Retrospective study: clinicopathological features and prognosis of idiopathic membranous nephropathy with seronegative anti-phospholipase A2 receptor antibody

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Background: To discuss the clinicopathological features and prognosis of patients with idiopathic membranous nephropathy (IMN) who are serum-negative for the anti-PLA2R antibody.

Method: Overall, 229 IMN patients were retrospectively collected in this study and classified into anti-PLA2R antibody-negative (PLA2R-, 59 cases) and antibody-positive (PLA2R+, 170 cases) groups. The clinical and pathological features of the PLA2R- group were analyzed; 162 patients in both groups were followed up, and the PLA2R antigen was detected in renal biopsies from the PLA2R- group. Kaplan-Meier and survival analyses were used to compare differences in prognosis.

Results: Serum albumin levels were higher and 24-hour urine protein, creatinine, and beta 2-microglobulin (BMG) levels were lower in the PLA2R- group than in the PLA2R+ group; the proportion of acute and chronic tubular lesions was also significantly lower in the PLA2R- group than in in the PLA2R+ group. After treatment, the remission rate was significantly higher in the negative group than in the positive group (93.02% vs 74.78%,), especially the rate of complete remission (51.16% vs 23.47%). Furthermore, the PLA2R antigen-positive staining rate of 43 patients in the PLA2R- group was 62.79%. Although not significant, the survival rate was higher in the PLA2R- group than in the PLA2R+ group. BMG, 24-hour urine protein and acute and chronic tubular lesions were risk factors for kidney death, and 24-hour urine protein was an independent risk factor for kidney death.

Conclusions: Compared with the PLA2R+ group, the PLA2R- group had mild clinical manifestations and pathological damage and a higher clinical treatment remission rate. Renal tissue PLA2R antigen testing can be considered for patients with seronegative IMN to increase the diagnostic rate.

1 **Retrospective study: clinicopathological features and prognosis of idiopathic membranous**

2 **nephropathy with seronegative anti-phospholipase A2 receptor antibody**

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28   **Abstract**

29   **Background:** To discuss the clinicopathological features and prognosis of patients with

30   idiopathic membranous nephropathy (IMN) who are serum-negative for the anti-PLA2R

31   antibody.

32   **Method:** Overall, 229 IMN patients were retrospectively collected in this study and classified

33   into anti-PLA2R antibody-negative (PLA2R-, 59 cases) and antibody-positive (PLA2R+, 170

34   cases) groups. The clinical and pathological features of the PLA2R- group were analyzed; 162

35   patients in both groups were followed up, and the PLA2R antigen was detected in renal biopsies

36   from the PLA2R- group. Kaplan-Meier and survival analyses were used to compare differences

37   in prognosis.

38   **Results:** Serum albumin levels were higher and 24-hour urine protein, creatinine, and beta 2-

39   microglobulin (BMG) levels were lower in the PLA2R- group than in the PLA2R+ group; the

40   proportion of acute and chronic tubular lesions was also significantly lower in the PLA2R- group

41   than in in the PLA2R+ group. After treatment, the remission rate was significantly higher in the

42   negative group than in the positive group (93.02% vs 74.78%,), especially the rate of complete

43   remission (51.16% vs 23.47%). Furthermore, the PLA2R antigen-positive staining rate of 43

44   patients in the PLA2R- group was 62.79%. Although not significant, the survival rate was higher

45   in the PLA2R- group than in the PLA2R+ group. BMG, 24-hour urine protein and acute and

46   chronic tubular lesions were risk factors for kidney death, and 24-hour urine protein was an

47   independent risk factor for kidney death.

48   **Conclusions:** Compared with the PLA2R+ group, the PLA2R- group had mild clinical

49   manifestations and pathological damage and a higher clinical treatment remission rate. Renal

50   tissue PLA2R antigen testing can be considered for patients with seronegative IMN to increase

51   the diagnostic rate.

52

Introduction

54 Membranous nephropathy with unclear etiology is called idiopathic membranous nephropathy

55 (IMN) and accounts for 30 ~ 40% of all cases of primary nephrotic syndrome (Braden et al.

56 2000; Couser 2017). Recent clinical observations show that the incidence of membranous

57 nephropathy is increasing, and the age of onset is becoming younger, which may be related to

58 various factors such as improvements in diagnostic factors and environmental pollution (Ayach

59 et al. 2011). Moreover, IMN is also a heterogeneous disease with a long natural course.

60 Approximately one-third of patients will exhibit variable degrees of persistent proteinuria

61 without deterioration in renal function, another third will experience progressive deterioration of

62 renal function and eventually progress to end-stage renal disease (ESRD), and the final third of

63 patients will experience spontaneous remission (Obrisca et al. 2015).

64   Currently, the method for diagnosing membranous nephropathy is mainly based on renal

65 biopsy, an invasive examination method that has inherent risks and cannot be used to diagnose

66 and treat diseases in a timely and effective manner. Therefore, the serological diagnosis of IMN

67 has become a hot topic in kidney disease research. In 2009, Beck et al. (Beck et al. 2009)

68 discovered autoantibodies against phospholipase A2 receptor antibodies in the glomerular

69 podocytes of IMN patients. PLA2R is a transmembrane protein located on the surface of human

70 podocytes and colocalizes with IgG4 in the immune deposits of glomeruli in patients with IMN.

71 Binding of the circulating anti-PLA2R antibody to the PLA2R antigen on glomerular podocytes

72 to form an in situ immune complex activates a complement to cause podocyte and immune

73 damage that results in urinary protein production, ultimately causing kidney damage (Glassock

74 2012). Additionally, Beck et al. found that serum anti-PLA2R antibodies were detectable in 70%

75 of patients with IMN and detected at a low rate in people without kidney disease and other

76 kidney patients (Hofstra & Wetzels 2014). Further studies have found that the antibody level is

77 related to the urine protein level, disease condition, and prognosis and has high sensitivity and

78 specificity in the clinic (Hofstra et al. 2011; Hoxha et al. 2011; Hoxha et al. 2014). In addition to

79 serum PLA2R antibodies, a number of clinical studies (Beck et al. 2009; Dai et al. 2015;

80 Svobodova et al. 2013) have found that the detection of PLA2R antigen in renal tissue also plays

81 an important role in the clinical diagnosis, guiding treatment and judgment of disease activity of

82 IMN. The expression of PLA2R antigen in renal tissue was significantly enhanced in IMN

83 patients, and low or no expression was observed in patients with secondary membranous

84 nephropathy (SMN) and other nonmembranous glomerular diseases, indicating that the PLA2R

85 antigen in renal tissue also has high specificity in the diagnosis of IMN.

86 Several studies have shown that the anti-PLA2R antibody titer reflects the activity of the

87 clinical disease. The low antibody titer in antibody-positive patients indicates a high disease

88 remission rate, and thus, this antibody can be used as a predictor of clinical efficacy (Hofstra et

89 al. 2012; Oh et al. 2013). However, there are few studies on the clinical and pathological

90 characteristics of patients with IMN who are negative for the serum anti-PLA2R antibody, and

91 the effect of a negative antibody on disease prognosis is unknown. Besides, some studies suggest

92 that patients with negative antibodies may have secondary factors, such as tumors and heavy

93 metals (Radice et al. 2018). Therefore, the characteristics of serum anti-PLA2R antibody-

94 negative IMN require further clinical studies.

95 In this study, we performed a two-step evaluation. First, we retrospectively compared the

96 clinical and pathological features of anti-PLA2R-negative (PLA2R-) and anti-PLA2R-positive

97 (PLA2R+) subjects, and the PLA2R antigen was detected in renal biopsies from the PLA2R-

98 group. Then, we evaluated the prognostic value of PLA2R-negative results using a longitudinal

99 design and analysis.

100 **Materials and Methods**

101 **1. Patient selection**

From January 2015 to December 2017, 229 adult patients at Shandong Provincial Hospital who were diagnosed with nephrotic syndrome confirmed by renal biopsy as IMN and had complete clinical data were included in this study. All patients had IMN, a condition that is universally diagnosed by kidney biopsy based on the presence of subepithelial spikes along capillary walls as determined by silver staining, granular IgG and C3 along capillary walls as determined by immunofluorescence, and subepithelial deposits as determined by electron microscopy (EM). The clinical study excluded patients with SMN caused by autoimmune disease, tumors, and hepatitis B virus- and metabolic-related diseases. At the time of selection, the patients' renal function was in the normal range, and 162 patients were followed up for more than 6 months with complete follow-up data. This study has obtained verbal consent from patients, as well as reviewed and approved by the Medical Ethics Committee of Shandong Provincial Hospital (LCYJ: no. 2019-105).

2. Clinical and laboratory data collection

2.1 General demographics (age, gender) and clinical (duration of disease, systolic and diastolic blood pressure, etc.) records were collected. Laboratory data included general tests (urinary red blood cells, hemoglobin, white blood cells, platelets, alanine aminotransferase (ALT), aspartate aminotransferase (AST), superoxide dismutase (SOD), serum total protein (TP), albumin (ALB), globulin (GLO), cystatin C (Cys-C), retinol-binding protein (RBP), β2 microglobulin (BMG), complement C1q, calcium, immunoglobulin, total cholesterol (CHOL), low-density lipoprotein (LDL-C), high-density lipoprotein (HDL-C), antibody, antinuclear antibody, ANCA antibody, blood glucose (GLU), etc.) and specific biomarkers of renal function and disease activity (urine protein, 24-hour urine protein, serum creatinine (CREA), and blood urea nitrogen (BUN)). The eGFR was calculated based on the modification of diet in renal disease (MDRD) formula.

2.2 Anti-PLA2R detection method: The anti-phospholipase A2 receptor antibody IgG detection kit (enzyme-linked immunosorbent assay) produced by EUROIMMUN (product no. EA 1254-9601 G) was used and performed according to the standard procedure in the manual using a

128   normal reference range of 0-20 RU/ml. The study also included antibody-positive components in

129   the low-titer group (20-180 RU/mL) and high-titer group (> 180 RU/mL) (Hofstra et al. 2012).

130   **2.3** PLA2R antigen and IgG4 detection: We collected paraffin sections of kidney biopsies from

131   43 patients negative for anti-PLA2R antibodies, used direct immunofluorescence to detect the

132   deposition of IgG4 in renal tissue and indirectly detected the PLA2R antigen in renal tissue. The

133   fluorescence intensity was semiquantitatively recorded by fluorescence microscopy and scored

134   as negative (-), suspicious positive (±), and positive (+).

135   **3. Renal biopsy**

136   Each patient's renal biopsy included at least 10 glomeruli for histopathological evaluation. We

137   performed light microscopy, electron microscopy(EM)and immunofluorescence on renal

138   puncture tissues. Two renal pathologists participated in the reading and review of the

139   pathological results. We divided membranous nephropathy into 4 stages. If two stages were

140   present at the same time, we defined the highest stage as the final stage and stages III and IV as

141   the advanced pathological stages. Simultaneously, this study observed the presence or absence of

142   focal segmental glomerular sclerosis, spheroidal sclerosis, mesangial proliferative lesions,

143   crescent incidence, balloon adhesions, endothelial hyperplasia, acute and chronic tubular lesions,

144   inflammatory cell-infiltrating lesions, small blood vessel lesions, etc. The pathological

145   manifestations of glomerular disease combined with focal segmental glomerulosclerosis (FSGS)

146   showed focal and segmental distribution of glomerular lesions under light microscopy, with an

147   increased mesangial matrix and balloon adhesions as the main manifestations, accompanied by a

148   small amount of mesangial hyperplasia, corresponding tubular atrophy and renal interstitium

149   fibrosis. Spherical sclerosis is defined based on the hardening of one glomerulus, and crescent

150   incidence is defined as the presence of a cellular or fibrous crescent in one glomerulus.

151   Mesangial proliferative disease is defined as the number of cells in the mesangial area being

152   greater than or equal to 4. Acute tubulointerstitial lesions are defined as tubular epithelial edema

153   or necrosis of at least 10%. Chronic tubulointerstitial lesions are defined as astubular atrophy and

154 interstitial fibrosis of at least 10%. Interstitial inflammatory disease mainly manifests as

155 neutrophil infiltration. Small vessel lesions include arterial intimal thickening, elastic layer

156 stratification, and hyaline degeneration.

4. Outcomes in followed up patients

157

158 We defined worsening renal conditions as doubling of the baseline Scr level; we defined ESRD

159 as a creatinine clearance rate of less than 15 ml/min at the last follow-up, start of dialysis or renal

160 transplantation. Complete remission (CR) was defined as a urine protein level <0.3 g/d based on

161 the premise of stable renal function. Partial remission (PR) was defined as a 50% reduction in the

162 urine protein to a proteinuria level <3.5 g/d. A simplified MDRD formula was used to calculate

163 eGFR as follows: eGFR $[ml \cdot min^{-1} \cdot (1.73\ m^2)^{-1}]$ =186×[Scr (μmol/L)/88.4]$^{-1.154}$×Age$^{-0.203}$× (if

164 female, 0.742). Serious complications included clinical death, serious lung infection, pulmonary

165 embolism, cerebral infarction, myocardial infarction, and tumors. All patients underwent eGFR

166 measurements at the beginning and after treatment.

5. Statistical methods

167

168 Statistical analysis was performed using the statistical software SPSS 19.0. Data with a normal

169 distribution were expressed as mean ± standard deviation (SD) and compared by t-tests, and data

170 with a non-normal distribution were presented as the median and quartile and compared by

171 nonparametric test. The categorical variables were expressed as rates and were compared by the

172 χ2 test. Correlation between several parameters was expressed as Spearman rank coefficient of

173 correlation. Patient and renal survival probabilities were estimated by the Kaplan- Meier method.

174 The relationships of the covariates to patient and renal survival were evaluated in the univariate

175 analysis with the log-rank test and in the multivariate analysis with the Cox proportional hazards

176 model; the Harrel C statistic was used for verification of the risk factors confirmed by the Cox

177 proportional hazards model. A Cox analysis of risk factors was performed in combination with

178 clinical significance. P < 0.05 was considered statistically significant.

179   **Results**

180   **1. General clinical data:** Two hundred twenty-nine patients with IMN were retrospectively

181   collected and analyzed in this study, including 59 (25.76%) patients in the PLA2R- group and

182   170 (74.24%) patients in the PLA2R + group. Among the antibody-positive group, there were

183   109 (64.12%) patients in the low-titer group and 61 (35.88%) patients in the high-titer group.

184   The comparisons of baseline clinical characteristics and laboratory examination data between the

185   two groups are shown in Table 1. In this study, the levels of serum creatinine (61.57±15.88 vs

186   65.90±15.64, df=226, P=0.033) and β2-microglobulin (2.13±0.74 vs 2.39±0.81, df=209,

187   P=0.045) in the PLA2R- group were lower than those in the PLA2R+ group, and the level of

188   ALB (26.11±6.02 vs 24.20±5.11, df=226, P=0.019) in the PLA2R- group was higher than that in

189   the PLA2R+ group. The average 24-hour urine protein quantification in the PLA2R- group was

190   slightly lower than that in the PLA2R+ group (4.91±3.18 vs 5.81±3.50), but the difference was

191   not statistically significant (df=224, P=0.086). At the same time, we found no correlation

192   between the anti-PLA2R antibody level and the serum creatinine level in the PLA2R+ patients

193   (r=-0.08, P=0.256). The anti-PLA2R antibody level was positively correlated with proteinuria

194   (r=0.151, P=0.05) and negatively correlated with serum albumin (r=-0.164, P=0.03). The 24-

195   hour urine protein levels of patients in the low-titer group were significantly lower than those in

196   patients in the high-titer group (5.28±3.42 vs 6.76±3.46, df=166, P=0.008).The eGFR values of

197   the antibody-negative and antibody-positive groups were 115.394 and 109.860 [ml·min-1· (1.73

198   m2)-1], respectively, but the difference was not statistically significant (df=225, P=0.172).

199   **2. Pathological data:** Renal biopsy immunofluorescence showed no significant differences in

200   IgG, IgM, IgA, C3, Fib, or C1q deposits between the two groups. There was no significant

201   difference in the proportion of renal pathological stages between the two groups (P>0.05). The

202   proportions of acute and chronic tubular lesions and inflammatory cell infiltration in the PLA2R-

203   group were significantly lower than those in the positive group (respectively, 3.39% vs 11.76%

204   df=180, P=0.015; 3.39% vs 13.53% df=183, P=0.01; and 5.08% vs 13.53% df=158, P=0.031)

205   (Table 2).

206   **3. Treatment and outcomes:** Among the 229 patients, 162 patients, including 43 patients in the

207   PLA2R- group and 119 patients in the PLA2R+ group, were followed up for more than 6

208   months, and the average follow-up time was 11.97±3.84 months. We treated patients with

209   individualized and specific treatments, including glucocorticoids plus immunosuppressants

210   (cyclophosphamide (CTX), cyclosporine A (CSA), tacrolimus). Our treatment plans were as

211   follows: patients on drug regimens were treated according to their blood pressure and blood

212   lipids. Acetylcholinesterase inhibitors (ACEIs) and angiotensin type 1 receptor blockers (ARBs)

213   were used to control blood pressure below 140/90 mmHg, and statins were used to control blood

214   lipids. Depending on the presence of edema, diuretics were sometimes used. Tacrolimus was

215   administered according to the following regimen: an initial oral dose of 0.5 mg/(kg·d), with

216   continued treatment for at least 6 months. The plasma concentration of tacrolimus was

217   determined for 1 week to maintain of value of 5~10 ng/ml. CSA was administered according to

218   the following regimen: an initial oral dose of 3-5 mg/(kg·d), with continued treatment for at least

219   6 months. The plasma concentration of CsA was monitored for 1 week to maintain a value of

220   100~200 ng/ml. CTX was administered according to the following regimen: administration of a

221   static dose of 750 mg/m2 body surface area for at least 6 months and a cumulative dose of 6-8 g.

222   All patients were given a sufficient dose of prednisone 1 mg/(kg·d). After 8 weeks of adequate

223   treatment, the dose size was reduced by 5 mg every 2 weeks and then held constant at a low dose

224   of 10 mg/d. The total course of treatment was at least 9 months. This study found that the overall

225   remission rate (including CR and PR) in the PLA2R- group was 93.02%, which was significantly

226   higher than that in the PLA2R+ group (74.78%), and the difference was statistically significant

227   (χ2=6.474, df=1, P= 0.01). The CR and PR rates in the negative group were 51.16% and 41.86%,

228   respectively, and the CR and PR rates in the positive group were 24.37% and 50.42%. In

229   addition, in the antibody-positive group, the low titer group had a higher remission rate than the

230   antibody high titer group(81.58%VS62.79%), and the difference was statistically significant

231 ($\chi 2$=5.142, df=1, P= 0.02). We further performed a subgroup analysis, which revealed that the

232 CR rate of patients in the PLA2R- group was significantly higher than that of patients in the

233 PLA2R+ group in the different treatment regimens (Table 3). Consistent with the above results,

234 the serum albumin levels of both groups were increased, and the quantified 24-hour urine protein

235 levels were decreased significantly after treatment. However, the levels of proteinuria (df=154,

236 P=0.01), serum creatinine and urea nitrogen (df=106, P=0.03) in the negative group were lower

237 than those in the positive group, and the albumin level (df=156, P=0.036) in the negative group

238 was higher than that in the positive group (Table 4). After treatment, the eGFR values of the

239 negative and positive groups were 107.77±24.431 and 105.20±28.09 [ml·min-1·(1.73 m2)-1],

240 respectively, but the difference was not statistically significant. Moreover, for patients with IMN

241 with follow-up data, the reduction rate of the anti-pla2r antibody after treatment was 92%. In

242 addition, we detected both the PLA2R antigen and IgG4 in the renal tissues of 43 patients with

243 negative antibodies by staining, revealing that 27 (62.79%) people were positive for the PLA2R

244 antigen, 29 (67.44%) people were positive for IgG4, and 25 (58.14%) people were positive for

245 both (Figure 1). By the end of the follow-up, a total of 5 patients had worsening renal conditions,

246 and all 5 patients were in the PLA2R+ group. Four of the patients were in the hormone plus

247 calcineurin inhibitor treatment group, and of these, 2 patients had excessively high blood drug

248 concentrations, 2 patients had some relief of proteinuria, and 2 patients had no improvement of

249 proteinuria. Another patient was assigned to the hormone plus CTX group, and the patient's

250 proteinuria did not turn negative. During the follow-up process, we found one case of a benign

251 lung tumor and three cases of severe complications, including 2 cases of severe pneumonia (1

252 death without renal failure) and 1 case of intracranial fungal infection, in the PLA2R+ group

253 (Table 5).

254   In this study, we used the Kaplan-Meier method to map the survival curve. The log-rank test

255 showed no significant difference in renal survival between the two groups ($\chi 2$=1.586, P=0.208)

256 (Figure 2). At the same time, we conducted risk factor analysis, revealing that BMG, 24-hour

257 urine protein and acute and chronic tubular lesions were risk factors for kidney death. The Cox

258   proportional hazard model analysis showed that 24-hour urine protein was an independent risk

259   factor for renal death (P=0.034) (Tables 6, 7).

260   **Discussion**

261   IMN is an autoimmune disease caused by endogenous antigens combined with specific antibodies

262   in the circulation to form in situ immune complexes that are deposited in renal tissues. The anti-

263   PLA2R antibody in circulation binds to PLA2R on the surface of podocytes to form an electron-

264   dense deposit under the foot process and epithelium, thereby activating the complement-forming

265   membrane attack complex, causing podocyte and kidney damage (Glassock 2012). Several studies

266   have demonstrated that the anti-PLA2R antibody is a key factor in the pathogenesis of IMN.

267   However, there are few studies on patients with IMN who are negative for the serum anti-PLA2R

268   antibody, and the pathogenesis and prognosis of patients with IMN who are negative for the anti-

269   PLA2R antibody are not very clear. In this study, we first studied the clinicopathological features

270   and prognosis of IMN patients who were seronegative for the anti-PLA2R antibody.

271   Herein, 74.23% of the 229 patients with IMN were positive for the antibody, which was

272   consistent with previous studies, as the rate generally ranges from 71.0-77.8% in IMN patients

273   (Kanigicherla et al. 2013). The 24-hour urine protein, BMG, and creatinine levels were

274   significantly lower in the negative group than in the positive group, and plasma albumin levels

275   were higher in the negative group than in the positive group. Other clinical studies also showed

276   that patients in the PLA2R- group had lower 24-hour urine protein and higher albumin levels than

277   those in the antibody-positive group, which is basically consistent with our findings (Hofstra et al.

278   2011; Oh et al. 2013; Radice et al. 2016). In this study, the antibody titer in the positive group was

279   positively correlated with the 24-hour urine protein level and negatively correlated with the serum

280   albumin level. J. M. Hofstra et al (Hofstra et al. 2011) also showed that the anti-PLA2R antibody

281   level was positively correlated with the urine protein level and negatively correlated with the

282   albumin level. However, their study also showed a positive correlation between antibodies and

283   serum creatinine, which was inconsistent with the results of our study. The reason for this

284 discrepancy may be related to the facts that the renal function of the patients in our study was

285 within the normal range and that the clinical symptoms were relatively mild. In terms of renal

286 pathology, the incidence of acute and chronic tubular injury was significantly lower in the negative

287 group than in the positive group, which was consistent with the results of Hara et al. (Hihara et al.

288 2016) and indicates that the pathological damage of patients in the antibody-negative group is less

289 severe than that of patients in the positive group.

290   In our study, we selected patients with an average daily urine protein level ≥ 6 g, and these

291 patients had at least a moderate or high risk of progressive disease. In addition, CR can lead to a

292 good long-term prognosis, and PR can independently reduce the risk of renal failure (Troyanov et

293 al. 2004). Therefore, early therapeutic intervention may be a more effective approach for treating

294 IMN patients with severe proteinuria. For newly diagnosed patients, on the basis of adjuvant

295 therapies such as antihypertensive, lipid-lowering and anticoagulative treatments, we preferred

296 hormone therapy with CTX or a semiquantitative hormone plus tacrolimus (FK506) and CSA.

297 Herein, the remission rate of the negative group was significantly higher than that of the positive

298 group, especially the CR rate. To the best of our knowledge, this study is the first to report that

299 patients in the negative group had a better clinical remission rate than those in the positive group.

300 Furthermore, in the positive group, patients with a low titer had low 24-hour proteinuria levels,

301 high albumin levels, mild pathological damage, and higher remission rates than those with a high

302 antibody titer, thus suggesting that the patients with low antibody titers were more likely to achieve

303 clinical remission. Lida M Rodas et al. (Rodas et al. 2019) found that spontaneous CR did not

304 occur in patients with PLA2R levels >40 IU/mL and was less frequently observed in patients with

305 a proteinuria level >8 g/day. Furthermore, patients with high antibody levels had a higher risk of

306 developing ESRD. The authors also found that patients with low anti-PLA2R antibody titers

307 (especially antibody titers <40 UI/mL) and proteinuria levels <4 g/day had high spontaneous

308 remission rates. These studies are consistent with our findings, indicating that the anti-PLA2R

309 antibodies titer is strongly correlated with the disease activity of IMN.

310   Survival analysis in this study showed that the survival rate of the PLA2R- group was higher

311   than that of the PLA2R+ group, but this difference was not statistically significant. Risk factor

312   analysis results showed that BMG, 24-hour urine protein, acute and chronic tubular disease were

313   risk factors, but only 24-hour urine protein was an independent risk factor for renal death. In a

314   predictive model of IMN progression risk, Cattran et al. (Cattran et al. 1997; Pei et al. 1992)

315   proposed that patients with abnormal or worsening serum creatinine or proteinuria, especially

316   those with a duration exceeding 6 months, had a 72% chance of developing end-stage renal failure.

317   Shiiki H and Ponticelli C et al. (Ponticelli et al. 1989; Shiiki et al. 2004) also showed that high

318   serum creatinine concentrations, severe proteinuria, and chronic tubulointerstitial lesions were

319   important predictors of the risk of IMN progressing to ESRD. Various clinical indicators, such as

320   severe proteinuria, decreased renal function at diagnosis, and development of renal tubular

321   interstitial lesions, can be considered risk factors for the progression of IMN to renal failure.

322   Herein, 43 of 162 IMN patients were negative for the serum anti-PLA2R antibody, potentially due

323   to the following reasons: 1. The serum anti-PLA2R antibody was negative, but the PLA2R antigen

324   in kidney tissue was positive; 2. other pathogenic antigens and specific autoantibodies may have

325   existed in the patients with IMN; 3. other secondary factors that have not yet been discovered

326   played roles; 4. the anti-PLA2R antibody in the sera of patients with IMN was indeed negative; 5.

327   the patients' conditions were in a stable state, and there was no immune activity in the body; and

328   6. the limitations of the detection technology itself. Therefore, we stained for the PLA2R antigen

329   in the kidney tissues of 43 patients who were negative for the serum anti-PLA2R antibody, and

330   the granular staining rate for the PLA2R antigen in kidney tissue was 62.79%. Consequently, for

331   patients with IMN who were negative for serum anti-PLA2R antibodies, we could routinely

332   perform renal tissue PLA2R antigen staining. This result is also consistent with the findings of Hill

333   et al. (Hill et al. 2016), who found that the PLA2R antigen was potentially positive in renal tissue

334   when serum antibodies were absent in IMN patients, and when the serum anti-PLA2R antibody

335   was detected in combination with the PLA2R antigen in renal tissue, the sensitivity of disease

336   diagnosis could be increased to 95.2%. Detection of the PLA2R antigen in renal tissue is more

337   sensitive and specific for the diagnosis of IMN and can be used as a supplementary detection

338   method for patients who have undergone renal biopsy and are negative for the serum anti-PLA2R

339   antibody (Hofstra & Wetzels 2014; Svobodova et al. 2013).

340   For patients with IMN who were negative for the anti-PLA2R antibody, we also considered the

341   presence of other antibodies with structural functions similar to those of the anti-PLA2R antibody.

342   Several clinical reports on other pathogenic antigens of IMN, including thrombospondin type-1

343   domain-containing 7A (THSD7A) (Tomas et al. 2014), superoxide dismutase (SOD2), α-enolase

344   (a-ENO), aldose reductase (AR) (Bruschi et al. 2011; Prunotto et al. 2010), neutral endopeptidase

345   (NEP), bovine serum albumin (BSA) and BSA antibodies (Murtas et al. 2012), have been

346   published. In 2014, Tomas et al. found THSD7A in the sera of patients with IMN who were

347   negative for the PLA2R antibody. This study suggested that antibodies specific for this antigen

348   were detectable only in the sera of anti-PLA2R antibody-negative IMN patients and were almost

349   undetectable in patients with SMN and other glomerular diseases. The anti-THSD7A antibody

350   may become a highly specific new indicator of disease diagnosis and activity monitoring in IMN

351   patients who are negative for the serum anti-PLA2R antibody (Tomas et al. 2014). In addition,

352   some studies showed that the serum anti-PLA2R antibody, anti-THSD7A antibody, anti-SOD2

353   antibody, and anti-ENO antibody were all negative in IMN patients with a low proportion of

354   nephrotic syndrome at onset, high plasma albumin level, and high disease remission rate (Murtas

355   et al. 2012). Therefore, the anti-THSD7A antibody, anti-SOD2 antibody, and anti-α ENO antibody

356   in the sera of patients with IMN who are negative for the anti-PLA2R antibody can potentially

357   indicate that the patient's clinical severity is less severe, and clinical remission can be achieved

358   more quickly. We also need to conduct a larger number of clinical data studies in the future to

359   confirm these results. In this study, we did not find new tumors in the antibody-negative group,

360   and a benign lung tumor occurred in one patient in the antibody-positive group. This finding was

361   not exactly the same as the relevant research results. Previous clinical studies showed that patients

362   with IMN were negative for the anti-PLA2R antibody, especially when they had antibodies against

363   THSD7A. Furthermore, 21% of patients with IMN associated with THSD7A were found to have

364   tumors within 3 months of being diagnosed with membranous nephropathy (Hoxha et al. 2016).

365   Qin W et al. detected serum anti-PLA2R autoantibodies in 10 patients with tumor-associated

366   membranous nephropathy. Seven of the 10 patients were negative for the anti-PLA2R antibody,

367   while only 3 patients were positive, and most patients with tumor-associated membranous

368   nephropathy were considered to be negative for the antibody in the study (Qin et al. 2011). In the

369   antibody-negative group in our study, there were no tumors (low tumor incidence rate), the clinical

370   remission rate was high, and the prognosis was good. The reasons for our results may be related

371   to the short follow-up time, loss of follow-up, and other pathogenic factors. We also need to further

372   follow-up with the negative group to observe the relationship between tumors and the anti-PLA2R

373   antibody.

374   Immunosuppressive therapy has been well proven in patients with IMN. However, because this

375   treatment regimen may cause potential side effects, it must be reserved for patients who truly

376   benefit from this treatment. This retrospective study also found that patients with IMN who were

377   negative for the anti-PLA2R antibody had fewer clinical manifestations and renal pathological

378   changes, and numerous side effects of early immunosuppressive therapy were observed in these

379   patients. Therefore, whether patients with IMN who are negative for the anti-PLA2R antibody and

380   without tumors and other secondary factors can actively use immunosuppressive therapy at an

381   early stage to achieve disease remission will be a new direction of our future research. In addition,

382   for patients with IMN who are negative for the anti-PLA2R antibody, if other specific antibodies

383   are not detected in their serum and the renal tissue PLA2R antigen staining result is also negative,

384   we should consider whether other secondary findings exist that have not yet been discovered, such

385   as tumors, heavy metals, and metabolic factors.

386   **Conclusions**

387   This study focused on the clinical significance of negativity for the serum anti-PLA2R antibody

388   in IMN. The patients who were negative for the serum anti-PLA2R antibody had mild clinical

389   manifestations, mild pathological damage, and relatively high clinical remission rates. Renal tissue

390   PLA2R antigen testing can be considered for patients with seronegative IMN to increase the

391   diagnostic rate of IMN. In addition, studies have shown that 24-hour urine protein is an

392   independent risk factor for kidney death. This retrospective study has some limitations, as the study

393   sample size was limited, and the follow-up time was relatively short. The long-term prognosis and

394   factors related to survival analysis need to be further studied.

395

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399

400   **Ethics approval and consent to participate**

401   The collection of data from related cases was approved by the Ethics

402   Committee of Shandong Provincial Hospital Affiliated to Shandong

403   University (LCYJ: no. 2019-105).

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Comparison of clinical characteristics and basic laboratory tests between the two groups

Note: SBP, systolic blood pressure; DBP, diastolic blood pressure; 24HUPRO, 24-hour urine; WBC, white blood cells; PLT, platelets; HGB, hemoglobin; AST, alanine aminotransferase; AST, aspartate aminotransferase; SOD, superoxide dismutase; TP, serum total protein; ALB, albumin; GLO, globulin; GLU, blood glucose; BUN, blood urea nitrogen; CREA, serum creatinine; Cys-C, cystatin C; BMG, β2 macroglobulin; RBP, retinol binding protein; CHOL, total cholesterol; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein; FSGS, focal glomerular sclerosis; IgG4, immunoglobulin G4; IgG, immunoglobulin G; IgM, immunoglobulin M; IgA, immunoglobulin A; C3 C4, complement 3 complement 4;PLA2R, phospholipase A2 receptors. Compared with the PLa2R- group and the PLA2R+ group, P *<0.05, P**<0.01.

1   Table 1.

	IMN(N=229)	APLA2R-ab(-)(N=59)	APLA2R-ab +(N=170)	P value
Age (year)	45.88±13.64	45.75±13.49	45.92±13.73	0.931
Sex (female (%))	91/229 (39.74%)	30/59 (50.84%)	61/170 (35.88%)	0.043*
Duration of illness (month)	117.72±175.40	129.57±210.58	112.44±160.84	0.542
SBP (mmHg)	137.70±20.48	135.63±23.30	140.90±21.03	0.190
DBP (mmHg)	87.10±13.89	88.93±16.66	88.08±13.93	0.626
Urine RBC/HPF	14.07±17.76	11.24±14.87	15.04±18.59	0.160
24HUPRO (g/L)	6.12±8.17	4.91±3.18	5.81±3.50	0.086
WBC (×10^9/L)	8.28±17.76	11.95±34.47	7.00±2.81	0.275
HGB (g/L)	134.28±30.47	132.18±38.80	135.01±27.05	0.605
PLT (×10^9/L)	268.09±71.73	270.17±95.40	267.36±61.64	0.833
AST (u/L)	22.67±8.20	23.26±8.61	22.46±8.07	0.525
ALT (u/L)	22.83±11.96	25.37±12.18	21.96±11.79	0.063
SOD (u/mL)	111.83±27.18	115.83±28.70	110.41±26.59	0.245
TP (g/L)	47.53±7.60	49.17±8.28	46.97±7.30	0.056
ALB (g/L)	24.69±5.42	26.11±6.02	24.20±5.11	0.019*
GLO (g/L)	22.92±4.61	23.62±4.67	22.68±4.58	0.183
GLU (mmol/L)	5.36±1.30	5.59±1.99	5.28±0.93	0.248
BUN (mmol/L)	5.36±1.92	5.44±1.93	5.33±1.93	0.702
CREA (μmol/L)	64.78±15.78	61.57±15.88	65.90±15.64	0.033*
Cys-C(mg/L)	0.99±0.48	1.06±0.81	0.97±0.28	0.427
BMG (mg/L)	2.32±0.80	2.13±0.74	2.39±0.81	0.045*
RBP (mg/L)	55.97±18.24	54.36±16.44	56.51±18.83	0.451
Ca (mmol/L)	2.17±0.21	2.17±0.21	2.17±0.22	0.972
CHOL (mmol/L)	8.84±2.68	8.62±2.68	8.92±2.69	0.485
HDL-C (mmol/L)	1.66±0.51	1.71±0.60	1.65±0.48	0.454
LDL-C (mmol/L)	5.45±2.25	5.14±1.95	5.56±2.34	0.243
FSGS	41/229 (17.9%)	9/59(15.25%)	32/170 (18.82%)	0.538
IgG4 (mg/L)	352.49±317.78	305.83±268.24	368.56±332.43	0.220
IgG (g/L)	5.74±2.51	5.71±2.27	5.74±2.59	0.942
IgM (g/L)	1.13±0.51	1.17±0.53	1.11±0.50	0.449
IgA (g/L)	2.34±0.91	2.24±0.86	2.37±0. 86	0.360
C3 (g/L)	1.19±0.23	1.19±0.18	1.18±0.24	0.937
C4 (g/L)	0.30±0.08	0.28±0.07	0.30±0.18	0.112
C1q (mg/L)	216.70±38.59	213.19±34.61	217.99±40.01	0.463

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Comparison of pathological characteristics between the two groups

Note: Compared with the PLa2R- group and the PLA2R+ group, P*<0.05, P**<0.01.

1   Table 2.

	APLA2R-ab (-)(N=59)	APLA2R-ab (+)(N=170)	P value
Pathological stage (%)			
I	45/59 (76.27%)	112/170 (65.88%)	0.454
II	14/59 (23.72%)	55/170 (32.35%)	0.321
III + IV	0	2/170 (1.18%)	0.293
Tissue IgG	2.46±0.83	2.37±0. 89	0.486
Tissue IgM	0.36±0.79	0.38±0.69	0.810
Tissue IgA	0.12±0.50	0.23±0.61	0.182
Tissue C3	1.09±0.87	1.19±0.94	0.459
Tissue Fib	0.08±0.39	0.15±0.52	0.264
Tissue C1q	0.53±0.80	0.50±0.71	0.796
Pathological characteristics			
Global sclerosis	35/59 (59.32%)	88/170 (51.76%)	0.337
Glomerular mesangial hyperplasia	16/59 (27.12%)	64/170 (37.65%)	0.124
Crescents	2/59(3.38%)	0	0.159
Hyperplasia endothelialitis	4/59 (6.78%)	7/170 (4.12%)	0.418
Acute renal tubular lesions	2/59(3.39%)	20/170 (11.76%)	0.015*
Chronic renal tubular lesions	2/59 (3.39%)	21/170 (13.53%)	0.010**
Inflammatory cell infiltration	3/59 (5.08%)	23/170 (13.53%)	0.031*
Vascular disease	21/59 (35.59%)	57/170 (33.53%)	0.796

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Comparison of remission rates after 1 year of follow-up and treatment in twogroups ofpatients

Note: CR, complete remission; PR, partial remission; CsA, cyclosporin; Pred, prednisone; TAC, tacrolimus; CTX, cyclophosphamide; MN, membranous nephropathy; Compared with the PLa2R- group and the PLA2R+ group, P*<0.05, P**<0.01.

1   Table3

Total	Complete remission rate (CR)	Partial remission rate (PR)	CR+PR
MN (N=162)	51/162(31.48%)	78/1629 (48.15%)	129/162 (79.63%)
PLA2R- (N=43)	22/43 (51.16%)	18/43 (41.86%)	40/43 (93.02%)*
PLA2R+ (N=119)	29/119 (24.37%)	60/119 (50.42%)	89/119 (74.78%)
PLA2R+			
low-titer(20-180)	22/76(28.95%)	40/76(52.63%)	62/76(81.58%)*
high-titer(>180)	7/43(16.28%)	20/43(46.51%)	27/43(62.79%)
Pred+Ctx			
PLA2R- (N=15)	9/15 (60%)	4/15 (26.67%)	13/15 (86.67%)**
PLA2R+ (N=55)	16/55 (29.09%)	24/55 (45.45%)	41/55 (74.54%)
Ped+Csa/ F506			
PLA2R- (N=25)	11/25 (44%)	13/25 (54.16%)	24/25 (96%)
PLA2R+ (N=61)	12/61 (19.67%)	34/61 (55.73)	46/61 (75.41%)
Ped+Csa			
PLA2R- (N=9)	6/9 (66.67%)	3/9 (33.33%)	9/9 (100%)*
PLA2R+ (N=31)	6/31 (19.35%)	16/31 (51.61%)	22/31 (70.97%)
Ped+F506			
PLA2R- (N=16)	5/16 (31.25%)	10/16 (62.5%)	15/16 (93.75)
PLA2R+ (N=30)	6/30 (20%)	18/30 (60%)	24/30 (80%)

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Comparison of data between the two groups of patients after 1 year of follow-up treatment

Note: eGFR: estimated glomerular filtration rateCompared with the PLa2R- group and the PLA2R+ group, P*<0.05, P**<0.01.

1   Table 4.

Total	24HUPRO (g/L)	ALB (g/L)	CREA (μmol/L)	BUN (mmol/L)	eGFR
PLA2R-(N=43)	0.78±1.10	38.21±6.14	64.18±14.37	5.37±1.50	107.77±24.43
PLA2R+(N=119)	2.22±2.10**	35.09±6.97*	71.47±23.91	6.68±2.72*	105.20±28.09
Pred+CTx					
PLA2R-(N=15)	0.79±1.24	37.37±6.80	59.54±12.89	4.83±1.23	118.65±27.28
PLA2R+(N=55)	2.24±2.02	34.28±6.46	63.01±23.25	5.75±1.68	114.90±26.31
Ped+Csa					
PLA2R-(N=9)	0.21±0.25	40.01±4.49	72.36±14.42	6.25±2.20	92.84±17.14
PLA2R+(N=31)	2.54±2.45**	35.02±7.41	80.85±23.66	7.41±2.62	93.74±23.15
Ped+F506					
PLA2R-(N=16)	1.18±0.61	38.18±5.94	67.77±15.70	5.59±1.55	115.02±28.47
PLA2R+(N=30)	1.80±1.77	36.66±7.08	73.19±22.95	7.08±3.75	114.75±33.04

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Comparison of prognosis between the two groups

1   Table 5.

Prognosis	PLA2R-(N=43)	PLA2R+(N=119)
Kidney death	0	5
Clinical death	0	1
Severe infection	0	3
Tumor	0	1
Complete remission	31	14
Partial remission	21	60

Factors related to kidney death in patients with IMN (Cox single factor analysis)

Note: CRTL, chronic renal tubular lesions; ARTL, acute renal tubular lesions; β2-MG, β2-microglobulin; 24HUPRO, 24-hour urine.

Table 6.

	B	SE	Ward	Degrees of freedom	Saliency	Exp (B)	95%Exp(B)'Cl Lower limit	Upper limit
24HUPRO	.236	.096	6.100	1	. 014	1.266	1.050	1.527
β2-MG	.651	.249	6.822	1	.009	1.917	1.176	3.123
CRTL	-1.880	.817	5.298	1	.021	.153	1.322	32.516
ARTL	-2.169	.818	7.038	1	.008	1.114	1.762	43.468

Multivariate Cox regression analysis of renal death risk in patients with IMN

Note: CRTL, chronic renal tubular lesions; ARTL, acute renal tubular lesions; β2-MG, β2-microglobulin; 24HUPRO, 24-hour urine.

1   Table 7

	B	SE	Ward	Degrees of freedom	Saliency	Exp (B)	95%Exp(B)'Cl Lower limit	Upper limit
24HUPRO	.291	.138	4.483	1	. 034	1.338	1.022	1.752
β2-MG	.971	.532	3.334	1	.068	2.641	.931	7.491
CRTL	-1.937	1.607	1.453	1	.228	.144	.006	3.360
ARTL	-.668	1.062	.395	1	.530	1.949	.243	15.636

3

Figure 1

Detection of PLA2R antigen and IgG4 in renal tissue.

Note: The expression of PLA2R and IgG4 in glomeruli was observed by immunofluorescence microscopy in patients with negative anti-PLA2R antibodies. Simultaneous staining of PLA2R and IgG4 is positive were shown in A and B; Simultaneous staining of PLA2R and IgG4 is negative were shown in C and D; E and F show that PLA2R staining is negative and igG4 staining is positive.

PIA2R   IgG4
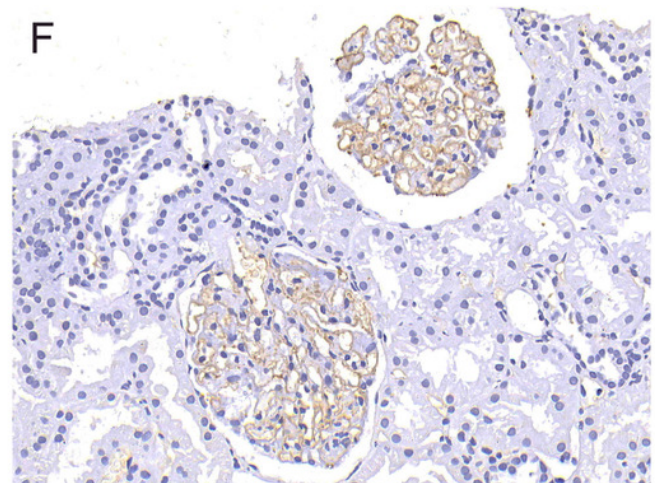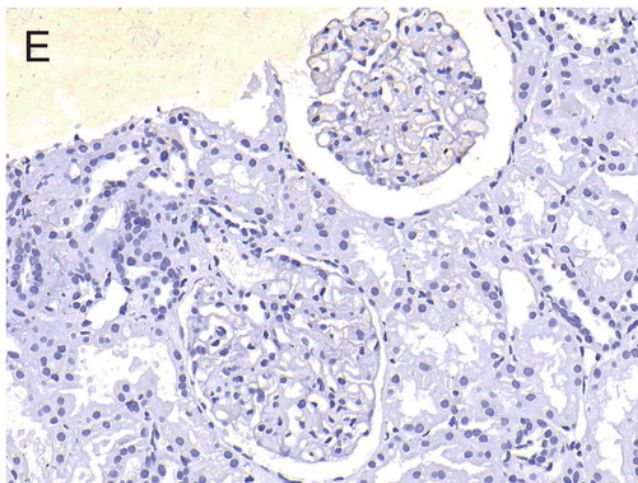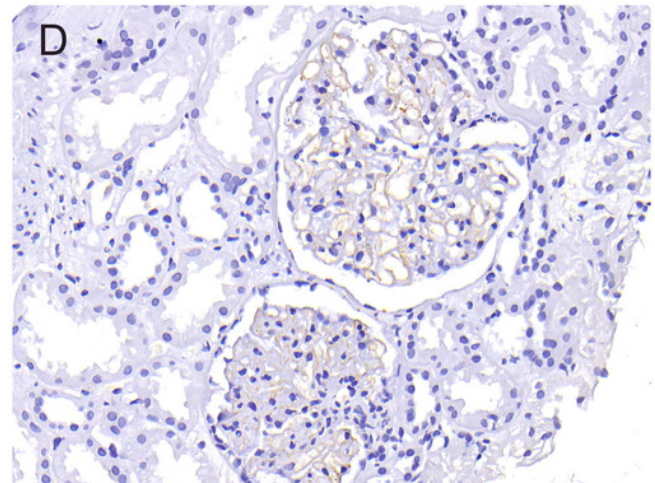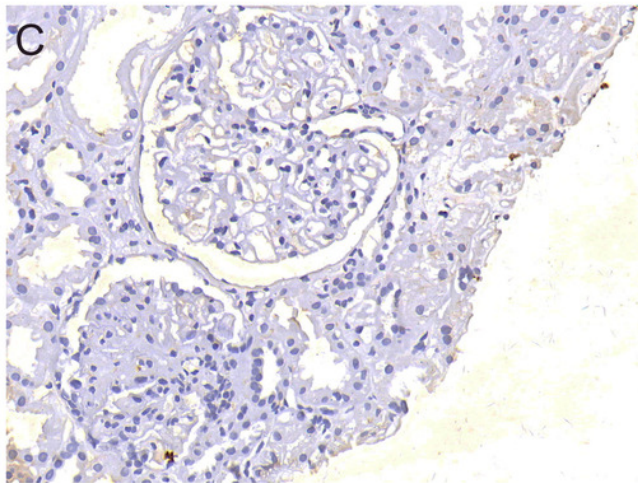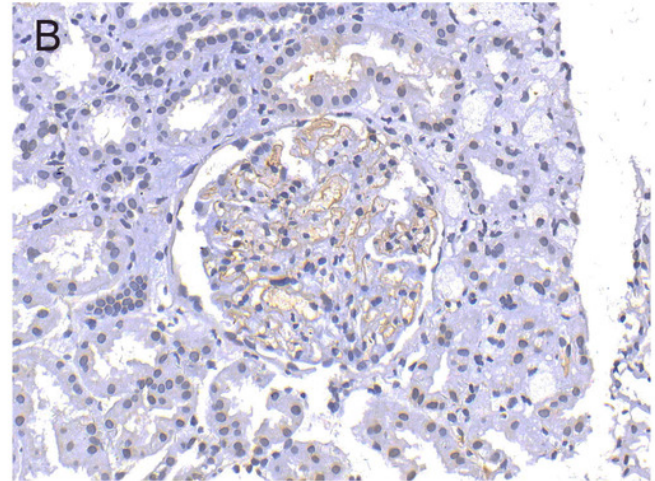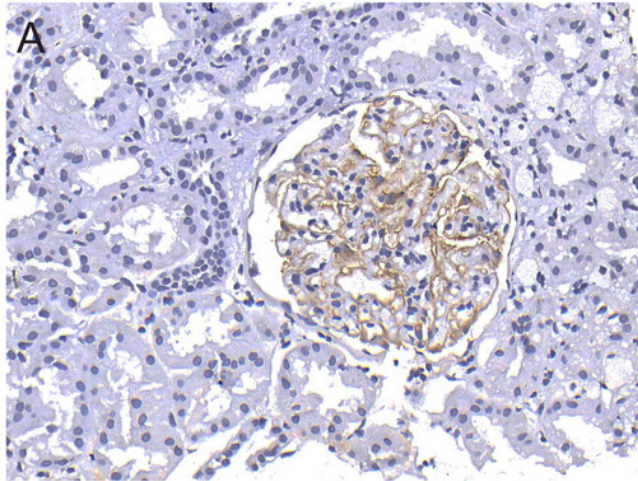
Figure 2

The log-rank test showed no significant difference in renal survival between the two groups.

The log-rank test showed no significant difference in renal survival between the two groups.