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# Compared staining of the phospholipase A2 receptor in the glomeruli of Chinese adults and children with idiopathic membranous nephropathy

Dan Zhang[a], Ying Wu[b], Chong Zhang[a], Wenzhu Zhang[c], Jun Zou[a,*], Gengru Jiang[a,*]

[a] Department of Nephrology, Xinhua Hospital, School of Medicine, Shanghai Jiao Tong University, Kong Jiang Road, 1665, 200092, Shanghai, China
[b] Department of Nephrology and Rheumatology, Children's Hospital of Shanghai, Shanghai Jiao Tong University, Shanghai, China
[c] Department of Histopathology, Xinhua Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China

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ABSTRACT

*Background:* The identification of the M-type phospholipase A2 receptor (PLA2R) is a breakthrough recognized as a major target for adults with idiopathic membranous nephropathy (IMN). However, the role PLA2R played in pediatric patients with IMN, particularly in Chinese, has yet to be determined.

*Methods:* This retrospective study included 187 adult patients and 38 pediatric patients aged 17 years or younger with biopsy proved IMN. The pediatric cohort consisted of 27 children aged from 1 to 12 years and 11 children aged from 13 to 17. Glomerular expression of PLA2R was analyzed in stored, formalin-fixed, paraffin-embedded kidney biopsy sections.

*Results:* PLA2R staining in glomerular deposits was observed in 82.7% and 42.1% of adult and pediatric patients with IMN, respectively. The PLA2R-positive staining patients with IMN presented with more severe clinical features than PLA2R-negative staining patients in both adult and pediatric cohorts. When compared to the young children patients with IMN, the adolescents exhibited a higher positive rate of PLA2R staining (81.8% versus 25.9%), similar to the adult patients.

*Conclusion:* The clinical features and prevalence of PLA2R positive staining in adolescent patients with IMN were similar to adult patients, suggesting that they probably have a close etiology and pathogenesis. However, most of the young children patients with IMN were PLA2R negative staining, suggesting a different underlying etiology.

## 1. Introduction

Idiopathic membranous nephropathy (IMN) is an autoimmune glomerular disease and is the most common glomerular disease associated with nephrotic syndrome (NS) in the adult Caucasian population [1]. The frequency of IMN occurrences in primary glomerular disease has also significantly increased in China over the past 10 years [2]. However, IMN rarely occured in the pediatric population and accounted for only 1–2% of childhood NS in the past [3] and still less than 2% of kidney biopsies for children in the NEPTUNE cohort in North America [4]. Nevertheless, it's prevalence in Chinese children was doubled in the past decades [5] and recently even reached to 6% of pediatric glomerular diseases [6].

In 2009, the M-type phospholipase A2 receptor (PLA2R), a membrane glycoprotein that localizes to podocytes, was identified as the pathogenic antigen in adult patients with IMN [7]. Circulating PLA2R autoantibodies were found in a majority (52–82%) of serum samples from patients with IMN [8–12]. Furthermore, PLA2R staining was assessed in renal biopsies and displayed a higher level of sensitivity than anti-PLA2R antibodies in IMN subjects [11–14]. This suggests that these measurements are valuable in the diagnosis and treatment of adult patients with IMN. However, data on PLA2R staining in renal biopsy tissues of pediatric patients with IMN was relatively limited and the positive staining rate was much lower than their adult counterparts, about 45% [15] but variable from 6% to 72% depending on different age and ethnicity [16–18]. Additionally, there are no studies comparing PLA2R staining in pediatric to adult patients with IMN. Therefore, we performed this retrospective study to determine the specificity and sensitivity of PLA2R staining in kidney biopsy samples of IMN patients, and to compare the value of glomerular PLA2R deposition and its relation to clinical features between adult and child patients with IMN. Moreover, The underlying causes of PLA2R-negative patients with IMN had been explored and thrombospondin type-1 domain-containing 7 A (THSD7 A) was identified recently as a new autoantigen related to IMN [19]. It had been reported that 10.5–16.0% of PLA2R-negative patients with IMN had elevated circulating THSD7 A autoantibodies levels or

* Corresponding authors.
*E-mail addresses:* zoujun@xinhuamed.com.cn (J. Zou), jianggengru@xinhuamed.com.cn (G. Jiang).

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positive glomerular THSD7 A deposits [20,21]. Hence, we also assessed THSD7 A staining in glomerular deposits in both adult and pediatric PLA2R-negative patients with IMN.

## 2. Materials and methods

### 2.1. Patients and samples

A retrospective database was searched for cases of biopsy-proven membranous glomerulopathy in patients at Xinhua Hospital from 2004 to 2016, and Children's Hospital of Shanghai from 2010 to 2016. The screening for secondary MN included a detailed medical history, serologic tests for lupus erythematosus and hepatitis, and a screening for malignancies dependent on the patient's age and additional risk factors. All lupus patients fulfilled the American College of Rheumatology revised classification criteria for SLE [22], and 4 of 11 must be present to establish the diagnosis of SLE. A total of 187 adult patients and 38 patients 17 years of age or younger biopsy proved with IMN were used for this analysis. Meanwhile, 33 patients with secondary MN and 27 patients with other glomerular disease were used as control. This study was approved by the Ethics Committee of Xinhua Hospital, School of Medicine, Shanghai Jiao Tong University and Children's Hospital of Shanghai, Shanghai Jiao Tong University. Written informed consents were obtained from the patients regarding the use of all specimens.

Patient demographics and presenting features included gender, age, disease duration, the presence or absence of NS and/or hypertension. Blood samples and urine samples at the time the renal biopsies were obtained were also collected from all of the patients. Laboratory features included urinalysis, 24-hour proteinuria, serum albumin, serum creatinine (Scr), estimated glomerular filtration rate (eGFR, calculated by Chronic Kidney Disease–Epidemiology Collaboration equation for adults and Schwartz formula for children) and serologic testing that included complement components 3 and 4 (C3 and C4), anti-nuclear antibody (ANA), and hepatitis B surface antigen.

### 2.2. Renal biopsy

Renal biopsies were performed on all patients at the time of diagnosis using routine biopsy processing techniques. Standard renal biopsies were processed with light, immunofluorescence (IF), and electron microscopy (EM). Paraffin-embedded tissues used for light microscopy were sectioned at 3 μmfor routine staining. All light microscopy samples were stained with hematoxylinand eosin (H&E), Jones methenamine silver, Masson trichrome, and periodic acid-Schiff reagent. For direct immunofluorescence, renal tissues were snap frozen in liquid nitrogen and cut into 3 μm sections. Antibodies against to IgG, IgA, IgM, C3, C4, C1q, and fibrinogen were examined in the biopsy specimens. In EM, the Ehrenreich and Churg classification was used for the ultrastructural staging of IMN [23].

### 2.3. Glomerular staining of PLA2R and THSD7A proteins

Sections of renal biopsies were embedded in paraffin and cut into 3 μm sections for immunohistochemical staining. Rabbit anti-PLA2R (Sigma-Aldrich, USA) and rabbit polyclonal anti-THSD7 A(Sigma-Aldrich, USA) were used as primary antibodies and a horseradishperoxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Changdao, Shanghai, China) was used as a secondary antibody. Detection of glomerular PLA2R and THD7 A deposition were performed using methods previously described [11,24]. Negative controls were obtained by staining with minimal change disease. Positive deposition of glomerular PLA2R and THSD7 A was defined as a strong pattern of staining distributed along the glomerular capillary wall in a granular pattern.

**Table 1**
The glomerular deposition of PLA2R in adult patients with IMN, secondary MN and non-MN.

| Group | PLA2R (+) | PLA2R (-) | Total | % |
|---|---|---|---|---|
| **IMN, n** | **155** | **32** | **187** | **82.9 %** |
| **Secondary MN, n** | **9** | **24** | **33** | **27.2 %** |
| Hepatitis B virus associated MN | 4 | 8 | 12 | |
| Lupus nephritis, class V | 0 | 7 | 7 | |
| Malignancy associated MN | 5 | 9 | 14 | |
| **Non-MN, n** | **0** | **27** | **27** | **0 %** |
| Minimal change disease | 0 | 5 | 5 | |
| Focal segmental glomerulosclerosis | 0 | 5 | 5 | |
| IgA nephropathy | 0 | 5 | 5 | |
| Diabetic nephropathy | 0 | 5 | 5 | |
| Lupus nephritis, classII-IV | 0 | 5 | 5 | |
| Anti-GBM glomerulonephritis | 0 | 2 | 2 | |

MN, membranous nephropathy; GBM, glomerular basement membrane.

### 2.4. Statistical analysis

All statistical analyses were performed using the SPSS 17.0 software package (SPSS Inc., Chicago, IL). Normally distributed variables were described as a mean ± SD. When applicable, the chi-square test or Fisher's exact test were used to compare distributions between groups. All p-values were two-tailed, with values $< 0.05$ being considered statistically significant.

## 3. Results

### 3.1. Comparison of clinical characteristics between PLA2R-positive and PLA2R-negative adult patients with IMN

As shown in Table 1, PLA2R staining was positive (Fig. 1a and b) in 155/187 (82.9%) adult patients with IMN, while positive in only 9/33 (27.2%) adult patients with secondary MN and no staining in all 27 adult patients with other glomerular diseases. For adult patients with IMN, PLA2R-positive patients had more male and hypertensive subjects, more proteinuria (5.20 vs. 3.85 g/24 h, $p = 0.007$) and lower serum albumin levels (25.39 vs. 28.22 g/L, $p = 0.010$) compared to PLA2R-negative patients (Table 2).

### 3.2. Comparison of clinical characteristics and PLA2R glomerular deposits between adult and pediatric patients with IMN

A total of 187 adult (55.2 ± 14.3 years) patients and 38 pediatric (9.4 ± 5.1 years) patients with IMN were eligible for this study. The clinical characteristics of the patients were shown in Table 3. Male subjects were more common in both the adult and pediatric patients with IMN. Hematuria was more common in the pediatric patients ($p < 0.001$), and the prevalence of hypertension was high in the adult patients ($p < 0.001$). Compared to the pediatric patients with IMN, the adult patients had more proteinuria (4.97 vs. 2.29 g/24 h; $p < 0.001$), a lower level of serum albumin (25.88 vs. 30.18 g/L; $p = 0.003$), a higher level of serum creatinine (73.49 vs. 38.48 μmol/L; $p < 0.001$) and a lower eGFR (93.31 vs. 180.90 ml/min/1.73m$^2$; $p < 0.001$). However, there was no significant difference observed in course of disease and lipid profiles. The proportions of stage I for the adult and pediatric groups were 9.6% and 44.7%, respectively, which was significantly different ($p < 0.001$). PLA2R staining was positive in only 16 of 38 (42.1%) pediatric patients with IMN, much less than their adult counterparts ($p < 0.001$), in both stage I and II, but not in stage III.

### 3.3. Comparison of clinical characteristics between PLA2R-positive and PLA2R-negative pediatric patients with IMN

We investigated the pediatric patients with IMN. Compared to

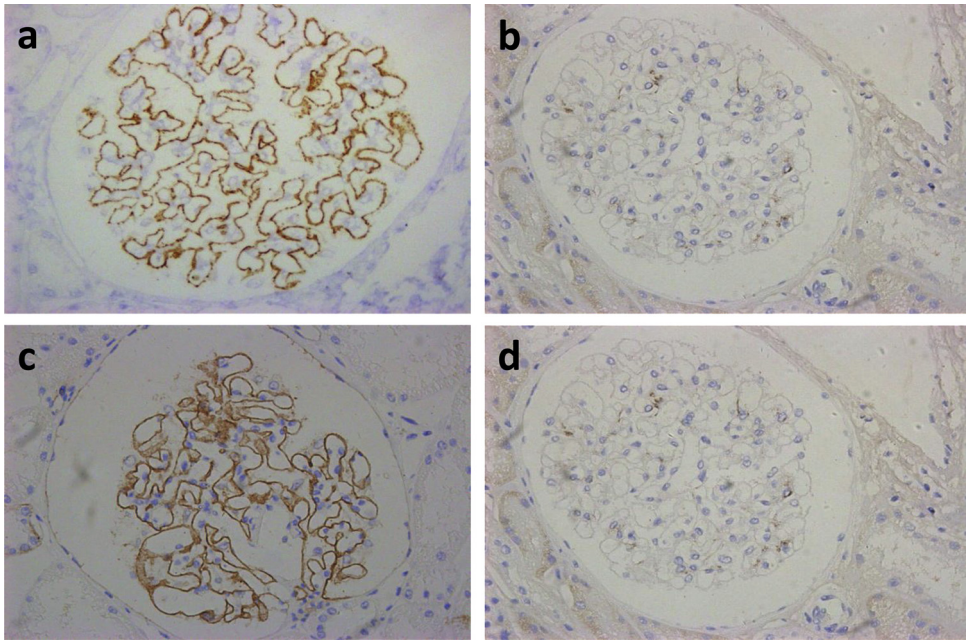


**Fig. 1.** Glomerular staining of PLA2R and THSD7 A in membranous nephropathy. PLA2R and THSD7 A were detected as granular deposits in the sub-epithelial space (a and c, magnification × 400). Staining in renal biopsy tissues of patients with minimal change disease was used as controls (b and d, magnification × 400).

**Table 2**
Comparison of clinical characteristics between PLA2R-positive and PLA2R-negative adult patients with IMN.

| | PLA2R(+) (n = 155) | PLA2R(-) (n = 32) | *P* |
|---|---|---|---|
| Male, n (%) | 105 (67.7%) | 15 (46.9%) | **0.019** |
| Age, years | 55.56 ± 14.49 | 53.59 ± 13.39 | NS |
| Course, months | 6.05 ± 13.10 | 6.48 ± 7.75 | NS |
| Proteinuria, g/24h | 5.20 ± 2.57 | 3.85 ± 2.36 | **0.007** |
| Serum albumin, g/L | 25.39 ± 5.39 | 28.22 ± 6.37 | **0.010** |
| Hematuria, n (%) | 45 (29.0%) | 9 (28.1%) | NS |
| Hypertension, n (%) | 95 (61.3%) | 11 (34.4%) | **0.006** |
| Serum creatinine, μmol/L | 75.10 ± 26.30 | 65.82 ± 23.53 | NS |
| eGFR, ml/min/1.73 $m^2$ | 92.30 ± 23.03 | 98.10 ± 19.22 | NS |
| Total cholesterol, mmol/L | 7.60 ± 2.29 | 7.09 ± 2.24 | NS |
| Triglyceride, mmol/L | 2.51 ± 1.52 | 2.10 ± 1.03 | NS |
| **Ehrenreich–Churg stage** | | | NS |
| stage I, n (%) | 13 (8.4%) | 5 (15.6%) | |
| stage II, n (%) | 118 (76.1%) | 25 (78.1%) | |
| stage III, n (%) | 24 (15.5%) | 2 (6.3%) | |

Values are mean ± SD or numbers of subjects (%).
PLA2R, phospholipase A2 receptor, IMN, idiopathic membranous nephropathy; eGFR, estimated glomerular filtration rate.

PLA2R-negative pediatric patients with IMN, PLA2R-positive patients had significantly more proteinuria and higher serum creatinine levels, and lower serum albumin level but did not reach significance (Table 4). We also found that PLA2R-positive pediatric patients were significantly older than PLA2R-negative patients (12.88 vs. 6.82, $p < 0.001$).

We further separated the pediatric patients with IMN. The pediatric patients with IMN consisted of 27 young children aged between 1 and 12 years and 11 adolescents between 13 and 17 years of age (Table 5). We compared some clinical features and the prevalence of PLA2R positive staining between the young children and the adolescents. A significantly higher rate of PLA2R staining was observed in adolescents compared to those young children patients (81.8% vs. 25.9%, $p = 0.003$). Additionally, the prevalence of hematuria, proteinuria, serum creatinine level and eGFR were all significantly different between the two subgroups.

**Table 3**
Comparison of clinical characteristics and glomerular PLA2R deposits between adult and pediatric patients with IMN.

| | Adults n = 187 | Children n = 38 | *P* |
|---|---|---|---|
| Male, n (%) | 120 (64.2%) | 25 (65.8%) | NS |
| Course, months | 6.12 ± 13.96 | 4.38 ± 7.10 | NS |
| Hematuria, n (%) | 52 (27.8%) | 30 (78.9%) | **< 0.001** |
| Hypertension, n (%) | 106 (56.7%) | 1 (2.6%) | **< 0.001** |
| Proteinuria, g/24h | 4.97 ± 2.58 | 2.29 ± 1.85 | **< 0.001** |
| Serum albumin, g/L | 25.88 ± 5.66 | 30.18 ± 8.10 | **0.003** |
| Serum creatinine, μmol/L | 73.49 ± 26.02 | 38.48 ± 15.13 | **< 0.001** |
| eGFR, ml/min/1.73 $m^2$ | 93.31 ± 2.48 | 180.90 ± 45.40 | **< 0.001** |
| Total cholesterol, mmol/L | 7.51 ± 2.21 | 7.03 ± 2.86 | NS |
| Triglyceride, mmol/L | 2.43 ± 1.45 | 1.78 ± 1.11 | NS |
| **Ehrenreich–Churg stage** | | | **< 0.001** |
| stage I, n (%) | 18 (9.6%) | 17 (44.7%) | |
| stage II, n (%) | 143 (76.5%) | 17 (44.7%) | |
| stage III, n (%) | 26 (13.9%) | 4 (10.5%) | |
| PLA2R(+), n (%) | 155 (82.9%) | 16 (42.1%) | **< 0.001** |
| PLA2R(+) in stage I | 13/18 (72.2%) | 6/17 (35.3%) | **0.004** |
| PLA2R(+) in stage II | 118/143 (82.5%) | 7/17 (41.2%) | **0.004** |
| PLA2R(+) in stage III | 24/26 (92.3%) | 3/4 (75%) | NS |

Values are mean ± SD or numbers of subjects (%).
PLA2R, phospholipase A2 receptor, IMN, idiopathic membranous nephropathy; eGFR, estimated glomerular filtration rate.

### *3.4. The glomerular deposition of THSD7A in PLA2R-negative patients with IMN*

We further performed THSD7 A staining in all of PLA2R-negative 22 adult and 22 pediatric patients with IMN. We found distinct granular THSD7 A deposits in only 3 (13.6%) adult patients with IMN (Fig. 1c and d), whereas no positive staining in all pediatric patients with IMN.

## 4. Discussion

MN is a disease predominantly found in middle-aged and elderly individuals, but is uncommon in children. It is interesting to note that children differ from adults in several important disease characteristics including etiology, demographics, clinical, and pathologic features. MN

**Table 4**
Comparison of clinical characteristics between PLA2R-positive and PLA2R-negative child patients with IMN.

| | PLA2R(+) n = 16 | PLA2R(-) n = 22 | *P* |
|---|---|---|---|
| Male, n (%) | 11 (68.8%) | 15 (68.2%) | NS |
| Age, years | 12.88 ± 3.36 | 6.82 ± 4.63 | **< 0.001** |
| Course, months | 4.02 ± 6.18 | 4.64 ± 7.83 | NS |
| Proteinuria, mg/24h | 3403.52 ± 1967.72 | 1474.21 ± 1262.25 | **0.002** |
| Serum albumin, g/L | 27.21 ± 6.69 | 32.34 ± 8.48 | NS |
| Hematuria, n (%) | 10 (62.5%) | 20 (90.9%) | NS |
| Hypertension, n (%) | 1 (6.3%) | 0 | NS |
| Serum creatinine, μmol/L | 46.26 ± 13.38 | 32.82 ± 14.00 | **0.005** |
| eGFR, ml/min/1.73 $m^2$ | 172.62 ± 46.22 | 186.92 ± 44.90 | NS |
| Total cholesterol, mmol/L | 7.59 ± 2.85 | 6.62 ± 2.86 | NS |
| Triglyceride, mmol/L | 1.90 ± 1.19 | 1.70 ± 1.07 | NS |
| **Ehrenreich–Churg stage** | | | NS |
| stage I, n (%) | 6 (37.5%) | 11 (50%) | |
| stage II, n (%) | 7 (43.8%) | 10 (45.5%) | |
| stage III, n (%) | 3 (18.8%) | 1 (4.5%) | |

Values are mean ± SD or numbers of subjects (%).
PLA2R, phospholipase A2 receptor, IMN, idiopathic membranous nephropathy; eGFR, estimated glomerular filtration rate.

**Table 5**
Comparison of clinical characteristics and glomerular PLA2R deposits between young children and adolescent with IMN.

| Age group | Young children (1 ~ 12 year) n = 27 | Adolescents (13 ~ 17 year) n = 11 | *P* |
|---|---|---|---|
| Male, n (%) | 17 (63.0%) | 8 (72.7%) | NS |
| Course, months | 4.24 ± 7.10 | 4.73 ± 7.43 | NS |
| Hematuria, n (%) | 25 (92.6%) | 5 (45.5%) | **0.004** |
| Hypertension, n (%) | 0 | 1 (0.9%) | NS |
| Proteinuria, g/24h | 1.66 ± 1.35 | 3.84 ± 2.04 | **< 0.001** |
| Serum albumin, g/L | 30.78 ± 8.41 | 28.70 ± 7.45 | NS |
| Serum creatinine, μmol/L | 31.44 ± 10.86 | 55.75 ± 8.90 | **< 0.001** |
| eGFR, ml/min/1.73 $m^2$ | 194.81 ± 45.79 | 146.75 ± 19.23 | **0.002** |
| Total cholesterol, mmol/L | 7.12 ± 2.94 | 6.78 ± 2.75 | NS |
| Triglyceride, mmol/L | 1.84 ± 1.25 | 1.63 ± 0.69 | NS |
| **Ehrenreich–Churg stage** | | | NS |
| stage I, n (%) | 13 (48.1%) | 4 (36.4%) | |
| stage II, n (%) | 13 (48.1%) | 4 (36.4%) | |
| stage III, n (%) | 1 (3.7%) | 3 (27.3%) | |
| PLA2R(+), n (%) | 7 (25.9%) | 9 (81.8%) | **0.004** |

Values are mean ± SD or numbers of subjects (%).
PLA2R, phospholipase A2 receptor, IMN, idiopathic membranous nephropathy; eGFR, estimated glomerular filtration rate.

in children is often associated with secondary causes, such as systemic lupus erythematosus and hepatitis B infection, while most adult MN cases are considered to be idiopathic in nature [18]. Our study found that when compared to adult patients with IMN, hematuria (78.9%) was observed frequently in children with IMN, which is consistent with other reports [25,26]. However, proteinuria was less, no hypertension was present and renal function was normal, suggesting that our child cohort had milder clinical symptoms than the adult patients, similar to other studies [25–27]. We also observed that 44.7% of the children in our groups with IMN presented as stage I by electron microscopy, while more adult patients were evaluated as stage II. This difference may explain the varying clinical features between the pediatric and adult cohorts. Although proteinuria was less in the child group, there was no significant difference in lipid profile between the two groups. The association between changes in serum albumin and cholesterol is unclear, as previously reported [28]. This could be due to the underlying metabolic problems observed in the young children.

Adolescents have often been included in the child category in previous IMN research [25–28]. However, significant differences in clinical and pathological features of adolescents with NS when compared to young children with NS do exist [29]. Therefore, children with IMN were also divided into two subgroups in our study. When compared to young children with IMN, adolescents displayed very similar profiles to those of adults, showing less hematuria, more proteinuria and lower eGFR.

Since Beck et al. first reported detecting PLA2R as a target antigen for human IMN in 2009 [7], subsequent studies have demonstrated that anti-PLA2R antibodies ranged from 52 to 82% in patients with IMN. This was a potentially useful marker for identifying IMN, monitoring disease activity and determining treatment options [8–12,30–32]. Furthermore, these antibodies can disappear after immunosuppressive therapy or spontaneous remission. Some research showed that the positive rate of glomerular PLA2R was higher than PLA2R antibodies in serum [11–14], suggesting that PLA2R staining could be more reliable for diagnosing PLA2R-related MN. In this study, 153 of the 187 adult patients with IMN (82.7%) were positive for glomerular PLA2R expression, which was similar to other Chinese studies [14,33]. However, until now the role of PLA2R in pediatric IMN has not been well characterized due to its uncommonness. In our child cohort, 16 of the 38 (42.1%) patients exhibited positive PLA2R staining as described in a previous study [15].This was much lower than adult. Nine out of 11 (81.8%) adolescents presented with positive PLA2R staining, comparable to what was seen in adults, whereas only 7 out of 27 young children were observed positive PLA2R deposits.

Due to the differences in clinical features and the varying positive PLA2R staining rates among adults, adolescents and young children, it was suggested that PLA2R staining cannot be identified as the etiology of disease for the majority of the young children with IMN. Debiec et al. has identified neutral endopeptidase in podocytes as an antigen for antenatal membranous nephropathy in infant patients with MN [34]. They also found antibodies to dietary cationic bovine serum albumin in young infants with MN [35]. Therefore, more diverse and underlying causes for IMN in the child population remain to be identified. Notably, previous research has suggested that PLA2R staining may be absent in early-stage IMN [36]. When compared to adults, the child patients presented more stage I with IMN, indicating that some PLA2R-related MN may be detectable during their follow-up. Additionally, PLA2R-related MN occurs more frequently in adolescents, closer to what was observed in the adult cohort, which implies that the etiology and pathogenesis of the adolescent patients with IMN may be similar to adults. Recent research has also reported that enhanced staining for PLA2R in glomeruli was observed in72% of adolescent patients with IMN [17]. This suggests that the diagnosis and therapy criteria used for adolescents should differ from those used for young children.

To date, there were few reports that suggested a correlation between glomerular PLA2R deposit and clinical manifestations in patients with IMN. In our study, adult patients with positive PLA2R staining displayed more proteinuria and lower serum albumin levels than those without PLA2R. In addition, the occurrence in males and hypertensive rates were both significantly higher in the PLA2R-positive patients, indicating that PLA2R-positive patients with IMN presented more severe clinical features compared to the PLA2R-negative patients. This was inconsistent with data from previous studies [11,12]. The differences may be due to the different study population. The positive PLA2R staining children with IMN in our study were also much older than the negative PLA2R children. Proteinuria and serum creatinine levels were significantly higher than in PLA2R-positive children as well, compared to PLA2R-negative children, which may be associated with the age difference.

In our cohort, 3 patients with positive THSD7 A staining in glomerular deposits were found in adult PLA2R-negative patients with IMN, whereas no one with positive THSD7 A staining in pediatric PLA2R-negative patients with IMN, which was consistent with the other

study reported [37]. This further suggests the different etiology between adult and pediatric patients with IMN. However, since a low prevalence of THSD7 A associated MN, more cases are needed to be investigated.

Due to its retrospective nature, this study had several limitations. First, the treatment and outcome of the disease were not analyzed. Second, the serum PLA2R antibody was not examined, and it could be better for monitoring disease activity. Despite these limitations, the present study included a large sample of adult and pediatric patients with IMN and demonstrates the sensitivity of PLA2R staining.

## 5. Conclusion

We observed clinical features and glomerular deposits of PLA2R in adults, adolescents and young children patients with IMN. The drastically low proportion of positive PLA2R staining in young children indicates that the majority of childhood patients with IMN may not be PLA2R-related MN. The etiology of IMN in young children may be more various and underlying in contrast to that observed in adults. However, the adolescents with IMN demonstrated similar clinical characteristics and prevalence of PLA2R positive staining compared to adult patients, which implies that adults and adolescents are likely to have a close etiology and pathogenesis of the disease.

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