University of Kerbala
College of Applied Medical Sciences
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Study of Some Immunological and Biochemical Parameters in Patients with Lupus Nephritis

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Submitted by

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيم

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We certify that this entitle thesis (**Study of Some Immunological and Biochemical Parameters in Patients with Lupus Nephritis)** was prepared under my supervision at the department of Clinical Laboratories, at the College of Applied Medical Sciences, University of Kerbala, as a partial requirements for the degree of Master in Clinical Laboratories.

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We, the examining committee, certify that we have read the thesis entitled **" Study of Some Immunological and Biochemical Parameters in Patients with Lupus Nephritis "** and have examined the student **(Asmaa Salah Essa Jafeer)** in its content and that in our opinion it is accepted as a thesis for degree of Master of **Clinical Laboratories.**

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Dedication

To this who have reached the message… He advised the nation… To the Prophet of mercy and the light of the Worlds

(Prophet Muhammad peace be upon him)

To my wounded country …

(Iraq)

To the angel of my life… To the meaning of love and devotion… To whom her prayers was the secret of my success and a balm for my surgeon … To my love.

(Dear mother)

To whom Allah keep prestige and dignity… Who taught me to tender without waiting … To whom I bear their names proudly… To whom how supported and encouraged me.

(Dear father)

To my spiritual twin and companion … To sweet heart and honest intentions … To who exist in my life earns me the power and infinite love.

(Dear husband)

I dedicate this work

Researcher
Asmaa

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Researcher
Asmaa

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List of Abbreviations

Symbol	Description
AB	Antibody
ACR	American College of Rheumatology
Ag	Antigen
ANA	Antinuclear Antibodies

Anti dsDNA	Anti-double stranded deoxyribose nucleic acid
Anti-Sm Ab	Anti-smith Ab
ASCs	Antibody secreting Cells
C	Complement
CAM	Cell Adhesion Molecular
CD	Cluster of differentiation
CIC	Circulating Immune Complexes
DNA	Deoxyribonucleic acid
EBV	Epstein Barr Virus
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme linked immunosorbent assay
ENA	Extractible nuclear antigen
ESR	Erythrocytes Sedimentation Rate
ESRD	End Stage Renal Disease
GN	Glomerular Nephritis
GRF	Glomerular Filtration Rate
HLA	Human Leukocyte Antigen
HRP	Horse Radish Peroxidase
IC	Immune Complexes
IFTA	Interstitial Fibrosis Tubular Atrophy
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin

IFN	Interferon
LN	Lupus Nephritis
MLN	Membrane Lupus Nephritis
NGAL	Neutrophil Gelatinase Associated Lipocalin
NIH	National Intitute of Health
NK	Natural Killer cells
PLA2R	Phospholipase A2 Receptor
PMN	Primary Membranous Nephropathy
PSS	Progressive Systemic Scleroderma
RA	Rheumatoid Arthritis
RNA	Ribonucleic acid
SD	Stander Deviation
SLE	Systemic Lupus Erythematosus
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
SLICC	Systemic lupus International collaborating clinic
Th	T helper cell
TLR2	Toll Like Receptor 2
TMB	Tetra Methyl Benzidine
TNF	Tumor Necrosis Factor
UEGF	Urinary Epidermal Growth Factor
UV	Ultra Violet light
VCAM	Vascular Cell Adhesion Molecular
WHO	Word Health Organization

Summary

Lupus nephritis is caused by the formation of an immune complex that develops as a complication of systemic lupus erythematosus. The pathogenesis of lupus nephritis involves a variety of disease-causing mechanisms. The extrinsic etiology of systemic lupus erythematosus depends on multiple groups of genetic variants that affect those mechanisms responsible for immune tolerance to self-nuclear antigens. The increasing incidence of end-stage renal failure underscores the importance of early diagnosis of this disease.

This study aims to investigate some immune markers, which included estimating the concentrations of the phospholipase A2 receptor (PLA2R), the neutrophil gelatinase associated Lipocaline (NGAL), vascular cellular adhesion molecules (VCAM), and urinary epidermal growth factor (UEGF), in addition to studying some biochemical parameters such as urea and creatinine. The study also included estimating the concentrations of Extract nuclear antigen (ENA)., antinuclear antibodies (ANA), antibodies to double-stranded DNA (dsDNA), and concentrations of complement components (C1q, C3, C4).

The results of the phospholipase A2 receptor test on blood samples showed that (5) samples from the women gave a positive result and that they had primary glomerulonephritis, so they were excluded from the study, while the remaining (80) samples showed a negative result for this test so that they had systemic lupus erythematosus. The disease samples were divided based on traditional tests. It was divided into three groups: women with severe lupus nephritis 30 samples, moderate lupus nephritis 20 samples, and systemic lupus erythematosus 30 samples.

The results of the analyzes showed an increase in the concentrations of antinuclear antibodies (ANA) and antibodies to double-stranded deoxyribonucleic acid (dsDNA) in patients with severe lupus nephritis

compared to patients with moderate lupus nephritis and patients with systemic lupus erythematosus, while the results of complement components showed a decrease in their concentrations in patients with Severe lupus nephritis compared to patients with moderate lupus nephritis and patients with systemic lupus erythematosus.

The results of the extracted nuclear antigen (ENA) study also showed that 77% of patients suffer from lupus nephritis alone, while 23% of patients suffer from lupus nephritis and other immune diseases.

The study showed an increase in the concentration of the neutrophil gelatinase associated lipocaline in patients with severe lupus nephritis compared to patients with moderate lupus nephritis and systemic lupus erythematosus, where the concentration reached (**1027.53±** 259.01, **768.82±** 228.8, **715.89±** 173.9 ng/ml), respectively. It also showed The study showed an increase in the concentration of vascular cellular adhesion molecules for the three groups, reaching (**21.43±** 5.83, **13.6±** 2.63, **13.56±** 12.28 ng/ml), respectively. The results also showed a significant decrease in urinary epidermal growth factor concentrations for patients with severe lupus nephritis compared to moderate lupus nephritis and systemic lupus erythematosus reaching (**145.97±** 45.55 , **195.78±**60.38, **339.15±** 85.59ng/ml), respectively.

The results showed that urea and creatinine concentrations increased significantly for patients with severe lupus nephritis compared to moderate lupus nephritis and systemic lupus erythematosus.

The results also showed a significant positive relationship between urinary epidermal growth factor and complement components, as well as a significant positive relationship between the neutrophils gelatinase associated Lipocaline concentration and vascular cellular adhesion molecules.

Decrease in urinary epidermal growth factor concentration in patients with moderate to severe lupus nephritis makes it a good marker for predicting kidney failure at an early stage.

Chapter One
Introduction

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease, characterized by hyperactive B cells, decreased clearance of apoptotic materials and production of anti-nuclear autoantibodies (ANA) and immune complex formation (Dubois and Wallace.,2013).

The disease has several phenotypes, with varying clinical presentations in patients ranging from mild mucocutaneous manifestations to multi organ and severe central nervous system involvement (Ramos., *et al*.,2021). The etiology of SLE is not completely understood, but a combination of environmental and hormonal factors is believed to trigger disease in genetically susceptible subjects (Ulff-Moller., *et al*.,2017). (SLE) characterized by overproduction of antibodies to self-antigens, which are mostly derived from cell components like the nucleus, cell membranes, cytoplasm and ribosomes, the disease most affected patients are women of childbearing age, one of the most frequent organ manifestations of SLE is Lupus Nephritis (LN), which in many cases leads to end -stage renal disease (Te *et al*., 2010). Autoantibodies play an important role in the pathogenesis of SLE, and the diverse clinical manifestations of the disease are caused by the deposition of immune complexes in blood vessels, leading to inflammation in the kidney, brain, and skin. Direct pathogenic effects of the autoantibodies contribute to hemolytic anemia and thrombocytopenia (Graham *et al*., 2007).

Lupus nephritis (LN) is a major cause of morbidity and mortality in patients with systemic lupus erythematosus, the general consensus is that 60% of lupus patients will develop clinically relevant nephritis at some time in the course of their illness (Appel *et al*., 2007). Furthermore, most SLE patients develop nephritis early in the course of their disease, the vast majority of patients who develop nephritis are younger than 55 years, and children are more likely to develop severe nephritis than are elderly patients (Mak *et al*., 2007).

Phospholipase A2 receptor (PLA2R) is a type I transmembrane protein abundantly expressed on glomerular podocytes. Antibodies against PLA2R were described by Beck as serum markers for idiopathic membranous nephritis and also shown in renal tissue (Beck *et al*., 2009). Anti-PLA2R antibody titers are associated with spontaneous remission and are important for monitoring the diagnosis and therapy of primary membranous nephropathy (PMN) (Medrano *et al*., 2015). It has been suggested that in patients with anti-PLA2R antibody-associated PMN, the antibody titer might be used to predict prognosis, supposing that there is a lag between the immunological and clinical response (De Vriese *et al*., 2017). The serum PLA2R antibody has diagnostic specificity and can be used to distinguish between PMN and other glomerular diseases (Radice *et al.*, 2018).

Extractible nuclear antigens ENA profile is useful for the diagnosis of systemic autoimmune rheumatic diseases such as systemic lupus SLE, Sjogren's syndrome, Sharp syndrome, polymyositis/dermatomyositis, or progressive systemic scleroderma (PSS) (Yurasov *et al*., 2006). Because antibodies against ENA have a partial marker function for the individual diseases, the isolated detection of these antibodies autoimmune diseases with the ENA profile allows serological differentiation of these diseases, autoimmune disease detection protocol starts with determination of ANA, and positive ANA test leads to further investigation of extractible nuclear antigens (Orton *et al*.,2004). Antibodies against extractable nuclear antigens (ENA) are auto-antibodies that interact with cellular nuclear proteins, which are referred to as "extractable" because they may be extracted from nucleus by saline, systemic autoimmune diseases are particularly associated with one or more ENA (Khater and Al Sheik;2022).

Complement is known as a mediator of inflammation; complement deficiency might predispose to the development of SLE, inherited complement C4 ,C2 and C1q deficiency, whether partial or complete, confer a high risk to

developing SLE, whereas C3 deficiency is only rarely associated with SLE-like illness (Cohen, 2004). The deficiency of complement leading to the inappropriate clearance of apoptotic cells which lead to formation of immune complexes. Impaired handling of immune complexes is a major pathogenetic factor in SLE (Horák *et al*., 2009). The complement protein C1q is the recognition component of the classical complement pathway and plays a role in efficient clearance of apoptotic cells, the rapid uptake of apoptotic materials by C1q prevents the release of intracellular materials, which activates the dendritic cells and leads to synthesis of cytokines maintaining the dendritic cells in an immature state (Cook & Botto, 2006).

Human epidermal growth factor (urinary EGF), is a growth factor that stimulates cell growth, proliferation, and differentiation by binding to its receptor (Epidermal growth factor receptor) EGFR, some studies found that urinary EGF has a role in the development of body organs such as the brain, lungs, blood vessels, and kidneys (Betsholtz ,2004).Urinary EGF is locally produced in several tissues, such as Henle's loop and the distal convoluted tubule in the kidney, salivary glands, and duodenum (Zeng and Harris.,2014).

Vascular cell adhesion molecule 1 (VCAM-1), or cluster of differentiation 106 (CD106), is the most abundant circulating CAM in the periphery. It is constitutively expressed in endothelial cells and glomerular parietal epithelial cells (Rabb *et al*., 1997) and has been shown to be substantially elevated in active SLE, during renal allograft rejection and in septic shock (Zonneveld *et al.,* 2014).

Neutrophil gelatinase-associated lipocalin (NG- AL) is a small molecular protein of 25 KD, can be used as an early diagnostic marker of acute kidney injury, diabetic nephropa- thy and contrast-induced nephropathy (Briguori *et al*.,2014). Moreover, NGAL level can be used as clinical standard reflecting LN disease activity (Rubinstein *et al*., 2010).

Aim of the study

The combination of traditional disease-specific biomarkers and novel (serum, urine) biomarkers may represent the best choice to correctly classify stage with lupus nephritis in SLE patients. The aim of current study was achieved by using the following objectives:

1. Estimation the levels of Phospholipase A2 Receptors (PLA2R).
2. Measuring The level of urinary epidermal growth factor.
3. Evaluate the serum levels of NGAL and VCAM.
4. Estimation the level of complement component C3, C4, C1q.
5. Find out the correlation between study parameters.

Chapter Two
Literature Review

2.1. Systemic Lupus Erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease characterized by the production of antibodies to components of the cell nucleus in association with a diverse array of clinical manifestations (Bernknopf *et al*., 2011). It is a type III hypersensitivity reaction caused by formation of antibody immune complex, the course of the disease is unpredictable, with remissions alternating with periods of illness (called flares) (Rahman & Isenberg, 2008). The first study relating to SLE in Iraq was in 1971 in Which there was a 10-year follow-up on 36 SLE cases in which clinical, laboratory and pathological studies were conducted (AL-Damlujii *et al*., 1971).

The SLE pathogenesis results from various immune abnormalities involving impaired clearance of apoptotic cells and immune complexes together with the reduction in the thresholds of activation of B and T lymphocytes, leading to loss of self-tolerance and overproduction of autoantibodies, which are mainly directed against DNA,RNA and other cell component(Belot and Cimaz, 2012).

The causes are believed to be environmental, hormonal, or genetic factors, which results in a misdirected immune response in people who are genetically susceptible. Lupus is characterized by the presence of autoantibodies that are directed against a person's own proteins, these are most commonly anti-nuclear antibodies, which are found in nearly all cases these antibodies lead to inflammation (Bartels, 2015).

2.2. The History of SLE:

The history of systemic lupus erythematous can be divided into three periods:

1-The classical period: began when the disease was first recognized in the middle ages and see the description of the dermatological manifestation of the disorder. Classical descriptions of the various dermatologic features of

lupus were made in the early nineteenth century (Kaposi, 2010).

2-The Neoclassical period: the history of lupus began in 1872 when the first described the systemic nature of the disorder. It proposed that there are two types of lupus erythematosus; the discoid form and a disseminated form. Over the last thirty years, pathological studies that documented the structural changes in the glomeruli of lupus patients (Klemperer *et al*.,2010).

3-The modern period: began in 1948 with the discovery of the Lupus Nephritis cell and this period was characterized by advances in the knowledge of the pathophysiology and clinical-laboratory features of the disease, as well as advances in treatment (Hochberg, 1991).

2.3. Classification Criteria of SLE

Formal classification criteria of SLE often used in clinical studies include the 1982 American College of Rheumatology (ACR) classification criteria and the Systemic Lupus International Collaborating Clinics (SLICC) classification criteria from 2012 (Petri *et al*.,2012).

There was, however, no breakthrough for these criteria because of the absence of a validation. To be classified as SLE according to the SLICC classification, the patients must fulfil at least 4 criteria, including at least one clinical and one immunologic criterion, or the patients must have biopsy-proven lupus nephritis (LN) in the presence of ANA or anti-double-stranded DNA antibodies it's shown in table (2-1)

Table (2-1) SLICC classification system for SLE (Petri *et al*.,2012)

Clinical Feature	**Immunological Feature**
Acute cutaneous lupus (maculopapular lupus rash ,malar rash , photosensitive lupus rash)	High ANA concentration
Chronic cutaneous lupus (discoid rash , mucosal lupus)	High anti –dsDNA antibody concentration
Oral or nasal ulcer	Presence of anti –Sm
Nonscarring alopecia	Positive APA
Synovitis in 2 joints	Low complements (C3 , C4 , CH50)
Serositis	Direct Coombs test
Renal (urine protein or RBC casts)	
Neurologic (seizure , psychosis , others)	
Hemolytic anemia	
Leukopenia or lymphopenia	
Thrombocytopenia	

2.4.Epidemiology:

There is a worldwide prevalence of lupus ranges from 14 to 172 cases per 100,000 people .This wide variance is relative to racial, ethnic, and geographic factors (Wallace, 2008). The incidence increased three times in the last 40 years of the 20th century due to the improvement of the detection of early disease abnormalities (Uramoto *et al*., 1999). Probable incidence rates are 1 to 25 per 100,000 in North America, South America, Europe and Asia (Danchenko *et al*., 2006).

The incidence is highest among women in childbearing age, with women to men ratio of about 9:1 commonly reported (Lewis and Jawad, 2017). the female predominance is less pronounced in juvenile and elderly populations. The reason for this is unknown but hormones as well as genetics, including the double X-chromosome, is likely to contribute (Rees *et al*., 2017).

Most SLE patients develop nephritis early in the course of their disease. The vast majority of patients who develop nephritis in young and children are more likely to develop severe nephritis than are elderly patients (Mak *et al.*, 2007). In a recent retrospective study, male sex, young age (<33 years), and non-European ancestry were found to be determinants of earlier renal disease in patients with SLE. Asian, African Caribbean, and African American ethnicities may present with more severe nephritis than other ethnic groups (Seligman *et al.*, 2002)

In LN disease, the incidence and prevalence of LN varies depending on the studied population, the LN cumulative incidence is higher in people of African (51%), Asian (55%) compared with Caucasians (14%) (Ortega *et al*., 2010).

Up to 25% of these patients still develop end-stage renal disease (ESRD) after 10 years' onset of SLE. In terms of outcome, the 5- and 10year renal survival rates of LN. So, LN develops early in the course of SLE thus becoming

a major predictor of poor prognosis. However, approximately 5% of the cases, LN may appear several years after the onset of SLE (Mok;2020).

2.5.Geographic and racial distribution:

The prevalence of the disease is higher among Asian Indians compared with Caucasians in Great Britain, Afro-Americans, Afro-Caribbean's, and Hispanic Americans compared with Americans of European decent in the United States (Rus *et al*., 2002). However, SLE occurs infrequently in Blacks in Africa (Symmons, 1995). The disease seems to be more common in urban than rural areas (Chakravarty *et al*., 2007).

In Iraq, the prevalence of SLE is about 1/1867 of the general population, and the first case of SLE was reported in 1971(Al-Qubaeissy *et al*., 2020).

2.6. The pathogenesis of SLE:

The lupus pathogenesis incorporates roles for genetic susceptibility based on a threshold model involving multiple genes; environmental triggers, including microbial infection, sunlight, and certain drugs; and altered immune system function, The individuals with clinical SLE usually experience over a period of many years, flares and remissions, associated with some organs damage related to chronic inflammation. Advance practical immunology has focused attention on the mechanisms that account for innate immune system activation. The genetic and environmental factors seem to play an important role in promotion of innate immune system activation and subsequent autoimmunity, while other etiological factors may contribute to inflammation and tissue damage (Goldman and Ausiello, 2007).

The pathogenesis of SLE is multifactorial including ethnicity, environmental and hormonal factors, but to a large extent it is unknown. The pathogenesis of SLE comprises dysregulated apoptosis and inefficient removal of apoptotic cellular material (Munoz *et al*., 2010). Such cellular debris will expose nuclear constituents as well as phospholipids on its surface, which under

certain conditions may undergo conformational changes, and become immunogenic. And this may result in loss of B cell tolerance and production of autoantibodies against nuclear (Andreoli *et al*., 2013).

A prominent production of autoantibodies and immune complexes, and an increased expression of type I interferon regulated genes, recognized as the IFN-signature is often seen (Bengtsson and Ronnblom, 2017). According to one study 50-75% of adults and 90% of children with SLE displayed a type I IFN signature; whereas circulating levels of IFN-α in adult with SLE may be considerable lower (Enocsson *et al*., 2014).

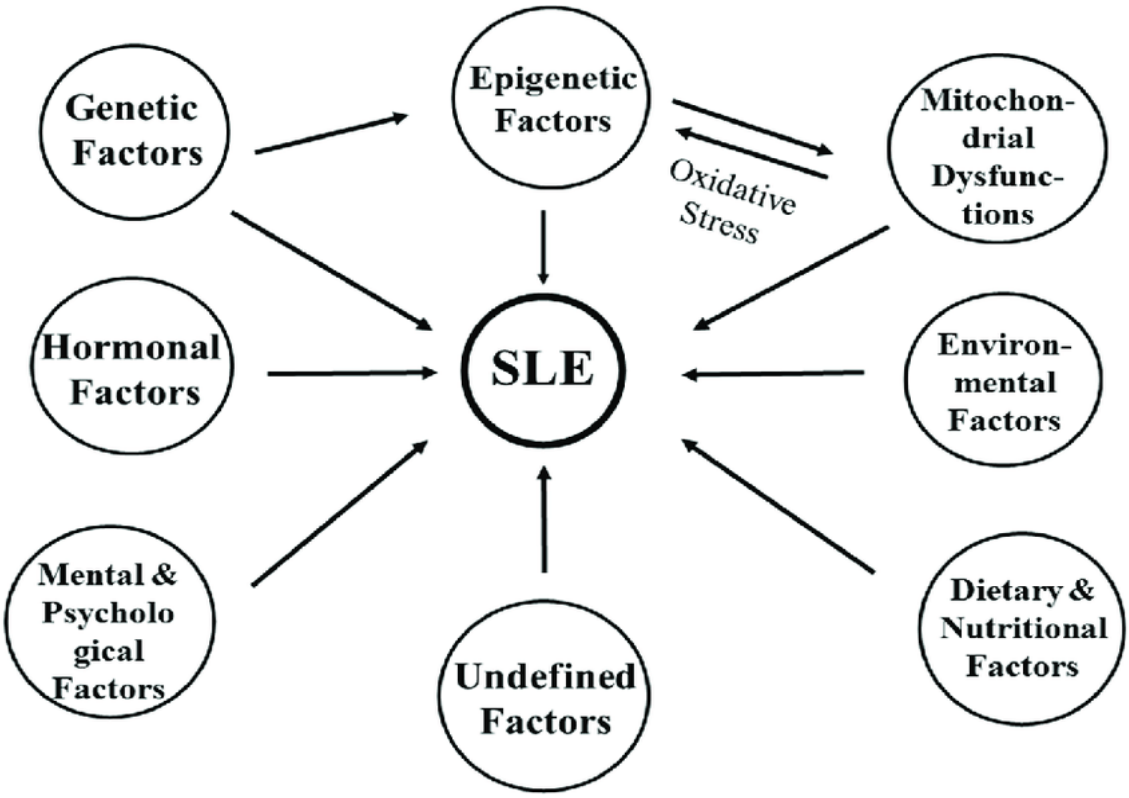


Figure (2.1) Factors associated with SLE pathogenesis (Tsai et al.,2019).

2.6.1. Genetic factors:

At least 30 susceptibility genes for SLE have been identified and their presence varies widely depending on race, ethnicity and geography (Croker and Kimberly, 2005). A genetic factor appears clearly from data obtained in first-degree relatives of SLE patients, the risk of SLE is about 20 times higher than in the

general population. Also, the rate in monozygotic twins range from 24 to 58% as compared with 3 to 10% for dizygotic twins (Hochberg, 1997).

SLE association with the major histocompatibility antigen (HLA) were extensively investigated since 1971, many studies have shown that SLE is associated with HLA-DR3 particularly in Caucasian individuals (Harley *et al.,* 1998).

2.6.2 The influence of the environment:

Many potential environmental triggers for lupus have been studied:

1. Ultraviolet light exposure: is a well-described trigger of lupus flares. Possible mechanisms that account for this observation include DNA damage and induction of apoptosis of keratinocytes, which results in concentration of nucleic acids and associated proteins in cell membrane blebs and increased availability of these self-antigens for processing by antigen-presenting cells. Exposure to ultraviolet light causes SLE flares in approximately 70% of patients (Goldman and Ausiello, 2007).

2. The role of infections: normal immune response to bacterial and viral infections may spin-off into a state of B-cell hyperactivity, triggering autoantibody. Another hypothesis is that a virus may infect the immune system, activating self-reactive T cells that would otherwise remain quiescent. A virus that infects immune cells and kidney cell can possibly be reactivated periodically and thus lead to glomerular nephritis (GN) (Gharavi *et al.*,2011)**.**

Epstein Barr virus (EBV) infection was found to become periodically reactivated, but a potential causative role in GN has been difficult to prove. Evidence supporting EBV infection as an etiologic factor includes first, long term studies demonstrate a higher association with LN in individuals with early presence of serum antibodies against specific EBV antigens and second high expression of EBV antigens within LN plaques (Hislop *et al.*,2013).Therefore, it is potential that EBV reactivation is an effect rather than a cause for example

instead of viral reactivation being the trigger for LN, reactivation might be an epiphenomenon of a dysregulated immune system. (Draborg *et al.*,2013)**.**

As a possible viral etiology that may lead to the disease, the best studied is the EBV. This virus infects B cells via binding of the viral envelope glycoprotein 350 to the B-cell type 2 complement receptor and remains dormant for the life of the individual, the titer of anti-EBV antibodies is also higher in LN, and the EBV viral load is 10–100 times higher than that in controls, there is also evidence of impaired T-cell reactivity to EBV viral proteins and biomarkers of latent EBV activation correlate with exacerbations of LN (Lech *et al.*,*2008*).

3. The role of drugs: Pharmaceuticals have multifaceted actions ranging from exacerbating or aggravating an immune process to inducing lupus. For example, a drug can alter DNA or render it immunogenic and lead to the production of autoantibodies (e.g., procainamide). Other agents promote autoreactive T or B lymphocytes (e.g., phenytoin). Hypo methylation of DNA results in altered DNA repair and autoantibody formation (e.g., fludarabine). Oxidized metabolites of certain agents in slow acetylators, for example, can induce an immune reaction (e.g., hydralazine, isonaizid). Certain drugs are sun-sensitizing (e.g., nonarylamine sulfa antibiotics, phenothiazines) and lead to a phototoxic inflammatory response (Wallace, 2008).

A lupus-like syndrome has been seen in patients with hematologic malignancies or hepatitis C infection who are receiving doses of recombinant interferon-α. In addition, immunotherapy with anti-TNF agents has induced lupus autoantibodies and occasionally clinical lupus in patients with rheumatoid arthritis (Goldman and Ausiello, 2007).

4. The influence of hormone: Several Studies dealing with SLE denoted the effect of sex hormones on the occurrence of this disease with increased incidence in female more than male which may reach to 10-20 fold (Hochberg, 1985).

Normally, women tend to mount more Th1 like responses than Th2 responses. But during pregnancy, some women showed increased Th2 like responses. It is believed that pregnancy-associated levels of sex steroid may promote an anti- inflammatory environment. As a result, diseases like SLE associated with high Th2 like responses, can be exacerbated during pregnancy, while diseases that involve inflammatory responses, such as rheumatoid arthritis, sometimes are ameliorated in pregnant women (Ryan and Mills,2022).

The sex steroids that are circulating throughout the body can alter the immune responses by altering patterns of gene expression. Thus, estrogen enters cells, binds to the estrogen receptor, and thus induces the binding of the estrogen receptor to a specific DNA sequence, which in turn results in the modulation of transcription (Goldspy *et al*., 2000).

5. Toxic substances: Certain studies revealed that toxic exposure increases risk of SLE is silica (Parks *et al.,* 2002). Smoking is a risk factor for SLE and, in addition, in patients with established SLE, smoking increases the risk of discoid lupus. Hair dyes, Pesticides and Solvents have been also identified to have some risk for SLE (Costenbader *et al.,* 2004).

2.7. Lupus nephritis:

Lupus nephritis is an immune complex Glomerular Nephritis (GN) that develops as a frequent complication of SLE (Bao *et al.*,2011).The morbidity and mortality of LN is considerable, with up to (10 %) of the patients developing end-stage renal disease (ESRD) defined as dialysis or transplantation (Hanly *et al.,* 2016).

Late diagnosis of lupus nephritis is correlated with a higher frequency of renal insufficiency (Esdaile *et al*., 1994). The increased incidence of ESRD underlines the importance of early diagnosis in this difficult to control disease with unpredictable course (Faurschou. *et al*., 2006).

This loss of tolerance becomes clinically detectable by the presence of antinuclear antibodies. In addition, nucleic acids released from netting or apoptotic neutrophils activate innate and adaptive immunity via viral nucleic acid-specific Toll-like receptors. Therefore, many clinical manifestations of systemic lupus resemble those of viral infection. In lupus, endogenous nuclear particles trigger IFN signaling just like viral particles during viral infection. As such, dendritic cells, T helper cells, B cells, and plasma cells all contribute to the aberrant polyclonal autoimmunity (Lech *et al.,* 2008).

The intra renal etiology of lupus nephritis involves antibody binding to multiple intra renal auto antigens rather than the deposition of circulating immune complexes. Tertiary lymphoid tissue formation and local antibody production add to intra renal complement activation as renal immunopathology progresses. An update on the pathogenic mechanisms that lead to LN and provide the rationale for the latest and novel treatment strategies (Ayodele *et al.*, 2010).

Lupus Nephritis is one of consequences of SLE, which is an autoimmune disease characterized by overproduction of antibodies to self-antigens, which are mostly derived from cell components like the nucleus, cytoplasm ribosomes, and cell membranes (Yu *et al.*,2010). The disease most affected patients are women of childbearing age ,and the symptoms of lupus nephritis are generally related to hypertension, proteinuria, and renal failure. Immune complexes identified in lupus nephritis include: DNA-anti-DNA nucleosomes, chromatin Sm La (SS-B) and Ro (SS-A) and ribosomes (Bao *et al.,* 2011).

Lupus nephritis is currently classified into six types based on kidney biopsy findings:

1. Class I: refers to presence of mesangial deposits without mesangial hypercellularity.
2. Class II refers to presence of mesangial deposits with mesangial hypercellularity.

3. Class III refers to focal glomerulonephritis (involving <50 percent of the total number of glomeruli).
4. Class IV refers to diffuse glomerulonephritis (involving >50 percent of the total number of glomeruli), segmental and global.
5. Class V refers to membranous nephropathy.
6. Class VI refers to advanced sclerosing lesions (Almaani *et al.,* 2017).

The ideal biomarker in SLE patients with suspicion or confirmation of LN should have the following properties: (1) be specific for renal involvement, (2) have a good correlation with kidney activity or damage, (3)be useful for serial monitoring, (4) be superior to conventional clinical or laboratory parameters, (5) possess the ability to assess the severity of renal involvement, (6) be cost-effective, and (7) easy to perform and available in most clinical laboratories (Reyes *et al*., 2011).

2.8 The Pathogenesis of Lupus nephritis:

The immune complex mediating renal immuno-pathology and nonspecific activation of autoreactive B cells explains the polyclonal autoantibody response leading to the LN, which involved IgM and IgG deposits (Schwartzman and Putterman , 2012).Immune complex deposits in the glomeruli are primarily responsible for the inflammatory process and lead to glomerular damage, if deposited in sub endothelial space and the mesangial, immune complexes will activate the complement system(cause hypocomplementemia),the result is an influx of neutrophils and mononuclear cells that secrete proteases, reactive oxygen species, and pro inflammatory cytokines and chemokines, causing glomerular injury(Fiehn *et al.,* 2003).

Glomerular thrombosis is another mechanism that may play important role in pathogenesis of lupus nephritis, mainly in patients with antiphospholipid antibody syndrome, and is believed to be the result of autoantibodies directed against negatively charged phospholipidprotein

complexes, and Intra renal activation of TLRs and IFN signaling. The nucleic acid component of immune complexes also activates intra renal inflammation by Toll like receptors (TLRs) in intra renal dendritic cells and macrophages (Feldman *et al.;* 2013). In addition, immune stimulatory nucleic acids activate mesangial cells and glomerular endothelium, and macrophages to produce large amounts of pro inflammatory cytokines such as IL-1, IL-6, TNF and IFN-B. Together, the ligation of TLRs, complement receptors, and FcRs activates renal cells to release chemokines pro inflammatory cytokines, and induces the luminal expression of selectins and adhesion molecules inside the micro-vasculature (Parikh *et al.*, 2015).

Chemokine-mediated recruitment of different leukocyte, cytotoxic T cells, T-helper17cells, and B-cells infiltrate the kidney in LN. The members of the chemokine family specifically direct different leukocyte subsets by distinct chemokine receptors into different renal compartments for example, the chemokine CCL2 recruits CCR2+proinflammatory macrophages and T- cells into the tubule interstitium and glomerulus , whereas CCR1+cells only recruit to the interstitial compartment and not to the glomerulus in LN*(*Malemud, 2010). Figure (2.2) showed the pathogenesis model of lupus nephritis.

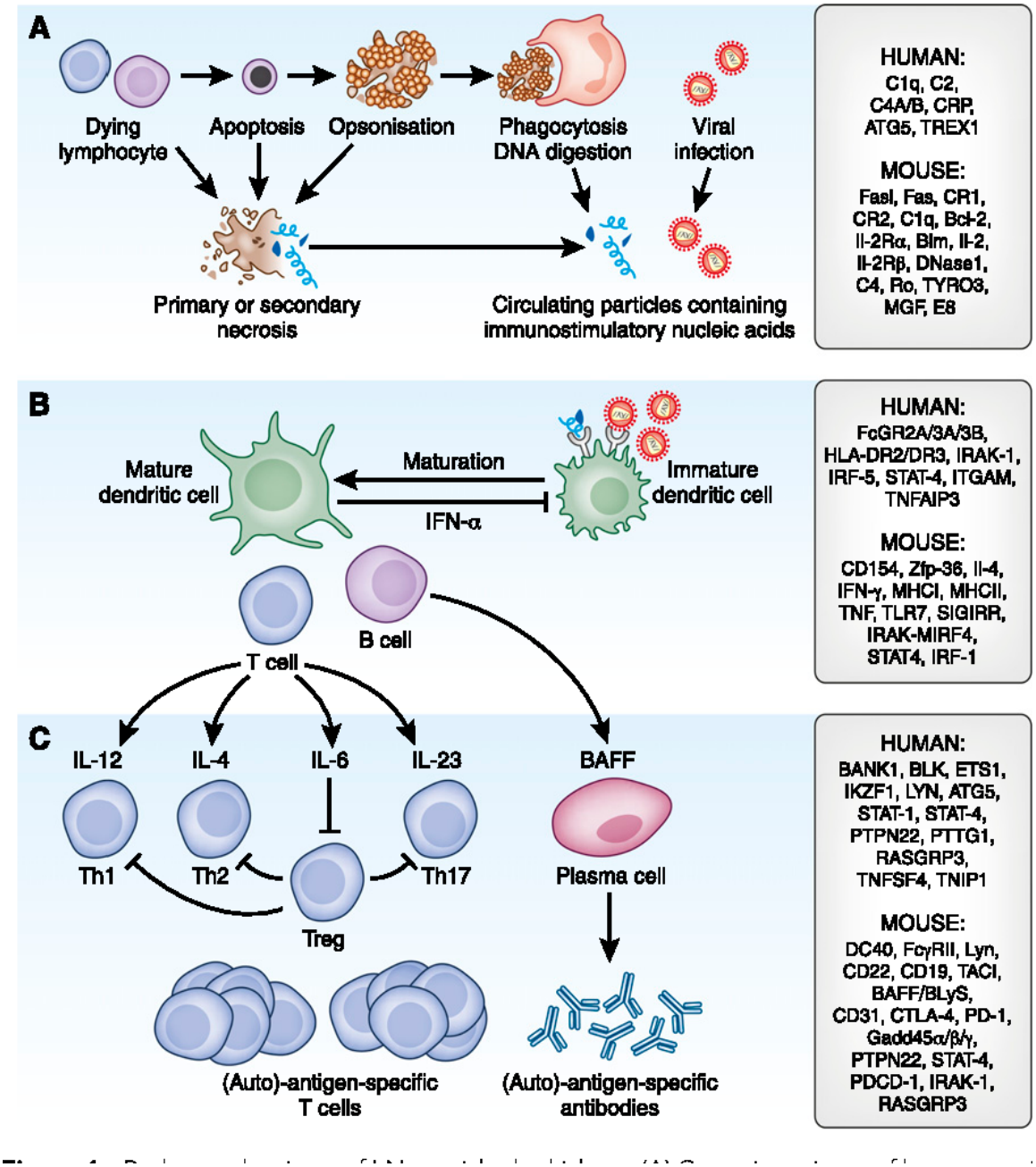


Figure (2.2) Pathogenesis model of Lupus nephritis *(*Maciej and Hans,2013)

2.9: Diagnosis: The diagnosis of SLE is based on clinical and laboratory criteria. The criteria set developed by the American College of Rheumatology (ACR) are most widely used (Hochberg ,1997).

2.9.1 Traditional diagnosis:

The study of chemical changes in the blood electrolytes that give indicators for people with this disease although biopsy is a harmful techniques that require surgical intervention and the insertion of a piece of kidney (Glomerular) and examined in the laboratory after long and complex procedures include fixation ,dehydration, clearing ,embedding, blocking , cutting, staining and examination under microscope by histo pathologist (Alvarado *et al.;* 2014).To detect the systemic lupus erythematosus by measuring antibody production using the ELISA technique.

2.9.1.1 -Phospholipase A2 receptor (PLA2R):

Phospholipase A2 receptor (PLA2R) is a type I transmembrane protein abundantly expressed on glomerular podocytes. Antibodies against PLA2R were described as serum markers for idiopathic membranous nephritis and also shown in renal tissue (Beck *et al*., 2009).

MLN (Membrane Lupus Nephritis) shares many features with idiopathic or primary membranous nephropathy (PMN), both are often associated with heavy proteinuria and the nephrotic syndrome, differentiating MLN from PMN can be challenging, especially when MLN is the first manifestation of SLE. However, distinguishing MLN from PMN has been made easier with the discovery of the phospholipase A2 receptor (PLA2R) in PMN. For example, approximately 70% of patients with PMN will have a circulating anti-PLA2R antibody. Meanwhile, MLN is typically anti-PLA2R negative though cases of PLA2R positive MLN have been reported (Gunnarsson *et al*., 2007).

The anti-PLA2R antibody has a higher positive rate in renal tissue, where it accumulates with IgG within immune deposits (Beck and Salant,2014).in addition Anti-PLA2R antibody titers are associated with spontaneous remission and important for monitoring the diagnosis and therapy of (primary membranous nephropathy) PMN (Medrano *et al*., 2015). It has been suggested that in the

patients with anti-PLA2R antibody-associated PMN, the antibody titer might be used to predict prognosis, supposing that there is a lag between the immunological and clinical response (De Vriese *et al*.,2017). Despite the fact that around 30% of patients with PMN are anti-PLA2R-negative, even in their renal tissue, little information is available on these patients (Jatem Escalante *et al*.,2015). Most studies using PLA2R to predict prognosis focused on the anti-PLA2R status of the patients, and few considered the glomerular PLA2R antigen (Wei *et al*.,2016).

As major podocyte autoantigen, phospholipase A2 receptor (PlA2R) was found, accounting for 50-80% of the cases of PMN (francis *et al*.,2016).

2.9.1.2 Anti-Nuclear Antibody (ANA)

Patients with SLE show positive ANAs test in about 95% of them using immunofluorescence screening, especially early in the disease, the sensitivity of ANA testing can be as low as 70% (Sjowall, et al., 2008). This fact is especially true in laboratories that are using enzyme immunoassays or other automated assays, which display marked intermanufacturer variation in performance and have a reported sensitivity of 70–98% for the detection of ANA, many individuals with positive ANA do not have SLE and have a low risk of developing the disease. so that, ANA screening has a low specificity for SLE, which lowers its use for diagnosis (Meroni and Schur, 2010)

2.9.1.3 Anti-double strand deoxyribonucleic acid (Anti-dsDNA)

Antibodies against double-stranded DNA which can be detected by the ELISA which is found in up to 70% of patients with SLE at some point during the course of their disease and have 95% specificity in established SLE cohorts, making them a valuable diagnostic marker (Craig and Ledue 2011). The ds-DNA is always the principle antigen for anti-dsDNA antibodies in SLE. First described in the late 1950s autoantibodies to deoxyribonucleic acid (DNA) are highly heterogeneous with respect to their avidity, immunoglobulin

subclass composition, cross-reactivity and complement fixing ability. In SLE, anti-dsDNA antibodies are classified according to their reactivity to ds-DNA; antibodies to single stranded DNA (ss-DNA) are not specific for SLE as they are found in sera from patients with both rheumatic and non-rheumatic diseases. (Feletar, 2003)

The prevalence of patients with SLE and a positive anti-DNA assay despite a negative ANA result has been reported to be 0–5.5% (Craig and Ledue 2011).

2.9.1.4 Extractible nuclear antigens ENA:

Extractible nuclear antigens ENA is useful for the diagnosis of systemic autoimmune rheumatic diseases such as systemic lupus SLE, Sjogren's syndrome, Sharp syndrome, polymyositis / dermatomycosis's, or progressive systemic scleroderma (PSS) (Yurasov *et al.,* 2006). Because antibodies against ENA have a partial marker function for the individual diseases, the isolated detection of these antibodies autoimmune diseases with the ENA allows serological differentiation of these diseases. Autoimmune disease detection protocol starts with determination of ANA. Positive ANA test leads to further investigation of extractible nuclear antigens (Orton *et al*.,2004).

Other prevalent Abs in SLE patients recognize extractable nuclear antigens (ENAs), including anti-Smith (Sm), -SSA/Ro, and -SSB/La ribonucleoproteins, among others. Consequently, antibody-secreting cells (ASCs) are now considered a relevant target for different therapies in SLE (Hiepe and Radbruch, 2016). Antibodies to ENA profile are the hallmark in the diagnosis of SLE and sometimes support to the diagnosis in spite of negative ANA in a SLE patients (Yamad *et al*.,2018).

Antibodies against extractable nuclear antigens (ENA) are auto-antibodies that interact with cellular nuclear proteins, which are referred to as "extractable" because they may be extracted from nucleus by saline, systemic

autoimmune diseases are particularly associated with one or more ENA (Khater and Al Sheik;2022).

Consequently, if ANA results are detected in patients with suspected autoimmune disorders, anti-ENA tests are carried out to screen the presence of certain diseases, however since it was detected that anti-ENAs could be detected in ANA negative samples, there is an argument that the anti-ENA detection should be more expanded (Bossuyt &Luyckx ,2005).

Autoantibodies against ENAs have been investigated most extensively in SLE patients. These autoantibodies to ENAs are linked to lupus nephritis pathogenesis and play a significant role in determining disease activity and severity (Emad *et al*.,2018).

2.9.1.5 The complement system

The complement system is a part of the immune system and consists of multiple complement components with biological functions such as defense against pathogens and immunomodulation. The complement system has three activation pathways: the classical pathway, the lectin pathway, and the alternative pathway. Increasing evidence indicates that the complement system plays a role in aging. Complement plays a role in inflammatory processes, metabolism, apoptosis, mitochondrial function, and Wingless Int-1 (Wnt) signaling pathways (Zheng *et al*., 2022).

The most common laboratory findings of SLE low complement about (88%) of SLE patient show low complement (Timlin *et al*., 2018). decreased C3 and/or C4 complements, may indicate disease activity (Siddiqui *et al*., 2018) (Çelik *et al*., 2022). complement increases during the acute phase response, individual variability in complement gene copy number and expression, and variability in protein synthesis and catabolism. all these can influence plasma/serum complement levels, Because of these factors, low complement levels perform poorly as diagnostic markers for SLE (Weinstein *et al*., 2021).

SLE is recognized as a disease where autoantibodies develop and fix to self-antigen resulting in complement activation and leading to inflammation and tissue damage (Weinstein *et al*., 2021). The complement system, which consists of about 40 soluble and membrane bound proteins. The classical and the lectin pathways are activated through pattern recognition. Serine proteases bound to a pattern-recognition molecule are activated upon recognition of a fitting pattern and through several enzymatic reactions this lead to the activation of the classical and lectin pathway C3-convertase (C4b2a). The alternative pathway is in a state of constant activation, but is at the same time inhibited. There is a continuous hydrolyzes of C3 in the circulation, which can lead to complement activation via the alternative pathway of complement activation. Under normal condition, this process is inhibited both in the circulation and at the cell surface (Troldborg *et al*., 2018). The complement system plays a major role in the systemic lupus erythematosus (SLE). However, the role of complement in SLE is complex since it may both prevent and monuments the disease (Leffler and Blom, 2014).The deficiency of complement leading to the inappropriate clearance of apoptotic cells which lead to formation of immune complexes. Impaired handling of immune complexes is a major pathogenetic factor in SLE (Horák *et al*., 2009). There was an elevated levels of the parameters; ANA, anti-dsDNA and IL-4 that measured in SLE patients which reflect the severity of disease comparing with controls group, also there was importance of ANA, anti-dsDNA, C3, C4 and IL4 in the predictions of disease activity. (Hassan., 2015).

C1q is the recognition and starter molecule of the classical pathway of the complement system. It is a 460 kDa glycoprotein consisting of 18 polypeptide chains that have an Nterminal collagen-like domain. These chains form six triple helices assembling to a structure that resembles a bouquet of tulips with the stalks being formed by the collagen-like regions while the Cterminal parts form the flower-like globular head regions of the molecule, which primarily mediate the binding of C1q. A comprehensive (but not exclusive) explanation for the role of C1q in SLE was provided by the so-called ‘waste disposal hypothesis (Botto and Walport,2002).

The complement protein C1q is the recognition component of the classical complement pathway and plays a role in efficient clearance of apoptotic cells. The rapid uptake of apoptotic materials by C1q prevents the

release of intracellular materials, which activates the dendritic cells and leads to synthesis of cytokines maintaining the dendritic cells in an immature state (Cook and Botto, 2006).

C1q deposition is a specific histological finding in severe lupus nephritis where it is detected in electron-dense deposits of the renal subendothelial space and/or the glomerular basement membrane (Sjowall *et al*., 2013).

2.9.1.6 Hematological finding

The complete blood count screens for anemia, autoimmune hemolytic anemia, leucopenia, lymphopenia, and thrombocytopenia (Wallace, 2008).

The erythrocyte sedimentation rate (ESR), though a very nonspecific indicator of systemic inflammation is often monitored and in many patients can provide an indication of disease activity. Interestingly, C-reactive protein, an acute phase reactant, is relatively uninformative in SLE because it is often low in comparison to an erythrocyte sedimentation rate performed on the same occasion (Goldman and Ausiello, 2007), except in the presence of Serositis, and infection (Colledge *et al.*, 2010).

2.9.2 New Trends Diagnosis

Non-invasive approaches might provide additional diagnostic criteria for patients for whom there is a high suspicion of an underlying Lupus Nephritis; these might include serum and urinary biomarkers. Combining biomarkers might increase the diagnostic sensitivity and specificity, providing an alternative to renal biopsy (Hastings *et al.;* 2013).

2.9.2.1 Human epidermal growth factor (urinary EGF):

Human epidermal growth factor (urinary EGF), a 6000 molecular weight polypeptide (Cohen and Carpenter.,1975), is a growth factor that stimulates cell growth, proliferation, and differentiation by binding to its receptor (Epidermal growth factor receptor) EGFR. Some studies found that urinary EGF has a role

in the development of body organs such as the brain, lungs, blood vessels, and kidneys (Betsholtz ,2004).

Urinary EGF is locally produced in several tissues, such as Henle's loop and the distal convoluted tubule in the kidney, salivary glands, and duodenum (Zeng and Harris.,2014), In the kidney, urinary EGF is involved in the repairing process of renal tissues (Chou *et al*., 1997).

High concentrations of urinary EGF can be found in the urine. Based on in vitro experiments, it has been previously suggested that urinary EGF originates from the ultra-filtrate. However, in vivo, it was shown in rats and in humans that the urinary EGF is mainly produced in the kidney itself. Therefore, it is generally accepted that the urinary EGF excretion reflects the renal EGF production (Grandaliano *et al*.,2000).

Reduced concentrations of urinary EGF in the urine have been previously observed in diabetes nephropathy, IgA nephropathy, adult polycystic kidney disease chronic renal failure (Meybosch *et al*.,2019). Also, the possibility that urinary EGF might serve as a surrogate marker for functional regeneration of the renal tubules, reflecting their ability to respond to future acute or chronic injury, was recently put forward (Ju *et al*.,2015). In the kidney, EGF exerts several biological functions such as regulation of cellular metabolism and glomerular hemodynamics, modulation of cell growth, and renal repair after injury (Chiarelli *et al*.,2009).

An experimental model of acute renal failure showed that EGF assisted with tubular injury recovery by activating regeneration pathways, resulting in re epithelialization of the injured tubules (Cao *et al*.,2005).Higher urinary levels of EGF (UEGF) are thought to reflect functional tubular mass and regeneration potential (Ju *et al*.,2015). In contrast, lower uEGF concentrations are associated with interstitial fibrosis and tubular atrophy (IFTA), besides, the EGF protein detected in the urine of kidney disease patients and reported in several studies may actually be proEGF (Mejia *et al*.,2021).

Urinary epidermal growth factor (UEGF) has been evaluated as a possible LN biomarker the urinary EGF levels in LN patients were determined in a longitudinal study. Its level was lower in patients with active LN compared to patients with active non-renal SLE, patients with inactive SLE and healthy kidney donors. In addition, the urinary EGF level was inversely correlated with the chronicity index assessed by kidney biopsy histology. In a follow up of the patients, it was corroborated that urinary EGF were lower at flare and were decreasing over time in the case of adverse long-term kidney damage (Mejia *et al*.,2021).

2.9.2.2 Vascular cell adhesion molecule-1 (VCAM-1)

Vascular Cell adhesion molecules (VCAMs) are known to be important components in the inflammatory process. They are expressed on the vascular endothelium and act as counter-receptors for leucocyte integrins, enabling leucocyte adhesion and rolling along endothelial cell surfaces, a process that eventually leads to extravasation of leukocytes into adjacent tissue (Brady and Tonks., 1994).

Vascular cell adhesion molecule is the most abundant of the circulating CAM, and shows the greatest variation in serum level across a number of inflammatory diseases, with the highest levels observed in active systemic lupus erythematosus (SLE), renal allograft and septic shock, it has been shown to be substantially elevated in active SLE, during renal allograft rejection and in septic shock (Zonneveld *et al*., 2014).Vascular cell adhesion molecule appears to be of particular interest in renal disease, as raised levels have been found in active nephritis (Singh *et al*.,2012). In SLE patient's previous studies have suggested a role of VCAM-1 in LN development (Sabry *et al*.,2007). In addition, Vascular cell adhesion molecule has been found to be elevated in patients with systemic lupus erythematosus (Constans *et al*., 2003).

Some, but not all studies, have also found correlations with disease activity and particular disease phenotypes (Egerer *et al*., 2000). Elevated levels of VCAM-1 have also been observed in SLE patients compared with controls and in LN patients with advanced renal damage (Abd-Elkareem *et al*.,2010: Mok *et al*.,2018). Several studies correlated the VCAM-1 levels with active LN (Wu *et al*.,2015). Patients with more advanced histological changes (class III, IV and V nephritis with greater kidney biopsy activity) had the highest values (Singh *et al*.,2012). In addition, a previous study showed that high levels of VCAM-1 may indicate patients at increased risk of long-term loss of renal function (Parodis et al.,2019).

2.9.2.3 Neutrophil gelatinase-associated lipocalin (NG- AL)

Neutrophil gelatinase-associated Lipocaline (NGAL) is a 25 kDa protein of the Lipocaline super family, this protein is secreted by immune cells, hepatocytes and renal tubular cells in several pathological conditions. NGAL has recently generated great interest as an early marker of renal injury. However, like many other endogenous biomarkers it is produced by several cell types and it exists in several molecular forms. Different pathological conditions may be involved in the production of this molecule (Makris *et al*., 2010).

NGAL can be used as an early diagnostic marker of acute kidney injury, diabetic nephropathy and contrast-induced nephropathy (Antonucci *et al*., 2014). moreover, NGAL level can be used as clinical standard reflecting LN disease activity (Rubinstein *et al*., 2010).

The NGAL, expressed in neutrophils. and this molecule is involved in the iron trafficking in the kidney and in the inflammatory responses by sequestering neutrophil chemo attractants (Bolignano *et al*., 2008).

NGAL is widely expressed following ischemic or nephrotoxic injury in humans. and is thought to mediate inflammatory responses by sequestering neutrophil chemo attractants such as leukotriene B4 and platelet-activating factor (Mishra *et al*., 2003). Several clinical studies found that NGAL

represented a very sensitive and highly predictive biomarker for progressive tubular and glomerular injury (Sirisopha *et al*., 2016), furthermore, in a previous study on SLE patients, an increase in NGAL levels correlated with other renal disease activity (Brunner *et al*., 2006).

The elevated level of NGAL is associated with injury to epithelial cells in the gastrointestinal tract, respiratory tract or renal tubules (Nakov, 2019). The relatively small size, secreted pattern, and reliable stability have made it a valuable diagnostic and prognostic biomarker in multiple diseases including acute or chronic kidney diseases, sepsis, cardiovascular diseases, inflammatory bowel diseases and cancer (Rysz *et al.*, 2017)

Chapter Three
Materials and Methods

3.1 Materials:

3.1.1. Instruments and Equipment's:

The equipment's and instruments used in this study were listed in the tables (3.1) and (3.2).

Table (3. 1): Instruments that used in the current study.

NO	Tools	Company	Country
1	Cylinders (different size)	China	China
2	Disposable sterile syringe	China	China
3	Disposable sterile tips	China	China
4	EDTA K3 tube 2 ml Vacuum	AFCO	Jordan
5	Eppendorff tube	Eppendorff	Germany
6	ESR racks	AFCO	Jordan
7	ESR tube	AFCO	Jordan
8	Micropipettes (different sizes)	Fisher Scientific	U.S.A
9	Multichannel pipettes	China	China
10	Plane tube	China	China
11	Rack Tube	China	China
12	Urine cup	China	China
13	Vacuum Gel Active tube 6ml	AFCO	Jordan

Table (3. 2): Equipment's that used in the current study.

NO	Tools	Company	Country
1	Centrifuge	Kokusan H-19F	Japan
2	Deep freeze -70 C	Ateko	Denmark
3	ELISA Human	Human	Germany
4	Genius	PA54	China
5	Incubator	Memmert	Germany
6	Refrigerator	Concord	Lebanon
7	Vortex	Thermolyne	USA

3.1.2. Prepared Kits:

The commercial kits used in the present study are shown in table (3.3).

Table (3. 3): commercial kits used in the study.

NO	Types of kits	Company	Country
1	ANA screen	Generic assay	Germany
2	Anti-dsDNA-NcX ELISA(IgG)	Generic assay	Germany
3	Anti-Phospholipase A2 receptor, (Anti PLA2R)	EUROIMMUN	Germany
4	Complement C3	Genus	China
5	Complement C4	Genus	China
6	Creatinine	Human	Germany
7	Human Complement 1q(C1Q)ELISA Kit	Shanghai YL Biont	China
8	Human Epidermal growth factor(EGF)ELISA Kit	Shanghai YL Biont	China
9	Human neutrophil gelatinase-associated lipocalin (NGAL) ELISA Kit	Shanghai YL Biont	China
10	Human Vascular cell adhesion molecule 1(VCAM-1)ELISA Kit	Shanghai YL Biont	China
11	IMTEC-ANA-LIA XL	IMTEC	Germany
12	Urea	Randox	United Kingdom

3.2 Methods

3.2.1. Classification Criteria: The classification criteria for SLE were sited in 1971, revised in 1982, and revised again in 1997 (Hochberg, 1997). These criteria are based on the common signs and symptoms of SLE. Lupus erythematosus is diagnosed when any four or more criteria are present (Smith and Gordon, 2010) as show in table (3.4)

Table (3.4) SLICC classification system for SLE (Petri *et al*.,2012)

Clinical Feature	**Immunological Feature**
Acute cutaneous lupus (maculopapular lupus rash ,malar rash , photosensitive lupus rash)	High ANA concentration
Chronic cutaneous lupus (discoid rash , mucosal lupus)	High anti –dsDNA antibody concentration
Oral or nasal ulcer	Presence of anti –Sm
Nonscarring alopecia	Positive APA
Synovitis in 2 joints	Low complements (C3 , C4 , CH50)
Serositis	Direct Coombs test
Renal (urine protein or RBC casts)	
Neurologic (seizure , psychosis , others)	
Hemolytic anemia	
Leukopenia or lymphopenia	
Thrombocytopenia	

3.2.2. Assessment of Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)

Several worldwide organ-specific activity indices are commonly used in the evaluation of SLE patients (Urowitz and Gladman, 1998).

3.2.3 Patients samples:

The current study has been performed on 85 female's patients suffering from SLE (30 SLE patient 20 moderate lupus nephritis patient and 35 sever lupus nephritis patient) including. Their ages range was 15-50 years old in the Imam AL Hasan Al Mujtaba Hospital in Karbala city during the period from November (2022) to March (2023). the study population was divided in to three groups first group include 30 patients with SLE while the second and third groups include 20 and 35 patients with moderate lupus nephritis and sever lupus nephritis respectively.

3.2.4 Exclusion Criteria

Patients with Tumors, Patients under 10 years old, Persons taking nutritional supplements, other inflammatory disease such as RA, Osteoporosis, Myositis vasculitis, history of hypersensitive, Treatment Cyclophosamide, chronic condition such as asthma or Cohn's disease, thyroid disease and pregnant

3.2.5 Ethical approval

This research was approved by the Institutional Higher Scientific and Ethical Committee, and before participation all women were given an idea about the study and their written informed consent was taken. A special questioner was designed for all patients.

3.2.6 Samples Collection:

Six milliliters of blood sample have been drawn from each patient by vein puncture using disposable syringes under aseptic technique. (Barenfanger *et al.*, 2004). Blood of each sample has been divided to two parts. tow milliliter is

transferred into EDTA tubes for measuring ESR. While remaining four milliliters have been transferred into vacuum gel tubes and left at room temperature for at least 30 minutes for clotting then centrifuge at 4000 rpm for 10 minutes. Then separated serum has been divided into Eppendorf tubes (for estimating the level of Urea, Creatinine, ANA, ds-DNA, ENA, C1q, C3, C4, VCAM, NGAL, and PLA2R) and stored at -20° C until used. Also the urine samples from each patient were collected in sterile tube (2 ml) and centrifuge at 2000-3000 rpm for approximately 20 minute then collect the supernatants carefully and saved in Eppendorff tubes and stored at -20° C until used for estimation the level of Urinary EGF.

3.2.7 Study design

The study design of current study was illustrated in figure (3.1)

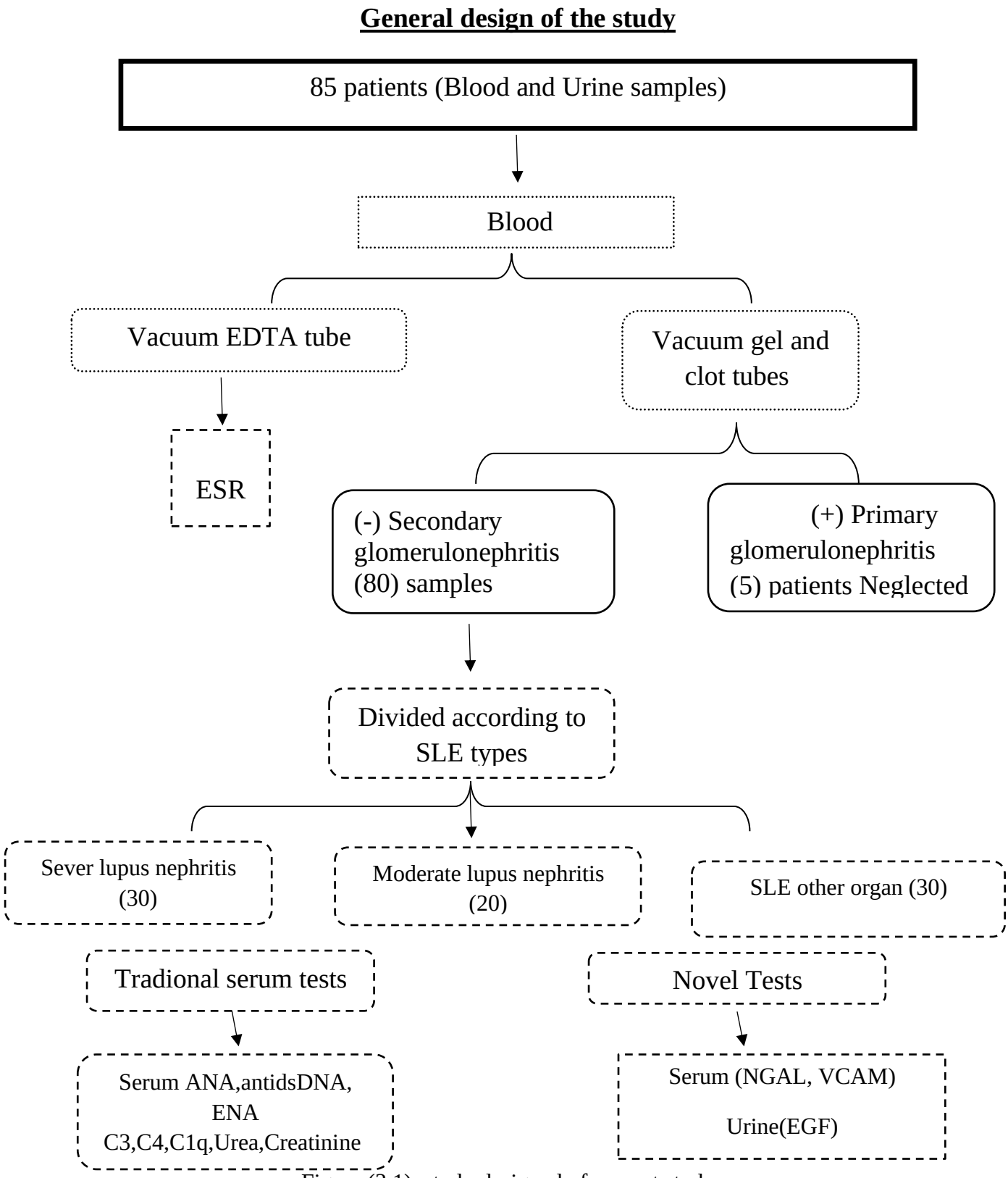


Figure (3.1): study designed of current study

3.3 Tradional Tests:

3.3.1 Evaluation the level of phospholipase A2 receptor (PLA2R):

Principle test

The ELISA test kit provides a semiquantitative or quantitative in vitro assay for human autoantibodies of the IgG class against PLA2Rin serum. The test kit contains microtiter strips each with 8 break-off reagent wells coated with PLA2R.In the first reaction step, diluted patient samples are incubated in the wells. The positive samples specific for IgG antibodies (also IgA and IgM) will bind to the antigens. the second incubation is carried out using an enzyme – labelled anti-human IgG (enzyme conjugate) catalyzing a colour reaction.

Assay procedure

Sample incubation: (1 step)

One hundred ml of the positive control have been transferred, negative control or diluted patient samples in to the individual microplate wells according to the pipetting protocol incubate for 30 minute at room temperature (+18 c to +25 c).

Washing: Manual: the wells have been emptied and subsequently wash 3 times using 300 ml of working strength wash buffer for each wash.

Automatic: The reagent wells have been washed 3 times with 450 ml of working strength wash buffer (program setting: e.g. TECAN Columbus washer overflow modus). Leave the wash buffer in each well for 30 to 60 second per washing cycle, and then empty the wells after washing (manual and automated testes) thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffers.

Conjugate incubation: (2 step)

Pipette 100 ml of enzyme conjugate (peroxidase-labelled anti-human IgG) into each of the microplate wells incubate for 30 minute at room temperature (+18 c to +25 c).

Washing: the wells have been emptied. Wash as described above.

Substrate incubation: (3 step)

Pipette 100 ml of chromogen/substrate solution in to each of the microplate wells incubation for 15 minute at room temperature (+18 c to +25 c) protect from direct sunlight.

Stopping the reaction: Pipette 100 ml of stop solution in to each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced.

Measurement:

Photometric measurement of the colour intensity should be made at a wave length of 450 nm and a reference wave length between 620 nm and 650 nm within 30 minute of adding the stop solution. Prior to measuring slightly shake the micro-plate to ensure a homogeneous distribution of the solution.

3.3.2 Biochemical Tests

A. Urea test Intended use: For the quantitative in vitro determination of urea in serum. This product is suitable for manual use.

Colorimetric method

. Principle

The method is based on the following reaction:

Urea + H2O ⟶ 2NH3 +CO2 Salicylate and hypochlorite in the reagent react with the ammonium ions to form a green complex.

Table (3.5) showed the content of reagent for urea test.

Contents		Initial concentration
RI a.	urease	≥ 5000 U/l
RI b.	phosphate buffer	120 mmol/l , pH 7.0
	Sodium salicylate	63.4 mmol/l
	Sodium nitroprusside	5.00mmol/l
	EDTA	1.5 mmol/l
R2.	Sodium hypochlorite	18 mmol/l
	Sodium hydroxide	750 mmol/l
CAL	Standard	See lot specific insert

. Stability and preparation of reagents

Urease Rla, phosphate buffer Rlb, sodium hypochlorite R2 and standard (CAL) are supplied ready to use. Stable up to the expiry date state when stored at +2-+8˚C.

. Working reagent

1 vial of urease Rla have been added to 1 bottle of phosphate buffer Rlb

Stable for 1month at +2 to +8 ˚C stored protected from light.

. Assay Procedure

Wavelength	600nm (Hg 578 nm – Hg 623 nm)
Cuvette	1 cm light path
Temperature	+ 25 , + 37 ˚C
Measurement	Against reagent blank

Pipette into test tubes:

	Reagent blank	Standard	Sample
Standard	--------	10µl	-------
Sample	--------	------	10µl
Working reagent (Rl)	1 ml	1 ml	1 ml

Shake. Incubate for at least 3min at 37˚C or 5 min at 20 to 25 ˚C.

. Calculation

Urea concentration= Asample /A standard * standard conc. (mmol/l) (mmol/l)

Urea concentration= A sample/A standard* standard conc.(mg/dl) (mg/dl)

Normal values

Serum or plasma 2.5 – 7.5 mmol/l (0.15 – 0.45 g/l)

Urine 338 – 538 mmol/24h (20 -35 g/24h)

B. Creatinine test

Creatinine forms in alkaline solution an orange-red coloured complex with picric acid. The absorbance of this complex is proportional to the creatinine concentration in the sample.

. Principle

Creatinine + picric acid ⟶ creatinine – picric complex

. Assay procedure

Wavelength	492 nm (Hg 490 nm – Hg 510 nm)
Cuvette	1 cm light path
Temperature	25 / 37 ˚C
Measurement	Against air

Pipette into test tubes:

	Reagent blank	Standard	Sample
Standard	--------	10µl	-------
Sample	--------	------	10µl
Working reagent (Rl)	1 ml	1 ml	1 ml

Mix and start the stopwatch. After 30 sec. read the absorbance A1. Read the absorbance A2 exactly after 2 min.

3.3.3 Antinuclear Antibody (ANA screen):

. Principle of the test

ANA screen is used for the semi-quantitative determination of autoantibodies to nuclear and cytoplasmic antigens in human serum and plasma. Antibodies of the controls and diluted patient samples react with nuclear and cytoplasmic antigens immobilized on the solid phase of microtiter plates. Using

complete HeLa nuclei enriched with recombinant and native antigens guarantees the specific binding of autoimmune antibodies of specimen under investigation. Following an incubation period of 60 min at room temperature (RT, 18-25 ºC), unbound sample components is removed by a wash step. The bound IgG antibodies react specifically with anti-Human-IgG conjugated to horseradish peroxidase (HRP). Within the incubation period of 30 min at RT, excessive conjugate is separated from the solid-phase immune complexes by the following wash step. HRP converts the colorless substrate solution of 3, 3', 5, 5'tetramethylbenzidine (TMB) added into a blue product. The enzyme reaction is stopped by dispensing an acidic solution into the wells after 15 min at room temperature turning the solution from blue to yellow. The optical density (OD) of the solution at 450 nm is directly proportional to the amount of specific antibodies bound. Patient ratios are calculated by dividing the respective OD of the specimen with the calculated cut-off OD.

. Assay procedure:

- patient's sera have been dilute with sample diluent (c) 1+100 (v/v),

e.g. 10µl sample +1 ml sample diluent (c).

the procedure steps as follow:

1- Bring all reagents to room temperature (18-25 ºC) before use.mix gently without causing foam.

2- Dispense:

One hundred µl controls P, CO, N

One hundred µl diluted patient samples into the respective wells.

3- Plate have been covered, incubate 60 min at room temperature (18…25 ºC).

4- Decant, then each well had wash three times using 300µl wash solution (made of B).

5- One hundred µl of conjugate (D) solution have been Added to each well.

6- Cover plate, incubate 30 min at room temperature (18…25 ºC).

7- Decant, then wash each well three times using 300µl wash solution (made of B).

8- One hundred µl of substrate have been Added (E) to each well.

9- Incubate 15 min protected from light at room temperature (18…25 ºC).

10- One hundred µl of stop solution (F) have been added to each well and mix gently.

11- Read the OD at 450 nm versus 620 or 690 nm within 30 min after adding the stop solution.

Anti-nuclear antibody was performed by indirect ELISA technique, for semi quantification of the results; each patient of optical density (OD) value can be expressed by the index-value which is calculated by dividing the patient-OD by the cut-off OD (1.0).

3.3.4 Anti-dsDNA-NcX ELISA (IgG) test instruction

Principles of the test

Anti-dsDNA is an enzyme immunoassay for the quantitative determination of lgG antibodies to dsDNA .The antibodies of the calibrators, control and diluted patient samples react with dsDNA immobilized on the solid phase of microtiter plates. Highly purified ds-DNA coated on the microtiter plate guarantees the specific binding of dsDNA lgG antibodies of the specimen under investigation. Following an incubation period of 60 min at room temperature (RT), unbound sample components are removed by a wash step.

The bound IgG antibodies react specifically with anti-human-lgG conjugated to horseradish peroxidase (HRP). Within the incubation period of 30 min at RT, excessive conjugate is separated from the solid-phase immune complexes by the following wash step.

Horseradish Peroxidase converts the colorless substrate solution of 3, 3', 5, 5'-tetramethlylbenzidine (TMB) added into a blue product. The enzyme reaction is stopped by dispensing an acidic solution into the wells after 15 min at room temperature turning the solution from blue to yellow. The optical density (OD) of the solution at 450 nm is directly proportional to the amount of specific antibodies bound. The standard curve is established by plotting the antibodies concentrations of the calibrators (x-axis) and their corresponding OD values (y-axis) measured. The antibody concentration of the specimen is directly read off the standard curve. Alternatively, result can be calculated by a semi-quantitative method too using calibrator 2 as cut-off calibrator.

Assay procedure

Dilute patient sera with sample diluent (c)1+ 100(v/v), e.g. 10 µl serum + 1.0 ml sample diluent (c).

The procedure steps as follow:

1. All reagents have been Bring to room temperature (18-25 ˚C) before use. Mix gently without causing foam.

2. Dispense: 100 µl calibrators 0 (optionally) 1-4 or 100 µl calibrator 2 (semiquantitative), 100 µl control P, N (N optionally), 100 µl diluted patient samples into the respective wells.

3. Cover plate; incubate 60 min at room temperature (18-25 ˚C).

4. Decant, then wash each well three times using 300 µl wash solution (made of B).

5. Add One hundred µl of conjugate (D) solution to each well.

6. Cover plate; incubate 30 min at room temperature (18-25 ˚C).

7. Decant, then wash each well three times using 300 µl wash solution (made of B).

8. Add One hundred µl of substrate (E) to each well.

9. Incubate 15 min protected from light at room temperature (18-25 ˚C).

10. Add One hundred µl of stop solution (F) to each well and mix gently.

11. Read the OD at 450 nm versus 690 nm within 30 min after adding the stop solution.

The construction of the standard curve for the measurement of antidsDNA in serum of SLE patients, for quantitative was carried by plotting the optical density of each calibrator with respect to the corresponding concentration values in IU/ml, figure (3.1).

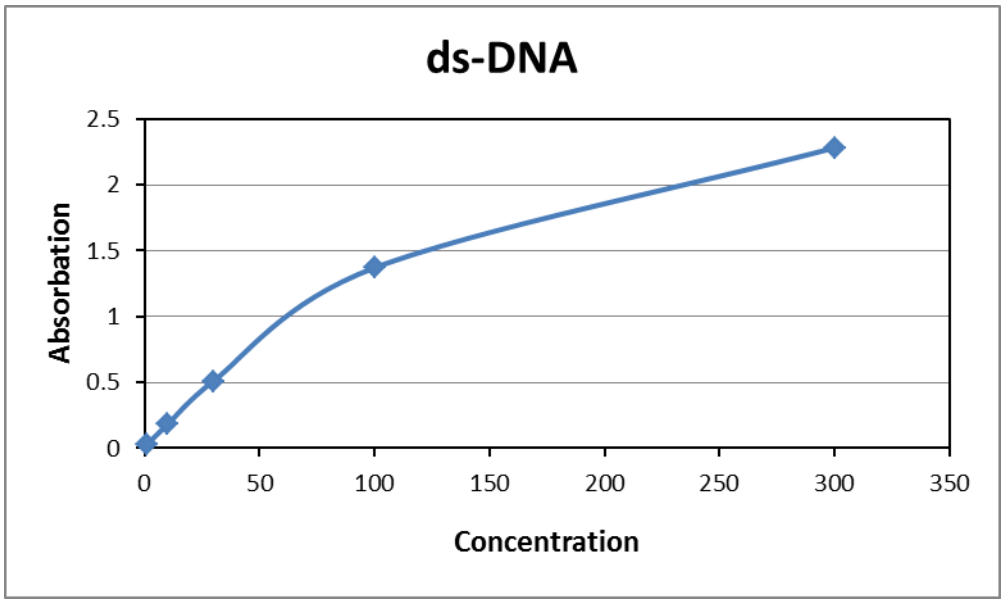


Figure 3.2: The stander curve of the anti-dsDNA.

3.3.5 Evaluation of Complements:

A. C3 and C4 Complements

Intended use for in vitro quantitative determination of complement componentS 3 and 4 content in human serum of patients of SLE and control groups by radial Genius technique.

. The principle of Complement assay

Complement components C3 and C4 in the sample encounters its corresponding antibody in liquid phase, they immediately form an antigen-antibody complex with certain turbidity. With a certain amount of antibody, the turbidity is proportional to the antigen content in the sample. By detecting the reaction change at specific wavelengths and referring to the multi-point calibration curve, the complement (C3, C4) content in the sample can be calculated. The reagents are pre-calibrated, each specific calibration curve has been recorded into the magcard and each detection kit is allocated with one magcard.

Assay Procedure

1- After startup, the instrument displays the main measurement interface, select the test item and sample type at the time column.

2- Click 'LOT ' at the batch No. column to enter the card-swiping interface. put the corresponding magcard onto the magnetic induction area, when a 'di ' sound heard , the magcard was successfully swiped, and the interface returns to the main measurement interface. For the same batch of reagents, it is no need to swipe the card again.

3- The instrument interface prompts ' Input Cupl '.

4- Take out cuvette, put one stirrer into it, and then use the pipettor to accurately add in 400 µl buffer solution, and then add in 3µl sample to C3 test and 4µl sample to C4 test.

5-Put the cuvette into the test channel, the instrument automatically stirs for one time.

6- The instrument prompts ' please add antiserum ' then use the pipettor to accurately add (60 for C3) and (20 for C4) µl antiserum

7- Immediately press the corresponding channel's start button, the instrument will stir automatically. When the test finished, the instrument will automatically display and print the test result.

8- After the test, take out the cuvette; the instrument prompts ' input cup ', do the next test.

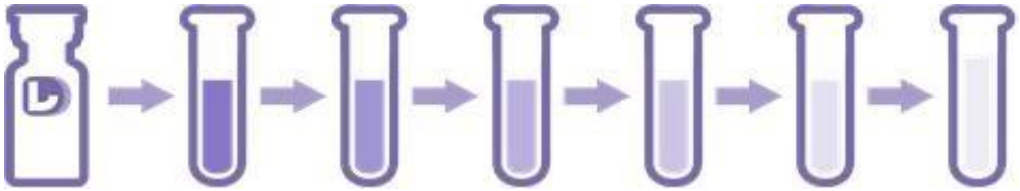
B. Human Complement component C1q The kit is a sandwich enzyme immunoassay for in vitro quantitative measurement of C1q in human serum.

. Principle of the test this kit uses enzyme-linked immune sorbent assay (ELISA) based on the Biotin double antibody sandwich technology to assay the Human Complement 1q (C1Q). Add Complement 1q(C1Q) to the wells, which are pre-coated with Complement 1q(C1Q) monoclonal antibody and then incubate. After that, add anti C1Q antibodies labeled with biotin to unite with streptavidin-HRP, which forms immune complex. Remove unbound enzymes after incubation and washing. Add substrate A and B. Then the solution will turn blue and change into yellow with the effect of acid. The shades of solution and the concentration of Human Complement 1q (C1Q) are positively correlated

. Assay procedure:

1. Dilution of standard solutions: (This kit has a standard of original concentration, which could be diluted in small tubes by user independently following the instruction.):

16μg/ml	Standard No.5	120μl Original Standard + 120μl Standard diluents
8μg/ml	Standard No.4	120μl Standard No.5 + 120μl Standard diluents
4μg/ml	Standard No.3	120μl Standard No.4 + 120μl Standard diluent
2μg/ml	Standard No.2	120μl Standard No.3 + 120μl Standard diluent
1μg/ml	Standard No.1	120μl Standard No.2 + 120μl Standard diluent

Stock standard						
Tube	Standard	S5	S4	S3	S2	S1
μg/ml	32	16	8	4	2	1

2. The number of stripes needed is determined by that of samples to be tested added by that of standards. It is suggested that each standard solution and each blank well should be arranged with three or more wells as much as possible.

3. Sample injection: 1) Blank well: Add only Chromogen solution A and B, and stop solution. 2) Standard solution well: Add 50μl standard and streptavidin-HRP 50μl. 3) Sample well to be tested: Add 40μl sample and then 10μl C1Q antibodies, 50μl streptavidin-HRP. Then cover it with seal plate membrane. Shake gently to mix them up. Incubate at 37℃ for 60 minutes.

4. Preparation of washing solution: Dilute the washing concentration (30X) with distilled water for later use.

5. Washing: Remove the seal plate membrane carefully, drain the liquid and shake off the remaining liquid. Fill each well with washing solution. Drain the liquid after 30 seconds' standing. Then repeat this procedure five times and blot the plate.

6. Color development: Add 50μl chromogen solution A firstly to each well and then add 50μl chromogen solution B to each well as well. Shake gently to mix them up. Incubate for 10 minutes at 37℃ away from light for color development.

7. Stop: Add 50μl Stop Solution to each well to stop the reaction (the blue color changes into yellow immediately at that moment).

8. Assay: Take blank well as zero, measure the absorbance (OD) of each well one by one under 450nm wavelength, which should be carried out within the 10 minutes after having added the stop solution.

9. According to standards' concentrations and the corresponding OD values, calculate the linear regression equation of the standard curve. Then according to the OD value of samples, calculate the concentration of the corresponding sample. Special software could be employed to calculate as well.

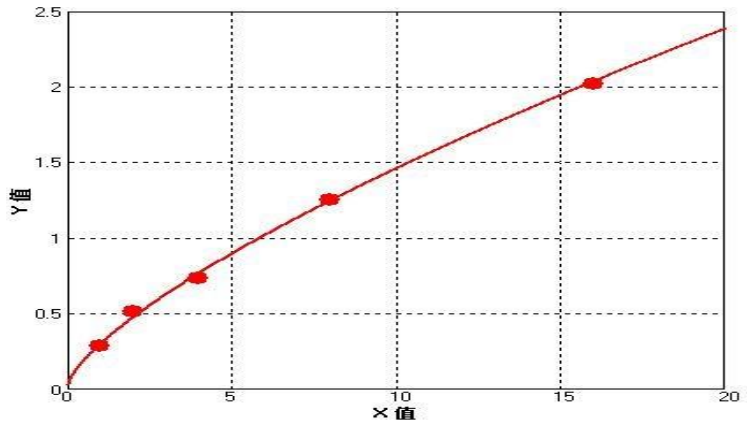


Figure (3.3) : The stander curve for estimation the level of the Complement component c1q

3.3.6 Evaluation the level of ENA

. Principle test

The ENA Screen assay is an indicator chemiluminescence immunoassay.

The sample (or calibrator/control, if application) buffer and magnatic microbeads coated with ENA(nRNP/Sm, Sm ,SS-A ,SS-B ,Scl-70 ,jo-1)are mixed thoroughly and incubated to form immune complexes after incubation material bound to the magnetic microbeads are held in a magnetic field while unbound material are washed away during awash cycle followed by adding ABEI labeled with mouse monoclonal anti –human IgG antibody incubate to form sandwich complexes after precipitation in amagnetic field the supernatant is decanted and then another wash cycle is performed subsequently the starter 1+2 are added to initiate a chemilurninescent reaction .The light signal is measured by a photomultiplier as relative light units (RLUs) which is proportional to the concentration of anti –ENA antibodies present in the sample (or calibrator/control ,if applicator).

. Assay procedure

Preparation of magnetic microbeads

- -the magnetic microbeads are provided in a lyophilized form. The vial containing the lyophilized magnetic microbeads must be opened carefully and reconstituted with the magnetic microbeads buffer.
- Remove 2 ml magnetic microbeads buffer from the magnetic microbeads tube (blue collar and serrated reagents tube on the bottom) in to the vial containing lyophilized magnetic microbeads before use, cover with a rubber stopper and gently shake. allow the dissolved magnetic microbeads to stand for 10-15 minute.
- Swirl gently to ensure homogeneity. avoid heavy shaking when dissolving (avoid formation of foam).
- Transfer all reconstituted magnetic microbeads in vial to the magnetic microbeads tube and mix it with the remaining magnetic microbeads buffer evenly, then place prepared kit on to the corresponding analyzer.
- After use, the kit including the reconstituted magnetic microbeads should be stored at 2-8 ℃ in an upright position.

3.3.7 Hematology Test

Specimens of venous blood about 2 ml in EDTA tube has been used to determine the Erythrocytes Sedimentation Rate (ESR)

Erythrocytes sedimentation rate was measured using westergren method (Lewis and Bain 2001). Using disposable ESR tube containing 0.5 ml of sodium citrate add 0.2Ml blood and mix the sample thoroughly and then draw it up into Westergren-tube to the 200 mm mark by means of mechanical device. The tube is placed in a ESR rack in a strictly vertical position for 1 hour at room temperature, at which time the distance from the lowest point of the surface meniscus to the upper limit of the red cell sediment is measured the distance of fall of erythrocytes, expressed as millimeters in 1 hour.

3.3.8 Evaluation the level of Human Neutrophil Gelatinase-Associated Lipocaline(NGAL)

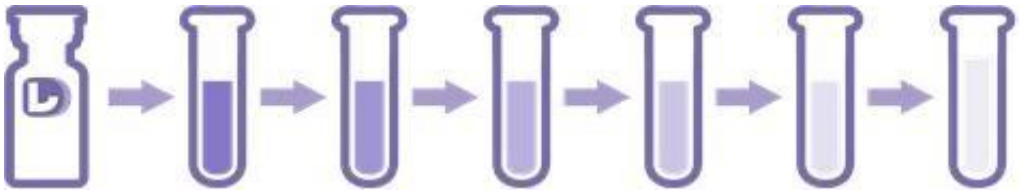
. Principle of the assay

This kit uses enzyme-linked immune sorbent assay (ELISA) based on the Biotin double antibody sandwich technology to assay the Human neutrophil gelatinase-associated lipocalin (NGAL). Add neutrophil gelatinase-associated lipocalin(NGAL)to the wells, which are pre-coated with neutrophil gelatinase-associated lipocalin(NGAL)monoclonal antibody and then incubate. After that, add anti NGAL antibodies labeled with biotin to unite with streptavidin-HRP, which forms immune complex. Remove unbound enzymes after incubation and washing. Add substrate A and B. Then the solution will turn blue and change into yellow with the effect of acid. The shades of solution and the concentration of Human neutrophil gelatinase-associated lipocalin (NGAL) are positively correlated.

. Assay Procedure

1. Dilution of standard solutions: This kit has a standard of original concentration, which could be diluted in small tubes by user independently following the instruction.:

1600ng/ml	Standard No.5	120µl Original Standard + 120µl Standard diluents
800ng/ml	Standard No.4	120µl Standard No.5 + 120µl Standard diluents
400ng/ml	Standard No.3	120µl Standard No.4 + 120µl Standard diluent
200ng/ml	Standard No.2	120µl Standard No.3 + 120µl Standard diluent
100ng/ml	Standard No.1	120µl Standard No.2 + 120µl Standard diluent

Stock standard						
Tube	Standard	S5	S4	S3	S2	S1
ng/ml	3200	1600	800	400	200	100

2. The number of stripes needed is determined by that of samples to be tested added by that of standards. It is suggested that each standard solution and each blank well should be arranged with three or more wells as much as possible.

3. Sample injection: 1) Blank well: only Chromogen solution A and B have been Added , and stop solution. 2) Standard solution well: Add 50µl standard and streptavidin-HRP 50µl. 3) Sample well to be tested: Add 40µl sample and then 10µl NGAL antibodies, 50µl streptavidin-HRP. Then cover it with seal plate membrane. Shake gently to mix them up. Incubate at 37℃ for 60 minutes.

4. Preparation of washing solution: Dilute the washing concentration (30X) with distilled water for later use.

5. Washing: Remove the seal plate membrane carefully, drain the liquid and shake off the remaining liquid. Fill each well with washing solution. Drain the liquid after 30 seconds' standing. Then repeat this procedure five times and blot the plate.

6. Color development: Add 50μl chromogen solution A firstly to each well and then add 50μl chromogen solution B to each well as well. Shake gently to mix them up. Incubate for 10 minutes at 37℃ away from light for color development.

7. Stop: Add 50μl Stop Solution to each well to stop the reaction (the blue color changes into yellow immediately at that moment).

8. Assay: Take blank well as zero, measure the absorbance (OD) of each well one by one under 450nm wavelength, which should be carried out within the 10 minutes after having added the stop solution.

9. According to standards' concentrations and the corresponding OD values, calculate the linear regression equation of the standard curve. Then according to the OD value of samples, calculate the concentration of the corresponding sample. Special software could be employed to calculate as well.

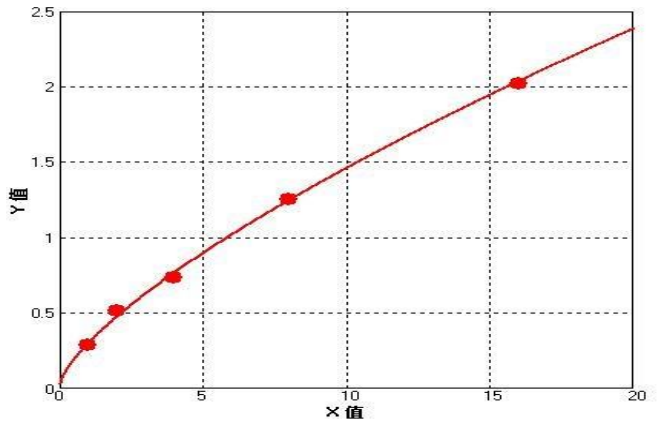


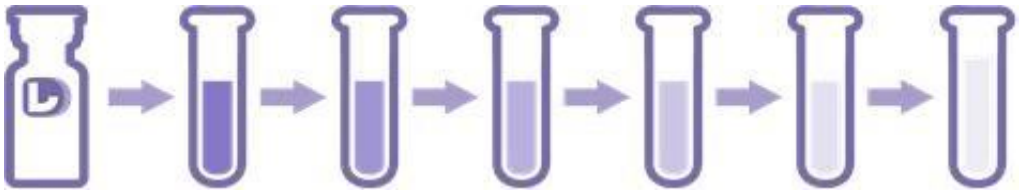
Figure 3.4 : The stander curve of the Neutrophil Gelatinase-Associated Lipocalin(NGAL)

3.3.9 Evaluation the level of Human Vascular cell adhesion molecule-1 (VCAM-1).

. Principle test This kit uses enzyme-linked immune sorbent assay (ELISA) based on the Biotin double antibody sandwich technology to assay the Human Vascular cell adhesion molecule 1 (VCAM-1). Add Vascular cell adhesion molecule 1(VCAM-1) to the wells, which are pre-coated with Vascular cell adhesion molecule 1(VCAM-1) monoclonal antibody and then incubate. After that, add anti VCAM-1 antibodies labeled with biotin to unite with streptavidin-HRP, which forms immune complex. Remove unbound enzymes after incubation and washing. Add substrate A and B. Then the solution will turn blue and change into yellow with the effect of acid. The shades of solution and the concentration of Human Vascular cell adhesion molecule 1 (VCAM-1) are positively correlated.

. Assay procedure 1. Dilution of standard solutions: This kit has a standard of original concentration, which could be diluted in small tubes by user independently following the instruction.:

32ng/ml	Standard No.5	120µl Original Standard + 120µl Standard diluents
16ng/ml	Standard No.4	120µl Standard No.5 + 120µl Standard diluents
8ng/ml	Standard No.3	120µl Standard No.4 + 120µl Standard diluent
4ng/ml	Standard No.2	120µl Standard No.3 + 120µl Standard diluent
2ng/ml	Standard No.1	120µl Standard No.2 + 120µl Standard diluent

Stock standard						
Tube	Standard	S5	S4	S3	S2	S1
ng/ml	64	32	16	8	4	2

2. The number of stripes needed is determined by that of samples to be tested added by that of standards. It is suggested that each standard solution and each blank well should be arranged with three or more wells as much as possible.

3. Sample injection: 1) Blank well: Add only Chromogen solution A and B, and stop solution. 2) Standard solution well: Add 50µl standard and streptavidin-HRP 50µl. 3) Sample well to be tested: Add 40µl sample and then 10µl VCAM-1 antibodies, 50µl streptavidin-HRP. Then cover it with seal plate membrane. Shake gently to mix them up. Incubate at 37℃ for 60 minutes.

4. Preparation of washing solution: Dilute the washing concentration (30X) with distilled water for later use.

5. Washing: Remove the seal plate membrane carefully, drain the liquid and shake off the remaining liquid. Fill each well with washing solution. Drain the

liquid after 30 seconds' standing. Then repeat this procedure five times and blot the plate.

6. Color development: Add 50µl chromogen solution A firstly to each well and then add 50µl chromogen solution B to each well as well. Shake gently to mix them up. Incubate for 10 minutes at 37℃ away from light for color development.

7. Stop: Add 50µl Stop Solution to each well to stop the reaction (the blue color changes into yellow immediately at that moment).

8. Assay: Take blank well as zero, measure the absorbance (OD) of each well one by one under 450nm wavelength, which should be carried out within the 10 minutes after having added the stop solution.

9. According to standards' concentrations and the corresponding OD values, calculate the linear regression equation of the standard curve. Then according to the OD value of samples, calculate the concentration of the corresponding sample. Special software could be employed to calculate as well.

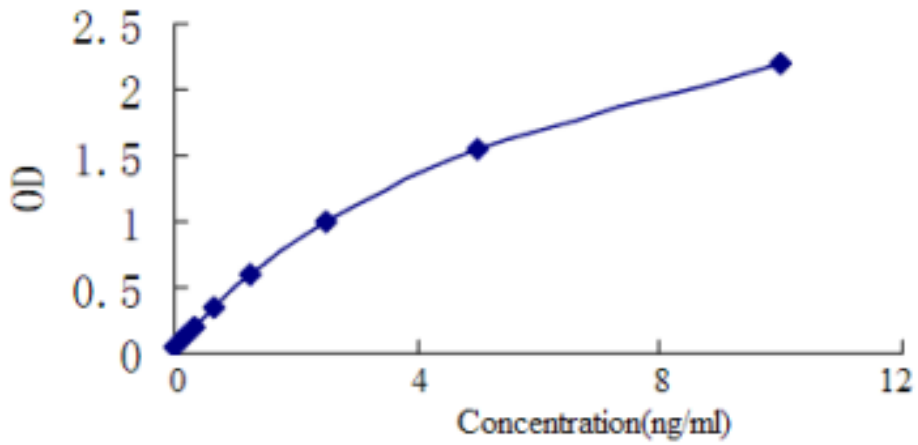


Figure 3.5: The stander curve of the Vascular cell adhesion molecular

3.3.10 Evaluation the level of Human Epidermal growth factor (EGF)

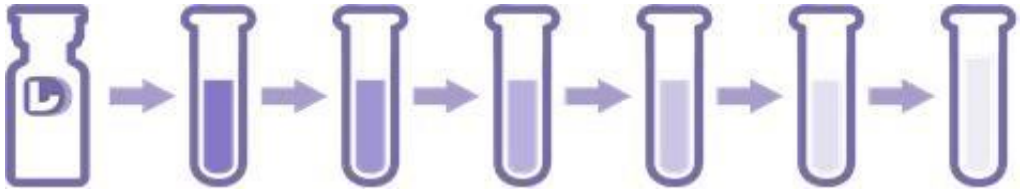
. Principles of the test:

This kit uses enzyme-linked immune sorbent assay (ELISA) based on the Biotin double antibody sandwich technology to assay the Human Epidermal growth factor (EGF). Add epidermal growth factor (EGF) to the wells, which are pre-coated with epidermal growth factor (EGF) monoclonal antibody and then incubate. After that, add anti EGF antibodies labeled with biotin to unite with streptavidin-HRP, which forms immune complex. Remove unbound enzymes after incubation and washing. Add substrate A and B. Then the solution will turn blue and change into yellow with the effect of acid. The shades of solution and the concentration of Human Epidermal growth factor (EGF) are positively correlated.

. Assay Procedure

1. Dilution of standard solutions: This kit has a standard of original concentration, which could be diluted in small tubes by user independently following the instruction:

1200ng/L	Standard No.5	120µl Original Standard + 120µl Standard diluents
600ng/L	Standard No.4	120µl Standard No.5 + 120µl Standard diluents
300ng/L	Standard No.3	120µl Standard No.4 + 120µl Standard diluent
150ng/L	Standard No.2	120µl Standard No.3 + 120µl Standard diluent
75ng/L	Standard No.1	120µl Standard No.2 + 120µl Standard diluent

Stock standard						
Tube	Standard	S5	S4	S3	S2	S1
ng/L	2400	1200	600	300	150	75

2. The number of stripes needed is determined by that of samples to be tested added by that of standards. It is suggested that each standard solution and each blank well should be arranged with three or more wells as much as possible.

3. Sample injection: 1) Blank well: Add only Chromogen solution A and B, and stop solution. 2) Standard solution well: Add 50μl standard and streptavidin-HRP 50μl. 3) Sample well to be tested: Add 40μl sample and then 10μl EGF antibodies, 50μl streptavidin-HRP. Then cover it with seal plate membrane. Shake gently to mix them up. Incubate at 37℃ for 60 minutes.

4. Preparation of washing solution: Dilute the washing concentration (30X) with distilled water for later use.

5. Washing: Remove the seal plate membrane carefully, drain the liquid and shake off the remaining liquid. Fill each well with washing solution. Drain the liquid after 30 seconds' standing. Then repeat this procedure five times and blot the plate.

6. Color development: Add 50μl chromogen solution A firstly to each well and then add 50μl chromogen solution B to each well as well. Shake gently to mix them up. Incubate for 10 minutes at 37℃ away from light for color development.

7. Stop: Add 50μl Stop Solution to each well to stop the reaction (the blue color changes into yellow immediately at that moment).

8. Assay: Take blank well as zero, measure the absorbance (OD) of each well one by one under 450nm wavelength, which should be carried out within the 10 minutes after having added the stop solution.

9. According to standards' concentrations and the corresponding OD values, calculate the linear regression equation of the standard curve. Then according to the OD value of samples, calculate the concentration of the corresponding saple. Special software could be employed to calculate as well.

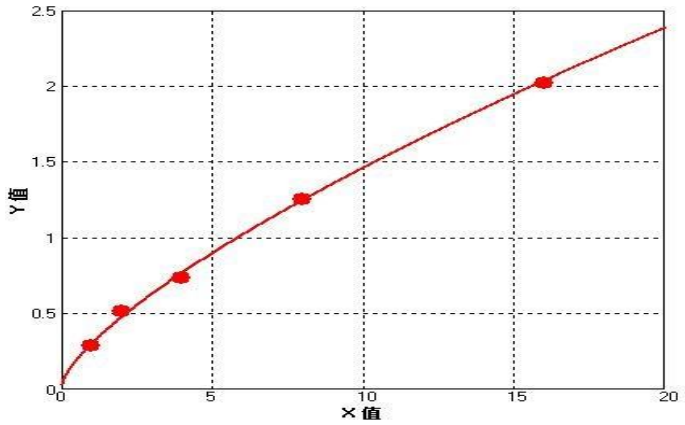


Figure 3.6: The stander curve of the Epidermal growth factor (EGF)

3.4 Statistical Analysis

By using Statistical Package for Social Science (SPSS 16 IBM, Armonk, USA), one-sample Kolmogorov-Smirnov test used to know how the values were distributed. If the values were distributed normally, then t- test is used and if the values are not distributed normally, so Mann-Whitney test should be applied. In this study the t-test was used for studying data. The results were expressed as mean ± standard deviation (SD), the differences in means of the variables between three patient groups (sever lupus nephritis , moderate lupus

nephritis , SLE groups) were analyzed by analysis of variance (one way ANOVA) test. Correlations between all of the studied variables were evaluated using Pearson's correlation coefficient (r) and linear regression analyses were used for the evaluation of data. P-value of < 0.05 was considered to be statistically significant.

Chapter Four

Results and Discussion

4.1. Sample distribution according to the severity of disease:

The current study included 80 Female patients with three main groups sever lupus nephritis, moderate lupus nephritis and other organ SLE [30 (**37.5%**), 20 (**25%**), 30 (**37.5%**)] respectively as show in table (4.1).

Table (4.1) SLE patients with Lupus Nephritis and other organs depend on traditional test in Female patients

	Sever LN (%)	**Moderate LN (%)**	**Other organ SLE (%)**	**Total Number**
Female patients	30 (37.5%)	20 (25%)	30 (37.5%)	**80**

All patients in the current study were female because found that female had an increased incidence of autoimmune inflammatory disease (Isgor *et al.*, 2003), these classification of groups according severity depend on Criteria of SLEDAI which mainly depend on the primary symptom presentation that varied widely from patient to patient and the essential diagnoses test to recognize active and inactive of SLE disease (Narayanan *et al.*, 2010).

The results of current study was agreed with most studies in the world showed wide range of female (Pradgan *et al.*, 2013).

The increased in female SLE patients suggested that sex factors modulate disease proclivity and development (Whitacre *et al.*, 1999), because this disease related with steroid hormones, especially estrogen and progesterone, influence the immune response. Low progesterone levels in women with lupus, suggesting that a relative imbalance in favor of estrogen may contribute to immune reactivity in some female patients (Shabanova, 2008).

4.2. Clinical manifestation

According to the American College of Rheumatology (ACR) clinical criteria of SLE patients, the arthritis was the most frequent feature, in the current study was observed in 95% of patients, hematological disorder 90%, oral ulcer 80%, immunological disorder 75%, photosensitivity 60%, molar rash 50%, kidney disorder 21.6%, Serositis 15% and Neurologic disorder 10%. As show in Figure (4.1).

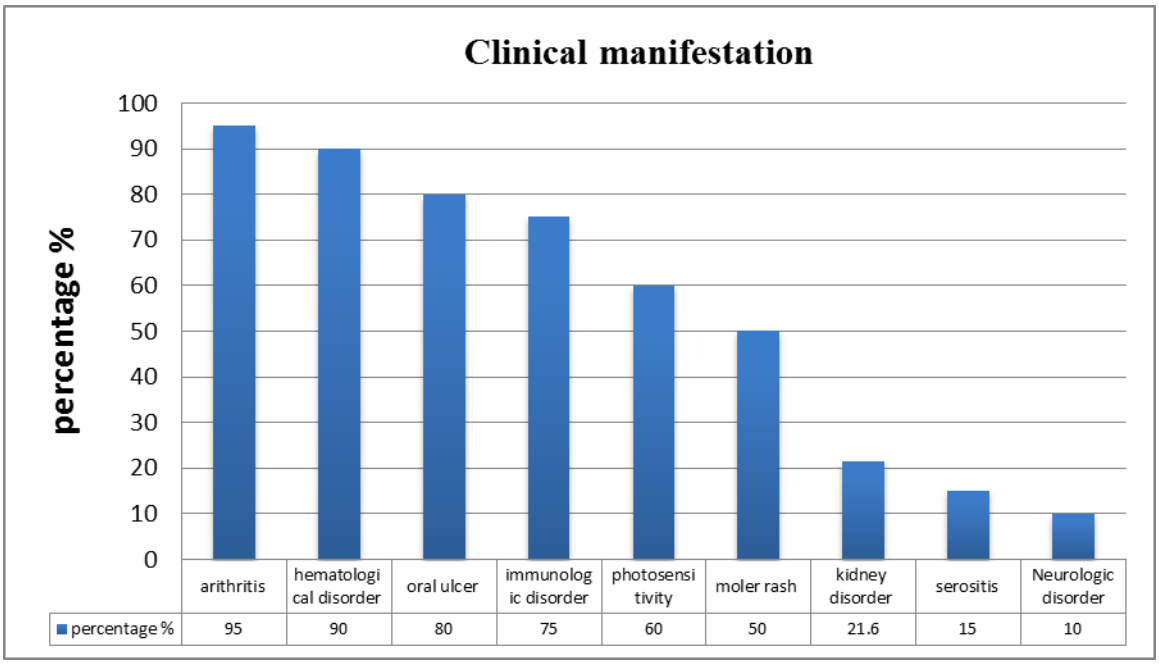


Figure 4. 1: The clinical manifestation of SLE patients during their disease duration.

The result of current study has shown that prevalence of some manifestation such as arthritis, malar rash and oral ulcer were high than other criteria. Arthritis is the most common American College of Rheumatology defined clinical criterion for SLE diagnosis, this finding was similar to other studies (AlSaleh *et al.*, 2008), also in Eastern Asia studies (Salido & Manapat-Reyes, 2010). In addition, of this criteria several symptoms were common in Iraq population, including fever, joint pain, fatigue and weight loss. And this finding was matches with some previous studies (Pradgan *et al.*, 2013).

4.3. Phospholipase A2 receptor (PLA2R)

The positive results of PLA2R was observed in **5** (5.9%) of patients which is present as the primary glomerulonephritis while negative PLA2R was observed in **80** (94.1%) patients that present as secondary glomerulonephritis (Systemic lupus Erythromatous), as shown in table (4.2).

Table (4.2) SLE patients with Lupus Nephritis and other organs depend on Phospholipase A2 receptor test

	Sever LN	**Moderate LN**	**Other organ SLE**	**Total Number**
Positive	5	0	0	5
Negative	30	20	30	80
Statistical analysis	X^2= 7.5; Df=2; P=0.022			

The current study was agreement with study that show detection of PLA_2R antibody in serum has an almost 100% specificity for the diagnosis of PLA_2R1-associated with membranous nephropathy (Svobodova*; et al* 2013), also the result of current study conformity to other that show serum PLA2R autoantibody was found only in Idiopathic memberance nephropathy but not in other renal diseases (Beck, *et al* 2009), while the results was disagree with the results of other studies that has been reported about the value of either serum PLA2R antibody or glomerular PLA2R antigen to diagnosis of primary MN with 50–80% sensitive and almost 100% specific (Dai H; et al 2015).

Phospholipase A2 receptor is overexpressed in renal tissue epithelial cells of Primary membranes nephropathy patients, the expression of anti-PLA2R antibodies also follows, and a series of studies has confirmed that the levels of

anti-PLA2R antibodies in PMN patients are significantly higher than those in normal and non-MN patients (Ramachandran *et al*.,2017).

The anti-PLA2R antibody has many biological characteristics and can cause complement system activation, podocyte injury, and basement membrane damage when combined with PLA2R on the glomerular podocyte membrane leading to the emergence of large amounts of proteinuria (Mcquarrie *et al*.,2012).

4.4. Traditional Tests

4.4.1 Estimation the serum levels of anti-ANA and anti –dsDNA antibodies.

The level of anti- nuclear antibodies (ANA) showed that highly significant increase in sever lupus nephritis patients in comparison with moderate lupus nephritis and other organ SLE patients in which the levels was reached to **6.22**± 2.41 , **2.11**± 0.77**, 1.03**± 0.19 (U/ml) respectively, also the levels of Anti-double strand deoxyribonucleic acid (anti-dsDNA) antibodies showed that highly significant increase in sever lupus nephritis, moderate Lupus Nephritis and other organ SLE and the mean of anti-dsDNA was reached to **425.47**± 180.33 ,**233.8**± 93.73 ,**77.13**± 30.91 (U/ml**)** respectively as shown in table (4.3).

Table (4.3) The serum levels of anti-ANA and anti –dsDNA antibodies in patients with Lupus Nephritis and SLE other organs

	Sever LN	**Moderate LN**	**Other organ SLE**	Normal range *
ANA Mean ±SD	A **6.22**± 2.41	B **2.11**± 0.77	B **1.03**± 0.19	<1.0Neg >1.0Pos U/ml
ds-DNA Mean ±SD	A **425.47**± 180.33	B **233.8**± 93.73	C **77.13**± 30.91	<30 neg >50 pos U/ml

Note: Normal range was obtained from protocol of kits according to Manufacture Company.

: Identical letter means non-significant difference.

: Different letter means significant difference.

Previous study demonstrated that high serum level of ANA is most often associated with active SLE, Kavanaugh *et al.*, (2000) show that ANA is one of the most common tests used by physicians to diagnosis SLE. The anti-nuclear antibody (ANA) is heterogeneous group of antibodies produced against variety of antigens within the cell nucleus.

A positive ANA test does not automatically mean lupus but it shows that immune system is making an antibody that reacts with components of body's cells, in addition ANA positive is not mandatory for the diagnosis, because most people with SLE have ANA, but most patients with ANA do not have SLE, and may not relate to the patient's symptoms but were indicated to other autoantibodies that may present in patient serum (Hyoun *et al.*, 2009),and these this finding was similar to other studies (Maher, 2013).

These results showed that increased in anti dsDNA antibody level prior to disease exacerbations of SLE is part of a restricted immune response or merely the consequence of polyclonal B cell activation (Ter Borg *et al*., 1991). Moreover, Giasuddin *et al*., (1991) have mentioned that the anti dsDNA antibodies are present in 85.3% of SLE patients.

For many years, the anti-dsDNA antibody assay has been regarded as the serological gold standard in the diagnosis and assessment of disease activity in patient with SLE (Isenberg, 2004). The prevalence of anti-dsDNA in this study was 75%. This is in consistent with previous data which reported that anti-dsDNA reactivity was between 40 -80% of SLE patients (Ravirajan *et al.*, 2001). increased levels of anti-dsDNA antibodies are related with disease flares (Linnik *et al*.,2005).

4.4.2 The Complements components (C3, C4, C1q)

The results showed that a significant decrease in the level of C3 in sever lupus nephritis patients compare to moderate lupus nephritis and other organ SLE, in which the mean reached to 0**.79**± 0.36, **0.85**± 0.47 and **1.04**± 0.35 (g/l) respectively. Also the level of C4 revealed a significant decrease in the sever lupus nephritis patients compare to moderate lupus nephritis and other organ SLE and the level of C4 was **0.14**± 0.1 ,**0.31**± 0.17 and **0.38**± 0.2 (g/l) respectively, the results of current study have illustrated that non-significant differences in the C1q between the studies groups in which the mean was **8.04**± 8.93 ,**8.44**± 1.46 and **9.43**± 2.52 (µg/ml**)** respectively as shown in table (4.4).

Table (4.4) The level of Complement components in patients with Lupus Nephritis and SLE other organs

	Sever LN	**Moderate LN**	**Other organ SLE**	Normal range *
C3 Mean ±SD	B **0.79**± 0.36	B **0.85**± 0.47	A **1.04**± 0.35	(0.9-1.8) g/L
C4 Mean ±SD	B **0.14**± 0.1	A **0.31**± 0.17	A **0.38**± 0.2	(0.15-0.45) g/L
C1q Mean ±SD	A **8.04**± 8.93	A **8.44**± 1.46	A **9.43**± 2.52	(2.5-9.5) µg/ml

Note: Normal range was obtained from protocol of kits according to Manufacture Company.

: Identical letter means non-significant difference.

: Different letter means significant difference.

These results of current study were agreement with results of Li *et al*., (2015), who have found low serum level of complement components C3 and C4 in Chinese patients with SLE. Also, the current study was agreement with Julkunen *et al*., (2012) that suggests complementC3 and C4 serum levels are correlated with disease activity and patients with active lupus nephritis had significantly decreased levels of C3 and C4 compared to patients with inactive lupus nephritis.

The results of present study were agreement with other result of which show a particularly C4 is critical for starting a renal flare, while C3 activation is implicated in the actual tissue damage (Birmingham *et al*., 2010)

In addition, Ho *et al*. (2001) have found that decreases in serum levels of complement components were not consistently related with SLE flares but also related with a concurrent increase in renal and hematologic SLE activity.

The current data was agreement with the results of Tang *et al.*, (2005) that showed the complement classical pathway is a key driver of the renal injury in LN. Immune complexes trigger the activation of the complement cascade through the classical pathway leading to tissue injury, there for the Sub-endothelial and sub-epithelial deposits of immunoglobulin (IgM ,IgG) and complement classical pathway components, such as C4 and C1q, are always found in LN patients.

The autoantibodies against C1q have strong correlation with the disease severity and immunological activity, contrary to those against C1r and C1s, which are rarer and show no correlation. Patients with LN usually have autoantibodies against C1q (Yin *et al.*, 2012), and Complement components of the classical pathway vary in relation to disease activity and low levels of C1q and C3 have been reported in active renal disease, Low C1q levels were determined to be the strongest predictor of subsequent development of renal flare compared to the levels of other complement factors (Jonsson *et al.*, 1995).

The findings of multiple apoptotic bodies in the glomeruli of C1q deficient, which develop SLE-like disease with a high incidence of nephritis, also indicates apoptosis as a contributing factor in the development of nephritis (Botto *et al.*, 1998).

The immune dysfunction is a hallmark of SLE during its pathogenesis in which the immune disorder is a main cause of productions of autoantibodies, deposition of immune complexes, and the defect of autoimmune tolerance, which in turn leads to injuries of multiple organs (Yap and Lai, 2015).

The complement system has been recognized to play crucial roles in scavenging immune complexes and autoantigens generated from cell apoptosis and prevent the autoimmune-induced tissue and organ injury (Elkon and Santer ,2012).

Owing to its opsonizing role in the clearance of autoantigens and apoptotic bodies under a physiological condition, a defective function of complement

system may lead to failure in properly identifying and promptly cleaning cell debris and autoantigens, which in turn activate immune response and produce autoantibodies. Sequentially, autoantibodies bind to complement fragments, which have a major functional consequence to cause tissue injury, especially in the kidney (Dragon-Durey *et al.*,.2013).

4.4.3 Hematological Criteria (Erythrocytes Sedimentation Rate)

The current study showed that SLE disease was associated with raised erythrocytes sedimentation rate and there were significant differences between the sever lupus nephritis, moderate lupus nephritis and the other organ SLE. The mean of ESR for these groups was **48.72**± 32.62, **33.42**± 30.18 and **27.83**± 19.24 (mm/hr) respectively as shown in table (4.5).

Table (4.5) Level of ESR in patients with Lupus Nephritis and other organs

Markers	**Sever LN**	**Moderate LN**	**Other organ SLE**	Normal range *
ESR Mean ±SD	A **48.72**± 32.62	B **33.42**± 30.18	C **27.83**± 19.24	(5-20) mm/hr

Note: Normal range was obtained from protocol of kits according to Manufacture Company

: Identical letter means non-significant difference.

: Different letter means significant difference.

The results showed increasing level of ESR in SLE patients, due to inflammation causes increase in ESR, and this results were match with other studies (Shaldonum *et al.,* 2012; Afify *et al.,* 2013 and Kusworini *et al.,* 2013).

4.4.4 Biochemical Test:

Biochemical investigations were done for measure the chemical substances carried by the blood. Urea and creatinine commonly used to assess kidney function as renal injury (glomerulonephritis). There was a significant difference in the levels of urea between the sever lupus nephritis, moderate lupus nephritis and other organ SLE. The mean of urea was **157.59**±65.83, **61.56**±26.4 and **30.43**± 8.91 (mg/dl) respectively, also the levels of creatinine showed that a significant difference in these groups which reached to **3.53**± 1.5, **1.26**± 0.5 and **0.73**± 0.26 (mg/dl) respectively as shown in table (4.6).

Table (4.6) Concentration of Biochemical test in patients with Lupus Nephritis and SLE other organs

Markers	**Sever LN**	**Moderate LN**	**Other organ SLE**	Normal range
Urea Mean ±SD	A **157.59**± 65.83	B **61. 56**± 26.4	C **30.43**± 8.91	(5-20) mg/dl
Creatinine Mean ±SD	A **3.53**± 1.5	B **1.26**± 0.5	C **0. 73**± 0.26	(0.7-1.3) mg/dl

Note: Normal range was obtained from protocol of kits according to Manufacture Company.

: Identical letter means non-significant difference. *

: Different letter means significant difference.

The results of current study was agreements with other study that shows in the state kidney failure, machines are needed to cleaning the blood of accumulated waste products in a process called dialysis ,and this may explain why the levels of urea and creatinine in the blood of SLE patients increased (Waldman and Maldman, 2005).This study appeared to explore the association of serum urea and creatinine with clinical and laboratory characteristics in SLE patients, independent of lupus renal involvement , although these two components in serum were all common marker of renal function, they showed

almost completely different associations with various clinical features in SLE patients.

4.5. Extractable Nuclear Antigen (ENA Screen)

The results showed that **23%** of patients was positive for anti-ENA which considered (SLE associated with other autoimmune disease) and **77%** of patients were negative for anti-ENA (lupus nephritis), as shown in figure (4.2).

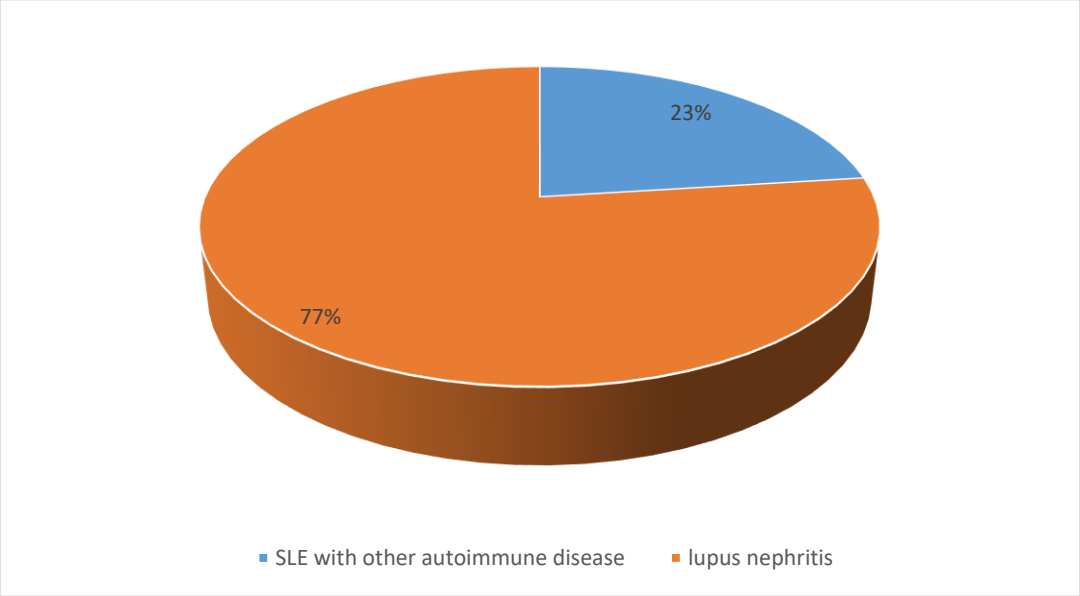


Figure (4.2) The percentage of anti-ENA antibody in SLE with other autoimmune disease and lupus nephritis

The result was agreement with other studies that showed the ENA can be used to diagnosed autoimmune diseases (Jeong *et al.*, 2018).

The current study was conformity to the study that showed ENA autoantibodies were crucial and need to be correlated with clinical diagnosis and other serological testing for early diagnosis and intervention of the autoimmune disease (Khater and Al Sheik ,2022).Certain autoimmune diseases are characteristically related to the existence of anti-ENA antibodies, these autoantibody associations can help to distinguish between different autoimmune diseases and aid in the diagnosis of autoimmune disorders (Orton *et al.*, 2004).

4.6. The Novel Test

4.6.1 Neutrophil Gelatinase Associated Lipocaline (NGAL)

The results of current study have illustrated a significant difference in levels of NGAL in sever lupus nephritis group compare with moderate lupus nephritis and SLE other organ groups, in which the **1027.53**± 259.01 ,**768.82**± 228.8 and **715.89**± 173.9 (ng/ml**)** respectively as shown in table (4.7).

The data of current study was agreement with the study of Nakhjavani *et al*; (2019) that found the serum NGAL was significantly higher in SLE individuals, Furthermore, NGAL was even more elevated in SLE patients with LN when compared to those without nephritis.

The current study was conformity to the study of that showed the NGAL has been the most widely studied biomarker in acute renal injury and has been demonstrated to possess an excellent diagnostic performance, Previous studies have shown that concentrations in urine and serum of NGAL represent sensitive, specific, and highly predictive biomarkers for acute renal injury after cardiac surgery (Parikh *et al*;2011). also the current study was in accordance with Xiang and Hogguist ;(2012) who documented increased level of serum NGAL in patients with chronic kidney disease (CKD) e.g. in polycystic kidney disease, IgA nephropathy, dysplasia, obstruction, Lupus Nephritis and glomerulonephritis.

Neutrophil Gelatinase Associated Lipocaline expressed from the tubular epithelial cells and tubular epithelium of the distal nephron after damage, therefore increase NGAL in the urine and serum reflect to the kidney disease (Bolignano *et al.*, 2008). In addition, the study of Soni *et al*;(2010) was found increase in the expression of NGAL in the present inflammation and injured epithelia for this considered the NGAL is one of the first proteins produced in the kidney after nephrotoxic.

4.6.2 Vascular Cell Adhesion Molecule-1(VCAM-1)

The results of current study showed that a significant differences in the level of VCAM in sever lupus nephritis compare with moderate lupus nephritis and other organ SLE group, which reached to **21.43**± 5.83, **13.6**± 2.63 and **13.56**± 12.28 (ng/ml**)** respectively as shown in table (4.7).

Vascular Cell Adhesion Molecule - 1 is expressed on endothelial cells and tubules and it participates in the migration and recruitment of leucocytes, it could distinguish active LN from inactive disease in adults and is sensitive to change in status (Stanley *et al.*, 2020).

The study of Parodis *et al*;(2020) indicated high U-sVCAM-1 levels appear to reflect SLE disease activity, U-sVCAM-1 showed ability to distinguish SLE patients with active renal involvement from patients with quiescent or no prior nephritis, High U-sVCAM-1 levels may indicate patients at increased risk for long-term renal function loss.

4.6.3 Urinary epidermal growth Factor (UEGF)

The results of current study have illustrated that a significant decrease in the level of UEGF in sever lupus nephritis compare with other groups, which reached to **145.97**±45.55, **195.78**±60.38 and **339.15**±85.59 (ng/L**)** respectively as shown in table (4.7).

The current study was agreement with the study of Isaka; (2016) that show the EGF has gained interest as a biomarker of renal disease because its decreased urinary excretion has been observed in nearly all human kidney diseases including diabetic nephropathy, IgA nephropathy, and lupus nephritis, also the current study was agreement with Ju *et al* ;(2015) that showed EGF tissue expression and urinary excretion decrease after kidney injury.

In proliferative LN, immune complex deposition leads to glomerular visceral epithelial cells (podocytes) damage and proteinuria. Recently, EGF has

been shown to promote podocyte proliferation and re-expression of differentiation markers after exposure to high glucose concentrations (Sun *et al.*,.2021).

Therefore, EGF could decrease proteinuria in LN by restoring glomerular function through its ability to repair podocytes. Since the generation of EGF is restricted mostly to the kidneys, levels of EGF in the urine may reflect local renal production. Consequently, LN patients with higher levels of EGF in the urine who responded to therapy may have less initial kidney injury and a higher ability to repair damaged podocytes and tubules (Ngamjanyaporn *et al.*,.2022).

Urinary EGF levels have been shown to correlate with the severity of tubulointerstitial fibrosis in primary glomerulonephritis (Worawichawong *et al.*,.2016), and low urinary EGF has been shown to be a risk predictor of kidney progression in non-diabetic kidney diseases (Ju *et al.*,.2015).

Table (4.7): Novel Tests for patients with Lupus Nephritis and SLE other organ

Marker	Sever LN	Moderate LN	Other organ SLE	Normal Range*
NGAL Mean ±SD	A **1027.53**± 259.01	B **768.82**± 228.8	B **715.89**± 173.9	(283-990) ng/ml
VCAM Mean ±SD	A **21.43**± 5.83	B **13.6**± 2.63	B **13.56**± 12.28	(5.1-19.2) ng/ml
UEGF Mean ±SD	C **145.97**± 45.55	B **195.78**±60.38	A **339.15**± 85.59	(216-740) ng/L

Note: Normal range was obtained from protocol of kits according to Manufacture Company.

: Identical letter means non-significant difference.

: Different letter means significant difference.

4.7 The correlation between study parameters.

4.7.1 The correlation between UEGF and complements components (C3, C4, C1q) in patient's groups.

The Table (4.8) showed that there was a significant positive correlation between UEGF and complement components (C3, C4, C1q) r= (0.773, 0.693, 0.514) respectively in sever lupus nephritis patients, also the Table showed there was significant positive correlation between UEGF and C3, C4 (0.625, 0.507) respectively in Moderate lupus nephritis, but non-significant correlation between UEGF and C1q (0.453) in addition, the results showed there was no significant correlation between UEGF and complements components (0.328, 0.484, 0.195) respectively in SLE other organ.

Table (4.8) Correlation between the Urinary epidermal growth factor and Complement factors (C3, C4, C1q) in patients groups

		C3	C4	C1q	UEGF
Sever LN	C3	1			
	C4	0.53801*	1		
	C1q	0.69824*	0.42678	1	
	UEGF	0.77361*	0.69345*	0.51432*	1
Moderate LN	C3	1			
	C4	0.41547	1		
	C1q	0.49453	0.43754	1	
	UEGF	0.62590*	0.50718*	0.45372	1
Other organ SLE	C3	1			
	C4	0.34978	1		
	C1q	0.46777	0.39167	1	
	UEGF	0.32853	0.48404	0.19591	1

*means significant correlation

The current study agreement with study that show decreased UEGF was associated with more rapid kidney function decline instead of less rapid decline. Moreover, decreased renal expression and urinary excretion of UEGF were found in human kidney disease such as lupus nephritis and was interpreted as a sign of insufficient repair (Azukaitis *et al.*,.2019).

The levels of C1q and C4 were positively correlated with the blood level of C3, as an upstream factor, C1q activates C3 with the help of C4 in the classical complement pathway (Liu *et al.*,.2021).Furthermore, the result of current study was agreement with Trouw, et al (2017) finding that show significant decrease of plasma C3 in the presence of LN is consistent with previous clinical which reported that complement activation leads to secondary decreases in the blood levels of C4 and C3 during the active period of SLE.

The current study was agreement with study of Chapman, et al (2003) that show urinary EGF excretion might be a reflection of the amount of healthy tubular mass, and thus may be an indicator of tubular regenerative repair capacity as others have suggested.

The prognosis of LN patients is poor when there is no C1q deposition in the kidneys (Kim *et al.*,.2020). A deficiency of C1q strongly predisposes individuals to SLE which is thought to be related to the role of C1q in the removal of apoptotic cells and the clearance of immune complex (Macedo and Isaac,2016).

Decreased serum levels of C1q protein and increased titers of C1q antibodies may be involved in the pathogenesis of SLE, especially LN (Mosaad *et al.*,.2015).

4.7.2 The correlation between NGAL, VCAM tests and ANA, anti –ds DNA in patient's groups.

Table (4.9) showed that there was a significant positive correlation between NGAL with VCAM, ANA, and ds- DNA r= (0.685, 0.551 ,0.628) respectively. also between VCAM with ANA and ds-DNA r= (0.612 ,0.724) respectively in sever lupus nephritis patients.

The results showed significant positive correlation between NGAL and ANA (0.505), and VCAM and ANA (0.513) in moderate lupus nephritis patients but non-significant between NGAL and VCAM r= (0.402).

There was non-significant correlation between NGAL and VCAM in SLE other organ r= (0.379).

Table (4.9) Correlation between NGAL, VCAM tests and ANA, anti-DNA in patient's groups.

		NGAL	VCAM	ANA	ds-DNA
Sever LN	NGAL	1			
	VCAM	0.68526*	1		
	ANA	0.55183*	0.61246*	1	
	ds-DNA	0.62866*	0.72431*	0.75535*	1
Moderate LN	NGAL	1			
	VCAM	0.40276	1		
	ANA	0.50509*	0.51395*	1	
	ds-DNA	0.42127	0.37441	0.55793*	1
Other organ SLE	NGAL	1			
	VCAM	0.37916	1		
	ANA	0.26862	0.12661	1	
	ds-DNA	0.42806	0.15357	0.57717*	1

*means significant correlation

The current results was agreement with several studies have shown elevated and strongly correlated serum VCAM-1 with lupus nephritis activity and severity (Parodis *et al.*,.2020).

In resonance with the studies in SLE, VCAM-1 was significantly elevated in active LN patients versus active non-renal, inactive SLE so that VCAM-1 was highly predictive of SLE disease activity (Soliman *et al.*,.2022).

The current study was agreement with Kiani, et al (2012) study that found the VCAM-1 had a strong correlation with descriptor of renal activity, also agreement with study that show the role of NGAL in promote inflammation and cause tubular apoptosis so that NGAL level correlates with the severity of renal damage (Mishra *et al.*,.2003). furthermore, higher NGAL levels in patients with LN suggest more severe kidney damage than in those without LN (Li *et al.*,.2019) , as well as , increased levels of plasma NGAL showed a predictive power for Chronic kidney disease progression and was reflective of renal disease severity (Wasung *et al.*,.2015).

Mahmoudi et al;(2017) revealed that ds-DNA was correlated positively with lupus nephritis activity, also Gheita et al;(2018) reported that ds-DNA level is found to be significantly higher in active lupus nephritis patients.

The current results were agreeing with study that showed the implication of the co-positivity of anti-dsDNA, as risk factor for the development of proliferative nephritis and for more active disease, so their detection may be considered as a prognostic marker for disease progression (Elsayed and Mohafez, (2020)

Manson and Rahman, (2006) showed over 90% of patients with SLE have positive anti-nuclear antibodies (ANA), in addition it is revealed that positive ANA level in 88.2% of the patients, and this parameter should be used to diagnose for most patients because it is positive in over 85% of cases, typically when symptoms first appear. However, positive ANA results do not always denote SLE, though. almost all patients with SLE are ANA positive insignificant titers (Carey *and adam* ., 2008).

Conclusions and Recommendations

Conclusions

1-Decrease the level of Human epidermal growth factor (urinary EGF) in moderate and sever Lupus nephritis patients may be make it good marker for predicting the kidney failure at early stage.

2- There was significant correlation NGAL with Tradional test in moderate lupus nephritis so it can be used as an early diagnostic marker for acute kidney injury.

3- Use the Extractible nuclear antigen screen test (ENA) recognized the Systemic lupus erythromatous from other autoimmune disease.

Recommendations

1.Using of human epidermal growth factor (urinary EGF) as predicting test for the kidney failure.

2.Study role of viral infections in SLE.

3.Using SNPs for detection genes related with SLE.

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Appendix

Appendix: (**5**)

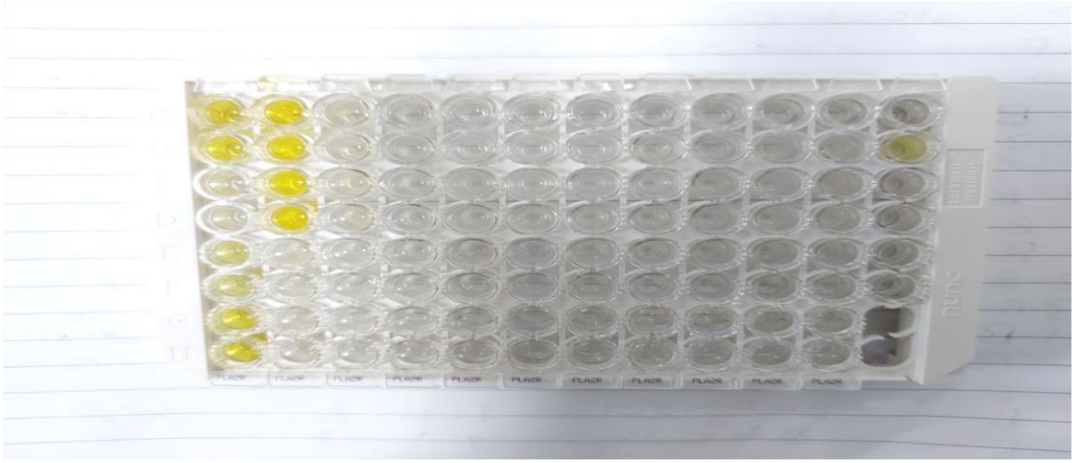


Figure (5.1): Phospholipase A2 receptor kit

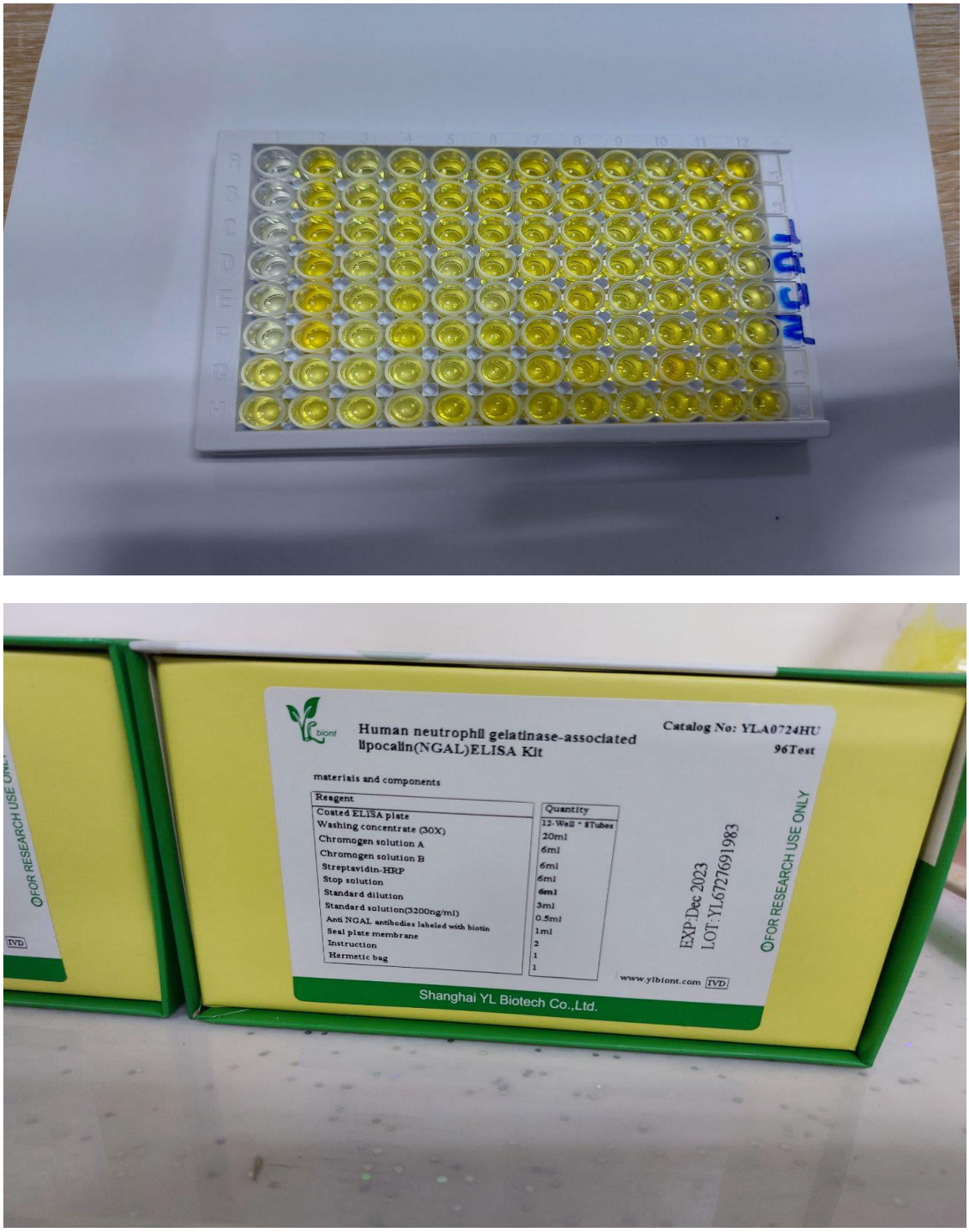
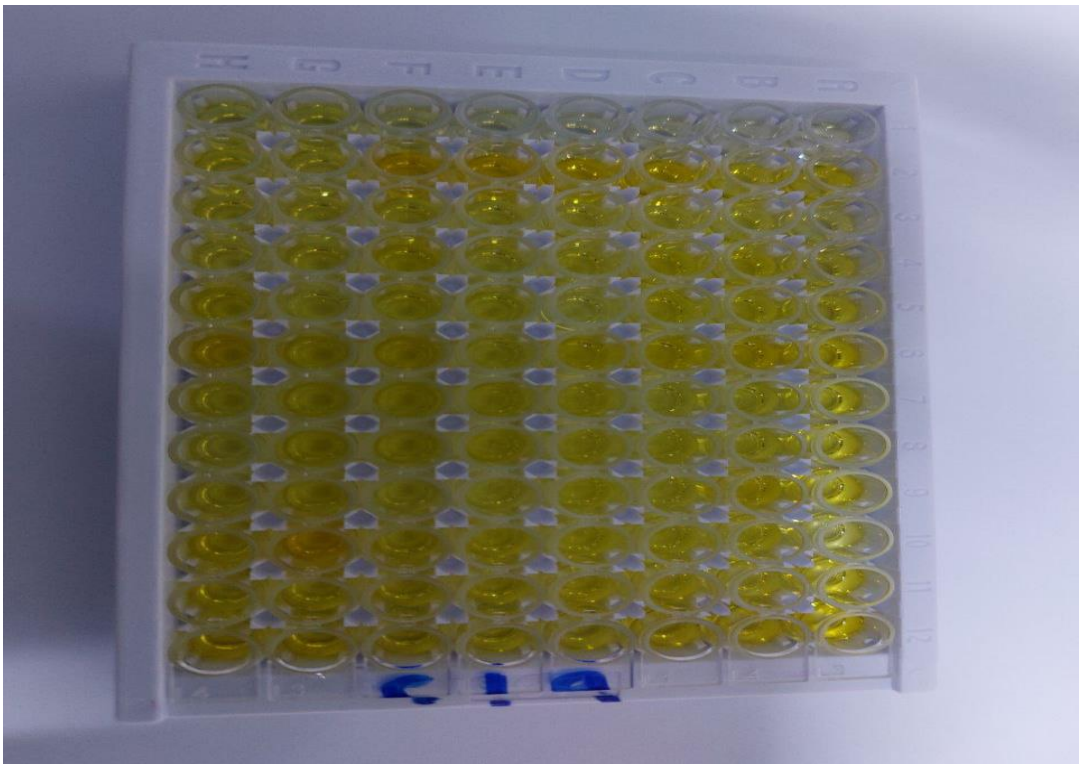


Figure (5.2): Neutrophil gelatinase associated Lipocaline kit



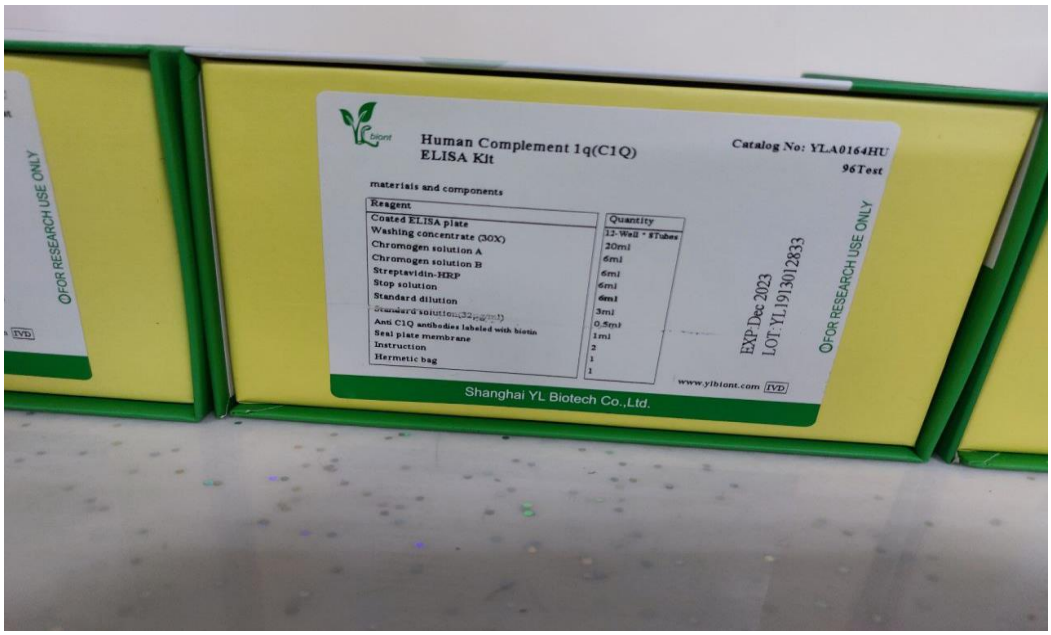


Figure (5.3): human complement C1q kit

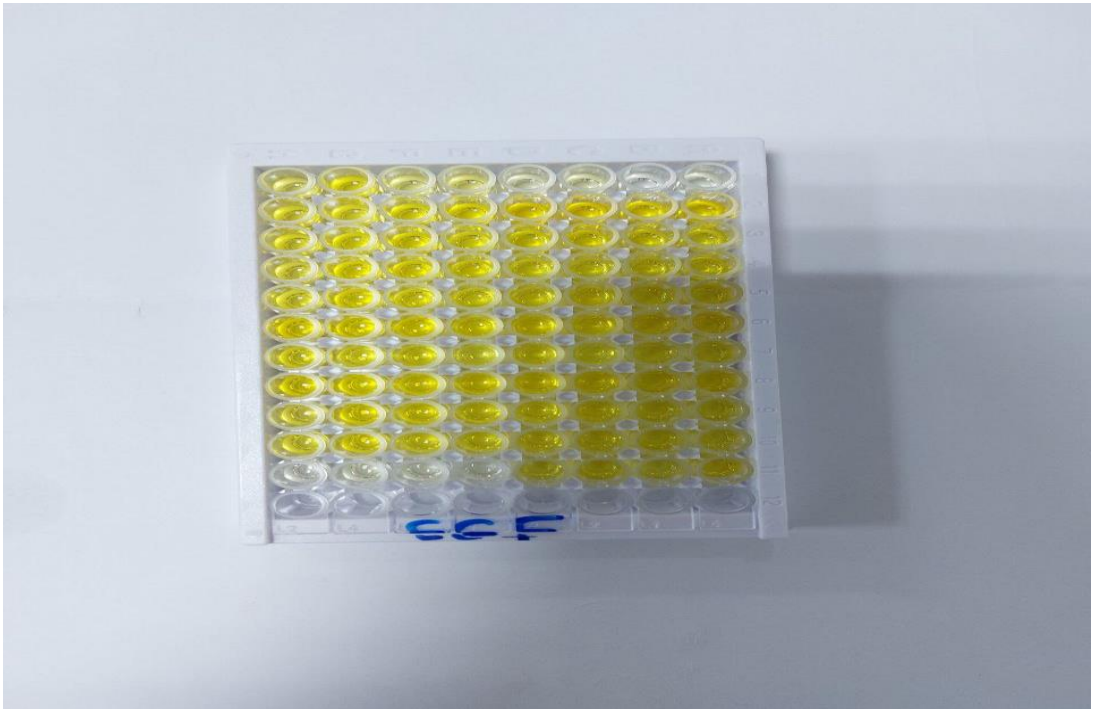
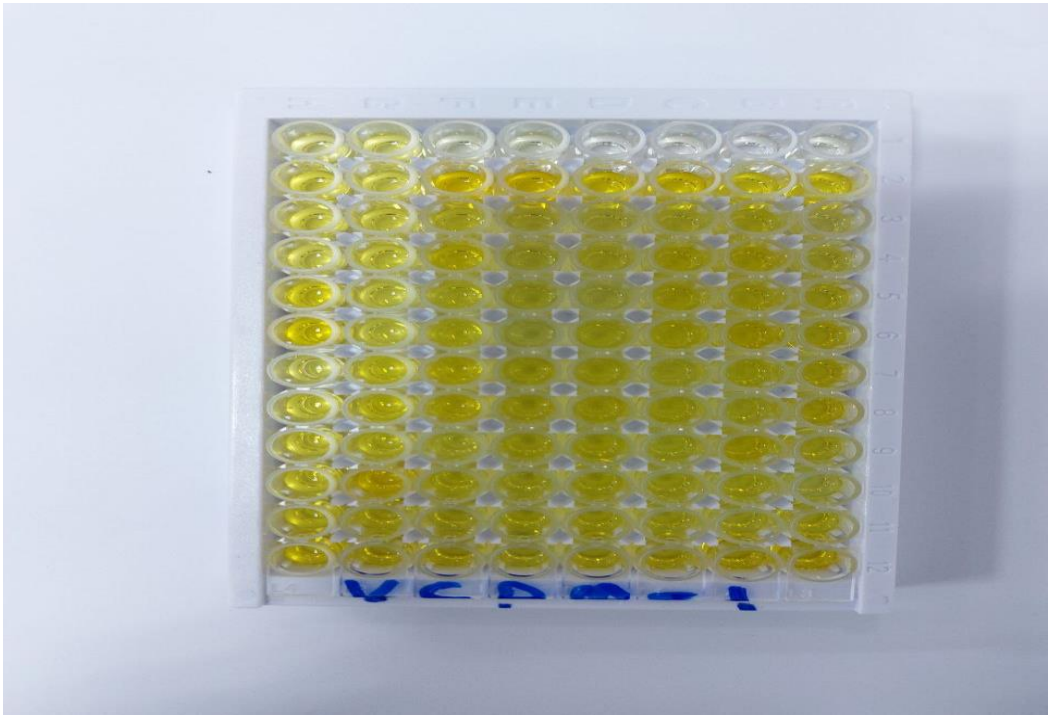


Figure (5.4) :human Epidermal Growth factor kit



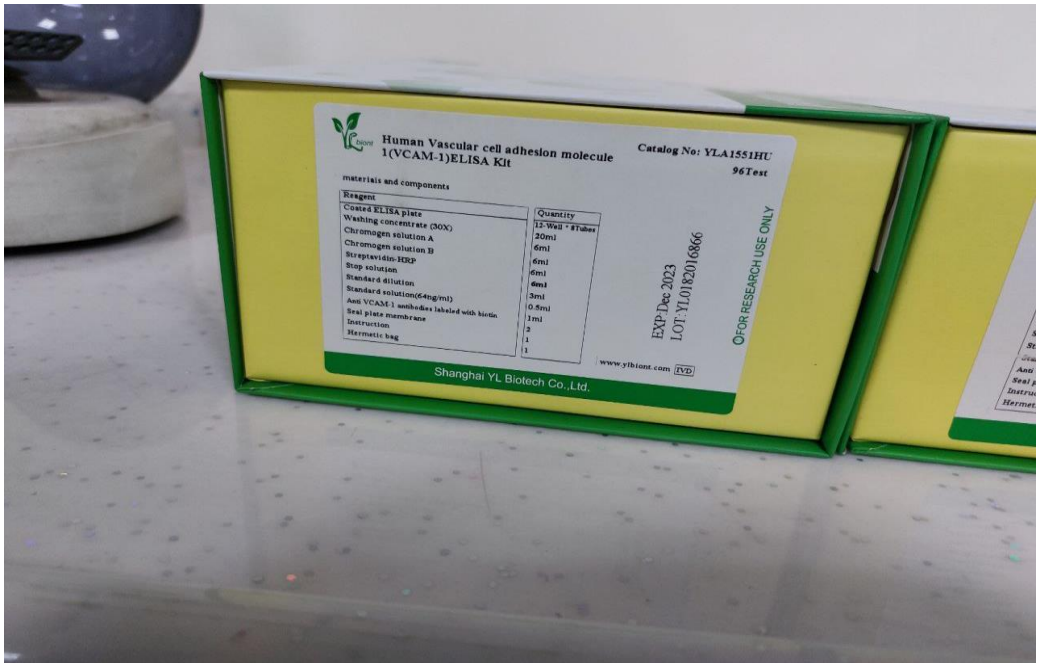


Figure (5.5) :Vascular cell adhesion molecular-1 kit

Figure (5.6): Complement (C3, C4) Kits

Patients a questionnaire survey

Sequence: ☐

hospital	
Full Name	
Age	
Gender	Male ☐ female ☐
home adress	
Telephone number	
Social status	
Main symptoms	joint pain ☐ molar rash ☐ photosensitivity ☐ Anemia ☐ Arthritis ☐ Hair Loss ☐ Oral ulcer ☐ renal failure ☐

chronic diseases	Pressure ☐ Heart ☐ Disease Sugar ☐ Kidney ☐ disease Diseases of the joints ☐ Fat content in the blood
Medicine used	
History of the disease	

الخلاصة

يحدث داء الكلية الكبيبي نتيجة تكون المعقد المناعي والذي يتطور كمضاعفات لداء الذئبة الاحمرارية الجهازية والتسبب في داء الكلية الذئبي ينطوي على مجموعة متنوعة من الاليات المسببة للمرض. تعتمد المسببات الخارجية لمرض الذئبة الاحمرارية الجهازية على مجموعات متعددة من المتغيرات الجينية التي تؤثر على تلك الاليات المسؤولة عن التحمل المناعي للمستضدات النووية الذاتية وتؤكد زيادة حدوث الفشل الكلوي بمراحله الأخيرة على أهمية التشخيص المبكر لهذا المرض.

تهدف هذه الدراسة الى التحقق من بعض الواسمات المناعية التي شملت تقدير تراكيز مستقبل انزيم ، الفوسفوليباز ، انزيم الجيلاتينيز للعدلات المرتبطة بالليبوكالين، الجزيئات الالتصاقية الخلوية الوعائية وعامل النمو البشري البولي، بالإضافة الى دراسة بعض المعايير الكيموحيوية مثل اليوريا والكرياتنين , كذلك شملت الدراسة تقدير تراكيز المستضد النووي المستخلص , الاجسام المضادة النووية , الاجسام المضادة الى الحامض النووي منقوص الاوكسجين مزدوج الشريط , وتراكيز مكونات المتمم

شملت الدراسة 85 مريضة تعاني من داء الذئبة الاحمرارية الجهازية حيث تم جمع عينات الدم والادرار من مستشفى الامام الحسن المجتبى في محافظة كربلاء من تشرين الثاني 2022 الى شهر اذار 2023

أظهرت نتائج اختبار مستقبل الفوسفوليباز على عينات الدم ان 5 عينات من النساء أعطت نتيجة موجبة وأنهن مصابات بالتهاب الكبيبات الكلى الاولي حيث تم استبعادهن من الدراسة في حين أظهرت 80 عينة المتبقية نتيجة سلبية لهذا الاختبار وأنهن مصابات بداء الذئبة الاحمرارية الجهازية قسمت عينات المرض اعتمادا على الاختبارات التقليدية الى ثلاثة مجاميع وهي المصابات بداء الكلية الذئبي الشديد 30 عينة، داء الكلية الذئبي المتوسط 20 عينة، داء الذئبة الاحمرارية الجهازية 30 عينة.

أظهرت نتائج التحاليل ارتفاعا في تركيزي الاجسام المضادة النووية، الاجسام المضادة الى الحامض النووي منقوص الاوكسجين مزدوج الشريط لدى مرضى داء التهاب الكلية الذئبي الشديد مقارنة مع مرضى داء التهاب الكلية المتوسط ومرضى داء الذئبة الاحمرارية الجهازية، بينما أظهرت نتائج مكونات المتمم انخفاضا في تراكيزها لدى مرضى داء الكلية الذئبي الشديد مقارنة بمرضى داء الكلية المتوسط ومرضى داء الذئبة الاحمرارية الجهازية.

كذلك أظهرت نتائج دراسة المستضد النووي المستخلص بان 77% من المرضى يعانون من مرض داء الكلية الذئبي لوحدها في حين 23% من المرضى يعانون من مرض داء الكلية الذئبي وامراض مناعية أخرى.

لقد أظهرت الدراسة زيادة في تركيز انزيم الجيلاتينيز للعدلات المرتبط بالليبوكالين بالنسبة لمرضى داء الكلية الذئبي الشديد مقارنة بمرضى داء الكلية الذئبي المتوسط وداء الذئبة الاحمرارية الجهازية حيث بلغ التركيز(259.01+**1027.53** ,173.9+**715.89** , 228.8+**768.82** نانوغرام /مل) على التوالي كذلك بينت الدراسة زيادة في تركيز الجزيئات الالتصاقية الخلوية الوعائية للمجاميع الثلاثة اذ بلغت (12.28+**13.56** ,2.63+**13.6**, 5.83+**21.43** نانوغرام /مل)على التوالي .كما أظهرت النتائج انخفاضا ملحوظا بتراكيز عامل النمو البشري البولي لمرضى داء الكلية الذئبي الشديد مقارنة بداء الكلية الذئبي المتوسط وداء الذئبة الاحمرارية الجهازية التي بلغت (45.55+**145.97** ,60.38+**195.78** , 85.59 +**339.59**نانوغرام /مل)على التوالي .

بينت النتائج زيادة تراكيز اليوريا والكرياتيين بشكل ملحوظ لمرضى داء الكلية الذئبي الشديد مقارنة بداء الكلية الذئبي المتوسط وداء الذئبة الاحمرارية الجهازية.

كما أظهرت النتائج وجود علاقة إيجابية معنوية بين عامل النمو البشري البولي ومكونات المتمم كذلك وجود علاقة إيجابية معنوية كبير بين تركيز انزيم الجيلاتينيز للعدلات المرتبطة بالليبوكالين والجزيئات الالتصاقية الخلوية الوعائية.

ان نقصان تركيز عامل النمو البشري البولي لدى مرضى داء الكلية الذئبي المتوسط والشديد يجعله علامة جيدة للتنبؤ بالفشل الكلوي في مرحلة مبكرة.

جامعة كربلاء

كلية العلوم الطبية التطبيقية

قسم التحليلات المرضية

دراسة بعض الواسمات المناعية والكيميائية الحيوية لدى مرضى التهاب الكلية الذئبي

رسالة مقدمة

الى مجلس كلية العلوم الطبية التطبيقية - جامعة كربلاء

وهي جزء من متطلبات نيل شهادة الماجستير في التحليلات المرضية

من قبل

أسماء صلاح عيسى جعفر

بكالوريوس تحليلات مرضية كلية العلوم – جامعة الكوفة/ 2014

بأشراف

ا.د هادي رسول حسن

2023 م **1445 ه**