SPECIAL ARTICLE www.jasn.org

Antigen Identification in Membranous Nephropathy Moves toward Targeted Monitoring and New Therapy

Pierre Ronco and Hanna Debiec

Université Pierre et Marie Curie University of Paris 06, Paris, France; Institut National de la Santé et de la Recherche Médicale, Unité Mixte de Recherche Scientifique 702, Paris, France; and Assistance Publique-Hôpitaux de Paris, Hôpital Tenon, Paris, France

ABSTRACT

Membranous nephropathy, a disease characterized by an accumulation of immune deposits on the outer aspect of the glomerular basement membrane, is the most common cause of idiopathic nephrotic syndrome in Caucasian adults. In the rat model described by Heymann in 1959, the target antigen of antibodies is megalin, a multiligand receptor expressed in the rat glomerulus but absent from the human glomerulus. In the past few years, two major antigens have been identified in human membranous nephropathy. The first is neutral endopeptidase, the alloantigen involved in neonatal cases of membranous nephropathy that occur in newborns from neutral endopeptidase-deficient mothers. The second is the type-M phospholipase A2 receptor (PLA_2R), the first autoantigen identified in idiopathic membranous nephropathy in the adult. Megalin, neutral endopeptidase, and PLA_2R are all expressed on the podocyte surface where they serve as targets for circulating antibodies, which lead to *in situ* immune complex formation, complement activation, and proteinuria. The recent discovery of neutral endopeptidase and PLA_2R provides new tools for monitoring human disease activity and should be of value in designing new antigen-driven therapeutic strategies.

J Am Soc Nephrol 21: 564–569, 2010. doi: 10.1681/ASN.2009121220

Membranous nephropathy is characterized by an accumulation of immune deposits on the outer aspect of the glomerular basement membrane that causes a membrane-like thickening. The immune deposits consist of IgG, mainly IgG4 and IgG1[1] (and unpublished personal data), against antigens that have long eluded identification and the membrane attack complex of complement C5b-9. The formation of subepithelial immune deposits and complement activation are responsible for functional impairment of the glomerular capillary wall, causing proteinuria.

Membranous nephropathy is the most common cause of idiopathic nephrotic syndrome in Caucasian adults, accounting for approximately 20% of cases. Although spontaneous remission of the nephrotic syndrome occurs in approximately one-third of patients, approximately 40% of patients develop end-stage renal failure after 10 years.[2,3] Eighty percent of patients are referred to as "idiopathic," whereas approximately 20% are classified as "secondary" because they occur in patients presenting with associated clinical conditions including infections, systemic lupus erythematosus-related diseases, cancers, and drug exposure. It is generally felt that idiopathic membranous nephropathy is an autoimmune disease whereas secondary forms involve exogenous antigens such as viral or tumor antigens.

Treatment of membranous nephropathy often is controversial and challenging.[4–6] This is in part because of the heterogeneity of disease and lack of reliable biomarkers because of ignorance of the target antigens and nephritogenic antibodies. New strategies to target B-lymphocytes with anti-CD20 antibody[7–9] and to inhibit complement[10] have been designed but with varying efficacy. The key to a specific pathophysiology-driven therapy is the understanding of initiating factors leading to the development of immune deposits, which first requires identification of the pathogenic antigens and then the ensuing events mediated by C5b-9.

In the past 10 years, considerable advances have been made in understanding the molecular pathophysiology of membranous nephropathy. This started in 2002 with the identification by our group of neutral endopeptidase as the responsible antigen in a rare subset of patients with alloimmune antenatal membranous nephropathy.[11,12] This discovery provided proof of concept that a human podocyte antigen could serve as target for nephritogenic antibodies, as shown for rat megalin some 20 years earlier by Kerjaschki and Farquhar in Heymann nephritis, the first experimental model of membranous nephropathy.[13,14] The identification of the podocyte neutral endopeptidase paved the way for that of M-type phos-

Published online ahead of print. Publication date available at www.jasn.org.

Correspondence: Dr. Pierre Ronco, INSERM Unit 702, Hôpital Tenon, 4 Rue de la Chine, 75020 Paris, France. Phone: 33-1-56-01-66-39; Fax: 33-1-56-01-69-99; E-mail: pierre.ronco@tnn.aphp.fr

pholipase A2 receptor (PLA_2R), the first podocyte antigen involved in autoimmune idiopathic membranous nephropathy.[15]

Here we focus on the antigens involved in membranous nephropathy, starting with Heymann nephritis because this experimental model provides the basis for molecular and kinetic concepts of immune deposit formation and glomerular capillary wall thickening. We also discuss new therapeutic approaches driven by novel pathophysiologic findings.

MEGALIN: THE AUTOANTIGEN OF HEYMANN NEPHRITIS

The active model of Heymann nephritis is induced by immunization of Lewis rats with preparations of brush-border proteins.[16] Initial studies of this model suggested that subepithelial deposits result from the glomerular trapping of circulating immune complexes formed by circulating brush-border-related antigens and corresponding antibodies. This hypothesis was based on the observation that glomerular disease could be induced by fractions of membrane prepared from rat renal brush border, not from glomerular extracts.

Subsequently, the development of the model of passive Heymann nephritis in rats that receive an injection of rabbit anti-rat brush-border antibodies led to the suggestion that subepithelial immune deposits form without the intervention of circulating immune complexes. Van Damme *et al.*[17] and Couser *et al.*,[18] using *ex vivo* and isolated perfused kidney systems, further demonstrated that anti-brush-border antibodies bind glomeruli in the absence of circulating brush-border-related antigen, which provided the proof of principle that immune deposit formation occurred *in situ*. Definitive evidence establishing the role of *in situ* immune deposit formation in the glomerular capillary wall required identification of the antigenic moiety.

Kerjaschki and Farquhar identified the target autoantigen in rats in the early 1980s[13,14] as the podocyte membrane protein now called megalin, an approximately 4600-amino-acid transmembrane protein with a molecular weight of approximately 600 kD[19,20] (Figure 1A). This polyspecific receptor, a member of the LDL receptor superfamily, expresses with clathrin at the sole of podocyte foot processes where immune deposits are formed. The system was dissected on a molecular level to the precise amino acid sequence for the pathogenic epitopes, located in a

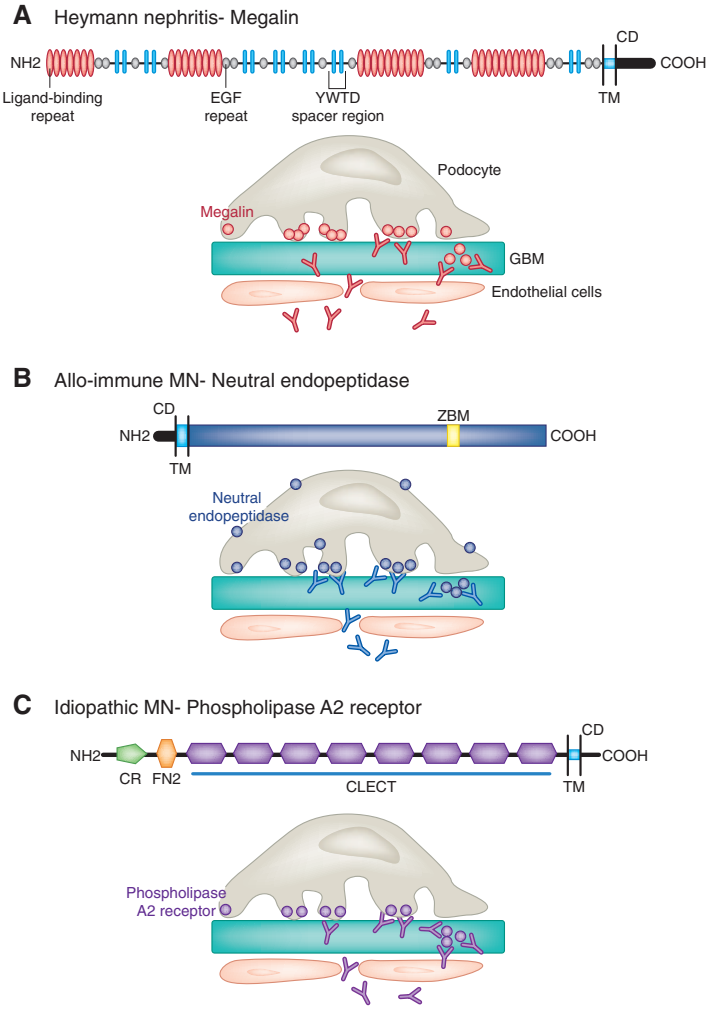

Figure 1. *In situ* formation of immune deposits in three forms of membranous nephropathy. The *in situ* formation of immune complexes involves circulating antibodies binding to a native podocyte protein. Once formed, these antigen-antibody complexes are capped then released into the subepithelial space. They attach to underlying glomerular basement membrane, resist degradation, and persist as immune deposits detectable by immunofluorescence and electron microscopy. Large immune deposits form by repeated cycles of this mechanism, which has been demonstrated in the experimental model of Heymann nephritis. The formation of subepithelial immune deposits and complement activation are responsible for functional impairment of the podocyte and proteinuria. Three antigenic targets have been identified. In all cases, the pathogenic antigens are integral glycoproteins of podocytes. (A) In Heymann nephritis, the polyspecific receptor megalin, a member of the LDL-receptor superfamily, is concentrated in clathrin-coated pits. (B) In alloimmune membranous nephropathy, neutral endopeptidase is more diffuse on the membrane outside of clathrin-coated pits. (C) In idiopathic membranous nephropathy, the exact localization of PLA_2R in membrane domains has not been identified yet. CD, cytoplasmic domain; TM, transmembrane domain; ZBM, zinc-binding domain; CR, cysteine-rich domain; FN2, fibronectin type II domain; CLECT, C-type lectin-like domain.

small glycosylated N-terminal fragment of megalin.[21] The continued growth of immune deposits seems to require the *de novo* synthesis of new molecules of megalin by the podocytes, which are assumed to be delivered by vesicles that eventually fuse with the cell membrane at the base of foot processes.[22] These findings provided the first evidence that podocytes actively contribute to the formation of glomerular immune deposits in membranous nephropathy.

Although considerable insight into the mechanisms of immune deposit formation and complement activation have been provided by studying Heymann nephritis, megalin is not responsible for human membranous nephropathy because it is not found in human glomeruli or podocytes, nor has it been detected in subepithelial immune deposits from patients with membranous nephropathy. In fact, the rat is the only species where megalin is detected in glomeruli, although megalin is found in the brush border in all species studied including humans.

NEUTRAL ENDOPEPTIDASE: THE ANTIGEN OF ALLOIMMUNE ANTENATAL MEMBRANOUS NEPHROPATHY

After 20 years of research since the discovery of megalin, we identified a human counterpart to the Heymann nephritis antigen in a patient with neonatal membranous nephropathy.[11] Because of early development of membranous nephropathy in this infant, we hypothesized pregnancy-induced immunization of the mother with transplacental passage of nephritogenic antibodies. The nature of the target antigen was suspected by indirect immunofluorescent examination of rabbit and rat kidney sections incubated with antibodies from the mother or infant. The same pattern as in human kidneys is observed in the rabbit, whereas in the rat, staining is restricted to cells of Bowman's capsule and to the brush border of deep cortical segments of proximal tubule. We had previously observed similar interspecies differences with anti-neutral endopeptidase antibodies.[23] This led us to identify neutral endopeptidase as the target antigen for antibodies from the mother and the infant by Western blot and immunoprecipitation.[11]

The anti-neutral endopeptidase antibodies produced by the mother were most likely responsible for the infant's membranous nephropathy, given that injection of rabbits with the serum IgG fraction from the mother induced intraglomerular deposits and proteinuria, whereas injection with the IgG fraction from the father did not. Furthermore, neutral endopeptidase was localized by confocal microscopy in immune deposits together with the membrane attack complex of complement in the infant and in the rabbits injected with maternal IgG.[11,24]

Because the mother had no apparent renal abnormalities despite high serum titers of anti- neutral endopeptidase antibody, we hypothesized she might be deficient in neutral endopeptidase and thus analyzed neutral endopeptidase expression on granulocytes from both parents.[11] Cell extracts prepared from maternal granulocytes failed to react with monoclonal or polyclonal antibodies against neutral endopeptidase. Moreover, the mother's serum reacted with the father's granulocytes but not with her own granulocytes, suggesting an alloimmunization process. Alloimmunization in the mother most likely occurred at the time of a previous miscarriage, given that a plasma sample obtained earlier did not show anti-neutral endopeptidase antibodies.[11] At that time, the mother's immune system was fully exposed to neutral endopeptidase expressed by syncytiotrophoblasts and fetal cells.

Since the description of the index case, we have identified two other families, one in The Netherlands and the other in Belgium but from Moroccan origin, with at least one infant born with membranous nephropathy following the same mechanism of disease.[12] We found four other anti-neutral endopeptidase immunized mothers from these Dutch and Moroccan families were also neutral endopeptidase deficient, which led us to search for mutations in the *MME* gene encoding neutral endopeptidase.[12] The *MME* gene is composed of 24 exons. Exons 3 to 24 encode a 749-amino-acid protein that consists of a short cytoplasmic domain, a transmembrane domain, and a large extracellular moiety with a zinc-binding motif required for enzymatic activity (Figure 1B). We identified two truncating mutations in these families located in exon 7 and exon 15, respectively.

We thus have characterized a novel fetal-maternal disease in which a genetic defect in the mother leads to the development of membranous nephropathy in her fetus. Currently, Rhesus incompatibility is the paradigm of fetal maternal diseases because of alloimmunization, and those diseases have been described only for red blood cells and platelets. Our findings raise the possibility that truncating mutations in other podocyte antigens, asymptomatic for the carrier mother, could lead to alloimmune early-onset glomerulopathies. Similarly, immunization against allovariants of proteins differentially expressed by the mother's cells, placental cells, and glomerular cells in the fetus might cause neonatal renal disease.

TYPE-M PLA_2R: THE FIRST ANTIGEN OF AUTOIMMUNE IDIOPATHIC MEMBRANOUS NEPHROPATHY

Searching for a target antigen in autoimmune idiopathic membranous nephropathy had been fruitless for many years. All attempts to identify anti-podocyte antibodies in the blood, podocyte antigens in immune deposits, and anti-podocyte reactivity in eluates from kidneys of patients with membranous nephropathy desperately failed. This is likely explained by low titers of circulating antibodies, which requires the development of highly sensitive assays, or by the longitudinal remodeling of the subepithelial immune deposits, which makes it difficult to detect the target antigen within the deposits.

Beck *et al.* resolved this difficult issue by using kidneys from deceased donors that were unsuitable for transplantation.[15] Glomeruli were isolated from the kidneys with the use of graded sieving, contaminating IgG was removed, and glomerular proteins were extracted and used for

Western blotting with circulating antibodies from adults with membranous nephropathy or with other nephropathies or autoimmune diseases as controls. Under nonreducing conditions, a 185-kD protein band was detected in samples from 26 of 37 patients (70%) with idiopathic membranous nephropathy. In contrast, serum from controls and from patients with secondary membranous nephropathy did not react with that protein band. Reactivity persisted after N-deglycosylation, which yielded a 145-kD antigen. The target antigen, PLA_2R, was then identified by mass spectrometry of the 145- and 185-kD bands. PLA2R is a type 1 transmembrane receptor for secretory phospholipase in the mannose receptor family. All members have a conserved structure and undergo endocytic recycling (Figure 1C).[25]

Four lines of evidence indicate that PLA_2R is recognized by antibodies in patients with membranous nephropathy: (*1*) antibodies to PLA_2R identify a glomerular protein band of the same size as that recognized by the serum of patients with membranous nephropathy; (*2*) conversely, all serum samples from patients with membranous nephropathy that react with the 185-kD glycoprotein from human glomeruli also recognize recombinant human PLA_2R; (*3*) immunoprecipitates of human glomerular extract incubated with serum samples from patients with membranous nephropathy yield a 185 -D protein that is also detected by anti-PLA_2R antibodies in Western blotting; and (*4*) recombinant PLA_2R and the native glomerular protein share the same reduction-sensitive epitope. Although IgG4 was the predominant subclass of anti-PLA_2R antibodies, other subclasses were also present in smaller amounts. It is noteworthy that under reducing conditions, the polyclonal antibodies against PLA_2R still detect recombinant and native glomerular PLA_2R, whereas autoantibodies from patients with membranous nephropathy only recognize a conformation-dependent epitope. A similar restricted immune response to a reduction-sensitive epitope has been described for the Goodpasture antigen in the noncollagenous NC1 domain of α3 type IV collagen.[26,27] It is likely that different conformations defined by disulfide bonding may play a role in exposure of the epitope.

The next question that Beck *et al.* addressed relates to the mechanism of immune deposition, assuming that like Heymann nephritis and alloimmune antenatal membranous nephropathy, autoimmune idiopathic membranous nephropathy involves expression of PLA_2R on the podocyte surface and accumulation of PLA_2R in immune deposits. PLA_2R was indeed detected in human glomeruli, apparently in podocytes. PLA_2R and IgG4 colocalize within subepithelial immune deposits in patients with idiopathic membranous nephropathy. Furthermore, IgG eluted from biopsy samples from four patients with idiopathic membranous nephropathy specifically reacts with cell-expressed recombinant PLA_2R and with a band of the appropriate size in human glomerular extracts, whereas the IgG eluted from the samples from patients with lupus membranous nephropathy and IgA nephropathy did not.

Taken together, these groundbreaking data suggest that in human idiopathic membranous nephropathy, subepithelial deposits form *in situ* through binding of circulating anti-PLA_2R autoantibodies to the PLA_2R antigen expressed on the surface of podocytes. This interaction potentially initiates complement activation and a cascade of events leading to the nephrotic syndrome. However, definitive demonstration of the implication of anti-PLA_2R in the pathogenesis of idiopathic membranous nephropathy would require transferring disease to nonhuman primates (because anti-PLA_2R autoantibodies do not detect PLA_2R in rodent or rabbit glomerular extracts[15]), or to the allografted kidney in patients with recurrent membranous nephropathy.

ARE THERE OTHER ANTIGENS ON THE HORIZON IN PATIENTS WITH IDIOPATHIC MEMBRANOUS NEPHROPATHY?

Circulating anti-PLA_2R antibodies were not detected in approximately 30% of patients with idiopathic membranous nephropathy, which likely means that idiopathic disease is heterogeneous, unless current assays used for the detection of anti-PLA_2R antibodies lack sensitivity. The autoimmune process may also target more than one antigen in the initial or later phases of the disease.

To test whether neutral endopeptidase is involved in autoimmune idiopathic membranous nephropathy, we recently developed a sensitive enzyme-linked immunosorbent assay using recombinant human neutral endopeptidase, which reveals low but significant levels of anti-neutral endopeptidase antibodies in a substantial proportion of adult patients with membranous nephropathy, whereas sera from patients with other nephropathies or healthy controls were negative. In addition, neutral endopeptidase is found in subepithelial immune deposits by confocal microscopy (H. Debiec and P. Ronco, unpublished observations).

The presence of PLA_2R and neutral endopeptidase in these immune deposits does not rule out a role for other antigens. One could speculate that after podocyte injury by C5b-9, intracellular proteins and cryptic epitopes are also exposed, which may induce a second wave of immunization. Patient sera analyzed by Western blot with human podocyte lysates indeed show various antibody profiles that are not observed with control sera. We identified several proteins for which reactivity with patient sera was confirmed with recombinant proteins (H. Debiec and P. Ronco, unpublished). Whether the circulating antibodies raised against those proteins are nephritogenic and involved in the perpetuation of the disease remains to be established.

UNRESOLVED QUESTIONS

One important question relates to the trigger developing the immune response. Anti-PLA_2R antibodies from unrelated patients react with a similar reduction-sensitive epitope. The conditions that lead to exposure of the PLA_2R epitope on the surface of podocytes or in other structures is unknown. Potential crossreactivity of that epitope with exogenous antigens, including bacterial antigens,

should be carefully investigated. Molecular mimicry, which was recently demonstrated by Kain and colleagues in pauci-immune focal necrotizing GN,[28] might also be involved in membranous nephropathy. Another point of importance is B cell epitope spreading, a process whereby the primary immune response against the dominant initiating epitope further extends to other epitopes within the same molecule or among different molecules. This phenomenon may be relevant to the pathogenesis of membranous disease, because in active Heymann nephritis, the onset of proteinuria correlates with intramolecular spreading.[29] In this context, epitope spreading could facilitate polyvalent crosslinkages, thus forming more stable deposits and enhancing complement fixation in the immune complex. B cell epitope spreading has also been observed in many other models of antibody-mediated autoimmune disease.[30,31] Furthermore, as observed in Heymann nephritis,[32] autoimmunity against a glomerular antigen might be followed by an anti-IgG response, which is frequently of the IgG4 subclass in humans.[33]

A second issue relates to the mechanisms of glomerular pathology and proteinuria, and to the pathogenic role of IgG4. Alloimmune membranous nephropathy is transmitted by maternal IgG1 and IgG4, not by IgG4 alone, and IgG1 and IgG4 are equally deposited in glomeruli.[12] IgG4 seems to play an important role in autoimmune idiopathic membranous nephropathy,[1] although it does not activate complement and is functionally monovalent,[34] so its role in the formation of subepithelial immune deposits is unclear. The IgG1 and low amounts of IgG3 present in immune deposits in idiopathic membranous nephropathy, of course, could synergize with IgG4 to induce antigen clustering and complement activation (Figure 1).

Additional mechanisms of proteinuria should also be considered. Megalin and PLA_2R are cell-surface receptors, and neutral endopeptidase cleaves biologically active peptides at the cell surface. It is conceivable that altering receptor-mediated pathways or blocking enzymatic activity causes part of the pathogenic effects of related antibodies. One should also not neglect a role for CD8 T cells.[35]

A third question concerns the role of circulating immune complexes. Although they could not be detected or exist at very low levels in patients with circulating anti-PLA_2R or anti-neutral endopeptidase antibodies, respectively, they still may play some role in the pathogenesis of secondary membranous nephropathy, in which various antigens including hepatitis, tumor, and thyroglobulin antigens have been detected in deposits.[36] In a patient with Pompe disease who developed membranous nephropathy after 10 months of high-dose enzymotherapy, granular staining was observed along capillary loops and in the mesangium with anti-α-glucosidase antibody.[37] It is likely that mesangial deposits are caused by circulating immune complexes whereas subepithelial deposits result from *in situ* immune deposit formation after trapping of α-glucosidase.

TOWARD ANTIGEN TARGETED MONITORING AND THERAPY

In their seminal paper, Beck *et al.*[15] present preliminary data suggesting an association of the presence or titer of anti-PLA_2R autoantibodies with disease activity. At the last meeting of the American Society of Nephrology in San Diego, they reported that in a Dutch cohort the presence of anti-PLA_2R antibodies correlates with clinical status as defined by proteinuria, decreasing with spontaneous or treatment-related remission and increasing with recurrence. A substantial decline in anti-PLA_2R associates with a clinical response to rituximab. If confirmed, these observations may be the first step toward better monitoring of disease activity and treatment efficacy than by proteinuria alone. Because several antigen-antibody systems may be involved, the next step will be to analyze serial serum samples from patients with idiopathic membranous nephropathy to establish antibody profiles, which may lead to identifying subsets of patients with different outcomes and responses to treatment.

Current treatment protocols for patients with membranous nephropathy are entirely empirical. The design of specific therapies for autoimmune disease is primarily directed toward the induction of specific immune tolerance. This requires, ideally, identification of pathogenic epitopes born by the antigen. Nasal administration of the recombinant NC1 domain of α3 type IV collagen, or an immunodominant peptide thereof, induces tolerance in a model of anti-glomerular basement membrane GN.[38,39] We have identified two immunodominant epitopes on the neutral endopeptidase antigen that are recognized specifically by maternal antibodies.[40] Because future pregnancies in neutral endopeptidase-immunized mothers are high risk for the fetus,[41] epitope-driven therapies, including neutralization of pathogenic antibodies with immunodominant peptides and induction of mucosal tolerance, are needed in addition to nonspecific immunosuppressive therapy. A similar approach could be of value in idiopathic membranous nephropathy once the implications of anti-PLA_2R antibodies as the initial pathogenic step are established firmly.

CONCLUSIONS

Substantial progress has recently been made in understanding the pathophysiology of human membranous nephropathy. The first human podocyte antigen, neutral endopeptidase, has been identified in a small subset of patients with neonatal alloimmune disease, followed by the identification of PLA_2R in autoimmune idiopathic membranous nephropathy. Translational research in this area should soon lead to assays of circulating pathogenic antibodies and to innovative targeted therapies.

DISCLOSURES

The research of the authors is supported by grants from Fondation pour la Recherche Médicale, Agence Nationale pour la Recherche (ANR-07-Physio-016–01), AURA (Association pour l'Utilisation du Rein Artificiel), and Coordination Theme 1 (Health) of the European Community's 7th Framework Program, Grant agreement number HEALTH-F2-2007-201590. Part of this work was supported by grants from AMGEN France.

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See related editorial, "Idiopathic Membranous Nephropathy: Getting Better by Itself," on pages 551–552.