The National Ribat University
Faculty of Graduate Studies
and Scientific Research

Assessment of the level and Phenotype of Haptoglobin and Association between the Polymorphisms of A disintegrin & metalloprotease (ADAM) 33, Interleukin-4 promoter (-590), Interleukin-4 receptor promoter (-576) and Glutathione S-transferase (GSTT1&GSTPi) genes in Sudanese with asthma

A thesis submitted in fulfillment of the requirement of the Ph.D degree in Human Physiology

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To my parents
To the soul of my brother Hafiz

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Abstract

Introduction:
Asthma is an inflammatory disease that results from interactions between multiple genetic and environmental factors that influence both its severity and its responsiveness to treatment. Haptoglobin (Hp) is an acute phase protein and functions as an immune system modulator. Recent studies have revealed evidence for linkage of human chromosomes 5q23, 11q13, 16p12.1, 20p13 and 22q11.2 as regions likely to contain genes related to asthma. Among the candidate genes in these regions are the genes encoding for IL4, GSTPi, IL4Rα, ADAM33 and GSTT1.

Objectives:
To assess the level and common phenotype of Hp among asthmatic and control subjects. Also to assess the frequency of the mutant ADAM 33, IL4 (-590), IL4R α (-576), GSTT1 and GSTPi genes in asthmatic patients in comparison with healthy controls.

**Methods:**
A total of 63 patients with allergic asthma and 53 normal subjects were studied. Serum levels of IgE, IL-4 and Hp were measured by ELISA method. Serum Hp phenotypes were detected by using Polyacrylamide Gel Electrophoresis. DNA was extracted from blood using salting out method. Polymorphisms were determined by PCR method for GSTT1, PCR-RFLP method for ADAM33, IL4 and GSTPi, and allele specific PCR for IL4Rα.

**Results:**
Our results indicate that total level of serum IgE and IL4 in patients were considerably higher than controls. Serum electrophoresis showed that the distribution of Hp phenotypes of Hp1-1, Hp2-1 and Hp2-2 among asthmatic patients were 17.5%, 50.8% and 31.7%, respectively. Distributions of different Hp phenotypes in healthy controls were 7.6%, 66% and 26.4%, respectively. Serum Hp level was higher in asthmatics than controls (0.001).

The results showed that GSTT1 null genotype was higher (54%) than control (24.5%) (P=0.001). The mutant ADAM33 allele was higher (81%) than control (55%) (P=0.000), IL4 (-590) allele was higher (69.8%) than control (42.9%) (P=0.000), IL4R α (-576) allele was higher (57.1%) than control (48.1%) (P=0.170) and no significant differences of GSTPi allele between asthmatics (34.8%) and controls (29.2%) (p=0.358).

**Conclusion:**
The level of serum IgE and IL4 in patients were higher in asthmatic patients. Hp phenotypes have no role in activation of the disease, while Hp level was higher in asthmatic patients and in all asthma classes.

Gene polymorphism of ADAM33, IL4 (-590) and GSTT1 may be associated with the development of asthma in Sudan while IL4R α (-576) and GSTPi genes appears to play no role in the occurrence of the disease.
تهدف هذه الدراسة لتقييم مستوى النمط الظاهري للهابتوقلوبين بين مرضى الربو ومجموعته التحكم. أيضاً تقييم الوترة المتحولة للجينات ADAM33، IL4، IL4Rα (576-590) و GSTT1، GSTP1 في المرضى المصاصبين بالربو مقارنة مع الاصحاب.

الطريق:
أجريت هذه الدراسة المقطعية التحليلية في عدد 116 شخص في ولاية الخرطوم (63 مجموعة الدراسة) و 33 مجموعة التحكم. أجريت قياسات مستوي IgE (الطاقة الامينتقلوبيلين E) و اللابتوقلوبين في السيرم بواسطة ELIZA. و نوع اللابتوقلوبين باستخدام جل بولي أكريلاميد الكهربائي PAGE. تم استخلاص DNA من الدم و تحديد التغيير الجيني بواسطة PCR لجين IL4، GSTP1 و ADAM33. و allele specific PCR لجين IL4Rα. تم استخدام برنامج SPSS للتحليل الإحصائي.

النتائج:
عند مقارنة الأشخاص المصاصبين بالربو الشعبى (مجموعة الدراسة) مع الأشخاص الطبيعيين (مجموعة التحكم) نجد أن قياسات مستوي IgE و Hp و IL4 و وعمر المرضى الربو الشعبى. توزيع الجين الظاهري لللابتوقلوبين 1-1 و 1-2 و 2-2 عند مرضى الربو الشعبى هو %17.5 و %50.8 و %31.7 على التوالي و عند مجموعة التحكم هو %7.6 و %66 و %26.4 على التوالي.

و أظهرت النتائج أن النمط الجيني المتحول GSTT1 أعلى عند مرضى الربو (54%) من مجموعة ADAM33 (24.5%) من مجموعة التحكم (P=0.001). كان الأليل المتحول IL4Rα أعلى (576) أعلى (9.8%) من التحكم (42.9%) على (55%) (P=0.000). وكأن الأليل المتحول (590) أعلى (69.8%) من التحكم (P=0.000) (P=0.170) (P=0.358). لا وجود لفروق ذات دلالة للالهيل المتحول GSTP1 بين الربو (34.8%) والتحكم (29.2%).

الخاتمة:
قياسات مستوي الهابتوقلوبين Hp و IgE و IL4 و لابتوقلوبين الاحصائيات الاستقرائية، وفي مرضى الربو و ليس هناك اختلاف في النمط الظاهري لللابتوقلوبين

تعدد أشكال الجينات ADAM33، IL4، GSTT1. قد تتداخل مع الإصابة بمرض الربو في السودان، بينما جينات GSTP1 IL4Rα (P=0.000) (P=0.358).
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<td>Figure (3.11):</td>
<td>Mutant Allelic frequencies of IL4 (-590) polymorphism for asthmatic and control (%)</td>
<td>73</td>
</tr>
<tr>
<td>Figure (3.12):</td>
<td>The IL4 (-590) PCR product on 3% agarose</td>
<td>73</td>
</tr>
<tr>
<td>Figure (3.13):</td>
<td>Mutant allelic frequencies of IL4Rα polymorphism for asthmatic and control (%)</td>
<td>75</td>
</tr>
<tr>
<td>Figure (3.14):</td>
<td>The IL4Rα (-576) PCR product and analysis of polymorphism on 3% agarose</td>
<td>75</td>
</tr>
<tr>
<td>Figure (3.15):</td>
<td>GSTT1 null genotype frequencies for asthmatic and control (%)</td>
<td>76</td>
</tr>
<tr>
<td>Figure (3.16):</td>
<td>Analysis of GSTT1 polymorphism on 3% agarose</td>
<td>77</td>
</tr>
<tr>
<td>Figure (3.17):</td>
<td>Allele and genotype frequencies for GSTPi SNPs</td>
<td>78</td>
</tr>
<tr>
<td>Figure (3.18):</td>
<td>The GSTPi Ile105Val PCR product on 3% agarose</td>
<td>79</td>
</tr>
<tr>
<td>Figure (3.19):</td>
<td>Analysis of Ile105Val polymorphism on 3% agarose</td>
<td>79</td>
</tr>
<tr>
<td>Figure (3.20):</td>
<td>Combination of various risk mutant genotypes of 5 genes</td>
<td>80</td>
</tr>
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</table>
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHR</td>
<td>airway hyperresponsiveness</td>
</tr>
<tr>
<td>GSTM1</td>
<td>glutathione S-transferase mu 2 (muscle)</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-Lymphocyte associated protein 4</td>
</tr>
<tr>
<td>SPINK5</td>
<td>Serine protease inhibitor Kazal-type 5</td>
</tr>
<tr>
<td>IL-4Rα</td>
<td>Interleukin 4 receptor alpha</td>
</tr>
<tr>
<td>ADAM 33</td>
<td>A disintegrin and metalloprotease 33</td>
</tr>
<tr>
<td>ADRB2</td>
<td>adrenergic beta-2-receptor</td>
</tr>
<tr>
<td>BHR</td>
<td>bronchial hyperactivity</td>
</tr>
<tr>
<td>SPT</td>
<td>skin prick test</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
</tr>
<tr>
<td>CC16</td>
<td>Clara cell 16</td>
</tr>
<tr>
<td>CCL</td>
<td>CC chemokine ligands</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>NOD1</td>
<td>nucleotide-binding oligomerization domain containing 1</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen cell</td>
</tr>
<tr>
<td>GSTP1</td>
<td>Glutathione S-transferase Pi 1</td>
</tr>
<tr>
<td>ALOX-5</td>
<td>arachidonate 5-lipoxygenase</td>
</tr>
<tr>
<td>NOS1</td>
<td>nitric oxide synthase 1</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ASM</td>
<td>airway smooth muscle MCs: mast cells</td>
</tr>
<tr>
<td>Hp</td>
<td>haptoglobin</td>
</tr>
<tr>
<td>FEV1</td>
<td>Forced expiratory volume in one second</td>
</tr>
<tr>
<td>HMC-1</td>
<td>human mast cell line HMC-1</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td><strong>SNPs</strong></td>
<td>single nucleotide polymorphisms</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td><strong>EMTU</strong></td>
<td>epithelial mesenchymal tropic unit</td>
</tr>
<tr>
<td><strong>EGF</strong></td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td><strong>TGF</strong></td>
<td>transforming growth factors</td>
</tr>
<tr>
<td><strong>VCAM-1</strong></td>
<td>vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td><strong>GM-CSF</strong></td>
<td>Granulocyte macrophage colony-stimulating factor</td>
</tr>
<tr>
<td><strong>MHC II</strong></td>
<td>major histocompatibility class II</td>
</tr>
<tr>
<td><strong>GSTPi</strong></td>
<td>Glutathione S-transferase Pi</td>
</tr>
<tr>
<td><strong>GSH</strong></td>
<td>Glutathione</td>
</tr>
<tr>
<td><strong>GST</strong></td>
<td>glutathione transferase</td>
</tr>
<tr>
<td><strong>NF-AT</strong></td>
<td>nuclear factor of activated T cell</td>
</tr>
<tr>
<td><strong>PEF</strong></td>
<td>Peak expiratory flow</td>
</tr>
<tr>
<td><strong>GINA</strong></td>
<td>Global Initiative for Asthma</td>
</tr>
<tr>
<td><strong>PAGE</strong></td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td><strong>SDS</strong></td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td><strong>TE</strong></td>
<td>Tris EDTA</td>
</tr>
<tr>
<td><strong>dH₂O</strong></td>
<td>distilled water</td>
</tr>
</tbody>
</table>

**Declaration**
I declare that this work has not been previously submitted in support of application for another degree at this or any other university and has been done in the department of physiology, Faculty of Medicine, The National Ribat University. Part of this work has been submitted to the 9th Scientific Conference- Sudanese chest Physicians society (5-6 April 2015) as a paper in abstract form:

1. RE Ibrahim, HB Eltahir, JE Abdelrahman, A O Gundi, O A Musa. Genetic polymorphisms of ADAM33, IL4 (-590), IL4Ra (-576), GSTT1 and GSTPi genes in Sudanese with asthma. Presented by Randa Ibrahim.
Table 2.1: Single-nucleotide polymorphisms genotyped in IL-4, IL-4ra, ADAM33, GSTT1 and GSTP1 genes:

<table>
<thead>
<tr>
<th>Gene</th>
<th>location</th>
<th>SNP &amp; SNP name</th>
<th>Sequence of primer</th>
<th>Method</th>
<th>PCR product</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL4</td>
<td>5q23</td>
<td>-590C&gt;T</td>
<td>F: 5’ACTAGGCCTCACCTGATACG-3’&lt;br&gt;R: 5’GTTGTAATGCACTCCTCCTG-3’</td>
<td>PCR+RE</td>
<td>254bp</td>
<td>BsmFI</td>
</tr>
<tr>
<td>IL4Ra</td>
<td>16p12.1</td>
<td>-Q576R T&gt;C</td>
<td>F:InnerGCTTACCGAGCTTCAGCACCT&lt;br&gt;R:Inner GACACGGTGACTGGCTCAGTG&lt;br&gt;F:OuterTGGACCTGCTCGAGAGGAGA&lt;br&gt;R:OuterTAACCAGCCTCTCCTGAGGC</td>
<td>PCR-allele specific</td>
<td>574 pb Internal control&lt;br&gt;324bp(T)&lt;br&gt;294bp(C)</td>
<td>-------</td>
</tr>
<tr>
<td>ADAM33</td>
<td>20p13 Intron6</td>
<td>F+1 G&gt;A</td>
<td>F:5’GTATCTATAGCCCTCCAAATCAGAAGAGCC-3’&lt;br&gt;R:5’GGACCTGAGTGGAAAGCTG-3’</td>
<td>PCR+RE</td>
<td>166bp</td>
<td>MspI</td>
</tr>
<tr>
<td>GSTT1</td>
<td>22q11.2</td>
<td>null allele</td>
<td>F:5’-TTCCCTACTGTCTCCTCACATCTCTC-3’&lt;br&gt;R:5’TCACGCGATCATGGCCAGCA-3</td>
<td>PCR</td>
<td>480bp</td>
<td>-------</td>
</tr>
<tr>
<td>GSTP1</td>
<td>11q13</td>
<td>Ile105Val 313A&gt;G</td>
<td>F:5’-ACG CACATC CTC TTC CCC TC-3’&lt;br&gt;R:5’-TAC TTG GCT GGTGA TGT CC-3</td>
<td>PCR+RE</td>
<td>440bp</td>
<td>BsmAI</td>
</tr>
</tbody>
</table>

PCR = polymerase chain reaction; RE = restriction enzyme

Introduction & Literature review

1. Introduction:

Asthma is one of the most common chronic diseases affecting an estimated 300 million people worldwide. \(^{(1)}\) Currently, it is recognized that asthma is a complex
disease that results from interactions between multiple genetic and environmental factors.  
(2, 3) During an asthma attack, the airways that carry air to the lungs are constricted, and as a result, less air is able to flow in and out of the lungs.  
(4) Asthma attacks can cause a multitude of symptoms ranging in severity from mild to life-threatening.  
(4) Asthma is not considered as a part of chronic obstructive pulmonary disease as this term refers specifically to combinations of disease that are irreversible such as bronchiectasis, chronic bronchitis, and emphysema.  
(5) Unlike these diseases, the airway obstruction in asthma is usually reversible, if left untreated, the chronic inflammation accompanying asthma can make the lungs to become irreversibly obstructed due to airway remodeling.  
(6) In contrast to emphysema, asthma affects the bronchi, not the alveoli.  

1.1 Asthma:  
1.1.1 Definition:  
Asthma is a common chronic inflammatory disease of the airways characterized by variable and recurring symptoms, reversible airflow obstruction, and bronchospasm.  
(8) Common symptoms include wheezing, coughing, chest tightness, and shortness of breath.  
(9) The definition of asthma, according to international guidelines, includes the three domains of symptoms: (1) variable airway obstruction, (2) airway hyperresponsiveness (AHR) (or bronchial hyperreactivity), and (3) airway inflammation.  

1.1.2 Epidemiology and global prevalence of asthma:  
The prevalence of asthma in different countries varies widely, but the disparity is narrowing due to rising prevalence in low and middle income countries and plateauing in high income countries.  
(11) Beginning in the 1960s, the prevalence, morbidity, and mortality associated with asthma have been on the rise.  
(12) It is estimated that the number of people with asthma will grow by more than 100 million by 2025.  
(13) The greatest rise in asthma rates in USA was among black
children (almost a 50% increase) from 2001 through 2009.\(^{(11)}\) In Africa in 1990, 74.4 million asthma cases was reported, this increased to 119.3 million in 2010.\(^{(14)}\) About 70% of asthmatics also have Allergies.\(^{(13)}\) Workplace conditions, such as exposure to fumes, gases or dust, are responsible for 11% of asthma cases worldwide.\(^{(13)}\) While asthma is twice as common in boys as girls,\(^{(10)}\) severe asthma occurs at equal rates.\(^{(15)}\) In contrast adult women have a higher rate of asthma than men.\(^{(10)}\)

### 1.1.3 Prevalence of asthma in Sudan:

The prevalence among Sudanese children by using ISAAC questionnaire and adults by using a modified ISAAC questionnaire is different in many areas of the Sudan (Table 1.1). The average prevalence of asthma in adults in Sudan was found to be 10.4%.\(^{(16)}\)

<table>
<thead>
<tr>
<th>Areas</th>
<th>Prevalence %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Children</strong></td>
<td></td>
</tr>
<tr>
<td>Khartoum(^{(17)})</td>
<td>12.2</td>
</tr>
<tr>
<td>Atbara(^{(18)})</td>
<td>4.2</td>
</tr>
<tr>
<td>Gadarif(^{(19)})</td>
<td>5</td>
</tr>
<tr>
<td>White Nile(^{(20)})</td>
<td>11.2</td>
</tr>
<tr>
<td><strong>Adults</strong></td>
<td></td>
</tr>
<tr>
<td>Khartoum(^{(16)})</td>
<td>10.7</td>
</tr>
</tbody>
</table>

Table (1.1): Prevalence of asthma symptoms in adults & children (aged 13-14) in different areas in Sudan.
1.1.4 Causes:

Asthma is caused by a combination of complex and incompletely understood environmental and genetic interactions. These factors influence both its severity and its responsiveness to treatment. It is believed that the recent increased rates of asthma are due to changing epigenetics (heritable factors other than those related to the DNA sequence) and a changing living environment.

1.1.4.1 Environmental causes:

Many environmental factors have been associated with asthma development and exacerbation including: allergens, air pollution, and other environmental chemicals. Asthma is associated with exposure to indoor allergens. Common indoor allergens include: dust mites, cockroaches, animal dander, and mold. Efforts to decrease dust mites have been found to be ineffective. Smoking during pregnancy and after delivery is associated with a greater risk of asthma-like symptoms. Exposure to indoor volatile organic compounds may be a trigger for asthma; formaldehyde exposure, for example, has a positive association. Certain viral respiratory infections may increase the risk of...
developing asthma when acquired in young children such as respiratory syncytial virus and rhinovirus. Certain other infections however may decrease the risk. Asthmatics in the Sudan are found to be sensitive to cockroach allergens, cat allergens, Betulaceae trees allergens and the house dust mite.

1.1.4.2 Genetic causes:

Family history is a risk factor for asthma with many different genes being implicated. If one identical twin is affected, the probability of the other having the disease is approximately 25%. Family studies indicated that the first degree relatives of individuals with asthma are at about five to six times risk of asthma than the individuals in the general population. Heritability, the proportion of phenotypic variances that are caused by genetic effects, varies from study to study in asthma. By the end of 2005, 25 genes had been associated with asthma in six or more separate populations including the genes GSTM1, IL10, CTLA-4, SPINK5, LTC4S, IL4R and ADAM33 among others. Many of these genes are related to the immune system or modulating inflammation. Even among this list of genes supported by highly replicated studies, results have not been consistent among all populations tested. In 2006 over 100 genes were associated with asthma in one genetic association study alone and more continue to be found. Some genetic variants may only cause asthma when they are combined with specific environmental exposures. The most highly replicated regions with obvious candidate genes are chromosome 5q31-33 including interleukins 5, 13, 4, CD14, and adrenergic beta-2-receptor (ADRB2) and chromosome 6p21. A recent meta-analysis of genome-wide linkage studies of asthma, bronchial hyperresponsiveness (BHR), positive allergen skin prick test (SPT), and total immunoglobulin E (IgE) identified overlapping regions for multiple phenotypes on chromosomes 5q and 6p as well as 3p and 7p. Positional cloning studies have identified six genes for asthma on chromosome 20p13.
1.1.4.2.1 Genetic Analysis of Asthma Susceptibility:

The numerous genome-wide linkage, candidate gene, and genome-wide association studies performed on asthma and asthma related phenotypes have resulted in an increasing large list of genes implicated in asthma susceptibility and pathogenesis. This list has been categorized into four broad functional groups by several recent reviewers. (40, 41)

1. Epithelial barrier function:

Studies of asthma genetics have raised new interest in the body’s first line of immune defense, the epithelial barrier, in the pathogenesis of asthma. Filaggrin (FLG), a protein involved in keratin aggregation, is not expressed in the bronchial mucosa, (42) which has lead others to suggest that asthma susceptibility in patients with loss-of-function FLG variants may be due to allergic sensitization that occurs after breakdown of the epithelial barrier. (43) Several epithelial genes with important roles in innate and acquired immune function have also been implicated in asthma. These genes include defensin-beta1 (an antimicrobial peptide), Clara cell 16-kD protein (CC16) (an inhibitor of dendritic cell-mediated Th2-cell differentiation), and several chemokines (CCL-5, -11, -24, and -26) involved in the recruitment of T-cells and eosinophils. (44, 45, 46)

2. Environmental sensing and immune detection:

A second class of associated genes is involved in detection of pathogens and allergens. These genes include pattern recognition receptors and extracellular receptors, such as CD14, toll-like receptor 2 (TLR2), TLR4, TLR6, TLR10, and intracellular receptors, such as nucleotide-binding oligomerization domain containing 1 (NOD1/CARD4). (47, 48, 49) Additional studies have strongly associated variations in the HLA class II genes with asthma and allergen-specific IgE responses. (50)

3. Th2-mediated cell response:
Th2 -cell mediated adaptive immune responses have been widely recognized as a crucial component of allergic disease. Genes involved in Th2 -cell differentiation and function have been extensively studied in asthma candidate-gene association studies, and as one might expect, SNPs in many of these genes have been associated with asthma and other allergic phenotypes. Genes important for Th1 versus Th2 T cell polarization, like IL4 (51), IL4RA (52) and STAT6, (53) have been implicated with asthma and allergy. The genes encoding IL-13 and the beta-chain of the IgE receptor FcεR1 are well replicated contributors to asthma susceptibility. (50, 54)

4. Tissue response:
A variety of genes involved in mediating the response to allergic inflammation and oxidant stress at the tissue level appear to be important contributors to asthma susceptibility. Genes important for tissue response include a disintegrin and metalloprotease, (39) leukotriene C4 synthase (LTC4S) (55) glutathione-S-transferase (GSTP1, GSTM1), (56) arachidonate 5-lipoxygenase (ALOX-5), and nitric oxide synthase 1 (NOS1). (57)

1.1.5 Inflammatory cells in asthma:
It is evident that no single inflammatory cell is able to account for the complex pathophysiology of allergic disease, but some cells predominate in asthmatic inflammation. (58)

Figure (1.1): Inflammatory cells in asthma : (58)
1.1.6 Pathophysiology:

The pathophysiologic hallmark of asthma is a reduction in airway diameter brought about by contraction of smooth muscles, vascular congestion, edema of the bronchial wall and thick tenacious secretions. Chronic asthma may lead to irreversible airway structural changes characterized by subepithelial fibrosis, extracellular matrix (ECM) deposition, smooth muscle hypertrophy, and goblet cell hyperplasia in the airways. Inflammatory cells such as T cells, eosinophils, and mast cell (MCs) are believed to cause irreversible airway structural changes by releasing pro-inflammatory cytokines and growth factors. Th2 cell-mediated immune response against ‘innocuous’ environmental allergens in the immune-pathogenesis of allergic asthma is an established fact. Th2 polarization is mainly because of the early production of IL-4 during the primary response. IL-4 could be produced by the naive T helper cell itself. Cytokines and chemokines produced by Th2 cells, including GM-colony stimulatory factors, IL-4, IL-5, IL-9, IL-13, and those produced by other cell types in response to Th2 cytokines or as a reaction to Th2-related tissue damage (CCL11) account for most...
pathophysiologic aspects of allergic disorders, such as production of IgE antibodies, recruitment and activation of mast cells, basophils, and eosinophils granulocytes, mucus hyper secretion, subepithelial fibrosis and tissue remodeling.

(66)

**Figure (1.2): Inflammatory pathways in asthma:**

![Inflammatory pathways in asthma](image)

1.2 Haptoglobin (Hp):

Haptoglobin (Hp) is an acute phase protein with a strong hemoglobin-binding capacity which was first identified in serum in 1940. (68) Three major phenotypes named Hp1-1, Hp2-1 and Hp2-2, have been identified so far. (69,70) The biological role of Hp1-1 phenotype represents the most effective factor in binding free hemoglobin and suppressing the inflammatory responses. Hp2-1 has a more moderate role and Hp2-2 has the least. (71) The liver is the principal organ responsible for synthesis of haptoglobin and secreted in response to IL-1, IL-6, IL-8, and tumor necrosis factor (TNF) (72) and as one of the innate immune protection
markers, has different biological roles. \(^{(73,74)}\) Hp is present in the serum of all mammals, but polymorphism is found only in humans.\(^{(7)}\) In addition to the liver, Hp is produced in other tissues including lung, skin, spleen, brain, intestine, arterial vessels and kidney, but to a lesser extent.\(^{(75, 76)}\) Also Hp gene is expressed in lung, skin, spleen, kidney, and adipose tissue in mice.\(^{(77, 78)}\)

**1.2.1 Haptoglobin (Hp) phenotypes:**

The Hp polymorphism is related to 2 codominant allele variants (Hp1 and Hp2) on chromosome 16q22, a common polymorphism of the haptoglobin gene, characterized by alleles Hp 1 and Hp 2, give rise to structurally and functionally distinct haptoglobin protein phenotypes, known as Hp 1-1, Hp 2-1, and Hp 2-2.\(^{(79)}\) The three major haptoglobin phenotypes have been identified by gel electrophoresis.\(^{(80)}\) Hp 1-1 is the smallest haptoglobin, and has a molecular weight of about 86 kd. In contrast, Hp 2-2 is the largest haptoglobin, with a molecular weight of 170 to 900 kd. The heterozygous Hp 2-1 has a molecular weight of 90 to 300 kd.\(^{(81)}\) Haptoglobin phenotyping is used commonly in forensic medicine for paternity determination.\(^{(82)}\)

**Figure (1.3): The structure of the different haptoglobin phenotype:**\(^{(83)}\)
1.2.2 Haptoglobin as a Diagnostic Marker:

1.2.2.1 Conditions Associated With an Elevated Plasma Hp Level:

An elevated plasma haptoglobin level is found in inflammation, trauma, burns and with tumors.\(^{84,85}\) The plasma level increases 4 to 6 days after the beginning of inflammation and returns to normal 2 weeks after elimination of the causative agent.\(^{86}\) The plasma haptoglobin level in the initial phase of acute myocardial infarction is high, but later, owing to hemolysis, the plasma level decreases temporarily.\(^{87}\)

1.2.2.2 Conditions Associated With Decreased plasma Hp Level:

The plasma haptoglobin level is decreased in hemolysis, malnutrition, ineffective erythropoiesis, hepatocellular disorders, late pregnancy, and newborn infants.\(^{86,88}\) In addition, the plasma haptoglobin level is lower in people with positive skin tests for pollens, high levels of IgE and specific IgE for pollens and house dust mites, rhinitis, and allergic asthma.\(^{89}\)

1.2.3 Physiology and Functions of Haptoglobin:

In healthy adults, the haptoglobin concentration in plasma is between 38 and 208 mg/dL (0.38 and 2.08 g/L).\(^{80}\) People with Hp 1-1 have the highest plasma concentrations, those with Hp 2-2 the lowest plasma concentrations, and those with Hp 2-1 have concentrations in the middle.\(^{80,84}\) The half-life of haptoglobin is 3.5 days, and the half-life of the haptoglobin- hemoglobin complex is approximately 10 minutes.\(^{90}\) Haptoglobin (Hp) is a potent antioxidant and a positive acute-phase reaction protein whose main function is to scavenge free hemoglobin that is toxic to cells. Hp also exerts direct angiogenic, anti-inflammatory, and immunomodulatory properties in extravascular tissues and body fluids. It is able to migrate through vessel walls and is expressed in different tissues in response to various stimuli.\(^{91}\) Hp can also be released from neutrophil granulocytes \(^{92}\) at sites
of injury or inflammation and dampens tissue damage locally.\(^{(93)}\) Hp receptors include CD163 expressed on the monocyte-macrophage system \(^{(94)}\) and CD11b (CR3) found on granulocytes, natural killer cells, and small subpopulations of lymphocytes. Hp has also been shown to bind to the majority of CD4+ and CD8+ T lymphocytes \(^{(80)}\), directly inhibiting their proliferation and modifying the Th1/Th2 balance. \(^{(95)}\)

1.2.3.1 The role of Hp in regulation of the immune system:

Haptoglobin functions as an immune system modulator. Th1-Th2 imbalance is responsible for the development of various pathologic conditions such as in parasitic and viral infections, allergies, and autoimmune disorders. By enhancing the Th1 cellular response, haptoglobin establishes Th1-Th2 balance in vitro. \(^{(95)}\) Haptoglobin inhibits cathepsin B and L and decreases neutrophil metabolism and antibody production in response to inflammation. \(^{(96)}\) Also it inhibits monocyte and macrophage functions. \(^{(97, 98)}\) Haptoglobin binds to different immunologic cells by specific receptors. It binds to human B lymphocytes via the CD22 surface receptor and is involved in immune and inflammatory responses. \(^{(99)}\) Also, haptoglobin binds to the human mast cell line HMC-1 through another specific receptor and inhibits its spontaneous proliferation. \(^{(100)}\) In populations with Hp 2-2, the B-cell and CD4+ T-lymphocyte counts in the peripheral blood are higher than in populations with Hp 1-1. People with Hp 2-2 have more CD4+ in the bone marrow than do people with Hp 1-1. \(^{(101)}\)

1.2.3.2 Inhibition of Prostaglandins:

Some of the prostaglandins such as the leukotrienes have a proinflammatory role in the body. \(^{(102)}\) Haptoglobin by binding to hemoglobin and limiting its access to the prostaglandin pathway enzymes such as prostaglandin synthase has an important anti-inflammatory function in the body. \(^{(103, 104)}\)

1.2.3.3 Antibacterial Activity:
Iron is one of the essential elements for bacterial growth. The combination of hemorrhagic injury and infection can have a fatal outcome because the presence of blood in injured tissue can provide the required iron to the invading microorganisms. Once bound to haptoglobin, hemoglobin and iron are no longer available to bacteria that require iron.\(^{105}\) In the lungs, haptoglobin is synthesized locally and is a major source of antimicrobial activity in the mucous layer and alveolar fluid and also has an important role in protecting against infection.\(^{91}\)

### 1.2.3.4 Antioxidant Activity:

In plasma, free Hb binds Hp with extremely high affinity but low dissociation speed, in a ratio of 1Hp:1Hb, to form the Hp-Hb complexes. These complexes are rapidly cleared and degraded by macrophages. Hb-Hp complexes once internalized by tissue macrophages are degraded in lysosomes. The heme fraction is converted by heme oxygenase into bilirubin, carbon monoxide and iron.\(^{106}\) So Hp prevents the oxidative damage caused by free Hb which is highly toxic.\(^{107}\) Hp also plays a role in cellular resistance to oxidative stress since its expression renders cells more resistant to damage by oxidative stress induced by hydrogen peroxide.\(^{108}\) The capacity of Hp to prevent oxidative stress is directly related to its phenotype. Hp1-1 has been demonstrated to provide superior protection against Hb-iron driven peroxidation than Hp2-2. The superior antioxidant capacity of Hp1-1 seems not to be related with a dissimilar affinity with Hb but the functional differences may be explained by the restricted distribution of Hp2-2 in extravascular fluids as a consequence of its high molecular mass.\(^{109}\)

### 1.2.3.5 Activation of neutrophils:

During exercise, injury, trauma or infection, the target cells secrete the primary pro-inflammatory cytokines IL-1β and TNF-α, which send signals to adjacent vascular cells to initiate activation of endothelial cells and neutrophils. The activated neutrophils, which are first in the line of defense, aid in the recruitment
of other inflammatory cells following extravasation. Neutrophils engage in generating reactive oxygen species (ROS) as well as recruiting other defense cells, particularly monocytes and macrophages. Hp is synthesized during granulocyte differentiation and stored for release when neutrophils are activated.

1.2.3.6 The role of Hp in angiogenesis:
Haptoglobin is an important angiogenic factor in the serum that promotes endothelial cell growth and differentiation of new vessels, induced accumulation of gelatin serves as a temporary matrix for cell migration and migration of adventitial fibroblasts and medial smooth muscle cells (SMCs) which is a fundamental aspect of arterial restructuring. In chronic systemic vasculitis and chronic inflammatory disorders, haptoglobin has an important role in improving the development of collateral vessels and tissue repair. Among the haptoglobin phenotypes, Hp 2-2 has more angiogenic ability than the others.

1.2.3.7 Hp is required to induce mucosal tolerance:
Reduced or absent responsiveness of the immune system against self-antigens is necessary to allow the immune system to mount an effective response to eliminate infectious invaders while leaving host tissues intact. This condition is known as immune tolerance. Mucosal tolerance induction is a naturally occurring immunological phenomenon that originates from mucosal contact with inhaled or ingested proteins. Regular contact of antigens with mucosal surfaces prevents harmful inflammatory responses to non-dangerous proteins, such as food components, harmless environmental inhaled antigens and symbiotic microorganisms. Oral and nasal tolerance has been used successfully to prevent a number of experimental autoimmune diseases including, arthritis, diabetes, uveitis, and experimental autoimmune encephalomyelitis. The majority of inhaled antigen detected in lymph nodes is associated with a variety of dendritic cells. The CD4+ T cell population that arises after harmless antigen administration in the
nose is able to transfer tolerance and to suppress specific immune responses in naïve animals.\textsuperscript{(117)} Importantly, Hp expression is increased in cervical lymph nodes shortly after nasal protein antigen instillation without adjuvant.\textsuperscript{(118)}

1.2.4 Hp and asthma:
Hp has been shown to decrease the reactivity of lymphocytes and neutrophils toward a variety of stimuli, and may act as a natural antagonist for receptor-ligand activation of the immune system.\textsuperscript{(119)} A change in the concentration of Hp at the site of inflammation can modulate the immune reactivity of the inflammatory cells. Haptoglobin can be expressed by eosinophils, and variable serum levels have been reported in asthma, where both elevated \textsuperscript{(120)} and reduced \textsuperscript{(121)} serum haptoglobin levels were described. Increases in haptoglobin are seen in uncontrolled asthma \textsuperscript{(122)} and 24 hours after allergen challenge in late responders.\textsuperscript{(123)} Mukadder et al reported that Hp levels were found to be significantly decreased in patients with asthma and/or rhinitis Consistent with its roles in modulating immune responses.\textsuperscript{(124)} Haptoglobin has also been correlated with FEV1.\textsuperscript{(120)} Wheezing was reported to be significantly related to low levels of Hp, and bronchial hyper-responsiveness related to high level.\textsuperscript{(120)} As part of its tissue repair function, haptoglobin can induce differentiation of fibroblast progenitor cells into lung fibroblasts \textsuperscript{(125)} and angiogenesis.\textsuperscript{(112)} Bronchial asthma was correlated with Hp1-1.\textsuperscript{(80)}

1.3 A disintegrin and metalloprotease (ADAM) 33:
A disintegrin and metalloprotease (ADAM) family consists of 34 members (ADAM1-ADAM34). ADAM is synthesized as a latent proprotein with its signal sequence in the endoplasmic reticulum. They have an active site in the metalloprotease domain, containing a zinc-binding catalytic site.\textsuperscript{(126, 127)} The active site sequences of ADAM33 like the other protease-type ADAM members, implying that ADAM33 can promote the process of growth factors, cytokines,
cytokine receptors, and various adhesion molecules. ADAM33 mRNA is preferentially expressed in smooth muscle, fibroblasts, and myofibroblasts, but not in the bronchial epithelium or in inflammatory or immune cells. The ADAM protein has been detected in lung tissue, smooth muscle cells and fibroblasts. It is an asthma susceptibility gene and plays a role in the pathophysiology of asthma.

1.3.1 Physiological functions of ADAM33:
ADAM33 consists of 22 exons that encode a signal sequence, and different domains structure. From a functional standpoint, these different domains translate into different functions of ADAM33, which include activation, proteolysis, adhesion, fusion, and intracellular signaling. Metalloprotease and ADAM proteins play an important role in branching morphogenesis of the lung by influencing the balance of growth factors at specific stages during development. Several ADAM33 protein isoforms occur in adult bronchial smooth muscle and in human embryonic bronchi and surrounding mesenchyme.

1.3.1.1 ADAM 33 as a morphogenetic gene:
Multiple forms of ADAM33 protein isoforms exist in human embryonic lung when assessed at 8 to 12 weeks of development. Although many of these variants were similar in size to those identified in adult airways and airway smooth muscles, fetal lung also expressed a unique, small 25-kD variant. Immunohistochemistry applied to tissue sections of human fetal lung demonstrated ADAM33 immunostaining not only in airway smooth muscles but also in primitive mesenchymal cells that formed a cuff at the end of the growing lung bud. Polymorphic variation in ADAM33 could influence lung function in infancy.

1.3.2 ADAM 33 as a biomarker of severe asthma:
Lee and colleagues recently described the presence of a soluble form of
ADAM33 of approximately 55 kD (sADAM33). The soluble form was reported to be over expressed in airway smooth muscles and basement membrane in subjects with asthma but not in normal control subjects. Applying an immunoassay for sADAM33 in bronchoalveolar lavage fluid, levels increased significantly in proportion to asthma severity, with ADAM33 protein levels correlating inversely with the predicted FEV1%. These exciting observations raise the possibility that sADAM33 is a biomarker of asthma severity and chronicity and in its soluble form could play an important role in asthma pathogenesis.  

1.3.3 ADAM 33 gene polymorphisms and asthma:
Disintegrin and metalloproteinase domain-containing protein 33 is an enzyme that in humans is encoded by the ADAM33 gene located on chromosome 20p13. It was the first identified as an asthma susceptibility gene by positional cloning approach in the year 2002 in a genome wide scan of a Caucasian population. A survey of 135 polymorphisms in 23 genes identified the ADAM33 gene as being significantly associated with asthma using case-control, transmission disequilibrium and haplotype analyses.

A study on the Genetics of Asthma, demonstrated positive association between single nucleotide polymorphisms (SNPs) of ADAM33 and asthma and BHR in African-American, Hispanic, and white populations. Simpson et al supported the hypothesis that ADAM33 polymorphisms influence lung function in early life and epithelial-mesenchymal dysfunction in the airways may predispose individuals toward asthma, being present in early childhood before asthma becomes clinically expressed. The ADAM33 gene was associated with a significant excess decline in baseline forced expiratory volume in first second (FEV1) of 23.7–30 ml/year. These data imply a role for ADAM33 in airway wall remodelling which is known to contribute to chronic airflow obstruction in moderate to severe asthma (figure1.4). Another study conducted on infants born
of allergic/asthmatic parents has revealed positive associations between SNPs of ADAM33 and increased airway resistance, with the strongest effect seen in the homozygotes. These data support that the alterations in the expression or function of ADAM33 is in some way involved in impairing lung function in early life and as a consequence, increasing the risk of asthma development. The substitution of thymine for cytosine in the T1 SNP which is located in exon 20, results in the substitution of methionine for threonine in the cytoplasmic domain of the protein and this may alter intracellular signaling, resulting in increased fibroblast and smooth muscle cells proliferation. Some of the significant SNPs were located in noncoding regions within introns and the 3' untranslated region (UTR) and may affect alternative splicing, splicing efficiency or messenger RNA turnover as reported for other disease-causing genes.

Figure (1.4): Key cells influenced by ADAM33 in airway remodelling in asthma:

1.3.4 Role of the gene-environment interaction and ADAM33 in development of asthma:

The activation of tissue-specific susceptibility genes provides a basis for explaining environmental factors that may be more closely associated with
Selective expression of the ADAM33 gene in mesenchymal cells suggests its potential to affect the epithelial mesenchymal tropic unit (EMTU) along with Th2 cytokines. Possible exposure to environmental factors such as pollutants, microbes, allergens, and oxidant stimuli reactivates the EMTU, which is involved in morphogenesis during fetal lung development. It has been shown that epithelium of patients with asthma is structurally and functionally abnormal and more susceptible to oxidant-induced apoptosis. Apoptotic cell loss is accompanied by increased expression of epidermal growth factor (EGF) receptors and transforming growth factors (TGF) β1 and β2. These growth factors play an important role in promoting differentiation of fibroblasts into myofibroblasts that secrete other growth factors such as endothelin1, which act as mitogens for smooth muscles and endothelial cells.

1.4 Interleukins and asthma:
1.4.1 Interleukin-4 (IL 4):
It is a potent lymphoid cell growth factor which can stimulate the growth, differentiation and survivability of and regulate the function of various cell types of hematopoietic (including B and T lymphocytes, mast cells, monocytes and macrophages) and non-hematopoietic (e.g., vascular endothelial cells and certain tumor cells) origin. Its effects depend upon binding to and signaling through a receptor complex consisting of the IL-4 receptor alpha chain (IL-4Rα) and the common gamma chain (γc).

1.4.1.1 Physiological functions of IL4:
IL-4, a pleiotropic cytokine produced by Th2 cells and mast cells, is a central mediator of allergic inflammation. Its plays an essential role in IgE regulation and plays a critical role in the induction and maintenance of allergy. IL-4 acts on Th0 cells to promote their differentiation into Th2 cells. It decreases the production of Th1 cells, macrophages, IFN-gamma, and dendritic cell IL-12.
Overproduction of IL-4 is associated with allergies.\(^{(155)}\) IL-4 also induces vascular cell adhesion molecule 1 (VCAM-1) on vascular endothelium and thus directs the migration of T lymphocytes, monocytes, basophils, and eosinophils to the inflammation site.\(^{(156)}\) In asthma, IL-4 contributes both to inflammation and to airway obstruction through the induction of mucin gene expression and the hypersecretion of mucus.\(^{(157)}\) It also inhibits eosinophil apoptosis and promotes eosinophilic inflammation by inducing chemotaxis and activation through the increased expression of eotaxin.\(^{(158)}\) Some of these effects may be mediated by bronchial fibroblasts that respond to IL-4 with increased expression of eotaxin and other inflammatory cytokines.\(^{(159)}\)

### 1.4.1.2 Interleukin-4 gene and asthma:

IL4 gene has been mapped to chromosome 5q31 where asthma and atopy have also been linked.\(^{(160)}\) The human IL-4 promoter exists in multiple allelic forms, one of which confers high transcriptional activity; causing over-expression of the IL-4 gene.\(^{(161)}\) Basehore et al\(^{(162)}\) reported that nine SNPs in the IL-4 gene were significantly associated with asthma or total serum IgE in whites and other allergy related phenotypes, although ethnical differences have been reported. The IL4 (590 C/T) single nucleotide polymorphism (SNP) presents on the promoter region of the gene (chromosome 5q23.3-31.2).\(^{(163)}\) Phylogenetic studies indicate that this polymorphism belongs to a conserved region in all primates except for humans. The derivative allele T has been related to elevated serum levels of IgE and asthma, High frequencies of this allele may be the result of positive selection.\(^{(164)}\) Walley et al\(^{(165)}\) reported that allelic variant T-590 is associated with higher promoter activity and increased production of IL-4 as compared to C-590 allele. The –590C/T polymorphism is located in one of the unique binding sites for the nuclear factor of activated T cell (NF-AT) which plays an important role in the transcription of several cytokine genes.\(^{(166)}\)
1.4.2 IL4 Receptor:
This receptor exists in different complexes throughout the body. IL-4Rα pairs with the common γ chain to form a type I IL-4R complex that is found predominantly in hematopoietic cells and is exclusive for IL-4. IL-4Rα also pairs with the IL-13Rα1 subunit to form a type II IL-4R. The type II receptor is expressed on both hematopoietic and nonhematopoietic cells such as airway epithelium. These type II receptors have the ability to bind both IL-4 and IL-13, two cytokines with closely related biological functions. The sequential binding sequence for this receptor complex where IL-13 first binds to IL-13Rα1 and then this complex recruits IL-4Rα to form a high affinity binding site. In the case of IL-4, this sequence of events is reversed, where IL-4 first binds to IL-4Rα with high affinity before associating with the second subunit.

1.4.2.1 Physiological functions of IL4Rα:
Receptors for both interleukin 4 and interleukin 13 couple to the JAK1/2/3-STAT6 pathway. The IL4 response is involved in promoting Th2 differentiation. The IL4/IL13 responses are involved in regulating IgE production, chemokine and mucus production at sites of allergic inflammation. In certain cell types, IL4Rα can signal through activation of insulin receptor substrates, IRS1/IRS2. The binding of IL-4 or IL-13 to the IL-4 receptor on the surface of macrophages results in the alternative activation of those macrophages. Alternative activated macrophages downregulate inflammatory mediators such as IFNγ during immune responses, particularly with regards to helminth infections.

Soluble IL-4Rs (sIL-4Rs) are present in biological fluids and contain only the extracellular portion of IL-4R and lack the transmembrane and intracellular domains. In vitro, sIL-4R blocks B cell binding of IL-4, B cell proliferation, and IgE and IgG1 secretion. In vivo, sIL-4R inhibits IgE production by up to 85% in anti-IgD-treated mice and reduces airway inflammation, suggesting that sIL-
4R may be useful in the treatment of IgE-mediated inflammatory diseases such as asthma. \(^{175, 176}\) One potential disadvantage of sIL-4R as a treatment is that it does not block the effects of IL-13, because, in asthma, the effects of allergic inflammation are mediated by both IL-4 and IL-13.\(^{156}\)

### 1.4.3 Interleukin 4 receptor (IL-4Rα) gene and asthma:

The IL-4 receptor alpha (IL-4Rα, also called CD124) gene encodes a single-pass transmembrane subunit protein. This gene is located on chromosome 16p (16p12.1). \(^{177}\) There is evidence that interleukin-4 (IL-4) and its receptor (IL-4R) are involved in the pathogenesis of asthma. \(^{178, 179}\) A recent meta-analysis indicated a modest risk associated with IL4R single nucleotide polymorphisms (SNPs) on occurrence of asthma, but other investigators found conflicting results. \(^{179}\) Analysis of asthma candidate genes in a genome-wide association study population showed that SNPs in IL4R were significantly related to asthma. \(^{180}\) African Americans experience higher rates of asthma prevalence and mortality than whites \(^{181}\), few genetic association studies for asthma have focused specifically on the roles of the IL-4 and IL-4Rα genes in this population. \(^{162, 182}\) Gene–gene interaction between IL-4 and IL-4Rα and asthma has been demonstrated in several populations. \(^{183, 184}\) The Q576R polymorphism that is associated with asthma susceptibility in outbred populations, especially severe asthma, this allele is overrepresented in the African-American population (70% allele frequency in African Americans vs. 20% in Caucasians. \(^{185,186,187}\) The Q576R (A/G) is gain-of-function mutation also enhancing signal transduction, which results in glutamine being substituted by arginine. \(^{188}\)

### 1.4.3 Interleukin 13:

IL-13 is a cytokine closely related to IL4 that binds to IL-4Rα. IL-13 and IL-4 exhibit a 30% of sequence similarity and have a similar structure. \(^{189}\) produced by CD4\(^+\) T cells, NK T cells, mast cells, basophils, eosinophils. \(^{190}\) It is implicated as a
central regulator in IgE synthesis, mucus hypersecretion, airway hyperresponsiveness (AHR), and fibrosis.\textsuperscript{(191)}

1.4.3.1 Physiological functions of IL13:
In vitro studies have demonstrated that IL-13 has a broad range of activities, including switching of immunoglobulin isotype from IgM to IgE,\textsuperscript{(192)} increase adhesion of eosinophils to the vascular endothelium\textsuperscript{(193)} and augmentation of cell survival.\textsuperscript{(194)} Proliferation and activation and of mast cells.\textsuperscript{(195)} Increased epithelial permeability.\textsuperscript{(196)} Induction of goblet cell differentiation and growth.\textsuperscript{(197)} Transformation of fibroblasts into myofibroblasts and production of collagen.\textsuperscript{(198)} Diminished relaxation in response to \(\beta\)-agonists\textsuperscript{(199)} and augmentation of contractility in response to acetylcholine in smooth muscles.\textsuperscript{(200)}

1.4.3.1.1 IL-13 role in mucus production:
Goblet cell hyperplasia and mucus overproduction are features of asthma and chronic obstructive pulmonary disease and can lead to airway plugging, a pathologic feature of fatal asthma.\textsuperscript{(201)} Animal models demonstrate that IL-13 induces goblet cell hyperplasia and mucus hypersecretion \textsuperscript{(202)} and human in vitro studies demonstrate that IL-13 induces goblet cell hyperplasia. Experiments suggest that these effects are mediated by IL-13 signaling through IL-13Ra1.\textsuperscript{(203,204)} Human bronchial epithelial cells (HBEs), stimulated by IL-4 and IL-13, can also undergo changes from a fluid absorptive state to a hypersecretory state independent of goblet cell density changes.\textsuperscript{(205,206)}

1.4.3.1.2 IL-13 in airway hyperresponsiveness:
Inflammation, remodeling and AHR in asthma, are induced by IL-13 over expression.\textsuperscript{(207)} Also IL-13 modulates Ca\textsuperscript{2+} responses in vitro in human airway smooth muscles.\textsuperscript{(208)} Saha SK et al\textsuperscript{(209)} reported that Sputum IL-13 concentration and the number of IL-13\textsuperscript{+} cells in the bronchial submucosa and airway smooth muscles bundle are increased in severe asthmatics.\textsuperscript{(209)}
1.4.3.1.3 Interleukin-13 in pulmonary fibrosis:
Experimental models identify IL-13 to be an important profibrotic mediator. (210,211) Both IL-4 and IL-13 have redundant signaling pathways with implications in pulmonary fibrosis. IL-4 and IL-13 are important in alternatively activated macrophage induction, which is believed to regulate fibrosis. (211)

1.4.3.2 IL 13 and inflammatory pathways in asthma:
Munitz et al (191) demonstrated that key pathogenic molecules associated with asthma severity, such as chitinase, are entirely dependent on IL-13 signaling through IL-13Rα1, a component of the type II receptor. Rhinovirus (RV), the virus responsible for the common cold, in children is a distinct risk factor for asthma exacerbations. (212) Among the cytokines studied, IL-13 was the most increased in the RV group versus the respiratory syncytial virus (RSV) group. (213)

1.4.3.3 Anti-interleukin-13 antibody therapy for asthma:
Piper et al. (214) reported that patients with poorly controlled asthma who received a humanized monoclonal antibody directed against IL-13 (tralokinumab) showed improvement. Analysis of patients with elevated sputum IL-13 (>10 pg/mL⁻¹) at study entry had numerically higher improvements in FEV1 compared with subjects whose values were lower than these thresholds, suggesting that the presence of residual IL-13 was associated with a larger FEV1 response. These data bear much in common with recently published findings from a trial of lebrikizumab, another anti-IL-13 monoclonal antibody, in patients with asthma. (215) The treatment group who received lebrikizumab had a significant improvement in FEV1, 5.5% higher than that in patients receiving placebo. (215)

1.5 Glutathione S-transferases (GSTs):
Glutathione S-transferases (GSTs) belong to a super family of phase II detoxification enzymes, which play an important role in protecting cells from damage caused by endogenous and exogenous compounds by conjugating reactive
intermediates with glutathione to produce less reactive water-soluble compounds. \(^{(216)}\) GSTs are a multi-gene family of enzymes involved in drug biotransformation and xenobiotic metabolism and, in a few instances, activation of a wide variety of chemicals. \(^{(217)}\) Conklin et al \(^{(218)}\) reported that GSTs represent a major group of detoxification enzymes and redox regulators important in host defense to numerous environmental toxins and reactive oxygen species (ROS) including those found in cigarette smoke and air pollution. \(^{(219)}\)

1.5 Functions of GSTs:

GSTs neutralize the electrophilic sites of compounds by conjugating them to the tripeptide thiol, glutathione (GSH). \(^{(220)}\) The compounds targeted in this manner by GSTs encompass a diverse range of environmental or exogenous toxins, including chemotherapeutic agents and other drugs, pesticides, herbicides, carcinogens, and variably-derived epoxides. \(^{(216)}\) GSTs are responsible for a reactive intermediate formed from aflatoxin B\(_1\), which is a crucial means of protection against the toxin in rodents. \(^{(216)}\)

1.5.3.1 The detoxication functions of GSTs:

As enzymes GSTs are involved in many different detoxication reactions. The substrates mentioned below are some of the physiologically interesting substrates. GST P1-1, GST M1-1 and GST A1-1 have been shown to catalyze the inactivation process of α, β unsaturated carbonyls. One example of α, β unsaturated carbonyls is acrolein, a cytotoxic compound present in tobacco smoke. Exposure of cells to acrolein produce single strand DNA breaks. \(^{(221)}\) Another class of α, β unsaturated carbonyls are propenals, which are generated by oxidative damage to DNA. \(^{(222)}\)

1.5.3.2 The metabolic function of GSTs:

GSTs are involved in the biosynthesis of prostaglandins (PGs). Human GST M2-2 has been isolated as a prostaglandin E synthase in the brain cortex. \(^{(223)}\) Human GST M3-3 also displays the same activity while GST M4-4 does not. \(^{(224)}\) The
Sigma class of GST was shown to catalyze the isomerization reaction of PGF2 to PGD2. PGD2, PGE2 and PGF2 act as hormones that bind to G-protein coupled receptors. These receptors in turn regulate other hormones and neurotransmitters. (224)

1.5.3.3 The regulatory function of GSTs:
The most recent studies on GSTs have demonstrated that GST P1-1 interacts with c-Jun N-terminal kinase 1 (JNK1) suppressing the basal kinase activity. (225) Introduction of GST P1-1 elicits protection and increased cell survival when the cells are exposed to hydrogen peroxide (H$_2$O$_2$). (226) GST P1-1 does not elicit the same protection against UV-induced apoptosis. (225) In contrast to GST P1-1, mouse GST M1-1 seems to protect cells against both UV-and H2O2-induced cell death. (227)

1.5.4 GSTs and asthma:
Several studies have highlighted that oxidative stress damages pulmonary function and might act as a key player in the worsening of asthma symptoms. (228) The damage caused by oxidative injury leads to an increase in airway reactivity and/or secretions, and influences the production of chemoattractants and increases vascular permeability. (229) All these factors exacerbate inflammation, which is the hallmark of asthma. Phase II detoxification enzymes, particularly classes of glutathione S-transferases (GSTs), play an important role in inflammatory responses triggered by xenobiotic or reactive oxygen compounds. (230) Studies have shown that individuals with lowered antioxidant capacity are at an increased risk of asthma. (231) In particular, GSTM1 and GSTT1 null polymorphisms and a single nucleotide polymorphism of GSTP1 (Ile105Val) may influence the pathogenesis of respiratory diseases. (230) There are several explanations for the role of GSTs in disease pathogenesis, the first being that xenobiotics are not discharged from the organism in the absence of these enzymes, and may trigger mast cell
degranulation. (216) Secondly, it is known that leukotrienes released by mast cells and eosinophils, important compounds in bronchial constriction, are inactivated by GSTs. (232) The absence of these enzymes may contribute to asthma by abnormalities in the transport of inflammatory inhibitors to bronchioles. (233)

1.5.5 Glutathione S-transferase Theta 1 (GSTT1):
The GSTT1 is an important phase II enzymes that protect the airways from oxidative stress. (216) It has lower glutathione binding activity, along with increased catalytic efficiency. They utilize as substrates a wide variety of products of oxidative stress. (234) The GSTT1 gene is located on chromosome 22q11 and it is one of the members of GST’s enzymes family. Human GST genes are polymorphic, either due to presence of single nucleotide polymorphisms (SNPs) or due to deletions. (235) Total gene deletion or null polymorphism leads to no functional enzymatic activity. (236) Enzymes of this group have a wide range of substrates including carcinogens in food, air or medications, tobacco smoke, combustion products. (237) Frequency of GSTT1 null polymorphism differs largely among different populations. It has highest frequency in Asians (50%) followed by African Americans (25%) and Caucasians (20%). (238) Deletion polymorphism of GSTT1 gene has been associated with asthma in children and adults. (229, 239, 240) The GSTT null gene leads to non-transcription of messenger RNA and non-translation of the protein, resulting in complete loss of functionality. (241) It is postulated that GSTT1 null gene may lead to a reduction in anti-inflammatory and anti-oxidative systems, possibly potentiating the pathogenesis of asthma. (242)

1.5.6 Glutathione S-transferase Pi 1 (GSTP1):
In human, GST-Pi was first detected in placenta. (243) GSTP1 is the predominant cytosolic GST expressed in lung epithelium. (244) GSTP1 enzyme plays a key role in biotransformation and bioactivation of certain environmental pollutants such as
benzo[a]pyrene-7, 8-diol-9, 10-epoxide (BPDE) and other diol epoxides of polycyclic aromatic hydrocarbons.\(^{(245)}\) GSTP1 is a gene located on chromosome 11q13, a known “hot spot” for asthma-related genes.\(^{(246)}\) A single nucleotide polymorphism at position 313 in GSTP1 results from conversion of an adenine to a guanine (A>G).\(^{(247)}\) The resulting isoleucine to valine substitution in codon 105 of exon 5 (Ile105_Val105) significantly lowers GST enzyme activity.\(^{(248)}\) This GSTP1 variant has been associated with asthma in some studies.\(^{(249,250,251)}\) but not all studies.\(^{(252,253)}\) This variant has been reported to be both protective \(^{(254,255,256)}\) and a risk factor\(^{(250,257,258)}\) for asthma. The inconsistency may have several explanations, including differences in asthma pathogenesis in young children and adults, as well as the effects of other common variants in GSTP1 coding and promoter regions.\(^{(250,257)}\)

1.6 **Diagnosis:**

A diagnosis of asthma should be suspected if there is a history of recurrent wheezing, coughing or difficulty of breathing and these symptoms occur or worsen due to exercise, viral infections, allergens or air pollution.\(^{(8)}\) Spirometry is then used to confirm the diagnosis.\(^{(8)}\) In children under the age of six the diagnosis is more difficult as they are too young for spirometry.\(^{(10)}\)

1.7 **Classification and severity:**

According to guidelines, evaluation of severity is necessary for appropriate and adequate treatment, especially the selection and dosing of medications.\(^{(259,260)}\) Based on symptoms and signs of severity, asthma can be classified into four clinical stages: intermittent, mild persistent, moderate persistent and severe persistent.\(^{(259,260)}\) Certain medications are indicated for each clinical stage.\(^{(261)}\) It is clinically classified according to the frequency of symptoms, forced expiratory volume in one second (\textit{FEV1}), and peak expiratory flow rate.\(^{(262)}\) Peak flow meter is necessary to be able to assess the severity of asthma at different times, measure
the peak expiratory flow (PEF which is a good indication of the degree of obstruction to air flow (GINA). The international union against tuberculosis and lung disease estimates the severity of the symptoms as follows:

**Intermittent:** symptoms disappear for long periods. When they return, they occur less than once a week (weekly). The periods of attacks last only a few hours or a few days. When there are nocturnal symptoms, they occur less than twice a month.

**Persistent:** symptoms never disappear for more than one week. When symptoms occur more than once a week, they are called persistent.

**Mild persistent:** symptoms occur less than once a day (weekly) and nocturnal symptoms occur more than twice a month.

**Moderate persistent:** symptoms are daily and attacks affect activity and sleep patterns more than once a week.

**Severe persistent:** symptoms are continuous with frequent attacks, limiting physical activity and often occurring at night.

<table>
<thead>
<tr>
<th>Severity</th>
<th>Symptom frequency</th>
<th>Night time symptoms</th>
<th>%FEV$_1$ of predicted</th>
<th>FEV$_1$ Variability</th>
<th>SABA use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermittent</td>
<td>≤2/week</td>
<td>≤2/month</td>
<td>≥80%</td>
<td>&lt;20%</td>
<td>≤2 days/week</td>
</tr>
<tr>
<td>Mild persistent</td>
<td>&gt;2/week</td>
<td>3–4/month</td>
<td>≥80%</td>
<td>20–30%</td>
<td>&gt;2 days/week</td>
</tr>
<tr>
<td>Moderate persistent</td>
<td>Daily</td>
<td>&gt;1/week</td>
<td>60–80%</td>
<td>&gt;30%</td>
<td>daily</td>
</tr>
</tbody>
</table>

Table (1.2) Clinical classification (≥12 years old):
<table>
<thead>
<tr>
<th>Severe persistent</th>
<th>Continuously (7×/week)</th>
<th>Frequent (&lt; 60%)</th>
<th>&gt; 30%</th>
<th>≥ twice/day</th>
</tr>
</thead>
</table>

1.8 *Prevention and management:*

Early pet exposure may be useful. Reducing or eliminating compounds (trigger factors) known to the sensitive people from the work place may be effective. Plan for proactively monitoring and managing symptoms should be created. This plan should include the reduction of exposure to allergens, testing to assess the severity of symptoms, and the usage of medications. The treatment plan and the advised adjustments to treatment according to changes in symptoms should be written down. The most effective treatment for asthma is identifying triggers, such as cigarette smoke, pets and aspirin and eliminating exposure to them. If trigger avoidance is insufficient, the use of medication is recommended. Pharmaceutical drugs are selected based on, among other things, the severity of illness and the frequency of symptoms. Specific medications for asthma are broadly classified into fast-acting and long-acting categories.

1.9 *Justification:*

Currently, there is no cure for asthma; however, people who have asthma can still lead productive lives if they control their asthma. Physician evaluations of asthma severity and medication requirements often rely on subjective indices reported by patients, including daily symptom scores and use of inhaled asthma medication. These measures are prone to inconsistencies due to variations in investigator and patient assessments. They can also be difficult to obtain, especially in young children, and most of them imperfectly reflect physiologic alterations involved with asthma. Patient symptoms may subside despite the presence of residual
disease in the form of reduced lung capacity and bronchial hyperreactivity. Patients with decreased pulmonary function who remain asymptomatic may later experience shortness of breath caused by severely restricted lung capacity. Studies point to IL4/IL4RA, ADAM33 and GSTs and clinical utility as an indicator of asthma severity and as a monitor for asthma therapy, and to investigate haptoglobin phenotype in asthmatic patients.

1.10 Objectives:

General objectives:
The overall aim of this study is to investigate the possible immunological and genetic markers that may correlate with the occurrence and the severity of asthma among Sudanese asthmatics.

Specific objectives:
The specific objectives of the study are to:

1. Assess the level and the common phenotype of haptoglobin among Sudanese asthmatic and healthy controls.
2. Measure the level of IL4 and IgE in normal and different classes of asthma in Sudan using ELISA technique.
3. Determine the frequency of the known alleles of IL4, IL4Rα, ADAM33 and GSTs in Sudanese asthmatic patients and healthy controls using PCR based methods.

4. Correlate between genetic polymorphisms of IL4 (-590)/IL4Rα (- 576), ADAM33 and GSTs (GSTT1, GSTPi) and the severity of asthma among Sudanese population.

Materials and Methods

2.1 Materials:
The following materials were used in this study.

2.1.1 Electronic Spirometer:
- Micro/Micro Plus Spirometers. CE120. was purchased from Micro Medical Limited 1998
  – P.O Box 6 Rochester Kent ME 1 2AZ. England.

It measures magnitude and velocity of air movement out of lungs.

The indices measured are:-

1. Forced vital capacity (FVC): the volume of gas that can be forcefully expelled from the lung after maximal inspiration.
2. The forced expiratory volume in the first second (FEV1): the volume of gas expelled in the first second of the FVC maneuver.

3. Peak expiratory flow rate (PEFR): the maximum air flow rate achieved in the FVC maneuver.

4. Maximum voluntary ventilation (MVV): the maximum volumes of gas that can be breathed in and out during 1 minute.

5. Slow vital capacity (SVC): the volume of gas that can be slowly exhaled after maximal inspiration.

6. FEV1/FVC: values of FEV1 and FVC that are over 80% of predicted are defined as within the normal range. Normally the FEV1 is about 80% of the FVC.

Specification of micro and micro-plus spirometers:

- Transducer: digital volume transducer
- Resolution: 10ml
- Accuracy: +/- 3% (To ATS recommendations standardization of spirometry 1994 update for flows and volumes)
- Volume range: 0.1 - 9.99 liters.
- Flow range: 30L/min – 1000L/min
- Display: custom 3 digit liquid crystal
- Power supply: 9v PP3
- Storage Temperature: 20 to + 70°C
- Storage Humidity: 10% - 90% RH

Mouth pieces:

Disposable mouth pieces. Micro Medical CE 120.

Figure (2.1) Electronic Spirometer and mouth pieces:
2.1.2 Gel electrophoresis cell:
- Horizontal gels within a single tank.
- Designed for rapid screening of DNA and PCR samples.
- CS Cleaver Scientific - www.Cleaverscientific.com

Figure (2.2) Gel electrophoresis cell:
1. Power supply:  - Model: MP -250V  
   - Serial number: 091202016  - 120~240V ~ 47/60 Hz

2. Casting dams: allow gels to be rapidly cast externally.

3. Combs (The number of samples can be maximized using high tooth number combs).

4. Tank:
   - Stock code: MSMINI  - Made in the UK
   - Max volts 250 VDC.  - Max current 250 mA.

2.1.3 PCR machine:

Alpha Laboratories Ltd  40 Parham Drive, Eastleigh
Hampshire, 5050 4NU – United Kingdom  Tel: 023 80 483000

Figure (2.3) PCR machine:
2.1.4 Primers:
Made in Korea  www.mocrogene.com
Tel: 82-2-2113-7037  Fax: 82-2-2113-7919
Figure (2.4): Forward and Reverse primers:

2.1.5 Maxime PCR PreMix Kit (i-Taq):
- Product Catalog No. 25025 (for 20µL rxn, 96 tubes)
- iNtRON biotechnology, Inc. www.intronbio.com/ info@intronbio.com
- Korea T. (0505) 550-5600/ F. (0505) 550- 5660

Figure (2.5) Maxime PCR PreMix Kit (i-Taq):

2.1.6 UV Transilluminator JY-02S analyzer:
- Made in china - Model number: JY- 02S - Serial number: 0903002D
- Input voltage: 220 ± 10 - Input frequency: 50 Hz - Fuse: Φ 5× 20 3A×2
- It is used to take and analyze the picture after the electrophoresis.
2.1.7 Microplate reader:

Model: RT-2100C - 451403041 FSEP
a.c. 110 V- 220V  50/60Hz  120 WATTS  T3.15 AL  250V

Rayto life and Analytical sciences Co., Ltd
C&D/4F, 7th Xinghua industrial Bldg, Nanhai Rd.
Shanghai International Holding Corp.GmbH (Europe)
Eiffestrasse 80, D- 20537, Hamburg, Germany.

- RT-2100C is a microprocessor –controlled, general purpose photometer system designed to read and calculate the result of assays, including contagion, tumorous mark, hemopathy, dyshormonism, which are read in microtiter plate.

Figure (2.7): Microplate reader:
2.1.8 ELIZA kits:
Made in: MINNEAPOLIS, MN USA
- www.RnDSystems.com
R&D Systems, Inc. USA & Canada R&D systems, Inc (800)343-7475

Figure (2.8): ELIZA kits for haptoglobin and IL4.

2.1.9 Vertical electrophoresis cell:
- For routine mini protein electrophoresis.
- Model: DYC2 – 24 DN  - Serial number: 028 – 01
2.2 Methods:

2.2.1 Study design: It is a cross sectional analytic and descriptive study.
2.2.2 Study area: the study was conducted during (2013-2014) in Khartoum state.

2.2.3 Study population:
Asthmatic patients if only they have documented physician-diagnosed asthma and healthy subjects with no symptoms of present illness of both sexes and aged between 16 to 60 years were included. Subjects with any chronic disease or chest deformity or smoking habits were excluded.

Sample size:
- One hundred and sixteen Sudanese subjects were selected. Sixty three were asthmatic patients and Fifty three were healthy volunteers.

2.2.4 Ethical considerations:
Ethical clearance has been obtained from the ethical committee of the National Ribat University and consent from participants.

2.2.5 Procedures:
The study was conducted through the following phases: Questionnaire, Clinical examination, Body Mass Index (BMI), Lung function tests, collection of blood sample, Molecular analysis and Laboratory Analysis.

2.2.5.1 Questionnaire:
All selected subjects were interviewed to fill a questionnaire (appendix 1) form including information about personal data, smoking habits, Family history, age of onset, past medical history, drug history, triggering factors, Major asthma symptoms such as wheeze, cough, chest tightness and shortness of breathing. The severity of asthma was assessed in accordance with the international UNION classification. Also the asthmatics patients were assessed by using Asthma control questionnaire (appendix 2).

Asthma Control Test:
There were five questions in total. The patient was requested to circle the appropriate score for each question. By adding up the numbers of the patient's responses, the total asthma control score was calculated.

2.2.5.2 Clinical examination:
The respiratory rate, pulse rate, blood pressure and any chest signs were recorded.

2.2.5.3 Body Mass Index (BMI):
Weight and height were measured with participants standing without shoes and wearing light clothing by using digital Weight scale and height scales (mobile digital stadiometer). BMI was calculated by weight in kilograms over height in meters squared.

2.2.5.4 Lung function tests:
Pulmonary function tests were performed by using electronic spirometer. The switch unit was moved to its first position (BLOW). The display unit was indicated (Blow) and three zeros. Measurements started with asking a subject to stand up, take a deep breath, put a mouthpiece connected to the spirometer in his mouth with the lips tightly around it, and then blow air out as hard and as fast as possible for at least 5 seconds. When the subject has completed this maneuver both the FEV1 and FVC measurements were displayed by pushing the switch upward to the (VIEW) position. This procedure was performed until three acceptable measurements are obtained from each subject according to standard criteria. The best was taken (266) according to the guidelines of the American Thoracic Society and European Respiratory Society.

2.2.5.5 Sampling technique:
Five mL of venous blood was collected from each subject. 2.5ml of collected blood immediately transferred into EDTA tubes (EDTA as anticoagulant) and
stored at 4°C for DNA extraction, and 2.5 ml transferred into plain tube for serum. Serum was separated from the sample of blood after clotting and after centrifugation and stored in eppendorf tube at -20°C.

2.2.5.6 Molecular analysis:

2.2.5.6.1 DNA extraction:

DNA extraction from human blood:

DNA was extracted from the peripheral blood leucocytes using salting out method.

Salting out method for DNA extraction from human blood: (267)

I. Solutions and buffers:

1. PBS (1 mM KH2P04, 154mM NaCl, 5.6 mM Na2HP04; pH7.4)- 1 liter
2. Sucrose Triton X-lysing Buffer
3. T20E5, pH8
4. Proteinase K (stock of 10 mg/mL)
5. Saturated NaCl (100 mL of sterile water was taken and 40g NaCl was added to it slowly until absolutely saturated. It was agitated before use and NaCl was left to precipitate out).

Purification of DNA from blood (2.5mL sample):

2.5 mL blood was brought to room temperature before use and then transferred to a sterile polypropylene tube. It was diluted with 2 volumes of PBS, mixed by inverting the tubes and centrifuged at 3000 g for 10 min. The supernatant was poured off. The pellet (reddish) is resuspend in 12.5ml of Sucrose Triton X-100 Lysing Buffer. The mixture was vortexed and then placed on ice for 5 minutes. The mixture was spun for 5 minutes at 3000 rpm in TH.4 rotor and the supernatant was poured off. The pellet (pinkish or white) was resuspend in 1.5 mL of T20E5 (0.6X volume of original blood). 10% SDS was added to a final concentration of 1% (100 μL) then Protienase K was added (10 mg/mL) (20 μL).
The mixture was mixed by inversion after adding each solution then the samples were incubated at 45°C overnight. 1 mL of saturated NaCl was added to each sample and mixed vigorously for 15 seconds then it was spun for 30 minutes at 3000rpm. A white pellet was formed which consisted of protein precipitated by salt, the supernatant that contained the DNA was transferred to a new tube. Precipitation of DNA was achieved by adding two volumes of absolute alcohol kept at room temperature. The solution was agitated gently and the DNA was spooled off and transfered to eppendorf tube. The DNA was washed in 70% ice-cold alcohol (1 mL), air dried and dissolved in the appropriate volume of TE (100 μL) and stored at 4 degrees overnight to dissolve. DNA was detected by electrophoresis on gel.

Figure (2.10) Precipitation of DNA:
2.2.5.6.2 Agarose gel electrophoresis requirements for DNA and PCR product:

- Agarose: forms an inert matrix utilized in separation.
- Ethidium bromide: for fluorescence under ultraviolet light
- TBE: stands for Tris Borate EDTA.
- Loading buffer: 1x TBE buffer is the loading buffer which gives color and density to the sample to make it easy to load into the wells.

**Agarose gel electrophoresis procedure:**

- Making the gel (for a 2% gel, 100mL volume):
  The mixture was heated until the agarose completely dissolved and then cooled to about 60°C and 4µL of ethidium bromide (10mg/mL) added and swirled to mix. The gel slowly poured into the tank. Any bubbles were pushed away to the side using a disposable tip. The combs were inserted, double check for being correctly positioned and left to solidify. 5µL DNA or PCR product was loaded on the gel (inside the well). 1X TBE buffer (running buffer) poured into the gel tank to submerge the gel. The gel was run at 100V for 20 minutes. After that it was viewed on the ultra-violet radiation system.
-Five SNPs were selected for screening (table 2.1).
2.2.5.6.3 Polymerase chain reaction (PCR):

**Standard PCR reaction:**

All components necessary to make new DNA in the PCR process were placed in 20µl total volume. The experiment consists of DNA in 0.5 ml maxime PCR Premix tube.
**Primers preparation:**
10 µL of primer added to 90 µL of sterile water. Forward and reverse primers were prepared in separate eppendorf tube.

**Preparation of PCR mixture:**
The mixture prepared by adding 1 µL of forward primer, 1 µL of reverse primer and 16 µL sterile water to PCR Premix tube and finally 2 µL of DNA.(appendix 3)

**Table (2.2) The Polymerase chain reaction protocol:**

<table>
<thead>
<tr>
<th>PCR cycle</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C</td>
<td>5 min</td>
<td>-</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>30 sec</td>
<td>30</td>
</tr>
<tr>
<td>Annealing</td>
<td>57°C</td>
<td>30 sec</td>
<td>30</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>30 sec</td>
<td>30</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>5 min</td>
<td>-</td>
</tr>
</tbody>
</table>

**Checking of PCR products:**
To confirm the presence of amplifiable DNA in the samples, by evaluating the production of the target fragment by gel electrophoresis of 5µL PCR products on 2% agarose gel stained with ethidium bromide.

**2.2.5.6.4Restriction Fragment Length Polymorphism Analysis (RFLPs):**
For restriction enzyme analysis: the mixture contains 10 µL of the PCR product, 0.5 µL (10 U) of restriction enzyme, 2.5 µL of 10X buffer and 12 µL of H₂O (total volume 25 µL). This mixture was incubated according to the enzyme. After the incubation was complete, the restriction analysis was carried out in an agarose gel electrophoresis with 1X TBE buffer.
Table (2.3) the restriction enzymes incubation protocol:

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Incubation</th>
<th>Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BsmFI</td>
<td>1 hour at 65°C</td>
<td>20 minutes at 80°C</td>
</tr>
<tr>
<td>BsmAI</td>
<td>2 hours at 55°C</td>
<td>20 minutes at 80°C</td>
</tr>
<tr>
<td>MspI</td>
<td>20 minute at 37°C</td>
<td>------</td>
</tr>
</tbody>
</table>

2.2.5.7 Laboratory Analysis:
2.2.5.7.1 Polyacrylamide Gel Electrophoresis (PAGE): (268)

Principle of the test:
Different samples will be loaded in wells or depressions at the top of the polyacrylamide gel. The proteins move into the gel when an electric field is applied. The gel minimizes convection currents caused by small temperature gradients, and it minimizes protein movements other than those induced by the electric field. Proteins can be visualized after electrophoresis by treating the gel with a stain, which binds to the proteins but not to the gel itself. Each band on the gel represents a different protein (or a protein subunit); smaller proteins are found near the bottom of the gel.

2.2.5.7.1.1 Preparation of the reagents:
- 30% acryl/bisacrylamide:
  30g of acrylamide and 0.8g of bis-acrylamide were dissolved in 100ml distilled water (dH₂O).
- 8X Tris·Cl, pH 8.8 (1.5 M Tris·Cl):
  182g Tris base was dissolved in 300 ml H₂O and Adjusted to pH 8.8 with 1 N HCl. H₂O was added to 500 ml total volume.
- 10% of ammonium persulphate:
1g of ammoniumpersulphate was dissolved in 100ml dH₂O.

- **4X Tris·Cl, pH 6.8 (0.5 M Tris·Cl):**
  6.05 g Tris base was dissolved in 40 ml H₂O and adjusted to pH 6.8 with 1 N HCl. H₂O was added to 100 ml total volume.

- **Sample buffer:**
  1ml glycerol, 1ml distilled H₂O and 0.001g bromophenol blue were mixed.

- **5XElectrophoresis buffer:**
  15.1g Tris base and 72.0g glycine were dissolved in 1000 ml H₂O.
  - Dilute to 1X for working solution.

- **Benzidine stain:**
  14.6 ml acetic acid and 1 gram benzinedihydrochloride were dissolved in 485.4 ml distilled H₂O
  - 1 drop H₂O₂ (2% = 5 µl/200 µl) was added just before use.

- **Hemoglobin (Hb) solution:**
  1ml of whole blood was washed by 5ml PBS five times .1ml of washed RBCs added to 9 ml distilled water, left at room temperature for 30 minute .Then centrifuged at 15000 for 1hour .The supernatant is the standard Hb solution.

### 2.2.5.7.1.2 Separating gel preparation:

The phenotypes of Hp was determined using 7% acrylamide gel, which was prepared by mixing 3.5ml of 30% acrylamide/bis-acrylamide , 3.75ml of 1.5 M Tris-Cl (pH 8.8) , 70µl of 10% ammonium persulphate and 20µl Tetramethylethylenediamine (TEMED) and then the volume was completed by addition of 7.75ml deionized water, mixed well and 4ml of this mixture was immediately poured with a Pasteur pipette into the tray of the instrument. One ml of butanol was added just after the addition of the gel to prevent bubbles formation. The gel was left to solidify for 20 min before the addition of the stacking gel.

### 2.2.5.7.1.3 Stacking gel preparation:
After the solidification of the separating gel, stacking gel was prepared by mixing 0.65 ml of 30% acrylamide/bisacrylamide, 1.25 ml of 0.5 M Tris-Cl, 30µl of 10% ammonium persulphate and 15µl Tetramethylethylene diamine (TEMED) and then the volume was completed by addition of 3.05 ml deionized water, mixed well and 2ml of this mixture was immediately poured with a Pasteur pipette above the separating gel, and immediately a 1.5mm thick comb was inserted, the gel was left to solidify for 20 min after that the combs were removed.

2.2.5.7.1.4 Sample preparation and loading:
From the serum, 10µl was mixed with 3µl of Hb solution and incubated for 5 min at room temperature then 10µl of the sample buffer was added and mixed. 10 µl of the sample were loaded into the gel. Running has been done with 1X electrophoresis buffer at 200V for 2 hrs.

2.2.5.7.1.4 Protein staining:
After the protein has been separated, the protein was stained by Benzidin stain. After the gel was removed from the casting glasses it was immersed in 30ml of the stain solution, the bands started to appear immediately and Hp phenotypes was determined according to the pattern of each band in the gel.

2.2.5.7.2 ELISA (enzyme-linked immuno assay):

2.2.5.7.2.1 Quantitative assay for total IgE antibodies:
The components of the kit are: (appendix 7)
- Microplate: 96 wells, pre-coated with anti-IgE.
- Reagent 1: buffered protein solution with antimicrobial agent.
- Reagent 2: wash buffer concentrate.
- Reagent 3: (conjugate) mouse anti human IgE conjugate (horseradish peroxidase).
- Reagent 4: TMB substrate (Tetramethylbenzidine).
- Reagent 5: stop solution.
- Standards: 0, 50, 150, 375, 1250IU/ml of 10mM tris-buffered saline containing human serum IgE ready to use.
- Positive control 1mL of 10mM tris-buffered saline containing human serum IgE ready to use.

**Principle of the test:**

Diluted serum samples incubated with monoclonal anti human IgE immobilized on microtitre wells. After washing away unbound serum components, monoclonal mouse anti-human conjugated to horseradish peroxidase is added to the wells, and this binds to surface-bound IgE during the second incubation. Unbound conjugate is removed by washing, and a solution containing 3, 3’, 5, 5’, -tetramethylbenzidine (TMB) and enzyme substrate is added to trace specific antibody binding. Addition to stop solution terminates the reaction and provides the appropriate pH color development. The optical densities of the standards, positive control and samples are measured using microplate reader at 450 nm.

**Procedure:**

-100µl of each standard and positive control were dispensed.
20µl of each sample and 80µl of sample diluent were dispensed into each sample well (to make a 1/5 dilution). The plate was tapped rapidly to mix the well contents. It was then incubated for 60 minutes at room temperature. During all incubations direct sunlight and close proximity of any heat sources were avoided. After 60 minutes, the well contents were decanted and washed 3 times using manual procedure.

**Manual wash procedure:**
The wells were emptied by inversion and blotted on absorbent paper. The wells were filled with wash buffer by wash bottle and then emptied again. This wash process was repeated 3 times.

After that, 100µl of conjugate was dispensed to each well then it was incubated for 30 minutes at room temperature. After incubation the well contents were discarded and carefully the wells were washed 4 times with wash buffer. 100µl of TMB substrate was rapidly dispensed into each well by a repeating dispenser. The plate was incubated for 15 minutes. 100µl of stop solution was added to each well. To allow equal reaction times, the stop solution should be added to the wells in the same order as the TMB substrate. The optical density of each well was read at 450nm by a microplate reader within 10 minutes.

2.2.5.7.2.2 Quantitative assay for total Haptoglobin:

The components of the kit are: (appendix 8)

- Microplate: 96 well coated with antibody against human Hp.
- Hp Conjugate: antibody against human Hp (horseradish peroxidase)
- Wash buffer concentrate: concentrated solution of buffered surfactant.
- Color reagent A: stabilized hydrogen peroxide.
- Color reagent B: stabilized chromogen (tertramethylbenzidine).
- Stop solution: 2N sulfuric acid.

Principle of the assay:

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human haptoglobin has been pre-coated onto a microplate. Standards and samples are pipette into the wells and any haptoglobin
present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human haptoglobin is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of haptoglobin bound in the initial step. The color development is stopped and the intensity of the color is measured.

**Reagent preparation:**
All reagents and samples were brought to room temperature before use. Wash buffer was mixed gently. Color reagent A and B were mixed together in equal volumes within 15 minutes of use. Calibrator diluent was prepared by adding 20μL of it to 80 20μL distilled water. (Appendix 9)

**Assay procedure:**
The excess microplate strips were removed from the plate frame and returned to the foil pouch containing the desicant pack and reseal. 200μL of assay diluents RD1-109 was added to each well. 20 μL of standard, control and samples were added per well and covered with adhesive strip provided and incubated for 2 hours at room temperature. After that aspirated and washed and repeated the process three times for a total four washes. Complete removal of liquid at each step is essential to good performance. After the last wash, removed any remaining wash buffer by aspirating or decanting. The plate was inverted and plotted against clean paper towels.
After washing 200 μL of Hp conjugate was added to each well and covered with anew adhesive strip and incubated for 2 hours at room temperature. The aspiration/wash as in step 5 was repeated. 200 μL of substrate solution was added to each well and incubated for 30 minute at room temperature on the penchtop and
protected from light. 50 μL of stop solution was added to each well. The optical density of each well was determined within 30 minutes, by a microplate reader set to 450nm.

2.2.5.7.2.3 **Quantitative assay for IL4:**

The components of the kit are: (appendix 10)

- Microplate: 96 well coated with antibody specific for human IL4.
- Human IL4 standard: recombinant human IL4 in a buffered protein base.
- IL4 Conjugate: antibody specific for IL4 (horseradish peroxidase)
- Calibrator diluent RD6-9: animal serum.
- Wash buffer concentrate: concentrated solution of buffered surfactant.
- Color reagent A: stabilized hydrogen peroxide.
- Color reagent B: stabilized chromogen (tertramethylbenzidine).
- Stop solution: 2N sulfuric acid.

**Principle of the assay:** it is the same principle as for haptoglobin.

**Reagent preparation:**

All reagents and samples were brought to room temperature before use.

Concentrated wash buffer was mixed gently and 20mL of it was added to 480 mL distilled water. Substrate solution was prepared by mixing color reagent A and B together in equal volumes within 15 minutes of use. Calibrator diluent RD5L was diluted (1-5) by adding 20μL of it to 80μL distilled water. (Appendix 11)

**Assay procedure:**

The excess microplate strips were removed from the plate frame and returned them to the foil pouch containing the desicant pack and reseal. 100μL of assay diluents RD1-32 was added to each well and 50 μL of standard, control, samples were...
added per well within 15 minutes and covered with adhesive strip provided and incubated for 2 hours at room temperature.

Aspirate and wash each well, and repeated the process twice for a total three washes. Wash by filling each well with wash buffer (400 µL) using a squirt bottle. Any remaining wash buffer was removed by aspirating or decanting after the last wash. The plate was inverted and plotted against clean paper towels.

200 µL of human IL4 conjugate was added to each well and covered with anew adhesive strip and incubated for 2 hours at room temperature. The aspiration/wash was repeated and 200 µL of substrate solution was added to each well and incubate for 30 minute at room temperature on the pencthop and protected from light.50 µL of stop solution was added to each well and the color in the well was changed from blue to yellow. The optical density of each well was determined within 30 minutes, by using a microplate reader set to 450nm.

2.3 Method of data analysis:
Results obtained were analyzed using the Statistical Package for the Social Sciences (SPSS). Data were expressed as means with the standard deviation (sd). The correlations were analyzed by Pearson correlations. Numerical data were compared using one way ANOVA for multiple groups. Categorical data were compared using chi-square testing and cases Cross tabulation for multiple groups. When three groups (two homozygote groups and one heterozygote group) were compared, only the overall p value was generated to determine whether an overall difference in the three groups existed. And a P value equal to or lower than 0.05 was considered statistically significant.
Results

A total of one hundred and sixteen Sudanese subjects participated in the study of different ages and sexes.

Figure (3.1): The percentage of females and males in the study group:

3.1 Study group:
The participants were distributed as:

Test group:
63 subjects were selected and classified as Asthmatic.
- 35 subjects were females.
- 28 subjects were males.

Control group:
53 subjects were selected and classified as normal.
- 16 subjects were females.
- 37 subjects were males.

Figure (3.2): The total number of females and males:

Table (3.1) Distribution of anthropometric characteristics among female subjects:

<table>
<thead>
<tr>
<th></th>
<th>Means ± sd</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>Normal (16)</td>
<td>Asthmatic (35)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>29.4 ± 6</td>
<td>34.77 ±13</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>163.5 ± 6.5</td>
<td>158.9 ± 5.9</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>69.75 ± 8.38</td>
<td>68.5 ± 15.8</td>
</tr>
<tr>
<td>Body mass index (Kg/m²)</td>
<td>26.3 ± 4.17</td>
<td>27.08 ± 6.4</td>
</tr>
</tbody>
</table>

Table (3.2) Distribution of anthropometric characteristics among male subjects:

<table>
<thead>
<tr>
<th></th>
<th>Means ± sd</th>
<th>P values</th>
</tr>
</thead>
</table>
Genetic studies do not influence by age.

The asthmatic group was divided according to International UNION classification. We have included 63 cases of asthma of which 10 (16%) had intermittent, 10 (16%) had mild persistent, 27(43%) had moderate persistent and 16(25%) had severe persistent subgroup of asthma.

**Figure (3.3): The classification of asthmatic group:**

<table>
<thead>
<tr>
<th>Number</th>
<th>Normal (37)</th>
<th>Asthmatic (28)</th>
<th>----------</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>30.05 ± 7.8</td>
<td>40.58 ± 16</td>
<td>0.002*</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>179 ± 7.9</td>
<td>169.9 ± 8.9</td>
<td>0.751</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>76 ± 11</td>
<td>69.1 ± 16</td>
<td>0.181</td>
</tr>
<tr>
<td>Body mass index (Kg/m²)</td>
<td>24.1 ± 3.6</td>
<td>23.5 ± 4.6</td>
<td>0.717</td>
</tr>
</tbody>
</table>

3.2 Asthma Control Test (ACT):

The ACT test showed decline in asthmatic patients score (table 3.3).

**Table (3.3): The asthma control test score in asthma patients:**
ACT score < 20- off target indicates that asthma was not controlled.

### 3.3 lung function tests:

All parameters (FEV1, FVC, PEFR,) were better in normal compared to asthmatic subjects. The difference between normal and asthmatic was highly significant (table 3.4).

#### Table (3.4) Distribution of lung functions test among the study group:

<table>
<thead>
<tr>
<th>Test</th>
<th>Means ± Sd</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal (53)</td>
<td>Asthmatic (63)</td>
</tr>
<tr>
<td>FEV1</td>
<td>3.16 ± 0.77</td>
<td>2.01 ± 0.73</td>
</tr>
<tr>
<td>FVC</td>
<td>3.66 ± 0.83</td>
<td>2.63 ± 0.88</td>
</tr>
<tr>
<td>PEFR</td>
<td>484 ± 147</td>
<td>312.8 ± 112.5</td>
</tr>
</tbody>
</table>

FVC: forced vital capacity   FEV1: forced expiratory volume in 1second   PEFR: peak expiratory flow rate.

### 3.4 Serum level of IgE and IL4 in asthmatic and control:

Serum level of IgE and IL4 were significantly higher in asthmatics (table 3.5).

#### Table (3.5) Distribution of serum level of IgE and IL4 among the study group:

<table>
<thead>
<tr>
<th>Test</th>
<th>Means ± sd</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Asthmatic</td>
</tr>
<tr>
<td>IgE (IU/ml)</td>
<td>334 ± 250.71</td>
<td>769 ± 377.6</td>
</tr>
</tbody>
</table>
IL4 (pg/mL) | 10.27 ±1.1 | 12.5 ± 2.1 | 0.000

Figure (3.4) IgE level in asthmatic and control group:

Figure (3.5): IL4 level in asthmatic and control:
3.4.1 Serum level of IgE in different classes of asthma:
Comparing serum IgE levels between each asthmatic group by using one way ANOVA showed that statistically there was no significant difference between the groups observed (P= 0.867)(table3.6).

**Table (3.6) Serum IgE in different classes of Asthma:**

<table>
<thead>
<tr>
<th>Asthma class</th>
<th>Mean (±Sd)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermittent</td>
<td>743.3 ± 405.3</td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>855 ± 387.3</td>
<td>0.867</td>
</tr>
<tr>
<td>Moderate</td>
<td>767.9 ± 362.8</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>722 ± 412.3</td>
<td></td>
</tr>
</tbody>
</table>

3.4.2 Serum level of IL4 in different classes of asthma:
Comparing serum IL4 levels between each asthmatic group by using one way ANOVA showed that statistically there was no significant difference between the groups observed (P=0.536) (table 3.7).

**Table (3.7) Serum IL4 in different classes of Asthma:**

<table>
<thead>
<tr>
<th>Asthma class</th>
<th>Mean ±Sd</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermittent</td>
<td>11.9 ± 2.3</td>
<td>0.536</td>
</tr>
<tr>
<td>Mild</td>
<td>13.3 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>12.6 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>12.3 ± 1.6</td>
<td></td>
</tr>
</tbody>
</table>

### 3.5 Haptoglobin phenotypes and Level:

The high haptoglobin phenotypes frequency of asthmatics was observed in 50.8% patients with Hp2-1. The Hp2-2 phenotype frequencies were found in 31.7% and Hp1-1 in 17.5%. In healthy control group, the high haptoglobin phenotype frequencies were seen in 66% with Hp2-1, whereas 26.4% for Hp2-2 and 7.6% for Hp1-1 were found (table 3.8). Figure (3.6) shows determination of serum haptoglobin phenotypes electrophoresis in polyacrylamid gel using benzidin stain. Comparison of each haptoglobin phenotype together by using Chi-Square Tests and cases Cross tabulation, the frequency difference of each phenotype in the two groups were analyzed and statistically there was no significant difference between the two groups observed (P=0.163).

**Table (3.8) Distribution of Hp phenotype among the study group:**
<table>
<thead>
<tr>
<th>Phenotype</th>
<th>% of Normal</th>
<th>% of Asthmatic</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>7.6</td>
<td>17.5</td>
<td></td>
</tr>
<tr>
<td>2-1</td>
<td>66</td>
<td>50.8</td>
<td>0.163</td>
</tr>
<tr>
<td>2-2</td>
<td>26.4</td>
<td>31.7</td>
<td></td>
</tr>
</tbody>
</table>

Figure (3.6) Determination of Haptoglobin phenotype electrophoresed in polyacrylamid gel:

Serum Hp level showed significant difference between asthmatic and control (table 3.9)(figure 3.7). Comparing serum Hp levels between patients and controls in
each phenotypic group showed that mean of Hp serum level was significantly higher in patients than controls in 1-1 and 2-1 phenotypes and no significant differences in 2-2 phenotype (table 3.10).

Table (3.9) Distribution of serum level of Hp among the study group:

<table>
<thead>
<tr>
<th>Test</th>
<th>Means ± sd</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Asthmatic</td>
</tr>
<tr>
<td>Hp (g/L)</td>
<td>2.72 ±1.4</td>
<td>4.17 ± 1.7</td>
</tr>
</tbody>
</table>

Figure (3.7): comparison of Hp level in serum of asthmatic and control:

Table (3.10) A comparison of serum Hp in Patients and healthy control with different haptoglobin phenotype:

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Group</th>
<th>Mean(g/L) ±Sd</th>
<th>P. Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Comparing serum Hp levels between each asthmatic group by using one way ANOVA showed that mean of Hp serum level was significantly different between all groups (table 3.11).

Table (3.11) Serum Hp in different classes of Asthma:

<table>
<thead>
<tr>
<th>Asthma class</th>
<th>Mean (g/L) ± sd</th>
<th>P. Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermittent</td>
<td>5.23 ± 1.5</td>
<td>0.034</td>
</tr>
<tr>
<td>Mild</td>
<td>3.59 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>3.68 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>4.79 ± 1.4</td>
<td></td>
</tr>
</tbody>
</table>

3.6 polymorphisms of ADAM33 in chromosome 20:
Analysis of both allele and genotype frequencies for ADAM33 polymorphism by using Chi-square calculations tests showed that ADAM33 polymorphism was significantly associated with asthma. The minor allele, A, of ADAM33 SNPs was significantly more frequent in asthmatics than in the control group. The major allele, G, was significantly more frequent in the control group than in asthmatics.
Concerning genotype frequencies, Cases Cross tabulation and Chi-square tests demonstrated significant differences between asthmatics and control subjects: The minor A/A genotype was more frequent in the asthmatics (71.4%) than in the control group (P-value = 0.000) (table 3.12). Figure (3.8) showed ADAM33 PCR product on 3% agarose and Figure (3.10) showed analysis of ADAM33 polymorphism on 3.8% agarose.

**Table (3.12) Allele and genotype frequencies for ADAM33 SNPs:**

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Allele or genotype</th>
<th>% of Asthmatics</th>
<th>% of Normal</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>F+1 G&gt;A</td>
<td>G</td>
<td>19</td>
<td>44.3</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>81</td>
<td>55.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>9.5</td>
<td>24.5</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>19.1</td>
<td>41.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>71.4</td>
<td>34</td>
<td></td>
</tr>
</tbody>
</table>

Figure (3.8): Mutant allelic frequencies of ADAM33 polymorphism for asthmatic and control (%):
Figure (3.9): The ADAM33 PCR product on 3% agarose:

The ADAM33 F+1 PCR product on 3% agarose. M: DNA Ladder (100pb); Lane’s 1-7 ADAM33 PCR products

Figure (3.10): Analysis of ADAM33 polymorphism on 3.8% agarose:

Analysis of the ADAM33 F+1 polymorphism on 3.8 % agarose. M: DNA Ladder (100pb); Lanes 1, 2, 3: Allele (GA) heterozygous; Lanes 4,5 :Allele (GG) homozygous; Lane 7: Allele (AA) homozygous

Comparing FEV1 between each ADAM33 genotype in asthmatics and control subjects by using one way ANOVA showed that mean of FEV1 was significantly
different between heterozygous (GA) and homozygous (GG) mutant genotypes (table3.11). While no significant difference with Homozygous (AA) genotype (P=0.074) (table 3.13).

**Table (3.13): A comparison of FEV1 in asthmatics and healthy control with different ADAM33 genotype:**

<table>
<thead>
<tr>
<th>ADAM33 genotype</th>
<th>FEV1 mean ± Sd</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Asthmatics</td>
<td>Control</td>
</tr>
<tr>
<td>GG</td>
<td>203 ± 86.6</td>
<td>300 ± 72</td>
</tr>
<tr>
<td>GA</td>
<td>167.9 ± 83</td>
<td>291 ± 57.8</td>
</tr>
<tr>
<td>AA</td>
<td>209.6 ±67.9</td>
<td>341.9 ±90</td>
</tr>
</tbody>
</table>

3.7 Polymorphisms of IL4 (-590 C/T) in chromosome 5:

Analysis of both allele and genotype frequencies for IL4 promoter (-590C/T) polymorphism by using Chi-square calculations tests showed that IL4 promoter (-590C/T) polymorphism was significantly associated with asthma (table 3.14). The minor allele, T, of IL4 promoter (-590C/T) SNPs was significantly more frequent in asthmatics than in the control group (figure 3.11). The major allele, C, was significantly more frequent in the control group than in asthmatics (P.value = 0.000) (table 3.14). Concerning genotype frequencies, Cases Cross tabulation and Chi-square tests demonstrated significant differences between asthmatics and control subjects: The minor T/T genotype was more frequent in the asthmatics (65.1%) than in the control group (P.value = 0.022) (table 3.14). Figure (3.12) showed IL4 (-590C/T) PCR product on 3% agarose.

**Table (3.14): Allele and genotype frequencies for IL4 (-590C/T) SNPs:**

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Allele or genotype</th>
<th>% of Asthmatics</th>
<th>% of Normal</th>
<th>P. value</th>
</tr>
</thead>
</table>

Table 3.11: Mutant Allelic frequencies of IL4 (-590C/T) polymorphism for asthmatic and control (%):

<table>
<thead>
<tr>
<th>Allele</th>
<th>Asthmatic</th>
<th>Control</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>30.2</td>
<td>57.1</td>
<td>0.00</td>
</tr>
<tr>
<td>T</td>
<td>69.8</td>
<td>42.9</td>
<td>0.00</td>
</tr>
<tr>
<td>CC</td>
<td>25.4</td>
<td>54.3</td>
<td>0.022</td>
</tr>
<tr>
<td>CT</td>
<td>9.5</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>65.1</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

Figure (3.11): Mutant Allelic frequencies of IL4 (-590C/T) polymorphism for asthmatic and control (%):

Figure (3.12): The IL4 (-590 C/T) PCR product on 3% agarose:

The IL4 (-590) C>T PCR product on 3% agarose. M: DNA Ladder (100pb); Lane’s 2-7 IL4 PCR products.
3.8 Polymorphisms of IL4Rα (-Q576R) in chromosome 16:
Analysis of both allele and genotype frequencies for IL4Rα (-576) polymorphism by using Chi-square calculations tests showed that statistically no significant difference between the minor allele, C, and the major allele, T in asthmatics and control group (P.value = 0.170) (table 3.15) (figure 3.13). Concerning genotype frequencies, Cases Cross tabulation and Chi-square tests demonstrated no significant differences between asthmatics and control subjects: The minor C/C genotype was (33.33%) in the asthmatics and (24.5%) in the control group (P-value = 0.402) (table 3.15). Figure (3.14) showed IL4Rα (-576) PCR product and analysis of polymorphism on 3% agarose.

Table (3.15) Allele and genotype frequencies for IL4Rα (-576) SNPs:

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Allele or genotype</th>
<th>% of Asthmatics</th>
<th>% of Normal</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Q576R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T &gt;C</td>
<td>T</td>
<td>42.9</td>
<td>51.9</td>
<td>0.170</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>57.1</td>
<td>48.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>19.05</td>
<td>28.3</td>
<td>0.402</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>47.62</td>
<td>47.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>33.33</td>
<td>24.5</td>
<td></td>
</tr>
</tbody>
</table>

Figure (3.13):
Mutant allelic frequencies of IL4Rα polymorphism for asthmatic and control (%):
Figure (3.14): The IL4Rα (-576) PCR product and analysis of polymorphism on 3% agarose.

Analysis of the IL4Rα T>C (Q576R) polymorphism on 3% agarose.

M: DNA Ladder (100pb); Lanes 1, 5: Allele (CC) homozygous; Lanes 6, 7: Allele (TT) homozygous; Lanes 2, 3, 4: Allele (TC) heterozygous.

Analysis of null genotype frequencies for GSTT1 polymorphism by using Chi-square calculations tests showed that higher frequency of GSTT1 deletion polymorphism was found in asthmatic (P= 0.001)(table 3.16) (figure 3.15) .Figure (3.16) showed analysis of GSTT1 polymorphism on 3% agarose.

Table (3.16): Genotype distribution of GSTT1 SNPs:
<table>
<thead>
<tr>
<th>SNPs</th>
<th>Allele</th>
<th>% of Asthmatics</th>
<th>% of Normal</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Null</td>
<td>Null</td>
<td>54</td>
<td>24.5</td>
<td>0.001</td>
</tr>
<tr>
<td>Present</td>
<td></td>
<td>46</td>
<td>75.5</td>
<td></td>
</tr>
</tbody>
</table>

GSTT1 null genotype frequencies for asthmatic and control (%):

Figure (3.15):

Figure (3.16): Analysis of GSTT1 polymorphism on 3% agarose:

Analysis of the GSTT1 Genotype on 3% agarose. A 350 bp fragment from the β-globin was coamplified for internal control purposes.

M: DNA Ladder (100pb); Lanes 1, 4: null genotype; Lanes 2, 3, 5, 6, 7...
3.9.2 Polymorphisms of GSTPi in chromosome 11:
Analysis of both allele and genotype frequencies for GSTPi Ile105Val polymorphism by using Chi-square calculations tests showed that statistically no significant difference between The minor allele, G, and major allele, A in asthmatics and control group (P.value = 0.358) (table 3.17) (figure 3.17). Concerning genotype frequencies, Cases Cross tabulation and Chi-square tests demonstrated no significant differences between asthmatics and control subjects: The minor G/G genotype was (19%) in the asthmatics and (16.98%) in the control group (P-value = 0.669) (table 3.17). Figure (3.18) showed GSTPi Ile105Val PCR product on 3% agarose and Figure (3.19) showed analysis of GSTPi Ile105Val polymorphism on 3% agarose.

Table (3.17) Allele and genotype frequencies for GSTPi SNPs:

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Allele or genotype</th>
<th>% of Asthmatics</th>
<th>% of Normal</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ile105Val 313A&gt;G</td>
<td>A</td>
<td>65.1</td>
<td>70.8</td>
<td>0.358</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>34.9</td>
<td>29.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>50.8</td>
<td>58.49</td>
<td>0.669</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>30.2</td>
<td>24.53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>19</td>
<td>16.98</td>
<td></td>
</tr>
</tbody>
</table>

Figure (3.17): Mutant allelic frequencies of GSTPi polymorphism for asthmatic and control (%):
Figure (3.18): The GSTPi Ile105Val PCR product on 3% agarose:

The GSTPi A>G (Ile_Val105) PCR product on 3% agarose
M: DNA Ladder (100pb); Lanes 2-7GSTPi PCR product

Figure (3.19): Analysis of Ile105Val polymorphism on 3% agarose:
3.10: Frequencies of mutant genotypes:
A combination of the double and triple genetic polymorphisms more frequent in asthmatic than control subjects (table 3.18) (figure 3.20).

Table (3.18): Combination of various risk mutant genotypes:

<table>
<thead>
<tr>
<th>Number of mutant genotype</th>
<th>% of Normal</th>
<th>% of Asthmatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>32.1</td>
<td>7.9</td>
</tr>
<tr>
<td>1</td>
<td>34</td>
<td>7.9</td>
</tr>
<tr>
<td>2</td>
<td>18.9</td>
<td>38.1</td>
</tr>
<tr>
<td>3</td>
<td>13.2</td>
<td>33.3</td>
</tr>
<tr>
<td>4</td>
<td>1.8</td>
<td>11.1</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Figure (3.20): Combination of various risk mutant genotypes of 5 genes:

Analysis of the GSTPi A>G (Ile_Val105) polymorphism on 3% agarose. M: DNA Ladder (100pb); Lanes 1, 3: Allele (AA) homozygous; Lane2: Allele (GG) homozygous; Lanes 4, 5: Allele (AG) heterozygous.
Discussion

Asthma is an inflammatory disease influenced by genetic and environmental factors and associated with alterations in the normal architecture of the airway walls, termed airways remodeling. The principal finding in this study is that IgE and IL4 were significantly higher in asthmatic subjects compared to control (P=0.000 and P=0.000 respectively) and no significant difference between asthma classes. A linkage between total serum IgE and IL4 gene has been shown. IL4 is a pleiotropic cytokine contributing to the maintenance of the Th2 lymphocyte profile that leads to the elevation of baseline IgE levels. The expression of IL-4 gene was significantly more in asthmatic patients compared to controls (table3.13) and the IL4 T-590 allele causes hyperproduction of IL-4.

The association of Hp phenotypes with a variety of pathological conditions has been interested by many investigators. Comparison of Hp phenotype frequency between study groups showed that Hp1-1 and Hp 2-2 were higher in asthmatics than controls, whereas Hp2-1 was higher in control group. Langlois MR et al reported that Bronchial asthma has been seen with Hp1-1.
In this study serum Hp level was found significantly higher in asthmatic patients compared to healthy controls (table 3.9) and significantly higher in all asthma classes (table 3.11). This is not unexpected, since other studies \(^{(120,120)}\) showed that asthma is associated with a higher Hp level. Association between specific protein expression in bronchoalveolar lavage during differentiation of circulating fibroblast progenitor cells in mild asthma has been reported by Larsen K et al. \(^{(125)}\) They described elevated levels of Hp in patients with mild asthma compared to the other subjects and issued a novel role for expression of Hp in airway remodeling in patients with asthma. Krasteva et al \(^{(269)}\) reported that salivary Hp level in children with allergic asthma was elevated when compared to the healthy subjects. This result is in disagreement with Piessens MF et al \(^{(89)}\) who reported that the Hp level was decreased in asthmatic subjects. Increase in Hp was seen in uncontrolled asthma. \(^{(122)}\) The increased Hp level in this study might be due to the poor control of asthma. The possible explanation for the high level is the especial functions of Hp as an immune system modulator. \(^{(95)}\) Also, Hp binds to the human mast cell line HMC-1 and inhibits its spontaneous proliferation. \(^{(100)}\) Although IgE is measured as an investigation for some asthmatics, Hp has not been used and it could be a useful marker for asthma control.

Comparison of Hp level in Patients and healthy controls with different Hp phenotypes showed that Hp level in asthmatics with 1-1 and 2-1 phenotypes was significantly higher than control with same phenotype, while Hp level in asthmatics with 2-2 phenotype was slightly increased but with no significant value (table 3.10). The possible explanation of the slight increase that the B-cells and CD4+ T-lymphocyte counts in the peripheral blood were higher in 2-2 Hp phenotype \(^{(101)}\) and Hp bind to the majority of CD4+ and CD8+ T lymphocytes \(^{(80)}\), directly inhibiting their proliferation and modifying the Th1/Th2 balance. \(^{(95)}\)
A study on the Genetics of Asthma demonstrated positive association between single nucleotide polymorphisms (SNPs) of ADAM33 and asthma in African-American, Hispanic, and white populations.\textsuperscript{(137)} In this study, we genotyped ADAM33 variants in asthmatic and control subjects to evaluate the potential influences of ADAM33 gene polymorphisms on asthma risk. As expected, significant associations were observed in asthmatic patients. We found that the homozygous mutant genotypes and allele frequencies of SNP F+1 was significantly associated with asthma (table 3.12). In previous studies, F+1 SNP polymorphism was reported in Indian adult populations \textsuperscript{(270)} and UK \textsuperscript{(39)} and German populations \textsuperscript{(271)} and these associations were also found in the study samples. In contrast, lack of association for ADAM33 gene in asthma have also been reported in populations from Korea \textsuperscript{(272)} and Australia.\textsuperscript{(273)} Comparing FEV1 between each ADAM33 genotype in asthmatics and control subjects showed that FEV1 was significantly decreased in heterozygous (GA) and homozygous (GG) mutant genotypes (table3.13) while no significant difference with Homozygous (AA) genotype (table 3.13). These results are supported by previous studies. Jongepier H et al \textsuperscript{(138)} reported that ADAM33 gene was associated with a significant excess decline in baseline forced expiratory volume in first second (FEV1). These data imply a role for ADAM33 in airway wall remodelling which is known to contribute to chronic airflow obstruction in moderate to severe asthma.\textsuperscript{(139)} Another study conducted in infants of allergic/asthmatic parents has revealed positive associations between SNPs of ADAM33 and increased airway resistance, with the strongest effect seen in the homozygotes.\textsuperscript{(140)} Thus, the present study adds to the growing list of population studies where the ADAM33 SNPs seems to play a role in the susceptibility to asthma.
Polymorphism in promoter region of the cytokine gene was analyzed (C-590T IL4). The derivative allele T has been related to elevated serum levels of IgE and asthma. Walley et al reported that allelic variant T-590 is associated with higher promoter activity and increased production of IL-4 as compared to C-590 allele. The analysis of cytokine level revealed a statistically significant increase of IL-4 in asthmatic as compared to the control (table 3.5). Hyper production of IL-4 leads to overproduction of IgE in asthmatic groups as compared with controls (table 3.5). In this study -590C/T IL4 polymorphism showed the high incidence of the TT homozygote mutant genotypes (65.1% in asthmatic and 40% in control) (P=0.022) (table 3.13). Also it was found that the T allele frequencies was significantly associated with asthma (table 3.13) (P=0.000). The results of this study are more in agreement with work reported in different population.

The IL-4 receptor alpha mediates in B lymphocytes the class-switch recombination mechanism and generation of IgE and IgG, which happens in response to IL4/IL-13 and allergens/antigens. In this study, it was found that the minor allele C was more frequently in asthmatics than in control subjects; likewise, the major allele T was found more frequently in the control subjects than in asthmatics (table 3.14) but statistically not significant (P=0.170). The CC genotype was more frequent in asthmatics and TT genotype was more frequent in control subjects (table 3.14) but statistically not significant (P=0.402). Association studies between IL4Rα polymorphisms and asthma have been reported in several ethnic groups. One study showed that the IL-4Rα Q576R mutant was not associated with serum IgE levels in Chinese asthmatic children. IL-4R α polymorphisms alone may not always influence the susceptibility to disease. The combined effect of IL-4Rα Q576R SNP with mutant alleles in other genes could contribute significantly to disease susceptibility. The consequence of these findings is that common variants alone cannot account for a given disease.
Phase II detoxification enzymes, particularly classes of glutathione S-transferases (GSTs), play an important role in inflammatory responses triggered by xenobiotic or reactive oxygen compounds.\(^{(203)}\) Studies have shown that individuals with lowered antioxidant capacity are at an increased risk of asthma.\(^{(204)}\) In particular, GSTT1 null polymorphisms and a single nucleotide polymorphism of GSTP1 (Ile105Val) may influence the pathogenesis of respiratory diseases.\(^{(203)}\) This study showed that the GSTT1 null genotype was more frequent in asthmatic patients compared with control subjects (table 3.15). Frequency of GSTT1 null polymorphism differs largely among different populations. It has highest frequency in Asians (50%) followed by African Americans (25%) and Caucasians (20%).\(^{(211)}\) Total gene deletion or null polymorphism leads to no functional enzymatic activity.\(^{(209)}\) On the other hand, genotyped GSTP1 Ile105Val SNP showed no significant difference between asthmatic and control subjects (table 3.16). The mutant G/G genotype was (19%) in the asthmatics and (16.98%) in the control group (P = 0.669) in agreement with work reporting that in different populations.\(^{(225,226)}\) Some studies reported association between GSTP1 variant and asthma.\(^{(222,223,224)}\) Asthma is a complex disease where many genes are involved and contain different phenotypes such as bronchoconstriction, airway remodeling, hyperactivity, mucus hypersecretion, and persistent inflammation.\(^{(276)}\) Given the complexity of asthma, it could be speculated that in an individual, multiple SNPs in several genes could act in concert or synergy to produce a significant effect. The current study confirmed that polymorphism of the ADAM33 gene is associated with asthma. Therefore, it is suggested that the ADAM33 gene may be an important gene for asthma development and remodeling processes. The results showed a significant association between the IL-4 promoter polymorphism -589C/T and asthma. Furthermore, this study indicated a possible involvement of a single nucleotide
polymorphism in the IL-4 gene in the development of asthma. Moreover the results showed that The GSTT1 null genotype was more frequent in asthmatic patient compared with control subjects. The CC genotype of IL4Rα gene was more frequent in asthmatics compared with control subjects. Table (3.17) showed that double and triple Mutant genotypes are more frequent in asthmatics compared with control subjects. This finding supports the view that asthma is a complex polygenic disease and that more combined genes are needed to predict the risk of asthma. Based on study findings, each candidate gene might modulate an association with asthma. But a combination of more the one gene modulate the different role of pathogenesis, such as IL4 for persistent inflammation, ADAM33 for airway remodeling and hyperactivity.

Conclusion & Recommendations

5.1 Conclusion:
The level of IgE and IL4 were higher in asthmatic subjects with no significant difference between asthma classes. Hp phenotypes have no role in activation of the disease, while Hp level was higher in asthmatic patients and in all asthma classes. Gene polymorphism of ADAM33, IL4 (-590) and GSTT1 may be associated with the development of asthma. While GSTPi gene appears to play no role in the occurrence of the disease. The present data suggests that IL4Rα polymorphisms exert only a minor effect and do not contribute significantly to the development of asthma. It cannot be excluded that IL4Rα polymorphisms interact with polymorphisms in other genes that may have effects on development of asthma.

A combination of more than one gene may play an important role in the susceptibility and development of asthma. Chromosome 20p13, chromosome 16p12.1 and chromosome 22q11.2 may be associated with the development of asthma in Sudan.

5.2 Recommendations:
- Cytokines play a key role in airway inflammation and structural changes of the respiratory tract in asthma, and thus become an important target for the development of therapeutic. Therefore, the discovery of new cytokine can afford other opportunity to control the disease.
- Investigation of haptoglobin polymorphism in asthmatic patients and its possible association to the presenting symptoms.
- **The Haptoglobin level** can be used as a biomarker for asthma control.
• Further studies on the complete genetic pathway including specific IgE receptor gene.
• Functional studies on the IL4, ADAM33 and IL4Rα single nucleotide polymorphisms are probably needed.

References


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in Egyptian Asthmatics with Different Clinical Phenotypes. J Allergy Ther.
5, 189.

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The association between the IL-4, ADRb2 and ADAM 33 gene polymorphisms
and asthma in the Taiwanese population. Journal of the Chinese Medical
Association: 75; 635-643.

Appendix (1): Questionnaire:
Assessment of the level and Phenotype of Haptoglobin and Association between the Polymorphisms of (ADAM) 33 gene, IL4 & IL-4R α in Sudanese with asthma

Candidate No: ……………………… Date: ………………………………………..

Name: (optional): ………………………………………. Age: ……………………

Tel No:-: …………………………… Relative Phone No……………………………

Gender: Male □ Female □

Tribe: ………………………….. Residence: ………………………………

Occupation: …………………………… Marital status: …………………….

1. Family history of Asthma (chest allergy):
   1st degree relative □ 2nd degree relatives □

2. Duration of asthma (chest allergy): ………………………………………

3. Type of treatment: Inhalers □ Specify…………………..
   Oral □ Specify…………………..

4. History of allergic rhinitis: Yes □ No □

5. History of drug allergy: Yes □ No □

6. Asthma symptoms trigger factors:
   Outdoor: □ Specify…………………..
   Indoor: □ Specify…………………..

7. Past medical history of:
   Diabetes: Yes □ No □ Hypertension: Yes □ No □

8. History of smoking:
   Non-smoker □ Current smoker □ ex-smoker □

9. Asthma symptoms:
   10. Wheezing (whistling) □ Shortness of breath □ Cough
       Chest tightness □ □
11. Asthma symptoms are more:
   - At night (nocturnal)  
   - at day time

12. Asthma symptoms frequency (classification of Persistent Asthma):
   - < Weekly  
   - Weekly but not daily  
   - Daily but not all day  
   - Continuous

13. Number of unplanned visits:
   - Emergency room visits
   - Hospital admissions

Weight: --------  Height: -----------  BMI:  -----------  Lung function test:

<table>
<thead>
<tr>
<th>Test</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Best</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FVC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEFR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix (2): Asthma control test:

Asthma Control Test

The following test can help people with asthma (12 years or older) assess their asthma control. Please circle the appropriate score for each question. There are FIVE questions in total.

You can calculate your total Asthma Control Test score by adding up the numbers for each of your responses. Be sure to review your results with your doctor or nurse.

Look down to find out what your score means.

1. In the past 4 weeks, how much of the time did your asthma keep you from getting as much done at work, **score**
   - 1 All of the time
   - 2 Most of the time
   - 3 Some of the time
   - 4 A little of the time
   - 5 None of the time

2. During the past 4 weeks, how often have you had shortness of breath?
   - 1 More than once a day
   - 2 Once a day
   - 3 3 to 6 times a week
   - 4 Once or twice a week
   - 5 Not at all

3. During the past 4 weeks, how often did your asthma symptoms (wheezing, coughing, shortness of breath, chest tightness or pain) wake you up at night or earlier than usual in the morning?
   - 1 4 or more nights a week
   - 2 2 to 3 nights a week
   - 3 Once a week
   - 4 Once or twice
   - 5 Not at all

4. During the past 4 weeks, how often have you used your rescue inhaler or nebulizer medication (such as salbutamol)?
   - 1 3 or more times per day
   - 2 1 or 2 times per day
   - 3 2 or 3 times a week
   - 4 Once a week or less
   - 5 Not at all

5. How would you rate your asthma control during the past 4 weeks?
   - 1 Not controlled at all
   - 2 Poorly controlled
   - 3 Somewhat controlled
   - 4 Well controlled
   - 5 Completely controlled

Know your asthma score

<table>
<thead>
<tr>
<th>Score</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>Congratulations! You have complete control of your asthma. You have no symptoms and no asthma-related limitations. See your doctor or nurse if this changes.</td>
</tr>
<tr>
<td>20 to 24</td>
<td>On Target Your asthma may be well controlled but not completely controlled. Your doctor or nurse may be able to help you aim for complete control.</td>
</tr>
<tr>
<td>Less than 20</td>
<td>Off Target Your asthma may not be controlled. Your doctor or nurse can recommend an asthma action plan to help improve your asthma control.</td>
</tr>
</tbody>
</table>

Asthma control test™ is a trademark of QualityMetric Incorporated 2002.

Appendix (3):
Maxime PCR PreMix Series

Maxime PCR PreMix Kit (i-Taq)
for 20μl rxn / 50μl rxn

Cat. No. 25025: (for 20μl rxn, 96 tubes) Cat. No. 25026: (for 20μl rxn, 480 tubes)
Cat. No. 25035: (for 50μl rxn, 96 tubes)

DESCRIPTION
INFRON’s Maxime PCR PreMix Kit has not only various kinds of PreMix Kit according to experience purpose, but also a 2X Master mix solution.
Maxime PCR PreMix Kit (i-Taq) is the product what is mixed every component: i-Taq™ DNA Polymerase, dNTP mixture, reaction buffer, and so on. in one tube for 1 rxn PCR. This is the product that can get the best result with the most convenience system. The first reason is that it has every components for PCR, so we can do PCR just add a template DNA, primer set and O.W. The second reason is that it has Gel loading buffer to do electrophoresis, so we can do gel loading without any treatment. In addition, each batches are checked by a thorough Q.C., so its reappearance is high. It is suitable for various samplers experience by fast and simple method using.

STORAGE
Store at 2-8°C. Under this condition, it is stable for at least a year.

CHARACTERISTICS
• High efficiency of the amplification
• Ready to use: only template and primers are needed
• Stable for over 1 year at -20°C
• Time-saving and cost-effective

CONTENTS
• Maxime PCR PreMix Kit (i-Taq, for 20μl rxn) 96 (480) tubes
• Maxime PCR PreMix Kit (i-Taq, for 50μl rxn) 96 tubes

Component λ 20 μl reaction 50 μl reaction
i-Taq™ DNA Polymerase (5U/μl) 2.5U 5U
dNTP mix (1mM each) 2.5mM each 2.5mM each
Reaction Buffer (10x) 1x 1x
Gel Loading buffer 1x 1x

Note: The PCR process is covered by patents issued and applicable in certain countries. INFRON Biotechnology does not encourage or support the unauthorized or Unlicensed use of the PCR process. Use of this product is recommended for persons that either have a license to perform PCR or are not required to obtain a license.

EXPERIMENTAL INFORMATION
• Comparison with different company kit

Figure 1. Comparison of Maxime PCR PreMix (i-Taq) and Company A’s PreMix system by amplifying 1 Kb DNA fragment.

PCR reaction was performed with Maxime PCR PreMix (i-Taq) and company’s product.
Lane M, 100-1000 DNA Marker; lane 1, undiluted cDNA; lane 2, 1/10 diluted cDNA; lane 3, 1/100 diluted cDNA; lane 4, 1/1000 diluted cDNA; lane 5, 1/5 diluted cDNA; lane 6, 1/50 diluted cDNA; lane 7, undiluted cDNA; lane NC, Negative control.

PROTOCOL
1. Add template DNA and primers into Maxime PCR PreMix tubes (i-Taq).
   Note 1: Recommended volume of template and primer: 3μl-9μl
   Appropriate amounts of DNA template samples
   • cDNA: 0.5-10% of first RT reaction volume
   • Plasmid DNA: 10pg-100ng
   • Genomic DNA: 0.1-1μg for single copy

   Note 2: Appropriate amounts of primers
   • Primer: 1-5pmol/μl each (sense and anti-sense)

2. Add distilled water into the tubes to a total volume of 20μl or 50μl.
   Do not calculate the dried components

   Example Total 20μl or 50μl reaction volume
   PCR reaction mixture Add Add
   Template DNA 1-2μl 2-4μl
   Primer (F): 10pmol/μl 1μl 2-4μl
   Primer (R): 10pmol/μl 1μl 2-4μl
   Distilled Water 16-17μl 44-45μl

   Total reaction volume 20 μl 58 μl

   Note: This example serves as a guideline for PCR amplification. Optional reaction conditions as amount of template DNA and amount of primer, may vary and must be individually determined.

3. Dissolve the blue pellet by pipetting.
   Note: If the mixture lets stand at RT for 1-2min after adding water, the pellet is easily dissolved

4. (Option) Add mineral oil.
   Note: This step is unnecessary when using a thermal cycler that employs a top heating method (general methods).

5. Perform PCR of samples.

6. Load samples on agarose gel without adding a loading dye buffer and perform electrophoresis.

SUGGESTED CYCLING PARAMETERS

<table>
<thead>
<tr>
<th>PCR cycle</th>
<th>Temp.</th>
<th>PCR product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C</td>
<td>3min</td>
</tr>
<tr>
<td>30-40 Cycles</td>
<td></td>
<td>29sec</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>29sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>60-65°C</td>
<td>10sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1min45sec</td>
</tr>
<tr>
<td>Final extension</td>
<td>Optional: Normal 2-5min</td>
<td></td>
</tr>
</tbody>
</table>

INFRON BIOTECHNOLOGY
www.infronbio.com / info@infronbio.com
T. (03)550-9600 / F. (03)550-9650

Appendix (4):
Appendix (5):
Appendix (7):

MspI

Features: CutSmart™, Recombinant, Time-Saver™

Reaction Conditions:
CutSmart Buffer, 37°C.

Time-Saver Protocol:
Restriction Enzyme ............................ 1 µl
DNA .............................................. 1 µg
10X NEBuffer .................................. 5 µl (1X)
Total Rxn Volume .............................. 50 µl
Incubation Temperature ..................... 37°C
Incubation Time .............................. 5-15 min.
Can also be used overnight with no star activity.

Buffer Performance:

<table>
<thead>
<tr>
<th>Buffer Performance</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEBuffer</td>
</tr>
<tr>
<td>% Activity</td>
</tr>
</tbody>
</table>

For detailed product information, scan the code below or visit www.neb.com/R0106

For Research Use Only

CUTSMART™ and TIME-SAVER™ are trademarks of New England Biolabs, Inc.
Appendix (8):

4. Materials included in the kit

- **Microplate**: 96 wells in 12 X 8 break-apart strips, pre-coated with monoclonal anti-IgE, with holder in a foil bag with desiccant

- **Reagent 1: Sample Diluent**: 10mM Buffered protein solution, pH 7.2 with antimicrobial agent, 50 ml (blue), ready to use

- **Reagent 2: Wash Buffer Concentrate**: (X 10), 100mM Tris-buffered saline with detergent, pH 7.2, 100ml

- **Reagent 3: Conjugate**: mouse anti-human IgE conjugate (horseradish peroxidase) in protein stabilising solution and antimicrobial agent, 12 ml, ready to use

- **Reagent 4: TMB Substrate**: aqueous solution of TMB and hydrogen peroxide, 12 ml, ready to use

- **Reagent 5: Stop Solution**: 0.25M sulphuric acid, 12 ml, ready to use

- **Standards**: 0, 50, 150, 375, 1250 IU/ml, 1ml of 10mM Tris-buffered saline containing human serum IgE, ready to use

- **Positive Control**: 1ml of 10mM Tris-buffered saline containing human serum IgE, ready to use
# MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

<table>
<thead>
<tr>
<th>PART</th>
<th>PART #</th>
<th>DESCRIPTION</th>
<th>STORAGE OF OPENED/RECONSTITUTED MATERIAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haptoglobin Microplate</td>
<td>894567</td>
<td>96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody against human Haptoglobin.</td>
<td>Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*</td>
</tr>
<tr>
<td>Haptoglobin Conjugate</td>
<td>894568</td>
<td>21 ml of a polyclonal antibody against human Haptoglobin conjugated to horseradish peroxidase with preservatives.</td>
<td></td>
</tr>
<tr>
<td>Haptoglobin Standard</td>
<td>894569</td>
<td>400 ng of human Haptoglobin in a buffered protein base with preservatives; lyophilized. Note: Human sourced material. See Precautions section.</td>
<td></td>
</tr>
<tr>
<td>Assay Diluent RD1-109</td>
<td>895966</td>
<td>2 vials (11 ml/vial) of a buffered protein base with preservatives.</td>
<td>May be stored for up to 1 month at 2-8 °C.*</td>
</tr>
<tr>
<td>Calibrator Diluent RD5-67</td>
<td>896011</td>
<td>21 ml of a buffered protein base with preservatives. Used diluted 1:5 in this assay.</td>
<td></td>
</tr>
<tr>
<td>Wash Buffer Concentrate</td>
<td>895003</td>
<td>21 ml of a 25-fold concentrated solution of buffered surfactant with preservative. May turn yellow over time.</td>
<td></td>
</tr>
<tr>
<td>Color Reagent A</td>
<td>895000</td>
<td>12 ml of stabilized hydrogen peroxide.</td>
<td></td>
</tr>
<tr>
<td>Color Reagent B</td>
<td>895001</td>
<td>12 ml of stabilized chromogen (tetramethylbenzidine).</td>
<td></td>
</tr>
<tr>
<td>Stop Solution</td>
<td>895032</td>
<td>6 ml of 2 N sulfuric acid.</td>
<td></td>
</tr>
<tr>
<td>Plate Sealer</td>
<td>N/A</td>
<td>4 adhesive strips.</td>
<td></td>
</tr>
</tbody>
</table>

* Provided this is within the expiration date of the kit.

Appendix (9):
REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: Haptoglobin is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RDS-67 (diluted 1:5) - Add 20 mL of Calibrator Diluent RDS-67 to 80 mL of deionized or distilled water to prepare 100 mL of Calibrator Diluent RDS-67 (diluted 1:5).

Haptoglobin Standard - Reconstitute the Haptoglobin Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 400 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 300 µL of Calibrator Diluent RDS-67 (diluted 1:5) into each tube. Pipette 300 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 200 ng/mL standard serves as the high standard. Calibrator Diluent RDS-67 (diluted 1:5) serves as the zero standard (0 ng/mL).

Appendix (10):
# MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

<table>
<thead>
<tr>
<th>PART</th>
<th>PART #</th>
<th>CATALOG # D4950</th>
<th>CATALOG # S4950</th>
<th>DESCRIPTION</th>
<th>STORAGE OF OPENED/RECONSTITUTED MATERIAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human IL-4 Microplate</td>
<td>890597</td>
<td>1 plate</td>
<td>6 plates</td>
<td>96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human IL-4.</td>
<td>Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of zip-seal. May be stored for up to 1 month at 2-8 °C.*</td>
</tr>
<tr>
<td>Human IL-4 Standard</td>
<td>890592</td>
<td>2 vials</td>
<td>12 vials</td>
<td>Recombinant human IL-4 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.</td>
<td>Discard after use. Use a fresh standard for each assay.</td>
</tr>
<tr>
<td>Human IL-4 Conjugate</td>
<td>890591</td>
<td>1 vial</td>
<td>6 vials</td>
<td>21 mL/vial of polyclonal antibody specific for human IL-4 conjugated to horseradish peroxidase with preservatives.</td>
<td></td>
</tr>
<tr>
<td>Assay Diluent RD1-32</td>
<td>895253</td>
<td>1 vial</td>
<td>6 vials</td>
<td>11 mL/vial of a buffered protein base with preservatives. Contains a precipitate. Mix well before and during use.</td>
<td></td>
</tr>
<tr>
<td>Calibrator Diluent RD5L</td>
<td>895028</td>
<td>1 vial</td>
<td>6 vials</td>
<td>21 mL/vial of a concentrated buffered protein base with preservatives. For cell culture supernate samples. Use diluted 1:5.</td>
<td>May be stored for up to 1 month at 2-8 °C.*</td>
</tr>
<tr>
<td>Calibrator Diluent RD6-9</td>
<td>895423</td>
<td>1 vial</td>
<td>6 vials</td>
<td>21 mL/vial of animal serum with preservatives. For serum/plasma samples.</td>
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</tr>
<tr>
<td>Wash Buffer Concentrate</td>
<td>895003</td>
<td>1 vial</td>
<td>6 vials</td>
<td>21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative. May turn yellow over time.</td>
<td></td>
</tr>
<tr>
<td>Color Reagent A</td>
<td>895000</td>
<td>1 vial</td>
<td>6 vials</td>
<td>12 mL/vial of stabilized hydrogen peroxide.</td>
<td></td>
</tr>
<tr>
<td>Color Reagent B</td>
<td>895001</td>
<td>1 vial</td>
<td>6 vials</td>
<td>12 mL/vial of stabilized chromogen (tetramethylbenzidine).</td>
<td></td>
</tr>
<tr>
<td>Stop Solution</td>
<td>895032</td>
<td>1 vial</td>
<td>6 vials</td>
<td>6 mL/vial of 2 N sulfuric acid.</td>
<td></td>
</tr>
<tr>
<td>Plate Sealers</td>
<td>N/A</td>
<td>4 strips</td>
<td>24 strips</td>
<td>Adhesive strips.</td>
<td></td>
</tr>
</tbody>
</table>

* Provided this is within the expiration date of the kit.

Appendix (11):
REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μL of the resultant mixture is required per well.

Calibrator Diluent RDSL (diluted 1:5) - Add 20 mL of Calibrator Diluent RDSL to deionized or distilled water to yield 100 mL of Calibrator Diluent RDSL (diluted 1:5).

Human IL-4 Standard - Refer to the vial label for reconstitution volume. Reconstitute the Human IL-4 Standard with Calibrator Diluent RDSL (diluted 1:5) (for cell culture supernate samples) or Calibrator Diluent RD6-9 (for serum/plasma samples). This reconstitution produces a stock solution of 2000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 500 μL of the appropriate Calibrator Diluent into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted standard serves as the high standard (2000 pg/mL). The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL).
CHAPTER ONE

INTRODUCTION

&

LITERATURE REVIEW
CHAPTER TWO

MATERIALS & METHODS

CHAPTER THREE

RESULTS
CHAPTER FOUR

DISCUSSION