Circulating antipodocyte antibodies in membranous nephropathy: pathophysiologic and clinical relevance.

Yukihiro Kimura, Hiroyuki Morita, Hanna Debiec, Harutaka Yamada, Naoto Miura, Shogo Banno, Pierre Ronco, Hirokazu Imai

Circulating antibodies to α-enolase and phospholipase A_2 receptor and composition of glomerular deposits in Japanese patients with primary or secondary membranous nephropathy

Yukihiro Kimura[1], Hiroyuki Morita[1], Hanna Debiec[2], Harutaka Yamada[3], Naoto Miura[1], Shogo Banno[1], Pierre Ronco[2], Hirokazu Imai[1]

[1]Division of Nephrology and Rheumatology, Department of Internal Medicine, Aichi Medical University School of Medicine, Nagakute, Japan;

[2]Université Pierre et Marie Curie, Paris 6, Assistance Publique–Hôpitaux de Paris (AP-HP), Tenon Hospital, Paris, France ;

[3]Department of Internal Medicine, Kawana Hospital, Nagoya, Japan

Correspondence and Offprint request to: Hirokazu Imai, E-mail: imaihiro@aichi-med-u.ac.jp

Division of Nephrology and Rheumatology,

Department of Internal Medicine,

Aichi Medical University School of Medicine,

Nagakute, Aichi, 480-1195, Japan

tel: +81-561-62-3311

Fax: +81-561-62-6694

Short title: autoantibodies in MN

Word count: Abstract, 298 words;

Manuscript body, 2450 words

Blue: questions for Hiro

Green: questions for Hanna

Abstract

Background. Phospholipase A_2 receptor (PLA_2R) is recognized as a target antigen in primary membranous nephropathy (MN); however, little is known about the potential contribution of α-enolase to the pathogenesis of MN.

Methods. We evaluated circulating antibodies to α-enolase and PLA_2R, and glomerular deposition of these proteins in 25 patients with primary MN, 20 patients with secondary MN, 44 patients with collagen disease or severe infection as disease control, and 20 healthy subjects, using a dot blotting system. We used deletion mutants of α-enolase to analyze epitopes recognized by antibodies.

Results. In primary MN, 18 of 25 sera (72%) showed anti-α-enolase antibody (IgG1 and IgG4, 11 pts; IgG4 alone, six pts; IgG1 alone, one pt). In secondary MN, 15 of 20 sera (75%) contained anti-α-enolase antibody (IgG1 and IgG3, 13 pts; IgG3 alone, two pts). Epitopes localized in amino-acid stretches 1–66 and 349–433, respectively in the N- and C-terminal regions of α-enolase were identified. No circulating anti-α-enolase antibody was found in 44 collagen diseases or septic patients without MN, and 20 healthy subjects. Twelve of 25 sera (48%) from patients with primary MN were positive

for anti-PLA_2R antibody, whereas all patients with secondary MN (19 pts) were negative~~. Four of the 16~~ *(Hanna give new numbers)* PLA_2R-positive patients with primary MN also had anti α-enolase antibody. Although PLA_2R antigen was present in a subepithelial pattern in 10 of 19 (52%) patients with primary MN, α-enolase was never detected in glomerular deposits in X and Y patients with primary and secondary MN, respectively.

Conclusions. Circulating anti-α-enolase antibodies are present in both primary and secondary MN, while anti-PLA_2R antibodies are specific for primary MN with a prevalence apparently lower in the Japanese population than in Chinese and Caucasian populations. The absence of α-enolase from subepithelial immune deposits suggests that anti-α-enolase antibodies do not contribute directly to deposit formation, although they may have other pathogenic effects. ~~However, anti-α-enolase antibodies can be involved in endothelial dysfunction when binding to glomerular endothelium as wel as in podocyte dysfunction after binding to cytoplasm enolase in altered podocytes after the initial immune complex injury.~~

Key words: α-enolase; phospholipase A2 receptor; membranous nephropathy

Introduction

Membranous nephropathy (MN)—a major glomerular disease and common cause of adult nephrotic syndrome—is characterized by glomerular subepithelial IgG deposits [1]. Primary MN is predominantly associated with glomerular deposition of the IgG4 subtype [2-6], whereas secondary MN is characterized by prevailing deposits of IgG1, IgG2, and IgG3 [6-8]. During the past decade, several breakthroughs have occurred with the identification of several candidate human antigens in MN. Wakui *et al.* identified anti-α-enolase antibodies in patients with primary or secondary MN [9]. In infants with MN, Debiec *et al.* detected anti-neutral endopeptidase antibodies that were produced by mothers who lacked this enzyme [10, 11]. Beck *et al.* demonstrated that IgG4 antibodies specific for M-type phospholipase A_2 receptor (PLA_2R) were present in

glomerular eluates and serum from adult patients with primary MN [12]. Other candidate autoantigens in patients with primary MN include superoxide dismutase 2 (SOD2) and aldose reductase [13]. Debiec *et al*. reported that some patients who develop MN in early childhood had circulating anti-bovine serum albumin (BSA) antibodies and cationic BSA as a component of glomerular immune deposits [14].

Interestingly, unlike other antibodies identified in patients with autoimmune MN, anti-α-enolase antibodies have been found in both primary and secondary MN. In the present study, we attempted to identify the epitope (s) recognized by anti-enolase autoantibodies, and we examined the potential relationship between anti-α-enolase antibodies and anti-PLA_2R antibodies, and the glomerular deposition of α-enolase and PLA_2R proteins in Japanese patients with primary and secondary MN.

Materials and methods

This study was approved by the Ethics Committee at Aichi Medical University.

Patients Characteristics

We included Japanese patients with MN who were admitted to Aichi Medical University Hospital and affiliated hospitals between 2003 and 2011. Diagnosis of MN

was based on light microscopy, immunofluorescence, and electron microscopy analyses of kidney biopsy specimens [15]. Primary MN was defined according to morphologic criteria after excluding known underlying diseases and drug exposure. Sera from 25 patients with primary MN, 20 patients with secondary MN, 44 patients with collagen diseases or severe infection, and 20 healthy subjects were stored at –80°C until use. Sera from all patients with MN were obtained before steroid and immunosuppressive treatment with the approval of the institutional ethics committee. Patients characteristics are summarized in Table 1. Complete remission was defined as urinary protein excretion of less than 300 mg/day as a result of therapeutic intervention (spontaneous remission?). Partial remission was defined as…….

Preparation of α-Enolase Deletion Mutants

We obtained sequence encoding full-length human α-enolase (433 amino acids; GenBank AK315417, Ensembl:ENSG00000074800). As a result of post-translational modification, human α-enolase is a 47-kDa glycoprotein. Complementary DNA (cDNA) cloning and production of fusion proteins were described elsewhere [16]. Briefly, full-length and truncated cDNA encoding human α-enolase (Fig. 2) was amplified in polymerase chain reactions, and ligated to sequence encoding glutamine S-transferase

(GST) (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). DNA was cloned into pGEX plasmids and transformed into JM109 cells (Promega, USA), and protein expression was induced using isopropyl-β-D-thiogalactopyranoside. After protein extraction from JM109 cells, tagged proteins were affinity purified using glutathione-Sepharose 4B (Amersham Pharmacia Biotech).

Western and Dot Blot Analysis

Please describe the WB and specify whether it was performed in reducing or non-reducing conditions.

Nitrocellulose membranes (GE Healthcare, UK) were washed in PBS (Wako Pure Chemical Industries, Japan) containing 10 μg/ml recombinant α-enolase protein and 5% (w/v) skim milk (BD Difco, USA) for 60 minutes at room temperature. The membrane was washed three times with PBS containing 0.05% Tween (Katayama Chemical Industries, Japan), and incubated with patient serum at a dilution of 1:200. After three washes in PBS containing 0.05% Tween, the membrane was incubated with horseradish peroxidase–conjugated anti-human IgG antibodies at a dilution of 1:1000 (Sigma-Aldrich, USA). After three washes, reaction product on the membrane was visualized using an enhanced chemiluminescence system (ChemiLumit Kit, GE Health

Care, UK). Photographic images were obtained using an LAS-1000 system (FujiFilm Co., Japan). Image Reader Lite for LAS-1000 plus Ver. 1.3 (FujiFilm Co.) was used to capture the images, which were edited using Adobe Photoshop, when necessary.

Anti-PLA_2R Antibodies

Anti-PLA_2R–specific autoantibody titers were measured as previously described based on indirect immunofluorescence in HEK 293 cells that were transiently transfected with full-length cDNA encoding PLA_2R (Euroimmun) [17]. Antibody positivity was defined as positive staining at serum dilutions of at least 1/10. Negative results for anti- PLA_2R antibodies were defined as an absence of detectable signals at an antibody dilution of 1/10. Antibody specificity was checked on Western blots under nonreducing conditions using glycoproteins extracted from normal human glomeruli after removing endogenous IgG. [18].

Glomerular Deposition of α-Enolase and PLA_2R Proteins

α-enolase and PLA_2R were detected in paraffin-embedded native biopsies under a confocal microscope using affinity-purified specific anti-rabbit α-enolase (is the source Atlas?) and PLA_2R antibodies (Atlas Antibodies, Stockholm, Sweden) followed by goat

Alexa 488–conjugated anti-rabbit IgG Fab fragments (Molecular Probes, Eugene, OR, USA) as previously described [17]. Staining with only secondary antibodies produced negative results for all biopsies.

Statistical Analysis

Data were analyzed using Microsoft Excel software. Normally distributed variables were described as mean standard deviation and compared across primary and secondary MN using analysis of variance. Age, amount of urinary protein, and serum creatinine were compared using Student's t-test. Mann–Whitney U-tests (nonparametric) were used to compare results for anti-α-enolase antibody positivity in patients with MN and control subjects. All *P*-values are two tailed, with <0.05 considered statistically significant.

Results

Patients Characteristics

The study comprised 25 patients with primary MN, and 20 patients with secondary MN (seven patients had bucillamine-induced nephropathy and 13 patients had lupus nephritis World Health Organization type V). Patients characteristics are summarized in

Table 1A and 1B. The median age of the patients with primary and secondary MN was 61.0 ± 14.4 years and 48.7 ± 16.3 years, respectively ($p<0.01$). ~~The average~~ (Median or Mean?) serum creatinine level was 0.77 ± 0.25 mg/dl and 0.67 ± 0.18 mg/dl ($p=0.07$), and ~~average~~ (median or mean?) proteinuria was 3.16 ± 2.39 g/day and 3.47 ± 3.13 mg/day ($p=0.6$), at the time of diagnosis in primary and secondary MN. Treatment modalities were not known in seven patients. One patient received dipyridamole alone, 37 ??? (29 in the tables??) patients were treated with prednisolone with or without other immunosuppressants including Mizoribine (a new immunosuppressive agent used in Japan), six patients were treated with angiotensin II receptor blocker (ARB) alone, and two patients received no therapy.

Detection of Anti-α-Enolase Antibody

We first examined whether sera from patients with MN contained antibodies specific for recombinant α-enolase. ~~Recombinant α-enolase fragments were prepared as described in the Methods. Schematic representations of full-length α-enolase cDNA and 10 different deletion mutants used in the present study are shown in Figure 1. Western blot analysis of a representative patient serum produced major bands in each lane, which corresponded with the predicted migration positions (Figure 2 upper left). When serum~~

~~from a normal control was used instead, no bands appeared (Figure 2 upper right). Dot blotting data were similar to those from Western blotting (Figure 2 lower left); the patient's serum but not the normal control serum produced a signal for human α-enolase. These data demonstrated that patients with MN have circulating antibodies specific for α-enolase~~. Sera from 18 of 25 patients with primary MN (72%) immunoreacted with α-enolase. Nine of 13 patients with lupus nephritis (World Health Organization class V), (69%) and six of seven patients with bucillamine-induced MN (86%) were positive for anti-α-enolase antibodies (Figure ~~3~~ should be Fig 1). In contrast, sera from 44 disease control and 20 healthy control subjects did not react with α-enolase.

Analysis of α-Enolase Epitopes in Patients with MN

We next examined the patients with circulating antibodies specific for human α-enolase. Recombinant α-enolase fragments were prepared as described in the Methods Schematic representations of full-length α-enolase cDNA and 10 different deletion mutants used in the present study are shown in Figure ~~1~~ becomes Fig 2. Western blot analysis (Please specify: in non-reducing or reducing conditions?) of a representative patient serum produced major bands in each lane, which corresponded with the predicted migration positions (Figure ~~2~~ becomes Fig 3,upper left). When serum from a

normal control was used instead, no bands appeared (Figure 3 upper right). Dot blotting data were similar to those from Western blotting (Figure 3 lower left); the patient serum but not the normal control serum produced a signal for human α-enolase. Serum from each patient reacted with both full-length and truncated α-enolase fragments in which either an N-terminal (remaining amino-acid positions: 29–433, 109–433, 189–433, 269–433, or 349–433) or C-terminal (remaining amino-acid positions: 1–66, 1–146, 1–256, 1–306, or 1–386) region was deleted (Figure 2). Data for 1142F fragment (AA 349-433) and 295R fragment (AA 1-66) are shown in Figure 4. Of note, each patient serum that produced positive results for full-length α-enolase also immunoreacted with truncated segments containing only amino-acid residues 349 to 433 (lane A) or 1 to 66 (lane B). Taken together, these data demonstrate that 37 of 45 (73%) patients with either primary or secondary MN have circulating anti-α-enolase antibodies, which likely bind an epitope located on both N- and C-terminal regions.

IgG Subclasses of Circulating Anti-α-Enolase Antibodies

We then characterized IgG subclasses of anti-α-enolase antibodies in both primary and secondary MN (Figure 5). In primary MN, most antibodies appeared to be IgG1 or IgG4; 72% of patients being positive for at least one of these subclasses on dot blots. In

secondary MN, however, IgG1 and IgG3 produced the strongest signals, with positive results observed in 75% of patients, contrasting very little or absent IgG4 reactivity. Again, serum from each patient that immunoreacted with full-length α-enolase also was positive with the N- and C-terminal fragments (amino-acid stretch 1–66 and 349–433, respectively). These observations are in line with other data showing that subepithelial immune deposits in primary MN primarily contain IgG4 antibodies, whereas IgG1 and IgG3 subclasses are most common in deposits associated with secondary MN [15].

Circulating Anti-α-Enolase Antibodies Before and After Treatment

We examined the effects of treatment on circulating antibodies specific for α-enolase in a patient with primary MN, a patient with lupus MN, and three patients with bucillamine-induced MN, who all attained complete remission after treatment. We also assessed three patients with primary MN and one patient with lupus MN who failed to achieve complete remission despite therapy. Notably, circulating antibodies were no longer detected in any of the patients who reached complete remission (Figure 6). Among the 4 patients who failed to attain complete remission, antibody titers were markedly decreased in the patient with lupus MN, and in two of three patients with primary MN, and unchanged in the remaining patient (Figure 6). IgG subclass analyses

confirmed that the affected antibody titers were associated with IgG1 and/or IgG4 in primary MN, and IgG1 and IgG3 in secondary MN (Figure 6).

Anti-PLA_2R Antibody Hanna please revise the numbers

Twelve of 25 (48%) tested sera from patients with primary MN had anti-PLA_2R antibodies Could be written like this: "Of the 12 PLA_2R-positive patients, X were positive for anti-α-enolase, Y were negative. Of the 13 PLA_2R- negative patients,……" *Four of 16 (25%) patients also had anti-α-enolase antibody. Three patients with anti-PLA2R antibodies had no anti-α-enolase antibodies (Table 1A). Six patients with anti-α-enolase antibodies were negative for anti-PLA_2R antibodies, and three patients showed neither antibody.* None of the 19 examined patients with secondary MN were positive for anti-PLA_2R antibodies.

Glomerular Deposition of α-Enolase and PLA_2R Proteins Hanna please revise the numbers

In paraffin-embedded kidney biopsy specimens, confocal microscopy showed the presence of PLA_2R in subepithelial deposits along glomerular capillary loops in 10 of 19 patients with primary MN. Eight of the 10 patients also had circulating anti- PLA_2R

antibodies, but two had not (Figure 7). PLA_2R was absent in the Z examined biopsy specimens from patients with lupus- and bucillamine-related MN (Figure 8).

α-enolase was strongly expressed in tubular epithelium and weakly positive in glomerular parietal cells and?? (Hanna: there are also spots of fluorescence in podocytes? and may be endothelial cells?) (Figure 8 left side), but in contrast to PLA_2R, α-enolase was never detected in subepithelial deposits in the N ??? biopsy specimens where this was examined (Figure 8 right side). The pattern of expression for α-enolase was the same in all groups, irrespective of the presence or absence of circulating anti α-enolase antibodies.

Discussion

Our study provides important data on immunopathology of MN. It first shows that circulating anti-α-enolase IgG4 antibodies, with or without IgG1 antibodies, were present in 72% of the patients with primary MN, while anti-α-enolase IgG3 antibodies, with or without IgG1 antibodies, were detected in 75% of those with secondary MN. Circulating antibodies disappeared in the few tested patients undergoing complete remission. Second, we found that anti-α-enolase antibodies recognized both N- and C-terminal regions of α-enolase, where they react with an epitope located in two short amino-acid stretches. Third, we showed a low prevalence (48%) of anti-PLA_2R

antibodies in this small cohort of Japanese patients with primary MN assessed before treatment, with four of 16 patients producing both antibodies (25%) (Hanna please revise the numbers), while we could not detect anti-PLA_2R antibodies in secondary MN such as bucillamine-induced MN where they had not been searched as yet. Fourth, we could not detect α-enolase in subepithelial immune deposits in any of the patient with circulating anti-α-enolase antibodies, which questions their role in the pathogenesis of MN, including PLA_2R -unrelated MN, whereas PLA_2R was detected in the deposits in all eight tested patients with anti-PLA_2R antibodies and in two patients without circulating antibodies.

Anti-α-enolase antibodies have been reported in patients with several autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and autoimmune mediated retinopathy (AR). A recent analysis revealed the prevalence of anti-α-enolase antibody in SLE, RA, and AR being 21%, 25%, and about 30%, respectively [Mosca M J rheumatol, 2006, Saulot V Arthitis rheum 2002, Adamus Exp mol pathol 2009]. Based on our results and those from Wakui *et al.* [9], approximately 70% patients with primary or secondary MN carry anti-α-enolase antibodies. We detected mostly IgG4 and IgG1 anti-α-enolase antibodies in primary MN, and IgG3 and IgG1 anti-α-enolase antibodies in secondary MN, while Wakui *et al.* predominantly

identified IgG1 and IgG3 anti-α-enolase antibodies. These discrepancies might be due to different affinities of the anti-subclass antibodies used for detection (ref Hanna). More recently, Bruschi *et al.* found that anti-α-enolase IgG4 antibody levels were high in only 25% of patients with primary (Hanna, check "primary") MN [19 : Bruschi j proteomics]. The different prevalence of anti-α-enolase antibodies between Japanese and Italian populations may reflect underlying genetic factors, including human leukocyte antigen polymorphisms. Like Bruschi *et al* (19 : bruschi), we could not detect anti-α-enolase antibodies in disease controls including those with connective disease. Do you have an explanation for that?

We identified α-enolase epitopes in two distant amino-acid stretches 1–66 and 349–433, respectively in the N- and C- terminal ends of the protein. According to the potential three-dimensional structural of α-enolase based on amino-acid sequence, given by the Cn3D program from the National Institute of Biotechnology Information (http://www.ncbi.nlm.nih.gov/Structure/MMDB/mmdb.shtml), the α-enolase C- and N-terminal ends from two subunits are close, and share an external hydrophilic sequence in the dimer (Hanna, could you look at this in detail?). This finding may explain how an epitope that potentially encompasses distant sequences could be formed by both N- and C-terminal regions of α-enolase and recognized by circulating

antibodies, although further studies are necessary to validate this hypothesis.

α-Enolase is a 47-kDa glycoprotein [20] which catalyzes the dehydration of 2-phosphoglycerate to phosphoenolpyruvate, and usually exists as a cytoplasmic multienzyme complex with other glycolytic enzymes, such as pyruvate kinase, phosphoglycerate mutase, and muscle creatine kinase [21]. Different cellular localizations of potential MN auto-antigens are seen under normal physiologic conditions. While neutral endopeptidase and PLA_2R are expressed on cell surface of podocytes [12, 22], aldose reductase and SOD2 are cytoplasmic/mitochondrial enzymes which can be enhanced at the cell surface after oxidative stress [13]. Similarly, α-enolase is usually cytoplasmic in renal tissue although it is strongly expressed on the cell surface of tubular epithelium [23] where it may function as a plasminogen receptor [24]. Cytoplasmic α-enolase can appear at the cell surface following two pathways: the first is secretory pathway, the second involves binding of α-enolase released from damaged cells (ref????). Enhanced expression at the cell surface may thus occur after initial immune complex injury and complement activation.

Although circulating anti-α-enolase antibodies were commonly found, unlike PLA_2R α-enolase was never detected in subepithelial immune deposits, in contrast with the observations made by Bruschi *et al.*(19). This finding suggests that anti-α-enolase

antibody does not directly contribute to the formation of subepithelial immune deposits and resulting complement activation. We cannot, however, exclude a pathogenic role for anti-α-enolase antibodies through binding to endothelial or podocyte cell surface, and subsequent alteration of cell functions. For instance, anti-α-enolase antibodies inhibit the binding of plasminogen to α-enolase on cell surface, which means protective effects from proteolytic activities of plasmin [Moscato S eur j immunol 2000]. Furthermore, intracellular anti-α-enolase antibodies up-taken by endocytosis interfere with glycolysis, decrease cellular ATP levels, and increase intracellular Ca^{2+}, which ultimately may induce apoptosis [Magrys, j clin immunol 2007]. These alterations induced by binding of anti-α-enolase antibodies to the surface of endothelial cells or podocytes may increase permeability of the capillary wall leading to enhanced access and accumulation of pathogenic antibodies such as anti- PLA_2R.

We found that the prevalence of anti-PLA_2R antibodies in previously untreated patients (48%) was lower than that previously reported Caucasian, African, and Chinese cohorts where it was comprised between XX and YY % [12, 26 add Hoxha and Svobodova], suggesting an effect of the genetic background (Hanna, prevalence of HLA-DQA1 in the Japanese?).

In conclusion, circulating anti-α-enolase antibody was detected in about 70% of

patients with both primary and secondary MN, while anti-PLA_2R antibody was restricted to primary MN. The absence of glomerular deposition of α-enolase in subepithelial area contrasting with the presence of PLA_2R in immune deposits, suggests that instead of being implicated in the formation of immune deposits, anti-α-enolase antibody could be involved in endothelial and podocyte dysfunction upon binding to the cell surface, thus increasing access of other antibodies to target antigens of the podocyte such as PLA_2R. Our results raise the possibility that binding of anti-α-enolase antibody could be an enhancing event in primary and secondary MN. Further studies on larger cohorts are needed to confirm this hypothesis and to delineate the value of anti-α-enolase antibody as biomarker for diagnosis and monitoring of MN patients and of α-enolase as potential therapeutic target.

ACKNOWLEDGMENTS

This work was partially supported by a grant from the Aichi Medical University Alumni Association. Hanna, could you please complete?

DISCLOSURES

None

REFERENCES

1. Glassock RJ. The pathogenesis of idiopathic membranous nephropathy: a 50-year odyssey. Am J Kidney Dis 2010; 56:157-167
2. Doi T, Mayumi M, Kanatsu K, Suehiro F, Hamashima Y. Distribution of IgG subclasses in membranous nephropathy. Clin Exp Immunol 1984; 58: 57–62
3. Noel L-H, Aucouturier P, Monterio RC, Preud'homme J-L, Lesavre P. Glomerular and serum immunoglobulin G subclasses in membranous nephropathy and anti-glomerular basement membrane nephritis. Clin Immunol Immunopathol 1988; 46: 186–194
4. Aucouturier P, Monteiro RC, Noel LH, Preud'homme JL, Lesavre P. Glomerular and serum immunoglobulin G subclasses in IgA nephropathy. Clin Immunol Immunopathol 1989; 51: 338–347
5. Haas M. IgG subclass deposits in glomeruli of lupus and nonlupus membranous nephropathies. Am J Kidney Dis 1994; 23: 358–364
6. Imai H, Hamai K, Komatsuda A, Ohtani H, Miura AB. IgG subclasses in patient with membranoproliferative glomerulonephritis, membranous nephropathy, and lupus nephritis. Kidney Int 1997; 51: 270–276
7. Nagahama K, Matsushita H, Hara M, Ubara Y, Hara S, Yamada A. Bucillamine

induces membranous glomerulonephritis. Am J Kidney Dis 2002; 39: 706–712

8. Ohtani H, Wakui H, Komatsuda A, et al. Distribution of glomerular IgG subclass deposits in malignancy-associated membranous nephropathy. Nephrol Dial Transplant 2004; 19: 574–579
9. Wakui H, Imai H, Komatsuda A, Miura AB. Circulating antibodies against alpha-enolase in patients with primary membranous nephropathy. Clin Exp Immunol. 1999; 118: 445–450.
10. Debiec H, Guigonis V, Mougenot B, et al: Antenatal membranous glomerulonephritis from to anti-neutral endopeptidase antibodies. N Engl J Med 2002; 346: 2053–2060
11. Debiec H, Nauta J, Coulet F, et al. Role of truncating mutations in MME gene in fetomaternal alloimmunisation and antenatal glomerulopathies. Lancet. 2004 364:1252-1259
12. Beck LH Jr, Bonegio RG, Lambeau G, et al. M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy. N Engl J Med. 2009 361:11-21.
13. Prunotto M, Carnevali ML, Candiano G, et al., Autoimmunity in membranous nephropathy targets aldose reductase and SOD2. J Am Soc Nephrol 2010;

21:507-519

14. Debiec H, Lefeu F, Kemper MJ, et al. Early-childhood membranous nephropathy due to cationic bovine serum albumin. N Engl J Med. 2011 364:2101-2110.
15. Schwartz MM. Membranous Glomerulonephritis. In: Jennette JC, Olson JL, Schwartz MM, and Silva FG, eds. Heptinstall's Pathology of The Kidney edn.6.vol.1. Philadelphia: Lippincott Williams & Wilkins ; 2007:205-251.
16. Yamada H, Miura N, Kitagawa W, et al: Membranous nephropathy and pulmonary alveolar proteinosis. Intern Med. 46:1441-1446, 2007
17. Debiec H, Ronco P. PLA2R autoantibodies and PLA2R glomerular deposits in membranous nephropathy. N Engl J Med 2011; 364: 689-690
18. Knehtl M, Debiec H, Kamgang P, et al. A case of phospholipase A2 receptor-positive membranous nephropathy preceding sarcoid-associated granulomatous tubulointerstitial nephritis. Am J Kidney Dis 2011; 57: 140–143.

Mosca M, Chimenti D, Pratesi C, et al. Prevalence and clinico-serological correlations of anti-α-enolase, anti-C1q, and anti-dsDNA antibodies in patients with systemic lupus erythematosus. J Rheumatol 2006; 33: 697-697

Saulot V, Vittecoq O, Charlionet R, et al. Prevelance of autoantibodies to glycolytic enzyme α- enolase in sera from patients with early rheumatoid arthritis. Arthritis Rheum 2002; 46:

1196-1201

Adamus G, Brown L, Weleber RG. Molecular biomarkers for autoimmune retinopathies: Significance of anti-transducin-alpha autoantibodies. Exper Molecular Pathol 2009; 87: 195-203

19. Bruschi M, Carnevali ML, Murtas C, et al. Direct characterization of target podocyte antigens and auto-antibodies in human membranous glomerulonephritis: Alfa-enolase and borderline antigens. J Proteomics 2011; 74:2008-2017
20. Verma M, Dutta SK. DNA sequences encoding enolase are remarkably conserved from yeast to mammals. Life Sci 1994; 55:893-899
21. Capello M, Ferri-Borgogno S, Cappello P, Novelli F. α-Enolase: a promising therapeutic and diagnostic tumor target. FEBS J. 2011; 278:1064-74
22. Ronco P, Debiec H. Advances in membranous nephropathy: Success stories of a long journey. Clin Exp Pharmacol Physiol. 2011;38:410-416
23. Sabbatini A, Dolcher MP, Marchini B, et al. Alpha-enolase is a renal-specific antigen associated with kidney involvement in mixed cryoglobulinemia. Clin Exp Rheumatol. 1997; 15:655-658.
24. Lopez-Alemany R, Suelves M, Diaz-Ramos A, Vidal B, Munoz-Canoves P. Alpha-enolase plasminogen receptor in myogenesis. Front Biosci. 2005; 10:30-6

Moscato S, Pratesi F, Sabbatini A, et al. Surface expression of a glycolytic enzyme, α-enolase, recognized by autoantibodies in connective tissue disorders. Eur J Immunol 2000; 30: 3575-3584

Magrys A, Anekonda T, Ren G, Adamus G. The role of anti-α enolase autoantibodies in pathogenecity of autoimmune-mediated retinopathy. J Clin Immunol 2007; 27: 181-192

25. Esgleas M, Li Y, Hancock MA, Harel J, Dubreuil JD, Gottschalk M. Isolation and characterization of a-enolase, a novel fibronectin-binding protein from Streptococcus suis. Microbiol 2008; 154: 2668–2679 **N'EST PAS CITE**
26. Qin W, Beck LH Jr, Zeng C, Chen Z, Li S, Zuo K, et al. Anti-phospholipase A2 receptor antibody in membranous nephropathy. J Am Soc Nephrol. 2011; 22:1137-43.

Figure legends

Figure 1 α-Enolase deletion mutants and the structure of α-Enolase New Fig 2

Upper: Schematics of full-length proteins, five 5'-truncated molecules, and five 3'-truncated molecules are shown. Full-length cDNA encodes a 433 amino-acids protein. Numbers in the parenthesis correspond to the amino-acid residues contained in the deletion mutants.

Lower: Images of α-enolase molecules consisting of two subunits. The N terminus of one subunit is close to the C terminus of the other subunit (from PubMed/Structure).

Figure 2 Binding of circulating antibodies to human α-enolase fragments.New Fig3

Upper left: A Western blot of human α-enolase fragments probed using a serum sample from patient No. 8. Samples loaded in the various lane are as follows: a, 433 amino acids (full length); b, amino acids 29–433 (182F); c, amino acids 109–433 (422F); d, amino acids 189–433 (662F); e, amino acids 269–433 (902F); f, amino acids 349–433 (1142F); g, amino acids 1–66 (295R); h, amino acids 1–146 (535R); i, amino acids 1–256 (775R); j, amino acids 1–306 (1015R); and k, amino acids 1–386 (1255R).

Lower left: the photograph shows dot blotting, with positive results for the following human α-enolase fragments form above "a" to "k".

Upper and lower right: When serum from a normal control subject was used, no signals were observed on Western blots (upper right) or dot blotting (lower right).

Figure 3 Immunoreactivity observed with full-length α-enolase in dot blot assays.New Fig 1

Upper: For primary MN, sera from 19 of 25 patients (76%) immunoreacted with α-enolase. Nine of 13 patients with lupus nephritis (World Health Organization class V)(69%) and 6 of 7 patients with bucillamine-induced MN (86%) were positive for anti-α-enolase antibodies.

Figure 4 Immunoreactivity observed with truncated α-enolase in dot blot assays.

The numbers correspond to size of the patient pool for each disease category (what do you mean by "numbers"? I guess you have changed the figure and not updated the legend. In blots above each number, the 1142F fragment was attached to nitrocellulose membrane (lane A), whereas, in blots below the number, the 295R fragment was attached to the membrane (lane B). Anti-α-enolase antibodies bind ~~an epitope that contains~~ epitopes located in both N- and C-terminal regions.

Figure 5 Circulating anti-α-enolase IgG antibody subclasses in primary and secondary MN.

Upper left: IgG1 subclass, Upper right: IgG2 subclass, Lower left: IgG3 subclass, Lower right: IgG4 subclass. For primary MN, most antibodies appeared to be IgG1 or IgG4; 72% of patients were positive for at least one of these subclasses on dot blots. For secondary MN, however, IgG1 and IgG3 produced the strongest signals, with positive results observed in 75% of patients.

Figure 6 Circulating antibodies specific for α-enolase before and after treatment for MN.

I think it would be easier for the reader to use A, B, C, D…..rather than "Upper left",….

Upper left: Anti-α-enolase antibodies in serum specimens from 5 patients with MN who attained complete remission were assessed before and after treatment (left panel). Notably, positive signals were not detected after treatment in all patients with complete remission. Serum specimens from four patients with incomplete remission, however, produced mixed results; no positive signals were observed in one patient, whereas signals were unchanged ??? (I think the signal is reduced) and reduced in one and two patients, respectively (right panel). The left and right ~~lanes~~ columns in the condition

"before" and "after" treatment are dots of the 1142F fragment ("f" in Figure 3) and of the 295R fragment ("g" in Figure 3), respectively.

Middle and lower left: All patients who attained complete remission initially carried IgG1 anti-α-enolase antibodies (middle left panel). Four patients with secondary MN carried IgG3 anti-α-enolase antibodies (lower left panel), and one patient with primary MN carried IgG4 antibodies (lower right panel), which were not detected after treatment.

Middle and lower right: Dot blot results for four patients who did not achieve complete remission. One patient with primary MN showed robust signals for IgG1 before treatment and weak signals after treatment (middle left panel). Another patient with primary MN showed strong signals for IgG1 before treatment that remained unchanged after treatment (middle ~~right~~ left panel). A patient with lupus MN carried antibodies of the IgG3 subclass, which were not observed after treatment (lower left panel). Two patients with primary MN carried IgG4 autoantibodies before treatment, which decreased after treatment (lower right panel). Patient characteristics are depicted in Tables 1 and 2.

Fig 7 Glomerular deposition of α-enolase and PLA2R proteins in primary and secondary MN Hanna

PLA2R was present in primary MN, but not in secondary MN due to lupus nephritis (LN) and bucillamine (BUC). α-enolase was strongly expressed in tubular epithelium and glomerular parietal cells, but was not present in subepithelial deposits along glomerular capillary loops as was shown for PLA2R in primary and secondary MN (magnification: x 100).

Fig 8 High magnification of α-enolase and PLA2R proteins in primary and secondary MN Hanna

α-enolase was strongly expressed in tubular epithelium and glomerular parietal cells, however, PLA2R is present in subepithelial space along glomerular capillary loops (magnification: x 400).