Biomarkers in Primary Membranous Nephropathy, A Guide to Precision Medicine

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Keywords. membranous nephropathy, biomarkers, proteomics, transcriptomes, microRNA, extracellular vesicle, podocalyxin, PI3K/AKT, PLA2R, THSD7A, NELL-1, podocyte

Membranous nephropathy (MN) as one of the most common glomerulonephritis still relies on an invasive procedure of kidney biopsy for precise recognition. Over the recent past years noninvasive methods using wide range of biomarkers have been developed in order to diagnose and estimating the final prognosis of MN. Plasma, urine and tissue are readily accessible specimens for identification of these biomarkers. In order to utilize a single biomarker or a panel of them for detection of a specific entity, many factors should taking into consideration like the accuracy, precision, and validity, accompanying with being available and cost effective. This review is focused on recently developed biomarkers and their application on the diagnosis besides determining the prognosis of MN. The clinical utilities and limitations of each biomarker are discussed in details.

IJKD 2020;14:335-47
www.ijkd.org

Review

INTRODUCTION

The diagnosis of membranous nephropathy (MN) as the most common primary glomerulopathy among non-diabetic adults,[1] already depends on kidney biopsy, an invasive procedure with complications such as hematoma, hematuria, need for transfusion, and rarely nephrectomy.[2] Formerly called idiopathic, nowadays it is assumed as a renal limited autoimmune disorder. M-type phospholipase A2 receptor (PLA2R) and thrombospondin type-1 domain-containing 7A (THSD7A) are well known antigens, and antibodies against them are positive in about 70 to 80% of MN cases, collectively.[3] Their utility in place of kidney biopsy has been proposed. Hence, an optimal cut-off value, valid way of measurement, and large clinical studies are required to validate them as a substitute for kidney biopsy. On the other hand, due to the fact that 30% of patients with MN would reach end stage renal disease (ESRD) in long term follow up, predicting response to a specific therapeutic regimen or the final outcome and prognosis of MN at the time of diagnosis might be helpful in tailoring treatment according to patients' need and precision in medicine. Numerous studies evaluated urine, serum and tissue biomarkers in diagnosis, prediction and prognosis of MN by the means of proteomics, metabolomics, and transcriptomics. An ideal biomarker should be noninvasive, easily measured and readily available, with high sensitivity and specificity, and above all, lead us to better understanding of the pathogenesis of diseases.

DIAGNOSTIC BIOMARKERS IN MEMBRANOUS NEPHROPATHY

Urine Proteomes

Urine is an easily accessible biofluid and its markers are most likely to be affected by the pathologic events in kidney. It might mirror the immunologic and structural changes in MN. Several studies evaluated the diagnostic biomarkers in MN, yet no single diagnostic urinary marker has been identified.

In the search for diagnostic urinary biomarkers, Pang *et al.* analyzed urine samples of patients

with biopsy proven MN (both anti-PLA2R positive and negative patients) in comparison with healthy controls using tandem mass tag (TMT) and Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS). 249 proteins were identified to be expressed differently between the studied groups. The upregulated proteins were in common between anti-PLA2R positive and negative patients with MN. Therefore, they could only differentiate between MN patients and the healthy controls. Proteins with the highest fold change were α_1-antitrypsin, serotransferrin, and ceruloplasmin. Pathway analysis showed that the majority of identified proteins were involved in the platelet degranulation and activation of the classic pathway of complement that are compatible with thromboembolic complications of MN and complement deposition in pathologic studies, respectively. Nevertheless, the two upregulated proteins validated by Western blot (Afamin and α_1-antitrypsin) have been reported in different forms of glomerular diseases such as focal and segmental glomerulosclerosis (FSGS) and IgA nephropathy.[4,5] Thus, they might not be specific to MN.

Extracellular Vesicles (EVs)

EVs contain cellular components (such as proteins, miRNA, mRNA, and plasma membrane) and reflect the biologic processes in kidney and are not affected by filtered proteins, all these characteristics make them ideal biomarkers.[6] Circulating EVs consisting of exosomes and microvesicles.[7]

In a study evaluating EVs among patients with various renal pathologies and healthy controls, Lu *et al.* found that urinary microparticles (MPs) containing podocalyxin and annexin V can differentiate patients from healthy controls accurately (AUC = 0.82). MPs were correlated positively with proteinuria and inversely with the degree of glomerulosclerosis. Patients with MN had higher urinary level of podocalyxin positive MPs which was significantly reduced following immunosuppressive treatment.[8] Although these MPs might be considered as a biomarker of early diagnosis and response to therapy in MN patients, they might not be specific for MN, as loss of negatively charged podocalyxin on podocytes causes podocyte effacement and proteinuria, which might occur in other glomerular diseases. Annexin V is a member of calcium-binding proteins that binds to phospholipids and actin. It has anticoagulant function, and inhibits phospholipase A2. Annexin V has been detected on podocytes and distal tubules, and following glomerular injury leaks in urine. Its presence in urine MPs might be cause or effect of glomerular damage in MN.[9]

In another study, urine microvesicles were analyzed by LC-MS/MS technique. The proteome analysis of urine microvesicles demonstrated 16 differentially expressed proteins between MN patients and FSGS and healthy controls. The highest fold change was belonged to lysosome membrane protein 2 (LIMP-2). Tissue proteomic analysis confirmed the marked increase of LIMP-2 in glomeruli of MN patients. As increased glomerular expression of LIMP-2 was not detected in other disease-controls, it might be specific for MN. However, the investigators failed to detect circulating antibody against LIMP-2, which might be due to the fact that LIMP-2 expression in glomeruli is the consequence of immunologic injury to glomerular basement membrane (GBM) and not the cause of injury. LIMP-2 is more a marker of tissue injury than a diagnostic marker of a specific disease.[10]

Circular RNAs (circRNA)

Circular RNAs (circRNA) are a subclass of noncoding RNAs that regulate gene expression and modulate transcription. The characteristics of circRNA such as stability, universality, specificity, and conservatism make them suitable as biomarkers.[11] Ma *et al.* evaluated circRNAs of exosomes in serum and urine samples of patients with biopsy proven MN. Using polymer formulation method, exosomes were isolated, and then circRNA sequencing was performed. In serum and urine samples, 89 and 60 differentially expressed circRNA were identified, respectively. Pathway analysis of involved genes demonstrated platelet activation pathways and PI3K-AKT pathway. Mucin 3A (MUC3A) gene was upregulated in serum samples of patients with MN. This gene is involved in the lectin pathway of complement, which has a significant role in pathogenesis of MN. Thus, authors suggested MUC3A as a diagnostic biomarker of MN with a role in pathogenesis of disease.[12]

In search for circRNA in peripheral blood as a diagnostic biomarker, samples of 30 patients with

the diagnosis of primary MN were compared with 30 healthy controls, and 5 differentially expressed circRNAs were validated. Among more than 900 circRNAs, circ_101319 had the highest level of expression and had a sensitivity and specificity of 93.33% and 70%, respectively for diagnosis of primary MN (area under ROC curve = 0.89). The role of circ_101319 along with four other circRNA (namely, circ_033475, circ_102355, circ_101854, and circ_102711) was found to be in regulating PI3K-AKT pathway and nuclear factor of activated T-cell (NFAT5). PI3K pathway has a pro-survival role in podocyte, whereas the increased nuclear translocation of NFAT by calcineurin results in proteinuria.[13] These findings suggested that dysregulations in PI3K-AKT pathway is involved in pathogenesis of MN, and MUC3A and circRNAs can be used as a diagnostic biomarkers and targets for treatment in MN.

Urinary Podocalyxin

Podocalyxin is an apical membrane protein of podocyte with intracellular interactions with actin cytoskeleton of podocyte via NHERF-1/2 and Ezrin. Its role in glomeruli development and slit diaphragm function has been well studied. Podocalyxin maintain the negative charge of podocytes, and if lost, foot process effacement and proteinuria would occur. Its urinary expression correlates with the degree of podocyte injury.[14] Accordingly, Imaizumi *et al.* used urinary podocalyxin in combination with clinical characteristics of age, glomerular filtration rate (GFR), and diabetes mellitus as a diagnostic indicator of MN. This combination model led to a threshold probability of 40 to 80% in decision curve analysis.[15] Therefore, it could not be utilized as a solo marker to diagnose MN.

Urine Metabolomes

In search for diagnostic urinary biomarkers, Taherkhani *et al.* employed nuclear magnetic resonance (NMR) and gas chromatography-tandem mass spectrometry (GC-MS/MS) to define specific urine metabolome in MN patients, impaired pathways in pathogenesis of MN, and correlation of biomarkers with histologic characteristics. They suggested a panel of seven metabolites including α- hydroxybutyric acid, 3,4-dihydroxymandelic acid, 5α-cholestanone, 2-hydroxyglutaric acid lactone, nicotinamide, epicoprostanol and palmitic acid. This panel had AUC of 1 with sensitivity and specificity of 100%. The most significant metabolic pathway was pyrimidine metabolism. Isobutyric acid was negatively correlated with serum creatinine level and interstitial fibrosis and tubular atrophy, therefore it can also predict the outcome.[16] Reduced fecal concentration of isobutyrate has been shown among MN patients, which was associated with changes in gut microbiota.[17] Validation of this panel would lead to better understanding of disease pathogenesis.

miRNAs

miRNAs are subgroup of noncoding RNAs that suppress target gene expression by inhibiting or degrading mRNAs. miRNAs were vastly studied as biomarkers in kidney disease. Their discovery might also shed light on the pathogenesis of diseases. About 20 differentially expressed miRNAs has been shown in peripheral blood samples of MN patients. miR-217 is the most downregulated marker. With a level of less than 750 copies/μL, miR-217 has a sensitivity of 88.9% and specificity of 75.9% (AUC = 0.94) in diagnosis of MN. Downregulation of miR-217 results in overexpression of TNFSF11 (TNF super family 11). TNFSF11 induces PLA2R expression and has role in cell apoptosis, and this might show the part miR-217 play in pathogenesis of MN.[18] Another study evaluated miRNA tissue expression in pathogenesis and diagnosis of MN. miRNA profiling was done by TaqMan low-density arrays (TLDAs). Fifteen differentially expressed miRNA was found between MN patients and controls, 10 of which were confirmed in validation cohort (9 upregulated and 1 downregulated miRNA). Of them, upregulation of miR-107, miR-423-5p, and Let-7 lead to inhibition of IL-6 and MYC mRNA. miR-107 has a negative correlation with Anti-PLA2R antibody. These data suggested a panel of miRNAs in diagnosis of MN.[19]

Zhou *et al.* reported upregulation of miR-195-5p and 192-3p and downregulation of miR-328-5p in peripheral blood and urine samples of patients with MN. These miRNAs are involved in inflammatory and apoptotic pathways.[20] There are several other transcriptomic studies focused on miRNAs in MN.[21] A summary of studies is shown in Table 1.

Tissue Proteomics Analysis

Two well-known antigens in the pathogenesis

Table 1. miRNAs as Biomarkers in MN

miRNA	Sample Type	Direction of Changes	Affected Protein	Possible Use	Reference
miR-217	Peripheral Blood	Downregulated	TNFSF11	Diagnosis	18
miR-107, miR-423-5p, Let-7	Tissue	Upregulated	IL-6, MYC	Diagnosis	19
miR-195-5p, miR-192-3p	Peripheral Blood and Urine	Upregulated	PPM1A, RAB1A	Diagnosis	20
miR-328-5p	Peripheral Blood and Urine	Downregulated	BRSK1	Diagnosis	20
miR-186	Tissue	Downregulated	$P2X_7$	Diagnosis	21
miR-193a	Urine	Upregulated	WT1/Podocalexin	Prognosis	33

Abbreviations: IL-6, interleukin-6; PPM1A, protein phosphatase, Mg2+/Mn2+ dependent 1A; RAB1A, Ras-related protein Rab-1A; BRSK1, BR serine/threonine kinase 1; TNFSF11, TNF super family 11; WT1, wilms' tumor

of primary MN are PLA2R and THSD7A, which count for approximately 70% and up to 5% of cases, respectively. MS/MS analysis of laser microdissection of paraffin embedded sections of biopsy samples, and IHC staining were done and protein identification was performed on PLA2R negative biopsy samples. Two new proteins, exostosin 1(EXT1) and exostosin 2 (EXT2) were identified, with granular IgG1 dominant deposition along GBM. EXT1/EXT2 have a coploymerase role and are involved in heparan sulfate synthesis. Nevertheless, in 70.8% of EXT1/EXT2 associated MN, autoimmune tests like anti-nuclear antibody, anti-double stranded DNA, anti-SSA/SSB or anti-ribonucleoprotein antibodies were positive, and the pathologic findings were suggestive of secondary MN. Thus, EXT1/EXT2 is proposed as a diagnostic biomarker in secondary autoimmune MN, although antibody against them has not been detected till now.[22]

In an effort to identify a diagnostic biomarker in PLA2R negative primary MN patients, Nano-flow liquid chromatography electrospray tandem MS/MS analysis of kidney biopsy samples was performed on laser microdissection slides of 35 pilot cases. A new protein, Neural tissue encoding protein with epidermal growth factor (EGF)-like repeats (NELL-1), was discovered. The presence of NELL-1 was confirmed by immunohistochemistry staining of samples in validation cohort. Unlike PLA2R, the most abundant IgG subclass accompanying NELL-1 was IgG1. Up to 16% of PLA2R-negative MN pathologies were positive for NELL-1 staining. Nevertheless, PLA2R positive cases and other glomerular diseases such as FSGS, IgA nephropathy and diabetic nephropathy, were negative for NELL-1. Sethi *et al*, evaluated circulating NELL-1 antibody in patients with MN, and demonstrated a decreasing pattern of it in response to treatment, which like PLA2R antibody titer was earlier than remission of proteinuria. Hence, NELL-1 is suggested as a diagnostic biomarker of primary MN in double negative cases. (PLA2R negative and THSD7A negative cases).[23]

One of the caveats in managing MN is differentiating primary from secondary forms of MN. With Matrix assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) applied on renal biopsy samples of patients with primary or secondary MN. An ion signal was detected at m/z 1459 that had the highest discriminatory power with an AUC of 0.85. Its intensity was higher in primary MN, independent of PLA2R or IgG4 positivity. The ion was identified as a tryptic peptide fragment of Serine/threonine-protein kinase MRCK γ by MALDI/TOF/TOF. This protein belongs to the Cdc42 group which has a significant role in podocyte cytoskeletal architecture.[24] Table 2 indicates tissue biomarkers and their role in diagnosis of MN.

To summarized, the most studied biomarkers with validation in diagnosis of primary MN are

Table 2. Tissue Biomarkers (Tissue Staining) in Membranous Nephropathy

Biomarker	Pathology Result	IgG Subtype	Percent of Primary MN	Sensitivity	Specificity	Reference
PLA2R	Primary MN	IgG4	70%	78%	91%	25
THSD7A/PLA2R	Primary MN	IgG4	5%	61.8%	94.6%	26
NELL-1	Primary MN	IgG1	5%	-	-	23
EXT1/EXT2	Secondary MN	IgG1	-	-	-	22

Abbreviations: EXT1/EXT2, exostosin 1 and exostosin 2; NELL-1, neural epidermal growth factor-like 1 protein; PLA2R, M-type phospholipase A2 receptor; THSD7A, thrombospondin type-1 domain-containing 7A

PLA2R, THSD7A, and NELL-1. In future, these 3 biomarkers might be utilized in diagnosis of primary MN, along side with EXT1/2 to rule out secondary MN without kidney biopsy requirement. Plus, based on the above-mentioned studies, involved pathways in pathogenesis of disease are well-studied complement pathway and coagulation and platelet activation pathways, and also recently introduced anti-apoptotic pathway of PI3K-AKT, podocalyxin and Cdc42. Downregulation of PI3K-AKT pathway leads to podocyte injury and proteinuria. Angiotensin II production causes PI3K-AKT pathway downregulation and increased expression of caspase 9, both of which induce podocyte apoptosis. Additionally, PI3K-AKT pathway interacts with nephrin and podocin, two critical proteins in slit diaphragm with a significant role in cytoskeletal structure, thus derangement in this pathway results in proteinuria.[27] Podocalyxin, an outer membrane protein on podocytes, is linked to actin cytoskeletal by its cytoplasmic tail. Podocalyxin redistributes actin filaments to the apical membrane by activation of RhoA.[28] RhoA, Cdc42, and Rac1 are members of small GTPases family that have an important role in podocyte cytoskeletal dynamics. They orchestrate podocyte motility and slit diaphragm function, and their derangement results in proteinuria, a mechanism that might not be specific for MN.

PROGNOSTIC BIOMARKERS

As 35% of patients with MN would reach ESRD over 10 years, identification of those at risk upon diagnosis leads to prompt initiation of immunosuppressive treatment and closer observation. Characteristics of older age, male gender, decreased GFR at presentation and persistent elevation of PLA2R antibody are well-studied risk factors of progression toward ESRD.[1] Novel biomarkers are under investigation.

Urinary Angiotensinogen

Urinary angiotensinogen is a marker of local renal activation of renin-angiotensin system (RAS) in patients with nephrotic syndrome. Urinary angiotensinogen is increased in patients with nephrotic syndrome including minimal change disease (MCD), FSGS, diabetic nephropathy, and MN; and it is not able to differentiate distinct pathologies.[29] However, its level was correlated with the severity of proteinuria among MN patients and not in patients with MCD.[30] This might be due to the fact in patients with MCD, urinary angiotensinogen is a factor of serum angiotensinogen and systemic activity of RAS, but in MN is a marker of local RAS activity.[29] Thus, urinary angiotensinogen could be adopted as a marker of severity of MN and a guide to early initiation of immunosuppressive treatment.

Urine Low Molecular Weight Proteins

Urinary low molecular weight proteins such as free retinol binding protein (RBP), α_1 microglobulin (α_1M), and β_2 microglobulin (β_2 M) have been suggested as markers of tubular function in glomerulopathies, as these proteins can pass the filtration barrier and almost fully reabsorbed by proximal tubule via megalin. Therefore, tubular dysfunction and its severity can be diagnosed by measuring these proteins in a random urine sample. A study by van den Brand *et al.* depicted α_1M/ urine creatinine > 50 µg/10 mmol and β_2M/ urine creatinine > 1µg/ 10 mmol as the cutoff value for prediction of kidney outcome of progressive renal dysfunction in MN.[31] Back in 2005, urinary β_2M excretion rate greater than 0.5 µg/min was shown to predict renal outcome in patients with primary MN, with a sensitivity and specificity of 88% and 91%; respectively.[32] As is the case with urinary α_1M and β_2M, the increased urinary excretion of RBP predicted poor renal outcome with an AUC of RBP/creatinine ratio of 0.78. The lower the RBP concentration in urine, the earlier is the achievement of remission.[33] Hence, markers of tubular injury can predict the outcome of MN as the pathologic findings of interstitial fibrosis and tubular atrophy would.

Other Markers of Renal Tubular Damage

Urinary kidney injury molecule- 1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) as novel biomarkers of tubular damage were evaluated for their prognostic value in MN. Although they predicted the outcome of renal dysfunction among MN patients with normal renal function, they did not add any value to the prognostic ability of urinary α_1M and β_2M.[34]

N-acetyl-β-glucosaminidase (NAG) is a lysosomal enzyme and a marker of tubular damage. The hazard ratio of progressive renal dysfunction with NAG/

creatinine ratio of more than 19.2 was 18.97 (95% CI: 1.70 to 211.86). With an AUC of 0.77, NAG/creatinine ratio can predict the progression of renal disease toward end stage renal disease (ESRD) or ≥ 50% reduction in GFR.[35]

More recently, An *et al.* studied the relevancy of urine markers of tubular damage with the pathologic findings of tubular injury. Urinary KIM-1, NGAL, NAG, and RBP were higher in patients with MN when compared to healthy controls. Patients with nephrotic syndrome had higher amount of KIM-1, NGAL, and NAG in urine; however urinary RBP was not different between nephrotic and non-nephrotic patients. Regarding the severity of pathologic tubular damage, urine markers were not different among various histological grades, even though renal function (by the means of serum creatinine and cystatin-c) was worse in those with higher histological grade.[36]

Soluble ST2 and IL-4

The role of immune system in development and progression of primary MN is obvious, and recent studies illustrated the possible antigens responsible for the disease. MN is a state of chronic inflammation.[37] IL-33 as member of IL-1 family function as a main player in chronic inflammatory states. IL-33 binds to ST2 transmembrane receptor on Th2 and activates it. On the other hand, soluble ST2 (sST2) is a decoy receptor that binds to IL-33 and prevents Th2 activation and anti-inflammatory IL-4 production. Zhang *et al*, showed increased serum level of sST2 and reduced IL-4 level in MN patients which was correlated with severity of proteinuria, the level of which was reduced significantly following treatment. Serum levels of sST2 may predict the severity of proteinuria and renal dysfunction among MN patients.[38]

miRNAs

Altered expression of miRNAs has been recognized in pathogenesis of renal diseases. miR193a is one the well-studied miRNAs in the pathogenesis podocytopathies. Over expression of miR193a results in podocytes undifferentiation.[39] Urine samples of patients with MN at different stages were compared with healthy controls, and patients were followed for up to 4 years. Patients with MN had significantly elevated urinary miR193a level; the more advanced the pathologic stage of MN, the higher the urinary level of miRNA193a. Patients with urinary miRNA193a level more than 4.91 had worse renal survival.[40] miRNA193a suppresses the Wilms' tumor (WT1) gene, which positively regulates podocalyxin and nephrin expression, thus its downregulation leads to impaired cellular architecture and abnormal slit diaphragm.[41] Combining increased urinary level of miRNA193a (> 4.91) and decreased urinary podocalyxin (≤ 5.03) and WT1 (≤ 1.41) can predict renal outcome (AUC = 0.82; with sensitivity of 72%, and specificity of 82%).[40] Downregulation of podocalyxin and nephrin results in cytoskeletal instability and slit diaphragm damage, both of them lead to proteinuria. In an experimental study, inhibition of miR-193a resulted in suppression of apoptosis, decreased IgG deposition in renal tissue, and increased expression of podocalyxin and nephrin.[42]

Table 3. Urinary Biomarkers Predicting Renal Prognosis in MN

Biomarker	Cut off	AUC	Reference
Urinary α_1M/cr	> 50 µ/10 mmol	0.81	31
Urinary β_2M/cr	> 1 µ/10 mmol	0.81	31
Urinary NAG/cr	> 19.2	0.77	32
Urinary RBP/cr	> 0.477 mg/mmol	0.77	33
Urinary miR 193-a + WT1 + PODO	> 4.91; ≤ 1.14; ≤ 5.03	0.82	40

Abbreviations: α_1M, α_1 microglobulin; β_2M, β_2 microglobulin; Cr, creatinine; miR193-a, microRNA193-a; NAG, N-acetyl-β-glucosaminidase; PODO, podocalexin; RBP, retinol binding protein; WT1, wilms' tumor

Overall, similar to pathologic findings of tubular atrophy and interstitial fibrosis and their role in predicting the prognosis of glomerular diseases, markers of tubular damage such as urinary α_1M, β_2M, NAG, RBP, and miR193-a are predictors of renal outcome (Table 3).

PREDICTIVE BIOMARKERS OF RESPONSE TO TREATMENT

The goal of treatment in MN is achieving complete or partial remission. The renal outcome of patients accomplishing any kind of remission is fair.[1] As the mainstem of treatment is immunosuppressive treatment, recognition of patients who might be resistant to treatment results in less exposure to toxic effects of specific immunosuppressive agents and helps to choose an effective therapeutic regimen. Regarding treatment with calcineurin inhibitors (CNIs) as a less gonadotoxic alternative

for cyclophosphamide, studies reported different response rate from 80% remission to not different from supportive care.[1,43] Hence, there is an urgent need for biomarkers that predict response to therapy in general and more importantly to a specific agent.

BAFF and APRIL

With identification of anti-PLA2R, anti- THSD7A, and anti-NELL1 antibodies in the pathogenesis of MN, the autoimmune mechanism and role of humoral immune system came into spotlight. BAFF (B cell-activating factor belonging to the tumor necrosis factor family) and APRIL (a proliferation-inducing ligand) are two members of humoral system with B-cell activating capacity, and autoimmunity. These cytokines were evaluated among biopsy-proven MN patients at the time of biopsy. BAFF and APRIL levels were elevated in PLA2R positive MN patients to the similar level in patients with lupus nephritis. However, the level was low in PLA2R negative samples, close to the level in healthy controls. The lower the serum level of BAFF and APRIL at baseline, the higher was the chance of achieving partial or complete remission with cyclophosphamide and prednisolone therapy. A cut-off of BAFF < 6.05 ng/mL and APRIL < 4.2 ng/mL can predict the remission outcome among PLA2R positive MN patients.[44] Identification of these two cytokines help for better understanding of pathogenesis of the disease and predict response to therapy.

Serum Amyloid A1 Protein (SAA1)

With different treatment protocols for MN treatment, the ability to predict response to therapy at initiation will guide the physician to practice precision medicine. Yu *et al.* applied nano-HPLC-MS/MS analysis on serum samples of patients with MN under treatment with CNIs to determine proteome profile of patients with complete or partial remission vs non remitting patients. They found 3 upregulated serum proteins and 5 downregulated ones. The diversely expressed proteins had a role in classic pathway of complement activation. Among them, SAA1 with highest fold change has been validated by ELISA. When combined with PLA2R antibody titer (144.65 RU/ml), SAA1 > 44.5 ng/mL, a sensitivity and specificity of 85.7% and 96.2%; respectively, is achieved in predicting non remitting patients.[45]

Macrophage Migration Inhibitory Factor (MIF)

With regard to predict response to treatment with standard Ponticelli regimen, proteomics analysis of kidney tissue samples of 13 patients with biopsy proven primary MN were analyze by MALDI-MSI technique. Further evaluation of proteomic alterations was done using nano-liquid chromatography coupled with electrospray ionization tandem mass spectrometry (nLC-ESI-MS/MS) and the findings were verified by immunohistochemistry (IHC). Three statistically significant signals with AUC > 0.7 were detected. *m/z* 1111 and *m/z* 1303 value upregulated in non-responder and *m/z* 1198 value upregulated in responder group. The signal 1303 *m/z* had the highest power with AUC of 0.81. This peptide was identified as macrophage migration inhibitory factor (MIF). IHC staining demonstrated immunoreactivity for MIF in podocytes and parietal epithelial cells among non-responding patients. MIF has been shown to counteract with anti-inflammatory effects of glucocorticoids and also induce PLA2 and prostaglandin release.[46]

Sonic Hedgehog (SHH) and α-Smooth Muscle Actin (α-SMA)

In an effort to further evaluate the pathways responsible for response to therapy in primary MN patients, Smith *et al,* carried out MALDI-MSI analysis on kidney biopsy samples and the spatial localization of the previously detected signals (*m/z* 1111 and *m/z*1198) was determined.[46] The signals at *m/z* 1111 and *m/z*1198 were identified as tryptic peptide of SHH and α-SMA, respectively. *m/z* 1111 (SHH) signal showed higher intensity glomerular capillary tuft and podocytes as well as tubulointerstitial tissue in non-responder patients, while in patients with good response to treatment SHH signal was detected solely in tubular epithelial cells and interstitium. SHH has a role in epithelial- mesangial transition (EMT) and fibrosis, and that might be the reason of poor response to therapy. Among patients without adequate response, *m/z*1198 (α-SMA) signal was localized in both glomeruli mesangial and tubular cells. In the meantime, responder had prominent localization of α-SMA only in mesangial cells and proximal tubules but not in other tubular cells. α-SMA expression in glomeruli an indicator of poor outcome in different glomerular pathologies, thus

Table 4. Biomarkers Predictor of not Achieving Partial or Complete Remission in Primary Membranous

Biomarker	Cut off	Sensitivity	Specificity	AUC	Reference
BAFF	> 6.05 ng/mL	82.9%	90%	0.88	44
APRIL	> 4.2 ng/mL	82.9%	90%	0.88	44
SAA1	> 44.5 ng/mL	78.3%	86.8%	0.90	45
SAA1+ PLA2R Antibody	44.5 ng/mL; 144.65 RU/mL	85.7%	96.2%	0.95	45

Abbreviations: APRIL, a proliferation-inducing ligand; AUC, area under the curve; BAFF, B-cell activating factor of the TNF family; PLA2R, M-type phospholipase A2 receptor; SAA1, serum amyloid A1 protein.

it might not be specific for predicting response to therapy in MN patients.[47]

Overall, biomarkers evolved in prediction of response to specific regimen could prevent unnecessary exposure to side effects of immunosuppressive drugs. Apart from high titer Anti-PLA2R antibody, elevated serum levels of BAFF, APRIL, and SAA1 proteins are suitable candidates for validation as predictors of achieving remission. Table 4 depicted the summary of available results.

POTENTIAL APPLICATION IN PATHOGENESIS, DIAGNOSIS, AND PROGNOSIS OF MEMBRANOUS NEPHROPATHY

Although MN is one of the most common causes of nephrotic syndrome in adults, its pathogenesis yet to be illuminated. Variabilities in presentation, rate of progression and response to therapy in primary membranous nephropathy leads to delay in diagnosis, uncertainty in prognosis, and inability to predict response to specific therapeutic regimen, and therefore, non-targeted treatments which expose patients to serious side effects.

Application of Biomarkers in Pathogenesis of MN

Based on biomarker studies PLA2R, TNFRSF, podocalyxin, and PI3K/AKT pathway are found to be the most involved factors in pathogenesis of MN. PLA2R as a member of mannose receptor family has been studied widely in a process of diagnosis, prediction, and prognosis of MN. Although its role in podocyte biology is still unclear, the upregulation of PLA2R has been shown in MN patients. In a search for factors that are involved in upregulation of PLA2R, Cuarental *et al.* identified TFNRSF and its ligand TWEAK as factors that drive PLA2R overexpression in MN. They demonstrated the inhibitory effect of tacrolimus on upregulation of PLA2R by TWEAK.[48] In another study, downregulation of miR-217 appeared to cause TNFRSF and consequently, PLA2R overexpression.[18] Thus, tacrolimus and miR-217 mimics might be therapeutic options in treatment of MN patients, especially those with increased PLA2R and TFNRSF expression. The PLA2 enzyme has to different subtypes: secretory PLA2 (sPLA2) and cytosolic PLA2 (cPLA2), and its enzymatic action leads to arachidonic acid (AA) production. Annexin V has the ability to inhibit phospholipid hydrolysis by sPLA2.[49] The excessive expression of annexin in urinary MPs might be due to shedding of annexin which reduces its inhibitory effect on PLA2. Increased urinary loss of podocalyxin and annexin 5 lead to foot process effacement, actin cytoskeletal disruption, and PLA2 overactivity. In such a way, in MN patients, both PLA2R expression and PLA2 activity are increased.

Apart from its enzymatic function, sPLA2 binding to PLA2R produces proinflammatory cytokines, such as TNFα and IL-6.[50] sPLA2 is involved in cell proliferation, cell migration and arachidonic acid production via its receptor. In a study, Pan *et al.* depicted increased podocyte apoptosis by sPLA2 which was mediated by PLA2R. sPLA2-PLA2R complex produces excessive intracellular AA amount, and results in podocyte apoptosis via ERK1/2 activation.[51]

Nephrin is one of the main components of slit diaphragm (SD), which transmits signals of SD to interior of podocyte by its intracellular domain proteins of SH2/SH3. These domains bind to podocin, CD2AP, and c-Abl. AA at physiologic levels enters cyclooxygenase (COX) pathway and induces protein kinase A (PKA) activation and nephrin phosphorylation, and maintenance of podocyte actin cytoskeletal. Nephrin phosphorylation is a requisite for podocyte survival and cytoskeletal maintenance. Phosphorylated nephrin on one hand activates PI3K/AKT pathway which is a pro-

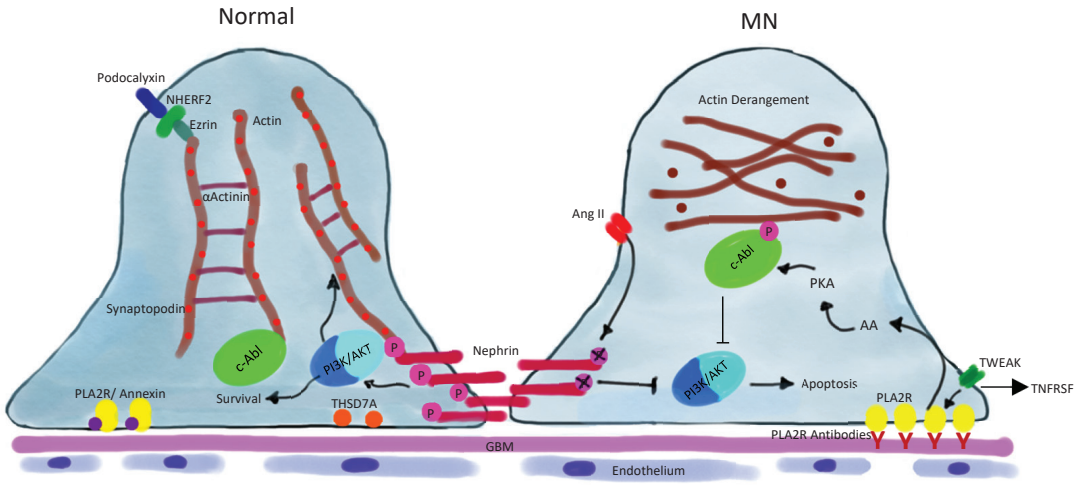

Figure 1. Pathways Involved in Podocyte Injury in MN (AA: Arachidonic Acid)

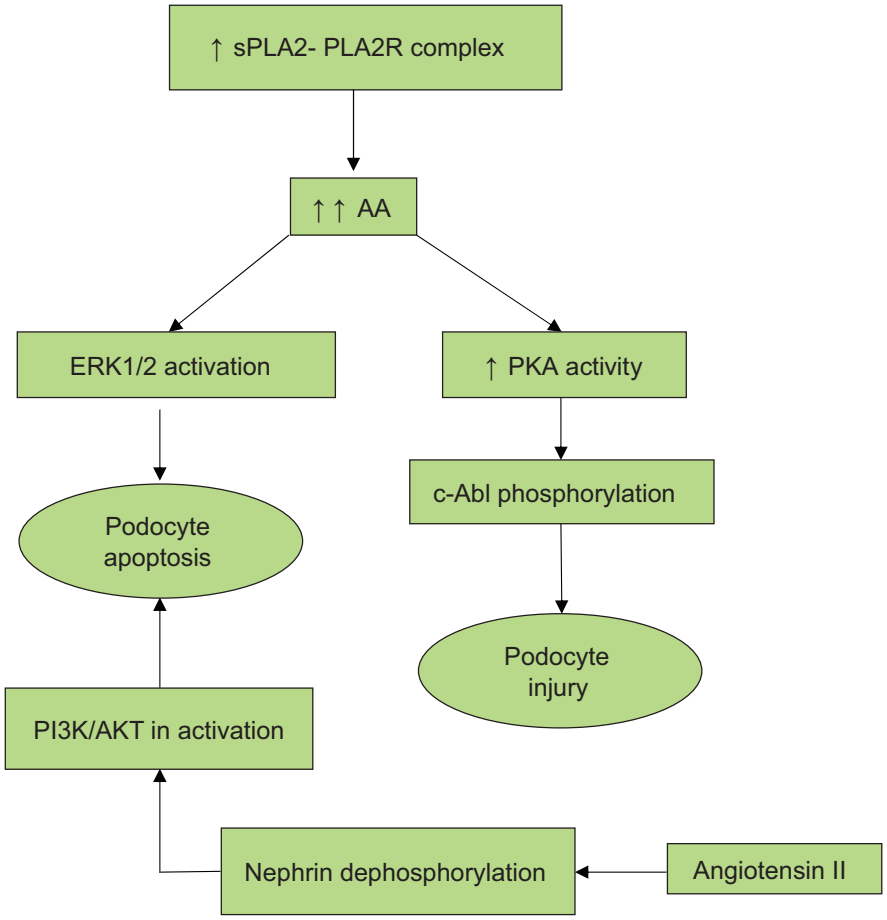

Figure 2. In Normal Physiology, Phosphorylated Nephrin Activates PI3K/ATK Pathway Which Mediates Actin Cytoskeletal Reorganization and Leads to Podocyte Survival. C-Abl binds to phosphorylated nephrin and facilitates F actin formation. Podocalyxin via NHERF2/Ezrin complex connects to the actin and play its role in cytoskeletal stability. In membranous nephropathy, loss of podocalyxin causes actin derangements. Moreover, PLA2R overexpression occurs in response to various factors including increased production of TNFRSF (due to miR-217 downregulation). TWEAK along with loss of annexin (an inhibitor of PLA2 activity) lead to overproduction of AA and activation of PKA, which in turn phosphorylates c-Abl. On the other hand, AngII dephosphorylates nephrin. Phosphorylated c-Abl and dephosphorylated nephrin result in reduced PI3K/AKT pathway activation and podocyte injury. (AA, arachidonic acid; MN, membranous nephropathy; PLA2R, M-type phospholipase A2 receptor; PKA, protein kinase A; THSD7A, thrombospondin type-1 domain-containing 7A; TNFRSF, TNF receptor super family)

survival pathway in podocyte and mediates actin reorganization via this pathway,[52] and on the other hand binds to c-Abl and results in F-actin formation. Angiotensin II via angiotensin II type 1 receptor (AT1R) results in nephrin dephosphorylation and PI3K/AKT inactivation and podocyte apoptosis. Nephrin dephosphorylation leads to c-Abl release from the intracellular domain and activation of SHIP2. SHIP2 overexpression reduces AKT phosphorylation and podocyte injury (Figure 1).[53-5]

However, overproduction AA by sPLA2-PLA2R complex in MN acts through PKA, which directly phosphorylates c-Abl. This PLA2/AA-PKA-cAbl pathway activation promotes podocyte contraction and injury.[56] Thus, both nephrin phosphorylation levels and its interaction with c-Abl are key components in maintaining podocyte health, which are affected by PLA2R upregulation and AA overproduction (Figure 2).

Application of Biomarkers in Diagnosis and Prognosis of MN

PLA2R antibody with diagnostic, prognostic and predictive abilities is one of the well-studied biomarkers in MN. THSD7A and NELL-1 are recently discovered diagnostic biomarkers of primary MN. Based on these finding one can propose a panel of biomarkers for initial evaluation of patients with nephrotic syndrome. This panel consist of serum PLA2R antibody, THSD7A antibody, and NELL-1 antibody to diagnose primary MN. On the contrary, secondary causes of MN could be ruled out by ANA, C3, C4, HBV, HCV, HIV evaluation and EXT1/EXT2 staining (Figure 3). Urinary low

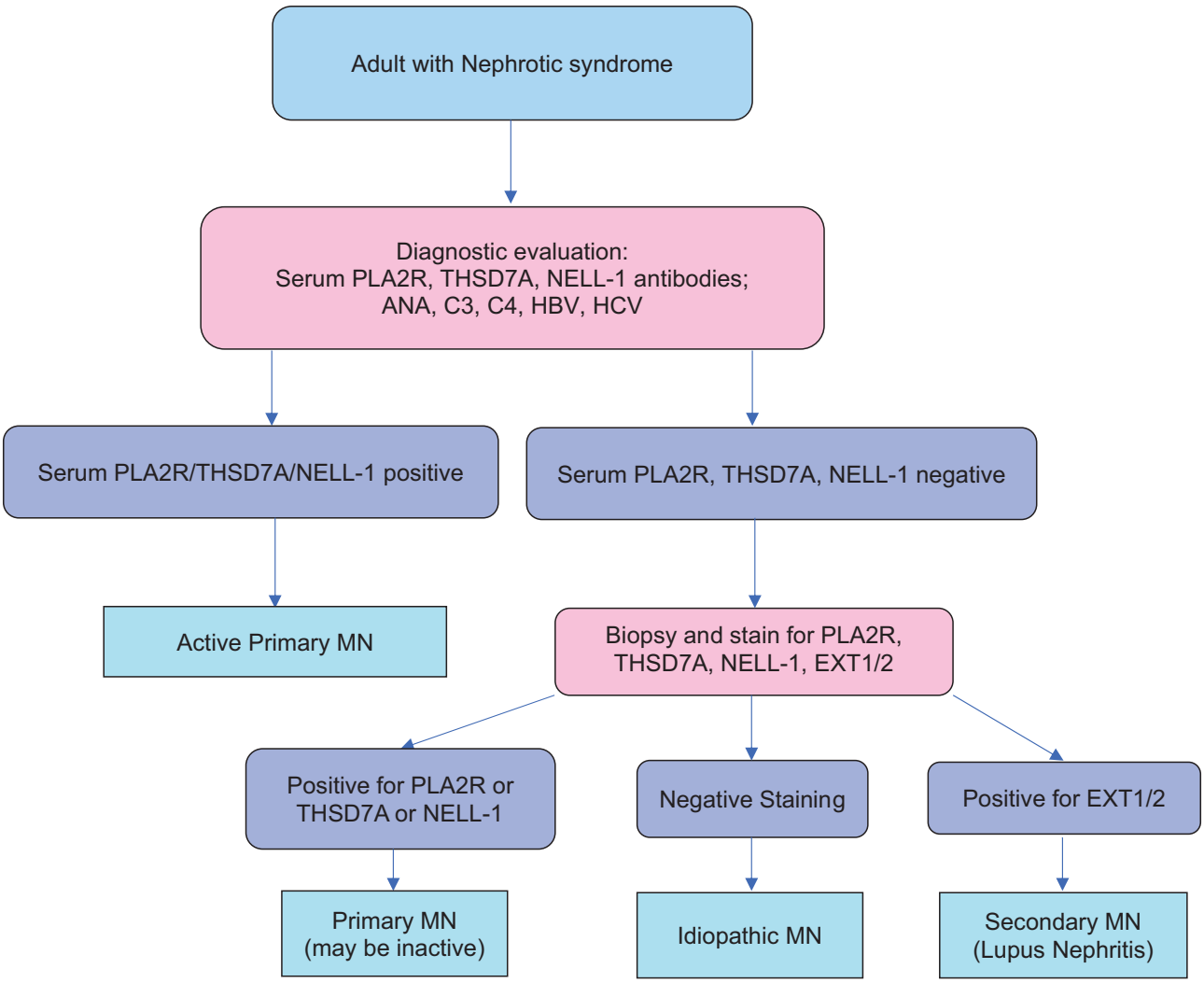

Figure 3. A Proposed Diagnostic Algorithm in an Adult Patient with Nephrotic Syndrome Suspected to Have Membranous Nephropathy

molecular proteins such as α_1 Microglobulin, β_2 Microglobulin, N-acetyl-β-glucosaminidase, and retinol binding protein are predictors of renal outcome. In search for predictive biomarkers, BAFF and APRIL have been shown to predict remission achievement. Thus, risk of progressive loss of renal function in patients with MN is associated with traditional risk factors of serum creatinine > 1.5 mg/dL, decrease in eGFR ≥ 20% in 12 months and proteinuria more than 8 g/d.[57] Novel risk factors of progression are low molecular weight proteinuria, urine IgG level, and serum BAFF and APRIL level. Patients categorized as high risk based on the above-mentioned factors are better to have a kidney biopsy for more accurate diagnosis. When physicians decide to choose treatment options for MN, serum level of PLA2R antibody, BAFF, APRIL, SAA1, and TNFRSF might help to pick more effective therapy. The higher the serum level of PLA2R antibody, BAFF and APRIL, the lower the probability of response to treatment. Interestingly, SAA1 can predict response to treatment with CNIs, and TFNRSF level may predict better response to tacrolimus. Increased tissue expression of MIF, SHH, and αSMA are predictors of non-responding to cyclophosphamide, in such a way along with pathologic findings of chronicity, IHC staining for these markers might help avoiding unnecessary toxic treatment.

CONCLUSION

Now we are at the start line in long road of biomarker discovery. There are too many unanswered questions on the use of biomarkers in MN. Do we have a biomarker precise enough to avoid biopsy? Is there a panel of biomarkers that predict prognosis at diagnosis? What are the roles of discovered biomarkers on podocyte physiology? Moving slowly, we need better understanding of methods of discovery, mechanism of disease and bioinformatics in order to verify and validate biomarkers to be utilized in clinical medicine.

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Abbreviations:
α_1M: α_1 Microglobulin
β_2M: β_2 Microglobulin
AA: Arachidonic Acid
APRIL: A Proliferation-inducing Ligand
AUC: Area Under the Curve
BAFF: B-cell Activating Factor of the TNF Family
BRSK1:BR Serine/Threonine Kinase 1
sBSA: Cationic Bovine Serum Albumin
circRNA: Circular RNA
CNIs: Calcineurin Inhibitors
EMT: Epithelial- Mesangial Transition
ESRD: End Stage Renal Disease
EXT1/EXT2: Exostosin 1 and Exostosin 2
FSGS: Focal and Segmental Glomerulosclerosis
GBM: Glomerular Basement Membrane
GFR: Glomerular Filtration Rate
GC-MS/MS: Gas Chromatography-Tandem Mass Spectrometry
IHC: Immunohistochemistry
LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry Analysis
LIMP-2: Lysosome Membrane Protein 2
MALDI-MSI: Matrix Assisted Laser Desorption/Ionization Mass Spectrometry Imaging
MCD: Minimal Change Disease
MN: Membranous Nephropathy
NAG: N-acetyl-β-glucosaminidase
NELL-1: Neural Epidermal Growth Factor-like 1 Protein
nLC-ESI-MS/MS: nano-Liquid Chromatography Coupled with Electrospray Ionisation Tandem Mass Spectrometry
NMR: Nuclear Magnetic Resonance
PHB 1: Prohibitin 1
PHB 2: Prohibitin 2
PLA2R: M-type Phospholipase A2 Receptor
PKA: Protein Kinase A
PODO: Podocalexin
PPM1A: Protein Phosphatase, Mg2+/Mn2+ Dependent 1A
RAB1A: Ras-Related Protein Rab-1A
RAS: Renin-Angiotensin System
RBP: Retinol Binding Protein
SAA1: Serum Amyloid A1 Protein
THSD7A: Thrombospondin Type-1 Domain-containing 7A
TMT: Tandem Mass Tag
TLDAs: TaqMan Low-density Arrays
TNFSF11: TNF Super Family 11
TOF/TOF: Tandem Time of Flight
WT1: Wilms' Tumor

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Received April 2020
Revised June 2020
Accepted August 2020