotion of IgE production and recruitment of eosinophils and mast cells (Shrestha Palikhe *et al.*, 2021). Asthma involves innate and adaptive immunity mediated by immune cells (Zhu *et al.*, 2020). Th2-polarized immune responses to harmless airborne allergens drive allergic asthma. However, some respiratory infections cause bronchiolitis in infancy and childhood wheezing and

established asthma exacerbation (Wills-Karp, 2007, Hansel *et al.*, 2013). Fundamental to innate immune responses to microbes are the interactions between pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs), which are associated with the type I interferon production, pro-inflammatory cytokines release, and the Th2 cell pathway in predisposed people (Hansel *et al.*, 2013).

Allergen exposure activates numerous immune system cells, including dendritic cells (DCs) and Th2 lymphocytes. DCs represent the most potent antigen-presenting cells (APC) of the immune system that bridge innate and adaptive immunity. DCs play a central role in initiating and maintaining allergen-driven Th2 immune responses in the airways. DCs in the airway epithelium and submucosa detect inhaled allergens (Kumar *et al.*, 2019, Morianos and Semitekolou, 2020).

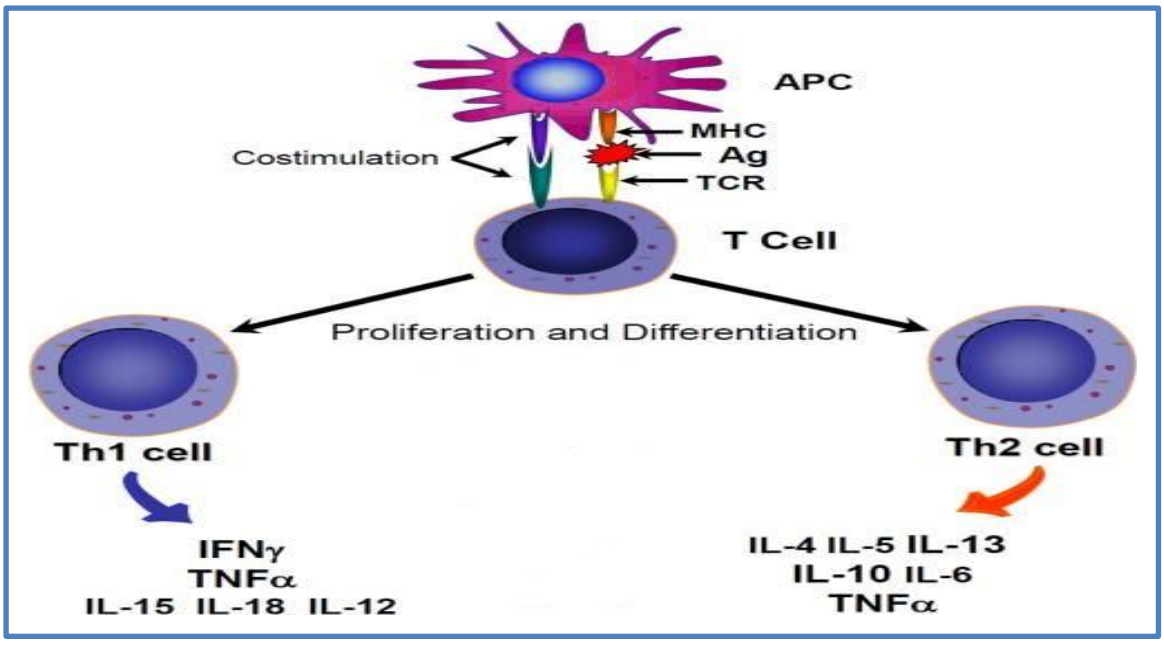

Figure (1-3): Dendritic cells as antigen-presenting cells (Finn and Bigby, 2009)

DCs then migrate to the secondary lymphatic systems where they process and present antigens via major histocompatibility complex class II (MHC II) to Th-cell, leading to the differentiation of Th0 to Th1 or Th2

cell types. In response to allergen presentation by DCs, Th cell control many aspects of the disease through the secretion of different cytokines such as IL-4, IL-5, IL-6, IL-10, IL-13, IL-15, IL-18, and IL-12 (Lambrecht and Hammad, 2013, Tai *et al.*, 2018).

1.2.9.2. Role of Th2 Cytokines in Asthma:

Cytokines of Th2 (IL-4, IL-5, and IL-13) are thought to drive the disease pathology of asthmatic patients and play a vital role in driving many of the hallmarks of allergic inflammation (Georas *et al.*, 2005, Lloyd and Hessel, 2010). Whereas IL-4 is essential for IgE production and allergic sensitization, and IL-5 is crucial for the survival of eosinophils, IL-13 has pleiotropic effects in the lungs, including a major role in the development of tissue remodeling and airway hyperresponsiveness (Finkelman *et al.*, 2010).

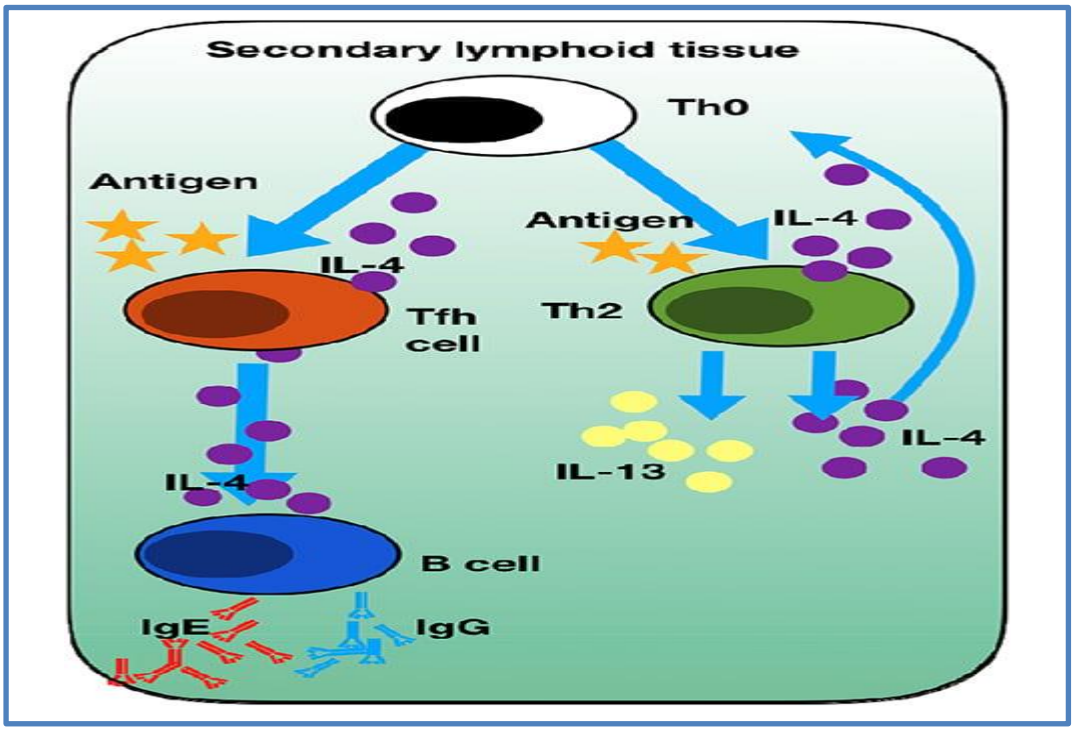

Figure (1-4): Role of IL-4 in asthma pathogenesis (Kariyawasam *et al.*, 2020)

IL-4 plays relevant roles in Th2 cell differentiation, IgE class switching, airway inflammation, and remodeling. The differentiation of Th2 cells derived from naïve CD4+ T cell. IL-4 suppresses regulatory T cells' immunomodulatory and tolerogenic functions, which do not allow Th2 cell maturation and clonal expansion in non-allergic subjects (Gandhi *et al.*, 2016, Palomares *et al.*, 2017, Tu *et al.*, 2017, Komlósi *et al.*, 2022).

IgE production by B cells requires the presence of IL-4 and a physical interaction between T and B cells, involving several surfaces and adhesion molecules such as CD40-CD40L and CD28/CD80. IL-4 plays an essential role in activating mature B cells as a cofactor for LPS, CD40L, and Ag stimulation to induce B cell differentiation, proliferation, and Ab secretion, mainly of IgG1 and IgE isotypes (Froidure *et al.*, 2016, Novosad and Krčmová, 2020).

1.2.9.3. Role of IgE in Asthma:

IgE is an antibody produced by B-cell after differentiation into plasma cells in lymph nodes. IgE is the most potent antibody classes and can trigger dramatic inflammatory reactions even when present in minute amounts. Despite a half-life of only a few days, there is evidence that the IgE response may last for years without allergen stimulation. It is likely caused by long-lived plasma cells which produce IgE (Poulsen and Hummelshoj, 2007, Laffleur *et al.*, 2017).

IgE class switching in B cells is regulated by stimuli transduced by cytokines and cell-cell contact. In addition to IL-4, IL-13 can induce isotype switching to IgE and IgG4 synthesis in immature human B cells. In addition, IL-5 has synergistic effects with IL-4 in the production of IgE. The class switch requires recombination in the Ig heavy-chain gene

locus. Class switch to IgE occurs in the nasal mucosa in allergic rhinitis (Takhar *et al.*, 2007, Zhang *et al.*, 2016, Lambrecht *et al.*, 2019).

AT first exposure, allergens are presented to T-cells by APCs. T-cells then signal for B-cell stimulation to produce IgE antibodies. Once IgE is released into the circulation, it binds to a high-affinity IgE receptor (FcεRI) located on the surface of effector cells (basophils, mast cells) (Rosado Ingelmo *et al.*, 2016, Palomares *et al.*, 2017, Eckl-Dorna *et al.*, 2019).

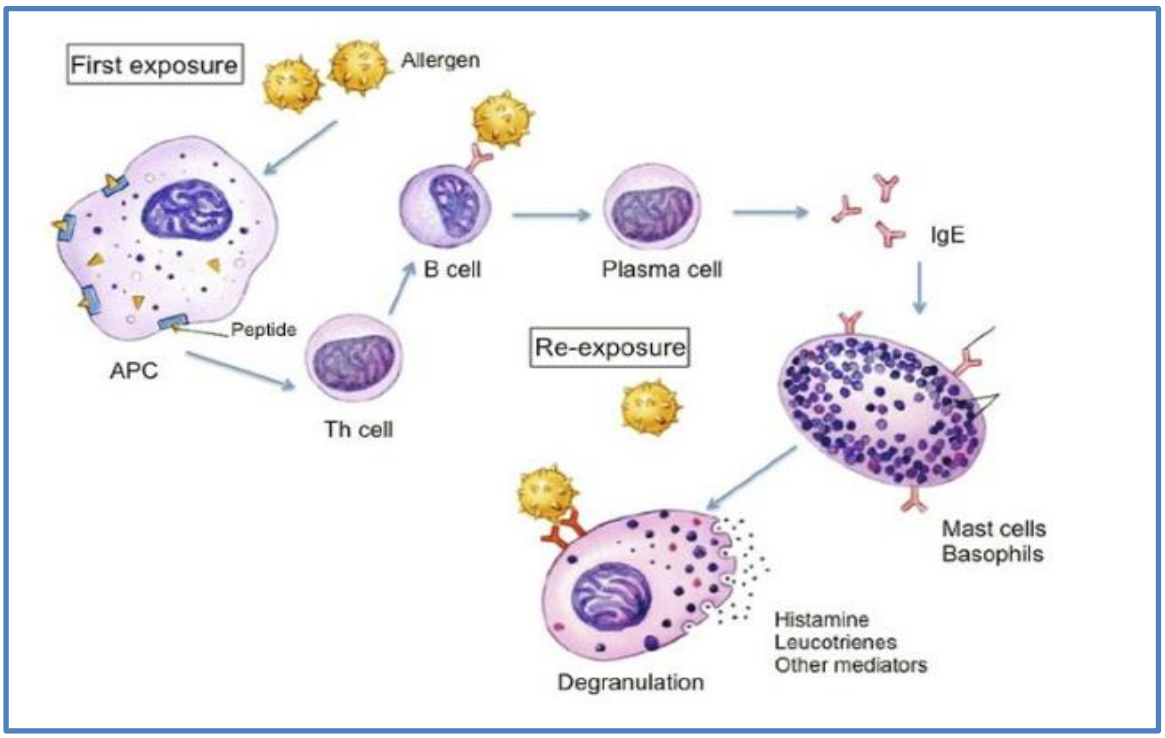

Figure (1-5): Role of IgE in asthma pathogenesis (Eckl-Dorna *et al.*, 2019)

At second exposure to the same allergen, the free antigen induces the crosslinking of these mast cells and basophil-attached IgE antibodies. These interactions trigger effector cell activation, releasing potent inflammatory mediators (histamine, prostaglandin, cytokines, leukotrienes, platelet-activating factors, tryptase, macrophage inflammatory proteins, etc.), recruitment of inflammatory cells, antigen

presentation, and production of allergen-specific antibody responses (Karagiannis *et al.*, 2015, Rosado Ingelmo *et al.*, 2016).

As a result, there is increased vascular permeability, peripheral vasodilation, and smooth muscle contraction, which can manifest in increased bronchospasm, mucous secretions, congestion, sneezing, wheezing, cough, runny nose, conjunctivitis, dyspnoea, and chest tightness (Palomares *et al.*, 2017).

1.2.10. Interleukin-4:

IL-4 is a cytokine produced by Th2 cells, basophils, mast cells, and eosinophils. The first report on IL-4 was in 1982. This cytokine is a multifunctional cytokine and has an essential regulator of inflammation. IL-4 mediates essential pro-inflammatory functions in asthma, including differentiation of Th2 cells leading to cytokine release, induction of the IgE isotype switch, promotion of eosinophil transmigration across the endothelium, expression of vascular cell adhesion molecule-1, and mucus secretion. IL-4 regulates T cell activation, proliferation, differentiation, and survival of different T cell types. IL-4 also immunomodulates B cells, macrophages, mast cells, and many cell types (Paul, 2015, Dong *et al.*, 2018, Quinnell *et al.*, 2020, Pelaia *et al.*, 2022)

IL-4 is considered an essential cytokine for tissue repair, anti-parasitic, wound healing, and counterbalancing the effects of proinflammatory type 1 cytokine. IL-4 cytokine also has been reported to promote the resolution of neutrophil-mediated acute lung injury (Information, 2022). Further, IL-4 activates the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling cascades, which may contribute to allergic responses (Seif *et al.*, 2017).

Human IL-4 has a molecular weight that forms between 15,000 and 19,000 daltons (Le *et al.*, 1988). IL-4 mediates its functions by binding to its receptor expressed on target cells. IL-4 receptor exists on freshly prepared B and T cells, macrophages, and other cell lines, including lymphoid cells, mast cells, various hematopoietic cell lines, stromal cell lines, and fibroblast (Gadani *et al.*, 2012, Junttila, 2018).

The human IL-4 gene is found on the long arm of chromosome 5 at 5q23.3-31.2. This gene consists of 4 exons spanning 10 kb. The IL-4 gene displays several cell-specific regulatory sequences in its promoter, which explain its restricted secretion pattern to activated T cells and mast cells. Genetic variation in IL-4 may be associated with the development of asthma. Polymorphisms in the IL-4 gene might confer susceptibilities to and modulate the severity of atopy and asthma (Beghé *et al.*, 2003, Tindall *et al.*, 2010, Sun *et al.*, 2017).

1.2.11. Single Nucleotide Polymorphisms (SNPs) in Asthma:

Polymorphism is a variation in the DNA sequence, which includes differences in genotypes ranging in size from a single nucleotide site to large nucleotide sequences visible at a chromosomal level (Information, 2005).

Single nucleotide polymorphisms (SNPs) are DNA sequence variations that happen when a single nucleotide in the genome sequence is altered (Morgil *et al.*, 2020).

SNPs can be essential in establishing ancestries and identifying genes involved in complex diseases. They are responsible for genetic effects that produce susceptibility to most autoimmune diseases. There are at least 3.1 million SNPs in the human genome, or about 1 SNP per kilobase

of sequence. Population frequencies of many polymorphic genes depend on race or ethnic specificity (Bell, 2002, Consortium, 2007, Ferguson *et al.*, 2007).

SNPs might be found in both coding and non-coding regions of DNA sequences. Gene-coding regions can lead to changes in the biological properties of the encoded protein. In contrast, SNPs in non-coding gene regulatory regions may affect gene expression levels in an allele-specific manner (Ramírez-Bello and Jiménez-Morales, 2017).

1.2.11.1. Detection of SNPs:

Methods to distinguish and detect SNPs should be highly specific and sensitive PCR-based methods. SNP detection is broadly categorized into two types- one type is a polymorphic allele-directed specific analysis using primers matched with substituted nucleotide or oligonucleotides to block or clamp the non-targeted template such as allele-specific PCR, RFLP, and AFLP. The second type is melting curve analysis, combined with real-time PCR techniques using hydrolysis probes, hybridization probes, or double-stranded DNA-binding fluorescent dyes (Matsuda, 2017). Furthermore, probe-based PCR is widely used for SNP genotyping and pathogen nucleic acid detection due to its simplicity, sensitivity, and cost-effectiveness; this technique is named Multiplex Probe Amplification (Fu *et al.*, 2012).

PCR-RFLP is a relatively inexpensive method for genotyping SNP, allows rapid detection of point mutations after PCR amplifies the genomic sequences, and it is a simple, economical method without the need for special equipment (Zhang *et al.*, 2005, Xiao *et al.*, 2006, Ota *et al.*, 2007).

1.2.11.2. Application of SNPs:

SNPs are essential for studying the human genome and can affect biological and therapeutic phenotypes (Drysdale *et al.*, 2000, Fucharoen, 2007). The association of these genes with person-to-person differences in drug effectiveness (resistance or hypersensitivity) and drug toxicity may also depend on the ethnic or racial characteristics of a population. In addition, SNPs mapping is the simplest and most reliable way to map genes because SNPs are highly dense and usually have no phenotype association, making them ideal markers for mapping (Davis and Hammarlund, 2006).

Further, SNPs were used for genetic epidemiology studies to scan for new polymorphisms and to determine the allele(s) of a known polymorphism in target sequences (Schork *et al.*, 2000, Kwok and Chen, 2003). Moreover, SNPs have shown massive potential in gene cloning and functional genomics (Liu and Zhang, 2006). Additionally, SNPs are a considerable source of mutation data for the development of molecular genetic markers in non-model species, with shared ancestral SNPs showing application within closely related species (Coates *et al.*, 2011). SNPs can be applied to identifying oncogenes, cancer predisposition genes, and tumor suppressor genes in specific types of tumors (Mao *et al.*, 2007).

1.2.11.3. Interleukin-4 C-589T Polymorphism:

The IL-4 gene on chromosome 5q31.1 encodes IL-4, a polyfunctional cytokine produced by activated T cells, type 2 innate lymphoid cells, and mast cells, which are involved in adaptive immunity. Several studies suggested that the -589T allele in rs2243250 is associated with increased serum or plasma IL-4 levels and is linked to total serum IgE levels.

Recently, the IL-4 -589T allele was associated with probable asthma, rhinitis, and atopy in a cohort of infants at risk for allergic disease. In addition, the IL-4 -589T allele was associated with lower FEV1 values in a population of white subjects with asthma. These data suggest that the -589T polymorphism may influence asthma severity (Zhu *et al.*, 2021).

Genetic variants in the IL-4 promoter can modify IL-4 gene transcription; these sequence variants can modify asthma severity. The influence and importance of IL-4 C-589T polymorphism is a single nucleotide polymorphism located within cytokine gene promoter regulatory sequences (Chiang *et al.*, 2007); the promoter mutation is known to cause functionally essential consequences for gene expression. Although promoter mutation analysis is complex, challenging to perform, and often laborious, it is an essential part of diagnosing disease caused by promoter mutations and improves understanding of the role of transcriptional regulation in human disease (De Vooght *et al.*, 2009).

CHAPTER TWO

Materials and Methods

2. Material and Methods:

2.1. Subjects and Study Design:

2.1.1. Subjects:

This study included consecutive asthmatic children attending the asthma clinic at Karbala Teaching Hospital for Children in the period extending from January 2022 to May 2022. All children had the European Respiratory Society/American Thoracic Society criteria for asthma (Chaplin, 2020). In this study, 79 asthmatic children had visited the clinic regularly and received regular treatment with either inhaled corticosteroids or montelukast, while eight patients were newly diagnosed. Their ages ranged from (1-16) years old. The control groups (87 person) included children of the same ages and sex of the patients randomly selected from the local community.

2.1.2. Inclusion and Exclusion Criteria:

2.1.2.1. Inclusion Criteria: Children were diagnosed with asthma at the asthma clinic of Karbala Teaching Hospital for Children, either newly diagnosed or on controller therapy.

2.1.2.2. Exclusion Criteria: Patients with liver disease, HBV and HCV infection, current acute infection (tuberculosis and other lung diseases), cardiopulmonary failure, and primary immune dysfunction.

2.1.3. Study Design: This case-control study was performed on 174 subject enrolled. However, after performing IgE tests, persons with IgE < 100 IU/ml were excluded (41 persons) because this study was designed for IgE-mediated asthma, and 100 IU/ml is the cut-off value of a positive IgE level. This study involved 87 asthmatic children with high total IgE (57 male and 30 female) and 87 non-asthmatic children (48 male and 39 female). The non-asthmatic children (control) had the age and sex of asthmatic children. The study design enrolled in this study is shown in Figure (2.1).

2.2. Ethical and Scientific Approval:

1. Ethical approval was obtained from the Ethical committee in Babylon medical college and the relevant ethical committee in the health directorate.

2. Verbal concent was obtained from patients and/or their parents prior to sampling.

3. Health measures and safety have been taken when sampling.

2.3. Data Collection:

Demographic and clinical data were collected through an interview conducted with patients and/or their parents through a questionnaire.

Socio-demographic and observed data: sex, age, weight, height, address, family history of asthma or another allergic disease, exposure to smoking,

presence of domestic and/or pet animal in the house, personal history of atopy, and triggers of asthma.

2.3.1. Questionnaire:

Questionnaires were designed to search from the children at Kerbala Teaching Hospital for Children and the Research Committee, taking the international and local standards into account, for collecting data from children with asthma and non-asthmatic children and /or their parent. (Appendix I and Appendix II).

Types of Treatment: Doctors prescribed two main types of treatment: montelukast and inhaled corticosteroids.

Asthma Severity and Control: The degree of asthma severity and control were identified based on the international standards diagnosed in the NAEPP/EPR 3 Guidelines (Program, 2007) by the specialist paediatrician.

2.4. Collection of Specimens:

Approximately three ml of venous blood were drawn from each participant. One ml of blood was dispensed into an EDTA tube for the molecular tests. Two ml of blood was dispensed into a gel tube and allowed to clot. Then serum was separated by centrifugation at 3000 rounds per minute (rpm) for 15 minutes. Then the serum was separated and transferred to four Eppendorf tubes and stored in deep freeze (-20°c) to be used for immunological assays.

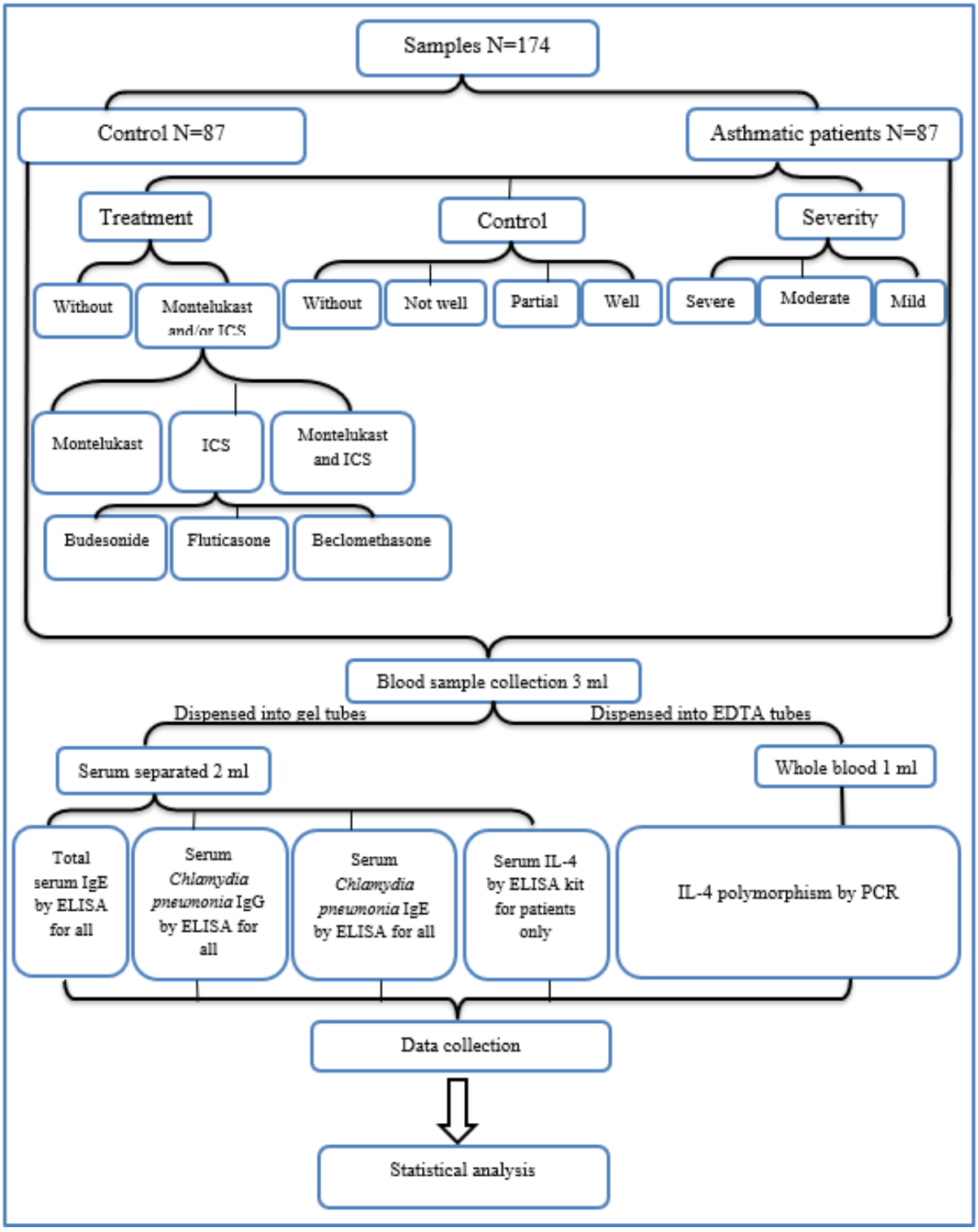

Figure (2-1): A flow chart illustrating the study design and methods

2.5. Materials:

2.5.1. Equipment and Instruments: The present study used the following Equipment and instruments (tables 2-1)

Table (2-1): Equipment and Instruments used in the Presented Study

Equipment and Instruments	Manufacturing Company	Country
Autoclave	Hirayama HVE-50	Japan
Biophotometer plus	Eppendorf	Germany
Centrifuge	Hettich	Germany
Cooling centrifuge (universal 320 R)	Hettich	Korea
Electronic balance	Sartorius	Japan
ELISA Devices (washer & reader)	Human	Germany
Gel Electrophoresis	VG-SYS Vari-gel MAXI System	United Kingdom
Gel documentation	Biometra	Germany
Incubator	Binder	Germany
Refrigerator	Philipp kirsch	Germany
Thermal and gradient thermal cyclers	Biometra	Germany
Thermomixer comfort	Eppendorf	Germany
U.V PCR cabinet	Chroma	Spanish
Vortex	Clay adams	Germany
Water distillatory	GFL	Germany

Table (2-2): Equipment and Instruments with their Country of Origin

Equipment and Instruments	Country
Cold medical box	China

Cylinders (250,500 ml)	Germany
EDTA tube	China
Eppendorf tube (0.5 ml & 1.5 ml)	China
Filter paper	China
Filter Tips for PCR (100 µl & 200 µl)	China
Flasks (different size)	China
Gel and Clot Activator Tube	China
Gloves	China
Micropipettes (different size)	Japan
Tips (Yellow & Blue)	China

2.5.2. Chemicals and Biological Materials: Table (2-3) showed the chemicals and biological materials used in the current study.

Table (2-3): Chemicals and Biological Materials used in the Current Study

Chemicals and biological materials	Manufacturing Company	Country
Agarose	Promega	USA
Ethidium bromide	Promega	USA
Ethanol	Ghadeerlab	Iraq
Proteinase K	Elabscience	USA
TBE Buffer 10X	Promega	USA
Water, nuclease free	Promega	USA

2.6. ELISA Kit: several kits used in this study as the following tables.

Table (2-4): ELISA Kits used in the Study

Type of ELISA Kit	Manufacturing Company	Country
Human *Chlamydia pneumoniae* IgG	SUNLONG	China
Human *Chlamydia pneumoniae* IgE	SUNLONG	China
Human serum IL-4	Elabscience	USA
Total serum IgE	AccuBind	USA

2.6.1. ELISA Kit Content of Total Serum IgE: It was listed in the table 2-5.

Table (2-5): ELISA Kit for Detection of Human Total Serum IgE

Components	Format
1. Antibody-coated micro plate wells	12×8
2. Biotin reagent	1×13 ml
3. Calibrator 1 0 IU/ml (IgE. Human)	1×1.0 ml
4. Calibrator 2 5 IU/ml (IgE. Human)	1×1.0 ml
5. Calibrator 3 25 IU/ml (IgE, human)	1×1.0 ml
6. Calibrator 4 50 IU/ml (IgE, human)	1×1.0 ml
7. Calibrator 5 150 IU/ml (IgE, human)	1×1.0 ml
8. Calibrator 6 400 IU/ml (IgE, human)	1×1.0 ml
9.Chromogen A/substrate solution TMB/H2O2	1×7 ml
10.Chromogen B/substrate solution TMB/H2O2	1×7 ml
11. Enzyme conjugate horse radish peroxidase (HRP)	1×13 ml
12. Stop solution	1×8 ml
13. Wash buffer 50x concentrated	1×20 ml

2.6.2. ELISA Kit Content of Serum IL-4: It was listed in table 2-6

Table (2-6): ELISA Kit for Detection Serum IL-4

Components	Format
1. Micro ELISA Plate (Dismountable)	96T: 8 wells ×12 strips
2. Reference standard	2 vials
3. Concentrated Biotinylated detection Ab (100x)	120 µl
4. Concentrated HRP conjugate (100x)	120 µl
5. Reference standard & sample diluent	20 ml
6. Biotinylated detection Ab diluent	14 ml
7. HRP conjugate diluent	14 ml
8. Concentrated Wash Buffer (25x)	30 ml
9. Substrate Reagent	10 ml
10. Stop solution	10 ml
11. Plate Sealer	5 pieces

2.6.3. ELISA Kit Content of Human *Chlamydia pneumoniae* IgG: It was listed in table 2-7

Table (2-7): ELISA Kit for Detection Human *Chlamydia pneumoniae* IgG

Components	Format
1. Closure plate membrane	2
2. Microelisa stripplate	1
3. Reference standard 270 ng/L	0.5ml×1 bottle
4. Standard diluent	1.5ml×1 bottle

5. HRP-Conjugate reagent	6ml×1 bottle
6. Sample diluent	6ml×1 bottle
7. Chromogen Solution A	6ml×1 bottle
8. Chromogen Solution B	6ml×1 bottle
11. Stop solution	6ml×1 bottle
12. Wash Solution	20ml (30X)×1bottle

2.6.4. ELISA Kit Content of Human *Chlamydia pneumoniae* IgE:

It was listed in table 2-8

Table (2-8): ELISA Kit for Detection Human *Chlamydia pneumoniae* IgE

Components	Format
1. Closure plate membrane	2
2. Microelisa stripplate	1
3. Reference standard 90 ng/L	0.5ml×1 bottle
4. Standard diluent	1.5ml×1 bottle
5. HRP-Conjugate reagent	6ml×1 bottle
6. Sample diluent	6ml×1 bottle
7. Chromogen Solution A	6ml×1 bottle
8. Chromogen Solution B	6ml×1 bottle
11. Stop solution	6ml×1 bottle
12. Wash Solution	20ml (30X)×1bottle

2.7. DNA Extraction Kit: The contents were mentioned below

Table (2-9): DNA Extraction Kit

DNA extraction kit contents	Volume	Manufacturing Company	Country
RBC lysis buffer	135 ml	Favorgen	Taiwan
FATG buffer	30 ml		
FABG buffer	40 ml		
W1 buffer	45 ml		
Wash buffer (concentrate)*	25 ml		
Elution buffer	30 ml		
FABG mini column	100 pcs		
Collection tube	200 pcs		
User manual	1		

***** Once used, 100 ml of (96-100%) ethanol was added to the wash buffer.

2.7.1. Polymerase Chain Reaction Kits:

Table (2-10): Reagent and Equipment Required for PCR

Reagent and Equipment of PCR	Volume	Company	Country
GoTaq® G2 Master Mixes	2 × 1.25 ml	Promega	USA
Nuclease free water	2 × 1.25 ml		
IL-4 forward primer	30 nmol	Macrogen	Korea
IL-4 reverse primer	30 nmol		
Ladder 100 bp	0.1 ml x5 ea	SolGent	Korea
Loading dye	1 ml	SolGent	Korea

2.7.2. Restriction Enzyme Kits: It was listed on table 2-11

Table (2-11): Restriction Enzyme Kits

Restriction enzyme kits contents	Volume	Company	Country
BslF I (Bacillus stearothermophilus FI)	100 unit	Sibenzyme	Russia
Buffer of BslF I	1 x SE		
BSA	100 µl		

2.8. Methods:

2.8.1. ELISA

2.8.1.1. Measurement of Total Serum IgE

Serum was analysed to determine the total IgE concentration by Combiwash Max-Planck-Ring 21 automated immunoassay analyzer (Human, Germany) using AccuBind total IgE ELISA kit (LOT NO. 25K1D1).

2.8.1.1.1. The Principle of the Test:

In this Sandwich-ELISA, immobilization occurs during the interaction of streptavidin-coated on the well and exogenously added biotinylated monoclonal anti-IgE antibody at the surface of a microplate well. Upon mixing a monoclonal biotinylated antibody, and a serum containing the native antigen, reaction results between the native antigen and the antibody, forming an antibody-antigen complex. Then a second incubation was carried out using an enzyme-labelled anti-human IgE (enzyme conjugate). The addition of substrate would catalyse a colour reaction. After that, a stop

solution would stop the reactions. Finally, IgE concentration was measured by the calibration curve at a wavelength of 450 nm.

2.8.1.1.2. Procedure of the Test:

1- Sample incubation: 25 µl of the appropriate serum reference calibrator, control, or specimen into the assigned well.

2- One hundred µl of the IgE Biotin reagent was added to each well and allowed to incubate for 30 min at room temperature.

3- Automatic wash was done three times with 350 µl of wash buffer.

4- One hundred µl of enzyme conjugate was added to each microplate well and left for 30 minutes at room temperature.

5- Automatic wash was done three times with 350 µl of wash buffer.

6- One hundred µl of the working substrate (50 µl from substrate A and 50 µl from substrate B) was added to each microplate and left for 15 min at room temperature.

7- Fifty µl of stop solution was added to each micro plate well.

8- Photometric measurement was done at 450 nm within 30 min of adding the stop solution.

2.8.1.1.3. Interpretation of Result:

1- The average absorbance value (450) 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 concentration on the horizontal or X-axis and absorbance on the vertical (y) axis.

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

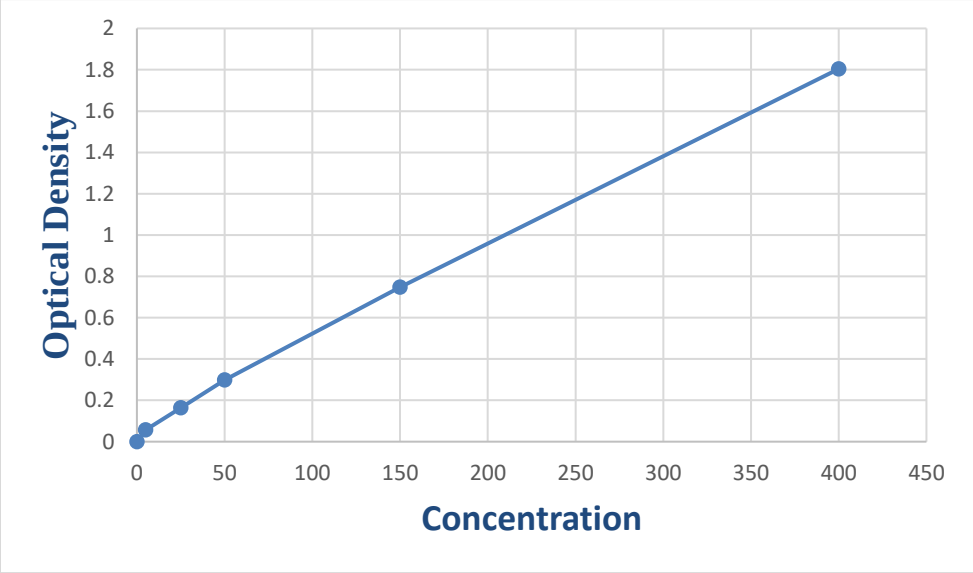

Figure (2-2): standard curve of total serum IgE in the present study

2.8.1.2. Measurement of Serum IL-4:

Serum IL-4 concentration was measured by Combiwash Max-Planck-Ring 21 automated immunoassay analyzer (Human, Germany) using Elabscience ELISA kit (LOT NO. 39CE12C5TF).

2.8.1.2.1. Principle of Test:

Sandwich-ELISA principle was used in this test, in which the microplate well had been pre-coated with an antibody specific to Human IL-

4. Samples and standards were added to the microtiter wells and combined with the specific antibody. Afterward, a biotinylated detection antibody specific for Human IL-4 and Avidin-Horse Radish Peroxidase (HRP) conjugate was added successively to each microtiter well and incubated free components were washed away. After that, substrate solution was added to each well and incubated. Finally, the enzyme-substrate reaction was terminated by adding a stop solution, and the color turned yellow. The IL-4 concentration was measured using the standard curve at 450 nm ± 2 nm wavelength.

2.8.1.2.2. Procedure of the Test:

1- The standard was centrifuged at 10,000 rpm for 1 min. One mL of Reference Standard &Sample Diluent was added and allowed to stand for 10 min. The Reference Standard &Sample Diluent was inverted several times gently. After it dissolved fully, it was mixed thoroughly with a pipette. This reconstitution produces a working solution of 2000 pg/mL.

Dilution method: 7 Eppendorf tubes were taken, and 500uL of Reference Standard & Sample Diluent was added to each tube. Five hundred uL of the 2000 pg/mL working solution were pipetted to the first tube and mixed up to produce a 1000 pg/mL working solution. Five hundred uL of the solution from the former tube was pipetted into the latter according to this step. Serial dilutions were made as follows: 2000, 1000, 500, 250, 125, 62.5, 31.25, 0 pg/mL.

2- Sample incubation: 100 µl of standard or sample was added to each well and incubated for 90 min at 37°C.

3- The liquid was removed without a wash.

4- One hundred µl of Biotinylated Detection Ab was added and incubated for 60 min at 37℃, then aspirated and washed three times with 350 µl of wash buffer.

5- One hundred µl of HRP Conjugate was added and incubated for 30 min at 37℃, then aspirated and washed five times with 350 µl of wash buffer.

6- Ninety µl of Substrate Reagent was added and incubated for 15 min at 37℃.

7- Fifty µl of Stop Solution was added and read at 450 nm immediately.

2.8.1.2.3. Interpretation of Result

1- A four-parameter logistic curve was plotted on log-log graph paper, with optical density values on the y-axis and standard concentration on the x-axis.

2- The concentration calculated from the standard curve must be multiplied by the dilution factor if the samples have been diluted. If the sample's optical density surpasses the standard curve's upper limit, it should re-test with appropriate dilution.

3-The actual concentration was the calculated concentration multiplied by the dilution factor.

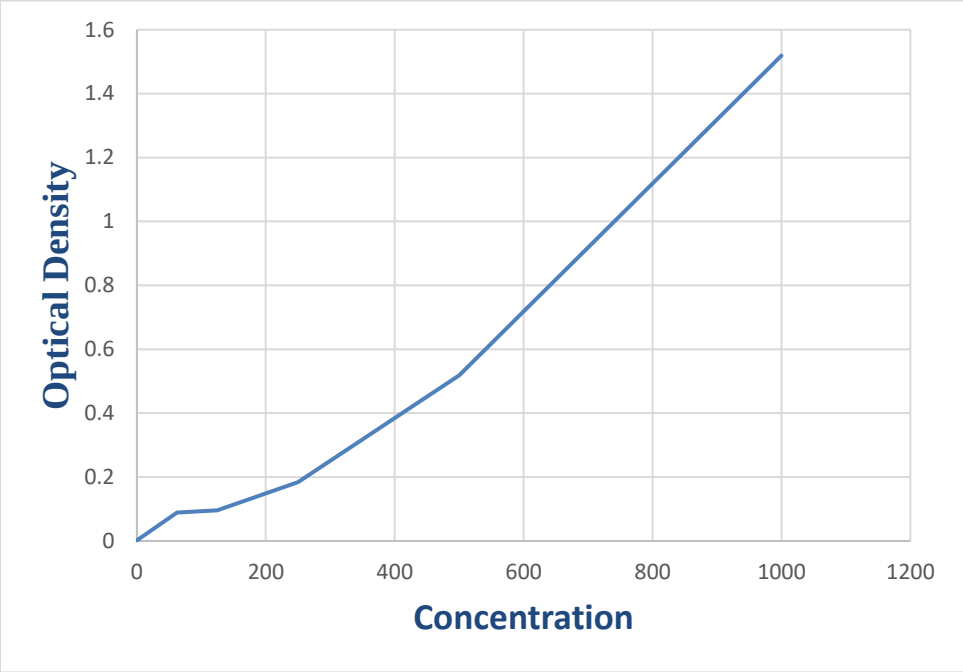

Figure (2-3): standard curve of IL-4 in this study

2.8.1.3. Measurement of Human *Chlamydia pneumoniae* IgG

Serum was analysed to determine the concentration of human *Chlamydia pneumoniae* IgG by Combiwash Max-Planck-Ring 21 automated immunoassay analyzer (Human, Germany) using SUNLONG human *Chlamydia pneumoniae* IgG ELISA kit (LOT NO. 20220426).

2.8.1.3.1. The Principle of the Test:

This kit used a Sandwich-ELISA principle. The microplate well provided in this kit has been pre-coated with an antigen specific to *Chlamydia pneumoniae* IgG. Standards or samples were added to the appropriate micro-ELISA strip plate wells and combined with the particular antigen. Afterward, a Horseradish Peroxidase (HRP)-conjugated antigen-specific for *Chlamydia pneumoniae* IgG was added to each micro-ELISA strip plate and incubated. Free components were washed away. Then, the TMB substrate

solution was added to each well. Only those wells containing *Chlamydia pneumoniae* IgG and HRP conjugated *Chlamydia pneumoniae* antigen will appear blue and then turn yellow after adding the stop solution. Finally, the optical density is measured spectrophotometrically at a wavelength of 450 nm.

2.8.1.3.2. Procedure of the Test:

1. The standard was diluted by small tubes, as in the table below.

Table (2-12): Standard Dilution of *Chlamydia pneumoniae* IgG ELISA Kit

180ng/L	Standard No.1	300µl Original Standard + 150µl Standard diluents
120ng/L	Standard No.2	300µl Standard No.1 + 150µl Standard diluents
60ng/L	Standard No.3	150µl Standard No.2 + 150µl Standard diluent
30ng/L	Standard No.4	150µl Standard No.3 + 150µl Standard diluent
15ng/	Standard No.5	150µl Standard No.4 + 150µl Standard diluent

2. Dilution of the sample: 40µl Sample dilution buffer and 10µl sample were added.

3. The standards and samples were added to each well of the micro-ELISA strip plate, left a well empty for blank control, then mixed well with gentile shaking.

4. The micro-ELISA strip plate was incubated for 30 min at 37℃, then was aspirated and washed five times with 350 µl of wash buffer.

5. Conjugate incubation: 50 µl of the HRP-Conjugate reagent was added to each well except for the blank control well.

6. The micro-ELISA strip plate was incubated for 30 min at 37℃, then was aspirated and washed five times with 350 µl of wash buffer.

7. Coloring: 50 µl Chromogen Solution A and 50 µl Chromogen Solution B was added to each well, were mixed with gently shaking, and were incubated at 37℃ for 15 minutes.

8. Termination: 50 µl stop solution was added to each well to terminate the reaction.

9. Read absorbance: optical density was read at 450 nm using a microtiter Plate Reader.

2.8.1.3.3. Interpretation of Result:

1. Known concentrations of Human *Chlamydia pneumoniae* IgG Standard and its corresponding reading optical density were plotted on the log scale (x-axis) and the log scale (y-axis), respectively.

2. Human *Chlamydia pneumoniae* IgG concentration in the sample was determined by plotting the sample's optical density on the Y-axis.

3. The original concentration was calculated by multiplying the dilution factor.

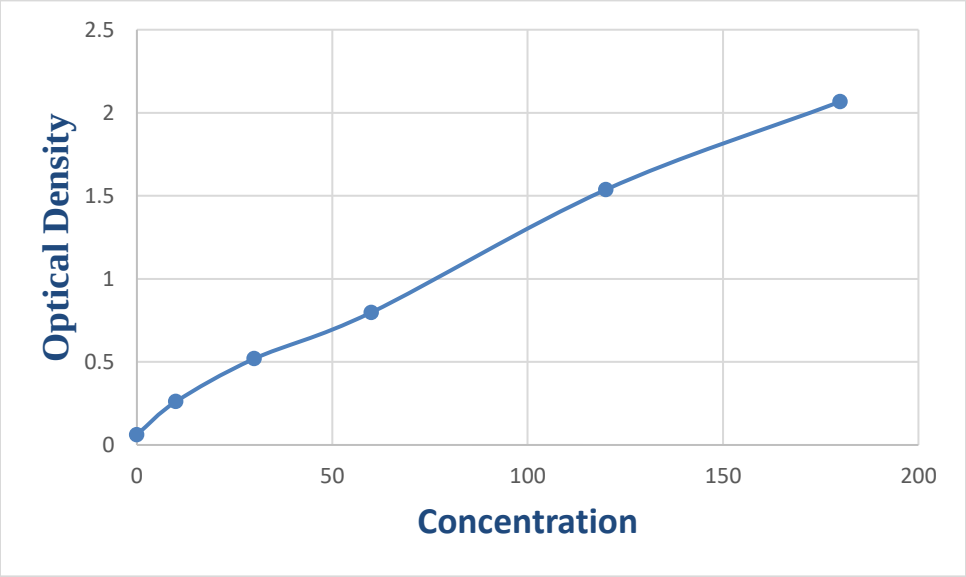

Figure (2-4): standard curve of Human *Chlamydia pneumoniae* IgG in this study

2.8.1.4. Measurement of Human *Chlamydia pneumoniae* IgE

Serum was analysed to determine the concentration of human *Chlamydia pneumoniae* IgE by Combiwash Max-Planck-Ring 21 automated immunoassay analyzer (Human, Germany) using SUNLONG human *Chlamydia pneumoniae* IgE ELISA kit (LOT NO. 20220426).

2.8.1.4.1. The Principle of the Test:

This kit used a Sandwich-ELISA principle. microplate well provided in this kit has been pre-coated with an antigen specific to *Chlamydia pneumoniae* IgE. Standards or samples were added to the appropriate micro-ELISA strip plate wells and combined with the particular antigen. Afterward, a Horseradish Peroxidase (HRP)-conjugated antigen-specific for *Chlamydia pneumoniae* IgE was added to each micro-ELISA strip plate and incubated. Free components were washed away. Then, the TMB substrate

solution was added to each well. Only those wells containing *Chlamydia pneumoniae* IgE and HRP conjugated *Chlamydia pneumoniae* antigen will appear blue and then turn yellow after adding the stop solution. Finally, the optical density is measured spectrophotometrically at a wavelength of 450 nm.

2.8.1.4.2. Procedure of the Test:

1. The standard was diluted by small tubes, as in the table below.

Table (2-13): Standard Dilution of *Chlamydia pneumoniae* IgE ELISA Kit

60 ng/L	Standard No.1	300µl Original Standard + 150µl Standard diluents
40 ng/L	Standard No.2	300µl Standard No.1 + 150µl Standard diluents
20 ng/L	Standard No.3	150µl Standard No.2 + 150µl Standard diluent
10 ng/L	Standard No.4	150µl Standard No.3 + 150µl Standard diluent
5 ng/L	Standard No.5	150µl Standard No.4 + 150µl Standard diluent

2. Dilution of the sample: 40µl Sample dilution buffer and 10µl sample were added.

3. The standards and samples were added to each well of the micro-ELISA strip plate, left a well empty for blank control, then mixed well with gentile shaking.

4. The micro-ELISA strip plate was incubated for 30 min at 37°C, then was aspirated and washed five times with 350 µl of wash buffer.

5. Conjugate incubation: 50 µl of the HRP-Conjugate reagent was added to each well except for the blank control well.

6. The micro-ELISA strip plate was incubated for 30 min at 37℃, then was aspirated and washed five times with 350 µl of wash buffer.

7. Coloring: 50 µl Chromogen Solution A and 50 µl Chromogen Solution B was added to each well, were mixed with gently shaking, and were incubated at 37℃ for 15 minutes.

8. Termination: 50 µl stop solution was added to each well to terminate the reaction.

9. Read absorbance: optical density was read at 450 nm using a spectrophotometry.

2.8.1.4.3. Interpretation of Result:

1. Known concentrations of Human *Chlamydia pneumoniae* IgE Standard and its corresponding reading optical density were plotted on the log scale (x-axis) and the log scale (y-axis), respectively.

2. Human *Chlamydia pneumoniae* IgE concentration in the sample was determined by plotting the sample's optical density on the Y-axis.

3. The original concentration was calculated by multiplying the dilution factor.

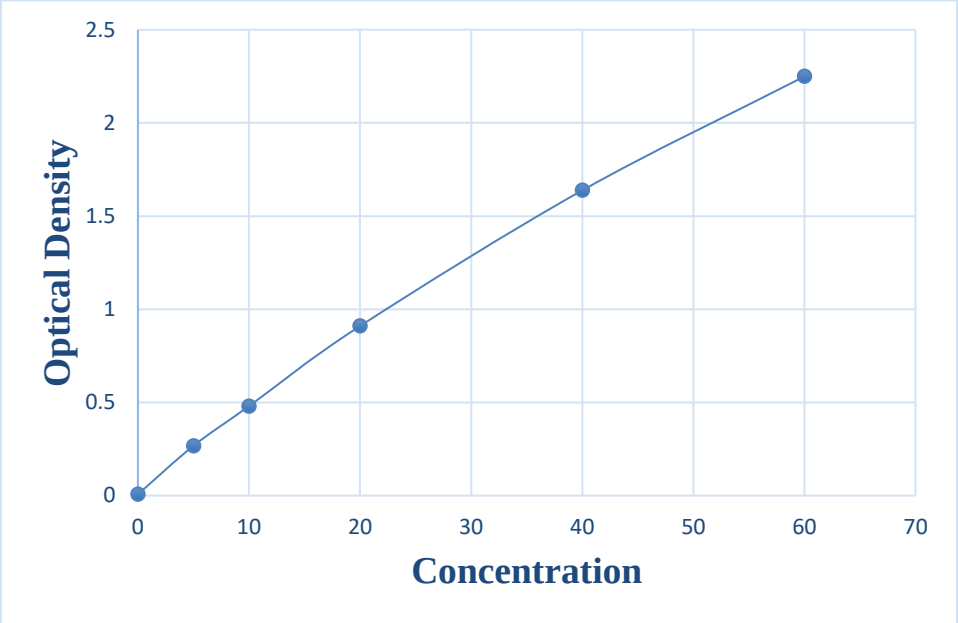

Figure (2-5): standard curve of Human *Chlamydia pneumoniae* IgE in this study

2.8.2. Molecular Procedure Steps:

2.8.2.1. DNA Extraction:

The genomic DNA was extracted from the nucleated cells of study groups under the aseptic condition and according to the protocol of FavorPrep™ Blood/ cultured cells genomic DNA, Favorgen, Taiwan.

2.8.2.1.1. DNA Extraction Procedure:

1. The blood sample was mixed thoroughly at room temperature for at least 10 minutes.

2. Twenty µl of Proteinase K (PK) solution was dispensed into a 1.5ml microcentrifuge tube.

3. Two hundred µl of blood was added to the tube containing the PK Solution, briefly mixed, and incubated for 15 min at 60 ºC

4. Two hundred µl of FABG Buffer 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 for 15 minutes.

6. While the blood sample was incubating, elution buffer was preheated at 70°C.

7. Two hundred µl of ethanol (96~100%) was added to the sample and mixed by vortex for 10 seconds.

8. A FABG column was placed into an empty collection tube. Then, the sample mixture was transferred carefully to FABG Column.

9. The FABG Column was caped and centrifuged for 1 minute at maximum speed. Then, the collection tube containing flow through was removed, and the liquid was discarded as hazardous waste.

10. The binding column was placed into a fresh collection tube, then 400µl of W1 buffer was added to the column, and centrifuged for 30 seconds at maximum speed.

11. The collection tube containing the flowthrough was removed, and the liquid was discarded. Then, the FABG Column was put back into the collection tube.

12. Six hundred µl of wash buffer was added to the FABG Column, centrifuged for 30 seconds at maximum speed, and then centrifuged for an extra 3 minutes at maximum speed.

13. The FABG Column was placed in a clean 1.5ml microcentrifuge tube.

14. One hundred µl of preheated elution buffer was added to the column and incubated at 37 °C for 10 minutes. Then, centrifuged for 60 seconds at maximum speed.

15. The FavoroPrep™ Binding Column was discarded, and the eluate stored in -20°C until used.

2.8.2.1.2. Detection of DNA Extraction Efficiency, Concentration, and Purity:

The extracted genomic DNA was checked by using a nanodrop, which measures the concentration of DNA by reading the absorbance at (260 /280 nm).

2.8.2.2. PCR Preparation:

2.8.2.2.1. Preparation 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.

2.8.2.2.2. Preparation of TBE 1 X Buffer Solution:

One hundred ml of 10 X TBE buffer was dissolved in 900 ml of water to form 1X of TBE buffer.

2.8.2.2.3. Polymerase Chain Reaction Protocol:

The polymorphism of IL-4 C589T gene promoter was identified by restriction fragment length polymorphism PCR (PCR-RFLP) method.

Restriction fragment length polymorphism (RFLP) detect SNPs by use restriction enzyme. Restriction endonuclease are enzyme that cut DNA into short pieces. Each restriction enzyme targets different nucleotide sequence in a DNA strand and therefore cuts at different sites (Mesh, 2019).

Table (2-14): The Specific Primers and their Sequences

Primer	Sequences	Product Size	Reference
IL-4 C-589T	F: 5-TGG GTA AGG ACC TTA TGG ACC-3	198	(Chiang *et al.*, 2007)
	R: 5-GGT GGC ATC TTG GAA ACT GT-3		

2.8.2.2.4. PCR Master Mix Preparation:

PCR master mix preparation was done according to promega protocol (Go Taq®Promega Green Master Mix, LOT: 0000319290) (as shown in the table 2-14). The addition of PCR master mix components was done in U.V PCR cabinet to prevent contaminations by nucleic acid.

Table (2-15): Protocol of PCR Mixture

Component	Volume in a 25 µl reaction
Go Taq®Promega Green Master Mix 2x	12.5 µl
Forward primer (10 pmol)	1 µl
Reverse primer (10 pmol)	1 µl
Template DNA	4 µl
Nuclease Free water	6.5 µl

2.8.2.2.5. PCR-Running Conditions:

PCR thermo cycling reaction for each gene was done by using convention PCR thermal cycler system. The running conditions were according to a previous study (Chiang *et al.*, 2007) (as shown in the table 2-16).

Table (2-16): PCR Thermo Cycling Condition for IL-4 C-589T Gene Polymorphism Detection

Step	Temperature	Time	Number of cycles
Initial Denaturation	94˚C	6 Minutes	
Denaturation	94˚C	15 Seconds	35 cycles
Annealing	60˚C	30 Seconds	
Extension	72˚C	30 Seconds	
Final Extension	72˚C	5 Minutes	
Hold	4 ˚C	∞	

2.8.2.2.6. Restriction Enzyme Preparation:

BslF I, sibenzyme:

This enzyme recognizes a sequence 5-GGGAC(N)10…3-, 3-CCCTG(N)14….5- of IL-4 C-589T gene . After PCR cycles had finished, one units of enzyme was added to 7.5 µl of IL-4 PCR prodect with 1 µl of enzyme buffer and 0.1 µl of BSA, then incubated for 2 hours in 37 ℃.

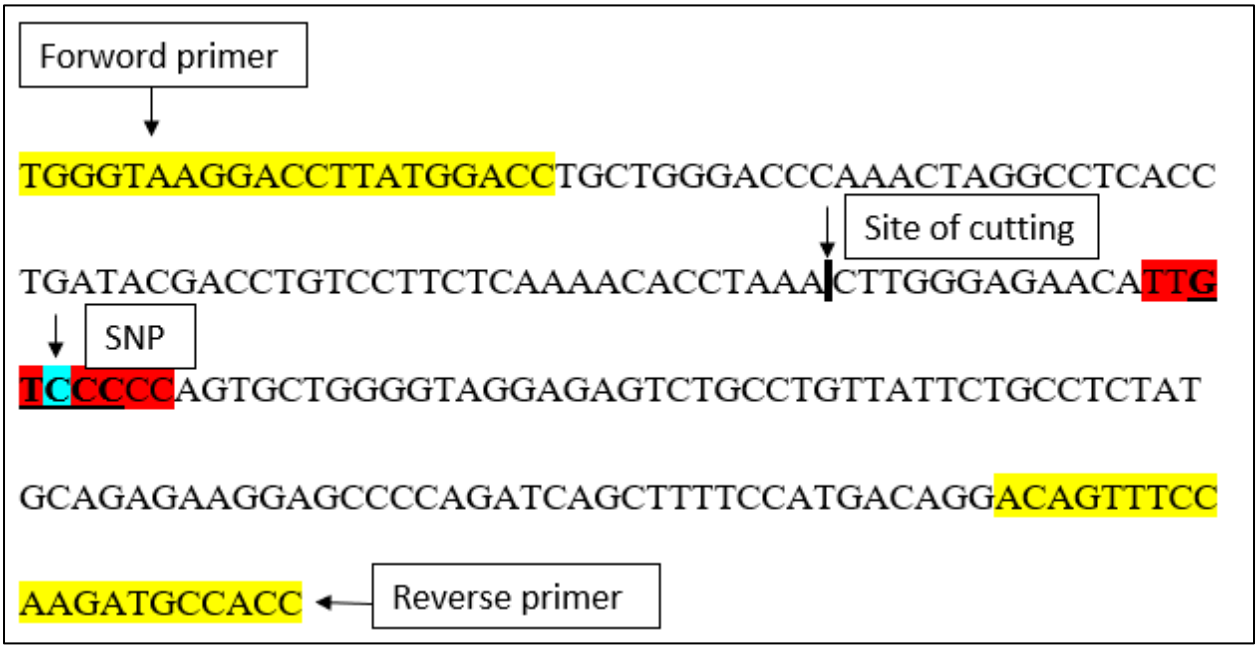

Figure (2-6): Site of Restriction Enzyme

3.8.2.2.7. Gel Electrophoresis:

A- Preparation of Gel Electrophoresis:

To prepare 1.5% agarose gel, one and a half grams of agarose powder was dissolved in 100 ml of TBE buffer in final concentration 1X and PH 8. This mixture was placed in a water bath until boiling at 100˚C to dissolve all agarose particles and allowed to cool at 50˚c. Then ten µl (final concentration 0.5 µg /ml) of ethidium bromide was added. The agarose-ethidium bromide solution was poured into the gel tray of the electrophoresis apparatus containing the combs, and the two ends of the gel tray were sealed. The agarose was allowed to solidify at room temperature for 10 min. The seal and the combs were removed gently from the tray.

The gel tray was fixed in the electrophoresis chamber, and 1X TBE buffer was added to the electrophoresis chamber until the surface of the gel

was covered. The electrodes were attached to the power supply then the DC power was turned on. Finally, the voltage was set at 70 volts for 90 minutes.

B-Agarose Gel Documentations:

The amplified PCR products and restriction enzyme products of IL-4 was 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 of the samples were equal to the target product size for IL-4 198 bp.

The result of IL-4 restriction enzyme products were distinguished when the DNA band of the sample separated into two bands with a length of 120 bp and 78 bp for the CC genotype, three bands with lengths of 198, 120, and 78 bp for the CT genotype, while only one band with a length 198 bp in TT genotype (DNA band was not separated).

2.9. Statistical Analysis:

Data was introduced into IBM SPSS Statistics Version 21 (San Diego, California, USA) for statistical analysis, while the figures constructed by EXCEL program of Microsoft Office 2010 (GraphPad prism Microsoft).

The results were expressed as Mean ± standrad deviation (SD). Comparisons between the two mean were performed using the T-test, while ANOVA was used to compare among mean. Kruskal Wallis Test is a non-parametric test used to compare the median ± Interquartile Range (IQR) when the p-value of Levene's test was less than 0.05. A p-value of <0.05 indicates the statistical significance and is highly significant if the p-value is

<0.001. Chi-square ($x^{\wedge 2}$) is used to compare two categorical variables. In addition, the Pearson correlation was used to explain the relation between IL-4 levels with total serum IgE levels.

Genotypes of IL-4 C-589T were presented as percentage frequencies, and one-tailed assessed significant differences between their distributions in asthmatic patients and controls‟s exact probability (P). In addition, the odds ratio (OR) was also estimated to define the association between a genotype with the disease.

Direct gene counting methods calculated allele frequencies of genes. At the same time, a significant departure from Hardy-Weinberg equilibrium (HWE) was estimated using the HWE calculator for two alleles, which is available on the online OEGE - Online Encyclopedia for Genetic Epidemiology studies http://www.oege.org/software/hwe-mr-calc.shtml.

2.10. Hardy Weinberg Equilibrium (HWE):

Testing for HWE is commonly used as a quality control filter in genome-wide scans for markers with experimentally determined genotypes (Shriner, 2011). HWE is an essential quality control step in population-based genetic association studies that have proven to be a powerful tool in identifying genes implicated in many complex human diseases that significantly impact public health (Namipashaki *et al.*, 2015). Statistical tests for HWE have been an essential tool for detecting genotyping errors in the past and remain important in the quality control of next-generation sequence data and permit evaluation of missingness. In addition, it can indicate inbreeding, population stratification, and even problems in genotyping (O'Rourke, 2018, Hui and Burt, 2020).

CHAPTER THREE

Results and Discussion

3. Results and Discussion

3.1. Demographical Distribution of Asthmatic Patients and Healthy Control:

Demographic characteristics for 174 children (87 asthmatic patients attending the asthma clinic at Karbala Teaching Hospital for Children and 87 healthy control) revealed the following results:

3.1.1. Age Distribution:

According to GINA, asthmatic patients' ages ranged from 1 to 16 years old, distributed into three age groups (less than six years, from 6 to 11 years, and more than 11 years). The results revealed that the disease was more frequent in 6 to 11 years (47.13%) than in other age groups (Figure 3-1).

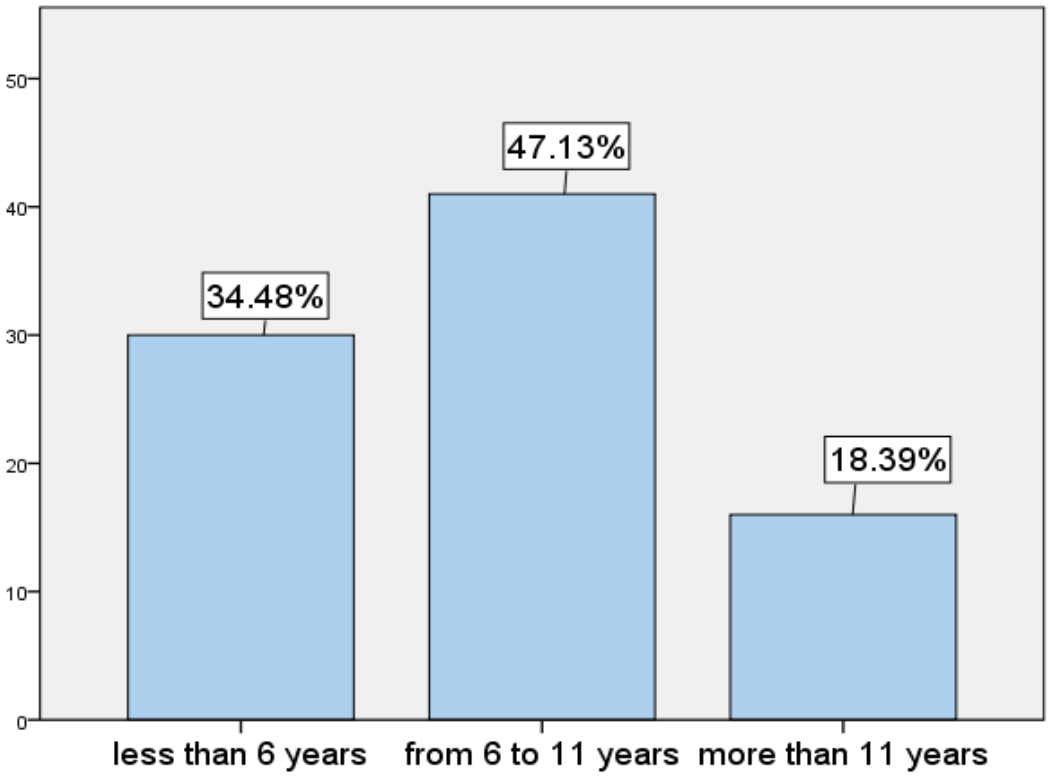

Figure (3-1): Age Distribution in Asthmatic Children

According to the Asthma Call-back Survey (ACBS) and Risk Factor Surveillance System (BRFSS) data from the participating states during 2006-2008, incidence among young children aged 0-4 years was more than five times greater than among youth aged 12-17 years (Winer *et al.*, 2012). Other data suggested that asthma-related hospitalizations might be higher in earlier childhood and middle age than in late adolescence (13–18 years old) and early adulthood (19-30 years old) (Wu *et al.*, 2014).

These results may be due to differences in risk factors that trigger asthma in different age groups. In children under five years old, the risk factors that trigger asthma are viral infections such as the respiratory syncytial virus and exposure to cold air. The most common asthma triggers in children over six years old are exercise, emotional changes, house dust, pollen, and pets. While in adolescence, animal dander, pollen, and irritants in the air, such as perfume, smoke, and air pollution, are the trigger of asthma. This age-related difference in risk factors raises the attention of patients and their parents, so they avoid the triggers.

3.1.2. Gender Distribution:

The study result revealed a higher prevalence of asthma in males, with whom the percentage of the male was 65.52%, whereas the percentage was 34.48% in females. There was no difference in gender distribution between the patients and the healthy control. However, there was a significant difference between females and males within the asthmatic group. The percentage of male /female were (65.52/34.48) % and (52.87/44.82) % for asthmatic and control, respectively (*P*-value= 0.163) (as shown in Table 3-1).

These findings were consistent with many previous studies, which detected that asthma was far more common in boys than girls during early childhood (Chowdhury *et al.*, 2021, Jenkins *et al.*, 2022). In practical

terms, the impact of asthma may differ according to gender in terms of daily activities for children.

Table (3-1): Difference in Gender between Patients and Healthy Control

Gender	Asthmatic patients	Healthy Children	x^2	*P-value*
Male	57 (65.52%)	48 (55.17%)	1.945	0.163
Female	30 (34.48%)	39 (44.82%)		
p-value	0.004**	0.335		

x^2: chi − square, **: highly significant (p<0.01)

3.1.3. Residence Distribution:

Most asthmatic children in this study lived in the urban area than the rural (as presented in Figure 3-2).

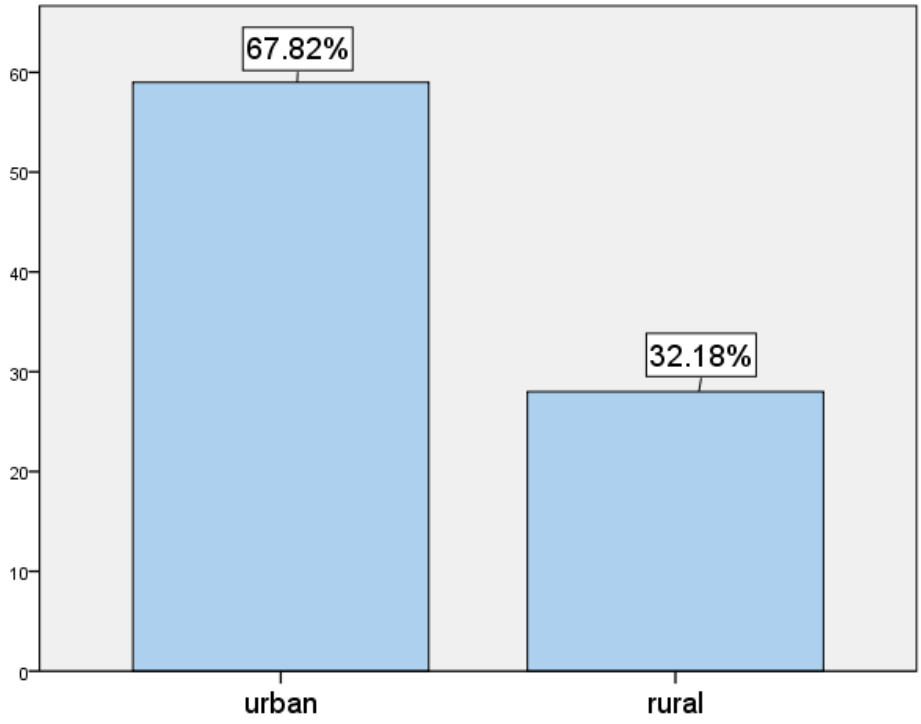

Figure (3-2): Residence Distribution of Asthmatic Children

This result was consistent with a previous systemic review, which showed a greater asthma prevalence in urban areas than in rural populations (Rodriguez *et al.*, 2019). Mostly, children from rural countries had fewer emergency department asthma visits than children from urban and suburban counties (Malik *et al.*, 2012). This difference may be correlated with environmental risk exposure and healthcare access. Urban people were more frequently exposed to environmental risk factors than rural people: dust mites, high levels of air pollution, and crowds. Further, the lower prevalence of asthma in rural children may be due to the beneficial effects of exposure to farm environments.

3.2. Major Characteristics of Asthmatic Children:

3.2.1. Severity of Asthma:

The study consists of (35.63%), (48.28%), and (11.29%) of asthmatic children with mild, moderate, and severe asthma, respectively (shown in Figure 3-3).

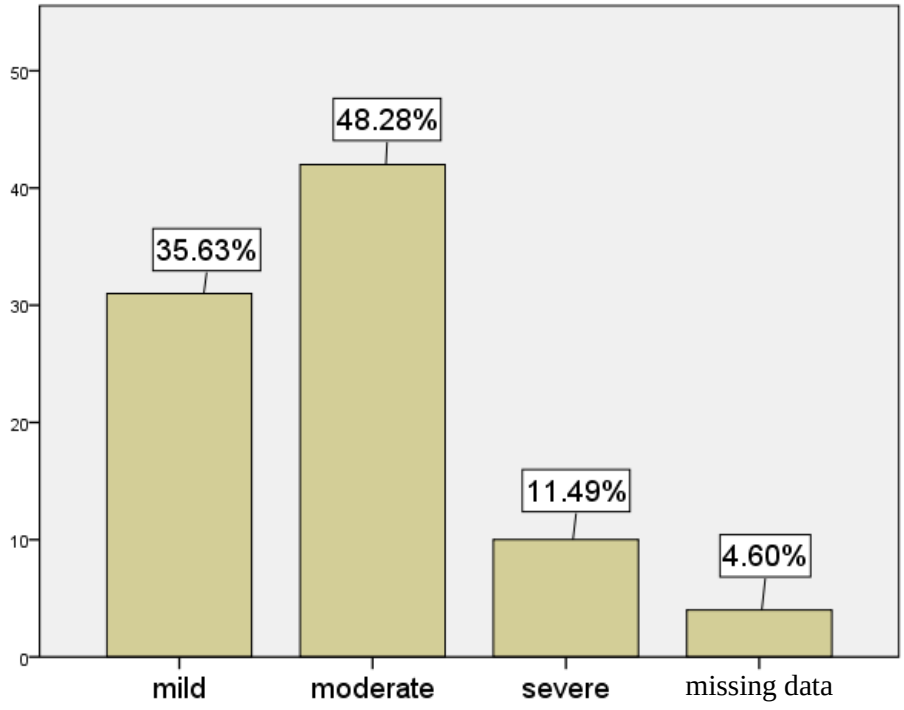

Figure (3-3): Distribution of Asthma according to the Severity

This result is consistent with previous studies, which thought severe asthma affected less percent of the total population with asthma than mild to moderate asthma (Wang *et al.*, 2020, Zafeiria *et al.*, 2021). The prevalence of uncontrolled asthma varies among samples categorized as severe asthma, and the variation is likely dependent on each study's design and the definition of asthma control.

3.2.2. Family History of Asthma and other Allergic Diseases:

The study showed a high association between positive family history of asthma/ allergic diseases and asthma in children (Figure 3-4). There were (43,68%) of asthmatic children with a close family history of asthma/ allergic diseases, (20.69%) of asthmatic patients have relatives with allergic disease, and only (35.63%) of children without a family history of allergy.

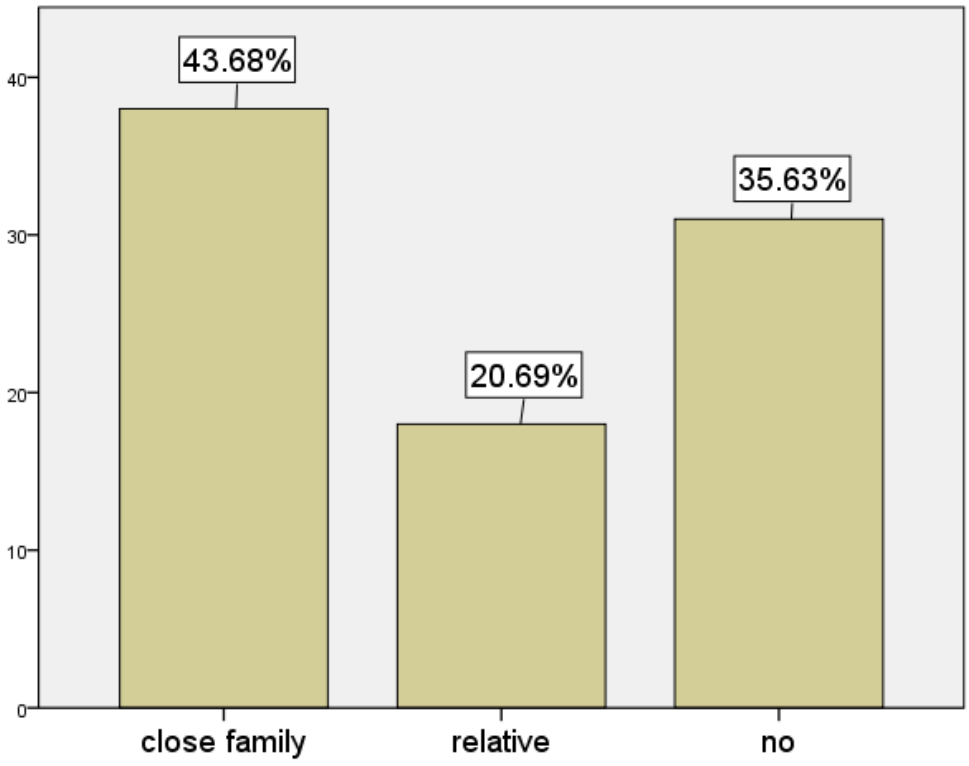

Figure (3-4): The association between Family History and Asthma

This finding was consistent with many asthma family history studies, which confirmed that asthma was highly associated with a family history of asthma and regarded as a risk factor (Paaso *et al.*, 2014, Yu *et al.*, 2021), the association may be due to genetic effects, could cause asthma and regulate the susceptibility to allergic and other environmentally induced inflammation. In addition, asthma was caused by multiple genes interaction. Some genes have a protective effect, and others contribute to the disease pathogenesis.

3.2.3. Type of Treatment and Control Status in Asthmatic Patients

In this study, 79 asthmatic children received regular treatment with either inhaled corticosteroids or Montelukast (35 take ICSs and 44 take montelukast), while eight patients were without treatment (Figure 3-5)**.**

The study consists of (41.77%), (41.77%), and (16.496%) of well-controlled, partially controlled, and not well-controlled, respectively (Figure 3-6).

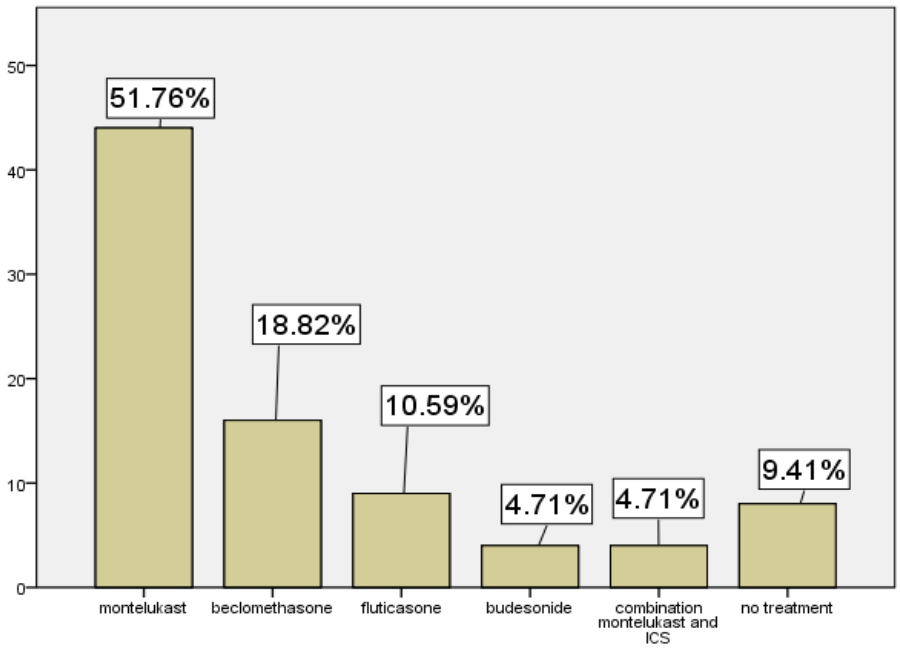

Figure (3-5): Treatment Distribution in Asthmatic Children

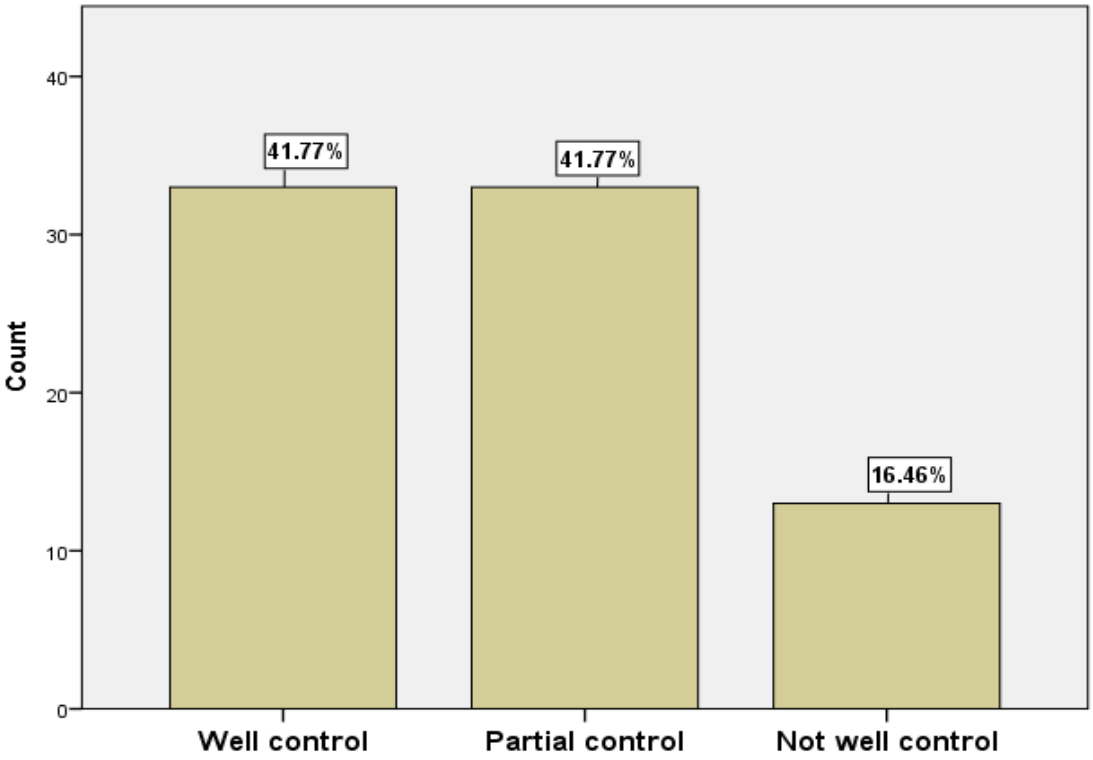

Figure (3-6): Characteristic of Control Status in Asthmatic Patients

Asthma treatment is based on risk factors, comorbidities, and on control status of asthmatic patients, which includes a cycle of assessment and re-evaluation of symptom control. Central to treatment is anti-inflammatory therapy with ICSs plus LABA (Papi *et al.*, 2018, Quirt *et al.*, 2018).

3.3. Immunological Study:

3.3.1. Comparison between Asthmatic Children and Control using Total Serum IgE Test:

Table (3-2) showed a highly significant difference in total serum IgE between asthmatic patients and control.

The result was consistent with previous studies in Iraq; one study (Altaii and Al-Tae, 2022) mentioned that the geometric means of total IgE were 316.87 IU/ml in allergic asthma compared to 16.90 IU/ml in healthy controls. Further, another study (Al Obaidi *et al.*, 2008) indicated that the

mean serum IgE level was 554 IU/ml in asthmatic patients, while that of the control was 69 IU/ml.

Table (3-2): Total Serum IgE Test between Asthmatic Children and Control

Comparison groups	Number	Mean IU/ml ± S.D.	T-test	*P-value*
Asthmatic patients	87	398.88 ± 227.156	12.388	0.000**
Healthy control	87	49.34 ± 66.831		

** significant at the 0.001 level (1-tailed).

Moreover, the result was consistent with previous international studies, which detected a robust positive relationship between total serum IgE levels and asthma in children. A previous study (Sandeep *et al.*, 2010) showed that IgE levels were high in asthmatics (1045.32 IU/ml) as compared to normal subjects (151.95 IU/ml). On the same line, the other researchers (Sharma *et al.*, 2006) showed that the mean IgE levels were the highest in Indian asthmatic patients and the lowest in the control group.

The possible explanation for this result was the presence of a genetic predisposition to immediate hypersensitivity reactions and a defect in the immune system development involving IgE production and early allergic sensitization. Allergic sensitization results from forming specific IgE in response to common inhalant allergens derived from house dust mites, pollens, animal dander, molds, and cockroaches. In addition to allergens acting as classical IgE inducers, viral infections, and air pollution may trigger the IgE pathway, notably resetting the threshold of IgE sensitivity by regulating FcεRI expression. In contrast, nonallergic individuals respond to the same antigen by producing IgG (There are no IgG receptors on mast cells).

3.3.2. Comparison between Asthmatic Children and Control using Human *Chlamydia pneumoniae* IgG Test:

The study showed a highly significant difference in the human *Chlamydia pneumoniae* IgG levels between patients and control (Table 3-3).

Table (3-3): Human *Chlamydia pneumoniae* IgG between Asthmatic Children and Control

Comparison groups	Number	Mean ng/L± S.D.	T-test	*P-value*
Asthmatic patients	87	24.89 ± 16.65	7.252	0.000**
Healthy control	87	10.93 ± 6.75		

** Significant at the 0.001 level (1-tailed).

This result was consistent with the previous studies that proved a relationship between *Chlamydia pneumoniae* infection and asthma. In Iraq, the authors (Al-Zaidi *et al.*, 2010) detected that a significantly (p<0.05) high number (73%) of patients with positive *Chlamydia pneumoniae* IgG, of which a significant number (p<0.05) of them (63%) were asthmatic, and (36%) were non-asthmatic chest infections. Further, the previous investigators (Al-Ghizzi *et al.*, 2020) found that IgG anti-chlamydia antibodies were positive in 21 (34.4%) of patients compared to 4 (13.8%) of controls, and the difference was significant (P=0.029).

In an international study, (Gencay *et al.*, 2001) showed that chronic infection with *Chlamydia pneumoniae* was more frequent in patients with asthma (18.2%) compared with control subjects (3.0%) (p < 0.01). In addition, others (Ferrari *et al.*, 2002) detected high IgG levels more frequently in atopic subjects (p = 0.04). In the same way, another study mentioned that 65% of the asthmatic population had serum IgG towards *Chlamydia pneumoniae* (Calmes *et al.*, 2021).

Many reasons may cause this result: Firstly, patients with asthma and other atopic conditions have significantly increased risks of severe infections with viruses and bacteria due to disruption and altered function of the airway epithelium and structural alterations of airways. Secondly, the inability of the host to completely eradicate the pathogens; as a result, *Chlamydia pneumoniae* can enter a state of dormancy with intermittent periods of replication.

Thirdly, ICS negatively affects many aspects of cell-mediated immunity and favours the shift from a T-helper-1-type toward a T-helper-2-type response. Because *Chlamydia pneumoniae* requires a T-helper-1-type immune reaction to be cleared thus, ICS may severely deteriorate the host's ability to eradicate it. Indeed, the associations between ICS use and bacterial respiratory infections remain controversial.

On the other hand, the occasional initiation of asthma by *Chlamydia pneumonia*e cannot be excluded since the evidence is available that this could be true. Acute *Chlamydia pneumoniae* infections in previously unexposed, non-asthmatic individuals can result in chronic asthma. Repeated or prolonged exposure to *Chlamydia pneumoniae* may have a causal association with wheezing, asthmatic bronchitis, and asthma.

3.3.3. Comparison between Asthmatic Children and Control According to of Human *Chlamydia pneumoniae* IgE Test:

The study showed a significant difference in the human *Chlamydia pneumoniae* IgE levels between patients and control (Table 3-4).

This result was consistent with the results of local and previous meta-analyses, which detected the possibility of some undetected antigens responsible for generating a cryptic IgE response in some asthma patients with *Chlamydia pneumoniae* (Al-Sabhany *et al.*, 2011, Hahn *et al.*, 2012).

Table (3-4): Human *Chlamydia pneumoniae* IgE between Asthmatic Children and Control

Comparison groups	Number	Mean ng/L ± S.D.	T-test	*P-value*
Asthmatic patients	87	8.037 ± 4.645	1.995	0.024*
Healthy control	87	6.52 ± 3.062		

* Significant at the 0.05 level (1-tailed).

In Iraq, (Al-Sabhany *et al.*, 2011) found that of 54 tested sera from asthmatic patients, 37 showed positive ELISA tests for IgE specific for *Chlamydia pneumoniae* antigen. In contrast, all tested sera (15) from the control group showed negative IgE *Chlamydia pneumoniae* antigen.

Chlamydia pneumoniae IgE detection was significantly (P = 0.001) associated with asthma compared to healthy blood donor controls (Hahn *et al.*, 2012). Similarly, others (Smith-Norowitz *et al.*, 2020) detected that *Chlamydia pneumoniae* IgE levels were higher in asthmatics than in non-asthmatic. Equally, researchers (Loeffler *et al.*, 2019) found that *Chlamydia pneumoniae* IgE levels were significantly higher in asthmatic patients compared with non-asthmatic subjects (1.015 ± 0.305 vs. 0.39 ± 0.340; P<0.001). One possible interpretation of these results is that four chlamydial antigens may induce *Chlamydia pneumoniae*-specific IgE antibody levels in asthmatic patients' serum (LPS, Crp A, HSP 60, and POMP).

3.3.4. Comparison of Immunological Parameters depending on Demographic Characteristics in Asthmatic Children

The result showed a significant difference in IL-4 levels between age groups in asthmatic patients (*p-value*=0.008). Further, there was a significant difference in *Chlamydia pneumoniae* IgE level (p=0.047). In contrast, there was no significant difference in both total IgE levels and

Chlamydia pneumoniae IgG between age groups of asthmatic children (*p*= 0.201, 0.862 respectively) (Table 3-5).

Table (3-5): Comparison of Immunological Parameter between Age Groups of Asthmatic Children

Parameter	Age groups	Number	Mean ± S.D	ANOVA	*P-value*
Total IgE IU/ml	<6	30	338.75±234.216	1.637	0.201
	6-11	41	432.94±236.127		
	>11	16	424.385±173.219		
Chlamydia pneumoniae IgG ng/L	<6	30	25.886±16.642	0.148	0.862
	6-11	41	24.897±19.15		
	>11	16	23.051±8.434		
Chlamydia pneumoniae IgE ng/L	<6	30	9.695±5.632	3.164	0.047*
	6-11	41	7.334±4.232		
	>11	16	6.73±2.446		
IL-4 level pg/mL [a]	<6	30	1.691± 5.183	9.739	0.008*
	6-11	41	1.921± 5.081		
	>11	16	6.185± 13.223		

* Significant at the 0.05 level, [a] used Kruskal Wallis Test

Table (3-6) showed a correlation between age and immunological parameters (total serum IgE, *Chlamydia pneumoniae* IgG, *Chlamydia pneumoniae* IgE, and IL-4 level). The results found a positive linear correlation between age and total IgE and IL-4, while a negative linear correlation between age and *Chlamydia pneumoniae* IgE. On the other hand, there was no correlation between age and *Chlamydia pneumoniae* IgG.

Table (3-7) showed no significant difference in immunological parameters (Total IgE and *Chlamydia pneumoniae* IgG) between urban and rural areas of asthmatic children aged from one to eleven years old. Paradoxically, there was a significant difference in *Chlamydia pneumoniae* IgE levels between urban and rural asthmatic children aged more than eleven years (*P*=0.036). In urban residents, the asthmatic children more than eleven years old had a low *Chlamydia pneumoniae* IgE level (5.845±

1.821 ng/L) compared with asthmatic children who lived in rural areas area (8.206 ± 2.793 ng/L).

Depending on age groups, there was a significant difference (*P*=0.047) in *Chlamydia pneumoniae* IgE level in asthmatic children who lived in urban areas. The *Chlamydia pneumoniae* IgE level in urban asthmatic children decreased with increased age. On the other hand, there was no significant difference in *Chlamydia pneumoniae* IgE level in rural asthmatic children depending on age groups (*P*=0.228), as shown in Figure (3-7).

Table (3-6): Correlation between Age and Parameter in Asthmatic Patients

	Parameter	Spearman's rank	*p-value*
Age	Total IgE IU/ml	0.255	0.017*
	Chlamydia pneumoniae IgG ng/L	0.012	0.910
	Chlamydia pneumoniae IgE ng/L	-0.233	0.030*
	IL-4 level pg/mL	0.283	0.008*

*Correlation is significant at the 0.05 level (2-tailed).

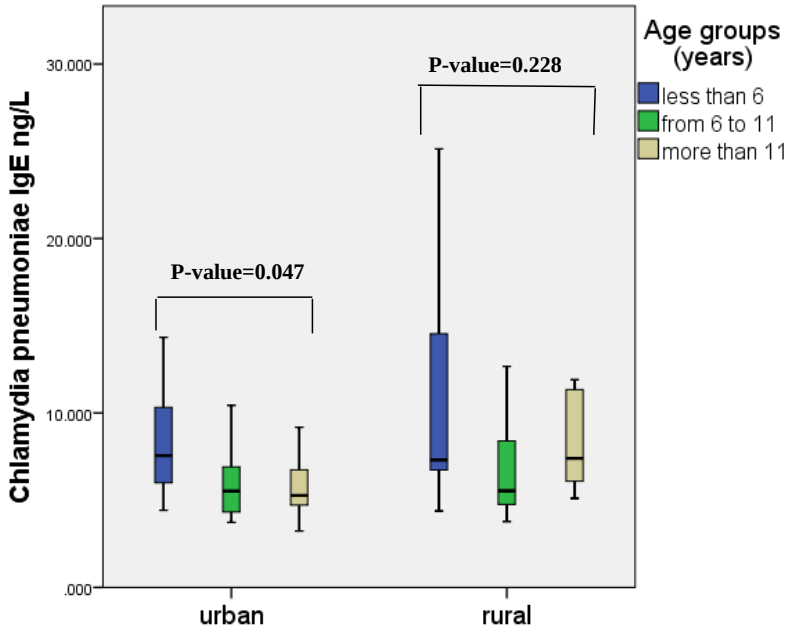

Figure (3-7) Comparison between *Chlamydia pneumoniae* IgE Levels in Asthmatic Children Depending on Age Groups and Geographic areas.

Table (3-7): Comparison between Immunological Parameters of Asthmatic Children Depending on Age Groups and Geographic Areas.

Age groups	Geographic areas		Total IgE IU/ml	*Chlamydia pneumoniae* IgG ng/L	*Chlamydia pneumoniae* IgE ng/L
Less than 6	Urban	Mean	304.261	25.814	9.228
		N	21	21	21
		Std. Deviation	206.828	17.138	5.008
	Rural	Mean	419.235	26.053	10.783
		N	9	9	9
		Std. Deviation	285.486	16.419	7.099
P-value			0.141	0.464	0.447
From 6 to 11	Urban	Mean	425.828	27.162	7.599
		N	28	28	28
		Std. Deviation	220.726	22.185	4.755
	Rural	Mean	448.261	20.018	6.765
		N	13	13	13
		Std. Deviation	275.429	8.791	2.886
P-value			0.418	0.191	0.439
More than 11	Urban	Mean	434.413	20.745	5.845
		N	10	10	10
		Std. Deviation	152.345	7.962	1.821
	Rural	Mean	407.673	26.894	8.206
		N	6	6	6
		Std. Deviation	218.408	8.4222	2.793
P-value			0.356	0.09	0.036*

*Significant difference at *p-value* 0.05

The present study revealed that the total IgE levels and IL-4 levels were increased with age in asthmatic children younger than sixteen. In contrast, *Chlamydia pneumoniae*-specific IgE decreased with the age (Table 3-6 and

3-7). This result was consistent with a previous study that mentioned that the total IgE increased with age, but allergen-specific IgE levels significantly decreased when age increased (De Amici and Ciprandi, 2013). Indeed, no one can say that *Chlamydia pneumoniae*-specific IgE decreases with age because this IgE was not affected by age in asthmatic patients who live in rural (Figure 3-7). The low specific IgE value was detected only in urban asthmatic children over eleven (Table 3-8). Thus, no possible explanation based on age since many factors could interfere with and affected the outcome, as shown in the present study, the effect of residency on specific IgE levels.

The other possible explanation was that reducing exposure to the allergen was associated with improved sensitization. In contrast, this study showed no decrease in Chlamydial infection since *Chlamydia pneumoniae* IgG has not changed, and there was no significant difference in this immunoglobulin value between age groups in rural and urban asthmatic children.

The third explanation is that each allergen exhibits a particular trend with age. House dust mites, for example, induced the earliest IgE response, while the response to *Alternaria* was characteristic of adolescents and young adults. In the same way, pollens and grasses induced early IgE production, which declined after 30 years. Indeed, this hypothesis could not be accepted since early adolescent rural asthmatic children had the same *Chlamydia pneumoniae*-specific IgE level as other age groups of asthmatic children.

The fourth explanation could be that early adolescent asthmatic in urban areas have been exposed more heavily or widely to different allergens, which is not occurring in the case of younger age categories or rural

asthmatic children. A significantly higher percentage of allergic rural rather than urban children was monosensitized or sensitized to two to four allergens. In contrast, an almost fourfold higher rate of allergic urban children was sensitized to five or more allergens. A positive relationship exists between cumulative exposure to an allergen and the risk of allergic sensitization. Depending on this, it may be a decrease in *Chlamydia pneumoniae*-specific IgE resulting from increased sensitization to other antigens or selected other allergens, or it is probably due to pollen exposure. The clinical sensitivity to pollen is higher among subjects living along roads with heavy traffic.

Indeed, the result still cannot determine the mechanism that redirected allergic responses toward one allergen or whether exposure to many allergens will reduce specific IgE to one allergen over another. All known is that previous observations suggested the operation of environment-specific regulatory mechanisms affects the risk of sensitization and the magnitude of specific IgE production. Further, different interactions of potential allergens with airway epithelial cells and other cells located within and below the epithelium will affect the outcome of antigen exposure.

3.3.5. Correlation between Total IgE and *Chlamydia pneumoniae* IgE in Asthmatic Patients:

The result showed a significant positive linear correlation between total IgE level and human *Chlamydia pneumoniae* IgE (P= 0.019) under age control (Table 3-8).

The present study detected a positive linear correlation between total and *Chlamydia pneumoniae*-specific IgE under age control. This result

agreed with previous research that suggested increased specific IgE can lead to increased total IgE (Chang *et al.*, 2015).

Table (3-8): Correlation between Total IgE and *Chlamydia pneumoniae* IgE in Asthmatic Patients under Age Control

Chlamydia pneumoniae IgE ng/L	Parameter	Partial correlation	*p-value*
	Total IgE IU/ml	0.225	.019*

*Correlation is significant at the 0.05 level (1-tailed).

A high level of total IgE may reveal the presence of food sensitization, indicate a high risk of eczema, and be associated with a high prevalence of mite sensitization and atopic diseases in early childhood. Thus, Total serum IgE is predictive of a positive specific IgE. B cells are induced selectively to produce specific IgE when allergens enter the body. The level of specific IgE depends on the body's reaction to the allergens. Allergens combined with specific IgE induce an allergic reaction. Thus, specific IgE is a vital objective index for allergic disease diagnosis.

3.3.6. Correlation between *Chlamydia pneumoniae* IgG and *Chlamydia pneumoniae* IgE in Asthmatic Patients

Table (3-9) showed a highly significant positive linear correlation between *Chlamydia pneumoniae* IgG and *Chlamydia pneumoniae* IgE in asthmatic children ($P<0.001$).

This result was consistent with previous studies (Smith-Norowitz *et al.*, 2020, Calmes *et al.*, 2021), which clarify that *Chlamydia pneumoniae* antigen stimulates persistence-specific IgE production unless using antibiotics are to eradicate the bacteria. The transition to IgE from IgM and IgG varies over time since the increase in IgG level is subsequently passed onto the IgE production. However, the IgE class switching from IgG

occurred later than the IgG production. The presence of *Chlamydia pneumoniae*-specific IgE Abs in stable asthmatics (without acute airway infection) with *Chlamydia pneumoniae* provides further evidence for ongoing stimulation of allergic responses by *Chlamydia pneumoniae.* Unless *Chlamydia pneumoniae* is eradicated by antibiotics, antigenic stimulation leading to specific IgE production may be persistent.

Table (3-9): Correlation between *Chlamydia pneumoniae* IgG and *Chlamydia pneumoniae* IgE in Asthmatic Patients

	Parameter	Spearman's rank	*p-value*
Chlamydia pneumoniae IgG ng/L	*Chlamydia pneumoniae* IgE ng/L	0.466	<0.001**

**Correlation is significant at the 0.01 level (1-tailed).

3.3.7. Correlation between IL-4 Level and Allergic Parameters in Asthmatic Patients:

Table (3-10): showed no significant linear correlation between IL-4 level and total IgE and *Chlamydia pneumoniae* IgE (*P*= 0.872 and 0.958, respectively).

Table (3-10): Correlation between IL-4 Level and Allergic Parameters in Asthmatic Patients

	Parameter	Spearman's rank	*p-value*
IL-4 level pg/Ml	Total IgE IU/ml	-0.018	0.872
	Chlamydia pneumoniae IgE ng/L	0.006	0.958

This result was consistent with the previous research (Cernescu *et al.*, 2021), which mentioned no significant correlation between serum levels of

IL-4 and total serum IgE. On the other hand, the result was inconsistent with another previous study, which detected a relation between levels of serum IgE and IL-4 in (73%) of cases (Afshari *et al.*, 2007).

This finding could result from many reasons: Firstly, in most asthmatic patients, there was a correlation between the expression of the IL-4 gene and the level of serum IgE while they had normal serum IL-4. Secondly, cytokines are transient and non-permanent. The cause could be because under some conditions, IgE could be produced independently of IL-4 and IL-13. The previous results suggested that even without IL-4 and IL-13, alternative pathways to IgE may exist, enhanced by immunization, resulting in IgE effector responses *in vivo*. Finally, IL-15 can trigger STAT6 phosphorylation in mast cells, raising the possibility that cytokines other than IL-4 or IL-13 could use STAT6 to drive IgE production.

There was no preliminary evidence for the correlation between IL-4 levels and *Chlamydia pneumoniae* IgE levels in Iraqi asthmatic children. Thus, this correlation in this study provides the first evidence for a non-significant correlation between IL-4 levels and *Chlamydia pneumoniae* IgE levels in Iraq.

The previous mouse model study detected the role of IL-4 in early-life *Chlamydia pneumoniae* infection. The chronic and repeated diseases with *Chlamydia pneumoniae* increased IL-4 gene expression and thickness of airway subepithelial basement membrane in mice. On the other hand, the stimulation of IL-4 production was seen with only three of the seven *Chlamydia pneumoniae* strains infecting asthmatic children.

3.3.8. Comparison between Asthma Severity Groups Depending on Immunological Parameters in Asthmatic Children:

The study showed a high mean IL-4 level in severely asthmatic children (6.17 pg/ml) compared with mild and moderate asthma (4.886 pg/ml and 4.533 pg/ml, respectively) *p-value*=0.007, as shown in Table (3-11). Otherwise, the study didn't detect any significant difference between the asthma severity groups according to other immunological parameters (total IgE, *Chlamydia pneumoniae* IgG, and *Chlamydia pneumoniae* IgE).

Table (3-11): Comparison between Asthma Severity Groups Depending on Immunological Parameters in Asthmatic Children

Parameter	Severity	Number[b]	Mean ± S.D	ANOVA	*P-value*
Total IgE IU/ml	Mild	31	400.788±216.345	0.009	0.991
	Moderate	42	397.746±238.307		
	Severe	10	408.653±236.34		
Chlamydia pneumoniae IgG ng/L	Mild	31	26.988±23.538	0.685	0.507
	Moderate	42	22.587±10.966		
	Severe	10	26.588±10.315		
Chlamydia pneumoniae IgE ng/L	Mild	31	8.279±5.448	0.623	0.539
	Moderate	42	8.300±4.514		
	Severe	10	6.503±2.978		
IL-4 level pg/mL	Mild	26	2.184± 2.852	5.260[a]	0.007*
	Moderate	39	3.116±3.166		
	Severe	10	6.171±4.779		

*. significant at the 0.05 level, [a]: 8-sample excluded because its outliers,
[b]: 4-Sample excluded because its new

The study showed no significant association between total serum IgE levels and asthma severity (*P-value*= 0.991). The results were consistent with previous study which did not find a significant association between serum total IgE levels and asthma severity or airflow limitation, except for a higher percentage of patients with IgE > 400 IU/mL in the severe subgroup (Davila *et al.*, 2015). On the other hand, the results were inconsistent with previous results (Sandeep *et al.*, 2010, Kumar *et al.*, 2017, Trivedi and Patel, 2020), which mentioned that the total serum IgE levels increased as the severity of asthma increased.

Compared with the previous results, the difference in the current results was because mast cell-bound IgE could be a more sensitive indicator of IgE production in vivo than serum IgE levels since the half-life of IgE in serum is only 8–12 h. Still, in tissues, it extends to 6 days.

However, global multicentre clinical trials, such as the European Network For Understanding Mechanisms of Severe Asthma (ENFUMOSA, 2003) and the Severe Asthma Research Program, showed no correlation between levels of total and antigen-specific IgE and asthma severity.

The current study showed a high IL-4 level in severe asthma groups compared with mild and moderate asthma groups (P=0.046). The results were consistent with the previous studies (Poon *et al.*, 2012, Colley *et al.*, 2016); the previous study mentioned that various lines of evidence demonstrate the importance of IL-4 in allergic asthma in a subset of severe asthmatics with allergen-associated symptoms, eosinophil, and high serum IgE levels (Poon *et al.*, 2012). Furthermore, another study showed that severe asthmatic patients had a high level of IL-4 has been observed (Colley *et al.*, 2016). This finding may result from the fact that IL-4 is

involved in differentiating and stimulating Th2 cells, synthesizing IgE, and activating macrophages.

Serum IL-4 levels indicate a predisposition to atopic status. As there was an association between serum IL-4 levels and the degree of asthma severity, serum IL-4 levels can provide helpful information regarding the severity of asthma and the persistence of bronchial hyperresponsiveness in later adulthood. Quantitative measurement of IL-4, when integrated with other clinical indicators, can be used to predict the development of asthma and risk stratification. It can help choose treatment modalities, including using anti-IL-4 therapy with pascolizumab. Pascolizumab is a humanized anti-IL-4 monoclonal antibody that can inhibit upstream and downstream events associated with asthma, including TH2 cell activation and IgE production.

The present study detected no significant difference in *Chlamydia pneumoniae* IgG and IgE between asthma severity. This finding was consistence with a previous study in Iraq which noticed that the difference was statistically insignificant between mild, moderate, and severe asthmatic children with *Chlamydia pneumoniae* infection (Iman Kadhim *et al*., 2020). Nevertheless, this result inconsistence with previous studies that mentioned that *Chlamydia pneumoniae* IgE was strongly and positively associated with asthma severity (Hahn *et al.*, 2011, Chaplin, 2020). The explanation of this difference may result from the earlier studies that used the immunoblotting technique (which is more sensitive to antibody levels) to measure *Chlamydia pneumoniae* IgE. In contrast, our study used the ELISA technique.

3.3.9. Comparison between Control Status of Asthma Groups Depending on Immunological Parameters in Asthmatic Children:

The study showed a high IL-4 level at not well-controlled asthmatic patients (11 pg/ml) compared with well and partially-controlled (4.844 pg/ml and 4.329 pg/ml, respectively) *p-value*=0.004, as shown in Table (3-12). On the other hand, the study didn't detect any significant difference between the asthma control groups according to other immunological parameters (Total IgE, *Chlamydia pneumoniae* IgG, and *Chlamydia pneumoniae* IgE).

This study found no association between total serum IgE levels and the control status of asthma. This result was inconsistent with other research, which detected those asthmatic patients with high IgE levels exhibited worse asthma control than patients with low IgE levels (Yuan *et al.*, 2021). This difference may be because this study was performed on asthmatic children with high total IgE levels only (more than 100 IU/ml).

The study results revealed that the level of IL-4 in asthmatic children on well and partially-controlled therapy was lower than in patients with not well-controlled (P = 0.004). Not well-controlled asthma is difficult-to-treatment asthma, uncontrolled despite prescribing medium or high ICS with a second controller (usually a LABA) or requires high dose treatment to maintain reasonable symptom control and reduce the risk of exacerbations. The cause of this result is that IL-4 levels were elevated in severe persistent asthma since severe persistent asthma was a subset of difficult-to-treat asthma (not well-controlled).

Table (3-12): Comparison between Control Status of Asthma Groups Depending on Immunological Parameters in Asthmatic Children

Parameter	Control	Number	Mean ± S.D	ANOVA	*P-value*
Total IgE IU/ml	Well controlled	33	416.66±230.947	0.139	0.936
	Partial controlled	33	386.243±240.887		
	Not well controlled	13	403.085±215.956		
	Without controlled	8	370.922±204.297		
Chlamydia pneumoniae IgG ng/L	Well controlled	33	24.338±17.972	0.255	0.858
	Partial controlled	33	26.767±18.107		
	Not well controlled	13	23.157±11.062		
	Without controlled	8	22.335±13.827		
Chlamydia pneumoniae IgE ng/L	Well controlled	33	7.612±4.901	0.764	0.517
	Partial controlled	33	8.951±5.170		
	Not well controlled	13	7.611±3.618		
	Without controlled	8	6.715±1.883		
IL-4 level pg/mL[b]	Well controlled	31	2.624±5.252	13.256	.004**
	Partial controlled	33	2.566±6.458		
	Not well controlled	12	5.625±10.410		
	Without controlled	8	.10±1.136		

* Significant at the 0.01 level, [b] used Kruskal Wallis Test

The present study indicated no effect of *Chlamydia pneumoniae* IgG and IgE levels on the control status of asthma. Those results were inconsistent with the previous meta-analysis research, which suggested that *Chlamydia pneumoniae*-specific IgE may have a quantitatively crucial pathogenetic

mechanism in asthma, particularly in moderate/partly controlled and severe/uncontrolled asthma (Hahn, 2021). However, this meta-analysis study has limitations, including issues with a diversity of diagnosis, patient selection, and biomarker methodology in the source material, all of which may contribute to analytic heterogeneity.

3.4. Molecular Characterization of SNPs (PCR-FFLP):

3.4.1. Genomic DNA Extraction:

DNA was extracted as mentioned in 2.8.2.1.1. The quality and integrity were checked by agarose gel electrophoresis before performing a PCR reaction (Figure 3-8). All DNA extracts showed a single bright band. No signal DNA degradation was seen, such as smearing.

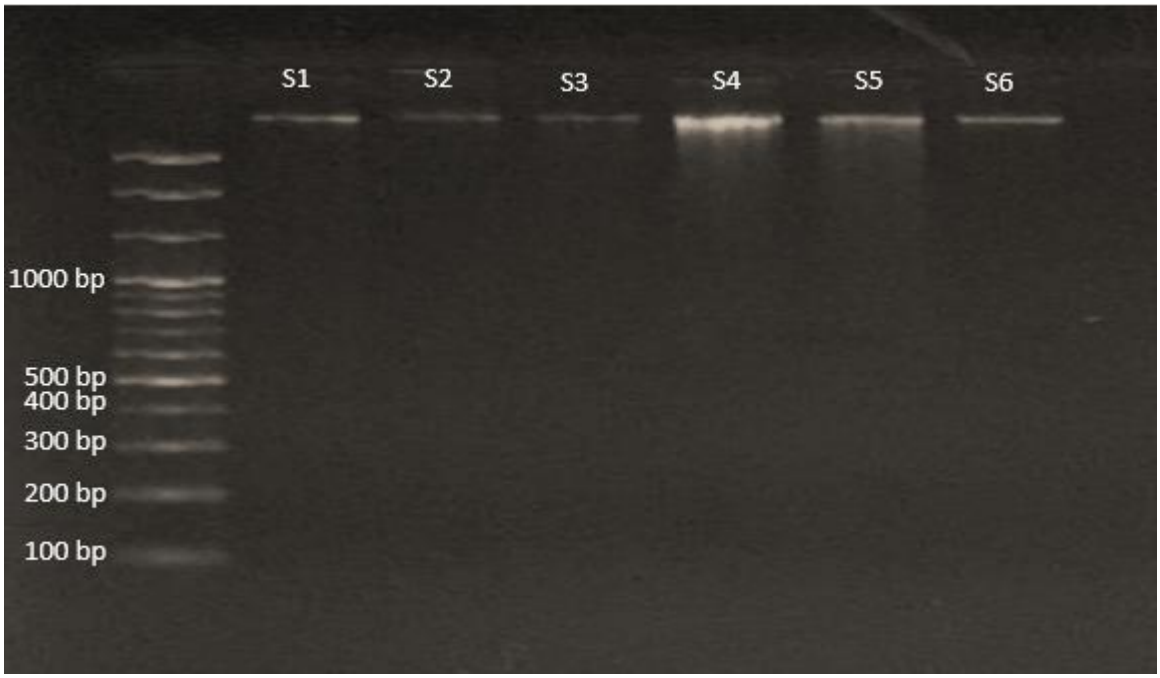

Figure (3-8): Evaluation of DNA Extract Quality and Integrity. 1.5 % Agarose Gel Electrophoresis of Genomic DNA in 70-Volt, 2-Hours

3.4.2. IL-4 C-589T Gene Polymorphism

3.4.2.1. IL-4 Gene Amplification:

The gene segment containing the possible single nucleotide polymorphism within the IL-4 gene region was amplified from extracted DNA for each asthma and healthy control patient (174 samples). By a specific primer, the polymerase chain reaction was performed under optimum conditions; then, the PCR product was electrophoresis on agarose gel 1.5%. The results were a single clear band with a molecular size of 198 bp. The size of the amplicon was determined by comparison with the DNA ladder 100 bp (as shown in Figure 3-9).

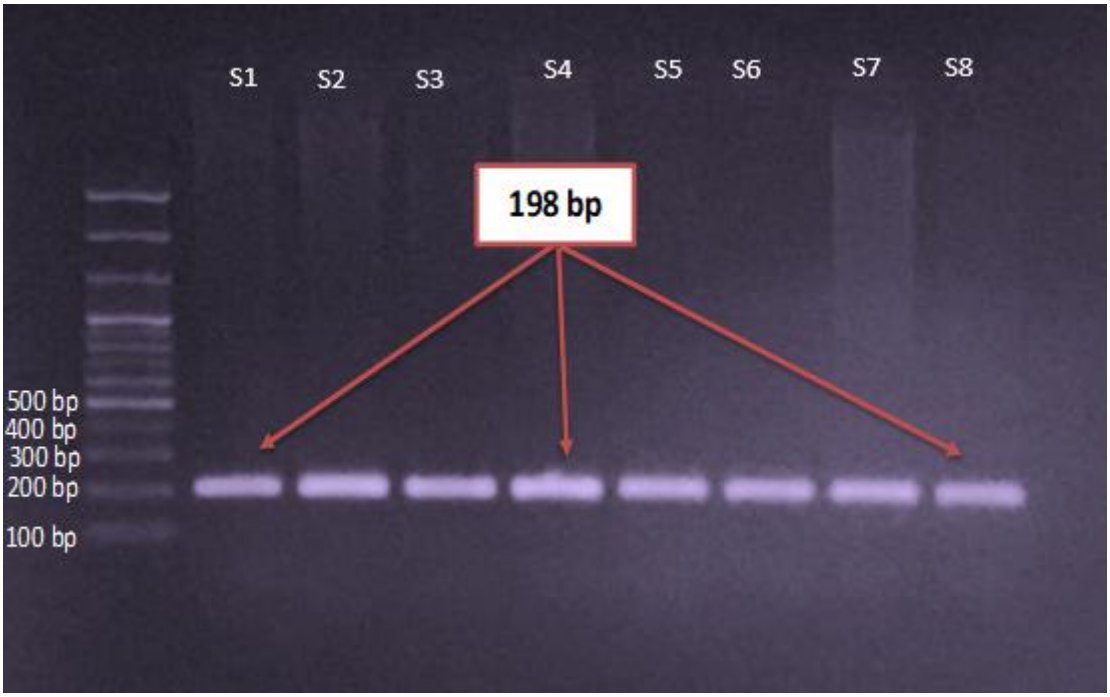

Figure (3-9): Gel Electrophoresis for PCR Product of IL-4 Gene. 198bp with DNA Ladder 100bp on Agarose Gel 1.5% in 70-Volt, 90 min.

3.4.2.2. Genotype of IL-4 C-589T Gene by PCR-RFLP:

IL-4 C-589T gene PCR product digested with BslF I, sibenzyme, the sequence of the restriction 5-GGGAC(N)10…3-, 3-CCCTG(N)14….5- in

C allele (Chiang *et al.*, 2007). The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) product was recognized on gel electrophoresis as a homozygote TT 198bp without any digestion, in the homozygote CC, two different fragments 120bp and 78bp, while three other fragments in the heterozygous form CT its (198, 120, and 78) bp (Figure 3-10).

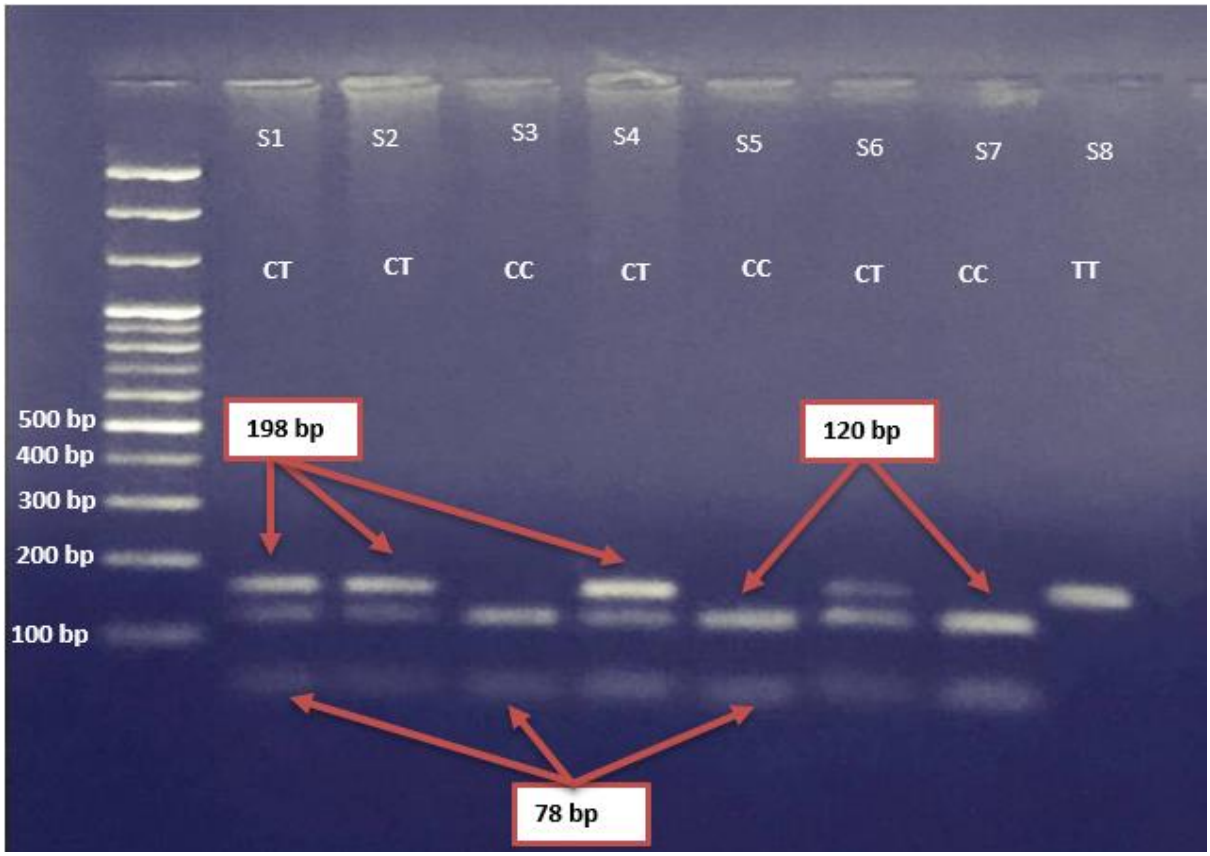

Figure (3-10): Gel electrophoresis of PCR-RFLP product, which illustrated genotype of IL-4 SNP on agarose 1.5% in 70 volts, 150 min. The restriction process showed three types of genotype CC (120, 78 bp), CT (198bp, 120 bp, and 78bp), and TT (198bp).

Genetic polymorphism of the IL-4 C589T gene was observed with three genotypes (CC, CT, and TT) (as shown in Table 3-13), which illustrated the distribution of genotypes of IL-4 in asthmatic patients and healthy control. The homozygous genotype CC recorded higher frequency

in healthy children (82.75%) than in asthmatic patients (64.4%), with a significant difference (*P-value*=0.006). Homozygote genotype TT frequency (1.1%) was non-significant in patients compared to the control subject (0%), and the genotype heterozygous CT frequency (34.5%) was significant in patients compared to control (17.24%). The allele frequency for allele C was (81.61%) in patients compared with control (91.38%), while for the allele T was (18.39%) in patients compared with control (8.6%) with a highly significant difference (*P-value*=0.01, as in Table 3-13).

Genotype and allele frequency (as shown in Table 3-13) found that, in the asthmatic children, the frequency of genotype CC recorded odds ratio (OR) of 0.376 with a confidence intervals (CI) value between 0.185-0.764 under 95%. The genotype CT recorded OR=2.526 with CI between 1.241-5.141 under 95%. The frequency of genotype TT recorded OR=3.041 with CI between 0.217-0.805 under 95%. The allele frequency of C showed OR=0.419 with a CI range between 0.217-0.805 under 95%. Thus, the T allele and CT genotype can be a risk factor for IgE-mediated asthma.

Table (3-13): Genotype and Allele Frequency of IL-4 SNP among Asthmatic Patients and Controls

IL-4 C-589T	Asthmatic patients N (%)	Healthy control N (%)	O. R^	*P-value*	(95%CI)
CC	56 (64.4%)	72 (82.75%)	0.376	0.006**	0.185-0.764
CT	30 (34.5%)	15 (17.24%)	2.526	0.01*	1.241-5.141
TT	1 (1.1%)	0 (0%)	3.041	0.49	0.122-75.867
C Allele	142 (81.61%)	159 (91.38%)	0.419	0.009**	0.217-0.805
T Allele	32 (18.39%)	15 (8.6%)			

^=Odds ratio with 95% confidential interval, ∗ Significance level at *p-value* ≤0.05, ** Highly Significance level at *p-value* ≤0.01

The current study indicated that a possible involvement of single nucleotide polymorphism in the IL-4 gene in the development of asthma, which was consistent with previous meta-analysis studies which revealed that IL-4 gene -589C/T polymorphism was a susceptibility risk of asthma (Jiang *et al.*, 2020, Kousha *et al.*, 2020, Jin and Zheng, 2021). However, contradictory studies were found regarding the association of IL-4 C-589T with asthma susceptibility (de Faria *et al.*, 2008, Jiang *et al.*, 2009, Liu *et al.*, 2022). This study, consistent with other studies in Iraq (Mahmood and Abdulla, 2021, Hassan *et al.*, 2022), showed significant differences in the frequency distributions of the heterozygous CT among asthmatic and healthy control.

On the other hand, other previous studies in Iraq revealed that the increased risk of asthma was associated with the C allele and the CC genotype (Hussein and Jaber, 2017, Abood and Mohanad, 2018). The present study was inconsistent with these previous findings, as the current study detected that the T allele and CT genotype were associated with asthma. The cause of this difference that the other authors performed their study on asthmatic adults, which may lead to increased interference of environmental factors in developing asthma more than gene factors.

However, the recent results were consistent with those reported by studies of asthmatic patients in Egypt (El-Shal *et al.*, 2022), Iran (Alyasin *et al.*, 2021), Taiwan (Chiang *et al.*, 2007), Russia (Gervaziev *et al.*, 2006), Italia (Ricciardolo *et al.*, 2013), European and African Americans (Donfack *et al.*, 2005, Haller *et al.*, 2009), China (Huang *et al.*, 2012), and Pakistan (Micheal *et al.*, 2013). These studies detected that the T allele frequency for the C-589T IL-4 gene promoter in asthma patients was higher than for normal subjects ($P < 0.001$).

In contrast, the present results were inconsistent with other previous studies which showed no or weakly significant association between IL-4 589 C <T polymorphism and asthma. In Kuwait (Hijazi and Haider, 2000), Egypt (Hussein *et al.*, 2020), Hong Kong Chinese (Mak *et al.*, 2007), Iceland (Hakonarson *et al.*, 2001), Chine (Liu *et al.*, 2022), India (Bijanzadeh *et al.*, 2010), and north Iran (Rad *et al.*, 2010).

On the other hand, some previous studies suggested polymorphism in a combination of multiple genes might magnify the impact on disease risk. Carriers of both IL-4 -590C/T TT and Arg130Gln locus AA genotypes significantly increased the risk of asthma compared with those who are carriers of either IL-4 -590C/T TT or Arg130Gln locus AA genotype (Zhang *et al.*, 2019). Further, only the combined analyses of genetic alterations in the IL-4 C589T/IL-13 C-1112T pathway reveal its actual significance to the development of atopy and childhood asthma (Kabesch *et al.*, 2004, Li *et al.*, 2014).

The differences in the age groups, race of study subjects, limited sample sizes, diversity in the diagnostic criteria of the patients, gene-to-gene interaction, and gene-to-environmental interaction may be the cause of such inconsistent results.

The results of IL-4 SNPs of asthmatic children and healthy control were in good agreement with the Hardy- Weinberg equilibrium (According to the website OEGE - Online Encyclopedia for Genetic Epidemiology studies) (Rodríguez *et al.*, 2006), and no significant variation between the observed and expected genotypes frequencies was observed, as shown in Table (3-14).

Table (3-14): Expected Frequencies of Genotypes of the IL-4 SNP using Hardy-Weinberg Equilibrium

Gene Position	Genotype	Observed asthmatic patients	Expected asthmatic patients	*p-value*	Observed healthy control	Expected healthy control	*p-value*
IL-4 C-589T	**CC**	56	57.49	0.38	72	72.65	0.67
	CT	30	26.11		15	13.71	
	TT	1	2.94		0	0.9	

3.4.2.3. Association between IL-4 C-589T Gene and Asthma Characteristics

3.4.2.3.1. Association between Asthma Severity and IL-4 C-589T Genotypes in Asthmatic Children:

Depending on asthma severity, there was a highly significant difference between asthma severity groups depending on IL-4 C589T genotypes (*p-value* <0.001). A high frequency of CC IL-4 C589T genotype was found in mild asthma compared with moderate and severe asthma (80.65%, 57.14%, and 40%, respectively). In contrast, a high frequency of CT IL-4 C598T genotype was found in severe asthma compared with mild and moderate asthma (60%, 19.35%, and 40.47%, respectively), as shown in Table (3-15).

Further, there was a highly significant difference between asthma severity groups depending on the IL-4 allele (*p-value* =0.002). A high frequency of the C allele was detected in mild asthma. In contrast, a high frequency of the T allele was seen in severe asthma (Table 3-15).

Table (3-15): Association between IL-4 C-589T Genotypes and Asthma Severity in Asthmatic Children

IL-4 C-589T genotypes	Asthma Severity[b]			Chi-square	*P-value*
	Mild N=31	Moderate N=42[a]	Severe N=10		
CC	25 (80.65%)	24 (57.14%)	4 (40%)	35.083	<0.001**
CT	6 (19.35%)	17 (40.47%)	6 (60%)		
C Allele	56 (90.32%)	65 (77.38%)	14 (70%)	12.417	0.002**
T Allele	6 (9.67%)	19 (22.62%)	6 (30%)		

** Highly Significance level at *p-value* ≤0.01, [a]: excluded 1 sample with TT genotype, [b]: four sample with missing data was excluded

This result was consistent with previous studies, which detected that the IL-4-589 T allele was significantly increased in the subjects with severe asthma compared with mild-to-moderate asthma subjects (Sandford *et al.*, 2000, Shang *et al.*, 2016). The reason for the involvement of the IL-4 gene polymorphism in modulating asthma severity remains to be determined.

3.4.2.3.2. Association between Asthma Controlling and IL-4 C-589T Genotypes in Asthmatic Children:

The present study showed that a highly significant difference in asthma-controlling status depending on IL-4 C589T genotypes (p =0.001), as shown in Table (3-16). The partially controlled groups of asthmatic

children had a high frequency of CC IL-4 C589T genotypes compared with well and not-well-controlled asthmatic groups (69.7%, 63.64, and 46.15%, respectively). A high frequency of CT IL-4 C589T genotypes was found in not well-controlled asthmatic groups compared with well and partial-controlled groups (53.85%, 36.36, and 27.27%, respectively).

Table (3-16): Association between Asthma Controlling and IL-4 C-589T Genotypes in Asthmatic Children

IL-4 C-589T genotypes	Asthma Controlling[b]			Chi-square	*P-value*
	Well controlled N=33	Partial controlled N=33[a]	Not well controlled N=13		
CC	21 (63.64%)	23 (69.7%)	6 (46.15%)	14.846	0.001**
CT	12 (36.36%)	9 (27.27%)	7 (53.85%)		
C Allele	54 (81.82%)	55 (83.33%)	19 (73.08%)	3.700	0.157
T Allele	12 (18.18%)	11 (16.67%)	7 (26.92%)		

** Highly Significance level at *p-value* ≤0.01, [a]: one sample with TT genotype was excluded, [b]: 8 sample without control was excluded

Additionally, there was no significant difference in asthma-controlling status groups depending on the IL-4 allele (*p-value* =0.157).

The cause of this result was that the CT genotype of the IL-4 gene was associated with severe asthma (Table 3-15) since severe persistent asthma is a subset of difficult-to-treat asthma (not controlled).

3.4.2.3.3. Association between Immunological Parameters and Genotype of IL-4 C-589T Gene in Asthmatic Children:

The study showed a high association between high total serum IgE levels and the CT genotype of IL-4 C589T (*p-value*=0.02). In contrast, high

Chlamydia pneumoniae IgE levels were found to be associated with CC genotypes (*p-value*= 0.01), as presented in Table (3-17).

On the other hand, there were no significant differences in serum IL-4 levels and *Chlamydia pneumoniae* IgG levels depending on different IL-4 C589T genotypes *(p-value*= 0.145 and 0.662, respectively).

The present study was consistent with previous studies which detected that the higher level of IgE was associated with the T allele. The total serum IgE level in the TT genotype was significantly higher than that in CT and CC genotypes (Korzycka-Zaborowska *et al.*, 2015, Zhang *et al.*, 2019). It has been shown that the T allele of this SNP leads to increased affinity of the binding of transcription factors compared to the C allele, leading to overexpression of IL-4 mRNA. IL-4 has the ability to promote B cells activation to secrete IgG1 and IgE, promote the transformation of IgG to IgE, and IgE-mediated immune response enhancement.

Table (3-17): Association between Immunological Parameters and Genotype of IL-4 C-589T Gene in Asthmatic Children

Parameters mean± S.D	**IL-4 C-589T genotypes[a]**		**T-test**	***p-value***
	CC 56 (64.36%)	**CT 30 (34.48%)**		
Total IgE IU/ml	359.85±205.027	478.99±249.18	-2.380	0.02*
IL-4 level pg/mL	3.202±4.125	4.788±5.488	-1.471	0.145
***Chlamydia pneumoniae* IgE ng/L**	8.555±4.889	6.459±2.403	2.649	0.01*
***Chlamydia pneumoniae* IgG ng/L**	22.895±10.839	21.834±10.068	0.439	0.662

*: significant difference at *P-value* ≤0.05, [a]: One sample was excluded because it is TT genotype

The current result was inconsistent with the previous study, which detected that 589T alleles of the IL-4 gene were likely related to a high level of IL-4 in serum (Shang *et al.*, 2016). This difference was because additional polymorphic loci in the IL-4 gene should be investigated to verify the role of polymorphism on IL-4 levels since other loci can affect IL-4 levels. Polymorphism at the −33 locus may be associated with the IL-4 level. IL-4R α2 controls the IL-4 signalling pathway in early-life *Chlamydia pneumoniae* infection.

This is the first study in Iraq and the world that detect the correlation between IL-4 C589T polymorphism and the presence of *Chlamydia pneumoniae* IgG and IgE antibodies in asthmatic children.

For the *Chlamydia pneumoniae* IgG antibody, there was no significant difference in antibody levels between IL-4 genotypes. Thus, the IL-4 polymorphism at the C589T site did not affect persistent *Chlamydia pneumoniae* infection, and it's not the cause to increase *Chlamydia pneumoniae* IgG antibody in asthmatic than in healthy children (Table 3-17).

On the other hand, the *Chlamydia pneumoniae* IgE antibody was at a higher level in the CC genotype. Since the CC genotype was in higher frequency in non-asthmatic children, therefore; the development of *Chlamydia pneumoniae* IgE antibody in asthmatic children didn't depend on IL-4 polymorphism. The possible explanation is that the other molecular mechanisms and genetic polymorphisms may influence IL-4 levels and IgE class switching.

Conclusions and Recommendations

Conclusions:

- Children with asthma have more persistent *Chlamydia pneumoniae* infections than non-asthmatic children.
- The presence of *Chlamydia pneumoniae*-specific IgE will provide ongoing stimulation of allergic responses by *Chlamydia pneumoniae*.
- Drooping of *Chlamydia pneumoniae* IgE level in asthmatic adolescents who live in urban.
- High IL-4 level was associated with more severe asthma and not welled control in children.
- Asthma in children has been associated with the site-specific IL-4 C589T genotype in Iraqi asthmatic children.
- CT genotype of IL-4 C589T association with high more severe asthma and not welled control in children.
- CT genotype of IL-4 C589T association with high total IgE levels but not IL-4.
- IL-4 C589T polymorphism was not implicated in developing high *Chlamydia pneumoniae* IgE antibodies in asthmatic children.

Recommendations:

- Measuring *Chlamydia pneumonia* infection in Iraqi children with asthma to prevent the development of asthma in the future and to better manage asthma.
- Use of *Chlamydia pneumoniae*-specific IgE in an allergy panel test in Iraq.
- The pathophysiology of *Chlamydia pneumoniae* infection in asthma could be studied.

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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 . . . .

1-Budesonide/F 2- Beclomethasone 3- Fluticasone/S

Asthma Severity:

A- Mild B- Moderate C- Severe

Control:

A-Well controlled B- Partially controlled C- Not well controlled

History : Personal (hx.)

Eczema Yes NO Shortness of breath Yes NO

Wheezing Yes NO Allergic Rhinitis Yes NO

Cough Yes NO Allergic conjunctivitis Yes NO

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

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

Family (hx.)

Asthma Father Mother

Allergic Rhinitis: Father Mother

Food allergy: Father Mother Type

Drug allergy: Father Mother Type

Animal in the house Yes NO Type: . . . . . .

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 - Fume Yes NO

-Others

Parameter:

Total serum IgE ……

Serum IL-4 level ……

Chlamydia pneumoniae IgG……...

Chlamydia pneumoniae IgE……...

IL-4 C589T polymorphism……...

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:

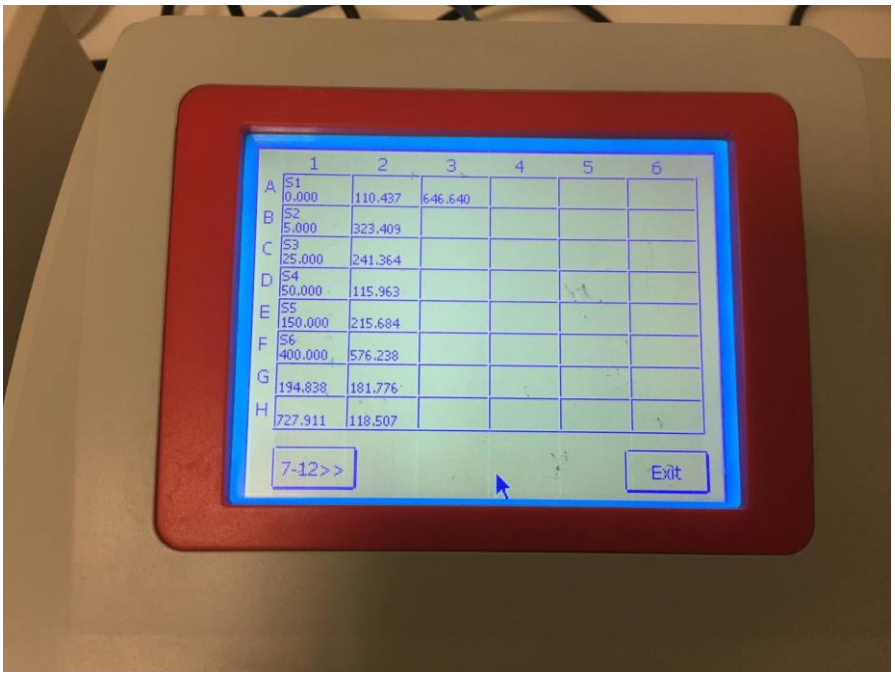
Total serum IgE ……

Chlamydia pneumoniae IgG……...

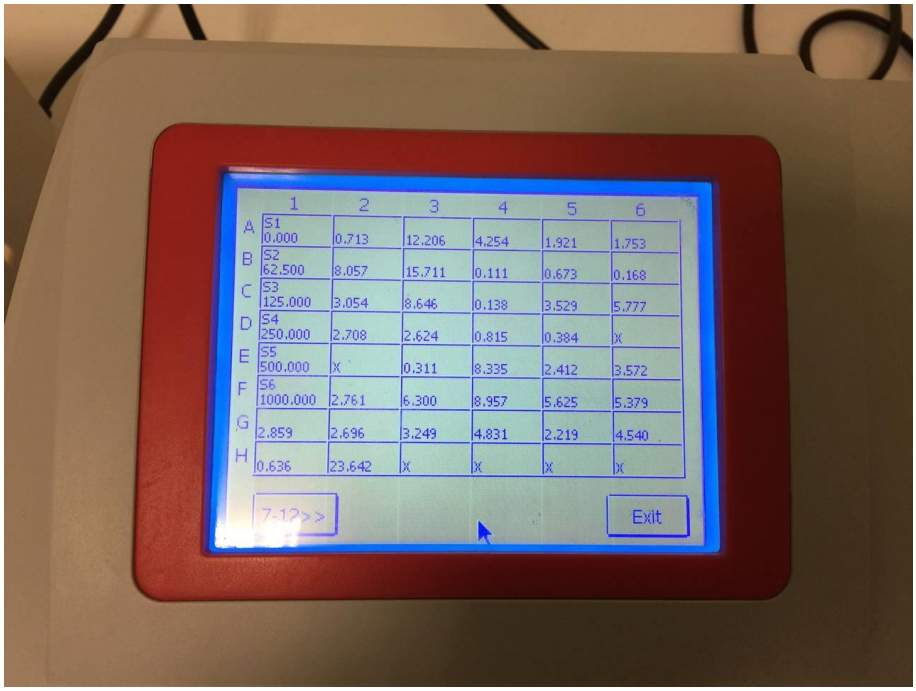
Chlamydia pneumoniae IgE……...

IL-4 C589T polymorphism……...

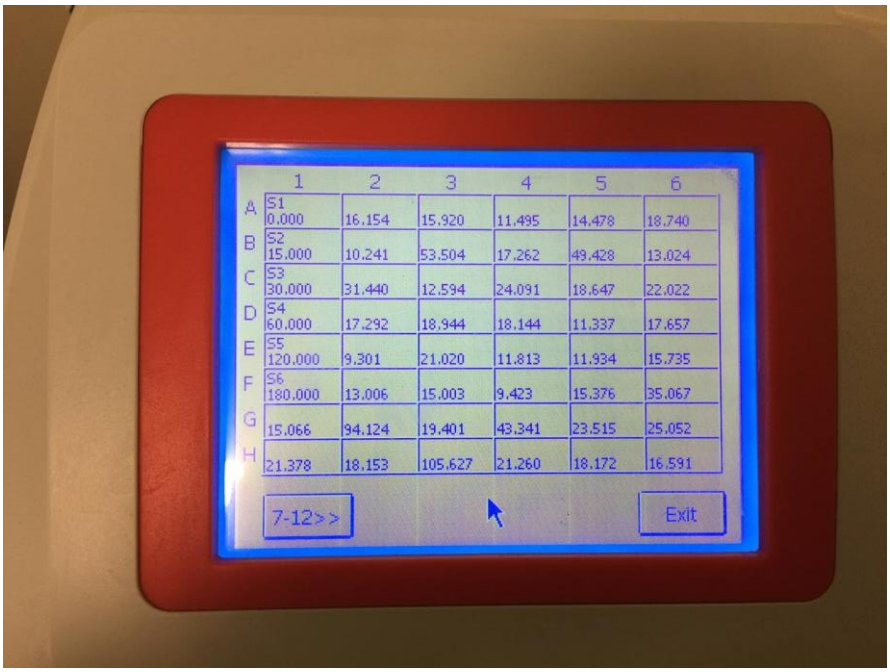
Appendix III: ELISA results of total serum IgE levels



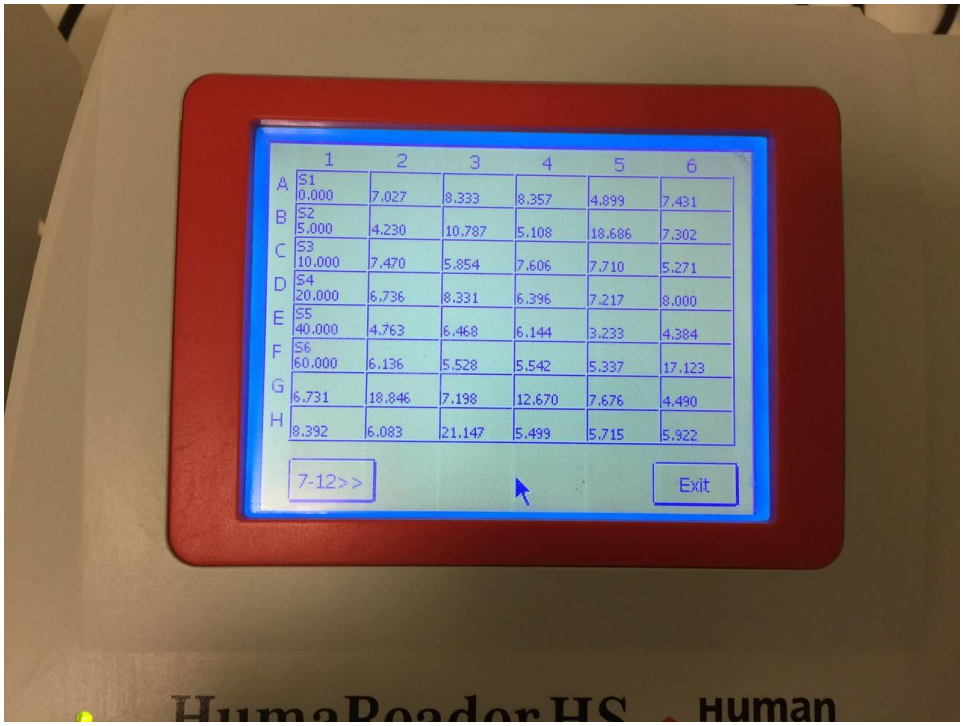
Appendix IV: ELISA results of serum IL-4 levels



Appendix V: ELISA results of *Chlamydia pneumoniae* IgG levels



Appendix VI: ELISA results of *Chlamydia pneumoniae* IgE levels

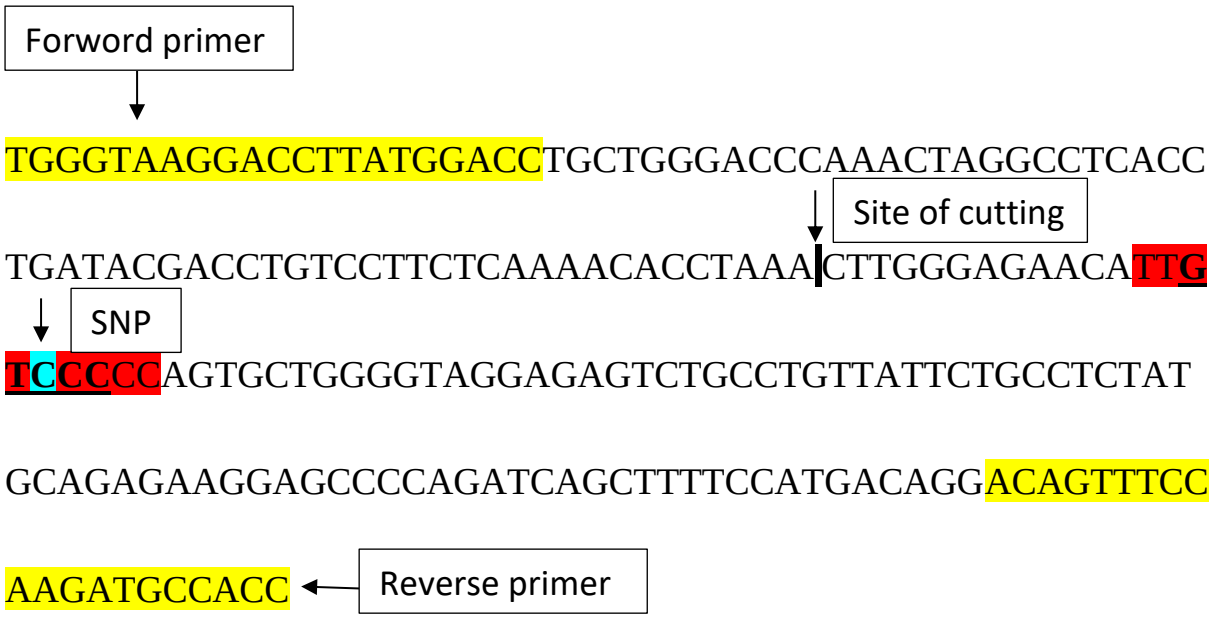


Appendix VII: Interleukin 4 gene [Homo sapiens (human)]

IL-4 C-590T rs2243250

Region of SNP: 132673367 to 132673565 (198pb)

Region of IL-4: 132673989 to 132682678 (8689pb)



الخلاصـــة

الربو هو اضطراب التهابي مزمن في الشعب الهوائية في الرئتين. تعتبر مشكلة صحية عالمية كبرى، فقد أثرت على ما يقدر بنحو 262 مليون شخص في عام 2019. تم إجراء دراسة جزيئية ومناعة للكشف عن العلاقة بين الربو وعدوى المتدثرة الرئوية. بالاضافة الى تحديد تأثيرات المتغيرات الجينية لجين IL-4 C589T على إجمالي مصل IgE ومستوى IL-4 في الدم ومستويات IgG و IgE لمتدثرة الرئوية وشدة الربو والتحكم في الربو.

تم إجراء دراسة الحالات والشواهد على 174 شخصا. بعد إجراء اختبارات IgE، تم استبعاد المرضى الذين لديهم IgE <100 IU / ml (41 مريضا) لأن هذه الدراسة مصممة للربو بوساطة IgE ، و 100 IU / ml هي القيمة الفاصلة لمستوى IgE الإيجابي. شملت هذه الدراسة 87 طفلاً مصابًا بالربو (57 ذكرًا و 30 أنثى) حضروا عيادة الربو في مستشفى كربلاء التعليمي للأطفال في الفترة الممتدة من كانون الثاني (يناير) 2022 إلى أيار (مايو) 2022. تراوحت أعمارهم بين سنة إلى ستة عشر عامًا. ضمت المجموعات الضابطة 87 طفلاً (46 ذكرًا و 39 أنثى) متطابقين في العمر والجنس مع المرضى ، تم اختيارهم عشوائيًا من المجتمع المحلي. جمع استبيان البيانات الديموغرافية والسريرية من المرضى و / أو والديهم. تم جمع الأمصال والدم الكامل من كل مشارك ، مع ملاحظة أنه تم استخدام المصل لتحديد مستويات IgE الكلية في الدم ، ومستويات IgG و IgE لمتدثرة الرئة البشرية لجميع العينات ومستوى IL-4 للأطفال المصابين بالربو فقط. بينما تم استخدام الدم الكامل لاستخراج الحمض النووي. تم استخدام استخراج الحمض النووي للكشف عن تعدد الأشكال في جين IL-4 (IL-4 C-589T) باستخدام تقنية RFLP-PCR، وتم تحليل البيانات إحصائيًا بواسطة برنامج SPSS الإصدار 21 .

أظهرت الدراسة فرقاً معنوياً عالياً في مستويات IgG و IgE لمتدثرة الرئة البشرية بين الأطفال المصابين بالربو والمجموعة الضابطة (القيمة الاحتمالية <0.001 و 0.024 على التوالي). علاوة على ذلك ، في الأطفال المصابين بالربو ، كان هناك ارتباط خطي إيجابي معنوي بين مستوى IgE الكلي في المصل و IgE للمتدثرة الرئوية تحت السيطرة على العمر (القيمة الاحتمالية p = 0.019). في نفس الخط ، كان هناك علاقة خطية إيجابية ذات دلالة إحصائية بين IgG المتدثرة الرئوية و IgE المتدثرة الرئوية في الأطفال المصابين بالربو (القيمة الاحتمالية p <0.001). بالإضافة إلى ذلك ، كان مستوى IL-4 أعلى عند الاطفال الذين لديهم ربو حاد واللذين ليس لديهم سيطرة جيدة على الربو مقارنة بالمجموعاتالاخرى من الاطفال المصابين بالربو (القيمة الاحتمالية = 0.007 و 0.004 ، على التوالي).

بالإضافة إلى ذلك ، أوضحت الدراسة أن تواتر النمط الجيني IL-4 CT متغاير الزيجوت كان معنويا في المرضى (34.5٪) مقارنة بمجموعة التحكم (17.24٪). لذلك ، يمكن اعتبار التركيب الوراثي المقطعي المحوسب عامل خطر للإصابة بالربو (نسبة الأرجحية = 2.526 ، القيمة الاحتمالية = 0.01). أظهر تحليل الأنماط الجينية باستخدام توزيع هاردي-واينبرغ عدم وجود فروق ذات دلالة إحصائية بين أعداد المرضى المرصودة والمتوقعة لجين IL-4 C589T. في الأطفال المصابين بالربو ، كانت هناك ارتباطات ذات دلالة إحصائية بين ارتفاع إجمالي مستوى IgE في الدم ، والربو الشديد ، وعدم السيطرة على الربو مع النمط الجيني CT لتعدد الأشكال IL-4 C589T (القيمة الاحتمالية 0.02 p، 0.001> ، و 0.001 ، على التوالي). في المقابل ، وجد أن مستويات IgE المرتفعة من المتدثرة الرئوية مرتبطة بالأنماط الجينية CC (القيمة الاحتمالية 0.01 = p).

الخلاصة ، يعاني الأطفال المصابون بالربو من عدوى الكلاميديا الرئوية المستمرة أكثر من الأطفال غير المصابين بالربو. قد يوفر وجود IgE الخاص بالكلاميديا الرئوية تحفيزًا مستمرًا للاستجابات التحسسية بواسطة المتدثرة الرئوية. انخفض مستوى IgE الخاص بالكلاميديا الرئوية في المراهقين المصابين بالربو الذين يعيشون في المناطق الحضرية. ارتبط ارتفاع مستوى IL 4 بربو أكثر حدة وعدم تحكم جيد في الأطفال. ارتبط الربو بالنمط الجيني IL 4 C589T لدى الأطفال العراقيين. يرتبط النمط الجيني IL 4 C589T بالربو الشديد وعدم التحكم الجيد في الأطفال. يرتبط النمط الوراثي IL 4 C589T بمستويات إجمالية عالية من IgE ولكن ليس IL-4. لم يكن IL-4 C589T متورطًا في تطوير أجسام مضادة نوع IgE خاصة بالكلاميديا الرئوية في الأطفال المصابين بالربو.

جـــمـهوريـة الـعـراق
وزارة التعليم العالي والبحث العلمي
جـــــامـــعـــة بـــابـــل
كـــلـــيـــة الـــطـــب

العلاقة بين تعدد الأشكال الوراثي والمتدثرة الرئوية عند الأطفال المصابين بالربو في العراق

أطروحـــة

مقدمة الى مجلس كلية الطب/ جامعة بابل وهي جزء من متطلبات نيل درجة دكتوراه الفلسفة في العلوم / الأحياء المجهرية الطبية

من قبل

رغده ميثم حميد مهدي

بكالوريوس كلية التقنيات الصحية والطبية / الجامعة التقنية الوسطى بغداد (٢٠١٤)

مـــاجـستـيـر / احياء مجهرية طبية / كلية الطب / جامعة كربلاء (٢٠١٩)

بأشراف

الأستاذ الدكتور

هدى هادي الحسناوي

فرع الأحياء المجهرية

كلية الطب/ جامعة بابل

الأستاذ الدكتور

حيدر عبد الأمير نجم عبود

فرع الأدوية

كلية الطب/ جامعة كربلاء

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