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Association of Biochemical, Immunological Parameters and Genetic Polymorphism of the Gene Encoding Protein Tyrosine Phosphatase Non Receptor 22 with Rheumatoid Arthritis in Iraqi Patients

A Thesis

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By

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

ٱقْرَأْ بِٱسْمِ رَبِّكَ ٱلَّذِى خَلَقَ ﴿١﴾ خَلَقَ ٱلْإِنسَٰنَ
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صَدَقَ اللهُ العَظِيمُ

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Dedication

To the women who raised me as a seed in life
and irrigated me by her kindness
To her dear and noble heart
My Mother

To the candle that burned to enlighten my way in life
To my greatest Honor
My Father

To those whom I live for their sake, and I owe them my happiness
My Brothers and Sisters

To every loving and loyal heart … I dedicate my work.

Ahmed Yaseen

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Summary

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by chronic inflammation and joint destruction. The aim of this study was to investigate some biochemical markers and immunological parameters in Iraqi RA patients and effects of medical treatments on these parameters. Also, the genetic polymorphism (rs2746601) for the gene encoding protein tyrosine phosphatase non-receptor 22 (*PTPN22)* was investigated in these patients.

Blood samples were obtained from 60 (newly diagnosed and treated) RA patients (males and females) referred to Rheumatology consultation clinic in Baghdad teaching hospital, Medical city. The RA patients included 10 newly diagnosed patients with a mean age of 28.4±2.27 years and 50 treated RA patients with a mean age of 42.96±14.59 years. On the other hand, blood samples were obtained from 30 healthy controls with a mean age of 37.33±11.72 years.

The Results showed a significant increase in erythrocyte sedimentation rate (ESR) in newly diagnosed and treated RA patients. Treatments given to these patients failed to normalize ESR level. C-Reactive protein (CRP), was detected in all serum samples taken from newly diagnosed patients (100% positive results), and in 68% of treated patients in comparison with 100% negative results in healthy controls. Also, results showed that Rheumatoid factor (RF), and Anti-CCP were detected in all serum samples taken from newly diagnosed patients (100% positive results), and 60% positive results in treated RA patients in comparison with 100% negative results in healthy controls. On the other hand, interleukins levels of IL-6 and IL-23 were significantly higher in newly diagnosed RA patients compared to control. Treatment offered to the patients managed to normalize these values. Results of oxidative stress (GSH and MDA) parameters revealed that the level of the

major endogenous antioxidant, glutathione (GSH) was significantly decreased in newly diagnosed RA patients. This was associated with comparable increase in level of lipid peroxidation byproduct, Malondialdehyde (MDA). Both parameters were normalized in RA treated patients.

Genetic polymorphism in *Protein Tyrosine Phosphatase non-receptor 22* (*PTPN22*) gene was studied for rs2476601 SNP in exon 14 within chromosome 1. This SNP (rs2476601) was regarded as a major risk factor associated with susceptibility and severity of rheumatoid arthritis. Genomic DNA was first extracted from blood samples for healthy controls and (treated and newly diagnosed) RA patients. Results showed that the concentration of extracted DNA ranged between 100-200 ng/µl with purity of 1.8-2.0. Then exon 14 was amplified by using specific primers designed to be used in this study. Results of amplification showed a single specific band of 684bp which represents the complete nucleotide sequence of exon 14 on electrophoresed agarose gel (1.8%). To examine genetic polymorphism (rs2476601) in this exon, the nucleotide sequence for this fragment was determined. Results showed that the rs2476601 SNP was non-polymorphic in both RA patients and healthy control subjects with total absence of the variant 'T' allele. Furthermore, the frequency of the 'T' allele was 0.0, with T/T, C/T and C/C genotype frequencies of 0.0, 0.0, and 1.0, respectively.

In conclusion, this study shows that the rs2476601 SNP of the *PTPN22* gene is non-polymorphic in Iraqi population and therefore not associated with RA. However, since variations in the rest of the gene were it is not investigated, these results do not exclude other *PTPN22* polymorphisms from playing a role in RA susceptibility in Iraq.

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List of Abbreviations

Abbreviation	***Meaning***
ACPA	Anti-Citrullinated Protein Antibody
ACR	American College of Rheumatology
APC	Antigen presenting cells
BCDF	B cell differentiation factor
CD	Crohn's disease
CIA	Collagen induced arthritis
CRP	C-reactive protein
CTLA4	Cytotoxic T lymphocyte-associated antigen 4
dNTP	Deoxynucleotide triphosphate
DZ	Dizygotic
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme- linked immune sorbent assay
ESR	Erythrocyte sedimentation rate
EtBr	Ethidium bromide
EULAR	European League of Arthritis and Rheumatism
FDR	First degree relatives
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
HCL	Hydrochloric acid
HLA	Human leukocyte antigen
HRP	Horseradish Peroxidase
IgE	Immunoglobulin E
IL-23	Interleukin 23
IL-23R	Interleukin 23 receptor
IL-6	Interleukin 6
JAK	Janus kinase
LSD	Last significant Difference

Lyp	Lymphoid Tyrosine Phosphatase
MDA	Malondialdehyde
$MgCl_2$	Magnesium chloride
MMPs	Matrix Metalloproteinase
MS	Multiple sclerosis
MZ	Monozygotic
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
OD	Optical density
PCR	Polymerase chain reaction
PTPN22	Protein Tyrosine Phosphatase Non-receptor 22
RA	Rheumatoid arthritis
RANKL	Receptor activator of NF-kappa B ligand
RBCs	Red blood cells
RF	Rheumatoid factors
RNS	Reactive Nitrogen Species
ROR	Retinoid orphan receptor
ROS	Reactive Oxygen Species
RPM	Round Per Minute
SE	Shared epitope
SNP	Single nucleotide polymorphism
STAT4	Signal Transducer and Activator of Transcription 4
TBA	Thiobarbituric acid
TBE	Tris-Borate-EDTA
TCA	Trichloroacetic acid
TGF	Transforming growth factor
T_h17	T helper 17 cells
TNF-α	Tumor necrosis factor alpha
UV	Ultra Violet

Chapter One

Introduction and Literature Review

1.1 Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by synovial inflammation and structural damage of joints (Westwood *et al.*, 2006). The diagnosis of rheumatoid arthritis is based on clinical manifestations of joints and serological markers (Arnett, *et al.*, 1988). Patients with Rheumatoid arthritis are characterized by presenting some circulating auto-antibodies in their blood. In clinical practice the most common diagnostic test is rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) and is generally accepted by the majority of rheumatologists and recommended by the European League of Arthritis and Rheumatism (EULAR) (Combe *et al.*, 2007). These autoantibodies are valuable biomarkers for the diagnosis of rheumatoid arthritis, articular manifestations and disease activity (Van der Helm-van Mil, 2005). C- reactive protein (CRP) an acute phase protein is synthesized by hepatocytes in response to pro-inflammatory cytokines, particularly IL-6. The better has been shown to be of great value as an inflammatory marker in RA and has been suggested to mediate part of the complement activation in RA (Molenaar *et al.*, 2001).

Although the cause of rheumatoid arthritis (RA) remains unknown, the excessive production of cytokines that play a fundamental role in the processes that cause inflammation, articular destruction and extra-articular manifestations associated with RA (Brennan & McInnes, 2008), such as interleukin-6 (IL-6), occupies a critical pathogenic role in the development and progression of the disease (Bencsath *et al.*, 2006; Andersson and Erlandsson-Harris, 2007). The pathological roles of IL-6 have also been clarified in various disease conditions, such as inflammatory, autoimmune, and malignant diseases (Cassim *et al.*, 2002). IL-23 is another cytokines involved in the pathogenesis of RA and other autoimmune and inflammatory diseases. This cytokine plays a potential role in immune modulation of

different subpopulations of T helper (Th) cells in conjunction with IL-12 (Lupardus & Garcia, 2008).

Several studies suggest that reactive oxygen species (ROS) and oxidative stress may be involved in the pathogenesis of various diseases, including rheumatoid arthritis. ROS have been implicated as mediators of tissue damage in patients with rheumatoid arthritis. Under normal conditions, a variety of antioxidant mechanisms serve to control this ROS production. In recent years, increasing attention has been given to the role of reactive oxygen metabolites in the pathogenesis of inflammatory disease such as rheumatoid arthritis. It has been suggested by several studies that enzymatic and/or non-enzymatic antioxidant systems are impaired in RA, and that RA patients are thus exposed to oxidant stress (Karatas *et al.,* 2003; Sarban *et al.,* 2005). Because of the impaired antioxidant system, it seems that RA patients are exposed to lipid peroxidation which is one of the indicators of oxidative stress (Kamanli *et al.,* 2004; Sezgin *et al.,* 2005). Most studies indicate that malondialdehyde (MDA) as a product of lipid peroxidation increases in the blood of RA patients (Pallinti *et al.,* 2009).

The etiology of RA, like other autoimmune disorders are not fully understood; however, RA is complex disorder that involves interactions of genetic and environmental factors. Genetic factors contribute 50% to 60% of the risk of developing RA. The major genetic risk factors that have been reproducibly shown strong association with susceptibility are Human leukocyte antigen of DRB1 alleles (HLA-DRB1), and Protein tyrosine phosphatases non- receptor 22 (*PTPN22*) genes. Among non-HLA genes, the main RA susceptibility factor is the tyrosine-phosphatase gene *PTPN22* on chromosome 1. A missense C→T substitution at nucleotide position 1858 of this gene leads to substitution of arginine (R) to tryptophan (W) at a residue 620 of the protein product. The resulting gain of function, with enhanced

regulation of T-cell receptor (TCR) signaling during thymic selection, permits autoantigen specific T cells to escape clonal deletion, thereby predisposing to autoimmunity. This *PTPN22* polymorphism was not seen in Asian populations (Begovich *et al.*, 2004).

This study aims to examine the association between biochemical, immunological parameters and genetic polymorphism of Protein Tyrosine Phosphatase Non Receptor 22 gene in patients with RA in Iraq. To the best of our knowledge, there was no such study on this topic to correlate the genetic polymorphism and relevant biochemical, immunological parameters in Iraqi patients with Rheumatoid arthritis. Therefore, the following research strategy put into place as follows:

1) Patients from different stages of RA are intended to be used to assess the biochemical, immunological and genetic polymorphism of PTPN22 in RA patients.
2) Biochemical parameters examined are Glutathione (GSH) as antioxidant and Malondialdehyde (MDA) as a byproduct of lipid peroxidation.
3) Immunological parameters studied are Rheumatoid Factor (RF), C-reactive protein (CRP) and Anti-Cyclic Citrullinated peptide (Anti-CCP).
4) Detection of genetic polymorphism of PTPN22 gene of exon 14 by PCR amplification and DNA sequencing.

1.2 Literature Review

1.2.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the joints that affects approximately 0.5% to 1% of the general population (Kvien *et al.*, 2004). It is an autoimmune disease that produces inflammatory response of synovium secondary to hyperplasia of the synovial cells. (Firestein 2003; Lipsky 2006). RA, like most autoimmune diseases has a predisposition for women, with a female to male ratio of 3:1 (Jawaheer *et al.*, 2006), with a peak incidence ranging 30 and 50 years (Ceccato *et al.*, 2006).

The pathology of the disease process leads to destruction of the articular cartilage and ankylosis of the joints, typically the joints of the hands, wrists, knees and feet are affected in a symmetric fashion (Hulsmans *et al.*, 2005). Rheumatoid arthritis can also produce extra-articular manifestations and diffuse inflammation in the lungs, pleura, pericardium and sclera and also nodular lesions mostly in the subcutaneous tissues and some studies indicated an increased mortality associated with extra-articular features compared with RA patients without extra-articular manifestations (Turesson *et al.*, 2007; Hannawi *et al.*, 2007).

Autoimmunity plays an important role in both chronicity and progression of RA. Its natural course is almost one of persistent painful symptoms and a progressive deterioration of joint structures leading to deformities and disability if not treated adequately (Firestein 2003). Environmental factors include smoking and a number of infectious diseases may be considered to be risk or precipitating factors for RA (Gossec *et al.*, 2004).

RA is a chronic, progressive inflammatory disease of the joints and surrounding tissues accompanied by intense pain, if untreated, irreversible joint destruction and systemic complications such as fatigue, anemia, and fever (McInnes and Schett, 2011). RA patients typically show immunological abnormalities leading to the production of autoantibodies such as RF and ACPA. The first immune abnormality described in patients with RA was the production of antibodies (Abs) so-called "rheumatoid factors, (RF)" directed against the constant region of IgG (Weyand and Goronzy , 2006). It is one of the seven classification criteria for RA proposed by American College of Rheumatology (ACR) (Spiritus *et al.*, 2004). While RF is elevated in ~75% of patients with RA this auto-antibody lack specificity (Mikuls *et al.*, 2004).

1.2.2 Epidemiology

The most frequent age between 40-50 years and from then on the incidence rises with age until the age of 80. The prevalence rate in Iraq is 1 % (Al-Rawi *et al.*, 1978). First degree relative's prevalence rate is 2-3%. All ethnic groups throughout the world can be affected. It is more common in smokers than non-smokers (Jones *et al.*, 1996; Sugiyama *et al.*, 2010).

1.2.3 Diagnosis and classification

The diagnosis of RA is reliant on clinical features, laboratory tests and/or radiological findings (Arnett *et al.*, 1988). In 2010, the American College of Rheumatology (ACR) together with the European League of Arthritis and Rheumatism (EULAR) published a update, with four categories (Table 1) (Aletaha *et al.*, 2010). The 2010 criteria include RF, anti-citrullinated protein antibody (ACPA), erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), all of which indicate an ongoing inflammation. A total score of 10 is possible and a score ≥6 is defined as being definitive of RA (Aletaha *et al.*, 2010).

Table 1-1| The 2010 American College of Rheumatology and the European League Against Rheumatism classification criteria for RA (Aletaha *et al.*, 2010).

The target population is: (1) patients who have at least one joint with clinical synovitis, or (2) a patient with synovitis not better explained by another disease.	
Category A – Joint Involvement	**Score**
1 large joint	0
2−10 large joints	1
1−3 small joints	2
4−10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint)	4
Category B – Serology (at least 1 test result is needed for classification)	
Negative RF and negative ACPA	0
Low-positive RF *or* low-positive ACPA	2
High-positive RF *or* high-positive ACPA	3
Category C – Acute-phase reactants (at least 1 test result is needed for classification)	
Normal CRP and normal ESR	0
Abnormal CRP *or* normal ESR	1
Category D – Duration of symptoms	
<6 weeks duration of symptoms	0
≥6 weeks duration of symptoms	1

1.2.4 Etiology

The etiology of RA is not fully understood (Alamanosa and Drosos, 2005). It is clear that both genetic and environmental factors play important roles and have been suggested to increase the risk for developing RA (Rindfleisch and Muller, 2005). These factors, or exposures, are also thought

to interact, sometimes during several years, making it a complex challenge to elucidate the etiology of RA.

1.2.4.1 Genetic risk factors

It has long been known that there is a genetic, i.e., an inheritable, component involved in the development of RA (Frisell *et al.*, 2013). This is supported by familial clustering seen by the greater disease occurrence in first degree relatives (FDR) of probands than of healthy controls, and by an increased relative risk of two or more for FDR (Frisell *et al.*, 2013). Furthermore, in several studies of twins, monozygotic twins (MZ) (identical twins sharing the exact same genetic background) have a higher probability of inheriting RA compared with dizygotic twins (DZ) (not sharing the exact same genetic background), i.e. larger disease concordance in MZ compared with DZ, estimated to be approximately 60% (MacGregor *et al.*, 2000).

1.2.4.1.1 HLA genes

The strongest and most replicated genetic risk factors are the human leukocyte antigen (HLA) alleles, identified in a series of studies of leukocytes from patients with RA and healthy controls during the 1970´s (Stastny, 1978). The human HLA region is located on chromosome 6, and comprises three clusters. Class I, class II coding for MHC class I and II, respectively, both having a central role in antigen present (Figure 1-1). The third cluster in the HLA-region codes for immune related components, such as cytokines and complement factors (Figure 1-1). Five main loci are located in the HLA class II cluster: HLA-DP, -DQ, -DR, -DM and –DO. The HLA-DR is the locus having the strongest association with RA disease, and in particular the so called “shared epitope” (SE) alleles (i.e. DRB1*04 and DRB1*01 alleles) (Gregersen *et al.*, 1987).

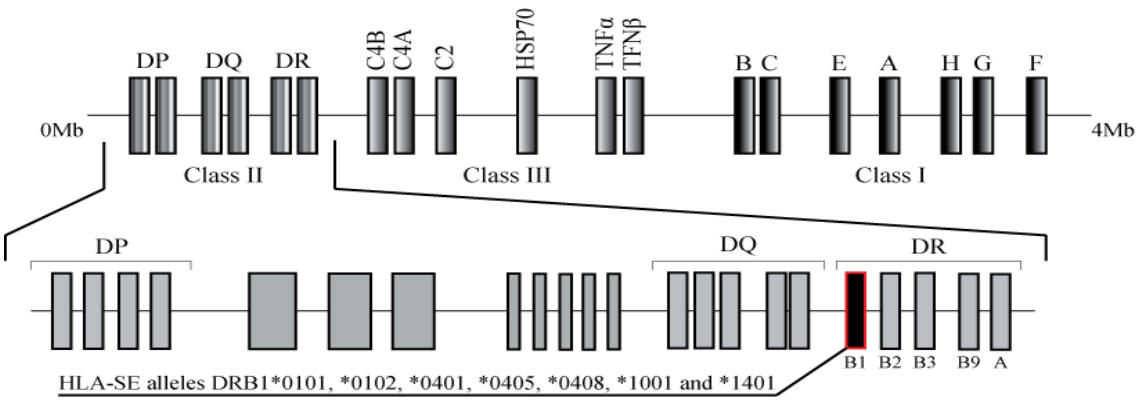

Figure 1-1| Simplified map of the HLA class I, class II and class III gene with a more detailed map of the HLA class II and the HLA-DRB1 'shared epitope' alleles associated with RA (Simmonds and Gough, 2005).

1.2.4.1.2 Non HLA-genes

1.2.4.1.2.1 Protein Tyrosine Phosphatase Non-receptor 22 (PTPN22)

The second strongest gene association with RA disease is the Protein Tyrosine Phosphatase Non-receptor type 22 *(PTPN22)* gene (Begovich *et al.*, 2004). The *PTPN22* gene encodes for the Lymphoid Tyrosine Phosphatase (Lyp) protein, involved in the negative regulation of T-cell activation (Hasegawa *et al.*, 2004). The minor allele of a non-synonymous SNP (rs2476601, 1858C→T, R620W) in the *PTPN22* gene, positioned on chromosome 1p13, has been found to be associated with RA. The *PTPN22* R620W polymorphism was increased in RA patients versus healthy controls in multiple studies North American and European Caucasian population, but not in Koreans (Kochi *et al.*, 2009). However, the rs2476601 SNP was not found to be associated with RA in the Asian population (Lee *et al.*, 2009; Ikari *et al.*, 2006). A substitution of two amino acids (Arginine to Tryptophan) at position 1858 (rs2476601) in the *PTPN22* gene has been shown to cause a gain-of-function of the Lyp protein, leading to a stronger suppression of the T-cell activation (Vang *et al.*, 2005). The important fact is that this polymorphism is not associated with RA in Asian populations, may be only with Asiatic Indians with RA positive (Mastana *et al.*, 2007).

TNF-α: Non-HLA genes within the MHC have also been examined for association with RA. TNF-α gene is located on chromosome 6 (6p21). This gene plays a central role in the inflammatory cascade in affected joints and the striking efficacy of TNF-α antagonists as therapeutic agents (Vossenaar *et al.,* 2004). Until now, five polymorphisms have been described within the *TNF-α* gene. Four polymorphisms consist of a guanine (G) to adenine (A) transition at positions –376, –308, –238 and –163 (Wilson *et al.,* 1993), and the fifth polymorphism consists of a cytosine (C) insertion in a C-stretch starting at position +70 (Brinkman *et al.,* 1995). All known *TNF-α* polymorphisms are situated in the inner region of the gene that is central to the transcriptional regulation of *TNF-α* expression. Single base alterations in such regions may have dynamic effects on gene expression (Matsuda *et al.,* 1992).

***STAT4*:** The signal transducer and activator of transcription 4 (STAT4) gene is located on chromosome 2 (2p32.2-32.3). STAT4 gene is another non-MHC gene associated with RA pathogenesis (Remmers *et al.,* 2007). Specifically, STAT4, which encodes STAT4, transmits signals induced by several key cytokines, including IL-12, IL-23, and type I interferons (IFNs) (Watford *et al.,* 2004). In contrast with HLA-DRB1 and *PTPN22*, the association of STAT4 with RA is more modest. Four polymorphisms in tight linkage disequilibrium (i.e. rs11889341, rs7574865, rs8179673 and rs10181656) form a susceptibility haplotype which is tagged by the T allele (rs7574865), have the strongest reported association with RA (Remmers *et al.,* 2007). Association of STAT4 variant (rs7574865) with RA was confirmed in patients from European, North American and Asian descent (Zervou *et al.,* 2008; Lee *et al.,* 2010).

1.2.4.2 Environmental factors

The most established environmental risk factor associated with RA, and in particular with sero-positive RA, is cigarette smoking. This association remains evident after adjustment for related factors such as body mass index, social class, age and self-perceived health (Stolt *et al.*, 2003). Smoking has also been shown to increase the risk of sero-positive RA in a dose dependent manner in combination with the strongest genetic risk factor (HLA-SE), and also showing that more citrullinated protein was found in bronchoalveolar lavage from smokers compared with non-smokers (Klareskog *et al.*, 2006). The association between smoking and RA has also been shown to be associated with sero-negative RA, but with a higher risk in the sero-positive group (Bang *et al.*, 2010). The gene-environment interaction between HLA-SE and smoking has been shown to be associated with a variety of antibodies against citrullinated peptides, indicating that smoking is associated with non-specific citrullination of several antigens rather than citrullination to only a few specific antigens (Willemze *et al.*, 2011). Furthermore, inflammation as a risk factor for ACPA-positive RA has previously shown in bronchiectasis (one of the most infectious diseases of the lung) irrespective of smoking status (Perry *et al.*, 2014).

In smokers, but not in non-smoking individuals, the intake of dietary salt has been shown to double the risk of developing RA (Sundström *et al.*, 2014). This finding is interesting since salt intake has been suggested to play a role in the development of several autoimmune disorders, and that in both *in vitro* and *in vivo* studies, increased salt levels has been demonstrated that enhanced Th17 cell differentiation and promoted IL-23R expression, promoting tissue inflammation (Wu *et al.*, 2013). Alcohol consumption, even in low quantities, has been shown to be protective for the development of RA,

with some studies showed that this effect is only present in ACPA-positive RA individuals (Scott *et al.*, 2013; Jin *et al.*, 2014).

1.2.4.3 Hormonal factors

Rheumatoid arthritis is 2-4 times more common in women than men but the mechanisms behind this are largely unknown. The use of oral contraceptives has been shown to be protective against future RA in several studies (Jorgensen *et al.*, 1996; Berglin *et al.*, 2010). Additionally, women have a lower risk of developing RA during pregnancy but they do have an increased risk during the first year postpartum (Peschken *et al.*, 2012). Other related factors have been shown with conflicting results, for example breast-feeding (Berglin *et al.*, 2010; Lahiri *et al.*, 2014), age at menarche, age at menopause and post-menopausal hormone use (Karlson *et al.*, 2004; Pikwer *et al.*, 2012).

1.2.5 Immunological Factors

1.2.5.1 Immunoregulatory Abnormalities and Autoimmunity in RA

Autoimmune diseases are disorders of immune regulation and inherently require that the affected individual have defect in the ability to distinguish self from non-self-molecule. The theory of autoimmune diseases suggests that some pathological organisms may cause an inappropriate immune response that result in the production of auto-antibodies. Also, the theory assumes that these antibodies are lacking sufficient specificity to that pathogen and theoretically mount an immune response against an inappropriate target the (synovium) because it is molecularly similar to identified molecule on the offending organism that generated the initial immune reaction. This Phenomenon is called Molecular Mimicry (Senkpiehl *et al.*, 2005).

Cytokines are produced mainly by monocytes, macrophages, lymphocytes and T-cells (Th1- and Th 2-cells), as well as mast cells, fibroblasts and endothelial cells (Evangelos *et al.,* 2002). Cytokines are involved in a wide range of activities, such as tissue breakdown and repair, cell growth and differentiation, inflammation and regulation of immune responses. The cytokines most important in arthritic diseases such as RA seem to be the pro-inflammatory cytokines, especially IL-1, IL-6 and TNFα, and possibly IL-8, being detected in elevated levels in the synovial membrane, synovial fluid and in the circulation (Al-Awadhi *et al.,* 2002). The quantity and profile of cytokines varies between different RA patients, and even between different joints of the same patient (Ulfgren *et al.,* 2000).

This inflammatory cascade of cytokines (IL-1, IL-6, TNF-α) play a pivotal role in the mechanism of bone and cartilage destruction. They are abundant in the inflamed joint and promote influx of inflammatory neutrophil and monocytes into the joint and stimulate cells in the inflamed synovium to produce proteolytic enzymes including collagenase and stromelysin that can degrade tissues (Dayer, 2004). IL-1 and TNFα have many important functions in RA. Both cytokines stimulate endothelial cells to express adhesion molecules, which is important for the attraction of inflammatory cells into the synovium (Choy and Panayi 2001). IL-1 and TNFα stimulate the proliferation of synovial cells resulting in pannus formation and they activate chondrocytes, fibroblasts and osteoclasts to secrete proteolytic enzymes which degrade cartilages and bones (Evangelos *et al.*, 2002). TNF-α produces similar effects to those of IL-1 including stimulation of the production of matrix degrading proteinases and suppression of cartilage matrix synthesis, the higher potency of IL-1compared with TNF-α in driving cartilage erosion is supported by studies showing that blockade of IL-1 is more effective than TNF-α neutralization in mice (Probert *et al.,* 1995).

IL-6 seems to perform a dual function by increasing the release of products that down regulate inflammation such as IL-1 receptor antagonist, soluble TNF receptor and tissue inhibitor of metalloproteinase. On the other hand, IL-6 enhances immune cell function (Connell and McInnes, 2006).

1.2.5.1.1 IL-6

1.2.5.1.1.1 IL-6 and Signaling Pathway of IL-6

IL-6 is a glycoprotein with a molecular weight of 26 kDa and pleiotropic activity. It was first identified as B cell differentiation factor (BCDF) or B cell stimulatory factor 2 (BSF-2), which is a T-cell-derived soluble factor that induces the differentiation of activated B cells into antibody producing cells (Kishimoto,1985). Subsequent studies also revealed that IL-6 performs multiple and essential functions in immune regulation, inflammation and could be a key mediator for the development of many chronic inflammatory or autoimmune diseases including RA (Kishimoto 2005). IL-6 triggers its signaling system through binding to an 80 kDa transmembrane IL-6 receptor (IL-6R) (Kishimoto *et al.*, 1992). After binding to IL-6R, the complex consisting of IL-6 and transmembrane IL-6R associates with signal-transducing molecule gp130, resulting in the activation of downstream signaling events via Janus kinase (JAK) in target cells (Taga *et al.*, 1998). This activation is known as classic signaling pathway. Accumulating evidence suggests that IL-6 trans-signaling is proinflammatory, whereas classic signaling is needed for regenerative or anti-inflammatory activities (Rose-John, 2012).

1.2.5.1.1.2 Pathological Role of IL-6 in RA

IL-6 has been shown to contribute to the production of autoantibodies by acting on plasma blasts (Suematsu *et al.,* 1989). Dysregulated persistent production of interleukin-6 (IL- 6) also plays a key role in the development of the main characteristics of RA (Tanaka, *et al.,* 2013). IL-6 signaling has been found to control proliferation and resistance of resting T cells against apoptosis by promoting IL-2 production and STAT3 activation (Rincon *et al.,* 1997).

It has further been demonstrated that IL-6 is involved in local inflammation causing joint destruction by inducing endothelial cells to produce IL-8 and monocyte chemo attractant protein-1 (MCP-1) and to activate expression of adhesion molecules and recruit leukocytes to involved joints (Suzuki *et al.,* 2010). Synoviocytes can produce IL-6, while IL-6 can induce synoviocyte proliferation and osteoclast differentiation through receptor activator of NF-kappa B ligand (RANKL) expression (Suzuki *et al.,* 2010). This stimulation by IL-6 is also associated with the development of osteoporosis and bone destruction. IL- 6 and IL-1 synergistically enhance the production of matrix metalloproteinase (MMPs) from synovial cells, which may lead to cartilage and joint destruction (Suzuki *et al.,* 2010).

Systemic inflammatory signs and symptoms related to RA include fever, malaise, sleep disturbance, muscle weakness, and anemia, while laboratory findings observed in patients with RA are CRP elevation, hypercoagulability, and hypoalbuminemia. These are thought to be mostly mediated by IL- 6 (Tanaka *et al.,* 2013). These findings prove that IL-6 plays a key role in the induction of immunological abnormalities and in the development of joint and systemic inflammation of RA. IL-6 was found to be elevated in serum as well as synovial fluid of patients with RA (Hirano *et al.,* 1988).

1.2.5.1.2 IL-23

Interleukin-23 (IL-23) is a heterodimeric cytokine belonging to the IL-6 /IL- 12 family that plays a key role in several autoimmune and inflammatory disorders. IL-12 family cytokines have not only proinflammatory effects but they also promote inflammatory responses. IL-23 is one of the essential factors required for the survival and/ or expansion of Th17 cells, which produce IL-17, IL-17F, IL-6 and TNF-alpha. The IL-23-IL-17 axis includes Th17 cells and plays a key role in the development of autoimmune arthritis (Paradowska-Gorycka *et al.*, 2010).

Functionally, IL-23 has been classified as a proinflammatory mediator responsible for keeping balance between effectors and regulatory T cell response and it is a necessary factor for the development of T cell-dependent inflammation (Izcue *et al.*, 2008). Recent reports have indicated that in humans increased amounts of IL-23 have been associated with the several autoimmune disease including RA, Lyme arthritis, inflammatory bowel disease, Crohn's disease (CD), psoriasis and multiple sclerosis (MS) (Beyer *et al.*, 2008; Brentano *et al.*, 2008; Izcue *et al.*, 2008). IL-23 can induce chronic inflammation through two independent pathways. A first pathway is by the activation of Th17 cells and the second by the induction of the secretion of IL-17 by non-T cells (Izcue *et al.*, 2008). Th17 cells produce cytokines, such as IL-17, IL-17F, IL-6, IL-21, IL-22 and TNF-a, which play an important role not only in the RA but also in the pathogenesis of experimental autoimmune encephalomyelitis (EAE) and collagen induced arthritis (CIA) (Lupardus and Garcia, 2008; Shahrara *et al.*, 2008; Kim *et al.*, 2007).

However, the serum level of IL-23, in patients with RA, correlated with number of swollen joint, DAS28 score and serum level of IL-17. Reduction of serum level of IL-23 after infliximab treatment correlated with number of

tender joint, DAS28 score and erythrocyte sedimentation rate, suggesting that IL-23 alone may play a role in pathogenesis of RA (Kageyama *et al.*, 2009).

1.2.5.2 Abnormal Immune Response

Abnormal immune responses implicate an altered threshold in the regulation of adaptive immune response. Also it has become clear that these genetic factors interact with the most clearly defined environmental factors for RA namely cigarette smoking (Sugiyama *et al.*, 2010).

When the abnormal immune response become established which may take several years before any symptoms occur, plasma cells derived from B-lymphocytes produce rheumatoid factors and ACPA of IgG and IgM classes in large quantities. These are not deposited in the same way they are in SLE, rather they appear to activate macrophages through FC receptor and complement binding and this contribute to synovial inflammation in the form of edema, vasodilatation and infiltration by activated T-cells (mainly CD4 in nodular aggregate and CD8 in diffuse infiltration) (Padyukov *et al.*, 2004).

Synovial macrophages and dendritic cells function as antigen presenting cells (APC) by expressing MHC class 2 molecules leading to local immune reaction. The disease progresses in concert with formation of granulation tissue at the edges of the synovial lining (pannus) with extensive angiogenesis and production of enzymes causing tissue damage (Sugiyama *et al.*, 2010).

1.2.6 Inflammatory markers in RA

While multiple blood markers of inflammation have been identified and shown to be useful in the evaluation and treatment of RA. To date, ESR and CRP have been most commonly studied and used in clinical practice.

1.2.6.1 ESR

The ESR is the most widely used laboratory measure of disease activity in clinical medicine and still remains a useful tool for monitoring inflammatory diseases, in particular, rheumatoid arthritis (Wolfe *et al.*, 2001). The ESR, an indirect assessment of inflammation, measures the distance that RBCs fall in a capillary tube over the course of an hour. The presence of inflammation causes the cells to fall more quickly due to the action of inflammatory proteins, such as fibrinogen or immunoglobulin, blocking the normal charge inhibition on RBCs. In many RA studies, an ESR level greater than 20 to 30 mm/h has been considered abnormal; however, considerable individual variability between normal and abnormal tests exists (Jonas and Deane, 2006).

In rheumatology, ESR plays an important role in different criteria assessing disease activity and improvement and as a laboratory activity measure in clinical trials (Cohen *et al.*, 2002). In RA, ESR has been shown to correlate with outcome and to influence radiological progression in many studies (Combe *et al.*, 2001).

1.2.6.2 C-Reactive protein (CRP)

C-reactive protein is a pentameric protein released in response to inflammatory stimuli. Because testing directly measures this protein, CRP levels are a more accurate measure of inflammation than the ESR. Measuring CRP in inflammatory conditions is preferred over the ESR as CRP responds much more quickly to inflammatory stimuli and can, therefore, be used as a timely marker of active inflammation (Jonas and Deane, 2006). CRP has been shown to be of great value as an inflammatory marker in RA, and its correlation with an increased rate of radiological progression has been shown in many studies (Scott, 2000; Combe *et al.*, 2001).

It is produced in the liver and is classified as an acute-phase protein on the basis of its increase in plasma concentration in response to inflammation and infection. CRP plays an important role in stimulating the complement system and cytokines production particularly IL-6.

C-reactive protein has been suggested to mediate part of the complement activation in RA (Molenaar *et al.*, 2001). It has been reported that CRP increases the expression of IL-1 and TNFα in human alveolar macrophages (Galve de Rochemonteix *et al.*, 1993), and induces the production of IL-1 receptor antagonist by human mononuclear cells (Tilag, 1993). The development of generalized bone loss in early RA has been shown to correlate closely with persistently elevated levels of CRP (Gough *et al.*, 1994). In clinical practice, a fall in CRP level represents the first objective sign of improvement in response to treatment with disease modifying drugs (Emery and Luqmani 1993).

While an elevated CRP is not specific for any condition, it is a sensitive index of ongoing inflammation and can be used in conjunction with the clinical assessment. The condition in which CRP may be positive includes RA, rheumatic fever, cancer, pneumonia and lupus. Preclinical elevations in CRP which occur 1-2 years prior to the diagnosis of RA have been observed (Nielen *et al.*, 2004).

1.2.7 Auto-antibodies

Auto-antibodies are proven as useful diagnostic tools for a variety of rheumatic and non-rheumatic autoimmune disorders. Several newly auto antibodies have become promising candidate as diagnostic indicators for RA (Vincent *et al.*, 2005). The following are the most important auto-antibodies in rheumatoid arthritis:

1.2.7.1 Rheumatoid factor (RF)

Autoantibodies directed against the Fc portion of IgG antibodies were discovered more than 50 years ago and are designated rheumatoid factor (RF). RF is primarily associated with RA, being present in between 70 and 80% of the patients, but can also be found in patients with other autoimmune diseases, e.g., Sjögren's syndrome (Van Boekel *et al.*, 2002), infectious diseases (e.g., tuberculosis and hepatitis) (Elkayam *et al.*, 2006) and occurs in about 5% of the healthy population (Van Boekel *et al.*, 2002).

The presence of RF in RA patients has been associated with a more severe disease including extra-articular manifestations, bone erosions and increased mortality, compared with RF negative patients (Sihvonen *et al.*, 2005; Berglin *et al.*, 2006). RF can be of several immunoglobulin isotypes (i.e., IgE, IgM, IgA and IgG) with RF of the IgM isotype as the most prevalent at the time of disease onset (Rantapää-Dahlqvist *et al.*, 2003). Also, several isotypes of RF, particularly IgA, but also IgM and IgG have been shown to precede the onset of symptoms in RA (Rantapää-Dahlqvist *et al.*, 2003).

The presence of RF of the IgM and IgA isotypes has been associated with exposure of tobacco smoke in both patients with RA and non-RA individuals (Jónsson *et al.*, 1998). A positive correlation has also been found between smoking and levels of IgA and IgM rheumatoid factors (Padyukov *et al.*, 2004).

Genetic susceptibility to the development of RF antibodies has been shown for carriage of the *PTPN22* R620W allele. Carriers with two risk alleles in *PTPN22* R620W have a higher risk of developing RF antibodies compared with carriers with only one risk allele (Lee *et al.*, 2005). HLA-SE,

also a risk factor for RA, has also been described as a risk factor for the development of RF antibodies in individuals with RA (Padyukov *et al.*, 2004).

1.2.7.2 Anti-citrullinated peptide antibodies (ACPA)

In 1998, the interesting finding that patients with RA produce antibodies against peptides and proteins containing citrulline, a modified form of the amino acid arginine, was first published (Schellekens *et al.*, 1998).

Anti-CCP belongs to a family of anti-filaggrin antibodies that react with the antigenic determinant that contains citrullinated arginine residues (Shmerling, 2009). Studies indicating that anti-CCP is a better diagnostic tool than RF have been reported for several years (Avouac *et al.*, 2006; Ates *et al.*, 2007). Anti-CCP has a sensitivity of 40% to 80%, a specificity of 81% to 100% (Avouac *et al.*, 2006), and an excellent positive and negative predictive value for RA diagnosis (Avouac *et al.*, 2006; Ates *et al.*, 2007). These features highlight the antibody's value for monitoring the pathogenesis of RA (Suzuki *et al.*, 2003). Schellekens *et al.*, (2000) reported that autoantibodies reactive with linear synthetic peptides containing the unusual amino acid citrulline were present in 76% of RA sera with specificity for RA of 96%. Furthermore, several studies have demonstrated that anti-cyclic citrullinated peptide (anti-CCP) antibodies can detect early RA (Kroot *et al.*, 2000; Schellekens *et al.*, 2000). The ability to diagnose early RA and detect RA in early synovitis patients, as well as differentiate RA from other connective tissue diseases such as SLE, further adds to the value of anti-CCP as bio-marker for RA (Pinheiro *et al.*, 2003; Tampoia *et al.*, 2005).

Native citrulline-containing peptides are only produced by enzymatic conversion of peptidylarginine to citrulline, because citrulline is a non-coded amino acid *in vivo*. The enzymes involved in this conversion are

peptidylarginine deiminases (PADIs). Five PADIs isoenzymes have been detected in humans, and two of them; PADIs2 and PADIs4 have been detected in human RA synovial tissues (Vossenaar *et al.*, 2003). PADIs4 gene was associated with RA (Jansen *et al.*, 2002). These facts strongly suggest that citrullination of self-proteins and production of autoantibodies against those citrullinated proteins play pathologic roles in RA.

1.2.8 Oxidative stress in Rheumatoid arthritis

Oxidative stress has been implicated in several physiological and pathological conditions (Vendemiale *et al.*, 1999), such as atherosclerosis, diabetes, aging, rheumatoid arthritis, osteoarthritis, cancer, inflammatory bowel disease, and many more (Halliwell and Gutteridge, 1999).

The consequent increase in ROS/RNS leads to changes in signal transduction and gene expression; a common phenomenon seen in disease. Signal transduction is a process enabling information to be transmitted from outside the cell to various functional elements inside the cell and it is also the mechanism by which cells communicate with each other. Tissues often respond to mild oxidative stress by producing extra antioxidants, but severe oxidative stress can cause tissue injury and consequently cell death (Halliwell, 1994).

Reactive oxygen species are produced during oxidative phosphorylation. When the production of ROS exceeds the physiological limit, it induces oxidative stress and damages proteins, lipids, and nucleic acids. Abundant amounts of ROS have been detected in the synovial fluid of inflamed rheumatoid joints (Biemond *et al.*, 1984).

Several lines of evidence are in agreement with the concept that an 'oxidative stress' contributes to the pathogenesis of RA. Several studies have been suggesting that the rheumatoid synovium is relatively ischemic and that ischemia-reperfusion has been implicated to be major factor in the injury occurring in RA (Han *et al.*, 2003). T cell stimulation leads to the production of ROS and cytokines (MacKenzie 2006). In rheumatoid arthritic synovial lymphocytes, Remans *et al.*, (2005) found intracellular ROS production. Hydrogen peroxide appears to be one of the ROS involved, since the addition of catalase suppressed the intracellular ROS production. It was concluded that chronic oxidative stress observed in synovial T lymphocytes originates from intracellular ROS production.

In other studies, Malondialdehyde (MDA) was measured as a marker of lipid peroxidation in rheumatoid arthritis patients. The levels of plasma (Kamanli *et al*, 2004) and serum (Ozkan *et al*, 2007) MDA were significantly higher in patients with RA compared to the control.

1.2.8. 1 Non-enzymatic antioxidants

1.2.8.1.1 Glutathione

Glutathione is a ubiquitous tripeptide, γ-glutamyl cysteinyl glycine, found in most plants, microorganisms, and all mammalian tissues. Glutathione exists in two forms the Thiol-reduced (GSH) and disulfide oxidized (GSSG) (DeLeve and Kaplowitz, 1991). GSSG is synthesized by the addition of two GSH molecules with the oxidation of the –SH groups in cysteine to form a disulphide bridge (-S-S-) (Halliwell and Gutteridge, 1999). Eukaryotic cells have three major reservoirs of GSH, cytosol (90%), mitochondria (10%) and small percentage in the endoplasmic reticulum (Hwang *et al.*, 1992).

Glutathione (GSH) is an important member of the antioxidant team as it has been shown to plays a key role in cellular resistance against oxidative damage; it destroys ROS and other free radicals by enzymatic as well as non-enzymatic mechanisms (Gambhir *et al.*, 1997). The glutathione system within the cells acts as a major homeostatic redox buffer and represents a primary antioxidant defense considering that it depends on the relative amounts of GSH/GSSG. Intracellular glutathione acts as an antioxidant in several ways. It can reduce peroxides to form H_2O (Martinez-Cayueta, 1995) or scavenge directly ROS (Cuzzocrea *et al.*, 2004). It can also act as a chelator of Cu^{2+} and reduces its activity to generate ROS (Halliwell and Gutteridge, 1999). Several studies have shown that the depletion of intracellular glutathione sensitizes cell populations to several circumstances such as aerobic ionizing radiation, cytotoxic drugs, and H_2O_2 (Dethmers and Meister, 1981).

Chapter Two

Materials and Methods

2. Materials and Methods

2.1. Materials

2.1.1. Apparatus

Apparatus used in this study are listed in Table (2-1).

Table (2-1): Apparatus used in this study.

Apparatus	Company	Origin
Vortex	Clay Adams	Germany
Centrifuge	Eppendorf	
Automatic Micropipette	Eppendorf	
Micro centrifuge	Eppendorf	
Incubator	American	USA
UV light Transilluminator	Ultraviolet Products institute	
Distillater	American	
Gel Electrophoresis Unit	Major Science	Japan
Digital Camera	Sony	
Refrigerator	Beko	Turkey
Hood	Telestar	Spain
Autoclave	P-Selecta/Mediclave	
Water bath	Precisterm	
Microwave Oven	Shownic	Thailand
Nano-drop Spectrophotometer	BioNeer	Korea
Micro ELISA system (reader)	Thermo	Germany
Thermal Cycler	Techne (tc-5000) Applied Biosystem	USA
Spectrophotometer	Cintra 5-GBC	France
Digital balance	Sartorius	Germany

2.1.2. Chemicals and Biological Materials

The chemicals and biological materials used in this study are listed in Table (2-2).

Table (2-2): Chemicals and Biological Materials used in this study.

Chemicals	Company	Origin
DNA Ladder Marker (100bp)	Promega	USA
Free Nuclease Distilled Water		
Loading Dye		
Master Mix		
TBE Buffer 10X (Tris-Borat EDTA)		
Ethidium Bromide	Promega	USA
Agarose		
Primer	Alpha DNA	Canada
HCL (Hydrochloric acid)	J.T. BAKER	USA
TBA (Thiobarbituric acid)	Alpha chemika	India
TCA (Trichloroacetic acid)		

2.1.3. Biological Kits

Kits used in this study are listed in Table (2-3).

Table (2-3): Kits used in this study

Kits	Company	Origin
DNA Extraction Kit	Promega	USA
Anti-CCP ELISA Kit	Elabscience Biotech	China
IL-6 ELISA Kit	GenAsia Biotech	
IL-23 ELISA Kit		
GSH (Glutathione) ELISA Kit		

2.2. Methods

2.2.1. Subjects

Blood samples were collected from 60 patients with rheumatoid arthritis (RA) referred to Rheumatology Consultation Clinic in Baghdad Teaching Hospital, Medical City for the period between November 2014 to June 2015.

The RA patients included 10 newly diagnosed (untreated) patients (2 men and 8 women) with a mean age of 28.4±2.27 year, and 50 already diagnosed confirmed having RA and currently receiving treatment included (5 men and 45 women) with a mean age of 42.96±14.59 year. Another blood samples were collected from 30 apparently healthy controls. They included 20 women and 10 men with a mean age of 37.33±11.72 year. Before blood sampling, all participants were informed about the study objectives, and their consent were obtained.

Treated patients were receiving several types of drugs, such as disease modifying anti-rheumatic drugs (DMARD) such as Methotrexate, Etanercept and Infliximab . While, some patients were affected only with Rheumatoid arthritis without receiving any treatment.

2.2.2. Biochemical and Immunological Biomarkers For Detection of Rheumatoid Arthritis

Blood samples (8-10 ml) were collected in plain tubes from 60 patients with Rheumatoid arthritis, and from 30 healthy control volunteers. Then blood samples were centrifuged at 3000 rpm for 15 minutes. After centrifugation, serum samples were taken and immediately stored at -20˚C for detection of biochemical and immunological biomarkers distinguishing the patient clinical status.

2.2.2.1. Detection of Glutathione (GSH)

2.2.2.1.1. Principle

Glutathione was detected in blood serum from patients and healthy controls by using GSH ELISA Kit. This kit uses enzyme linked immuno sorbent assay (ELISA) based on biotin double antibody sandwich technology to assay human glutathione (GSH). Glutathione (GSH) was added to wells that are pre-coated with glutathione (GSH) monoclonal antibody and then incubated. After incubation, anti-glutathione (GSH) antibodies labeled with biotin were added to unite with Streptavidin-Horse radish peroxidase, which forms an immune complex. Unbound enzymes were removed after incubation and washing, then substrates A and B were added. The solution will turn blue and change to yellow due to the effect of the acid. Addition the shades of solution and the concentration of human glutathione (GSH) are positively correlated.

2.2.2.1.2. GSH Assay Procedure

1. Dilution of standard solutions: the standard solution was diluted in small tubes following the manufacture instructions (Figure 2-1).

24ng/ml	Standard No.5	120µl Original Standard + 120µl Standard diluents
12ng/ml	Standard No.4	120µl Standard No.5 + 120µl Standard diluents
6ng/ml	Standard No.3	120µl Standard No.4 + 120µl Standard diluent
3ng/ml	Standard No.2	120µl Standard No.3 + 120µl Standard diluent
1.5ng/ml	Standard No.1	120µl Standard No.2 + 120µl Standard diluent

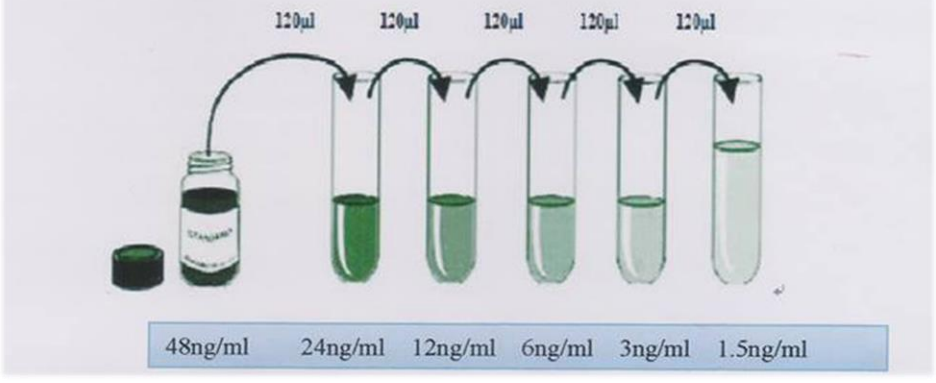

Figure (2-1): Standard Solution Preparation via Serial Dilutions

2. Sample injection:

A. Blank well: no sample, anti-Human GSH antibody labeled with biotin or Streptavidin-HRP was added in comparison with blank well; chromogen solution A and B and stop solution were added.

B. Standard solution well: 50µl standard and streptomycin-HRP 50µl were added (biotin antibodies have united in advance in the standard so no biotin antibodies are added).

C. Sample well to be tested: 40µl sample and then 10µl human GSH antibodies, 50µl Streptavidin-HRP were added. Then well was covered with seal plate membrane. Shook plate gently to mix and incubated at 37˚C for 60 minutes.

3. Preparation of washing solution: the washing concentration was diluted (30X) with distilled water for later use.

4. Washing: The seal plate membrane was carefully removed, liquid was drained and placed on filter paper for air dry. Each well was filled with washing solution, let stand for 30 seconds, then drained. This procedure was repeated five times then the plate was blotted.

5. Color development: First 50µl chromogen solution A was added to each well, and then 50µl chromogen solution B was added to each well. Shook gently to mix, then incubated for 10 minutes at 37˚C in the dark for color development.

6. Stop: 50µl Stop Solution was added to each well to stop the reaction. Color changes from blue to yellow immediately at that moment.

7. Assay: blank well was considered as zero, and then the absorbance (OD) of each well one by one was measured under 450nm wavelength. This step should be conducted within10 minutes after having added stop solution.

8. According to standards concentrations and corresponding OD values, the linear regression equation of the standard curve was calculated. Then according to the OD value of samples, the concentration of the corresponding sample was calculated.

2.2.2.1.3. Calculation of standard curve concentration of GSH

Concentration of standards was made the abscissa and OD value the ordinate. The standard curve was drawn on the graph paper. According to the OD value of the sample, its corresponding concentration (which is the concentration of the sample) was located. The linear regression equation of the standard curve was calculated according to the standard concentration and the OD value (Figure 2-2).

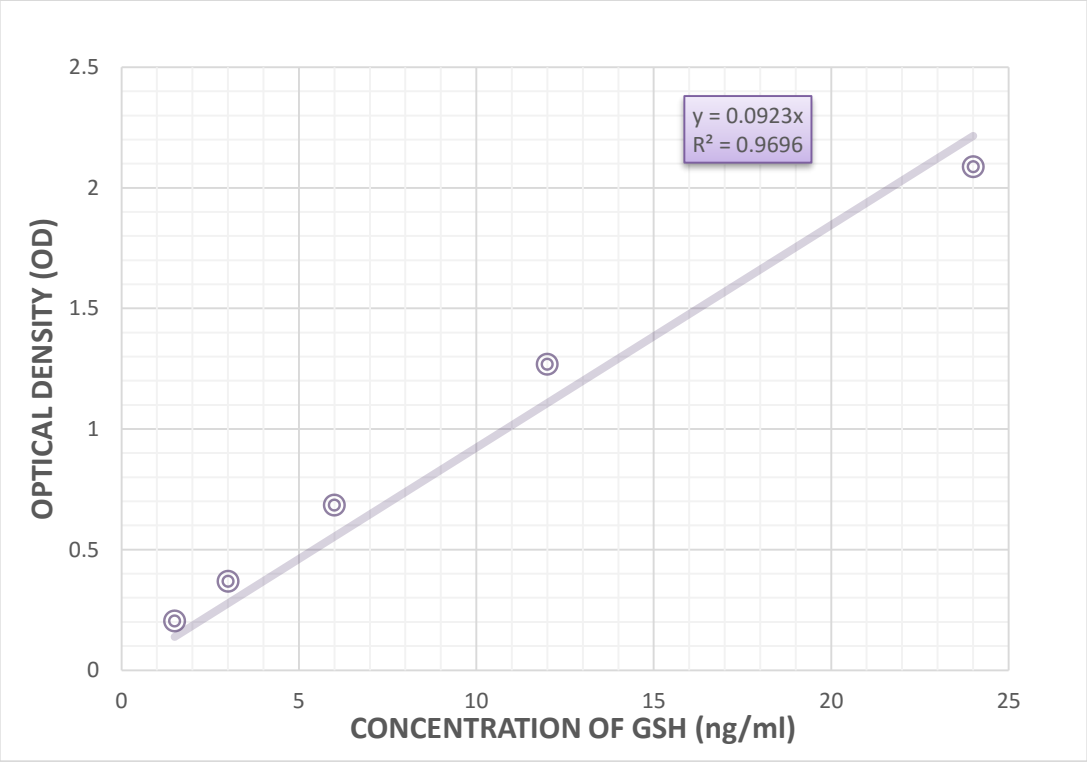

Figure (2-2): Standard Curve for Determining of GSH Levels

2.2.2.2. Detection of Malondialdehyde (MDA)

2.2.2.2.1. HCL Solution (0.25 N)

This solution was prepared by adding 2.08ml of concentrated hydrochloric acid to 98 ml of distilled water.

2.2.2.2.2. Thiobarbituric Acid Reagent (TBA)

This reagent was prepared by dissolving 0.375 g of Thiobarbituric acid (TBA) and 15 g of Trichloroacetic acid (TCA) in 100 ml of 0.25 N of hydrochloric acid solution.

2.2.2.2.3. MDA Assay Procedure

Malondialdehyde concentration in serum samples were assayed as follows:

1. Aliquot of 0.5 ml of serum samples were added to 1 ml of TBA reagent and mixed thoroughly, then the mixture was incubated at 100˚C for 15 minutes.

2. The mixture was centrifuged at 14000 rpm for 10 minutes.

3. After centrifugation, optical density for supernatants was measured at 532 nm against blank of distilled water (0.5 ml) and TBA reagent (1 ml).

4. Malondialdehyde concentration was calculated according to the following formula (Benge and Aust, 1969).

$$\text{MDA nmol/L} = \frac{OD\ (532nm) \times 100}{1.56 \times 10\text{^}5\ ^*}$$

*1.56×10^5 represents the molar absorption of MDA.

2.2.2.3. Rheumatoid Factors (RF)

2.2.2.3.1. Principle of the Method

Serum rheumatoid factor (RF) causes a visible agglutination on slide of suspension of latex particles coated with human gamma-globulin 1.

2.2.2.3.2. Composition

- A. Reagent: Suspension of latex particles coated with human gamma globulin, sodium azide 0.95 g/L, glycine buffer 100 mmol/L, pH 8.2.
- Negative Control: Serum containing RF < 30 IU/mL.
- Positive Control: Human serum containing RF > 30 IU/mL.

2.2.2.3.3. Procedure

1. Test reagents and samples are bought to room temperature.
2. Fifty µL of the sample and 1 drop of each control are placed into separate circles on the test card.
3. The latex vial (A) is shook gently before using. The Reagent vial (A) is held in vertical position and added 1 drop of Reagent (A) to each circle next to the sample to be tested.
4. Then mixed with a disposable stirrer stick and spread over the entire area enclosed by the ring. A new stirrer stick must be used for each sample.
5. Cards are rotated at 100 rpm for 2 minutes.

2.2.2.3.4. Measurement

The presence of visible agglutination is examined within a minute after removing the card from the rotator.

Positive results: The presence of a visible agglutination indicates an RF concentration 30 IU/ml.

Negative results: The absence of a visible agglutination indicates an RF concentration < 30 IU/ml.

2.2.2.4. C- Reactive protein (CRP)

2.2.2.4.1. Principle of the Method

Serum C-reactive protein (CRP) at 6 mg/L or higher causes a visible agglutination on slide of a suspension of latex particles coated with anti-CRP.

2.2.2.4.2. Composition

A: Reagent: Suspension of latex particles coated with anti-human C-reactive protein, sodium azide 0.95 g/L, glycine buffer 100 mmol/L, pH 8.2.

- C-: Negative Control: Serum containing CRP<6 mg/L.
- C+: Positive Control: Human serum containing CRP>6 mg/L.

2.2.2.4.3. Procedure

1. Test reagents and samples are brought to room temperature.
2. 50µL of the sample and 1 drop of each control are placed into separate circles on the test card.
3. The latex vial (A) is shook gently before using. The Reagent vial (A) is held in vertical position and added 1 drop of Reagent (A) to each circle next to the sample to be tested.
4. Then mixed with a disposable stirrer stick and spread over the entire area enclosed by the ring. A new stirrer stick is used for each sample.
5. Cards are rotated at 100 rpm for 2 minutes.

2.2.2.4.4. Measurement

The presence of visible agglutination is examined within a minute after removing the card from the rotator.

Positive results: The presence of a visible agglutination indicates a CRP concentration in the sample 6 mg/L.

Negative results: The absence of a visible agglutination indicates a content of CRP < 6 mg/L.

2.2.2.5. Human ACCPA ELISA Kit

2.2.2.5.1. Principle

This ELISA kit uses Sandwich-ELISA as the method. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to ACCPA. Standards or samples are added to the appropriate micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for ACCPA and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro plate well successively and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain ACCPA, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of ACCPA. One can calculate the concentration of ACCPA in the samples by comparing the OD of the samples to the standard curve.

2.2.2.5.2. Reagent preparation

All reagents were brought to room temperature (18-25℃) before use.

1. **Wash Buffer** - 30 mL of Concentrated Wash Buffer were diluted into 750 mL of Wash Buffer with deionized or distilled water. Unused solution was put back at 4℃.
2. **Standard** – Standard was prepared within 15 minutes before use. Centrifuged at 10,000×g for 1 minute. The Standard reconstituted with **1.0mL** of Reference Standard and Sample Diluent. The lid was tightened, let it stand for 10 minutes and turned it upside down for several times. After it dissolved fully, mix it thoroughly with a pipette. This reconstitution produced a stock solution of 400U/ml. Then serial dilutions were made as needed (making serial dilution in the wells directly is not permitted). The recommended concentrations were as follows: **400**, **200**, **100**, **50**, **25**, **12.5**, **6.25** and **0 U/ml** (Figure 2-3).

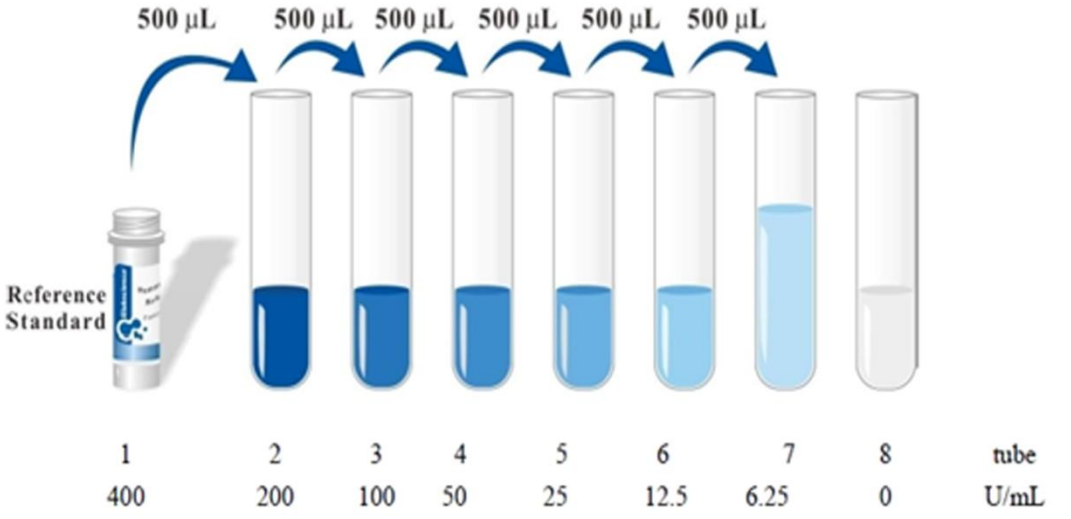

Figure (2-3): Serial dilutions of reference standards.

The undiluted standard serves as the highest standard (400U/mL). The Reference Standard &Sample Diluent serves as the zero (0 U/mL). (Standards can also be diluted according to the actual amount such as 200µL/tube)

3. **Biotinylated Detection Antibody -** The required amount was calculated before experiment (100µL/well). In actual preparation, one should prepare 100~200µL more. The stock tube was centrifuged before use. The concentrated Biotinylated Detection Ab was diluted to the working concentration using Biotinylated Detection Ab Diluent (1:100).

4. **Concentrated HRP Conjugate –**The required amount was calculated before experiment (100µL/well). In actual preparation, one should prepare 100~200µL more. The concentrated HRP conjugate was diluted to the working concentration using concentrated HRP conjugate diluent (1:100).

5. **Substrate Reagent**: As it is sensitive to light and contaminants, so one shouldn't open the vial until it is needed The needed dosage of the reagent can be aspirated with sterilized tips and the unused residual reagent shouldn't be dumped back into the vial again.

2.2.2.5.3. Washing Procedure

1. **Automated washer:** 350µL wash buffer was added into each well. The interval between injection and suction should be set about 60s.
2. **Manual wash:** 350µLWash Buffer was added into each well and soaked for 1~2 minutes. After the last wash, any remaining Wash Buffer was decanted by inverting the plate and blotting dry by rapping it firmly against clean and toweling absorbent paper on a hard surface.

2.2.2.5.4. Assay procedure

All reagents and samples were bought to room temperature before use. The sample centrifuged again after thawing before the assay. **All the reagents should be mixed thoroughly by gently swirling before**

pipetting. Foaming should be avoided. All samples and standards were assayed in duplicate.

1. **Sample:** 100µL of Standard, Blank, or Sample were added per well. The blank well was added with Reference Standard & Sample diluent. Solutions were added to the bottom of micro ELISA plate well, inside wall touching and foaming were avoided as possible, and mixed gently. The plate covered with sealer and incubated for 90 minutes at 37℃.
2. **Biotinylated detection antibody:** The liquid removed of each well, without washing. 100µL of Biotinylated Detection Ab working solution was immediately added to each well. Then covered with the Plate sealer. Then, gently tapped the plate to ensure thorough mixing and incubate for 1 hour at 37°C.
3. **Wash:** Each well was aspirated and washed, repeating the process three times. Washing was carried out by filling each well with Wash Buffer (approximately 350µL). Complete removal of liquid at each step is essential. After the last wash, remained Wash Buffer was removed by aspirating or decanting. The plate inverted and patted against thick clean absorbent paper.
4. **HRP Conjugate:** 100µL of HRP Conjugate working solution added to each well, covered with the Plate sealer and incubated for 30 minutes at 37°C.
5. **Wash:** The wash process was repeated for five times as conducted in step 3.
6. **Substrate:** 90µL of Substrate Solution was added to each well. Then covered with a new Plate sealer and incubated for about 15 minutes at 37°C. The plate was protected from light. The reaction time can be shortened or extended according to the actual color change, but not

more than 30minutes. When apparent gradient appeared in standard wells, user should terminate the reaction.

7. **Stop**: 50μLof Stop Solution added to each well. Then, the color turned to yellow immediately. The order to add stop solution should be the same as the substrate solution.
8. **OD Measurement**: The optical density (OD value) of each well was determined at once, using a micro-plate reader set to 450 nm.

2.2.2.5.5. Calculation of Standard Curve Concentration of ACCPA

The duplicate readings were averaged for each standard and samples, then subtracted the average zero standard optical density. A standard curve was created by plotting the mean OD values for each standard on the y-axis against the concentration on the x-axis and a best fit curve was drawn through the points on the graph. If samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample surpasses the upper limit of the standard curve, you should re-test it after appropriate dilution. The actual concentration is the calculated concentration multiplied dilution factor.

2.2.2.6. Detection of Interleukin-6 (IL-6)

2.2.2.6.1. Principle

Interleukin 6 (IL-6) was determined in serum samples by using IL-6 ELIAS Kit. This kit uses enzyme- linked immune sorbent assay (ELISA) based on biotin double antibody sandwich technology to assay human IL-6. IL-6 was added to wells that are pre-coated with IL-6 monoclonal antibody and then incubate. After incubation, anti- IL-6 antibodies labeled with biotin was added to unite with Streptavidin-HRP, which forms the immune complex. Unbound enzymes were removed after incubation and washing, and then substrate A and B were added. The solution will turn blue and

change to yellow with the effect of acid. The shades of solution and the concentration of human IL-6 are positively correlated.

2.2.2.6.2. IL-6 Assay Procedure

1. Dilution of standard solutions: Standard was diluted in small tubes following the manual (Figure 2-4).

320ng/L	**Standard No.5**	**120µl Original Standard + 120µl Standard diluents**
160ng/L	Standard No.4	120µl Standard No.5 + 120µl Standard diluents
80ng/L	Standard No.3	120µl Standard No.4 + 120µl Standard diluent
40ng/L	Standard No.2	120µl Standard No.3 + 120µl Standard diluent
20ng/L	Standard No.1	120µl Standard No.2 + 120µl Standard diluent

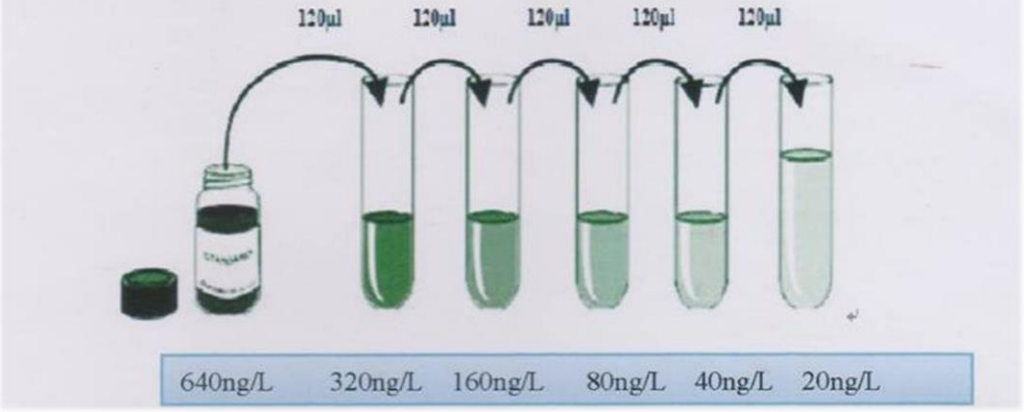

Figure (2-4): IL-6 Standard Serial Dilution

2. Sample injection:
   - **A.** Blank well: no sample, anti-human IL-6 antibody labeled with biotin or Streptavidin-HRP was added to comparison blank well. Chromogen solutions A and B were added followed by addition stop solution. Each other step operation was the same.
   - **B.** Standard solution well: 50µl standard and streptomycin-HRP 50µl was added (biotin antibodies have united in advance in the standard so no biotin antibodies are added).
   - **C.** Sample well to be tested: 40µl sample and then 10µl Human IL-6 antibodies, 50µl Streptavidin-HRP were added. Then well was

covered with seal plate membrane. Shake gently to mix and then incubate at 37˚C for 60 minutes.

3. Preparation of washing solution: The washing concentration was diluted (30X) with distilled water for later use.

4. The seal plate membrane was carefully removed; the liquid was drained and shake off the remainder. Each well was filled with washing solution, let stand for 30 seconds, then drained. This procedure was repeated five times then the plate was blotted.

5. Color development: First 50µl chromogen solution A was added to each well, and then 50µl chromogen solution B was added to each well. Shake gently to mix and then incubated for 10 minutes at 37˚C in dark for color development.

6. Stop: 50µl Stop Solution was added to each well to stop the reaction (color changes from blue to yellow immediately at that moment).

7. Assay: blank well was considered as zero, the absorbance (OD) of each well one by one was measured under 450nm wavelength. This should be conducted within 10 minutes after having added stop solution.

8. According to standards concentrations and corresponding OD values, the linear regression equation of the standard curve was calculated. Then according to the OD value of samples, the concentration of the corresponding sample was calculated.

2.2.2.6.3. Calculation of Standard Curve Concentration of IL-6

Concentration of standards was made the abscissa and OD value the ordinate. The standard curve was drawn on the graph paper. According to the OD value of the sample, its corresponding concentration (which is the concentration of the sample) was located; or the linear regression equation of the standard curve was calculated according to the standard concentration and the OD value. Then substitute with the OD value of the sample to calculate its concentration (Figure 2-5).

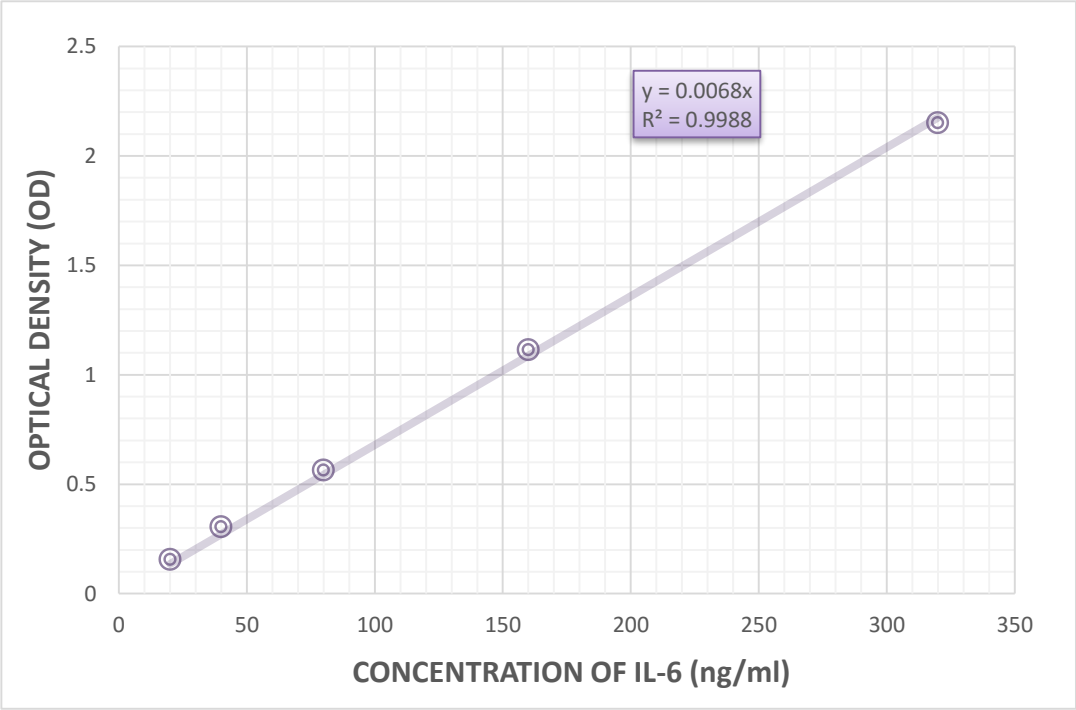

Figure (2-5): Standard Curve for Determining of IL-6 Levels

2.2.2.7. Detection of Interleukin-23 (IL-23)

2.2.2.7.1. Principle

Interleukin 23 (IL-23) was determined in serum samples by using IL-23 ELIAS Kit. This kit uses enzyme- linked immune sorbent assay (ELISA) based on biotin double antibody sandwich technology to assay human IL-23. IL-23 was added to wells that are pre-coated with IL-23 monoclonal antibody and then incubated. After incubation, anti- IL-23 antibodies labeled

with biotin were added to unite with Streptavidin-HRP, which forms the immune complex. Unbound enzymes were removed after incubation and washing, and then substrate A and B were added. The solution will turn blue and change to yellow with the effect of acid. The shades of solution and the concentration of human IL-23 are positively correlated.

2.2.2.7.2. IL-23 Assay Procedure

1. Dilution of standard solutions: Standard was diluted in small tubes following the manufacture instructions (Figure 2-6).

480ng/L	Standard No.5	120µl Original Standard + 120µl Standard diluents
240ng/L	Standard No.4	120µl Standard No.5 + 120µl Standard diluents
120ng/L	Standard No.3	120µl Standard No.4 + 120µl Standard diluent
60ng/L	Standard No.2	120µl Standard No.3 + 120µl Standard diluent
30ng/L	Standard No.1	120µl Standard No.2 + 120µl Standard diluent

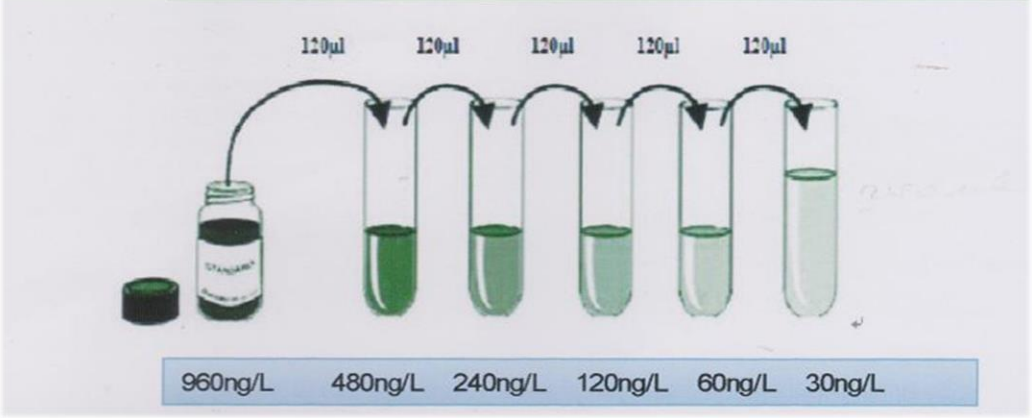

Figure (2-6): Serial Dilutions of Standard Solutions for IL-23 Measurement.

2. Procedure detail is the same as this described for IL-6 (see page 38).

2.2.2.7.3. Calculation of standard curve concentration of IL-23

Concentration of standards was made the abscissa and OD value the ordinate. The standard curve was drawn on the graph paper. According to the OD value of the sample, its corresponding concentration (which is the concentration of the sample) was located; or the linear regression equation of

the standard curve was calculated according to the standard concentration and the OD value (Figure 2-7).

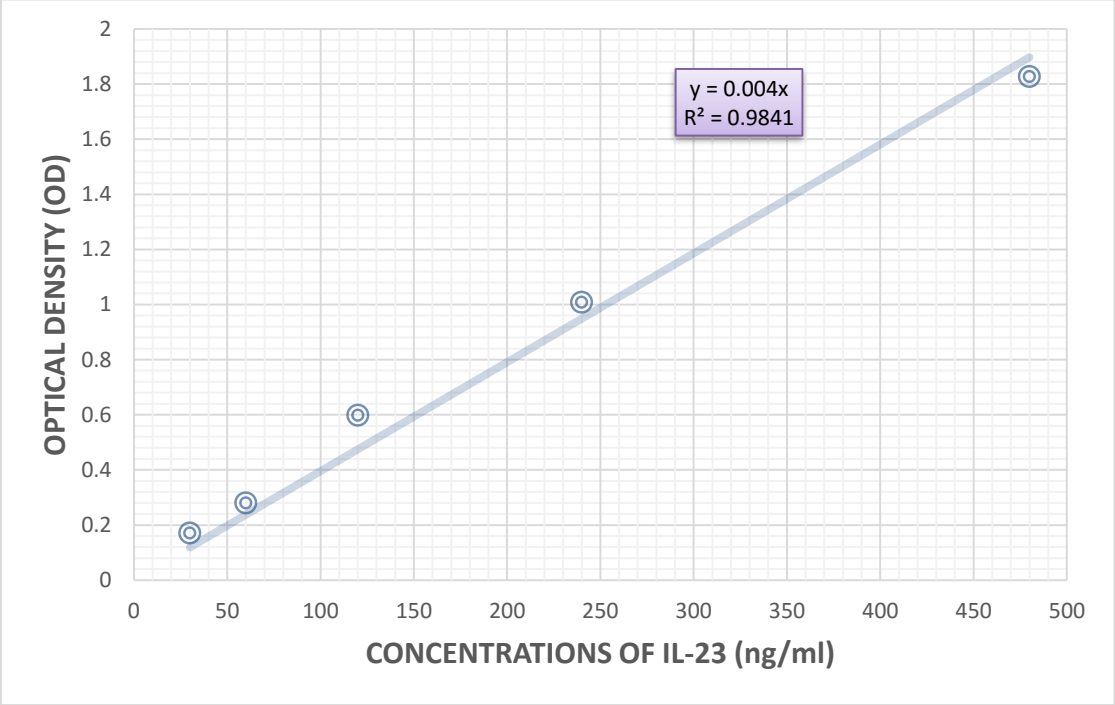

Figure (2-7): Standard Curve for Determining of IL-23 Levels

2.2.3. Genetic polymorphism for detection of Rheumatoid arthritis

Blood samples (3-5 ml) were collected in EDTA anticoagulant tubes from 25 patients with Rheumatoid arthritis, and from 10 healthy control volunteers. Then DNA was isolated from these blood samples. After DNA extraction, Exon 14 of *PTPN22* gene was amplified by using specific primers in a thermal cycler. Then, amplification products for exon 14 of the gene *PTPN22* was sent for sequencing to Macrogen Company (USA).

2.2.3.1. DNA Extraction

Total genomic DNA was extracted from 25 blood samples collected from newly diagnosed and treated RA patients, and from 10 blood samples collected from healthy volunteers. DNA extraction was carried out by using

ReliaPrep™ Blood gDNA Miniprep System supplied by Promega (USA) according to the following protocol:

1. A volume of 0.2 ml of blood samples were mixed thoroughly by vortexing for 10 minutes in microcentrifuge tubes (1.5ml) at room temperature, then aliquots of 20 µl of proteinase K were added and mixed gently.
2. Microcentrifuge tubes (1.5ml) were left to stand for few minutes, and then 200µl of cell lysis buffer solution was added to each tube, and mixed by vortexing for seconds. Then tubes were incubated at 56˚C for 10 minutes.
3. After incubation, volume of 0.25ml of binding buffer was added to each tube, and mixed by vortexing for few seconds.
4. The contents of microcentrifuge tubes (1.5ml) were transferred into the ReliaPrep™ binding column. Capped carefully and centrifuged at 10000 rpm for two minutes.
5. Collection tubes were removed and the flow through was discarded, then the binding columns were placed into a new collection tubes.
6. A volume of 0.5ml of washing column solution was added to the column, and then collection tubes were centrifuged at 10000 rpm for three minutes.
7. After centrifugation, flow through was discarded. Step 6 was repeated twice for a total of three washes, then columns were placed in a new clean microcentrifuge tubes.
8. A volume of 200µl of nuclease free water was added to the column and centrifuged at 10000 rpm for 1 minute.
9. The ReliaPrep™ binding column was discarded, and the elute represents the DNA solution was kept at -20˚C until use.

2.2.3.2. Quantitation of DNA concentration

Purity and concentration of DNA solution extracted from blood samples were determined by measuring the absorbence of DNA solutions at 260nm (A260) and 280nm (A280) by using Nano drop spectrophotometer.

$$\text{DNA Purity} = \frac{A^{260}}{A^{280}}$$

Note: This ratio measures the purity of DNA which is normally between 1.8 and 2.0 (Sambrook et al., 1989).

DNA concentration (µg/ml) = A260 X 50 µg/ml

Note: Absorbency at 260nm is equal to 50 µg/ml of pure double strand DNA.

2.2.3.3. Agarose Gel Electrophoresis

After genomic DNA extraction, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA.

2.2.3.3.1. Preparation of 1X TBE Buffer

The 1X TBE Buffer was prepared from 10X TBE buffer (as stock solution) by adding 100 ml of this stock solution to 900 ml of distilled water.

2.2.3.3.2. Preparation of agarose gel (0.8% and 1.8%)

1. 100ml of 1X TBE buffer solution were placed in 250ml conical flasks.
2. Agarose gel of 0.8gm and 1.8gm was added to TBE buffer in conical flasks to get final concentrations of 0.8% and1.8%, respectively.
3. Contents of each flask were heated to boiling in microwave oven until the gel particles were completely dissolved.

4. Agarose gel solutions were cooled to 55-60˚C, aliquots of 2 µl of 10 mg/ml Ethidium bromide (EtBr) was added to each flask, and then the contents were mixed thoroughly.

2.2.3.3.3. Casting of the horizontal agarose gel

Agarose gel solution was poured into the gel tray, and left to stand at room temperature for 30 minutes until complete solidification, then fixed comb was carefully removed and the tray was placed in the tank filled with 1X TBE buffer until it reached 2-3 mm over the surface of the gel.

2.2.3.3.4. Gel electrophoresis

Aliquot of 5 µl of pure DNA solution was mixed first with 2 µl of bromophenol blue loading dye, then DNA sample were loaded into the wells and the electrophoresis was carried out for 1-2 hours at 5 volts/cm. DNA bands were visualized under a UV-light transilluminator.

2.2.3.4. Amplification of Protein Tyrosine Phosphatase non Receptor Type 22 (PTPN22) Exon 14

2.2.3.4.1. PCR Primers

Amplification of *PTPN22* was achieved by using specific primers designed by Blast analysis (NCBI) indicated in Table (2-4). These primers were supplied by Alpha DNA (USA) in a lyophilized form of different picomols concentrations. Lyophilized primers were dissolved in a DNase/RNase free water to give a final concentration of 100 pmol/µl as a stock solution. The source of the PTPN22 gene was obtained from NCBI (Gregory *et al.*, 2006).

A working solution of 10 pmol/µl of these primers was prepared by adding 10 µl of primer stock solution to 90 µl of deionized distilled water to get a final concentration of 10 pmol/µl.

Table (2-4): Oligonucleotide primers used for the amplification of *PTPN22*.

Primers	Sequence (5'- 3')	Product Size (bp)
Forward	AAGAATAAGCAAAAACCTCCTGGG	684
Reverse	AACATTGAAAGGACCTGAGAAGT	

PCR was carried out in a total volume of 50ul. The reaction components are indicated in Table (2-5).

Exon 14 of *PTPN22* gene was amplified by using specific primers in a thermal cycler. Polymerase chain reaction was carried out according to the amplification program shown in Table (2-6), and then amplification products were analyzed on agarose gel (1.8%) in presence of 100bp DNA ladder marker.

Table (2-5): Components of reaction mixture for amplification of exon 14 of *PTPN22*.

Component	Volume (µl)
Master Mix: *Taq* DNA Polymerase, dNTPs, MgCl2, and reaction buffer.	25
Forward Primer	2
Reverse Primer	2
DNA Template	4
D.W.	17
Total volume	50

Table (2-6): PCR amplification conditions for Exon 14 of *PTPN22*.

Steps	Temperature (˚C)	Time	No. of Cycles
Initial Denaturation	95	4 min.	1
Denaturation	95	30 sec.	30
Annealing	60	30 sec.	
Extension	72	30 sec.	
Final Extension	72	7 min.	1

2.2.3.4.2. Sequencing of amplified products

Amplification products for exon 14 of the gene *PTPN22* was sent for sequencing to Macrogen Company (USA). Then, the sequencing for these products was compared with the information in gene bank of the National Center for Biotechnology Information (NCBI) for standard *PTPN22* gene, using Bioedit software.

2.2.4. Statistical analysis

The Statistical Analysis System- SAS (2012) program was used to evaluate different factors and parameters in this study. The least significant difference (LSD) test was used to significant compare between means of the present parameters. The level of significance was at $P<0.05$.

Chapter Three

Results and Discussion

3. Results and Discussion

3.1. Erythrocyte Sedimentation Rate (ESR)

This study demonstrated the presence of increased ESR levels in (newly diagnosed and treated) RA patients compared to controls (Table 3-1), suggesting the presence of an inflammatory response by the immune system against RA. Previous finding (Kadir*et al.*, 2003; Pasupathi *et al.*, 2009 and Vatutin *et al.*, 2015) demonstrated higher ESR levels in RA patients compared to controls. The failure of various treatments to reverse normalize ESR value is suggestive of presence other mechanisms responsible for the pathogenesis of RA not corrected by the current therapy.

Table 3-1| Erythrocyte Sedimentation Rate levels in healthy controls and RA Patients (newly diagnosed and treated).

Parameter	Controls N=30	RA Patients	
		Newly diagnosed N=10	Treated N=50
ESR (mm/hr)	22.20 ± 1.12^{a}	56.60 ± 2.33^{b}	43.89 ± 4.79^{b}

Values are expressed as Mean ± SE. N=Number; (ESR) Erythrocyte sedimentation rate; (RA) Rheumatoid arthritis; Values between a and b are significantly different ($P< 0.05$).

3.2. Biochemical Parameters (Oxidative Stress Biomarkers)

3.2.1. Serum levels of Glutathione (GSH)

The serum levels of GSH showed a significant decrease in newly diagnosed RA patients compared to controls. Furthermore, treatment of these patients managed to normalize GSH levels (Table 3-2). Palanisamy *et al.*, (2009) and Shankarlal *et al.*, (2014) demonstrated significant lower GSH levels in serum of newly diagnosed RA patients compared to controls. These results are consistent with the underlying hypothesis that there is an imbalance between reactive oxygen species production and the antioxidant defense system in inflammatory RA disease.

Glutathione (GSH) is an important non-enzymatic antioxidant preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides (Pompella *et al*., 2003). GSH protect cells against toxic free radicals and serves as a reductant antioxidant in oxidation reduction resulting in the formation of oxidized glutathione (GSSH) (Wu *et al.*, 2004).

3.2.2. Serum levels of Malondialdehyde (MDA)

Malondialdehyde (MDA) was measured as a marker of lipid peroxidation in (newly diagnosed and treated) RA patients and control subjects. The serum levels of MDA showed a significant ($P<0.05$) increase in newly diagnosed RA patients compared to controls, suggesting the presence of measured endogenous lipid peroxidation. This finding agrees with several studies who reported similar higher MDA levels in serum Rheumatoid arthritis patients (Walwadkar *et al.*, 2006; Desai *et al.*, 2010; Manju and Madhur, 2013; Shankarlal *et al.*, 2014). Furthermore, treatment of these patients reversed these changes in (MDA) levels to a value comparable to that seen with the controls (Table 3-2). This finding differed from other studies, which found that RA patients with active disease had an increase in the levels of MDA (Cimen *et al.*, 2000; Taysi *et al.*, 2002). Most of our treated RA patients were not in the active stage of disease on the day the blood was drawn as checked according to DAS28.

Table 3-2| Serum levels of GSH and MDA in healthy controls and RA patients (treated and newly diagnosed).

Parameters	Controls N=30	RA patients	
		Newly diagnosed N=10	Treated N=50
GSH (ng/ml)	6.35 ± 0.46^{a}	1.73 ± 0.11^{b}	6.32 ± 0.70^{a}
MDA (nmol/L)	0.250 ± 0.01^{a}	1.52 ± 0.12^{b}	0.250 ± 0.006^{a}

Values are expressed as Mean ± SE. N=Number; Values between a and b are significantly different ($P< 0.05$).

3.3. Immunological Parameters

3.3.1. Rheumatoid Factor (RF)

In this study, all the newly diagnosed RA patients showed positive results for the rheumatoid factor. Samples from treated RA patients 60% patients (30) also showed positive result for RF, while 40% patients (20) showed negative results for RF (Table 3-3).

Rheumatoid Factor has been widely used as a screening test for patients with arthritis. RF is prognostically useful and one recent study revealed that RF titer reflected RA disease activity (Geng *et al.,* 2012). RF constitutes one of the classification criteria proposed by the American College of Rheumatology (ACR). However, RF is present in patients with other autoimmune and infectious diseases, and even in a noticeable proportion of normal healthy subjects, particularly in aged individuals (Westwood *et al.,* 2006). In clinical practice, high positive result of RF levels in treated RA patients represents the objective sign for non-responding patients to treatment with disease modifying RA drugs.

Rheumatoid factor values in patients seems inconsistent, where Artur, and Brikena, (2013) demonstrated that (77.1 %) of RA patients with positive and (22.9 %) patients were negative, but Manhal *et al.,* (2009) demonstrated that all patients were positive result for RF. High serum levels of RF are a hallmark of rheumatoid arthritis and can be used to monitor disease activity (Meyer *et al.,* 1997). In other study the presence of RF has been proved to be predictive of radiological disease progression, which is a clinical hallmark of aggressive disease (Ringold and Singer, 2008).

Table 3-3| Immunological Parameters in healthy controls and RA patients (treated and newly diagnosed).

Risk Factor	Controls NT=30 (%)	RA patients	
		Newly diagnosed NT=10 (%)	Treated NT=50 (%)
RF			
Positive	0 (0%)	10 (100%)	30(60%)
Negative	30 (100%)	0 (0%)	20(40%)
CRP			
Positive	0 (0%)	10 (100%)	34(68%)
Negative	30 (100%)	0 (0%)	16(32%)
Anti-CCP			
Positive	0 (0%)	10 (100%)	30(60%)
Negative	30 (100%)	0 (0%)	20(40%)

NT= Total Number; (%) = Percentage; (RF) Rheumatoid Factor, (CRP) C-Reactive Protein and (Anti-CCP) Anti-Cyclic Citrullinated Peptides.

3.3.2. C- Reactive Protein (CRP)

In this study, all of newly diagnosed RA patients were positive for CRP. On the other hand, in treated RA patients 34/50 (68%) showed positive for CRP, while 16/50 (32 %) were negative for CRP (Table 3-3).

C-reactive protein, an acute phase protein, is synthesized by hepatocytes in response to pro-inflammatory cytokines, in particular IL-6. It has been shown to be of a great value as an inflammatory marker in RA, and has been suggested to mediate part of the complement activation in RA (Molenaar *et al.*, 2001). In clinical practice, a fall in CRP level represents the first objective sign of improvement in response to treatment with disease modifying drugs (Emery and Luqmani, 1993).

Most studies concluded that there were elevations in CRP levels in patients (Surekha *et al.*, 2006; Inmaculada *et al.*, 2003). It is indicated that CRP can be used as a serum marker for RA (Kadir *et al.*, 2004) .It is concluded, therefore, that C-reactive protein and rheumatoid factor in patients with rheumatoid arthritis might be sensitive inflammation markers for reflecting the presence and activity of the disease.

3.3.3. Anti-Cyclic Citrullinated Peptides (Anti-CCP) antibodies

All the newly diagnosed RA patients showed positive there were result for Anti-CCP. In treated RA patients 30/50 (60%) showed positive results for Anti-CCP (Table 3-3). This present is consistent with that of Binesh *et al.*, (2014) who demonstrated that 71% of RA patients showed positive Anti-CCP and 29% of RA patients were negative.

Anti-CCP testing is particularly useful in the diagnosis and treatment of RA as well as the predication of the severity of the disease causing irreversible damage induced by it (Hayashi *et al.*, 2010; Schellekens, *et al.*, 2000; Van der Woude, *et al.*, 2010; Lee *el al.*, 2003). Accordingly, Anti-CCPs have recently been added as one of the criteria in the 2010 American College of Rheumatology (ACR) /European League Against Rheumatism (EULAR) classification of RA (Aletaha *et al.*, 2010). Therefore, results of this study supports the importance of Anti-CCP measurement as a useful test for diagnosing rheumatoid arthritis.

3.3.4. Interleukin-6 (IL-6)

The serum levels of IL-6 showed a significant increase in newly diagnosed RA patients compared to controls. Treatment of these patients normalized IL-6 levels (Table 3-4).

The present findings of increased serum IL-6 levels in newly diagnosed RA patients suggest the presence of an inflammatory response by the immune system against RA. This finding is consistent with previous studies that revealed that IL-6 may contribute to the development of RA due to their pro-inflammatory effects (Smolen and Aletaha, 2011; Upadhyay *et al.*, 2011). The ability of the current therapy to normalize IL-6 levels proposed a potential role for IL-6 family cytokines in the pathogenesis of RA (Chung *et al.*, 2011) and is suggestive of its effectiveness to correct the mechanisms responsible for increased production of IL-6. The decrease in serum IL-6 concentration during the treatment was showed to be the best prognostic marker for the clinical outcome following disease modifying anti-rheumatic drugs (DMARDs) such as Methotrexate, Etanercept and Infliximab (Straub et al., 1997).

Table 3-4| Serum levels of IL-6 and IL-23 in healthy controls and RA patients (newly diagnosed and treated).

		RA patients	
Parameters	**Controls N=30**	**Newly diagnosed N=10**	**Treated N=50**
IL-6 (ng/ml)	63.70 ± 4.03^{a}	234.41 ± 13.46^{b}	68.15 ± 7.37^{a}
IL-23 (ng/ml)	126.53 ± 7.90^{a}	457.60 ± 12.69^{b}	122.85 ± 12.3^{a}

Values are expressed as Mean ± SE. N=Number; Values between a and b are significantly different ($P < 0.05$).

3.3.5. Interleukin-23 (IL-23)

The serum levels of IL-23 showed a significant increase in newly diagnosed RA patients compared to controls. Similar to IL-6 response, patients who received treatments showed was capable of correcting IL-23 levels to a value comparable to that seen with controls (Table 3-4). This indicates that treatment against RA affective in reversing IL-23 levels to the normal status.

The present finding of increased serum IL-23 levels in newly diagnosed RA patients suggests the presence of an inflammatory response by the immune system against RA. The serum IL-23 levels in RA were correlated with the CRP (Guo *et al.*, 2013). Similarly finding of higher IL-23 levels in serum of RA patients compared to controls were observed (Esam *et al.*, 2013; Guo *et al.*, 2013). They suggested that IL-23 reflects RA disease activity, and concluded that elevated serum IL-23 level may be a useful marker to detect active RA and disease progression in patients with RA.

3.4. Genomic DNA extraction

In order to study genetic polymorphism in Iraqi patients with rheumatoid arthritis and healthy controls, genomic DNA was extracted from blood samples of those subjects under study by using ReliaPrep™ Blood gDNA Miniprep System (Promega, USA).

Results in (Figure 3-1) showed high moleculat weight DNA bands typical of genomic DNA were obtained after electrophoresis of extracted DNA from healthy controls and pateints with RA on an agarose gel (0.7%). The concentration of DNA extracted from all samples ranged between 100-200 ng/µl, while the purity ranged between 1.8-2.0. This DNA was used in further genetic analysis such as PCR technique and DNA sequencing (Boesenberg-Smith*et al.*, 2012).

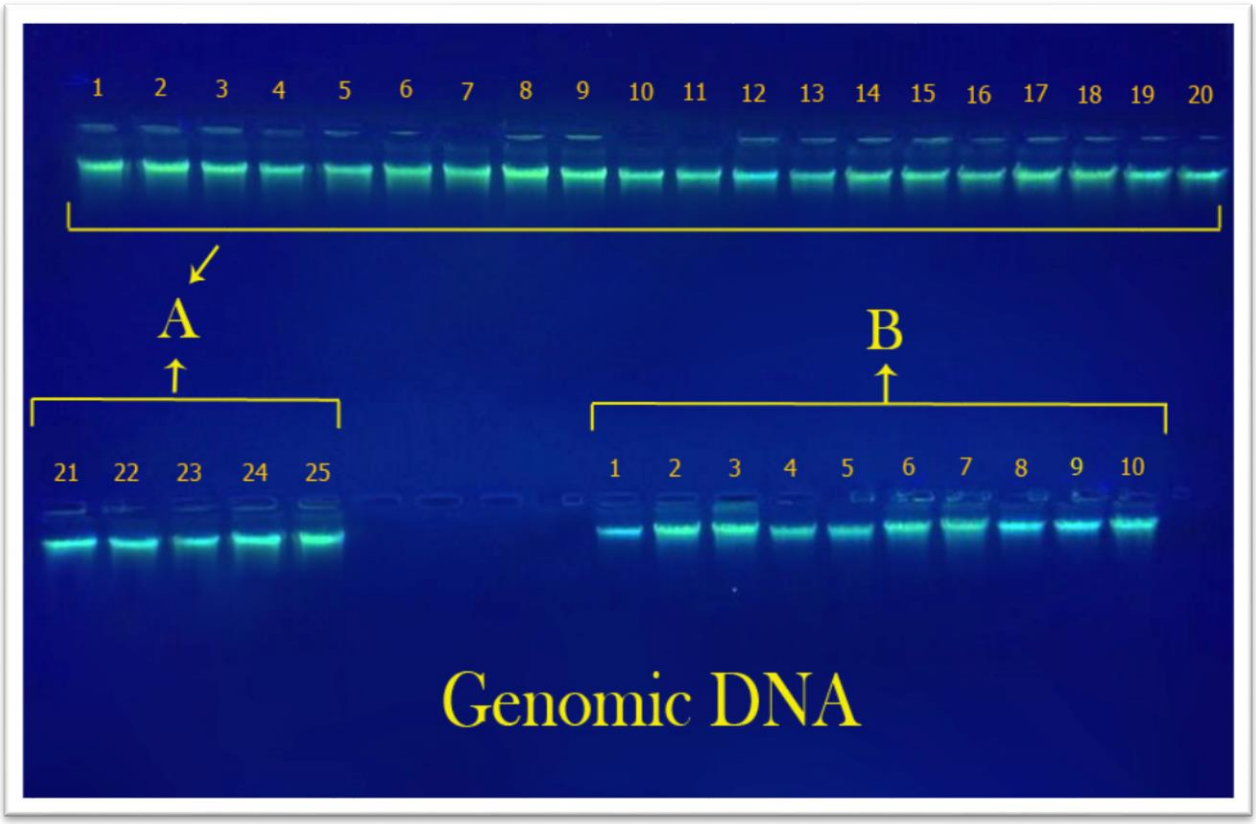

Figure (3-1): Quality of Genomic DNA extracted from RA patients (A) and healthy controls (B). Gel electrophoresis of DNA on agarose gel (0.8%) for 1 hour at 5 v/cm^2, then exposed to UV light and photographed.

3.5. Genetic polymorphism of PTPN22

Results illustrated in Figure (3-2) showed a single band of amplified products with a molecular size of 684 base pairs after electrophoresis on agarose gel 1.8%. These DNA fragments represent the region of exon 14 for *PTPN22* gene located on chromosome 1p13 (Kochi *et al.*, 2009).

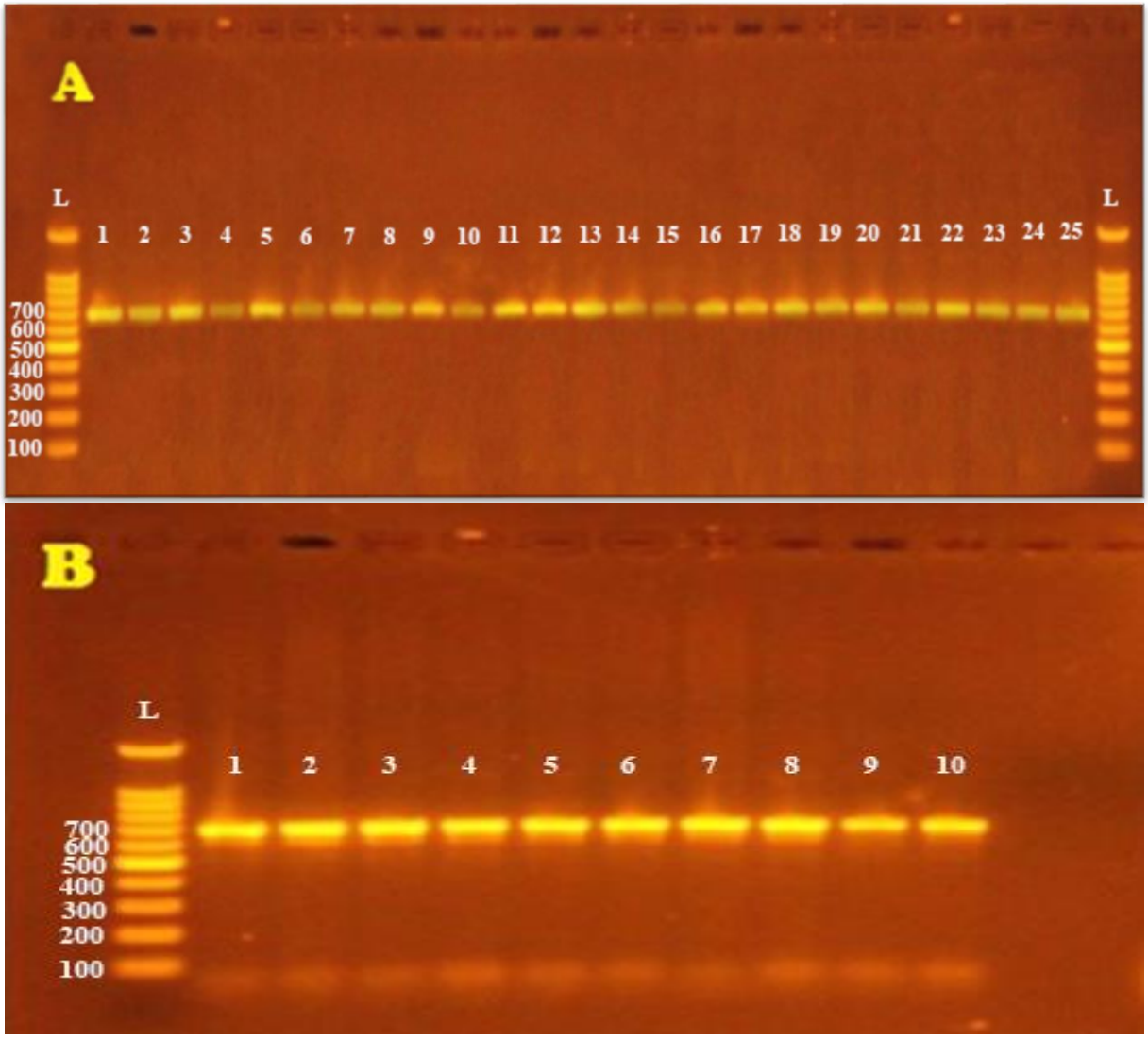

Figure | 3-2: Amplification of PTPN22 gene of exon 14. Gel electrophoresis for PCR products run on an agarose gel (1.8%) for 1 hour at 5 v/cm^2 in the presence of 1 kb DNA Ladder marker. (A) PCR products for DNA extracted from blood samples of patients with Rheumatoid arthritis. (B) PCR products for DNA extracted from blood samples of healthy controls.

3.6. Sequencing of Amplified Exon 14 of PTPN22 gene

In order to study the association of genetic polymorphism in *PTPN22* R620W (rs2476601) with RA susceptibility in Iraqi population, PCR products for exon 14 were sequenced. Results illustrated in Figure (3-3) showed the complete nucleotide sequence of this exon in healthy controls and RA patients. The position of the expected SNP (rs2476601) that may be associated with the incidence of the disease was defined.

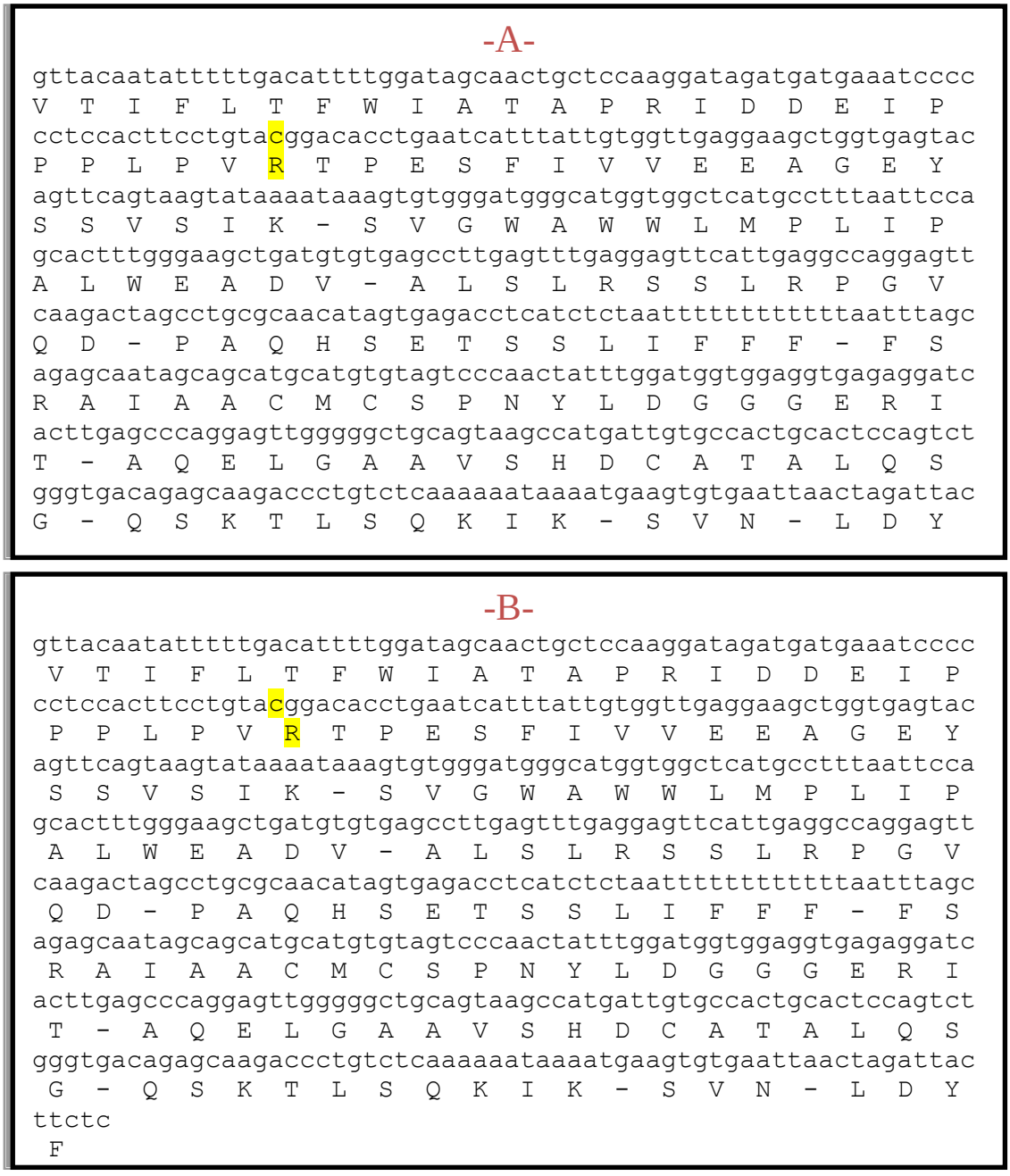

Figure | 3-3: Nucleotide sequences and amino acid of *PTPN22* gene of exon 14. Yellow letter indicates the position of expected *PTPN22* SNP in RA patients in Iraq. A: Sequence from healthy controls group, B: Sequence from RA patients group.

3.7. Alignment of Amplified Exon 14 of PTPN22 gene

The result of the sequence analysis was analyzed by blastn of the National Center Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.gov) and BioEdit program to detect rs2476601 polymorphism in exon 14 of *PTPN22* gene. Results illustrated in Figure (3-4) showed the nucleotide sequence alignment and the position of the cytosine (C) nucleotide of healthy control group matched with cytosine (C) nucleotide in reference sequence of NCBI, that showed 100% identity, and was given ID: gb|EF064714.1| from 38554-39158 number of nucleotide from *Homo sapiens* protein tyrosine phosphatase, non-receptor type 22 (lymphoid) (*PTPN22*) gene. Complete cds, from Gene Bank, with score (1118) and expect (0.0). The expectation value is defined to give an estimate of the number of times expected to get the same similarity coincidental and the lower the value of E (expect). This indicates that the degree of similarity was high between sequences which give greater confidence. The value of a very close to zero means that these sequences are identical. The bit Score: statistical measure of the moral similarity and the higher value indicates that the high degree of similarity, and if dropped from the class of 50 points, the sense that there is no similarity available.

On the other hand, results of sequencing of *PTPN22 of* exon 14 in Iraqi patients with RA illustrated in Figure (3-5) and Table (3-5) showed that nucleotide sequence alignment of RA patients group compared with reference sequence obtained from NCBI, with 100% identity, under sequence ID: ref|NG_011432.1|from 41613-42218 number of nucleotide from Homo sapiens Protein Tyrosine Phosphatase, non-receptor type 22 (*PTPN22*), Ref Seq Gene on chromosome 1.

Homo sapiens protein tyrosine phosphatase, non-receptor type 22 (lymphoid) (*PTPN22*) gene, complete cds Sequence ID: gb|EF064714.1|.

Score	Expect	Identities	Gaps	Strand
1118 bits(605)	0.0	605/605(100%)	0/605(0%)	Plus/Plus

```
Query 1      TGAACAAGTGTCAACTTTACTGATAATGTTGCTTCAACGGAATTTAAATATAAATTATGG 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 38554  TGAACAAGTGTCAACTTTACTGATAATGTTGCTTCAACGGAATTTAAATATAAATTATGG 38613

Query 61     TAAATTTATATTTAATATTAGAATATAAGAATTTCCTTTGGATTGTTCTAATTAACAATT 120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 38614  TAAATTTATATTTAATATTAGAATATAAGAATTTCCTTTGGATTGTTCTAATTAACAATT 38673

Query 121    GTTACAATATTTTTGACATTTTGGATAGCAACTGCTCCAAGGATAGATGATGAAATCCCC 180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 38674  GTTACAATATTTTTGACATTTTGGATAGCAACTGCTCCAAGGATAGATGATGAAATCCCC 38733

Query 181    CCTCCACTTCCTGTACGGACACCTGAATCATTTATTGTGGTTGAGGAAGCTGGTGAGTAC 240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 38734  CCTCCACTTCCTGTACGGACACCTGAATCATTTATTGTGGTTGAGGAAGCTGGTGAGTAC 38793

Query 241    AGTTCAGTAAGTATAAAATAAAGTGTGGGATGGGCATGGTGGCTCATGCCTTTAATTCCA 300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 38794  AGTTCAGTAAGTATAAAATAAAGTGTGGGATGGGCATGGTGGCTCATGCCTTTAATTCCA 38853

Query 301    GCACTTTGGGAAGCTGATGTGTGAGCCTTGAGTTTGAGGAGTTCATTGAGGCCAGGAGTT 360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 38854  GCACTTTGGGAAGCTGATGTGTGAGCCTTGAGTTTGAGGAGTTCATTGAGGCCAGGAGTT 38913

Query 361    CAAGACTAGCCTGCGCAACATAGTGAGACCTCATCTCTAATTTTTTTTTTTTAATTTAGC 420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 38914  CAAGACTAGCCTGCGCAACATAGTGAGACCTCATCTCTAATTTTTTTTTTTTAATTTAGC 38973

Query 421    AGAGCAATAGCAGCATGCATGTGTAGTCCCAACTATTTGGATGGTGGAGGTGAGAGGATC 480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 38974  AGAGCAATAGCAGCATGCATGTGTAGTCCCAACTATTTGGATGGTGGAGGTGAGAGGATC 39033

Query 481    ACTTGAGCCCAGGAGTTGGGGGCTGCAGTAAGCCATGATTGTGCCACTGCACTCCAGTCT 540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 39034  ACTTGAGCCCAGGAGTTGGGGGCTGCAGTAAGCCATGATTGTGCCACTGCACTCCAGTCT 39093

Query 541    GGGTGACAGAGCAAGACCCTGTCTCAAAAAATAAAATGAAGTGTGAATTAACTAGATTAC 600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 39094  GGGTGACAGAGCAAGACCCTGTCTCAAAAAATAAAATGAAGTGTGAATTAACTAGATTAC 39153

Query 601    TTCTC 605
             |||||
Sbjct 39154  TTCTC 39158
```

Figure | 3-4: A representative sequence alignment of *PTPN22* gene of exon 14 of 10 healthy control samples compared with standard *PTPN22* gene exon 14, obtained from Gene Bank. "Query" represents samples from this study; "Sbject" is a reference sequence from the National Center Biotechnology Information (NCBI). Yellow letters indicate the position of expected SNP.

Homo sapiens protein tyrosine phosphatase, non-receptor type 22 (lymphoid) (PTPN22) gene, Sequence ID: ref|NG_011432.1|

Score	Expect	Identities	Gaps	Strand
1120 bits(606)	0.0	606/606(100%)	0/606(0%)	Plus/Plus

```
Query 1     TGAACAAGTGTCAACTTTACTGATAATGTTGCTTCAACGGAATTTAAATATAAATTATGG 60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 41613 TGAACAAGTGTCAACTTTACTGATAATGTTGCTTCAACGGAATTTAAATATAAATTATGG 41672

Query 61    TAAATTTATATTTAATATTAGAATATAAGAATTTCCTTTGGATTGTTCTAATTAACAATT 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 41673 TAAATTTATATTTAATATTAGAATATAAGAATTTCCTTTGGATTGTTCTAATTAACAATT 41732

Query 121   GTTACAATATTTTTGACATTTTGGATAGCAACTGCTCCAAGGATAGATGATGAAATCCCC 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 41733 GTTACAATATTTTTGACATTTTGGATAGCAACTGCTCCAAGGATAGATGATGAAATCCCC 41792

Query 181   CCTCCACTTCCTGTACGGACACCTGAATCATTTATTGTGGTTGAGGAAGCTGGTGAGTAC 240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 41793 CCTCCACTTCCTGTACGGACACCTGAATCATTTATTGTGGTTGAGGAAGCTGGTGAGTAC 41852

Query 241   AGTTCAGTAAGTATAAAATAAAGTGTGGGATGGGCATGGTGGCTCATGCCTTTAATTCCA 300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 41853 AGTTCAGTAAGTATAAAATAAAGTGTGGGATGGGCATGGTGGCTCATGCCTTTAATTCCA 41912

Query 301   GCACTTTGGGAAGCTGATGTGTGAGCCTTGAGTTTGAGGAGTTCATTGAGGCCAGGAGTT 360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 41913 GCACTTTGGGAAGCTGATGTGTGAGCCTTGAGTTTGAGGAGTTCATTGAGGCCAGGAGTT 41972

Query 361   CAAGACTAGCCTGCGCAACATAGTGAGACCTCATCTCTAATTTTTTTTTTTTAATTTAGC 420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 41973 CAAGACTAGCCTGCGCAACATAGTGAGACCTCATCTCTAATTTTTTTTTTTTAATTTAGC 42032

Query 421   AGAGCAATAGCAGCATGCATGTGTAGTCCCAACTATTTGGATGGTGGAGGTGAGAGGATC 480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 42033 AGAGCAATAGCAGCATGCATGTGTAGTCCCAACTATTTGGATGGTGGAGGTGAGAGGATC 42092

Query 481   ACTTGAGCCCAGGAGTTGGGGGCTGCAGTAAGCCATGATTGTGCCACTGCACTCCAGTCT 540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 42093 ACTTGAGCCCAGGAGTTGGGGGCTGCAGTAAGCCATGATTGTGCCACTGCACTCCAGTCT 42152

Query 541   GGGTGACAGAGCAAGACCCTGTCTCAAAAAATAAAATGAAGTGTGAATTAACTAGATTAC 600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 42153 GGGTGACAGAGCAAGACCCTGTCTCAAAAAATAAAATGAAGTGTGAATTAACTAGATTAC 42212

Query 601   TTCTCA  606
            ||||||
Sbjct 42213 TTCTCA  42218
```

Figure | 3-5: A representative sequence alignment of *PTPN22* gene exon 14 of 25 RA patient samples compared with standard *PTPN22* gene of exon 14, obtained from Gene Bank. "Query" represents a sequence from this study; "Sbject" is reference sequence from National Center Biotechnology Information (NCBI). Yellow letters indicate the position of expected SNP.

Table | 3-5: Sequencing ID, Score, Expect, and Identities for *PTPN22* gene of exon 14.

Number of samples	Accession	Identities	Expect	Score	Number of Nucleotide
10 Controls	gb|EF064714.1|	100 %	0.0	1118	38554 - 39158
25 Patients	ref|NG_011432.1|	100 %	0.0	1120	41613 - 42218

In summary, 35 samples (25 RA patients and 10 healthy controls) were analyzed for the rs2476601 of *PTPN22* gene. Results of analyzed sequence showed that all samples were genotyped as homozygous CC (Table | 3-6) and (Figure | 3-6).

Table |3-6: Genotypes and allele frequency of *PTPN22* SNP in RA patients and controls.

Samples	No.	Genotypes			Alleles Frequency	
		C/C	C/T	T/T	C	T
Controls	10	10	0	0	1.00	0.00
RA Patients	25	25	0	0	1.00	0.00
No.= Number of subject studied						

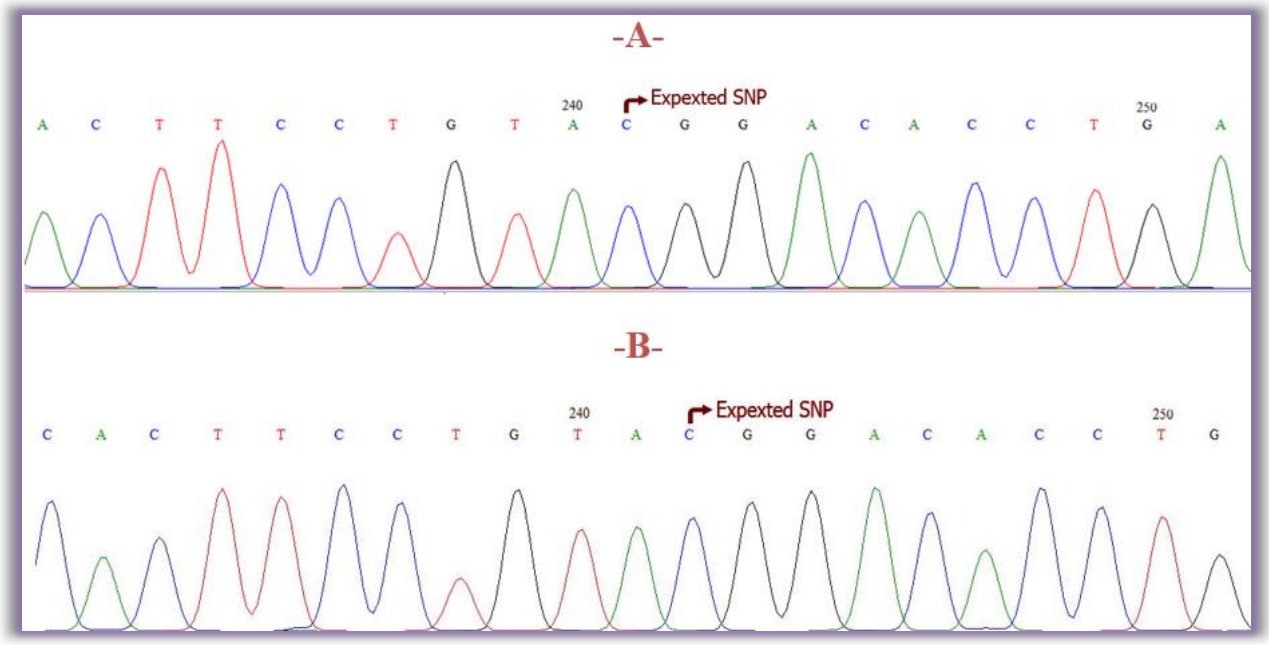

Figure |3-6: Chromatogram shows homozygote CC genotype for rs2476601 of *PTPN22* gene. A: Healthy Control group, B: RA Patient group.

In this study, the role of expected missense SNP (rs2476601) in a gene encoding PTPN22 was studied for its role in RA disease susceptibility and severity in Iraq population. Results in Table (3-6) showed that the genetic variant in the position of the expected SNP (rs2476601) was found to be non-polymorphic in all Iraqi population under study. These results are consistent with those obtained by Mastana *et al.,* (2007) and Somayeh *et al.,* (2015) who found that the rs2476601was non-polymorphic and play no role in susceptibility to RA and other autoimmune diseases in Asian Iranian population, respectively. In contrast, Song, *et al.*, (2013) showed that *PTPN22* polymorphism (rs2476601) confers susceptibility to RA in populations with different ethnicities, especially with the Europeans. However, this does not mean that functional polymorphisms of the *PTPN22*, other than rs2476601 do not play a role in the incidence of RA in Iraqi population. In the Japanese, *PTPN22* allelic heterogeneity was studied, but none of the 8 other SNPs present in the European populations, were found to be polymorphic (Ikari *et al.*, 2006).

Another explanation may be that the variants in the *PTPN22* gene may reflect genuine differences in the pathogenesis of RA between European and Iraqi patients. However, this can only be said with certainty once all variants of the gene have been studied. Such inconsistent associations might reflect differences in number of samples, although this is unlikely in this study as the rs2476601 was non-polymorphic in both RA patients and controls. These differences might also relate to clinical phenotypic differences between populations (Maritz *et al.*,2003).

In conclusion, the presence of imbalance of cytokine production oxidative stress in the absence of the *PTPN22* high risk allele of SNP (rs2476601), demonstrates the possible role of these parameters in the pathogenesis of joint damage in Iraqi patients with RA. Furthermore, the possibility of the role of other genetic variants, not yet identified, in Iraqi RA patients. The findings show conclusively that the functional R620W variant, a major risk factor for RA in

European populations, is not present and therefore does not play a role in the pathogenesis of RA in Iraq. The present results agree with those obtained by (Govind, 2011) who demonstrated that the functional R620W variant (rs2476601) in *PTPN22*geneis not present and therefore does not play a role in the pathogenesis of RA in Black South African population.

Chapter Four

Conclusions and Recommendations

4. Conclusions and Recommendations

4.1. Conclusions

1) The decline of serum GSH levels and the increase in MDA levels in serum of newly diagnosed RA patients suggest the presence of increased susceptibility to oxidative stress in RA. The ability of therapy to normalize most of the observed changes is suggestive of a possible antioxidant activity of the implemented therapy.

2) The results revealed the presence of alterations in the immune system of the RA state, manifested by an elevation in the IL-6, IL-23, RF, CRP, and Anti-CCP levels in the newly diagnosed RA patients.

3) The *PTPN22* gene sequencing of exon 14 from healthy controls and RA patients is 100% compatible with standard *PTPN22* gene of Gene Bank.

4) The rs2476601 of *PTPN22* gene is a non-polymorphic, having only C allele in Iraqi population; also this SNP may play no role in susceptibility to RA and other autoimmune diseases in Iraqi population.

4.2. Recommendations

1) Study the correlation between IL-17, IL-1 and IL-10 levels and the disease activity of RA.

2) Detection of single nucleotide polymorphisms in other genes associated with the incidence of RA in Iraqi patients, such as: TNF-alpha, HLA-DR1 and STAT4 genes.

3) Investigate the gene expression of *PTPN22* polymorphic in Iraqi patients with RA.

4) Studying the role of environmental and genetic factors in the susceptibility and severity of RA Iraqi patients.

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14 بأستخدام بادئات نيوكلوتيدية متخصصة تم تصميمها في هذه الدراسة , وقد تم الحصول من خلال التضخيم على قطعة دنا بحجم جزيئي مقداره 684 زوج قاعدي بعد الترحيل الكهرباء على هلام الاكاروز (1.8%) تمثل التعاقب النيوكلوتيدي للاكزون 14. ولمعرفة التباين الوراثي (rs2476601) في ذلك الاكزون, فقد اجريت عملية تحديد التعاقب النيوكلوتيدي الكامل للقطعة المضخمة. وقد اشارت النتائج الى ان التباين الوراثي للتغاير (rs2476601) هو غير متباين في عينات المرضى المصابين والسيطرة الاصحاء, مع غياب تام لتغاير اليل T. تردد اليل T في عينات المرضى المصابين والاصحاء هو 0.0 وتردد النمط الجيني لـ T/T, C/T and C/C هو 0.0, 0.0 and 1.0 على التوالي. في الاستنتاج , اظهرت النتائج بأن التغاير rs2476601 في جين *PTPN22* هو غير متباين في المجتمع العراقي وليس لها علاقة بمرض التهاب المفاصل الرثياني.

المعالجين طبياً مقارنة ب 100% نتيجة سالبة في عينات السيطرة الاصحاء. من ناحية اخرى فقد بينت النتائج ظهور الاجسام المضادة للسترولين (Anti-CCP) في مصل جميع العينات المأخوذة من الاشخاص حديثي الاصابة بالمرض (100% نتيجة موجبة) و60% نتيجة موجبة في المرضى المعالجين طبياً مقارنة ب100% نتيجة موجبة في السيطرة الاصحاء. اما الانترلوكينات (IL-6 و IL-23) فقد اشارت النتائج الى وجود زيادة معنوية ($P<0.05$) في مستوى كليهما في عينات المصل المأخوذة من الاشخاص حديثي الاصابة بالمرض مقارنة بالسيطرة الاصحاء والمرضى المعالجين طبياً التي لم يظهر في مابينهما اي اختلاف معنوي في مستوى الانترلوكينات في عينات المصل المأخوذة من كلا المجموعتين. اما بالنسبة لمؤشرات الاجهاد التأكسدي فقد اشارت النتائج الى حدوث انخفاض معنوي ($P<0.05$) في مستوى الكلوتاثايون (GSH) في عينات المصل للاشخاص حديثي الاصابة بالمرض مقارنة بمستواه في عينات السيطرة الاصحاء والمرضى المعالجين طبياً والتي لم يظهر في ما بينهما اي اختلاف معنوي ($P<0.05$) في ما اشارت النتائج ايضأ الى حدوث زيادة معنوية في مستوى المالونداي الديهايد (MDA) في عينات المصل للاشخاص حديثي الاصابة بالمرض مقارنة بمستواه في عينات السيطرة الاصحاء والمرض المعالجين طبياً والتي لم يظهر في ما بينهما اي اختلاف معنوي بمستواه في عينات المصل المأخوذة من كلا المجموعتين .

درس التباين الوراثي في الجين (*PTPN22*) المشفر لبروتين الفوسفاتيز التايروسين للتغاير الوراثي rs2476601 في الاكزون 14 ضمن الكروموسوم 1 , الذي يعد احد العوامل الرئيسية المصاحبة للاصابة بمرض التهاب المفاصل الرثياني. تم اولاً استخلاص الدنا المجيني من عينات الدم للاشخاص الاصحاء والمصابين بالمرض. وقد اشارت النتائج الى ان تركيز الدنا المستخلص من تلك العينات قد تراوح بين 100-200 نانوغرام/مايكروليتر وبنقاوة تراوحت بين 1.8- 2.0. بعدها تم تضخيم الاكزون

الملخص

يعد مرض التهاب المفاصل الرثياني (Rheumatoid arthritis) من الامراض المناعية الذاتية الشائعة المتميزة بالالتهاب الحاد وتضرر المفاصل. وكان الهدف من هذه الدراسة هو التحري عن بعض المؤشرات الكيمائية الحياتية والعوامل المناعية في المرضى العراقيين المصابين بألتهاب المفاصل الرثياني ومعرفة تأثير العلاج على هذه العوامل. فضلاً عن التحري عن التباين الوراثي (rs2746601) في الجين (*PTPN22*) المشفر لبروتين فوسفاتيز التايروسين (*Protein Tyrosine Phosphatase*) في هؤلاء المرضى.

جمعت 60 عينة دم من مرضى ذكور واناث مصابين بمرض التهاب المفاصل الرثياني مراجعين لاستشارية المفاصل في مستشفى بغداد التعليمي/ مدينة الطب. وقد تضمنت العينة حديثي الاصابة بالمرض ومعالجين. وتتراوح معدل اعمار المرضى حديثي الاصابة (10) 2.27 ± 28.4 سنة واخرون معالجون طبياً (50) بمعدل أعمار تراوحت 14.59 ± 42.96 سنة . كما جمعت 30 عينة دم من اشخاص اصحاء (سيطرة) بمعدل أعمار تراوحت 11.72 ± 37.33 سنة .

اشارت النتائج الى وجود زيادة معنوية في معدل ترسيب كريات الدم الحمر في الاشخاص حديثي الاصابة والمرضى المعالجين طبياً. اما بالنسبة للمؤشرات المناعية فقد اوضحت النتائج ظهور بروتين – C الفعال (CRP) في المصل لجميع العينات المؤخوذة من الاشخاص حديثي الاصابة بالمرض (100%) و68% نتيجة موجبة في المرضى المعالجين طبياً مقارنة ب 100% نتيجة سالبة في عينات السيطرة الاصحاء. أيضا أظهرت النتائج أن العامل الرثوي (RF) كان موجباً في المصل لجميع العينات المؤخوذة من الاشخاص حديثي الاصابة بالمرض (100%) و60% نتيجة موجبة في المرضى

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العلاقة بين المعايير الكيميائية الحياتية والمناعية والتباين الوراثي للجين المشفر لبروتين فوسفاتيز التايروسين في المرضى العراقيين المصابين بألتهاب المفاصل الرثياني

رسالة

مقدمة إلى كلية العلوم/جامعة النهرين
كجزء من متطلبات نيل درجة الماجستير في علوم التقنيات الاحيائية

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