"EVALUATE URINE IL -12p40 LEVELS IN DIFFERENT STAGES OF LUPUS NEPHRITIS" – ONE-YEAR CROSS-SECTIONAL STUDY

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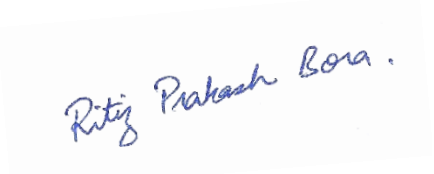

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LIST OF ABBREVIATIONS USED

S. No.	Abbreviations	Expansion of the Abbreviations
1.	SLE	Systemic Lupus Erythematosus
2.	LN	Lupus Nephritis
3.	PRRs	Pattern recognition receptors
4.	TLRs	Toll-like receptors
5.	PAMPs	Pathogen-associated molecular patterns
6.	Tregs	Regulatory T cells
7.	NK	Natural killer
8.	MS	Multiple sclerosis
9.	RA	Rheumatoid Arthritis
10.	ANA	Anti-nuclear antibodies
11.	RF	Rheumatoid factor
12.	CTLs	Cytotoxic T lymphocytes
13.	Th cells	Helper T cells
14.	T1D	Type 1 diabetes mellitus
15.	IBD	Inflammatory bowel disease
16.	HLA	Human leukocyte antigen
17.	FOX3P	Forkhead box protein P3
18.	AIRE	Autoimmune regulator gene
19.	EBV	Epstein-Barr virus
20.	dsDNA	Double-stranded DNA
21.	ACR	American College of Rheumatology
22.	SLICC	Systemic Lupus International Collaborating Clinics
23.	ISN/RPS	International Society of Nephrology/Renal Pathology Society
24.	IFN- γ	Interferon-gamma
25.	TNF	Tumour necrosis factor
26.	IL	Interleukin

27.	TWEAK	Tumour necrosis factor-like weak inducer of apoptosis
28.	NGAL	Neutrophil gelatinase-associated lipocalin
29.	uMCP-1	Urinary monocyte chemoattractant protein-1
30.	miRNAs	MicroRNAs
31.	PTX3	Pentraxin-3
32.	HIST1H4A	Histone H4 gene
33.	TNFR	Tumour Necrosis Factor receptor
34.	IGFBP	Insulin like growth factor binding protein
35.	CD163	Cluster of Differentiation
36.	PGRN	Progranulin
37.	VCAM	Vascular cell adhesion molecule
38.	AXL	'Anexelekto' (Receptor for tyrosine kinases)
39.	uAPRIL	Urinary proliferation-inducing ligand
40.	uBAFF	Urinary B-cell activating factor
41.	APCs	Antigen-presenting cells
42.	TRAF	Tumour necrosis factor receptor – associated factor
43.	IRAK-1	Interlukin-1 receptor-associated kinase 1
44.	qRT-PCR	Quantitative real-time polymerase chain reaction
45.	CLIA	Chemiluminescence Immunoassay
46.	SS-A (anti-Ro)	Anti Sjögren's syndrome type A
47.	SS-B (anti-La)	Anti Sjögren's syndrome type B
48.	SM/nRNP	Anti Smith (Sm) and ribonucleoprotein (RNP)
49.	PM Scl	Polymyositis/scleroderma
50.	PCNA	Proliferating cell nuclear antigen

ABSTRACT

EVALUATE URINE IL -12p40 LEVELS IN DIFFERENT STAGES OF LUPUS NEPHRITIS"– ONE-YEAR CROSS-SECTIONAL STUDY

Background:

Systemic Lupus Erythematosus (SLE) is an autoimmune disorder characterized by immune dysregulation and multi-organ involvement. Lupus nephritis (LN) is a severe complication of SLE, often requiring precise biomarkers for early detection and disease monitoring. IL-12p40, a subunit of the pro-inflammatory cytokine IL-12, has emerged as a potential biomarker for LN, influencing Th1 immune responses and inflammation.

Objective:

- To estimate IL-12 p40 level in urine among patients with lupus nephritis by ELISA to correlate the clinical presentation of SLE patients.
- To evaluate the association of variation of IL-12p40 levels with other biomarkers, in assessment of lupus nephritis.

Methodology:

A cross-sectional study was conducted on 35 SLE positive patients. Urine samples were collected and analysed for IL-12p40 levels using ELISA. Complement levels (C3, C4), ANA profile, and renal biopsy classifications were assessed. Statistical correlations between IL-12p40, disease severity, and laboratory parameters were analysed.

Results:

IL-12p40 was detected in 10 patients, predominantly in LN Class III (50%) and Class IV (40%). While IL-12p40 positivity showed some correlation with complement levels (C3, C4), statistical significance was not established ($p > 0.05$). No significant association was found between IL-12p40 levels and proteinuria severity. Anti-dsDNA demonstrated high sensitivity (>85%) in Class III and IV LN but lacked specificity in differentiating LN stages.

Conclusion:

Urine IL-12p40 may serve as a potential marker for LN, particularly in advanced disease stages. However, its clinical utility requires further validation due to inconsistent statistical significance. A multi-marker approach, integrating IL-12p40 with traditional and emerging biomarkers, is necessary for comprehensive LN assessment. Future longitudinal studies should explore its predictive value in disease progression and treatment response.

Keywords:

Systemic Lupus erythematosus, Lupus Nephritis, proteinuria, complement, Interleukin-12

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INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a multifaceted autoimmune disease marked by the presence of autoantibodies that trigger widespread inflammation and tissue damage. Initial research underscored that immune system dysregulation in SLE involves both inmate and adaptive immune responses, which results in chronic inflammation and multi-organ involvement (1). Advances in immunology have since broadened the understanding of autoimmune diseases, highlighting the necessity for innovative diagnostic and therapeutic approaches (2). Autoimmune conditions place a considerable strain on healthcare systems, emphasizing the need for more efficient strategies for early detection and management (3). The immune system's intricate network, encompassing both inmate and acquired immunity, carries a pivotal role in the pathogenesis of autoimmune disorders like SLE (4).

Diagnosing and treating SLE remain challenging due to its heterogeneous nature and unpredictable progression. The disease's development is driven by abnormal immune activation, resulting in absence of tolerance and the generation of pathogenic autoantibodies (5). Recent research into autoimmune mechanisms has illuminated the role of various immune cells and inflammatory mediators in disease progression (6). The interplay between genetic predisposition and environmental factors further complicates SLE pathogenesis, reinforcing the demand for improved diagnostic markers (7). Amongst the most severe of complications of SLE is lupus nephritis, which arises from immune complex deposition in the kidneys, leading to inflammation and subsequent organ damage (8). Clinical and immunological profiling of SLE patients has revealed significant variability, underscoring the importance of personalized treatment strategies (9).

Serum and urinary biomarkers have gained increasing attention for their potential role in

enhancing SLE diagnosis and disease monitoring. Investigations into cytokine levels in urine and serum suggest that specific cytokines may serve as indicators of disease activity and correlate with severity (10). A comprehensive review of lupus nephritis biomarkers further highlighted their utility in distinguishing active disease from remission, presenting promising candidates for clinical implementation (11). Additionally, novel molecular markers are being explored for potential inclusion in diagnostic panels, offering prospects for more accurate disease assessment (12).

Despite these advancements, there remains a critical need for further exploration of novel SLE biomarkers, as many existing markers lack the specificity and sensitivity required for routine clinical application. Gaining deeper insights into immune dysregulation in SLE could facilitate the development of even more precise diagnostic tools and targeted treatment. The identification of new biomarkers has the potential to improve early detection, disease monitoring, and treatment outcomes, ultimately benefiting patient care.

One biomarker currently under investigation is interleukin-12p40 (IL-12p40), a subunit of the cytokine IL-12, which has been implicated in the pathogenesis of lupus nephritis. Studies indicate that IL-12 overexpression is linked to a T-helper 1 (Th1)-dominant immune response, contributing to disease progression (13). Given its role in immune activation, IL-12p40 holds promise as a biomarker for lupus, offering insights into disease activity and therapeutic responses. Further research into its diagnostic and prognostic potential could significantly impact SLE management, enabling a more tailored approach to treatment.

OBJECTIVES

1. To estimate IL-12 p40 level in urine among patients with lupus nephritis by ELISA to correlate the clinical presentation of SLE patients.
2. To evaluate the association of variation of IL-12p40 levels with other biomarkers, in assessment of lupus nephritis.

REVIEW OF LITERATURE

Immunology of Autoimmunity

Autoimmunity is a phenomenon in which the immune system, which is primarily designed to defend against foreign pathogens, mistakenly attacks the body's self-tissues and cells. This erroneous autoimmune response results from a breakdown in self-tolerance, a fundamental mechanism that ensures immune cells do not react against the host's own antigens (1). Both inmate and adaptive immunity, which are components of the immune system, are essential for preserving immunological homeostasis and averting self-reactivity (2). However, when these regulatory mechanisms fail, autoimmune diseases emerge, leading to chronic inflammation and tissue damage (3).

The inmate immune system serves as the first line of defense against invasive infections through the combination of physical barriers, solvable regulators like complement proteins and cytokines, and phagocytic cells like neutrophils and macrophages. (4). Pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) detect pathogen-associated molecular patterns (PAMPs), leading to the activation of inmate immune responses. In autoimmune conditions, this system can become dysregulated, leading to inappropriate activation of immune cells and an inflammatory cascade those damages self-tissues (5). Recent studies have introduced the concept of trained immunity, where inmate immune cells, for example monocytes and macrophages develop a memory-like state after exposure to certain stimuli. This trained immunity, while beneficial in enhancing host defenses against recurrent infections, may also contribute to the persistent inflammation seen in autoimmune diseases (6).

The adaptive immunity is very specific and is mediated by lymphocytes, including T and B cells. Under normal conditions, central tolerance mechanisms in the thymus and bone marrow eliminate autoreactive T and B cells before they mature (1). Peripheral mechanisms of tolerance, including regulatory T cells (Tregs), help suppress autoreactive immune responses that escape central deletion (2)

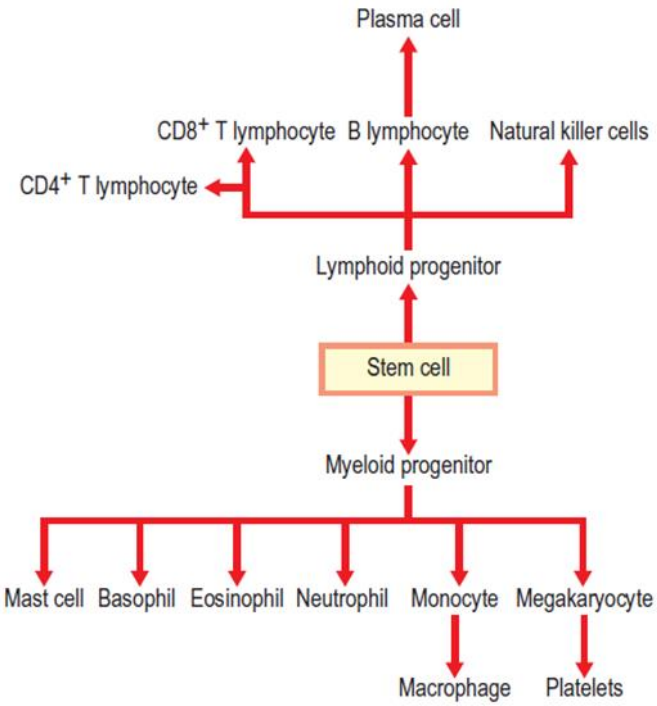


Figure 1: Cells of immune system
(Stewart J. Inmate and Acquired Immunity. Greenwood. 2011.)

However, defects in these tolerance pathways can result in survival and activation of T cells and B cells that are self-reactive, and then target the tissues of their hosts (3). Multiple sclerosis (MS), systemic lupus Erythematosus (SLE), and rheumatoid arthritis (RA) are among the autoimmune disorders that have been linked to Tregs' loss of regulatory control (4). Moreover, the presence of autoantibodies, which are produced by autoreactive B cells, is a hallmark of many autoimmune diseases, further highlighting the importance of adaptive immune dysregulation in autoimmunity (5)

Types of Immunity

Our immune system is broadly differentiated into inmate and adaptive immunity, each serving distinct yet interconnected roles in immune defence and regulation (6). The body's quick, general defence against infections is called inmate immunity, and it depends on soluble substances like complement proteins and inflammatory cytokines, cellular reactions mediated by neutrophils, macrophages, and natural killer (NK) cells, and physical barriers like the skin and mucosal surfaces (7). Inmate immunity does not confer long-term immunity but provides crucial early protection against infections. Importantly, inmate immune responses are also involved in the initiation of autoimmune diseases, particularly through dysregulated activation of PRRs and cytokines that promote inflammation (1).

Adaptive immunity, in contrast, is highly specific and involves antigen recognition by T and B lymphocytes (2). This type of immunity is characterized by immunological memory, enabling a faster and more effective reaction when the same infection is encountered again (3). The two subtypes of adaptive immunity are humoral and cell-mediated immunity. B cells, which generate antibodies that destroy infections and designate them for death, mediate humoral immunity (4). In autoimmunity, autoantibodies, such as anti-nuclear antibodies (ANA) in lupus and rheumatoid factor (RF) in RA, contributes to disease pathology (5). Cell-mediated immunity, on the other hand, involves T cells, particularly cytotoxic T lymphocytes (CTLs) that directly attack infected or damaged or abnormal cells, and helper T cells (Th cells) that regulate immune responses (6). The development of autoimmune disorders has been connected to dysregulation in T cell subsets, such as a disparity between pro-inflammatory Th17 cells and anti-inflammatory Treg (7).

Emerging research suggests that trained immunity bridges the gap between inmate and adaptive immunity. Epigenetic and metabolic reprogramming of inmate immune cells results

in a heightened state of responsiveness upon secondary exposure to stimuli. While this mechanism is advantageous in fighting infections, it may also contribute to autoimmunity by sustaining chronic inflammation and breaking immune tolerance. Understanding the interplay between inmate, adaptive, and trained immunity is essential for developing targeted therapies for autoimmune disorders.

Complement

Since the 1930s, it has been recognized that there is a heat-labile component in serum that may destroy red blood cells and kill Gram-negative rods. Early researchers did not understand the phenomenon's molecular intricacy and attributed the action to a single component known as complement. In actuality, complement is made up of several distinct serum proteins that are found in normal serum in little amounts. The result of the first reaction serves as the catalyst for the subsequent one, and so on. These molecules remain in a non-active state but might be triggered to create an enzyme cascade. (4)

The complement system involves around 30 proteins, some of which are structural proteins devoid of enzymatic activity, some are enzymes, and some of which are regulatory molecules (4).

The glomerular accumulation of immune complexes which induce a series of inflammatory events that cause substantial damage to tissues is the mechanism by which lupus nephritis (LN), a serious consequence of systemic lupus Erythematosus (SLE), exacerbates its morbidity and mortality. Although the exact mechanism or processes of their harmful involvement are unknown, the deposition of anti-dsDNA antibodies is an early event in LN

that proceeds by local generation of cytokines and chemokines that cause glomerular inflammation and eventually lead to irreparable renal damage (6,17).

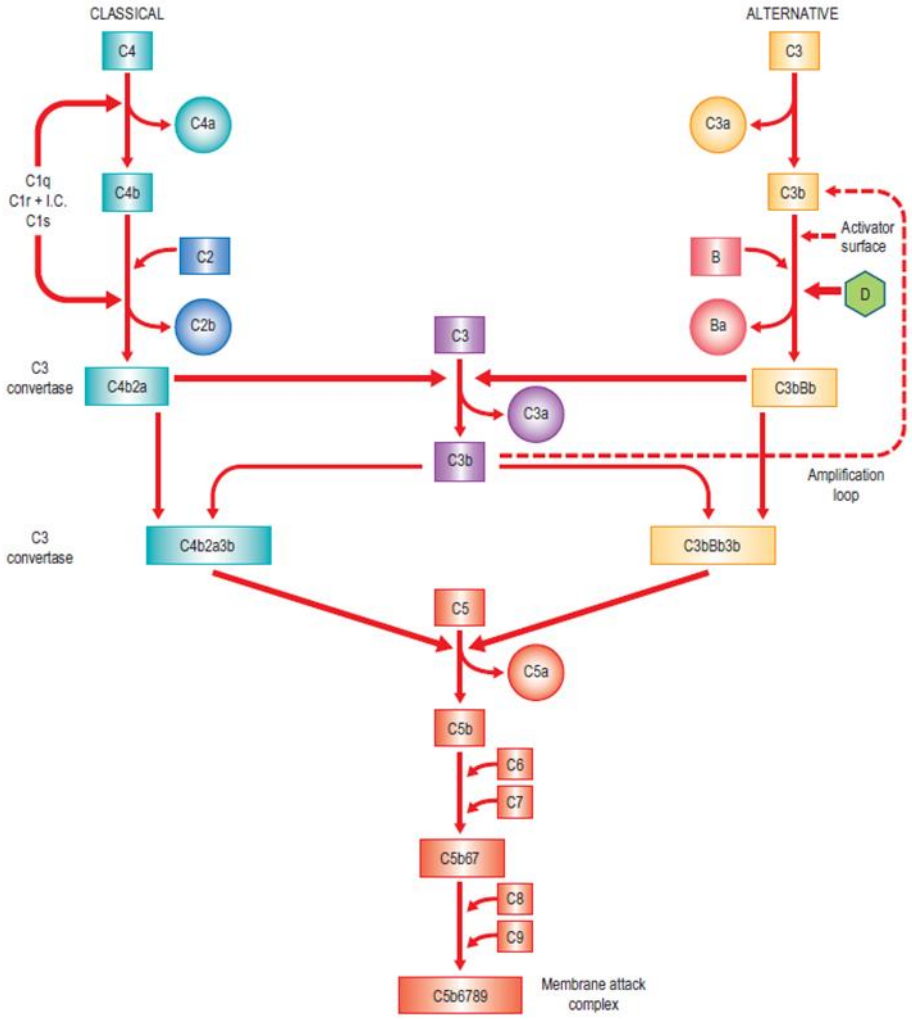


Figure 2: Complement activation via classical and alternative pathways
(Stewart J. Inmate and Acquired Immunity. Greenwood. 2011.)

Examples of Autoimmune Diseases

Autoimmune diseases are classified as either organ-specific, where the immune attack is directed against a single organ, or systemic, where multiple organs are affected (1). Organ-specific autoimmune diseases include type 1 diabetes mellitus (T1D), autoimmune thyroid diseases like Graves' disease and Hashimoto's thyroiditis, and neurological conditions such

as multiple sclerosis (MS) (2). In T1D, autoreactive T cells target pancreatic beta cells, leading to insulin deficiency and hyperglycaemia. Autoantibodies cause activation of the thyroid-stimulating hormone receptor in Graves' disease, which results in hyperthyroidism and increased thyroid hormone production. In MS, immune-mediated damage to the myelin sheath disrupts neural communication, resulting in neurological deficits.

Systemic autoimmune diseases involve widespread immune activation against multiple tissues and organs. Systemic lupus Erythematosus (SLE) is characterized by the generation of auto-antibodies against nuclear antigens, leading to immune complex deposition and multi-organ inflammation. Rheumatoid arthritis (RA) involves chronic joint inflammation due to autoreactive T and B cell activation, resulting in joint destruction and systemic complications. Sjögren's syndrome targets exocrine glands, leading to dry mouth and eyes, while inflammatory bowel disease (IBD) consists of conditions like Crohn's disease and ulcerative colitis, where immune dysregulation affects the gastrointestinal tract.

Autoimmunity - Pathogenesis

Multiple factors, including immunological dysregulation, triggers from the environment, and genetic predisposition, contribute to the onset of autoimmune disorders (3). Genetic factors play a critical role, with specific human leukocyte antigen (HLA) alleles being strongly associated with autoimmunity. (4) For example, HLA-DR4 is linked to RA, while HLA-DR3 and HLA-DR2 are associated with SLE and MS, respectively. Other genetic mutations, such as those affecting FOXP3 (a key regulator of Tregs) and AIRE (which governs central tolerance in the thymus), contribute to immune dysregulation and autoimmune disease susceptibility.

In people who are genetically susceptible, environmental variables including infections, UV rays, and nutritional effects can cause autoimmunity. Autoreactive T lymphocytes can be activated via molecular mimicry, in which microbial antigens imitate self-antigens. For instance, Epstein-Barr virus (EBV) has been implicated in triggering lupus, while Coxsackievirus is associated with T1D. Dysbiosis, or an imbalance in gut microbiome, has also been associated with autoimmune conditions, as the gut microbiome plays a role in immune regulation.

By changing the patterns of gene expression in immune cells, epigenetic changes such as DNA methylation and histone acetylation contribute to the pathophysiology of autoimmunity. These changes can sustain chronic inflammation and impair immune tolerance mechanisms. Understanding these pathogenic pathways is crucial for developing novel therapeutic strategies aimed at restoring immune balance and preventing autoimmunity progression.

Systemic Lupus Erythematosus (SLE) as an Autoimmune Disease

The production of antibodies against cytoplasmic and nuclear elements is a hallmark of systemic lupus Erythematosus (SLE), a chronic, multisystem autoimmune condition that results in tissue death and extensive inflammation. SLE mostly affects women, with a 9:1 female to male ratio, and typically occurs during the second to fourth decades of life (8). The disease is marked by periods of flares and remission, with patients often experiencing end-organ damage, particularly in the kidneys, skin, joints, and cardiovascular system (8,9).

The autoimmune nature of SLE is driven by absence of immune tolerance, leading to the production of autoantibodies such as double-stranded DNA (dsDNA), nucleosomes, and

ribonucleoproteins (8). These autoantibodies form immune complexes that deposit in various tissues, particularly the kidneys, leading to extensive inflammation and multi-organ destruction. The pathogenesis of SLE involves the whole immune system, with dysregulation of B and T cells, complement activation, and cytokine dysregulation playing key roles (8, 9).

Pathogenesis of SLE

SLE has a complicated pathophysiology that includes several immunological mechanisms. The collapse of immunological tolerance, which results in the generation of self-antibodies against nuclear antigens such dsDNA, histones, and nucleosomes, is a crucial characteristic (8). These autoantibodies form immune complexes that deposit in tissues, particularly the kidneys, leading to inflammation and tissue damage.

1. **Role of Complement System**

The complement system plays an important function in the pathogenesis of SLE. Complement components, particularly C1q, C2, and C4, are involved in the clearance of immune complexes. Deficiencies in these components are strongly associated with the development of SLE, as impaired clearance of immune complexes leads to their deposition in tissues, particularly the kidneys (8). Autoantibodies against C1q are commonly found in SLE patients and are strongly associated with lupus nephritis (LN). These autoantibodies bind to the collagen-like region of C1q, leading to complement activation and inflammation (8).

2. **Apoptosis and Autoantibody Formation**

Apoptosis, also known as programmed cell death, plays a very important role in pathogenesis of SLE. During apoptosis, nuclear components such as nucleosomes and dsDNA are exposed, which can act as autoantigens. In SLE, improper clearance of apoptotic cells accumulates nuclear debris, which triggers the production of autoantibodies (8). These autoantibodies create immune complexes that accumulate in organs, especially the kidneys, causing tissue damage and inflammation.

3. Role of Adaptive Immunity

The adaptive immune system, particularly B and T cells, plays a central role in the pathogenesis of SLE. Dysregulated B cells produce autoantibodies, while T cells, particularly Th1 and Th17 cells, contribute to inflammation and tissue damage (8). Toll-like receptors (TLRs), particularly TLR7 and TLR9, recognize nucleic acids and play a role in the activation of autoreactive B cells and the production of autoantibodies (8).

Clinical Presentation of SLE, Diagnosis, and Classification of Lupus

Clinical Presentation

SLE is a multisystem disease with a variable range of signs and symptoms which include fatigue, fever, weight loss, and joint pain. Skin manifestations such as malar rash, photosensitivity, and discoid lesions are also common (9). Renal involvement, known as lupus nephritis (LN), is a serious complication and occurs in 40-60% of SLE patients (8). Other manifestations include haematological abnormalities (anaemia, leukopenia, thrombocytopenia), serositis (pleuritis, pericarditis), and neurological involvement (seizures, psychosis) (9).

Diagnosis

The diagnosis of SLE is based on clinical criteria and laboratory findings. The American College of Rheumatology (ACR) and the Systemic Lupus International Collaborating Clinics (SLICC), these criteria are commonly used for diagnosis. Clinical manifestations including malar rash, arthritis, renal involvement, and haematological abnormalities are among these requirements, as are test results like low complement levels, antinuclear antibodies (ANA), and anti-dsDNA antibodies (9).

Classification of Lupus Nephritis

Lupus nephritis (LN) is classified based on renal biopsy findings according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) classification. The classification "includes six classes: Class I (minimal mesangial LN), Class II (mesangial proliferative LN), Class III (focal LN), Class IV (diffuse LN), Class V (membranous LN), and Class VI (advanced sclerosing LN)" (Sterner et al., 2014). Class IV LN is associated with poor renal outcomes (8).

Importance of Serum and Urinary Cytokine Levels in the Pathogenesis of Lupus Nephritis

1. **Role of Cytokines in SLE and Lupus Nephritis**

The pathophysiology of lupus nephritis and SLE is significantly influenced by cytokines. They mediate tissue injury, inflammation, and immune cell activation. Autoimmune complex formation, autoantibody synthesis, and autoreactive B and T cell activation are caused by dysregulation of cytokine production in SLE (10).

2. Serum Cytokine Levels

Patients with SLE, especially those with lupus nephritis, frequently have elevated levels of pro-inflammatory cytokines such interleukin-6 (IL-6), interleukin-1 (IL-1), and interferon-gamma (IFN-γ). These cytokines play a role in renal tissue injury and inflammation. In SLE, it has been demonstrated that IL-6 in particular correlates with activity of the disease and renal involvement (10).

3. Urinary Cytokine Levels

Urinary cytokine levels are increasingly recognized as important biomarkers for lupus nephritis. Elevated levels of cytokines such as IL-1, TNF-α, and IFN-γ have been observed in urine of SLE patients with renal involvement. These cytokines reflect local inflammation in the kidneys and may be used to monitor disease activity and response to treatment (10).

4. Cytokines as Biomarkers

Cytokines like IL-6, IL-1, and IFN-γ have been proposed as potential biomarkers for lupus nephritis. Elevated levels of these cytokines in the serum and urine are associated with active renal disease and can help predict renal flares (10). Furthermore, the combination of 'anti-dsDNA antibodies' and raised levels of cytokine has been demonstrated to correlate with greater renal disease activity synonymous to poor renal outcomes (8).

Role of Biomarkers in Lupus

Systemic Lupus Erythematosus (SLE) is a multifactorial autoimmune disorder that marks itself through a range of clinical stages affecting multiple organ systems. Traditional diagnostic markers, such as anti-dsDNA antibodies and complement levels, have limitations in sensitivity and specificity (11). Emerging biomarkers, including cytokines, chemokines,

and cellular molecules, provide an improved understanding of disease pathophysiology and hold potential for early diagnosis, disease monitoring, and prognostic evaluation (11).

Studies conducted in the last few years have highlighted the role of fluid-based biomarkers, such as "tumour necrosis factor-like weak inducer of apoptosis" (TWEAK), "neutrophil gelatinase-associated lipocalin" (NGAL), and "urinary monocyte chemoattractant protein-1" (uMCP-1), in lupus nephritis (LN). These biomarkers offer a less invasive approach for assessing renal involvement (11). Moreover, microRNAs (miRNAs), such as miR-21 and miR-146a, have demonstrated promising results in distinguishing between lupus patients with and without nephritis (12)

Laboratory Diagnosis: Evolving Concept with Biomarkers in SLE

Laboratory testing for SLE has evolved significantly with advancements in proteomics and metabolomics. Traditional serological tests, including ANA, anti-dsDNA, and complement levels, are still widely used, but their limitations necessitate the inclusion of novel biomarkers (11). Omics technologies have identified promising candidates, such as IL-6, IL-17, and pentraxin-3 (PTX3), which exhibit high sensitivity and specificity in distinguishing active SLE from remission (13).

A biomarker panel approach has been suggested as an improvement over single-marker diagnostic. Panels comprising anti-HIST1H4A, S100A4, C3dg, TNFRII, and IGFBP2 have demonstrated high diagnostic accuracy (12). Additionally, urine-based markers such as CD163, PGRN, VCAM1, and angiostatin have been used to differentiate between activity and inactivity in lupus nephritis (12). This evolving concept in laboratory diagnosis underscores the need for integrating these markers into routine clinical practice.

Novel Biomarkers for SLE

Recent research has identified several novel biomarkers for SLE that offer improved diagnostic and prognostic value.

- Cytokines and Chemokines: IL-10, IL-17, MCP-1, and IP-10 have been associated with disease activity (11).
- MicroRNAs: Circulating miR-21, miR-146a, and miR-29c have demonstrated potential in differentiating lupus nephritis from non-nephritis SLE (12).
- Autoantibodies: Emerging autoantibodies, such as anti-C1q and anti-α-enolase, have shown strong correlations with lupus nephritis progression (11).
- Serum Proteins: Elevated levels of AXL, ferritin, and sTNFRII have been noted in active lupus nephritis (13).

These novel biomarkers not only enhance diagnostic accuracy but also provide potential therapeutic targets for personalized medicine.

Role of Serum and Urinary IL as Potential Biomarkers for SLE Diagnosis

Interleukin (IL)-12 has been implicated in lupus nephritis pathogenesis. Studies indicate that both serum and urinary IL-12 levels correlate with lupus nephritis severity and Th1 immune polarization (13). Elevated urinary IL-12 reflects glomerular accumulation, making it a strong candidate for non-invasive monitoring (13).

Similarly, IL-17 and IL-23 have been associated with lupus nephritis activity. ROC curve analyses have demonstrated that these cytokines exhibit high predictive values for active

lupus nephritis (14). Urinary markers such as uAPRIL and uBAFF also show promise in distinguishing nephritic from non-nephritic SLE (14)

1. IL-12 and its Role in Immune Response

Dendritic cells and macrophages, which functions as antigen-presenting cells (APCs), are the major producers of interleukin-12 (IL-12), a heterodimeric cytokine made up of p35 and p40 subunits. By encouraging Th1 differentiation and increasing the production of interferon-gamma (IFN-γ), it plays a crucial role in coordinating immunological responses (15). This cytokine facilitates the activation of cytotoxic T lymphocytes and natural killer (NK) cells, contributing to pathogen clearance (15).

IL-12 is functionally linked to the IL-23/Th17 axis, which has emerged as a critical pathway in autoimmune diseases, including SLE (15). While IL-12 favours Th1 differentiation, IL-23 promotes Th17 activation, leading to IL-17 secretion. These pathways are implicated in inflammatory and autoimmune responses, and their dysregulation has been associated with SLE pathogenesis (15).

2. Role of IL-12 in Lupus Nephritis

A longitudinal comparison of cytokine profiles in SLE patients revealed significantly higher IL-12 levels in childhood-onset SLE as compared to adult-onset SLE. This suggests a stronger Th1-driven immune response in younger patients, which could influence disease severity and progression (16). Moreover, IL-12 levels were associated with leukopenia and alopecia in adult-onset SLE, emphasizing its role in systemic immune dysregulation (16).

One of the most dangerous complications of SLE is Lupus Nephritis, characterized by immune complex deposition and subsequent renal inflammation. Quite a few studies have

highlighted the function of IL-12 in the pathogenesis of LN. IL-12 quantities have been found to be increased in serum and urine of LN patients, correlating with disease activity (13).

A study by Tucci et al. showed increased IL-12 expression in glomerular mononuclear cells in LN patients, particularly in class IV and V LN. The findings indicated that IL-12 accumulation in renal tissue correlates with Th1 polarization, contributing to renal damage (13). Additionally, IL-12 enhances IFN-γ production, which further exacerbates inflammation and tissue injury (13).

3. Serum and Urinary IL-12p40 as a Potential Biomarker

A common component of IL-12 and IL-23, IL-12p40, has drawn interest as a possible biomarker for disease activity in SLE and LN. While some studies have found elevated IL-12p40 levels in active LN, others have reported inconsistent associations (17).

A study evaluating biomarkers for clinical activity in SLE found no statistically significant elevation in IL-12p40 levels in SLE patients as compared to patients without the disease (17). However, another investigation highlighted IL-12p40 as a novel biomarker for predicting minimal change disease relapse after treatment with glucocorticoid, suggesting its broader relevance in nephropathies (18).

Despite these conflicting findings, urinary IL-12p40 remains a promising non-invasive marker for LN assessment. Elevated urinary IL-12 levels correlate with glomerular inflammation, and its detection could serve as an early indicator of nephritic activity (17). Future studies should focus on validating IL-12p40 as a biomarker through multi-center clinical trials.

miRNA Definition and Role in Immunity

Definition: "MicroRNAs (miRNAs) are small, non-coding RNA molecules (~22 nucleotides) that regulate gene expression at the post-transcriptional level by binding to target messenger RNA (mRNA) molecules, leading to translational repression" (19).

Discovered in the early 1990s, miRNAs play critical roles in immune system regulation, influencing both the types of immune responses (19).

In inmate immunity, miRNAs modulate Toll-like receptor (TLR) signalling, cytokine production, and macrophage differentiation (19). miRNA-146a, for example, acts as a negative regulator of pro-inflammatory cytokines such as IL-6 and TNF-α by targeting TRAF6 and IRAK1(20). miRNA-155, on the other hand, enhances inflammatory responses by promoting cytokine secretion and antigen presentation (20). In adaptive immunity, miRNAs control T and B cell differentiation, proliferation, and function. For instance, miRNA-142-3p is very important for T cell homeostasis, and its dysregulation has a connection to autoimmune disorders (21).

miRNA and Role in Autoimmunity

Systemic lupus Erythematosus (SLE) is one of the autoimmune illnesses characterized by dysregulated miRNA expression. Patients with lupus have abnormal miRNA profiles, which may indicate a role in the development and course of the disease (19).

Several miRNAs have been seen to have key role in SLE pathogenesis. miRNA-146a, for instance, is downregulated in SLE, leading to excessive inflammatory responses due to impaired regulation of NF-κB signalling (20). Conversely, miRNA-155 is upregulated in

SLE, contributing to hyperactivity of B cells and autoantibody production (20). Similarly, miRNA-142-3p has been observed to be expressed differentially in lupus nephritis (LN) patients, highlighting its potential involvement in renal inflammation (21).

Moreover, miRNA-130b-3p was found to be increased in early-stage LN patients, correlating with proteinuria and renal damage (22). This suggests that miRNA-130b-3p might serve as a potential marker for early detection of kidney involvement in SLE (22)

Detection of miRNA and Role in Diagnosis of Lupus as a Novel Marker

miRNA profiling has emerged as a promising tool for detection and monitoring lupus. Detection methods include "quantitative real-time polymerase chain reaction" (qRT-PCR), microarrays, and "next-generation sequencing" (19). These techniques allow for the identification of specific miRNA expression patterns associated with SLE activity and severity (19).

For the diagnosis and prognosis of lupus, a number of miRNAs have been suggested as new biomarkers. It's been observed that serum levels of 'miRNA-146a' and 'miRNA-155' correlate with the activity of lupus nephritis, indicating that they may be used to monitor the condition (20). Similarly, miRNA-142-3p expression levels can distinguish between lupus patients with and without nephritis, indicating its diagnostic value (21).

Furthermore, miRNA-199 expression is significantly decreased in lupus patients, particularly in those with nephritis, suggesting its role in disease progression (19). The evaluation of miRNA-199 levels could aid in the early diagnosis of lupus nephritis, enabling timely intervention (19)

METHODOLOGY

Study design: One year cross- sectional study

Study population: Materials of the study are collected, from SLE positive patients attending medicine OPD or admitted in medicine ward of Dr Prabhakar Kore Charitable Hospital, Belagavi.

- Urine sample was collected in an universal container.
- 2ml was aliquoted and deep freezed at -80°C.

Processing: The urine samples were collected from patients diagnosed positive for Systemic Lupus Erythematosus, attending medicine OPD or admitted in medicine ward or in Nephrology ward of Dr. Prabhakar Kore Charitable Hospital, Belagavi. The samples were stored in -80°C, and were brought to room temperature at the time of processing.

KIT details:

Manufacturer: KRISGEN BioSystems

Name: Human IL-12/23 P40 GENLISA^{TM} ELISA kit

Ref no: KB1074

LOT: IL12P400324

Date of expiry: 28.02.2025

Inclusion criteria:

All clinically diagnosed SLE patients attending Medicine OPD or admitted in medicine ward Dr Prabhakar Kore Charitable Hospital, Belagavi.

Exclusion criteria : NIL

Sampling size:

$$n = \left\lfloor \frac{Z_\alpha + Z_\beta}{c} \right\rfloor^2 + 3 \qquad c = 0.5 \ln \left\lfloor \frac{1+r}{1-r} \right\rfloor$$

From previous article M. Tucci 2008, there was found to be a positive correlation between serum IL-12p40 and IFN-**γ**, r = 0.59.

Substituting r = 0.59 in above equation, we get c = 0.675
For α = 5% and β = 5% we get,

$$n = \left\lfloor \frac{1.96+1.644}{0.675} \right\rfloor^2 + 3$$

$$= (5.33)^2 + 3$$

$$= 28.40 + 3$$

$$= 31.4 \sim \mathbf{32}$$

After thawing the sample, following the kit insert manual, reconstitution of the standard, detection antibody, and wash buffer was done. After preparation of the middle stock solution, serial dilution of the standard was done to achieve the following standard concentration.

Standard concentration	**Standard number**
1 µg/ml	Standard - lyophilized
10 ng/ml	Middle Stock
2000 pg/ml	Standard No. 7
1000 pg/ml	Standard No. 6
500 pg/ml	Standard No. 5
250 pg/ml	Standard No. 4
125 pg/ml	Standard No. 3
62.5 pg/ml	Standard No. 2
31.3 pg/ml	Standard No. 1

Table 1 : serial dilution of the standard

Assay Procedure:

Add 100µl of **Standard and sample** to the plate, then add 50µl of diluted **Detection Antibody**. Seal plate.

↓

Incubate it at 37°C for 2 hours

↓

Aspirate and Wash plate 4 times with **wash Buffer (1X)**

↓

Add 100µl of diluted **Streptavidin-HRP** solution to each well and then seal the plate.

↓

Incubate it at 37°C for 30 minutes

↓

Aspirate and Wash plate 4 times with **wash Buffer (1X)**

↓

Add 100µl of ready-to-use TMB Substrate solution. Incubate the plate in dark at 37°C for 30 minutes

↓

Add 100µl **Stop solution.** Positive wells should turn from blue to yellow.

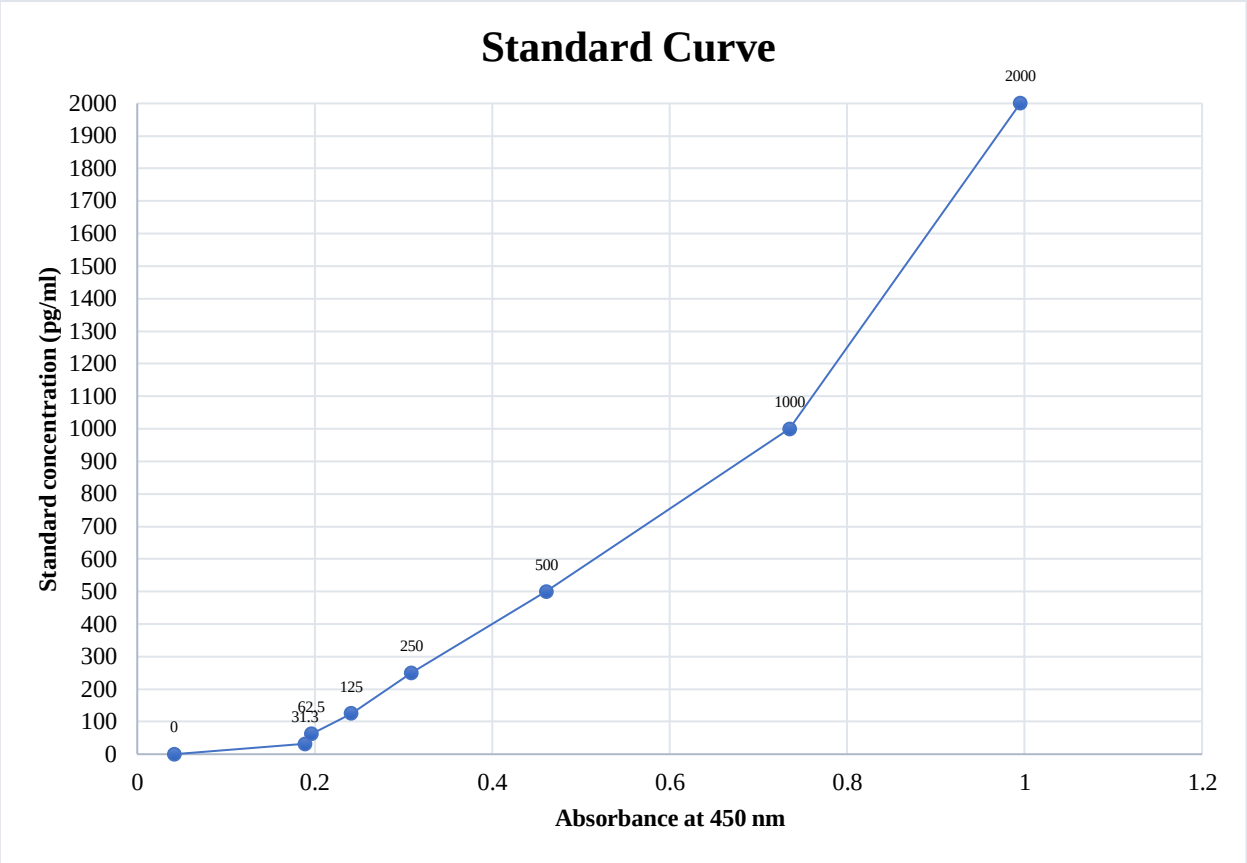
↓

Read the Absorbance at 450 nm within 30 mins of adding stop solution

The samples were processed using ***KRISHGEN BioSystems*** Human IL-12/23 P40 GENLISA™ ELISA kit as mentioned in the methodology. The Standards were run in duplicates as per the instructions in the manual.

The mean absorbance, for each set of duplicates of the standards, were taken and the absorbance value of the zero standards were subtracted. Standard curve was plotted

as per the kit insert instruction, by plotting cytokine concentration on the y-axis and plotting the absorbance values on x-axis (Graph 1). The serum samples were collected from the patient clinically relating to SLE and sent to the laboratory for **ANA profile**, which was done using IMMUNIBLOT technique (EUROIMMUN). **Complement levels** were measured using CLIA. **Renal biopsies** were categorized according WHO/ ISN/ RPS Classification.



Graph 1 : Plotting of different concentration of standards against the absorbance value at 450 nm.

Inferential Statistics:

These were the formulas used to find the association between two variables in our study:

1. **Chi-square test:**

'The chi-squared test is done to check if there is any difference between the observed value and expected value'. The formula for chi-square can be written as;

$$X^2 = \sum \frac{(\text{Observed value} - \text{Expected value})^2}{\text{Expected value}}$$

2. **Fisher's exact Test:**

$$P = \frac{(a+b)!\,(c+d)!\,(a+c)!\,(b+d)!}{(a!\,b!\,c!\,d!\,n!)}$$

Outcome category 1	Outcome category 2	Total	
Exposure category 1	**A**	**b**	**a + b**
Exposure category 2	**C**	**d**	**c + d**
Total	**a + c**	**b + d**	**a + b + c + d = n**

RESULTS:

Out of 35 patients who were included in the study, 30 were females. Gender distribution is depicted in Table.2.

Gender	No of Cases	Percent
Male	5	**14.3**
Female	30	**85.7**
Total	**35**	**100.00**

Table 2: Gender distribution of SLE patients

In the present study, amongst the patients diagnosed for SLE, females were predominant in number 30 (85.7%), as compared to males 5 (14.3%), as demonstrated in Table 2. The male: female ratio is **1: 6.**

Age (in years)	No of Cases	Percent
10-20	5	14.3
21-30	12	34.3
31-40	8	22.9
41-50	7	20.0
51-60	1	2.9
61-70	2	5.7
Total	35	100.0

Table 3: Age distribution of SLE patients

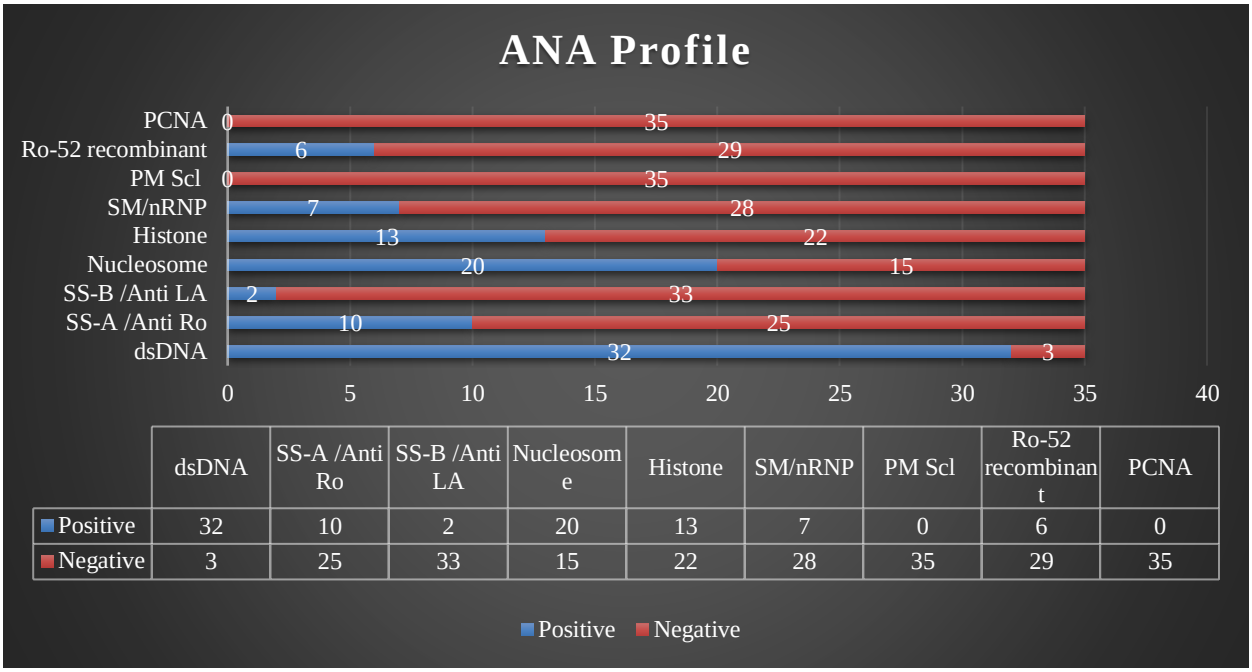
Amongst the samples, most patients belonged to the age bracket of 20-30 years of age, 12 in number (34.3%), followed by people belonging in the age group of 30-40 years of age (22.9%) and 40-50 years (20%). There were no patients below 20 years of age, as shown in Table 3.

Staging	No of Cases	Percent
II	2	5.7
III	16	45.7
IV	10	28.7
V	4	11.4
CKD – Stage V	2	5.7
Biopsy Not done	1	2.8
Total	35	100.0

Table 4: Distribution of Lupus nephritis according to classes based on histopathology reports

The samples were divided into different classes of Lupus Nephritis, as mentioned in methodology, which are shown in table. Out of 35 samples, 16 belonged to Class III, 10 belonged to Class IV, 4 belonged to class V and 2 patients belonged to Class II. 2 patients who had had biopsy report showing CKD – Stage V, were also found to be negative for ANA Profile.

Out of the 35 patients who were tested for ANA profile, serum biomarker **dsDNA** was positive in = 32 (88.6%) of them, followed by nucleosome = 20 (57.1%), histone = 13 (37.1%), SS-A = 10 (28.6%), SM/nRNP = 7 (20%), Ro-52 recombinant = 6 (17.1%), and SS-B = 2 (5.7%). None of the patients showed positive for biomarkers PM Scl and PCNA, as shown in Table 4.



	dsDNA	SS-A /Anti Ro	SS-B /Anti LA	Nucleosome	Histone	SM/nRNP	PM Scl	Ro-52 recombinant	PCNA
Positive	32	10	2	20	13	7	0	6	0
Negative	3	25	33	15	22	28	35	29	35

Graph 2 : ANA Profile distribution

Anti-dsDNA as a Sensitive or Specific Marker in Lupus Nephritis:

The sensitivity and specificity of anti-dsDNA in our study were analysed as follows: **The Anti-dsDNA Positivity in Different Lupus Classes in our study were as follows**: **Class III:** 87.5% (14/16) **Class IV:** 90% (9/10) **Class V:** 75% (3/4) **Class II:** 50% (1/2) **CKD Stage V:** 0% (0/2)

Taking the assay range of IL-12p40 From 31.3 pg/ml to 2000 pg/ml, and cutoff value of IL-12p40 as 108.78 pg/ml (5)**, 10 samples were positive for urine IL-12p40**

Staging	Urine IL-12p40 (pg/ml)		Chi Square test	
	Positive		$\chi 2$ Value	P Value
	Number (%)	%		
II	0	0	1.338	0.72
III	5	50		
IV	4	40		
V	1	10		
Total	10	100		

Table 5: Staging and Urine IL-12p40 positives (pg/ml)

Amongst the 10 positive samples for IL-12p40, 5 patients were from Class III, which consists of 50% of the overall positive samples for IL-12p40, 4 patients were from Class IV, which comprises of 40 % of overall positive samples for IL-12p40, and 1 from Class V (10%). Most IL-12p40 positive cases were in **stage III (50%)** and **stage IV (40%)**. **p-value = 0.72**, indicating no significant association, as seen in table 5.

Urine ILp40 (pg/ml)	C3 Level		Total
	Positive	Negative	
Positive	6	4	10
Negative	12	13	25
Total	18	17	35

Table 6 : Association between number of urine IL-12p40 positive patients with C3 positivity

Urine ILp40 (pg/ml)	C4 Level		Total
	Positive	Negative	
Positive	8	2	10
Negative	14	11	25
Total	22	13	35

Table 7 : Association between number of urine IL-12p40 positive patients with C4 positivity

A comparison was done between C3 and C4 levels in the overall patients which showed C3 levels being more out of normal range as compared to C4 levels, which shows that C3 is more specific than C4 in detecting disease activity in these patients.

The cut-off values for C3 and C4 were taken as <90 and <10 respectively. Any value below these were considered to be low (taken as positive), which signified positive disease activity at the time of sample collection.

The total sample consists of **35 patients**, divided into **18 (51.4 %)** with positive C3 levels and **17 (48.6 %)** with negative C3 levels. The distribution of lupus classes does not significantly differ between those with positive and negative C3 levels.

The total sample consists of **35 patients**, divided into **22 (62.8 %)** with positive C4 levels and **13 (37.2 %)** with negative C4 levels. Class III and IV show a 45% distribution in both positive and negative C4 levels. Class V has a higher proportion of negative C4 levels (25%) compared to positive (5%). Since this is **greater than 0.05**, it indicates that the differences observed in C3 levels across lupus classes **are not statistically significant**. **Correlation:** There is No statistically significant association ($p > 0.05$) between lupus class and C4 levels.

Urine ILp40 (pg/ml) - Positive								
Proteinuria							Chi Square test	
	Class III		Class-IV		Class-V		$\chi 2$ Value	P Value
	No	%	No	%	No	%		
1+	0	0	0	0	0	0	7	0.321
2+	1	17	0	0	0	0		
3+	4	67	2	67	0	0		
4+	1	17	1	33	1	100		
Traces	0	0	0	0	0	0		
Negative	0	0	0	0	0	0		
Total	6	100	3	100	1	100		

Table 8: Association between number of urine IL-12p40 positive patients with Proteinuria positivity

The table categorizes lupus into Classes II, III, IV, and V while analysing the presence or absence of proteinuria. The association between urine IL-12p40 positivity and proteinuria in lupus nephritis (LN) was analysed across different histological classes (III, IV, and V). While Class III had the highest proportion of proteinuria cases (67% at 3+ level), followed by Class IV (67% at 3+ and 33% at 4+), Class V was exclusively associated with severe proteinuria (100% at 4+). Despite these findings, the chi-square test indicates no statistically significant correlation ($p = 0.321$), suggesting that urine IL-12p40 levels may not directly influence proteinuria severity.

DISCUSSION:

1. Gender and Age Distribution in Lupus Nephritis

Our study found a strong female predominance (85.7%) among lupus nephritis (LN) patients, with the most affected age group being **21-30 years**. This aligns with previous findings in '**Shivaprasad et al.' (2016)** and '**Talukdar et al.' (2020)**, which also reported a higher prevalence of LN in females due to hormonal influences and immune system differences. Lupus nephritis (LN) is more common in women primarily due to **hormonal (**Estrogen enhances B-cell activity, causing autoantibodies to be produced, which are a hallmark of systemic lupus Erythematosus)**, genetic** (Women have two X chromosomes, whereas men have one, which have been shown to carry immune-related genes, such as those involved in the regulation of Toll-like receptors (TLRs) and cytokine production)**, and immunological factors** (Women are evidenced to have **higher levels of B cells and antibodies**, raising the chance of developing autoimmune)

However, some studies, such as **Palazzo et al. (2022)**, show that although lupus is more prevalent in women, the disease tends to proceed more severely in men. This aspect was not explored in our study and could be an area for further investigation.

2. Distribution of Lupus Nephritis Classes

Our results showed that Class **III (45.7%)** and **IV (28.7%)** were the most common forms of LN, with lower frequencies of Class **V (11.4%)** and Class **II (5.7%)**. These findings are consistent with **Mok (2010)** and **Aragón et al. (2020)**, who also noted that proliferative forms (Classes III & IV) dominate LN cases, while Class V occurs less frequently but presents with **higher proteinuria levels**, a sign of chronic kidney damage (**Zandi-Nejad et al., 2004**).

The gold standard for lupus nephritis (LN) diagnosis and staging is kidney biopsy. In lupus nephritis, kidney biopsy staging is essential for establishing a treatment plan, tracking the course of the illness, and forecasting renal survival. For example: **Class III & IV (Proliferative LN)** shows high risk of **renal failure** if untreated. It requires aggressive immunosuppression. **Class V (Membranous LN)** is associated with **severe proteinuria**, but progression to kidney failure is slower than Class IV. It is often treated with immunosuppression. **Class VI (Advanced Sclerosis)** indicates **chronic irreversible damage**

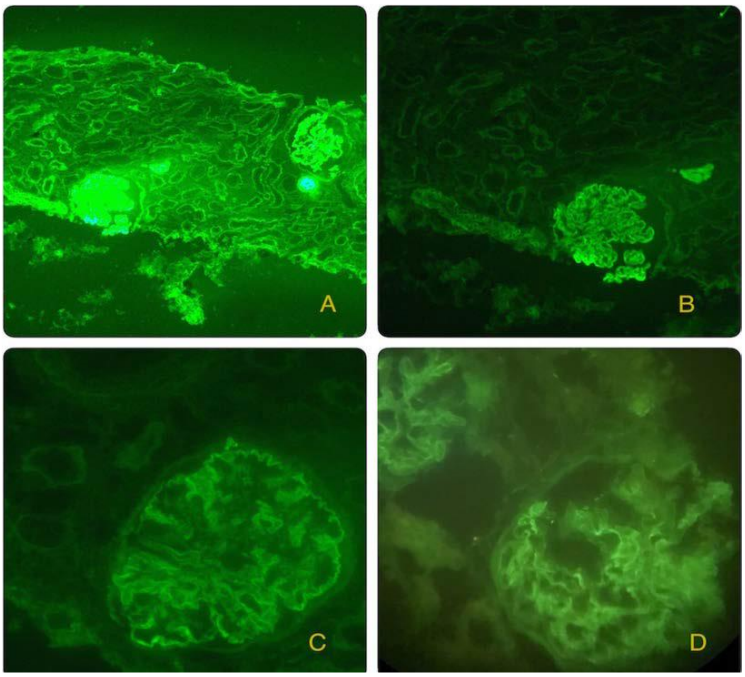


Figure 3: Image of Immunofluorescence in nephritis due to Lupus A: IgG; B: C 3 ; C: IgM. ("Buch A, et al. Role of renal biopsy in evaluation of morphological spectrum and pathogenesis of lupus nephritis. Annals of Pathology and Laboratory Medicine" (2017).)

3. Role of Anti-dsDNA in Lupus Nephritis

Our study confirmed **high sensitivity (>85%) of anti-dsDNA** not only in Class III but also in Class IV LN, with decreasing sensitivity in Classes V and II. This matches findings by **Brunmer et al. (2017)** and **Stanley et al. (2020)**, which also found anti-dsDNA to be a strong predictor of **active LN**, particularly in proliferative forms. However, our results indicate that anti-dsDNA is **not specific for differentiating between LN classes**, a limitation also discussed in **Mok (2010)**.

Anti-dsDNA is **highly sensitive (>85%) in Class III and IV** lupus nephritis, supporting its role as a useful marker in active disease.

Furthermore, **Peliçari et al. (2022)** suggest that while anti-dsDNA is useful in diagnosis, its fluctuations may not always correspond to disease severity, reinforcing our conclusion that **multiple biomarkers are necessary** for precise disease monitoring.

However, the **specificity of anti-dsDNA was limited in Class V (75%) and Class II (50%)**, suggesting that while it is **valuable for diagnosing lupus nephritis**, it may not reliably differentiate **between different classes.**

4. Complement Levels (C3 and C4) and Their Correlation with Lupus Nephritis Severity

Our study showed that **C3 and C4 depletion correlated with severe lupus nephritis** (Class IV and V) but lacked statistical significance in differentiating disease stages (**$p > 0.05$**). These findings align with **Tucci et al. (2008)** and **Nawata et al. (2023)**, who reported that

while low C3 and C4 levels indicate **immune complex activation**, they do not always correlate with histopathological severity.

C3 positivity was highest in Class III lupus nephritis (52.9%), suggesting that C3 remains detectable in early to moderate disease. C3 depletion was most significant in Class IV (80%) and Class V (75%), indicating severe disease progression associated with complement consumption due to immune complex deposition. C4 Levels and Disease Severity C4 depletion was observed across all lupus nephritis classes, with the highest negative rates in Class V (75%) and Class IV (55%).

Interestingly, our findings contrast with **Guo et al. (2024)**, who identified **urinary complement biomarkers** as more accurate indicators of LN progression than serum C3 and C4 levels. This suggests that additional studies incorporating **urinary biomarkers** may provide a clearer correlation.

5. Cytokine and Autoimmune Biomarkers in LN

Our study primarily focused on **anti-dsDNA and complement levels**, whereas recent literature emphasizes **cytokines and novel biomarkers**. For example:

IL-12 and Th17 cytokines have been identified as key mediators of LN progression (**Larosa et al., 2019; Wong et al., 2000**).

MicroRNAs (miR-124-3p, miR-377-3p) are emerging as LN biomarkers (**Yan et al., 2022**). **Urinary cytokines (IL-18, IL-17, IL-12)** were elevated in lupus patients (**Pacheco et al., 2017**), suggesting they may complement traditional markers like C3/C4.

6. Urinary IL-12: A Proinflammatory Cytokine in Lupus Nephritis

Th1 cell differentiation is significantly influenced by the cytokine IL-12, which is mostly generated by antigen-presenting cells. Elevated IL-12 levels indicate an inflammatory response in SLE. Specifically:

- IL-12p40, a subunit of IL-12, has been found elevated in urine samples of lupus nephritis patients, as shown by Bai et al. (2022).
- Tucci et al. (2008) reported increased expression of IL-12 in lupus nephritis, linking it to increased Th1-mediated inflammation and immune complex deposition.
- "Aragón et al." (2020) discussed the role of biomarkers in the urine samples of patients with lupus nephritis, where IL-12p40 was identified as a potential marker of inflammation of the renal parenchyma.

The presence of **IL-12 in urine suggests its role in local kidney inflammation**, as cytokines like IL-12 are not typically excreted in urine unless there is **significant renal damage or immune activation**. The two subunits that make up the heterodimeric structure of IL-12 are p40 (IL-12B) and p35 (IL-12A). The production of the physiologically active cytokines is dependent on the expression of p40 and is strictly controlled by the p35 subunit. In contrast, p40 subunit levels is greater than that needed for p35p40 assembly. (15)

7. Correlation Between C3, C4, and Urinary IL-12 in Lupus Nephritis

The interaction between complement proteins and IL-12 levels plays an important role in lupus nephritis pathophysiology:

❖ 60% of C3 positive cases had IL-12p40 positivity. **Sensitivity = 33%,** Specificity = 76%, PPV = 60%, NPV = 52%, Diagnostic Accuracy = 54%

- 80% of C4 positive cases had IL-12p40 positivity. **Sensitivity = 36%,** Specificity = 85%, PPV = 80%, NPV = 44%, Diagnostic Accuracy = 54%

❖ **C3 and C4 Consumption and IL-12 Elevation in Active Disease**

- When C3 and C4 levels drop, it often indicates active complement activation and immune complex deposition in lupus nephritis.
- This process triggers macrophages and dendritic cells, leading to increased IL-12 secretion (Tucci et al., 2008; Bai et al., 2022).

❖ **IL-12 and Th1/Th17 Activation Leading to Further Complement Activation**

- Elevated IL-12p40 promotes Th1 responses, leading to an increase in pro-inflammatory cytokines like IFN-γ and TNF-α, which further activate the complement cascade (Larosa et al., 2019).
- This creates a vicious cycle, where inflammation leads to more complement activation, further depleting C3 and C4 levels (Mok, 2010).

❖ **A Measure of Clinical Activity in Patients with Low C 3 and C 4 Levels: Urinary IL-12**

- Patients with active lupus nephritis exhibit both low C3/C4 and high urinary IL-12 levels, as evidenced from our study, which is in line with the studies of Stanley et al. (2020) and "Guo et al." (2024). We can relate, although inconclusively, that urinary biomarkers including IL-12 could complement traditional serum biomarkers like C3/C4 in predicting disease flares.

Since our study did not assess most of the cytokines, it may be beneficial to integrate these markers in to improve disease monitoring in the future, if evident with more positive results.

8. Association of Urine IL-12p40 with Proteinuria

Tucci et al. (2008) highlighted the overexpression of IL-12 in LN, linking it to Th1-dominated immune responses that drive renal inflammation and tissue damage. However, proteinuria results from glomerular injury and podocyte dysfunction, processes influenced by multiple cytokines beyond IL-12p40. The findings of our study, which **indicate no strong correlation between urine IL-12p40 and proteinuria**, suggest that while IL-12p40 is involved in immune activation, it may not be a direct contributor to glomerular permeability changes leading to proteinuria.

Zandi-Nejad et al. (2004) emphasized that proteinuria is a key marker of progressive kidney disease, often linked to podocyte injury and loss of glomerular integrity. While cytokines like IL-12p40 contribute to inflammation, proteinuria is more closely tied to direct glomerular injury, mesangial expansion, and complement activation. The findings from our study suggest that IL-12p40, while relevant in inflammatory cascades, does not have a definitive role in predicting proteinuria severity.

CONCLUSION

Our study reinforces the role of **anti-dsDNA as a highly sensitive marker (>85%)** in active LN (particularly in **Class III and IV**), but its **specificity remains limited** in differentiating between classes. **IL-12p40** appears to be associated **advanced disease stages (III and IV)**, but these associations were not statistically significant in most cases. **Complement levels (C3 and C4)** showed some correlation with IL-12p40 positivity, but the diagnostic accuracy was moderate, suggesting that IL-12p40 may not be a standalone biomarker for lupus nephritis.

Our findings highlight the **limitations of relying solely on complement levels (C3/C4) and serum ANA markers as markers of disease severity** in lupus nephritis. Although **C 3 and C 4 depletion are classically linked with active SLE**, our results indicate that **their correlation with renal biopsy findings is inconsistent**. The lack of strong statistical significance suggests that additional biomarkers may provide a comprehensive assessment of disease activity.

Literature supports the function of IL-12 in inflammation in LN, but its presence in urine **may reflect broader immune activation.**

The lack of significant associations in many analyses highlights the **complexity and multifactorial nature** of lupus nephritis, where multiple biomarkers and clinical factors interact to influence disease activity and progression.

Implications for Future Research:

- The findings in our study suggest that **IL-12p40** might be a useful biomarker in lupus nephritis, particularly in **advanced disease stages** and in **females**, but it should be used in conjunction with other biomarkers (e.g., proteinuria, complement levels, autoantibodies) for a more comprehensive assessment.
- The **near-significant trend** with **SS-A/ Anti Ro** warrants further investigation in larger studies to determine its potential as a predictive biomarker.
- Longitudinal studies should be the prime focus of future studies, to estimate the dynamic changes in IL-12p40 levels over time and their correlation with activity of the disease, treatment response, and outcomes.

STRENGTH AND LIMITATIONS

Strengths of the Study

1. **Novel Biomarker Evaluation**

    - The study investigates **IL-12p40 as a potential urinary biomarker** for lupus nephritis, which is a relatively unexplored area. This contributes to the ongoing search for more reliable markers beyond traditional ones like anti-dsDNA and complement levels.

2. **Comprehensive Analysis of Biomarkers**

    - The study correlates IL-12p40 levels with **ANA profile, C3, and C4 complement expression**, allowing for a broader understanding of its role in lupus nephritis severity.

3. **Use of ELISA for Biomarker Quantification**

    - The use of a **standardized ELISA-based approach** ensures precision in measuring IL-12p40 levels, increasing the study's reliability.

4. **Clinical Relevance**

    - The study includes **patients with varying stages of lupus nephritis**, providing insight into how IL-12p40 behaves across different severity grades.

5. **Well-Defined Inclusion Criteria**

- The study includes all **clinically diagnosed SLE patients**, ensuring a uniform patient population for better comparability.

6. **Correlation with Disease Severity**

   - The study attempts to **link IL-12p40 levels with lupus nephritis severity**, adding potential clinical value in predicting disease progression.

7. **Gender and Age Considerations**

   - The study acknowledges the **gender disparity in lupus nephritis**, reinforcing the fact that lupus predominantly affects females.

Limitations of the Study

1. **Sample Size (N=35)**

    - The study is conducted on **a limited number of patients**, which may reduce its statistical power and generalizability.

2. **Lack of Longitudinal Data**

    - The study is **cross-sectional**, meaning it captures only a snapshot of IL-12p40 levels rather than tracking changes over time, which would have provided stronger clinical insights.

3. **No Control Group for Comparison**

    - Since there is no healthy control group or non-lupus nephritis SLE patients in the research, it is challenging to evaluate IL-12p40's specificity for lupus nephritis.

4. **Limited Statistical Significance in Correlations**

    - The results showed **no strong statistical significance** in the correlation between IL-12p40 levels and complement (C3, C4), making it challenging to draw definitive conclusions.

5. **Lack of Other Biomarkers for Comparison**

    - While the study evaluates IL-12p40, it does not include **other cytokines like IL-6, IL-17, or TNF-α**, which could have strengthened the comparison.

6. **Potential Confounding Factors Not Addressed**

    - Factors such as **medications, disease duration, and co-existing infections** were not extensively controlled, which could impact IL-12p40 levels.

7. **Urine IL-12p40 Not Directly Linked to Proteinuria**

    - The study finds **no significant association between urine IL-12p40 and proteinuria**, limiting its utility as a standalone marker for renal disease severity.

8. **Single-Center Study**

    - The study is conducted at **a single institution**, which may not fully represent diverse populations or account for regional variations in lupus nephritis presentation.

SUMMARY

C3 and C4 depletion are common in lupus nephritis but are not always reliable for disease staging. Anti-dsDNA is a highly sensitive marker (88%) but lacks specificity in certain lupus nephritis classes. Our thesis provides valuable insights into the role of **IL-12p40** in lupus nephritis, highlighting its potential as a biomarker while also underscoring the need for a **multi-marker approach** to better understand and manage this complex disease. The results show potential for more study in this field and add to the ever-expanding corpus of knowledge on lupus biomarkers.

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ANNEXURE – I

KAHERs JNMC BELAGAVI

INFORMED CONSENT FORM

"EVALUATE IL-12P40 LEVELS IN DIFFERENT STAGES OF LUPUS NEPHRITIS" – ONE YEAR CROSS-SECTIONAL STUDY.

Name of Student/Principal Investigator:

Name of Guide/Co Investigators:

Introduction: Systemic lupus erythematosus (SLE) is an inflammatory autoimmune disease characterized by diverse autoantibodies and varied clinical features. In clinical practice complement, autoantibodies including anti-ds DNA antibody, leukocyte count and urinalysis, are used to monitor the progress of lupus nephritis. These markers correlate better with certain clinical manifestations of the disease (especially nephritis) than the disease activity itself.

Explanation of procedure: Urine sample is collected in an universal container, stored in -80°C and Urine IL-12p40 values are estimated and corelated with serum ANA values.

Withdrawal from participation in the study: Participation in this study in voluntary. You will be free to decide whether to participate in this study or continue participation once enrolled. In case you decide to withdraw your participation, you are free to do so. However, please convey the decision to the principal investigator.

Possible benefits from participating in the study: You will know your Urine IL-12p40 value by participating in this study, so a prognostic value can be given along with treatment. The data gathered will help population at large.

Possible risks from participating in the study: There are no risks involved in participating in this study.

Privacy and confidentiality: The information collected from you will be coded, to prevent any person to identify you. Your identity will never be revealed. The data collected from you will be kept confidential and only processed or aggregated data will be used for publication.

Financial incentives: You will not receive any payment for participating in this study.

Cost of investigations done during the course of study will be paid by the **principal investigator.**

Authorization for publication of aggregated data: Results obtained after processing of the aggregated data will be published for scientific purpose and or presented to scientific groups. However, your identity will never be revealed.

Questions: In case of any questions with regard to this study, you are free to contact:

If you have any question or complaints with regard to your right as study participant you may contact Dr Harsha Hegde, Chairperson, Ethical committee of JNMC, 0831-2473777 Extension 4052.

Legal rights: By signing this consent form, we are not waving any of your legal rights

CONSENT STATEMENT

I am making a voluntary decision to participate in the study **"EVALUATE IL-12P40 LEVELS IN DIFFERENT STAGES OF LUPUS NEPHRITIS".** My signature below indicates that I have decided to participate and I have read the information provided above or the information provided above has been read to me in the language that I understand best. I was given the opportunity to ask questions and that they have been answered to my satisfaction.

Name of the participant:

Signature or left thumb impression of the participant:

Name of the witness:

Signature or left thumb impression of the witness:

Name of the investigator:

Signature of the investigator:

ANNEXURE – II

QUESTIONNAIRE (PROFORMA) USED FOR COLLECTING DATA

PERSONAL DETAILS:

Name:	Sex /Age:
Economic status & Income per annum:	IP No:
Address:	Nature Of Sample:

DIAGNOSIS:

H/O PRESENTING COMPLAINS:

PAST MEDICAL HISTORY (IF ANY):

COMORBIDITIES (IF ANY):

FAMILIAL H/O SIMILAR ILLNESS:

H/O MEDICATION (OF ONGOING ILLNESS) & IT'S OUTCOME:

MEDICATION	OUTCOME

HISTORY OR FINDING OF ANY RELEVANT INVESTIGATION:

STAGING (GRADING) OF LUPUS NEPHRITIS:

ANA PROFILE:

SERUM BIOMARKERS	POSITIVE/NEGATIVE
dsDNA	
SS-A/Anti Ro	
SS-B/Anti LA	
Nucleosome	
Histone	
SmD1 (Anti Smith Antibodies)	
PM Scl (Polymyositis/Scleroderma)	
SCL-70	
PCNA (Proliferating Cell Nuclear Antigen)	

ANNEXURE – III

The GENLISA Human Interleukin 12/23 P40 (IL-12P40 / IL12P40) ELISA kit

Principle: The method employs sandwich ELISA technique. Monoclonal antibodies are pre-coated onto microwells. Samples and standards are pipetted into microwells and the analyte present in the standard and sample are bound by the antibodies. Biotin labeled antibody is added and followed by Streptavidin:HRP is pipetted and incubated to form a complex. After washing microwells in order to remove any unbound proteins, TMB substrate solution is added to microwells and color develops proportionally to the amount of analyte present in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.

Materials Provided:

- Coated Microtiter Plate (12 x 8 wells) - 1 no
- Standard (lyophilized, concentrated) - 2 vials
- Biotinylated Detection Antibody (concentrated) - 120 ul
- Streptavidin:HRP Conjugate (concentrated) - 120 ul
- Standard Diluent - 20 ml
- Biotin Antibody Dilution Buffer - 12 ml
- HRP Conjugate Dilution Buffer - 12 ml
- (25X) Wash Buffer - 20 ml • TMB Substrate - 12 ml
- Stop Solution - 12 ml
- Instruction Manual

Materials to be provided by the End-User:

- Microtiter Plate Reader able to measure absorbance at 450 nm.
- Adjustable pipettes and multichannel pipettor to measure volumes ranging from 25 ul to 1000 ul
- Deionized (DI) water
- Wash bottle or automated microplate washer
- Clean tubes and Eppendorf tubes
- Precision single and multi-channel pipette and disposable tips.
- 37°C incubator
- Timer.

Reagent Preparation (all reagents should be diluted immediately prior to use):

- Label any aliquots made with the kit Lot No and Expiration date and store it at appropriate conditions mentioned.
- Bring all reagents to Room temperature before use.
- To make **Wash Buffer** (1X) 500 ml; dilute 20 ml of (25X) Wash Buffer in 480 ml of DI water.
- **Streptavidin: HRP Conjugate & Biotinylated Antibody Working Solution** - Briefly spin or centrifuge the Streptavidin: HRP Conjugate & Biotinylated eEF2 Antibody before use. Dilute them to the working concentration 100-fold with HRP Conjugate Dilution Buffer & Biotin Antibody Dilution Buffer, respectively.

- **Standards Preparation:** Reconstitute original lyophilized standard with 1.0 ml of Standard Diluent. Keep the standard for 10 mins with gentle agitation before making further dilutions. Prepare the additional Standards by serially diluting the standard stock solution as per accompanying sheet available in the kit.

Assay Range: 7.82 - 500 pg/ml

Sensitivity: 2.8 pg/ml

Procedural Notes:

- In order to achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess un-reacted reagents is essential.

- High Dose Hook Effect may be observed in samples with very high concentrations of the analyte. High Dose Hook Effect is due to excess of antibody for very high concentrations of the analyte present in the sample.

- If the analyte concentration of the undiluted sample is less than the diluted sample, this may be indicative of the Hook Effect.

- Avoid assay of Samples containing sodium azide (NaN3), as it could destroy the HRP activity resulting in under-estimation of the amount of the analyte.

- It is recommended that all Standards and Samples be assayed in duplicates or triplicates.

- Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.

- If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to compromisation of the sensitivity of the assay.

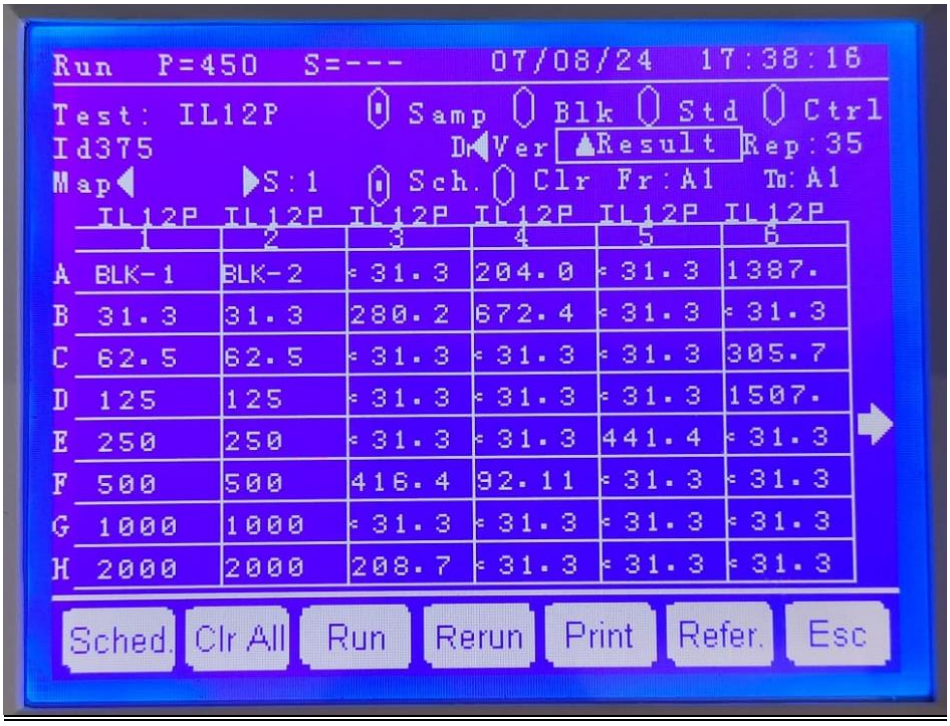
- The plates should be read within 30 minutes after adding the Stop Solution.

- Make a work list in order to identify the location of Standards and Samples.

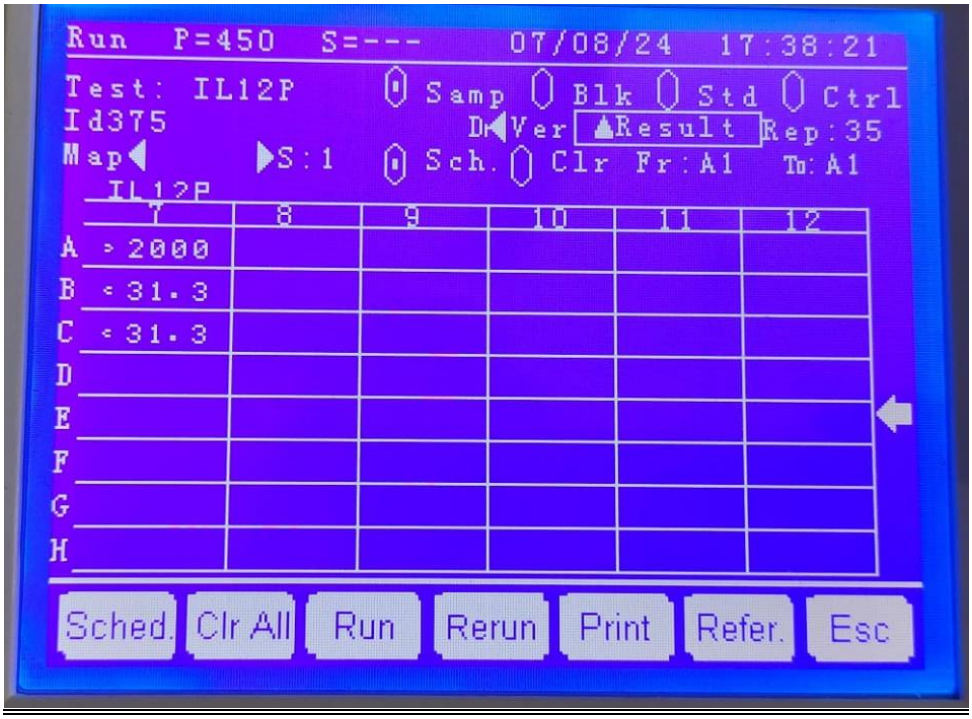
Calculation of Results:

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using Graph Paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown analyte concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the analyte concentration. If samples were diluted, multiply by the appropriate dilution factor.

Figure 4: Final observation of ELISA after adding stop solution



(a)



(b)

Figure 5: (a) and (b) showing readings of the ELISA

ANNEXURE IV - MASTERCHART

Serial No.	Age	Gender	Diagnosis	Staging	ANA Profile	Urine protein	C3 level	C4 level	dsDNA	SS-A /Anti Ro	SS-B /Anti LA	Nucleosome	Histone	SM/nRNP	PM Scl	Ro-52 recombinant	PCNA	Urine ILp40 (pg/ml)	OD Value
1	28 years	Female	Lupus Nephritis with RVD	Class IV	POSITIVE	4 +	31	3	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	-ve	<31.3	0.129
2	41 years	Female	SLE	Class IV	POSITIVE	3 +	54	15	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	**280.2**	0.328
3	19 years	Female	SLE with Class V Lupus Nephritis	Class V	POSITIVE	Trace	163	26	+ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	<31.3	0.102
4	46 years	Female	SLE with Glomerular nephritis	Class III	POSITIVE	2+	110	40	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	<31.3	0.167
5	68 years	Male	Leucocytoclastic vasculistis with AKI	Biopsy not done	NEGATIVE	1+	129	31	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	<31.3	0.0602
6	23 years	Female	SLE with Lupus Nephritis	Class V	POSITIVE	1 +	131	18	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	**416.4**	0.41
7	15 years	Female	Acute Intermittent Porphyria with SLE	Class IV	POSITIVE	1+	58.9	17	+ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	<31.3	0.079
8	42 years	Female	SLE Nephritis with Pneumonia	Class III	POSITIVE	3+	70	10	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	**208.7**	0.287
9	18 years	Female	Lupus Nephritis	Class III	POSITIVE	Negative	131	18	+ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	**204.01**	0.284
10	34 years	Female	SLE	Class IV	POSITIVE	3+	70	12	-ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	**672.43**	0.556
11	28 years	Male	SLE with Class III Lupus Nephritis	Class III	POSITIVE	2 +	58.9	8.9	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	<31.3	0.033
12	24 years	Female	SLE with Lupus Nephritis	Class IV	POSITIVE	Negative	108	18	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	<31.3	0.05
13	43 years	Female	Lupus Nephritis	Class III	POSITIVE	2 +	200	35	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	<31.3	0.084
14	65 years	Male	HTN Nephrosclerosis with severe CIN	CKD - Stage 5	NEGATIVE	1 +	58	8	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	92.11	0.217
15	42 years	Female	Lupus Nephritis Class V	Class V	POSITIVE	4 +	37	6	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	<31.3	0.063
16	23 years	Female	Lupus Nephritis Class IV-G	Class IV	POSITIVE	2+	66	8	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	<31.3	0.067
17	60 years	Male	RPGN	CKD - Stage 5	NEGATIVE	1+	60	19	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	<31.3	0.0828
18	22 years	Male	SLE with Lupus Nephritis	Class III	POSITIVE	3 +	48	7	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	<31.3	0.0812
19	22 years	Female	SLE with Renal Tubular Acidosis	Class II	POSITIVE	Trace	110	18	+ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	<31.3	0.0611
20	36 years	Female	SLE with Lupus Nephritis	Class V	POSITIVE	Trace	108	18	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	<31.3	0.0615
21	16 years	Female	SLE with Lupus Nephritis	Class III	POSITIVE	2 +	20	6	+ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	**441.45**	0.426
22	24 years	Female	SLE with CKD with RHD	Class III	POSITIVE	2 +	118	26	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	<31.3	0.0646
23	27 years	Female	SLE with Nephrotic syndrome	Class III	POSITIVE	1 +	105	12	+ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	<31.3	0.0894
24	29 years	Female	SLE with Nephrotic Syndrome	Class II	POSITIVE	3+	37	3	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	<31.3	0.0974
25	42 years	Female	SLE with Lupus Nephritis	Class IV	POSITIVE	1+	70	10	+ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	**1387.6**	0.8677
26	26 years	Female	SLE with Lupus Nephritis	Class IV	POSITIVE	Negative	80	10	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	<31.3	0.0961
27	16 years	Female	SLE with Lupus Nephritis	Class III	POSITIVE	3 +	66	8	+ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	**305.7**	0.3436
28	21 years	Female	SLE	Class III	POSITIVE	3+	68	8	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	**1507.1**	0.8677
29	31 years	Female	Lupus Nephritis	Class IV	POSITIVE	Negative	64.4	7.3	+ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	<31.3	0.1031
30	46 years	Female	SLE with Lupus Nephritis	Class III	POSITIVE	3 +	60	8	+ve	-ve	-ve	+ve	+ve	+ve	-ve	+ve	-ve	<31.3	0.1198
31	34 years	Female	SLE	Class III	POSITIVE	Negative	110	18	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	<31.3	0.0574
32	35 years	Female	SLE with Lupus Nephritis	Class III	POSITIVE	1 +	72	10	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	<31.3	0.0903
33	39 years	Female	SLE	Class IV	POSITIVE	Negative	48	8	+ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	**>2000**	1.4184
34	34 years	Female	SLE	Class III	POSITIVE	1 +	80	10	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	<31.3	0.0728
35	33 years	Female	SLE	Class III	POSITIVE	2 +	94	18	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	<31.3	0.0728