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सेवा में,

The Dean (Academics)
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Subject: Submission of M.D thesis.

आदरणीय महोदय,

This is to submit that the M.D thesis by the Academic Junior Resident (January 2020 Batch) of our Department has been duly completed and signed and is ready for submission. Please accept the same.

Details of her thesis are attached herein:

Name of candidate	Thesis topic
Dr. Nivedita A	PROFILING OF MEMBRANOUS NEPHROPATHY BASED ON IMMUNOHISTOCHEMICAL EXPRESSION OF ANTIGENS NELL-1, PLA2R AND THSD7A

धन्यवाद,

भवदीया

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आचार्या एवं विभागाध्यक्षा
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PROFILING OF MEMBRANOUS NEPHROPATHY BASED ON IMMUNOHISTOCHEMICAL EXPRESSION OF ANTIGENS NELL-1, PLA2R AND THSD7A

Thesis

Submitted to

All India Institute of Medical Sciences, Jodhpur

In partial fulfilment of the requirement for the degree of

DOCTOR OF MEDICINE (MD)

(PATHOLOGY)

JANUARY 2020

AIIMS, JODHPUR

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DECLARATION

I, hereby declare that the work reported in the thesis entitled "**Profiling of Membranous Nephropathy based on Immunohistochemical Expression of Antigens NELL-1, PLA2R and THSD7A"** embodies the result of original research work carried out by undersigned in the Department of Pathology and Lab Medicine, All India Institute of Medical Sciences, Jodhpur.

I further state that no part of the thesis has been submitted either in part or in full for any other degree of All India Institute of Medical Sciences or any other institution/University.

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ALL INDIA INSTITUTE OF MEDICAL SCIENCES, JODHPUR

CERTIFICATE

This is to certify that this thesis entitled "**Profiling of Membranous Nephropathy based on Immunohistochemical Expression of Antigens NELL-1, PLA2R and THSD7A**" is an original work of **Dr. Nivedita A,** carried out under our direct supervision and guidance at Department of Pathology and Lab Medicine, All India Institute of Medical Sciences, Jodhpur.

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I bow in respect to all the participants of my study.

Dr. Nivedita A.

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LIST OF ABBREVIATIONS

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MN	Membranous nephropathy
GBM	Glomerular basement membrane
Ig	Immunoglobulin
IF	Immunofluorescence
PLA2R	Phospholipase A2 receptor
THSD7A	Thrombospondin type I domain containing 7A
NELL-1	Neural epidermal growth factor like protein 1
SLE	Systemic lupus erythematosus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
PAS	Periodic acid Schiff
JMS	Jones Methenamine stain
EM	Electron microscopy
PM	Particulate matter
MAC	Membrane attack complex
iMN	Idiopathic Membranous nephropathy
MCTD	Mixed connective tissue disease
GFR	Glomerular Filtration Rate
FSGS	Focal segmental glomerulosclerosis
HLA	Human leukocyte antigen
Cys-R	Cysteine rich
CDLD	C type lectin domains
MS	Mass spectrometry
EXT1	Exostosin 1
EXT2	Exostosin 2

IHC	Immunohistochemistry
SEMA3B	Semaphorin 3B
PCDH7	Protocadherin 7
NCAM1	Neural cell adhesion molecule 1
HTRA1	High temperature recombinant protein A1
TGFBR3	Transforming growth factor beta receptor 3
CNTN1	Contactin 1
AB	Antibody
sMN	Segmental MN
H&E	Hematoxylin and Eosin
MT	Masson Trichrome
PBS	Phosphate buffered saline
EDTA	Ethylene diamine tetracetic acid
PLL	Poly-L-Lysine
FITC	Fluorescein isothicyanate
FFPE	Formalin Fixed paraffin embedded
ARB	Antibody retrieval buffer
HRP	Horseradish peroxidase
DAB	Diaminobenzidine tetrahydrochloride

Synopsis

Background: Membranous nephropathy (MN) is the leading causes of nephrotic syndrome in adults. Phospholipase A2 receptor (PLA2R) and Thrombospondin type - I Domain containing 7A (THSD7A) accounts for approximately 80% cases of primary MN. Neural epidermal growth factor like protein (NELL-1) is a novel antigen seen in primary MN. Data on NELL-1 related MN in Indian population remains unexplored.

Objectives: This study aimed to profile MN based on immunohistochemical expression of NELL-1, PLA2R and THSD7A and to correlate with the clinicopathological parameters.

Materials and methods: Expression of NELL-1, PLA2R and THSD7A was assessed immunohistochemically on 47 cases of membranous nephropathy.

Results: Among 47 cases of MN (32 primary, 15 secondary), the expression of NELL-1, PLA2R and THSD7A were seen in 27.7%, 78.7%, 14.8% cases respectively. Dual positivity was seen in 12 cases, of which NELL-1 and PLA2R co-expression were more common (9/12). Isolated NELL-1 positive MN was seen only in 2 cases. Triple negativity was seen common in secondary MN. High urinary protein levels were noted in PLA2R positive cases. No history of malignancy was seen in cases expressing NELL-1.

Conclusion: This study demonstrated the prevalence of NELL-1, PLA2R & THSD7A related MN among the Indian population. The results of this study highlighted a few characteristics feature that are strongly related to the expression of the profiled antigens in membranous nephropathy.

Chapter 1: Introduction

Membranous nephropathy (MN) is one of the commonest causes of nephrotic syndrome in adults. It is an immune complex mediated disease characterized by deposition of antigen-antibody immune complexes in the subepithelium of the glomerular basement membrane (GBM) (1).

MN is broadly classified as primary and secondary depending on the cause. Primary membranous nephropathy earlier considered to be idiopathic has now been characterized by deposition of M-type phospholipase A2 receptor (PLA2R) and Thrombospondin type - I Domain containing 7A (THSD7A) which accounts for approximately 75% and 5% of cases respectively (2–4). There are still 15% of cases of primary MN in which causative target antigens are yet to be identified (5). Recently, a novel antigen called Neural epidermal growth factor like protein (NELL-1) was identified in PLA2R and THSD7A negative MN cases by mass spectrometry (6). Secondary MN is associated with autoimmune diseases commonest being systemic lupus erythematosus (SLE), malignancies, infections like Hepatitis B (HBV) and Hepatitis C (HCV) and various drugs (7).

NELL-1 is a gene that is strongly expressed in neural tissue encoding a protein with EGF-like repeats. In the kidney, the tubules have the highest expression of NELL-1. But it may present as an extra cellular component and deposited in the GBM. NELL-1 is a cytoplasmic 90kDa secreted protein (6). NELL-1 associated MN is commonly associated with malignancy and has unique histopathologic findings characterized by segmental to incomplete capillary loop IgG staining, IgG1-predominance and lack of staining for other

immune reactants (IgA, IgM and C1q). In recent times, many studies have found out new autoantigens using mass spectrometry such as Sema3B associated MN, Protocadherin 7 associated MN, serine protease HTRA1 (5).

Data on PLA2R related and THSD7A related MN in Indian literature were limited and there have been outliers showing PLA2R/THSD7A positivity in secondary MN in this population (8). Role of NELL-1 in MN cases has been described in few studies in world literature till date and in India only one case report has been published (6,9–11).

The present study is being undertaken to identify the prevalence of NELL-1, PLA2R/ THSD7A in Indian population and to profile membranous nephropathy based on expression of NELL-1, PLA2R and THSD7A immunohistochemically and also aimed to observe the clinicopathological association with NELL-1, PLA2R and THSD7A related membranous nephropathy.

Chapter 2: Aim and Objectives

2.1 Aim

- To study the glomerular immunohistochemical expression of antigens PLA2R, NELL-1 and THSD7A in Membranous nephropathy.

2.2 Objectives

- Immunohistochemical profiling of MN cases based on PLA2R, NELL-1 and THSD7A.
- To compare expression of PLA2R, NELL-1 and THSD7A in primary versus secondary Membranous nephropathy.
- Clinicopathologic comparison of cases positive for either PLA2R, NELL-1 or THSD7A with PLA2R, NELL-1 and THSD7A negative cases.

Chapter 3: Review of Literature

Membranous nephropathy (MN) is defined by "the presence of subepithelial immune deposits that induce a spectrum of changes in the GBM". The diagnostic features of MN are GBM extensions or "spikes" projecting into the urinary space, best appreciated with the periodic acid-Schiff (PAS) stain and Jones methenamine silver (JMS) stain on light microscopy, bright granular Ig staining along GBM on immunofluorescence microscopy and sub-epithelial electron dense deposits on electron microscopy (EM) (1).

3.1 Epidemiology

Membranous Nephropathy (MN) is stated as one of the most common causes of nephrotic syndrome, found in all races and ethnicities (12). The incidence of the disease is around 1 per 100,000 cases in a year, and its peak is observed between the fourth and fifth decade of life, with a preposition for males (2:1) (13). Large population-based studies representative across the globe are not available. But, temporal changes in prevalence are reported across various regions. In a study encompassing all age groups in China, it was noted that the prevalence of MN increased by 13% annually, while the proportions of other glomerulopathies remained stable. Primary forms of MN are very rare in the paediatric age group (<7% of biopsies), but is the most common cause of idiopathic nephrotic syndrome in non-diabetic adults (12). Among the paediatric age group, MN is to be considered when it is associated with HBV infection or lupus. An interesting association noted in the recent studies is the high rates of MN in areas with higher levels of fine particulate matter of diameters $\leq 2.5\mu m$ (PM2.5) (12).

3.2 Pathogenesis

It occurs due to immune-complex formation on the outer aspect of the basement membrane, which results in thickening of the glomerular capillary walls. The immune deposits are made up of IgG, relevant antigens and complement components, including the membrane attack complex (MAC) (14). In consequence, these deposits lead to proteinuria (mediated by the podocyte injury) and progresses to nephrotic syndrome (1).

3.2.1 Subtypes of membranous nephropathy

The term primary *idiopathic* MN (iMN) is no longer used as the majority of cases are mediated by an autoantibody to PLA2R expressed on podocytes, hence it is replaced by specific term primary PLA2R-associated MN. Circulating IgG4 anti-PLA2r is detected in 70-75% of adults and adolescents with primary MN. The most common aetiology for secondary MN is systemic lupus erythematosus (SLE). Other best established etiologies are listed in the Table 3.1.

3.3 Clinical Features

MN displays the features of nephrotic syndrome, i.e., heavy proteinuria, hypoalbuminemia, edema or anasarca, hyperlipidaemia, and lipiduria. Other presentations include renal insufficiency, haematuria and hypertension (1). While most patients report fatigue, the disease usually has an indolent course, wherein the clinical features could be overlooked for months. This is mainly because of gradual and progressive accumulation of the immune deposits and resultant podocyte injury (12).

Table 3.1

Established secondary aetiologies of membranous nephropathy

Autoimmune diseases	• Systemic lupus erythematosus • Rheumatoid arthritis • Sjogren syndrome • Mixed connective tissue disease (MCTD) • Autoimmune thyroid disease
Malignancy	• Carcinoma of lung • Gastrointestinal cancer • Prostate cancer • Breast cancer
Infections	• Hepatitis B and C • Syphilis • Filariasis • Hydatid disease
Therapeutic agents	• Gold salts • Penicillamine, bucillamine • Captopril • Mercury • NSAIDs • Tiopronin • Trimethadione
Others	• Sarcoidosis • IgG4 disease • Allogenic hematopoietic stem cell transplant • Guillian-Barré syndrome

Adapted from Heptinstall's Pathology of the Kidney, Volume 1, 7th Edition (1)

The blood pressure remains normal and the glomerular filtration rate (GFR) is usually preserved for almost 70% of the patients at presentation. Reduction in the GFR warrants search of a coexisting diagnosis. Proteinuria in these patients could go unnoticed for years

together, as they are at a subclinical level, and are only found incidentally in most cases. Microscopic hematuria is seen in half of the patients (13).

Primary MN is idiopathic (~80% of patients) however, secondary MNs are usually associated with medications (NSAIDs), lupus, hepatitis B or C infections, and malignancies. Despite MN being a histopathologic diagnosis, there are some clinical cues to differentiate between the primary and secondary causes of MN, obtained by careful history taking. In secondary MN, the infectious agent or the secondary disease can predate the diagnosis of MN, while in malignancy or lupus, the nephrotic state induced by MN may be the presenting feature of the underlying disease (13). Distinguishing between primary and secondary MN is important, as the management depends on it. While primary MN is treated with immunosuppressive therapy, identifying and treating the underlying cause is pertinent for remission of secondary MN (2).

The ‘rule of thirds’ has been used to explain the patient outcomes for MN. About one third of the patients undergo spontaneous remission, the further one third have variable levels of proteinuria and the remaining third progress to advanced kidney failure (12). Withadvancements in immunosuppressive therapy, the rate of kidney replacement therapy has come down (to approximately 10%), with cyclophosphamide treatment. Other therapies such as rituximab are also tried. The disease recurs in approximately 30-45% of the patients after transplantation, leading to an increased risk of graft failure (15).

3.4 Pathologic Findings (1)

3.4.1 Light Microscopy

The glomeruli appear normal to enlarged in size and GBM appear normal in thickness in early stages. The podocytes appear swollen with enlarged cell bodies. As the disease progresses, the glomerular capillary walls appear thicker and more rigid with formation of subepithelial spike formation or vacuolated (holes), highlighted by PAS or JMS stains. There is little or no increase in mesangial hypercellularity.

Segmental or global glomerulosclerosis is observed in many cases with poorer prognosis in patients having accompanying focal segmental sclerotic (FSGS) lesions (16). Endocapillary hypercellularity, GBM duplication, mesangial hypercellularity, fibrinoid necrosis and crescent formation are uncommon in primary MN. When present, these findings suggest either a secondary form of MN or the concurrence of MN with an additional glomerular disease process.

Tubules show protein resorption droplets and can exhibit acute tubular injury characterized by luminal ectasia, cytoplasmic simplification and vacuolization, irregular luminal contours, loss of brush borders, prominent nucleoli and mitotic figures. As the disease progresses, tubular atrophy and interstitial fibrosis ensue and correlates with decreased renal survival.

There is mild interstitial inflammation which is more prominent in the areas of tubular atrophy and interstitial fibrosis. Interstitial foam cells are also seen. Arteriosclerosis and

arteriolosclerosis are seen commonly and they reflect patient's age and presence of systemic hypertension. Vascular inflammation is not a feature of MN.

3.4.2 Immunofluorescence

The characteristic pattern of staining is diffuse, uniform and granular deposits along the glomerular capillary loops against the antisera for IgG kappa and lambda light chains, the complement Cs may or may not stain. The dominant subclass is IgG4. Staining for IgM, IgA and C1q are usually not prominent in primary MN.

In membranous lupus nephritis, the hallmark pattern is full house staining for immunoglobulins and complements, characterized by positive staining for IgG, IgA, IgM, complements C3 and C1q along with kappa &lambda light chains.

Multiple studies found out that the staining for the subclasses of IgG can be used as a means of differentiating primary from secondary MN. In membranous lupus nephritis, IgG1, IgG2 and IgG3 predominate and have more intense staining than IgG4. In MN secondary to malignancy, mercury exposure and in de novo MN in the allograft, IgG1 predominates.

Mesangial deposits and extraglomerular deposits including tubular basement membranes are rare in primary MN and their presence favours a secondary form of disease. Mesangial deposits stain intensely for IgA with minimal to absent IgG staining.

Table 3.2
IgG subclass staining in primary versus secondary MN (17)

IgG subclass	Primary MN (n=114)	Secondary MN (n=43)	p value
IgG1	46%	60%	0.12
IgG2	3%	5%	0.62
IgG3	18%	23%	0.42
IgG4	75%	49%	0.0018

3.4.3 Electron Microscopy

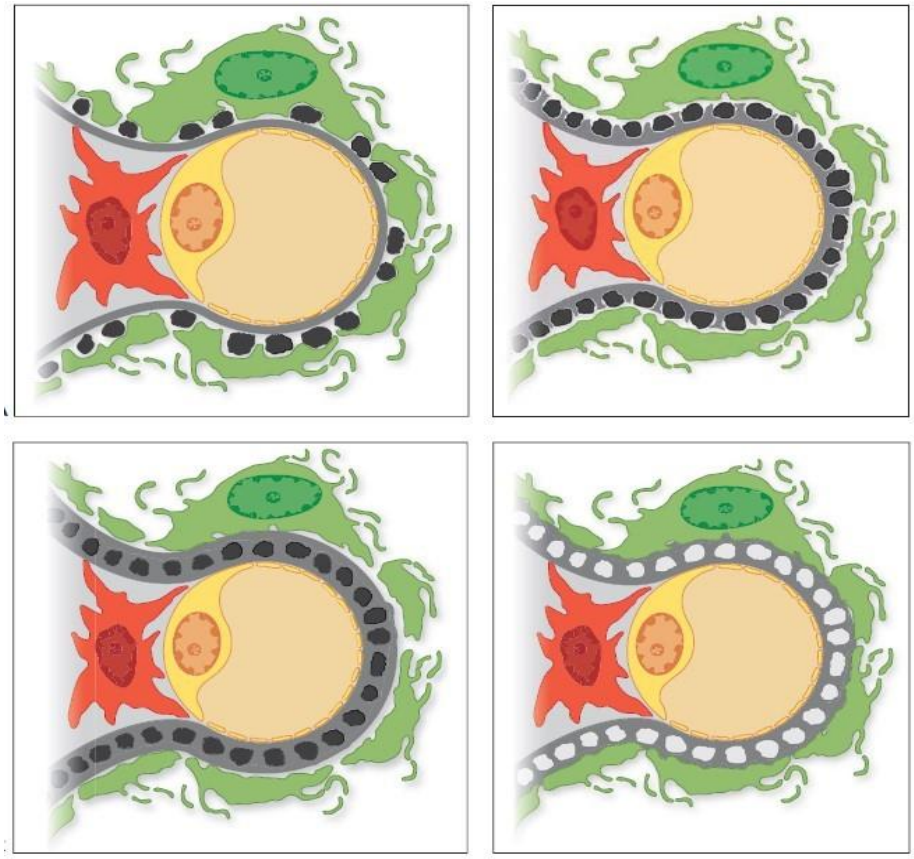


Figure 3.1: Stages of ultrastructural changes in membranous nephropathy observed under electron microscopy. (Adapted from Heptinstall's Pathology of Kidney, Volume 1, 7th edition) (1)

EM plays a vital role in establishing the diagnosis of MN. It also determines the stage of MN accurately. EM can demonstrate the electron dense deposits along with the GBM changes. Based on the nature of dense deposits and GBM changes, Ehrenreich and Chrug classified MN into four ultrastructural stages.

Stage I

Small, sparsely distributed electron dense deposits seen in the subepithelial region along with no intervening GBM spike formation. No significant abnormality detected in light microscopy.

Stage II

Numerous large subepithelial electron dense deposits in the subepithelial region surrounded by intervening projections of the GBM forming a characteristic "spike and dome" appearance, along with global thickening of the GBM and diffuse foot processes effacement.

Stage III

A new membrane material surrounds the subepithelial electron dense deposits and forms a neomembrane. Now the deposits have intramembranous appearance. More pronounced thickening of GBM noted.

Stage IV

GBM is irregularly thickened with presence of electron lucent deposits, owing to remodelling of the extracellular matrix.

There is also Stage V of MN described by Ehrenreich and Chrug which is not based mainly on EM findings and is characterized by capillary collapse, sclerosis, capsular adhesion and crescents.

This staging system has certain limitations. They provide insight into the evolution of disease but not all cases progress from stage I to stage IV. The stages may overlap and do not correlate with the level of proteinuria and renal function. Progression of one stage to another can be associated with clinical improvement or worsening of disease. Clinical remission has no relationship with the appearance of deposits.

Mesangial electron dense deposits, subendothelial and extraglomerular deposits are rare in primary MN, when present, secondary MN is strongly suspected.

3.5 Role of Genetic Factors in Membranous Nephropathy

MN is not a hereditary disease but has a strong genetic component. MN has a strong affinity for the human leukocyte antigen (HLA) class II antigens like HLA-DR3 and HLA-DQA1. Studies have detected single nucleotide polymorphism in noncoding regions of PLA2R gene. Homozygosity for high risk alleles in both HLA and PLA2R genes increases risk for primary MN (18).

3.6 Evolution of Concepts in Pathogenesis

Over the past two decades, significant progress has been made in our understanding of the pathogenesis of MN, which is a disorder of the kidney glomerulus. Several studies done in Heymann models of MN in rats detected subepithelial deposits of IgG, which resulted

from insitu immune complex formation involving megalin, a podocyte membrane antigen (19). However, megalin is not expressed by human podocyte; in 2002, Debiec et al identified neural endopeptidase in a rare form of alloimmune antenatal MN(20).

Later in 2009, Beck et al (3) demonstrated a 185kDa glycoprotein in serum of idiopathic MN (iMN) patients by western blot and identified a novel podocyte target antigen, which is mainly an M-type phospholipase A2 receptor (PLA2R) in 70% of idiopathic MN cases using mass spectrometry, that belongs to the mannose receptor family and is expressed by the human podocytes. It is also expressed in type II pneumocytes and splenic lymphocytes (21). It has conserved extracellular structure consisting of the cysteine-rich (Cys-R) domain, a fibronectin II domain and a tandem repeat of 8 C type lectin domains (CDLD 1-8) (14). Anti-PLA2R autoantibodies are mostly IgG4 type (3).

In 2014, Tomas et al, found another podocyte antigen, Thrombospondin type I domain containing 7A (THSD7A) that serves as a target antigen in 2-3% of patients with primary MN. In this study, anti-THSD7A antibodies were detected in the serum of 8-14% of iMN cases by western blot. THSD7A is a 250kDa multidomain transmembrane glycoprotein expressed at the basal surface of the podocyte but not in glomerular endothelial and mesangial cells. It is also expressed by certain malignancies, which initiate humoral response leading to MN. Initially THSD7A was identified as an endothelial protein, which was expressed in the placental vasculature (4). A major epitope of THSD7A has been discovered in the N terminal region, similar to the PLA2R motif. Thus this common epitope was found to the cause for cross reactivity of both the antibodies (22).

In the past two decades, many new novel antigens have been discovered owing to the technological advances in combining laser microdissection of glomeruli and tandem mass spectrometry (MS/MS). In 2019, Sethi et al identified two novel antigens. One was Exostosin 1(EXT1) /Exostosin 2 (EXT2) which is seen in 11.6% cases of PLA2R negative MN and showed bright granular glomerular staining in IHC. 80.7% of these cases showed features of autoimmunity (23).

Another protein was Neural epidermal growth factor like 1 protein (NELL-1), a 90kDa secreted protein, identified in 16.2% of cases of primary MN – PLA2R negative MN. He did a pilot study in 35 cases and found out that 6 cases were positive for NELL-1 by MS/ MS and IHC. Also in his discovery cohort, he performed IHC in 91 biopsies of PLA2R negative MN and found out 23 were NELL-1 positive and further MS/MS showed only 14 NELL-1 positive cases in that. Hence, 29 of 126 cases were positive for NELL-1. He also found five more NELL-1 positive cases out of 84 PLA2R and THSD7A negative cases in two validation cohorts from France and Belgium. All the NELL-1 associated MN showed thickened GBM on light microscopy, bright IgG and C3 staining along the capillary walls on IF microscopy and subepithelial deposits on electron microscopy. There were no subendothelial and mesangial deposits noted. NELL-1 associated MN is common seen in older patients with no gender predilection and presents with nephrotic syndrome (6).

Many more novel antigens such as Semaphorin (SEMA 3B), Protocadherin 7 (PCDH7), neural cell adhesion molecule 1 (NCAM1), serine protease high temperature recombinant protein A1 (HTRA1), Transforming growth factor beta receptor 3 (TGFBR3) and

contactin 1 (CNTN1) were identified in the recent years. These antigens were associated either with primary MN or with a specific cause of secondary MN (5, 24-26).

3.7 Summary of Studies in the Literature

3.7.1 PLA2R & THSD7A:

- Hoxha E et al. in 2012 did a prospective study and analysed PLA2R expression in glomeruli and serum PLA2R-antibodies (PLA2R-AB) levels in 88 patients with biopsy proven MN and found out 61 (69%) has strong PLA2R expression in glomeruli by IHC and PLA2R autoantibodies were detected in 60 of those patients. Out of 27 serum negative patients, 15 patients had a secondary cause. This study concluded that enhanced glomerular PLA2R staining and detection of PLA2R-AB helps in differential diagnosis between primary and secondary MN since PLA2R-AB and PLA2R antigen were not found in secondary MN (27).

- Larsen CP et al. in 2013 examined 165 cases of MN which includes 85 primary and 80 secondary cases and found out that tissue staining of PLA2R by immunofluorescence method had a sensitivity of 75% and specificity of 83% in primary MN. In secondary MN, PLA2R positivity was seen in 17.5% (14/80) of cases in which 7 had HCV, 3 had sarcoidosis, 3 had neoplasm and 1 had autoimmune aetiology. All the secondary MN cases with positive PLA2R showed IgG4 predominance which increases the probability that these cases are more pathogenically related to primary MN than secondary (28).

- Iwakura T et al. in 2015 evaluated 92 diagnosed cases of iMN in Japanese patients and found out that the prevalence of enhanced granular expression of THSD7A and PLA2R

were detected in 9.1% and 52.7% respectively in iMN patients. But in secondary MN none of the patients showed THSD7A expression and 5.4% of them showed PLA2R expression (29).

- Larsen CP et al. in 2016 investigated THSD7A associated MN using IHC in 258 biopsy proven MN with exception of lupus nephritis. They found out that 3% of cases showed THSD7A only positivity, 55% cases showed PLA2R only positivity and 1% cases showed dual positivity for THSD7A and PLA2R. In few patients, serologic testing was done and showed 100% correlation between the positive THSD7A and /or PLA2R tissue staining and the presence of respective serum autoantibodies, even in dual positive cases. This study highlighted the significance of panel-based approach in subtyping the cases of MN (30).

- Kanda et al. in 2016 studied 34 paediatric iMN cases and found out PLA2R positivity in 2 cases with weak intensity than that of PLA2R positive adult iMN cases. Thus PLA2R plays a vital role in adolescent and preteen patients and rarely associated with childhood iMN (31).

- Roy S et al. in 2017 did a retrospective study in an Indian cohort with clinicopathological correlations wherein 153 MN patients (99 primary and 54 secondary) were evaluated along with 37 controls with clinical follow-up. They found that the anti-PLA2R IHC is a rapid, accessible and specific marker with a sensitivity of 70.2% and specificity of 96.6%. PLA2R positivity was defined by the complete positive staining of the glomerular capillary walls and faint granular peripheral staining of the

podocytes were considered as a negative control. This study validated the use of PLA2R IHC for the identification of primary MN and also studied clinicopathological parameters. Severe nephrotic syndrome, impaired renal function and hypertension were more common in primary MN. However, mesangial matrix expansion is more common in secondary MN. It is also seen that for prognostication in primary MN, PLA2R positivity is not useful. The most important histological prognostic marker remains to be the tubulointerstitial compartment lesions (32).

- Xu NX et al. in 2017 compared clinical features between PLA2R associated and non PLA2R associated iMN and identified that there is no significant difference between them, while non PLA2R associated iMN showed some abnormal serological tests and quickly respond to immunosuppressive therapy and shows complete and partial remission (33).

- Uppin M et al. in 2017 analysed PLA2R expression immunohistochemically on 82 biopsy cases of MN which included 51 primary, 31 secondary MN. The sensitivity and specificity of PLA2R IHC were 91.8% and 95.1% respectively. They concluded that IHC for anti-PLA2R antibody can be used as a standard diagnostic approach to identify idiopathic MN. This offered scope for individualized treatment and reduced the need for a thorough workup to rule out secondary causes. This study also found out that there is no significant differences between iMN and sMN (34).

- L'Imperio et al. in 2018 aimed to validate and standardize IHC technique by using a 3 biomarkers panel (includes PLA2R, THSD7A and IgG4) to improve diagnostic

performance in MN. This study included 95 biopsy proven MN patients (72 primary and 23 secondary), PLA2R IHC showed a sensitivity and specificity of 71% and 83% respectively; IgG4 showed a sensitivity and specificity of 61% and 83% respectively and THSD7A IHC was found to be positive in only 1 case. Based on these combination of results the patients were classified into two main groups – Double positive (PLAR+/ IgG4+/THSD7A-) and triple negative (PLA2R-/IgG4-/THSD7A-). This study concluded that when combination of IHC is being used in a tandem fashion, the sensitivity and specificity increases upto 79% and 83% respectively and the risk of false positive or false negative reduces (35).

- Subramanian P et al in 2020 did a retrospective study of all cases of MN between 2014 and 2017 and aimed to characterize the baseline PLA2R and THSD7A profile of adult and paediatric MN in a large Indian institutional cohort. They did PLA2R direct IF and THSD7A IHC on biopsy and Anti PLA2R ELISA on baseline sera. 216 cases of MN were enrolled in this study which had adequate tissue and clinical data. This study showed that the glomerular capillary wall staining for PLA2R using direct IF was seen in 52% cases of primary MN and 11.5% of secondary MN and the sensitivity and specificity of tissue PLA2R staining for primary MN was 52.4% and 88.5% respectively. Also, significant serum anti-PLA2R antibodies were found in 59.7% cases of primary MN and 11.1% of secondary MN. Thus, on combining both the tests, the prevalence of PLA2R related MN was found out to be 72.8% for primary MN and 16.7% for secondary MN. They also did THSD7A IHC on 176 cases of MN and found out 3.4% cases were positive for THSD7A and only one case of membranous lupus nephritis was positive for THSD7A. Also, dual positivity was noted in 2 cases. Among

paediatric cases, PLA2R were detected in 44% cases of primary MN but not in any of the secondary MN (8).

3.7.2 New Antigens

- In 2019, Sethi et al studied 224 cases of biopsy proven PLA2R-negative MN and 102 controls in pilot and discovery cohorts and he identified a novel antigens – Exostosin 1(EXT1) / Exostosin 2 (EXT2) in 26 cases by mass spectrometry and laser microdissection and were localised by bright granular glomerular staining on IHC. In these, 80.7% of cases showed features of autoimmunity. They also evaluated 48 cases of PLA2R negative primary MN and lupus MN in a validation cohort and confirmed that Ext1/Ext2 staining was seen in 12 cases which included pure class 5 lupus, presumed primary MN associated with autoimmunity and mixed lupus. EXT1/EXT2 serves as a biomarker protein of secondary MN. This significant finding helps in classifying MN at the molecular level and also predict the occurrence of lupus in primary MN patients with autoimmunity (23).

- Sethi et al in 2020 studied 70 biopsies of PLA2R negative MN cases in a discovery cohort, out of which 2 cases were Semaphorin 3B positive by mass spectrometry and confirmed by IHC. They also screened an additional of 90 biopsies for Sema3B associated MN and found 1 positive case that was confirmed by mass spectrometry. Three validation cohorts from France and Italy showed that 8 cases were positive for Semaphorin3B out of 118 biopsies of PLA2R, THSD7A, EXT1/EXT2 and NELL-1 negative MN by immunofluorescence. They also found out that 8 (72.7%) of the 11 Sema3B-associated MN were paediatric patients (36).

- Kudose S et al in 2020 studied the clinicopathologic spectrum of segmental membranous nephropathy (sMN). They analyzed 50 cases of sMN (2.5% of MN), of which 21 cases had an alternative disease process and remaining 29 had isolated sMN with early stage MN . Staining for PLA2R, THSD7A and exostosin 1 was negative an all biopsies. NELL-1 immunofluorescence staining was positive in 5 of 17 cases (29.4%). Follow up was available in 21 cases, of whom 7 received immunosuppression, 86% had stable kidney function, 45% achieved complete remission and 15% achieved partial remission (37)

- Caza et al in 2020 evaluated 349 MN cases which includes 83 pure class V lupus nephritis and 266 primary MN cases in a nine-month consecutive case series and found out 181 were PLA2R positive (51.9%), 10 were THSD7A positive (2.9%), 33 were EXT2 positive (9.5%) and 6 cases were NELL-1 positive (1.7%). 3.8% of PLA2R and THSD7A negative cases were NELL-1 positive. The author studied the clinical and histologic associations of NELL-1 associated MN over a period of 5 years. 91 NELL-1 associated MN were identified and they had unique histopathology presentation characterized by segmental to incomplete global capillary loop staining pattern (93.4%), IgG1-predominance (95.5%), and were more often associated with malignancy (33%). Anti NELL-1 antibodies were detected in the serum of the 71.4% patients (9).

- Wang G et al in 2021 studied the prevalence and clinical characteristics of NELL-1 positive MN in Chinese individuals. They analyzed 832 patients with biopsy proven primary MN and glomerular expression of PLA2R, THSD7A and NELL-1 were noted.

They found that 94% (778/832) patients showed PLA2R expression, 20% (11/54) patients with PLA2R MN showed THSD7A positivity. Two patients had dual positivity. NELL-1 IHC was done in 43 patients of PLA2R and THSD7A negative MN, 31 patients with PLA2R positive MN and 2 patients with PLA2R and THSD7A double positivity. About one third cases with PLA2R and THSD7A negativity were NELL-1 positive and one patient showed dual positivity for PLA2R and NELL1. Most of the NELL-1 positive patients were women. No evidence of malignancy was seen in NELL-1 positive patients. They also found that patients with different antigen positivity had varied glomerular deposition of IgG subclasses (10).

- Iwakura et al in 2022 did a single centre retrospective study for the prevalence of NELL-1 and EXT1/EXT2 associated MN in Japan. They examined 107 patients out of which 69 had primary MN and 35 had secondary MN and 3 cases were excluded due to insufficient sample. They found enhanced glomerular staining of PLA2R, THSD7A, NELL-1 and EXT1 /EXT2 in 53.6%, 8.7%, 1.5% and 13.0% respectively in patients with primary MN. In secondary MN, PLA2R, NELL1 andEXT1/EXT2 were detected in 5.7%, 8.6% and 22.9% of patients respectively. The prevalence of glomerular staining of NELL1 and EXT1/EXT2 in PLA2R/THSD7A negative, non-lupus MN was 8.9% and 26.7%. This study showed that the assessment of PLA2R, THSD7A and NELL1 helps in distinguishing secondary from primary MN in addition to the clinical information (38).

Chapter 4: Methodology

The study design was a prospective and retrospective observational study, conducted in the Departments of Pathology and Lab Medicine, Nephrology, and Paediatrics, All India Institute of Medical Sciences, Jodhpur. The study was conducted from 2020 to 2022, with recruitment of cases from the year 2016.

4.1 Source of Samples

The study included renal core biopsy specimens of the cases diagnosed as membranous nephropathy. These were received in the Department of Pathology and Lab Medicine at AIIMS Jodhpur for histopathological examination over a period of six years (July 2016 to October 2022). A total of 50 biopsy specimens were diagnosed as membranous nephropathy, of which 47 with adequate tissue for immunohistochemistry (IHC) were used in the present study. Those with inadequate renal biopsy sample or insufficient tissue for IHC were excluded

4.2 Study Tools

1. A semi-structured questionnaire for socio-demographic details like age and gender, presenting complaints and symptoms of nephrotic syndrome, co-morbidities such as diabetes, hypertension, previous infections, autoimmune symptoms, history of malignancy, and vital signs.
2. Laboratory investigations included urine microscopy for proteinuria, casts, hematuria, biochemical examination (ANA, dsDNA, ANCA).
3. Serum investigations included urea, creatinine, BUN, creatinine clearance, serum albumin, blood glucose levels, HbA1c, serum complement levels, serum cholesterol.

4. Viral markers – Hepatitis B, Hepatitis C and HIV.
5. Light microscopy for kidney biopsy specimens (collected from HMIS)
6. Special stains and routine immunofluorescence details for the kidney biopsy specimens.
7. Immunohistochemistry for profiling the antigens NELL-1, PLA2R, and THSD7A.

4.3 Grossing of Renal Biopsy Specimens

Two renal biopsy cores were received, one in 10% formalin-fixed and other in normal saline. The specimens were measured and processed. Formalin fixed biopsy were processed, and paraffin blocks were prepared using routine histopathological techniques. Thin sections (2.5 - 3 µm) were stained with routine Haematoxylin and Eosin (H&E) and special stains such as Periodic Acid Schiff, Jones" Methenamine silver and Masson Trichrome stains were done. Light microscopy results were recorded. `

4.4 Processing of the Tissue (39)

4.4.1 Steps of block preparation and section cutting

1. Dehydration was carried out by passing the sections through a series of ascending grades of ethyl alcohol, from 50%, 70%, 95% to absolute alcohol.
2. The clearing was done by passing the tissue through two changes of xylene.
3. Impregnation was done in molten paraffin wax which had a melting point of 54-62˚C.
4. Embedding: Embedding station (Leica EG 1150 H) was used through which a small amount of liquid paraffin was layered into aluminium moulds. Properly oriented tissues were placed inside the moulds, which were then filled with liquid paraffin

60-62˚C and allowed to cool and harden. The lower portion of the cassette with an identification number was used as the final block.

5. Microtomy: Microtome (Leica-RM2255) was used and thin ribbons (2.5-3 µm) were cut and floated in warm water (~56˚C) for expansion of the curled sections. These sections were then collected on frosted glass slides and kept for drying.

4.4.2 Staining of Sections (for H&E Stain) (39)

1. Deparaffinization – The glass slides containing the tissue sections were kept over the hot plate at 60 ˚C for 10 minutes, followed by two changes in xylene (Xylene I & Xylene II), 10 minutes each.
2. Hydration – Through graded alcohol (100%, 95%, 70%, 50%) to water, 10 minutes respectively.
3. Haematoxylin – The sections were kept in Harris's Haematoxylin for 5 minutes.
4. Washing – The sections were washed well in water for 2 minutes.
5. Differentiation – Done in 1% acid alcohol (1% HCl in 70% alcohol) for 10 seconds.
6. Washing – Done under running tap water (usually for 15 – 20 minutes) until the sections 'blue'.
7. Eosin – Stained in 1% Eosin Y for 10 seconds.
8. Washing – Done in running tap water for 2 minutes.
9. Dehydration – Through graded alcohol (50%, 70%, 95%, 100%), 10 minutes each.
10. Clearing –Through xylene (Xylene II & Xylene I), 2 minutes each.
11. Mounting – The sections were mounted in DPX with a coverslip.

4.4.3 Special Stains

A. Periodic Acid Schiff stain

1. The glass slides were kept over the hot plate at 60 ˚C for 10 minutes, followed by dewaxing in xylene and rehydration through graded ethanol to distilled water.
2. The slides were treated with 1% periodic acid for 10 mins.
3. Washed with distilled water.
4. In Schiff reagent for 30 minutes.
5. Rinsed the slides in running tap water for 10minutes
6. Harris Haematoxylin was used as a counter stain to differentiate and blue the sections.
7. Dehydration done through graded ethanol and clearing done with xylene.
8. The sections were air dried and mounted in DPX with a coverslip.

B. Jone's Methenamine Silver stain

1. The sections were deparaffinized with two changes of xylene and rehydrated to distilled water.
2. The sections were treated with 1% periodic acid for 15 minutes.
3. Rinsed in distilled water.
4. Treated the section with working methenamine silver nitrate solution at 60°C (microwave oven) for 1hr and checked frequently until the desired staining intensity was achieved.
5. The sections were brought to room temperature and washed with distilled water.
6. The sections were toned with 0.02% gold chloride for 30 seconds.
7. Rinsed with distilled water.
8. The sections were treated with 2% sodium thiosulfate for 1 minute.

9. Washed in tap water.
10. Counterstaining done with the working light green solution for 1 minute 30 seconds.
11. Dehydration done through graded alcohol, cleared with xylene and mounted with DPX.

C. <u>Masson Trichome stain</u>

1. Deparaffinization of sections were done and brought to water.
2. The sections were treated with Bouin's solution overnight.
3. Washed in running water until yellow colour disappears.
4. Stained with haematoxylin for 10 minutes.
5. Washed in running water for 5 minutes.
6. One dip in 1% acid alcohol.
7. Washed in running water for 5 minutes.
8. Stained with acid fuchsin solution for 5 minutes.
9. Rinsed in distilled water.
10. Treated with phosphomolybdic acid solution for 5 minutes.
11. Washed with distilled water.
12. Stained with aniline blue for 2-5 minutes.
13. Rinsed in distilled water.
14. Treated with 1% acetic acid for 2 minutes.
15. Dehydration done through ascending grades of alcohol and clearing with xylene and mount with DPX.

4.5 Processing of the Tissue for Direct Immunofluorescence (39)

The renal biopsy core received in normal saline were processed for freezing as soon as possible.

- **Preparation of phosphate buffered saline (PBS)**: Prepared by adding 0.42g of potassium phosphate monobasic, 1.42gm of sodium phosphate dibasic anhydrous and 7.2g of sodium chloride in 1 litre of distilled water solution
- **Preparation of Tris-EDTA solution:** Prepared by adding 1.21 gm of Tris(hydroxymethyl)aminomethane and 0.4gm of Ethylene diamine tetracetic acid in 1 litre of distilled water.
- **Preparation of Poly-L-Lysine Solution (PLL Solution):** 1 ml of PLL was diluted with 9 ml of distilled water (1 in 10 dilutions).

Slide Coating Procedure

Step 1: Diluted PLL solution was taken in a clean container / Coplin jar

Step 2: Both sides of the glass slides were cleaned with tissue paper

Step 3: The clean slides were immersed in a PLL solution for 5 minutes

Step 4: After 5 minutes, the coated slides were removed and kept overnight for air drying. The coated slides were kept at room temperature. Tissue sections of 2.5-3 µ thickness were obtained on the PLL coated slides.

Antibody preparation

Antibodies such as Anti-IgG, IgA, IgM, C3, C1q, Fibrinogen, kappa and lambda light chains – purified rabbit polygonal antibody labelled with fluorescein isothiocyanate (FITC), Bio SB, concentrated antibody, 1:15µl dilution done in dark room.

Preparation of frozen tissue sections

1. The biopsy should be removed from the normal saline and placed in PBS solution for 3-4 hours and stored in freezer at 4-8°C.
2. Remove the tissue from wash solution and place onto a glass slide to remove excess wash solution.
3. Embedding - A small amount of OCT compound was placed on a metal chuck which was pre-cooled to -25°C. The biopsy tissue was placed in the OCT inside the Leica CM cryostat and more compound was added to form mound over the top of the tissue.
4. The chuck with tissue was left at -25°C for a minimum of 30 minutes before cutting.
5. The tissue was cut at 2.5µm thickness and were obtained in PLL coated slides.
6. The cut sections were stored in the freezer (-20°C to -25°C).

Staining procedure for direct immunofluorescence

1. The slides were kept outside until they reach room temperature.
2. The slides were washed with PBS solution – 3 changes, 5 minutes each.
3. Applied diluted FITC conjugate antibodies IgG, IgA, IgM, C3, C1q, Fibrinogen, Kappa and lambda (1:15 µl) on each slide and kept in a dark room for 1 and half hour.
4. The slides were washed in PBS solution -3 changes, 5 minutes each.
5. All the slides were mounted in buffered glycerol using coverslip.
6. The slides were examined under the fluorescent microscope in a dark environment.
7. Pictures were taken using the camera and preserved in the computer for future reference.

   Result: Green granular fluorescence – Positive

   Background - black

Immunofluorescence on Formalin Fixed paraffin embedded sections (IF-P)

1. Formalin fixed paraffin embedded (FFPE) tissues were cut at 2.5µ thickness and were obtained on PLL coated slides.
2. The sections were air dried overnight.
3. The sections were dewaxed in two changes of xylene and rehydrated with acetone and 70% alcohol for 2 minutes each.
4. Washed in running water for 5 minutes.
5. The slides were immersed in Tris EDTA (pH-9) for 30 minutes at room temperature
6. Enzymatic digestion – Done with 1.25mg/ml proteinase K and incubated in moist chamber for 20 minutes at room temperature.
7. The digestion was stopped by immersing the slides in cold Tris EDTA at 4°C for 40 minutes.
8. The sections were immersed in PBS for 10 minutes
9. FITC conjugated primary antibodies were added to the sections and then processed as for routine DIF staining.

Interpretation of IF:

The intensity of IF was graded as a score 0-4

4.6 Immunohistochemistry

Antibodies used

Primary antibody:

- **PLA2R (Phospholipase A2 receptor)** – Concentrated antibody (0.2mg/ml), one vial containing 100µl, 1:500 dilution, Rabbit polyclonal antibody, Company: Sigma Aldrich
- **NELL1 (Neural epidermal growth factor like 1 protein)** – Concentrated antibody (0.1mg/ml), one vial containing 100µl, 1:100, Rabbit polyclonal antibody, Company: Sigma Aldrich)
- **THSD7A (Thrombospondin type I domain containing 7A)** - Concentrated antibody (0.4mg/ml), one vial containing 100µl, 1:500 dilution, Rabbit polyclonal antibody, Company: Sigma Aldrich

Secondary Antibody: Bond Polymer Refine Detection, Leica

- Peroxide block, 3-4%(v/v)
- Post Primary, Rabbit anti-mouse IgG in 10% (v/v) animal serum in tris-buffered saline
- Polymer, Anti-rabbit Poly-HRP-IgG containing 10% (v/v) animal serum in tris-buffered saline
- DAB Part 1, in stabilizer solution
- DAB Part B ≤0.1% (V/V) Hydrogen peroxide in stabilizer solution
- DAB Part B ≤0.1% (V/V) Hydrogen peroxide in stabilizer solution
- Haematoxylin, 0.1%

Steps of IHC staining (39)**:**

A. Preparation of Buffer–Two types of buffers were used.

1. Wash Buffer

2. Antigen Retrieval Buffer (ARB)

- Wash buffer preparation: 6 gm powdered TRIS buffer salt was dissolved into 1 litre of distilled water and pH was set at 7.4.
- ARB preparation: Citrate Buffer**:** 29.40 grams of sodium citrate in 1 litre of distilled water, and the pH set at 6.0. The citrate buffer was used for NELL1, PLA2R and THSD7A immunohistochemical staining.

Note:

- To increase the pH, NaOH solution was added drop by drop and pH was titrated.
- To decrease the pH, HCl was added drop by drop and pH was titrated.

B. Preparation of PLL coated slides: Tissue sections of 2.5-3 µ thickness were obtained on the PLL coated slides.

C. Baking: The slides were kept at 60˚C for 1 hour and then cooled to room temperature.

D. IHC staining procedure:

1. Deparaffinization – The slides were kept in Xylene I (10 minutes), followed by Xylene II (10 minutes).
2. Rehydration – The slides were kept in 100%, 70%and 50% alcohol for 5 minutes each followed by running tap water for 5 minutes.
3. Antigen retrieval – by pressure cooker method. 200 ml of clean tap water was taken in the empty pressure cooker and heated up to the steam formation. The slides were placed in a rack. 300 ml of ARB was put in the container and the rack with slides was placed inside the container. Then the container containing the rack with slides, was

placed inside the pressure cooker and the lid was closed. After two whistles the pressure was released by lifting the air vent and allowed to cool till it reached room temperature.

4. Wash – Slides were washed in Wash Buffer (pH7.4) thrice at a 1-minute interval.
5. Peroxide blocking – Blocking reagent was added to the sections and incubated for 10 minutes in the Humidity chamber at room temperature. This step prevents unwanted, non-specific background staining.
6. The peroxide was decanted and not washed with buffer.
7. Primary antibody – PLA2R, NELL1 and THSD7A was added to the sections and incubated in the Humidity chamber for one hour.
8. Wash – After that slides were washed in Wash Buffer (pH 7.4) thrice at a 1-minute interval.
9. Amplifier – Amplifier was added over the sections and incubated for 30 minutes in the Humidity chamber at room temperature.
10. Wash – The slides were washed in Wash Buffer (pH 7.4) thrice at a 1-minute interval.
11. HRP label – The HRP was added and incubated for 30 minutes in the Humidity chamber at room temperature.
12. Wash – The slides were washed in Wash Buffer (pH 7.4) thrice at a 1-minute interval.
13. DAB – The DAB chromogen was applied to the sections and incubated in the Humidity chamber for 10 minutes, avoiding light exposure as much as possible.
14. Wash – The sections were washed in distilled water twice at a 1-minute interval.
15. Counterstain – Slides were counterstained using Harris Haematoxylin for 2-3 minutes.
16. Wash – The slides were washed in running tap water for 5 minutes.
17. Dehydration – was done in graded alcohol (50%, 70%, 95%, 100%), 1 minute each.

18. Mounting – Slides were air-dried, mounted with DPX and examined under the microscope.

Interpretation of Immunohistochemical stain

- **Neural epidermal growth factor like 1 protein (NELL-1):**

  Positive – Bright granular capillary wall staining

  Internal control – Strong cytoplasmic positivity in cells in the tubules

- **Phospholipase A2 receptor (PLA2R):**

  Positive – Granular staining of glomerular capillary walls

  Internal control– isolated faint granular peripheral staining of the podocytes

- **Thrombospondin type I containing domain (THSD7A)**

  Positive – Diffuse granular capillary wall staining

  Internal control – Paranuclear dot like staining in podocytes and luminal surface staining in proximal tubules

4.7 Statistical Analyses

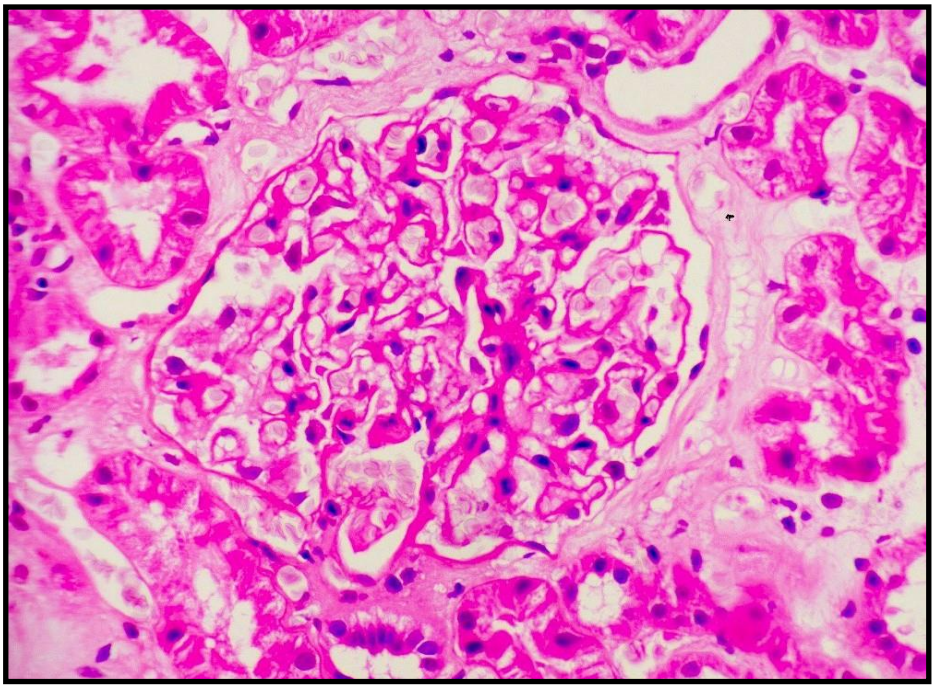
The data was entered in Microsoft Excel and all entries were checked again for errors. The data was analysed using Jamovi ver.1.8.2.0 and Statistical Package for Social Sciences ver. 23.0. (IBM SPSS, Inc., Chicago, IL). The data was checked for normality using Shapiro-Wilk test. Parametric and non-parametric tests of significance were applied for the outcome variables. Quantitative variables are presented in mean and standard deviation, or median and inter-quartile range, and analysed using the Mann-Whitney-U test. Categorical variables are presented in proportions and percentages, and analysed using the Chi-square test or Fischer-Exact's test.

4.8 Ethical Considerations

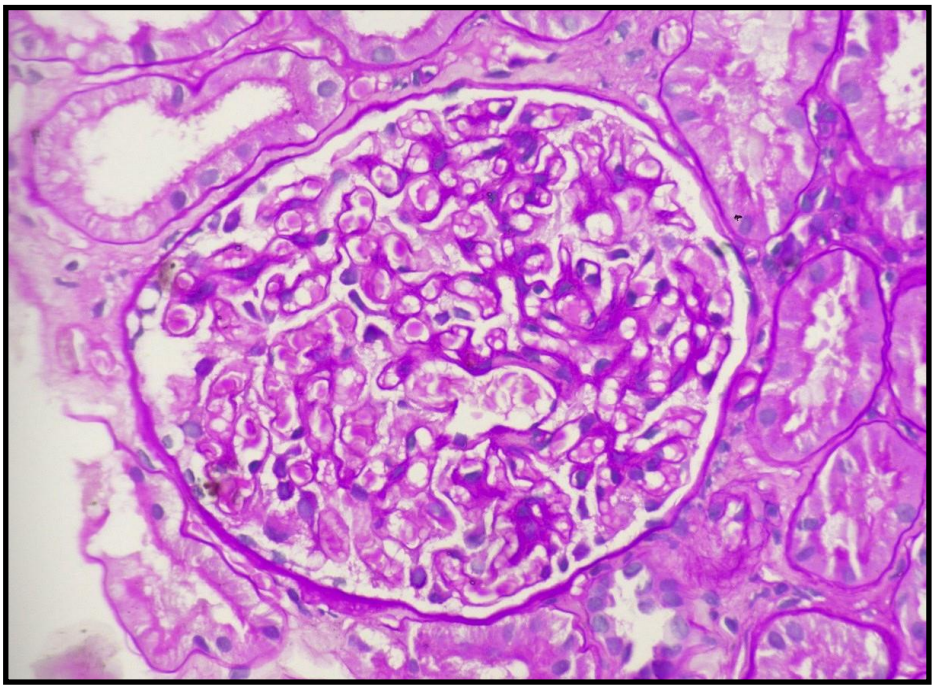
Ethical approval was obtained from AIIMS Jodhpur, Institutional Ethical Committee vide their letter No. **AIIMS/IEC/2020/3142**, dated 23/09/2020. All participants were informed about the objectives of the study and benefits of participating in the study. Informed consent was obtained from all the participants, before recruitment into the study. Participant information sheet was given to all participants and their role in the study were explained before administering the study questionnaire. They were assured of complete confidentiality of information and were explained the option of withdrawing from the study at any point of time if they desired to do so. All the data collected were kept confidential.

Chapter 5: Results

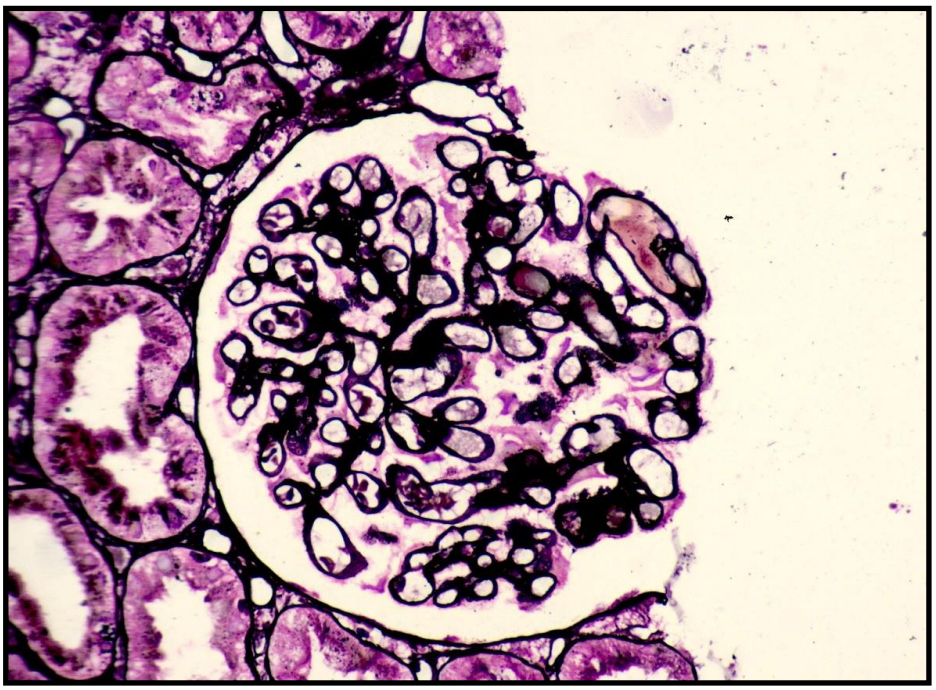
Photomicrographs



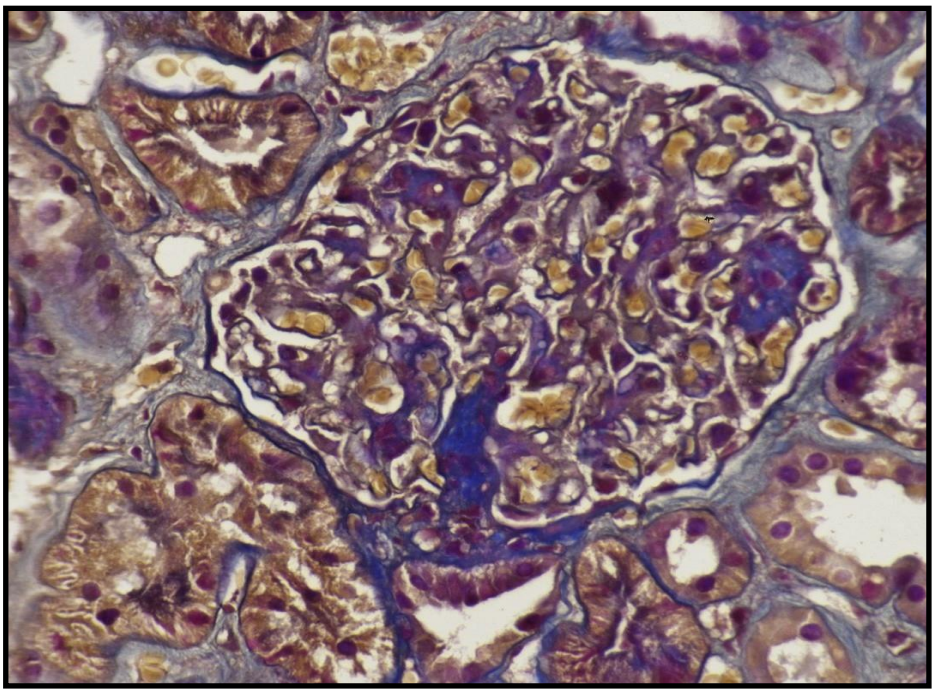
Photomicrograph 1 – Uniform thickening of glomerular basement membrane (400x; H&E)



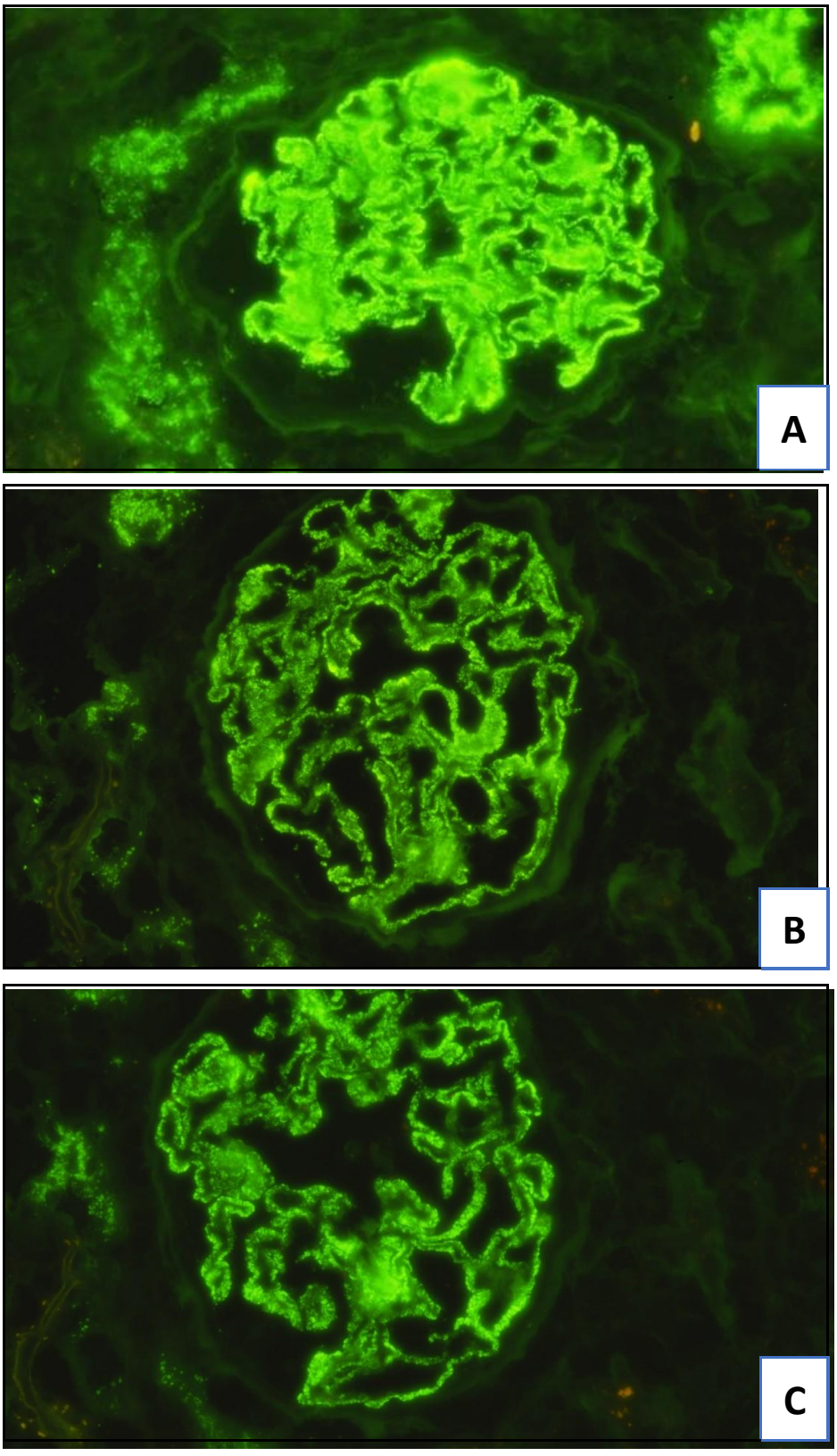
Photomicrograph 2 – PAS stain highlighting the thickened glomerular basement membrane along with subepithelial spikes (400x; PAS)



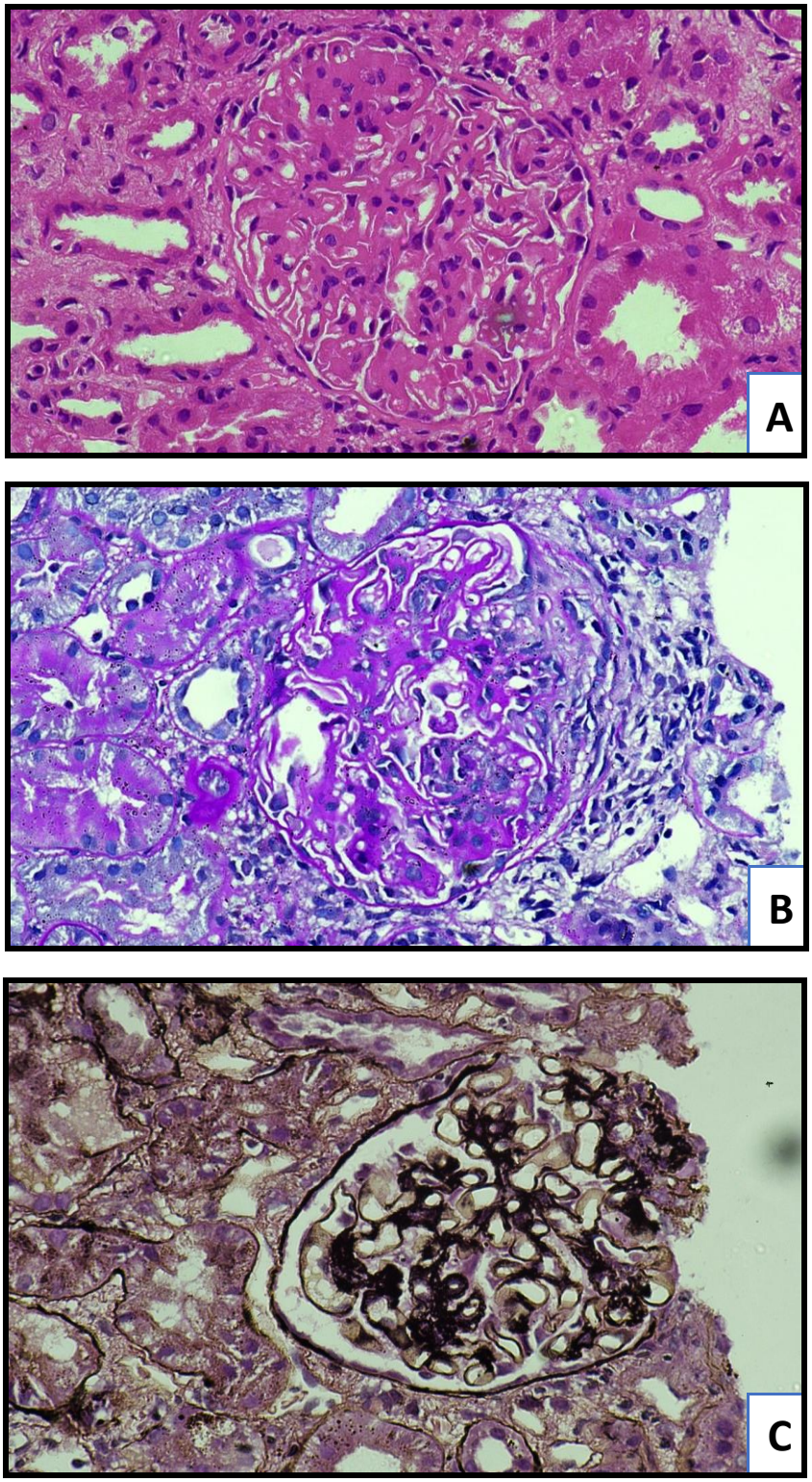
Photomicrograph 3 – JMS showing subepithelial spikes and craters (400x; JMS)



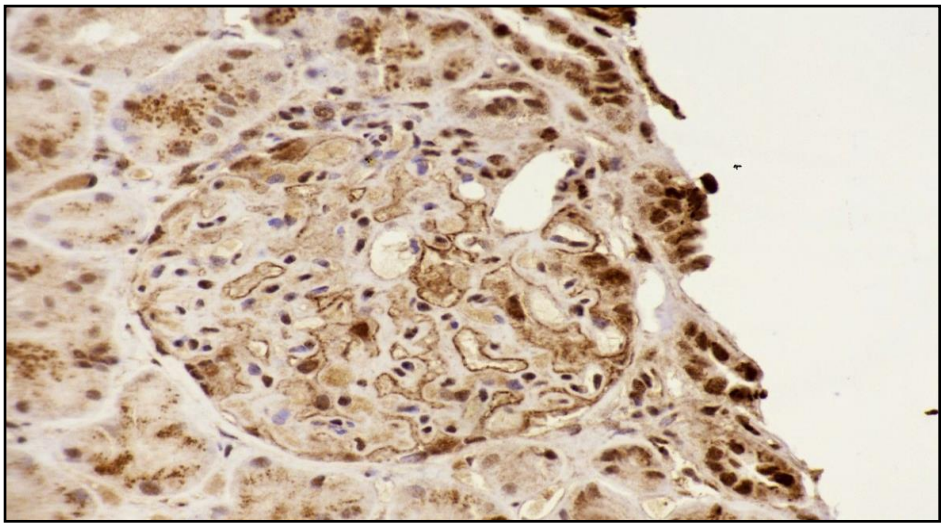
Photomicrograph 4 – MT stain demonstrates fuchsinophilic deposits along the subepithelial aspect of GBM (400x; MT).



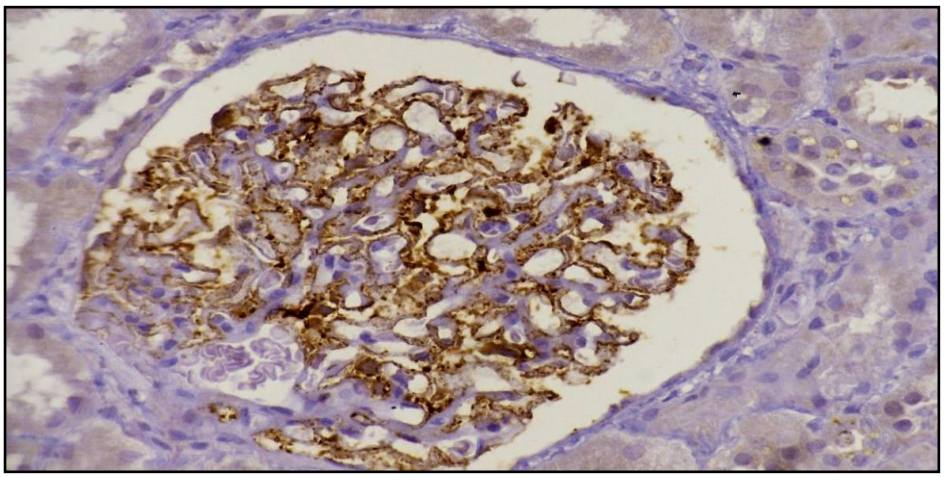
Photomicrograph 5 – Intense granular staining for (A) IgG, (B) Kappa, (C) Lambda respectively along the GBM (400x; DIF).



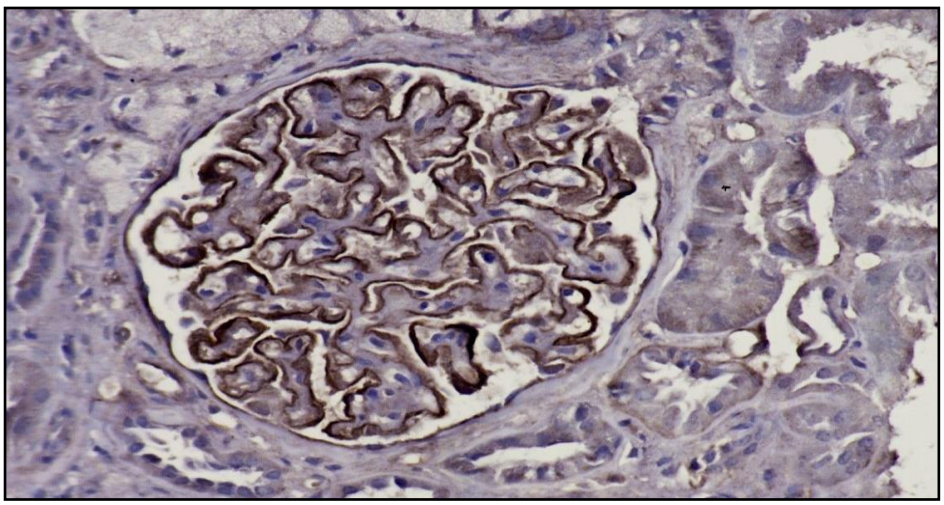
Photomicrograph 6 – (A) Marked thickening of glomerular capillary loops with mesangial and endocapillary hypercellularity (400x; H&E); (B) PAS highlights thickened GBM; (C) JMS showing sub-epithelial spikes and craters with wire loop lesions.



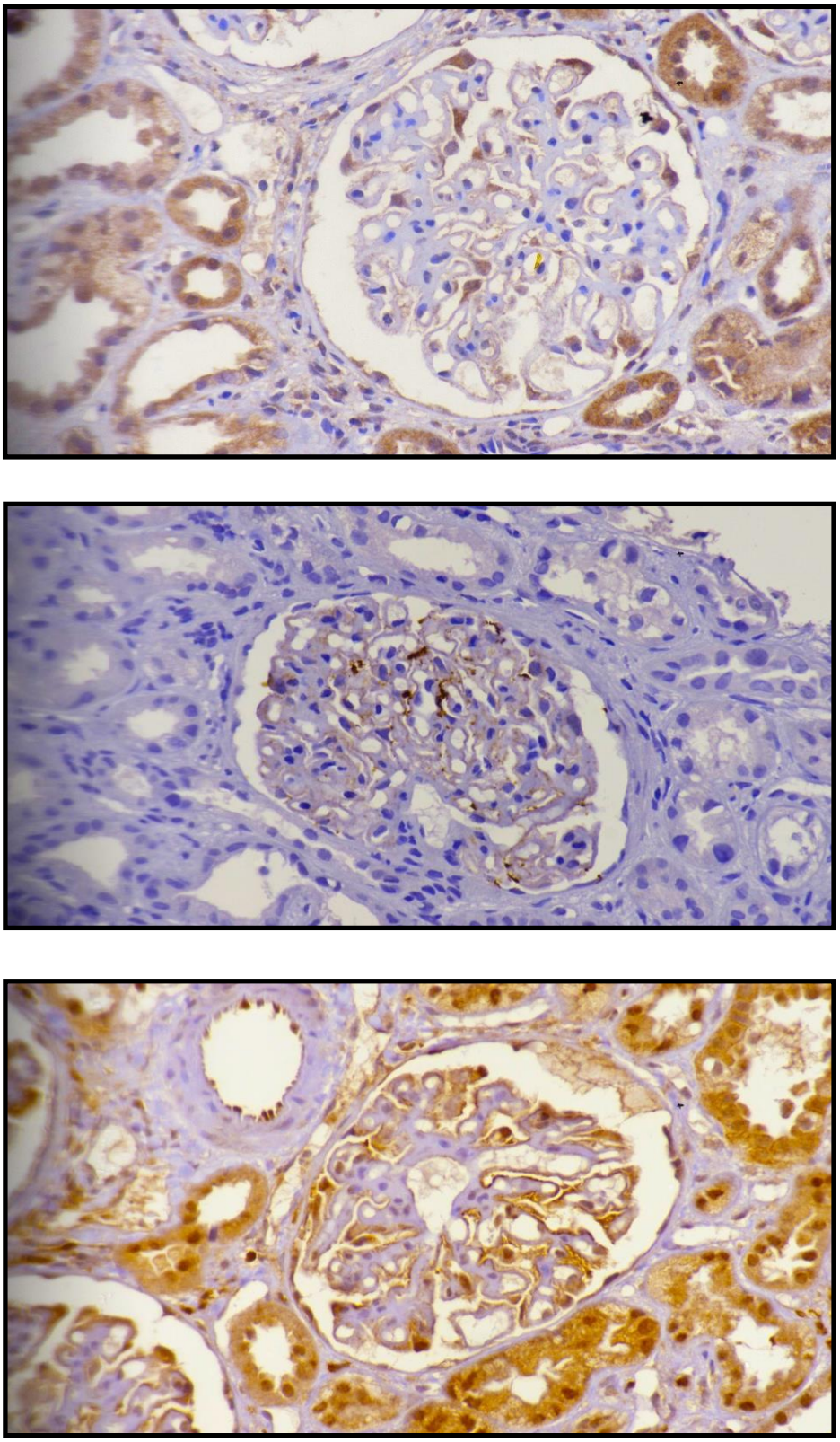
Photomicrograph 7 – Bright granular capillary wall staining of NELL-1 along glomerular capillary loops with positive internal control (400x, IHC)



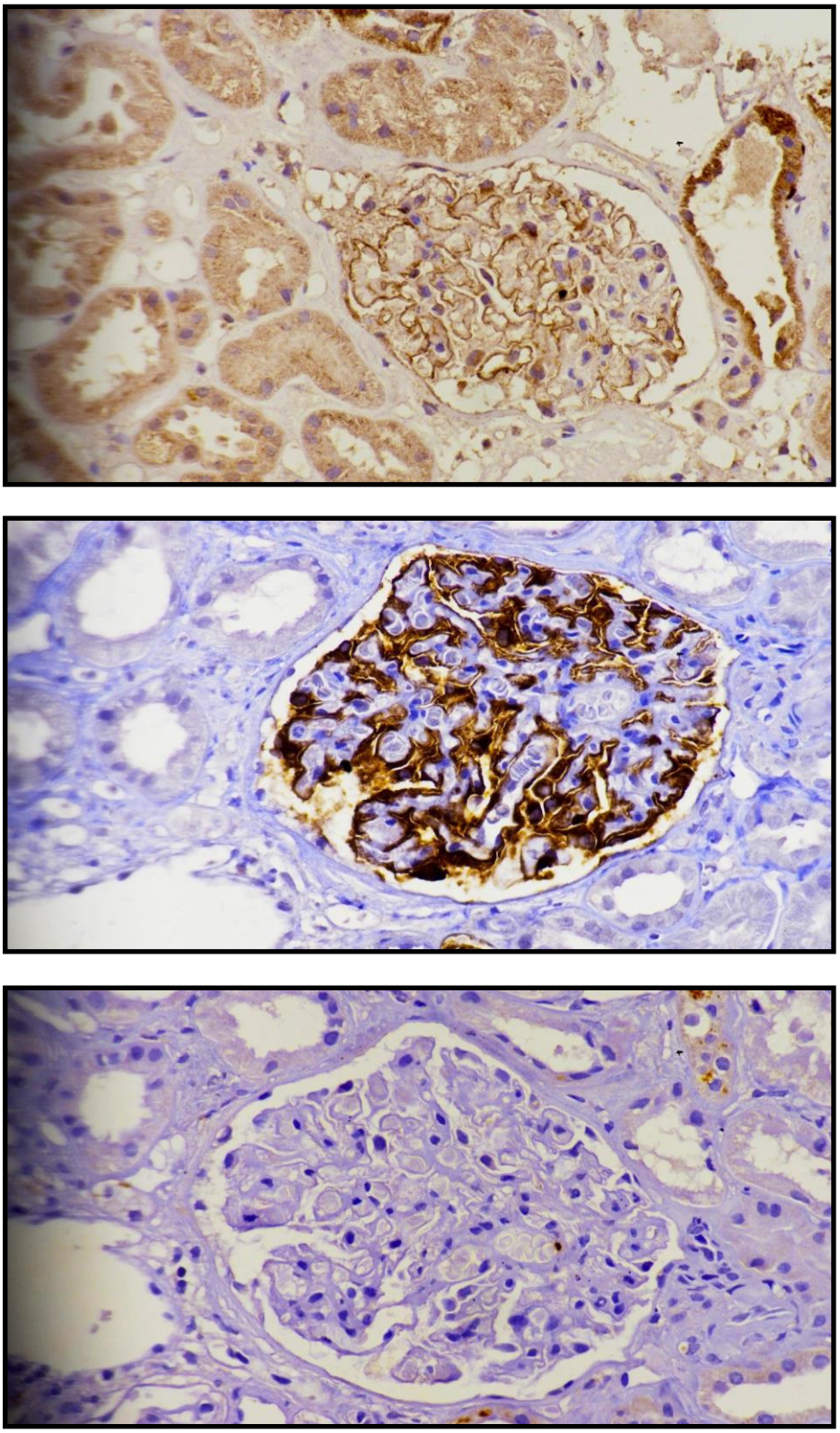
Photomicrograph 8 – Bright granular staining for PLA2R of glomerular capillary walls (400x, IHC)



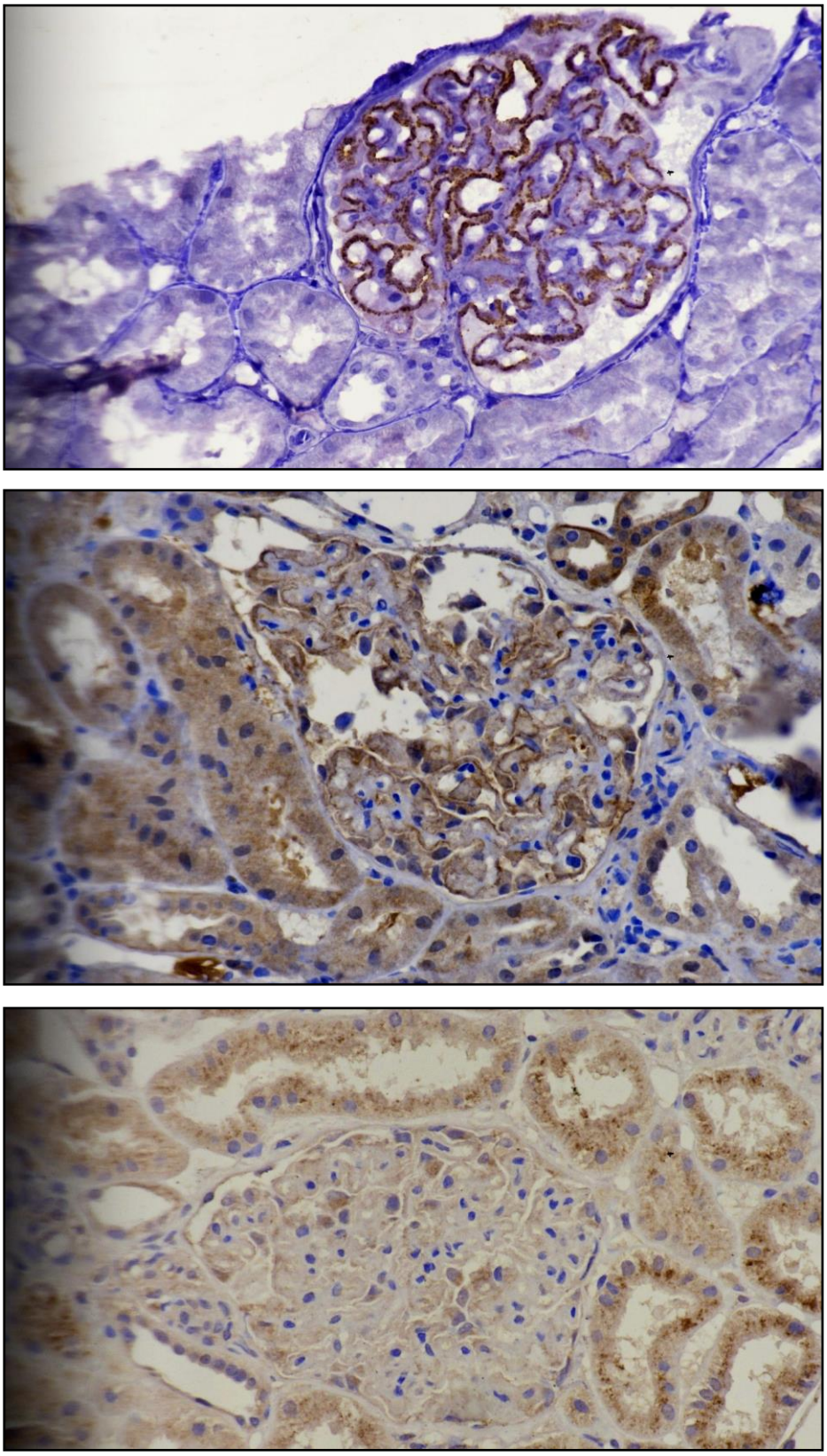
Photomicrograph 9 - Diffuse granular capillary wall staining of THSD7A with positive internal control (400x, IHC).



Photomicrograph 10 - Triple positivity for NELL-1, PLA2R and THSD7A (top tobottom) in a patient with membranous nephropathy (400x, IHC).



Photomicrograph 11 - Granular staining for NELL-1, PLA2R (upper and middle image respectively) along the glomerular capillary loops and negative staining forTHSD7A (lower image) (400x, IHC).



Photomicrograph 12 - Granular staining for PLA2R and THSD7A (upper and middle image respectively) along the glomerular capillary loops and negative stainingfor NELL-1 (lower image) (400x, IHC).

The study was conducted from 2020 to 2022, with recruitment of cases from the year 2016. Among the total number of 474 renal biopsies received in the laboratory over a period of six years, 50 cases were diagnosed as membranous nephropathy (10.5% prevalence). From the total number of cases, 47 patients with membranous nephropathy with adequate tissue and clinicopathologic data were profiled to study the glomerular immunohistochemical expression of antigens, viz., NELL-1, PLA2R, and THSD7A. The median age of the patients in the study was 40 years (IQR - 20.5 years). Among the 47 patients in the study, 20 were females (42.6%) and 27 were males (57.4%). Thirty-two patients (68.08%) had primary membranous nephropathy, while fifteen (31.92%) had secondary membranous nephropathy.

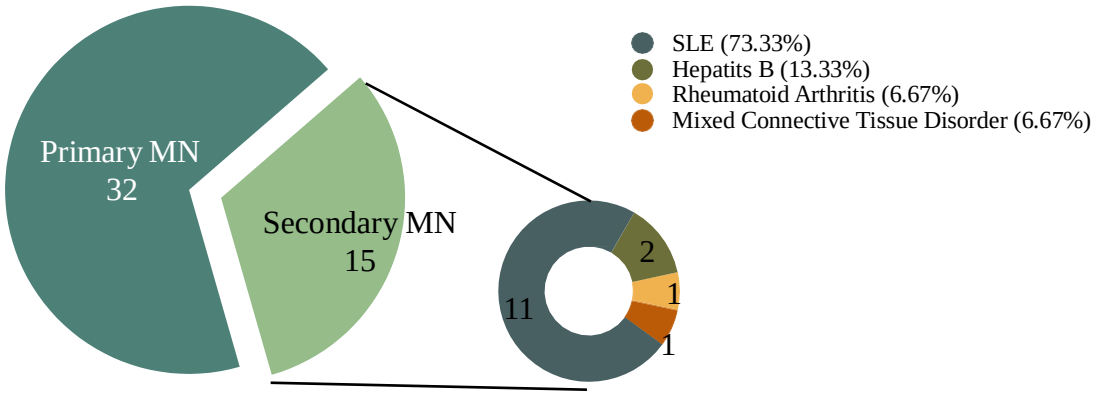


Figure 5.1 - Causes of membranous nephropathy among patients in the study.

From figure 5.1, it is observed that among the secondary causes for membranous nephropathy, 11 (73.33%) participants were diagnosed with systemic lupus erythematosus (SLE), while two (13.33%) had Hepatitis B infection. Other diagnoses in the group were rheumatoid arthritis (1, 6.67%), and mixed connective tissue disorder (1, 6.67%).

Table 5.1
Co-morbidity status of patients with membranous nephropathy

Variables	n(%) [N=47]
Hypertension	12 (25.50)
Diabetes mellitus	3 (6.40)
Systemic Lupus Erythematosus	11 (23.40)
Hepatitis B infection	2 (4.30)
Malignancies	0 (0.0)
Dyslipidemia	33 (70.20)
Hematuria	12 (25.50)

From table 5.1, it is observed that a quarter of the cases with membranous nephropathy had hypertension (12, 25.5%), and three (6.4%) had diabetes mellitus. There were no malignancies identified or reported among study participants. About 70% of the participants had dyslipidemia. Two-thirds of the participants (31, 66.0%) had proteinuria in the range of 3+ (Figure 5.2), while a quarter of the participants had hematuria (12, 25.5%).

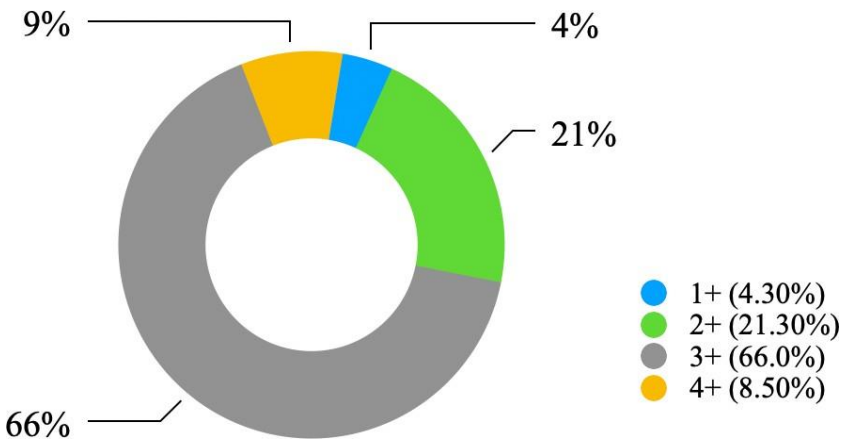


Figure 5.2 – Distribution of Proteinuria among study participants

The figure 5.3 below describes the pattern of immunofluorescence staining for immunoglobulins (IgA, IgG, IgM) and complements (C1q and C3) among the patients. Among the three antibodies (IgA, IgG, IgM) studied, it was observed that more than 80% (38 out of 44) of the participants had granular staining of IgG (≥3+). IgA staining was not found in more than 50% of the participants (23 out of 44).

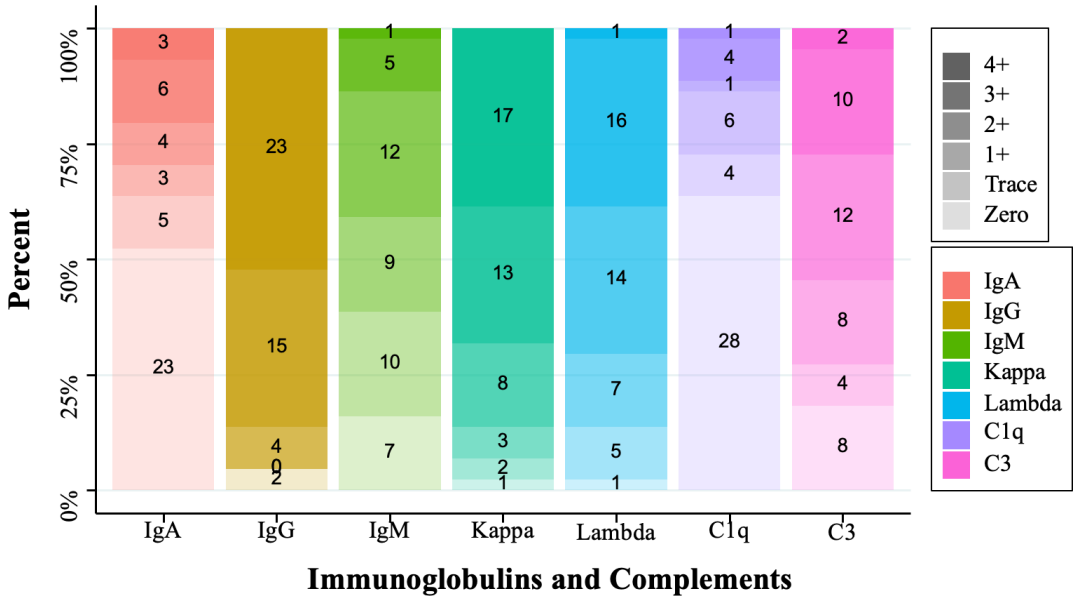


Figure 5.3 – Pattern of immunofluorescence staining among the patients with membranous nephropathy (n=44)

Profiling of the antigens among the cases of membranous nephropathy revealed that one patient of primary MN exhibited triple antigen positivity, while there were no such cases in secondary MN. Among the five triple antigen negative patients, there were four cases among secondary MN and one in primary MN. More than 50% of the participants (25 out of 47) had only PLA2R antigen positive, of which 19 had primary MN (59.38% among primary MN cases), and six had secondary MN (40.0% among secondary MN cases). Only NELL1 was found positive among two cases, of which one was primary and the

other being secondary MN (lupus class V). An exact similar picture was found for only THSD7A antigen positive patients. Among the double antigen positives, NELL-1 and PLA2R were more commonly exhibited among the participants (9, 19.14%), with seven (21.89% among primary MN cases) being primary MN and the other two (13.32% among secondary MN cases) being secondary MN. NELL-1 and THSD7A was found positive only among one patient with secondary MN, while PLA2R and THSD7A was found positive only among two patients with primary MN. (Table 5.2)

Table 5.2
Profiling of antigens in membranous nephropathy

	Primary MN [N=32] n (%)	**Secondary MN [N=15] n (%)**	**Total [N=47] n (%)**
Single antigen positives			
Only NELL-1	1 (3.12)	1 (6.67)	2 (4.24)
Only PLA2R	19 (59.38)	6 (40.0)	25 (53.19)
Only THSD7A	1 (3.12)	1 (6.67)	2 (4.24)
Double antigen positives			
NELL-1 and PLA2R	7 (21.89)	2 (13.32)	9 (19.14)
NELL-1 and THSD7A	0 (0.0)	1 (6.67)	1 (2.12)
PLA2R and THSD7A	2 (6.25)	0 (0.0)	2 (4.24)
Triple antigen positive	1 (3.12)	0 (0.0)	1 (2.12)
Triple antigen negative	1 (3.12)	4 (26.67)	5 (10.63)

Table 5.3
Immunohistochemical expression of antigens in primary vs. secondary membranous nephropathy

Antigens	Primary MN [N=32] n (%)	Secondary MN [N=15] n (%)	Total [N=47] N (%)	χ^2 value	p value
NELL-1	9 (28.1)	4 (25.7)	13 (27.65)	0.011	1.000
PLA2R	29 (90.6)	8 (53.3)	37 (78.72)	8.48	0.007
THSD7A	4 (12.5)	2 (13.3)	6 (14.89)	0.006	1.000

Fischer's-Exact test

From the table 5.3 above, it can be observed that PLA2R antigen is found significantly associated with primary membranous nephropathy, while no such association is appreciated for the other two antigens studied, i.e., NELL-1 and THSD7A.

It is observed that IgG staining was found to be ≥3+ in about 90% of the patients with primary MN, with a statistically significant association. Almost 90% of the patients with primary MN showed IgA staining of less than 3+, with a statistically significant association. There were no patients with IgM staining ≥3+ among primary MN. There was no statistically significant association observed between Kappa or Lambda levels and the type of membranous nephropathy. Among the complements, it was found that no patient with primary MN showed C1q staining of ≥3+. Also, there was no statistical association of membranous nephropathy with C3 staining. No patients with primary MN showed positivity for extraglomerular staining or presence of fibrinogen. (Table 5.4)

Table 5.4
Immunofluorescence staining in primary vs. secondary membranous nephropathy

Variables		Primary MN [N=29] n (%)	Secondary MN [N=15] n (%)	χ^2 value	p value
Immunoglobulins					
IgA	<3+	26 (89.7)	9 (60.0)	5.34	0.044*
	≥3+	3 (10.3)	6 (40.0)		
IgG	<3+	3 (10.3)	3 (20.0)	0.783	0.394*
	≥3+	26 (89.7)	12 (80.0)		
IgM	<3+	29 (100.0)	9 (60.0)	-	-
	≥3+	0 (0.0)	6 (40.0)		
Kappa	<3+	8 (27.6)	6 (40.0)	0.702	0.402
	≥3+	21 (72.4)	9 (60.0)		
Lambda	<3+	13 (44.8)	4 (26.7)	1.10	0.295
	≥3+	16 (55.2)	11 (73.3)		
Complements					
C1q	<3+	29 (100.0)	10 (66.67)	-	-
	≥3+	0 (0.0)	5 (33.33)		
C3	<3+	21 (72.4)	11 (73.3)	0.004	0.771*
	≥3+	8 (27.6)	4 (26.7)		
Fibrinogen		0 (0.0)	2 (13.33)	-	-
Extraglomerular staining		0 (0.0)	7 (46.7)	-	-

**Fischer's-Exact test; Empty boxes indicate that chi-square is not applicable*

Table 5.5
Light microscopy findings in primary vs. secondary membranous nephropathy

Variables	Primary MN [N=32] n (%)	Secondary MN [N=15] n (%)	χ^2 value	p value
Mesangial hypercellularity	4 (12.5)	10 (66.7)	14.3	<0.001
Endocapillary hypercellularity	0 (0.0)	7 (46.7)	-	-
Crescent formation	0 (0.0)	2 (13.3)	-	-
Focal segment glomerulosclerosis	17 (53.1)	7 (46.7)	0.75	0.686
Arteriosclerosis	18 (56.3)	8 (53.3)	0.03	0.851
Arteriolosclerosis	12 (37.5)	5 (33.3)	0.07	0.782

Empty boxes indicate that chi-square is not applicable

Table 5.5 shows the association of light microscopy findings with the type of membranous nephropathy. While diffuse and global capillary wall thickening with formation of spikes and craters (which is characteristic of MN) was found in all the cases, it is observed that no patients with primary MN exhibited endocapillary hypercellularity or crescent formation, but on the other hand, mesangial hypercellularity was significantly associated with secondary MN with more than 60% patients being positive for it. Other features such as focal segment glomerulosclerosis (FSGS), arteriosclerosis or arteriolosclerosis had no statistical association.

Table 5.6
Association of socio-demographic variables and co-morbidities with expression of NELL-1, PLA2R, THSD7A antigens

Variables		NELL-1 antigen				PLA2R antigen				THSD7A antigen			
		Positive (N=13) n (%)	Negative (N=34) n (%)	χ^2 value	p value	Positive (N=37) n (%)	Negative (N=10) n (%)	χ^2 value	p value	Positive (N=6) n (%)	Negative (N=41) n (%)	χ^2 value	p value
Socio-demographic variables													
Age (in years) (Mean±SD)		41.2±18.0	39.4±13.9	-	0.285*	42.1±15.1	32.0±12.1	-	0.059*	39.7±14.7	40.0±15.2	-	0.966*
Gender	Male	9 (69.2)	18 (52.9)	1.02	0.312	25 (67.6)	2 (20.0)	7.29	0.007	3 (50.0)	24 (58.5)	0.15	0.693
	Female	4 (30.8)	16 (47.1)			12 (32.4)	8 (80.0)			3 (50.0)	17 (41.5)		
Co-morbidities													
Hypertension		4 (30.8)	8 (23.5)	0.25	0.713#	9 (24.3)	3 (30.0)	0.13	0.700#	3 (50.0)	9 (22.0)	2.17	0.164#
Systemic Lupus Erythematosus		3 (23.1)	8 (23.5)	0.001	1.000#	4 (10.8)	7 (70.0)	15.4	<0.001#	2 (33.3)	9 (22.0)	0.38	0.614#
Dyslipidemia		11 (84.6)	22 (64.7)	1.78	0.288#	26 (70.3)	7 (70.0)			4 (66.7)	29 (70.7)	0.04	1.000#

**Independent samples t-test; #Fischer's Exact test; Empty boxes indicate that chi-square is not applicable for those variables.*

Table 5.6 shows the association of socio-demographic variables and co-morbidities with each of the immunohistochemical antigens considered in the present study. It was observed that there was no statistically significant difference in mean age between those patients who expressed either of these antigens as compared to the ones with no expression. PLA2R antigen is found to be expressed more among the males, with a statistically significant association. Gender-based association was not observed for the other two antigens. It could be seen that only four patients with SLE (which is the most important cause for secondary MN) express PLA2R antigen, and the statistical significance affirms the finding from table 5.3 that PLA2R antigen is expressed more commonly among patients with primary MN. There is no statistically significant association of these antigens with other co-morbidities.

Among the microscopy findings (Table 5.7), it was observed that crescent formation was not seen in patients who expressed either of the three antigens. Also, endocapillary hypercellularity was not observed in about 95% of patients who expressed PLA2R antigen, with a statistically significant association, while expression of other two antigens showed no such association for endocapillary proliferation. FSGS was found more commonly among patients who did not express NELL-1 antigen (21, 61.8%), as compared to those who expressed NELL-1 (3, 23.1%), with a statistically significant association. Other microscopy findings such as mesangial hypercellularity, arteriosclerosis or arteriolosclerosis had no statistical association with expression of either antigen (NELL-1, PLA2R, THSD7A)

Table 5.7
Association of laboratory and microscopy findings with expression of NELL-1, PLA2R, THSD7A antigens

Variables		NELL-1 antigen				PLA2R antigen				THSD7A antigen			
		Positive (N=13) n (%)	Negative (N=34) n (%)	□² value	p value	Positive (N=37) n (%)	Negative (N=10) n (%)	□² value	p value	Positive (N=6) n (%)	Negative (N=41) n (%)	□² value	p value
Laboratory findings													
Hematuria		2 (15.4)	10 (29.4)	0.97	0.464#	11 (29.7)	1 (10.0)	1.61	0.414#	2 (33.3)	10 (24.4)	0.22	0.637#
Proteinuria	<3+	2 (15.4)	10 (29.4)	0.97	0.540#	9 (24.3)	3 (30.0)	0.13	0.964#	3 (50.0)	9 (22.0)	2.16	0.331#
	≥3+	11 (84.6)	24 (70.6)			28 (75.7)	7 (70.0)			3 (50.0)	32 (78.0)		
Microscopy findings													
Mesangial hypercellularity		4 (30.8)	10 (29.4)	0.01	0.927	9 (24.3)	5 (50.0)	2.48	0.115	3 (50.0)	11 (26.8)	1.34	0.344#
Endocapillary hypercellularity		1 (7.7)	6 (17.6)	0.735	0.655#	2 (5.4)	5 (50.0)	12.4	0.003#	1 (16.7)	6 (14.6)	0.01	1.000#
Crescent formation		0 (0.0)	2 (5.9)			0 (0.0)	2 (20.0)			0 (0.0)	2 (4.9)		
Focal segment glomerulosclerosis		3 (23.1)	21 (61.8)	5.63	0.018	20 (54.1)	4 (40.0)	0.62	0.430	4 (66.7)	20 (48.8)	0.67	0.666#
Arteriosclerosis		10 (76.9)	16 (47.1)	3.39	0.065	21 (56.8)	5 (50.0)	0.14	0.703	3 (50.0)	23 (56.1)	0.07	1.000#
Arteriolosclerosis		7 (53.8)	10 (29.4)	2.43	0.119	13 (35.1)	4 (40.0)	0.08	0.776	3 (50.0)	14 (34.1)	0.57	0.653#

#Fischer's Exact test; Empty boxes indicate that chi-square is not applicable for those variables.

Table 5.8
Comparison of laboratory parameters with expression of NELL-1, PLA2R, THSD7A antigens

Variables	NELL-1 antigen [Median(IQR)]			PLA2R antigen [Median(IQR)]			THSD7A antigen [Median(IQR)]		
	Positive (n=13)	Negative (n=34)	p value	Positive (n=37)	Negative (n=10)	p value	Positive (n=6)	Negative (n=39)	p value
Urinary protein (24 hr) (mg/dL)	6.01 (6.30)	5.06 (3.52)	0.918	6.29 (5.13)	2.85 (2.48)	**0.006**	5.63 (1.77)	4.93 (5.84)	0.878
Blood urea nitrogen (mg/dL)	26.0 (13.0)	27.0 (17.0)	0.703	26.0 (13.0)	31.0 (29.0)	0.286	18.5 (4.0)	30.0 (13.0)	0.111
Serum creatinine (mg/dL)	0.85 (0.22)	0.86 (0.50)	0.625	0.85 (0.52)	0.81 (0.45)	0.584	0.82 (0.39)	0.85 (0.52)	0.962
Albumin (g/dL)	1.58 (0.75)	2.12 (0.83)	0.050	2.0 (0.75)	2.50 (1.05)	0.795	2.51 (0.79)	1.93 (0.84)	0.091
Glucose (mg/dL)	112.0 (29.8)	94.0 (12.3)	0.196	96.0 (18.5)	92.0 (33.0)	0.765	110.0 (50.0)	96.0 (16.0)	0.632
HbA1C (%)	5.60 (1.25)	5.80 (0.70)	0.775	5.80 (0.63)	5.40 (1.15)	0.445	6.0 (1.05)	5.80 (0.75)	0.611
Cholesterol (mg/dL)	297.0 (246.0)	292.0 (212.0)	0.730	349.0 (224.0)	232.0 (119.0)	0.120	275.0 (27.0)	325.0 (257.0)	0.320
Triglycerides (mg/dL)	209.0 (86.50)	205.50 (118.0)	0.883	205.0 (101.0)	213.0 (137.0)	0.403	252.0 (73.0)	205.0 (92.5)	0.278

**Mann-Whitney U test*

The laboratory parameters were found to be not normally distributed. Hence, non-parametric tests of significance (Mann-Whitney U test) was applied to test the significance of median values between two groups. It was found that the median 24hr urinary protein was higher among those who expressed PLA2R antigen as compared to those with no PLA2R antigen with a statistically significant difference [6.29 (5.13) vs. 2.85 (2.48), p=0.006]. Apart from that, none of the laboratory parameters had a statistically significant difference in median values between the patients expressing any of the antigens and those who did not. (Table 5.8).

Chapter 6: Discussion

The present study was conducted for immunohistochemical profiling of membranous nephropathy for expression of NELL-1, PLA2R, and THSD7A. In the present study, the clinicopathologic factors related to the expression of antigens were also assessed.

The median age of the patients with membranous nephropathy was found to be 40 years in the present study, in line with the literature that states the peak to occur between the fourth and fifth decades of life (1,40,41). There were no paediatric cases of membranous nephropathy reported in the present study. This could inherently be due to the rarity of the disease in children (primary forms of MN are reported in <7% of the biopsies) (12). The study findings also ascertained that the expression of the antigens (NELL-1, PLA2R, THSD7A) were independent of the age of the patient. As for gender preposition, more number of cases in the present study were reported among males, concordant with reported findings from the literature (12,13). Also, male predisposition was observed among patients with primary MN with a positivity for PLA2R antigen. This finding in the present study opens up the prospects of a genetic link for the disease or the expression of the antigen, which is not yet established in the literature.

Globally, many studies have shown that the primary MN and secondary MN accounts for approximately 75-80% and 20-25% of MN cases respectively (26,42). In our study, primary MN was reported among two-thirds of the patients, while the secondary MN was

associated with best established auto-immune causes such as Systemic Lupus Erythematosus (SLE), Rheumatoid Arthritis, and Mixed Connective Tissue Disorder (7). Hepatitis B infection was reported among two cases of secondary MN. While the literature suggests strong association of malignancies with secondary MN (7,43), no malignancies were observed among the study patients. Similarly, the strength of association for NELL-1 and THSD7A antigens with malignancy is established in the literature (9,44,45), while the present study could not second it, as there were no patients with malignancy. With the disease entity in itself being a rare occurrence, the co-existence of malignancy with it, could not be appreciated in the present study due to a smaller sample size and a time bound nature of the study. The role of therapeutic agents such as gold salts, penicillamine, and NSAIDs as causal agents for secondary MN were not assessed in the present study.

Among the study patients, it was observed that almost three-fourths exhibited proteinuria in the ranges of ≥3+ and dyslipidemia, while only a quarter of them exhibited hematuria, and also reported to be hypertensives. MN being one of the most common causes for nephrotic syndrome, these clinical presentations are expected of the patients (41). It was also observed that the disease has been indolently progressive among the patients of the study, which could be attributed to the gradual and progressive accumulation of sub-epithelial immune deposits in the glomerulus, resulting in podocyte injury (13). Furthermore, based on the results of the present study, it appears that these clinico-

pathological features also do not depend upon the expression of the profiled antigens (NELL-1, PLA2R, THSD7A), as these features are not associated with it.

The levels of 24-hour urinary protein were found to be significantly higher among those patients who expressed PLA2R antigen, making it a notable clinical feature which could be used in the predictive analysis of the antigens, similar result was found in the studies done by Subramanian P et al and Gudipati A et al (8,34). The other laboratory features assessed in the study exhibited no significant difference in their values, depending upon the expression of the antigens, which is in line with the studies done by Xu NX et al and Iwakura T et al (29,33).

In concordance with the studies from the literature (1,8,32), it was observed that mesangial hypercellularity was associated with secondary MN, and endocapillary hypercellularity and crescent formation was completely absent among patients with primary MN. Their presence almost always conforms with a secondary form of MN or the co-existence of an additional glomerular disease process (7). Another interesting feature that was noted among the light microscopy findings was the absence of crescent formation among the patients who expressed positivity for either of the three antigens. The link between absence of crescent formation in both primary MN and the expression of these antigens should further be explored. The absence of endocapillary hypercellularity is associated with the expression of the PLA2R antigen, reaffirming that

it is a feature of the secondary MN, as PLA2R antigen is more commonly expressed among patients with primary MN. While focal segmental glomerulosclerosis could indicate an ongoing additional glomerular process, it was found that there was no association of the feature with the type of membranous nephropathy, but with the non-expression of the NELL-1 antigen and not others. While arteriosclerosis and arteriolosclerosis are a reflection of the patient's age, it was neither associated with the type of membranous nephropathy nor with the expression of the antigens profiled (NELL-1, PLA2R, THSD7A).

The manifestation of higher levels of IgG and C3 in immunofluorescence staining among majority of patients affirms the fact that the immune-complexes formed in the patients of membranous nephropathy contains these antibodies and its relevant antigens, and complement components (1,18,41). But the lack of a significant statistical association of these antibodies and complements with the type of membranous nephropathy could be due to a lesser sample size in the present study. IgA antibodies were not expressed or expressed in small quantities, in a majority of the patients with primary MN. Also, it was found that no patient with primary MN had fibrinogen or extraglomerular deposits. These findings substantiate the evidences from the literature that their presence shall favour secondary causes for the disease (1) .

As for the profiling of the antigens in the present study, it was found that THSD7A was expressed the least among the patients, the rarity of its expression also discussed by

Tomas et al. in 2014 (4). PLA2R antigen was the most expressed, especially among patients of primary MN, very similar to the demonstrations of Beck et al., back in 2009 (3) and recent other studies of Subramanian P et al, Roy S et al, Gudipati A et al (8,32,34).Expression of IgG in higher proportions among the patients in the present study also supports the literature that anti-PLA2R autoantibodies are mostly of the IgG4 type (35,38). While the most of the studies in the literature suggests that NELL-1 antigen is more commonly expressed among PLA2R negative patients (6,9,37), only one study done by Wang G et al in 2021 showed that expression of NELL-1 can also be seen in PLA2R positive cases of MN (10). Similar to that, in the present study it was observed that the proportion for NELL-1 antigen expression (10 out of 13 patients) was higher among those who also expressed PLA2R antigen. In a study by Caza et al (9), it is seen that 33% of NELL-1 positive cases was found associated with malignancy, but in the present study, such an association could not be commented upon as there were no reported cases of malignancy. However, it was observed that expression of nell-1 was seen even in patients with no malignancy. In this regard, the findings by Wang et al. (10) is also to be noted, which reports no such association with malignancy. These differing conclusions between studies warrant further exploration of this association among patients with malignancy. Double antigens were found positive among a quarter of the patients in the study. Such dual positivity are described in literature (8,10,28,29), though the expression of these antigens are believed to be mutually exclusive. While the reasons for such dual expressions are to be explored further in depth,it is believed that PLA2R and THSD7A are expressed together due to a common antigenic motif in the N terminal region in these

antigens that activates B cells to produce antibodies. These antibodies may be directed against both the antigens or any one of the two (8,22). Recent studies (8,13,28) have shown that these profiled antigens are expressedeven in the secondary form of MN, which is also affirmed by the findings in the present study.

Chapter 7: Summary of Findings and Conclusion

7.1 Summary of Findings

This was an ambispective study on 47 cases of membranous nephropathy (MN). In the present study, the glomerular immunohistochemical expression of NELL-1, PLA2R and THSD7A was profiled. And their expression was correlated with various clinicopathological parameters.

- The mean age of presentation of MN was 40 years. 68.08% had primary MN.
- About two-thirds of the participants presented with proteinuria in the range of 3+. About 70% patients had dyslipidemia. The identified secondary causes in the present studywere SLE, HBV infection, RA and MCTD.
- All cases of MN showed diffuse and uniform capillary wall thickening with 66.7% of secondary showed mesangial hypercellularity.
- Among 47 cases of membranous nephropathy, the expression of NELL-1 was seen in 13 patients (27.65%). PLA2R and THSD7A expression were seen in 78.72% and 14.89% of MN respectively.
- 12 cases showed dual positivity with 9 cases having NELL-1 and PLA2R dual positivity. NELL-1 antigen was found to express along with PLA2R.
- One patient of primary MN exhibited triple antigen positivity. 26.67% (4/15) cases of secondary MN did not show expression of any of these antigens (triple negative).
- PLA2R positive cases were predominantly males and had higher median 24hr urinary protein levels.
- NELL-1 positivity was seen even in cases with no history of malignancy
- No statistically significant association of the NELL-1 & THSD7A with the socio-demographic variables, co-morbidities, laboratory & microscopic parameters was noted.

7.2 Conclusion

The findings of the present study, despite consolidating the evidences from the literature, also brings out certain features that are significantly associated with the expression of the profiled antigens in membranous nephropathy, such as higher 24 hour proteinuria in PLA2R positive cases. Also, to our present knowledge, this is the first observational study to profile NELL-1 antigen, alongside PLA2R and THSD7A among cases of membranous nephropathy in the Indian setting. The present study opens up a prospective path to study gender as an important non-modifiable risk factor of the disease and also its role in expression of antigens. Also, findings of the light microscopy in association with the laboratory parameters (such as 24-hour urinary protein) could be used as potential tools in devising a predictive algorithm for expression of the antigens. Lack of statistical associations in the present study cannot be considered conclusive, given the smaller sample size of the present study. Hence, future studies should beplanned to incorporate more number of patients, with extensive studying of features that could help differentiate the types of membranous nephropathy and expression of antigens.

Chapter 8: References

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Annexures

Annexure A

Institutional Ethics Committee Clearance Certificate

अखिल भारतीय आयुर्विज्ञान संस्थान, जोधपुर
All India Institute of Medical Sciences, Jodhpur
संस्थागत नैतिकता समिति
Institutional Ethics Committee

No. AIIMS/IEC/2020/3177 **Date: 23/09/2020**

ETHICAL CLEARANCE CERTIFICATE

Certificate Reference Number: **AIIMS/IEC/2020/**3142

Project title: **"Profiling of Membranous Nephropathy based on Immunohistochemical expression of antigens NELL-1, PLA2R and THSD7A."**

Nature of Project:	**Research Project Submitted for Expedited Review**
Submitted as:	**M.D. Dissertation**
Student Name:	**Dr. Nivedita A**
Guide:	**Dr. Aasma Nalwa**
Co-Guide:	**Dr. Vikarn Vishwajeet, Dr. Poonam Abhay Elhence, Dr. Manish Chaturvedy, Dr. Nitin Kumar Bajpai & Dr. Aliza Mittal**

Institutional Ethics Committee after thorough consideration accorded its approval on above project.

The investigator may therefore commence the research from the date of this certificate, using the reference number indicated above.

Please note that the AIIMS IEC must be informed immediately of:

- Any material change in the conditions or undertakings mentioned in the document.
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research.

The Principal Investigator must report to the AIIMS IEC in the prescribed format, where applicable, bi-annually, and at the end of the project, in respect of ethical compliance.

AIIMS IEC retains the right to withdraw or amend this if:

- Any unethical principle or practices are revealed or suspected
- Relevant information has been withheld or misrepresented

AIIMS IEC shall have an access to any information or data at any time during the course or after completion of the project.

Please Note that this approval will be rectified whenever it is possible to hold a meeting in person of the Institutional Ethics Committee. It is possible that the PI may be asked to give more clarifications or the Institutional Ethics Committee may withhold the project. The Institutional Ethics Committee is adopting this procedure due to COVID-19 (Corona Virus) situation.

If the Institutional Ethics Committee does not get back to you, this means your project has been cleared by the IEC.

On behalf of Ethics Committee, I wish you success in your research.

Dr. Praveen Sharma
Member Secretary

Member secretary
Institutional Ethics Committee
AIIMS,Jodhpur

Basni Phase-2, Jodhpur, Rajasthan-342005; **Website:** www.aiimsjodhpur.edu.in; **Phone:** 0291-2740741 Extn. 3109
E-mail : ethicscommittee@aiimsjodhpur.edu.in; ethicscommitteeaiimsjdh@gmail.com

Annexure B

Informed Consent Form - English

All India Institute of Medical Sciences (AIIMS)
Jodhpur, Rajasthan
Informed consent form (English)

Title of the project: **Profiling of Membranous nephropathy based on Immunohistochemical expression of antigens NELL-1, PLA2R and THSD7A.**

Name of the Principal Investigator: **Dr. Nivedita A** Tel. No. :8438331473
Patient/Volunteer Identification No.: _____
I, _____ S/o or D/o _____
R/o_____
give my full, free, voluntary consent to be a part of the study "**Profiling of Membranous nephropathy based on Immunohistochemical expression of antigens NELL-1, PLA2R and THSD7A**", the procedure and nature of which has been explained to me in my own language to my full satisfaction. I confirm that I have had the opportunity to ask questions.

I understand that my participation is voluntary and is aware of my right to opt out of the study
at any time without giving any reason.

I understand that the information collected about me and any of my medical records may be looked at by Doctors from AIIMS, Jodhpur. I give permission for these individuals to have access to my records.

Date: _____ _____
Place: _____ Signature/Left thumb impression

This to certify that the above consent has been obtained in my presence.

Date: _____ _____
Place: _____ Signature of Principal Investigator

Witness 1 Witness 2

_____ _____
Signature/ Left thumb impression Signature/ Left thumb impression

Name: _____ Name: _____
Address: _____ Address: _____

Annexure C

Informed Consent Form - Hindi

All India Institute of Medical Sciences (AIIMS)
Jodhpur, Rajasthan
Informed consent form (Hindi)

थीसिस / निबंधकाशीर्षक: एंटीजन एनईएल -1, पीएलए 2 आर और, टीएचएस 7 ए की इम्यूनोहिस्टोकेमिकल अभिव्यक्ति पर आधारित मेम्ब्रेनस नेफ्रोपैथी की रूपरेखा।

पीजीछात्रकानाम: डॉ. निवेदिता ए टेलीफोन: 8438331473

रोगी / स्वयंसेवक पहचान संख्या: _____

मैं, _____ पुत्र/पुत्री _____ पता _____

अध्ययन के एक हिस्से होने के लिए मेरी पूर्ण, नि: शुल्क, स्वैच्छिक सहमति दें " एंटीजन एनईएल -1, पीएलए 2 आर और, टीएचएस 7 ए की इम्यूनोहिस्टोकेमिकल अभिव्यक्ति पर आधारित मेम्ब्रेनस नेफ्रोपैथी की रूपरेखा। " का एक भाग बनने के लिए मेरी पूर्ण, स्वतंत्र, स्वैच्छिक सहमति दें, जिसकी प्रक्रिया और प्रकृति मुझे अपनी पूरी संतुष्टि के लिए अपनी भाषा में समझाई गई है। मैं पुष्टि करता हूं कि मुझे प्रश्न पूछने का अवसर मिला है।

मैं समझता हूं कि मेरी भागीदारी स्वैच्छिक है और मुझे किसी भी कारण दिए बिना किसी भी समय अध्ययन से बाहर निकलने के मेरे अधिकार की जानकारी है।

मैं समझता हूं कि मेरे और मेरे मेडिकल रिकॉर्ड के बारे में एकत्र की गई जानकारी को एम्स, जोधपुर के डॉक्टरों द्वारा देखी जा सकती है।

मैं इन व्यक्तियों को अपने अभिलेखों तक पहुंच के लिए अनुमति देता हूं ।

तारीख : _____

जगह: _____ हस्ताक्षर / बाएं अंगूठे का छाप _____

यह प्रमाणित करने के लिए कि मेरी उपस्थिति में उपरोक्त सहमति प्राप्त की गई है।

तारीख : _____

जगह: _____ पीजी छात्र के हस्ताक्षर _____

गवाह 1: _____ **गवाह 2:**

हस्ताक्षर: _____ हस्ताक्षर:

तारीख: _____ तारीख:

Annexure D

Patient Information Sheet - English

1. Risks to the patients: No interventions or life-threatening procedure will be done.
2. Confidentiality: Your participation will be kept confidential. Your medical records will be treated with confidentiality and will be revealed only to doctors/ scientists involved in this study. The results of this study may be published in a scientific journal, but you will not be identified by name.
3. Provision of free treatment for research related injury - Not applicable.
4. Compensation of subjects for disability or death resulting from such injury - Not Applicable.
5. Freedom of individual to participate and to withdraw from research at any time without penalty or loss of benefits to which the subject would otherwise be entitled.
6. You have complete freedom to participate and to withdraw from research at any time without penalty or loss of benefits to which you would otherwise be entitled.
7. Your participation in the study is optional and voluntary.
8. The copy of the results of the investigations performed will be provided to you for your record.
9. You can withdraw from the project at any time, and this will not affect your subsequent medical treatment or relationship with the treating physician.
10. Any additional expense for the project, other than your regular expenses, will not be charged from you.

Annexure E

Patient Information Sheet - Hindi

रोगी सूचना पत्रक

1. रोगियों के लिए जोखिम: कोई हस्तक्षेप या जीवन-धमकी प्रक्रिया नहीं की जाएगी।
2. गोपनीयता: आपकी भागीदारी गोपनीय रखी जाएगी। आपके मेडिकल रिकॉर्ड का इलाज गोपनीयता के साथ किया जाएगा और इस अध्ययन में शामिल डॉक्टरों / वैज्ञानिकों के लिए ही खुलासा किया जाएगा। इस अध्ययन के परिणाम वैज्ञानिक पत्रिका में प्रकाशित किए जा सकते हैं, लेकिन आपको नाम से पहचाना नहीं जाएगा।
3. अनुसंधान से संबंधित चोट के लिए नि: शुल्क उपचार की व्यवस्था - लागू नहीं है।
4. ऐसी चोट से होने वाली विकलांगता या मृत्यु के लिए विषयों का मुआवजा - लागू नहीं है।
5. किसी भी समय जुर्माना या लाभ के नुकसान के बिना व्यक्तिगत रूप से भाग लेने और अनुसंधान से वापस लेने के लिए स्वतंत्रता की स्वतंत्रता, जिसके विषय में अन्यथा हकदार होगा।
6. आपके पास किसी भी समय जुर्माना या लाभ के नुकसान के बिना भाग लेने और अनुसंधान से वापस लेने की पूर्ण स्वतंत्रता है जिसके लिए आप अन्यथा हकदार होंगे।
7. अध्ययन में आपकी भागीदारी वैकल्पिक और स्वैच्छिक है।
8. किए गए जांच के परिणामों की प्रति आपके रिकॉर्ड के लिए आपको प्रदान की जाएगी।
9. आप किसी भी समय परियोजना से वापस ले सकते हैं, और यह आपके बाद के चिकित्सा उपचार या उपचार चिकित्सक के साथ संबंधों को प्रभावित नहीं करेगा।
10. आपके नियमित खर्चों के अलावा, परियोजना के लिए कोई भी अतिरिक्त खर्च आपसे शुल्क नहीं लिया जाएगा।

Annexure F

Case Record Form

All India Institute of Medical Sciences (AIIMS), Jodhpur
Department of Pathology
CASE RECORD FORM

Date:

Name:

Age: **Sex**: **I.D**:

Address:

Relevant clinical History:

Diabetes mellitus (Y/N) Obesity (Y/N) Hypertension (Y/N) SLE (Y/N)

Chronic infections (Y/N) Malignancy (Y/N) Drug intake (Y/N)

Symptoms and Signs:

Blood pressure: _____ mm/Hg Edema: _____

Arthritis /Arthralgias: _____ Skin lesion: _____

Laboratory Data:

a. Urine:

24 hr proteinuria	_____g/24 hour	Sediment	
UPC ratio		Casts	
Hematuria		Proteinuria	

b. Serum

Cholesterol		Creatinine	
ANA		BUN	
Anti- DNA		Creatinine clearance	
ANCA		Albumin	
Hep B infection		Glucose	
Hep C infection		HbA1C	
PLA2R		Complement levels	

Histological Diagnosis (Light microscopy and Immunofluorescence microscopy):

Results of Immunohistochemistry markers:

PLA2R : Positive ☐ Negative ☐

NELL-1 : Positive ☐ Negative ☐

THSD7A : Positive ☐ Negative ☐