Molecular Pathomechanisms of Membranous Nephropathy: From Heymann Nephritis to Alloimmunization

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Membranous nephropathy (MN), the most common cause of idiopathic nephrotic syndrome in white adults, is characterized by an accumulation of immune deposits on the outer aspect of the glomerular basement membrane. In Heymann nephritis, the rat experimental model for MN, megalin—the target antigen of the nephritogenic antibodies—is expressed on the surface of podocytes, where immune complexes are formed, leading to complement activation and nephrotic-range proteinuria. However, megalin cannot be held responsible for human MN because it has not been found in human podocytes or detected in subepithelial immune deposits in patients with MN. Several potential antigens have been identified in so-called secondary forms of MN, but there is no real proof that these antigens are pathogenic. In a subgroup of infants with antenatal MN, neutral endopeptidase (NEP) has been identified as the first protein target on human podocytes of nephritogenic antibodies. The infants’ mothers became immunized during pregnancy against NEP expressed on syncytiotrophoblastic cells because they were NEP deficient as a result of truncating mutations in the MME gene. Severity of neonatal renal disease was determined by the mothers’ IgG response that led to the formation of the membrane attack complex of complement in the subepithelial deposits. Alloimmunization against NEP is a novel pathomechanism of MN that might also account for some cases of MN after renal or bone marrow transplantation. Other types of alloimmunization should be investigated in MN but also in other renal and nonrenal diseases, particularly those that affect the pediatric age.

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

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ISSN: 1046-6673/1605-1205
merular extracts. Subsequently, the development of the model of passive HN in rats that received an injection of rabbit anti-rat BB antibodies led to the suggestion that subepithelial immune deposits could be formed without the intervention of circulating immune complexes. Van Damme et al. (10) and Couser et al. (11), using ex vivo and isolated perfused kidney systems, further demonstrated that anti-BB antibodies could bind glomeruli in the absence of circulating BB-related antigen, which provided the proof of principle that immune complex formation occurred in situ. Definitive evidence establishing the role of in situ immune complex formation in the glomerular capillary wall required identification of the antigen moiety.

The autoantigenic target in the rat disease was identified by Kerjaschki and Farquhar (12,13) in the early 1980s as the podocyte membrane protein now called megalin. The polyspecific receptor megalin, a member of the LDL-receptor superfamily, is expressed with clathrin at the sole of podocyte foot processes (where immune complexes are formed). The system was dissected on a molecular level to the precise amino acid sequences of pathogenic epitopes (14,15) and provided the first evidence that podocytes actively contribute to the formation of glomerular immune deposits in MN (Figure 1). The continued growth of immune deposits seems to require the de novo synthesis by the podocytes of new molecules of megalin, which are assumed to be delivered via vesicles that eventually fuse with the cell membrane at the base of the foot processes (16).

Although considerable insight into the mechanisms of immune complex formation have been provided by studies of HN, megalin cannot be held responsible for human MN because it has not been found in human glomeruli or podocytes or detected in subepithelial immune deposits in patients with MN. In fact, the rat is the only species in which megalin is detected in glomeruli, although megalin is found in the BB in all species as yet studied, including humans.

Other podocyte membrane proteins, such as dipeptidyl peptidase IV (DPPIV) (17), NEP (18), and aminopeptidase A (19), were shown to serve as target antigens for circulating antibodies in rats, rabbit, and mice, respectively. At variance with megalin, DPPIV is evenly distributed on the membrane of podocytes, is further expressed on endothelial cells that line the glomerular capillary wall, and assumes a wide extrarenal distribution, including transport epithelia, capillary endothelia, and the vast majority of normal lymphocytes (20,21). The kinetics of immune deposits observed in the rat glomerulus after intravenous injection of anti-DPPIV monoclonal or polyclonal antibody sharply contrast with those noticed in rats that are given anti-megalin antibody (17). Glomerular binding is maximum 4 h after injection but from then on decreases dramatically to be absent or very weak 72 h later. In the mouse, the kinetics of the heterologous phase after injection of anti-DPPIV antibody bear close similarity to those reported in the rat, but the amount of antibody that remains in the glomerulus is sufficient to induce the development of an autologous phase that causes MN (22,23). DPPIV–anti-DPPIV immune complexes formed at the surface of glomerular endothelial cells may be shed in the capillary lumen, partly dissociate, then be filtered through the glomerular capillary wall, and finally reassociate on the outer aspect of the glomerular basement membrane. This pathophysiologic scenario occurs in a model of MN induced in the rabbit by polyclonal antibodies to angiotensin-converting enzyme, which is expressed by glomerular endothelial cells but not by rabbit’s podocytes (24).

The second antigen, NEP, has the same distribution in the rabbit and human kidneys as DPPIV, whereas in the rat, it is found on cells of the Bowman’s capsule and in the distal segment of the proximal tubule (pars recta) (25). Glomerular deposits observed after injection in rabbit of monoclonal antibody to NEP are particularly transient. Their almost complete disappearance within 24 h coincides with the appearance of the antibody on the BB of some proximal convoluted tubules, as noted in the rat with anti-DPPIV. Because both DPPIV and NEP are expressed on the human podocyte, we hypothesized approximately 15 yr ago that these two enzymatic antigens might play some role in the pathogenesis of MN in humans (18).
Antigens in Human MN

In the past 20 yr, considerable efforts have been devoted to the identification of antigens involved in human MN. In so-called secondary forms of MN (Table 1) (26–33), hepatitis B, hepatitis C, and Helicobacter pylori antigens; tumor antigens; thyroglobulin; and DNA-containing material have been detected in the subepithelial deposits, but there is no real proof that these antigens are pathogenic (reviewed in 33,34). Because of the increased permeability to proteins of the glomerular capillary wall, they may have been trapped passively between the lamina rara externa and the slit diaphragm, as is the case for albumin. Some similarities, such as glomerular deposition of renal tubular epithelial antigens, have been found between experimental HN and individual cases of MN, but the antigens could not be characterized at the molecular level (35–37).

Childhood MN occasionally may be associated with linear or granular deposits of IgG and C3 along the basement membrane. This rare subgroup of patients may have proximal tubule impairment and extrarenal manifestations, including lung hemorrhage, diarrhea because of intestinal villous atrophy or autoimmune enteropathy, and cornea and neurologic symptoms (38). Antibodies to the 58-kD tubulointerstitial nephritis (TIN) antigen were reported in five of the 11 reported cases (38). The TIN antigen is a glycoprotein molecule (39) that has the highest expression in the basement membrane of the proximal tubules, whereas it is absent from the glomerular basement membrane and the mesangial matrix (40). Therefore, the TIN antigen cannot serve as a target glomerular antigen for circulating antibodies. Moreover, careful analysis of the reports strongly suggests that in childhood MN with anti-TBM nephritis, the glomerular disease is the primary lesion, and the formation of anti-TBM antibodies and their fixation to the TBM and the development of the tubulointerstitial disease are secondary phenomena. Because some cases are associated with anti-BB antibodies (41), identification of the relevant antigen(s) would be of great value.

Most eluates from kidneys of patients with MN do not react with normal kidney (42), which should not be taken as an argument against in situ formation of immune complexes implicating podocyte antigens because eluates were usually obtained in late stage of the nephropathy. At that stage, immune deposits differ significantly from initial immune reactants because the immune deposits are being perpetuated by a secondary anti-idiotypic antibody response directed against the original antibody and also because the composition of immune deposits is continuously being altered by incorporation of passively trapped molecules that have traversed the diseased glomerular capillary wall.

NEP, the First Podocytic Antigen Responsible for Human MN: From Bedside to Bench

After 20 yr of research since the discovery of megalin, we identified a human counterpart to the HN antigen in a patient with neonatal MN (8). The male infant who was born at 38 wk of gestation presented with oligoanuria, massive proteinuria, and respiratory distress on the first day of life. His parents were unrelated, healthy individuals without a family history of renal or autoimmune disease. The mother, aged 24, had had a miscarriage at 14 wk of gestation 2 mo before this pregnancy. Her BP, urinalysis, and serum creatinine concentration were normal throughout and after the pregnancy, and she took no medications. However, antenatal echography showed oligohydramnios and enlarged fetal kidneys from the 34th week of gestation. Her levels of antineutrophil cytoplasmic antibodies, antinuclear and anti-DNA antibodies, and complement were normal.

Identification of NEP as the Target Antigen of Nephritogenic Antibodies

Because of the early development of MN in this infant, we suspected pregnancy-induced immunization of the mother with transplacental passage of nephritogenic antibodies. This hypothesis was first tested by indirect immunofluorescence examination of normal human kidney sections. A serum sample obtained 9 mo before pregnancy (7 mo before the miscarriage) was negative. Serum samples obtained at 3 mo of gestation and after delivery showed reactivity on the glomerular capillary walls and the BB on all kidney biopsy specimens, as did the serum obtained from the infant 13 d after birth. No

Table 1. Antigens identified in immune deposits in patients with secondary MN

<table>
<thead>
<tr>
<th>Groups</th>
<th>Disease or Agent</th>
<th>Antigen</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune diseases</td>
<td>systemic lupus thyroiditis</td>
<td>dsDNA, nucleosomes, histones</td>
<td>26, 27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>thyroglobulin, microsomal</td>
<td>28</td>
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<tr>
<td></td>
<td></td>
<td>antigens</td>
<td></td>
</tr>
<tr>
<td>Infectious or parasitic disease</td>
<td>hepatitis B syphilis</td>
<td>Hbe antigen</td>
<td>29, 30</td>
</tr>
<tr>
<td>Cancer</td>
<td>gastric infection lung, colon, stomach, palate, kidney, prostate, melanoma</td>
<td>Helicobacter pylori</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CEA, PSA, RTE, “tumor antigen”</td>
<td>review in 33</td>
</tr>
</tbody>
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*MN, membranous nephropathy; dsDNA, double stranded DNA; CEA, carcinoembryonic antigen; PSA, prostatic-specific antigen; RTE, renal tubule epithelium antigen*
reactivity was detected in the infant’s serum 40 d after birth, which confirmed that “anti-kidney” antibodies circulating in the infant’s serum were of maternal origin (8).

The nature of the target antigen was suspected by indirect immunofluorescence examination of rabbit and rat kidney sections incubated with the mother’s or the infant’s antibody. The same pattern as in human kidneys was observed in the rabbit, whereas in the rat, staining was restricted to the cells of Bowman’s capsule and to the BB of deep cortical segments of the proximal tubule. We had previously observed similar interspecies differences with anti-NEP antibodies, whereas distribution of DPPIV is not species dependent (25). The mother’s IgG antibody and the infant’s IgG antibody recognized by Western blotting a single antigen of approximately 90 kD in protein extracts from rat BB, rabbit kidney cortex, and cultured human podocytes. This antigen had the same electrophoretic mobility as NEP. Furthermore, NEP antigen and enzymatic activity were specifically immunoprecipitated from rat BB with the mother’s IgG (8).

The anti-NEP antibodies produced by the mother, which were found in the infant’s serum 13 d after birth, were most likely responsible for the infant’s MN, given that the injection of rabbits with the serum IgG fraction from the father did not. Furthermore, NEP was localized by confocal microscopy in immune deposits together with the membrane attack complex of complement, both in the infant (Figure 2) and in the rabbits that received an injection of the mother’s IgG.

Since the description of the index case, we have identified two other families, one in The Netherlands and one in Belgium but from Moroccan origin, with at least one infant born with MN and the same mechanism of disease (9). It is interesting that the Dutch case was reported in 1990; at that time, the mother’s serum had been tested for the presence of anti-DPPIV antibodies but not for that of anti-NEP antibodies (43).

Mechanisms of Immune Complex Deposition in the Infants’ Glomeruli

The four cases of antenatal MN because of transplacental transfer of anti-NEP antibodies led us to revisit the concept of in situ formed versus circulating preformed immune complexes that has remained a debated issue for the past 30 to 40 yr or so. It is most likely that in these cases, immune complexes were predominantly formed in situ at the sole of podocyte foot process, where NEP is expressed (Figure 1) (44). However, contrary to megalin, NEP is expressed in a diffuse pattern on the membrane of podocytes, as is angiotensin-converting enzyme on the plasma membrane of mature oocytes (45). In vivo interaction of angiotensin-converting enzyme with divalent antibodies induces the formation of granular immune deposits through a mechanism of “patching” and “shedding” of immune complexes (45). A similar mechanism may be implicated in the formation of immune deposits in the infants’ glomeruli. One can speculate that the immune complexes that are shed from the foot processes are sequestered between the lamina rara externa of the glomerular capillary wall and the podocytes’ slit diaphragms, whereas those that are shed from the podocyte cell bodies are excreted in the infant’s urine. However, transient low levels of circulating immune complexes were detected in the infant’s serum at day 13. The immune complexes isolated from the serum sample contained NEP, as demonstrated by Western blotting (8). Their contribution to the formation of subepithelial immune deposits is uncertain, because levels of circulating immune complexes were low, manifestations of serum sickness were absent, and subendothelial and mesangial immune deposits were not seen. However, the two mechanisms of immune complex formation (in situ versus preformed) are not mutually exclusive.

Mechanisms of Proteinuria: Role of the Membrane Attack Complex of Complement

Heavy deposits of the membrane attack complex of the complement C5b-9 were found on the outer aspect of glomerular capillary walls in the infants’ biopsy specimens (Figure 2) (8). These deposits might account for the unusual presence of annular formations within the electron-dense deposits (6,43). Similar structures were previously found in the zona pellucida of rabbit oocytes after the injection of antibodies against angiotensin-converting enzyme (45). The finding of heavy C5b-9 deposits recalls previous observations made in HN in the rat, which also requires formation of C5b-9 to increase glomerular permeability (46). In the rat model, glomerular damage is presumably mediated by the formation by podocytes of oxygen radicals that are released into the glomerular basement membrane and into immune deposits, causing sublytic damage of podocytes (47,48). Although these reactive compounds can directly damage matrix proteins, their effect is further potentiated by local peroxidation of lipids, which can be suppressed by treatment with the scavenger probucol (49). Because C5b-9 was also
found within the immune deposits in MN induced by anti-NEP antibody, it is possible that a sequence of complement activation, oxygen radical formation, and lipid peroxidation contributes to glomerular damage and proteinuria (50).

C5b-9, directly or via the production of reactive oxygen species, can induce DNA damage in podocytes (51). It can also enhance expression by podocytes of matrix metalloproteinase-9 (52,53), a matrix-degrading enzyme with targeted activity on collagen type IV (a main component of the glomerular basement membrane) and alter nephrin expression (54). In glomeruli of patients with MN, a more granular pattern or a loss of staining of nephrin was observed (55).

We suggest that the binding of the nephritogenic anti-NEP antibodies and ensuing complement activation may result in alterations of the podocytic phenotype, shedding of important podocytic proteins such as nephrin from plasma membrane to an extracellular site, and production of toxic mediators including collagenases and oxygen radicals. Further research in this area should help to elucidate the pathogenic mechanisms involved in the induction of albuminuria. Complement, oxygen radicals, and lipid peroxidation could provide a therapeutic target, at least for symptomatic treatment, of the nephrotic syndrome in MN.

**Mechanisms of Proteinuria: Role of IgG Subclasses**

The glomerular deposition of IgG1 and IgG4 subclasses is a characteristic feature of MN (3,4). Both subclasses were found in the diseased infants' biopsy specimens (9). However, the expression of the renal disease was variable in the infants who were born to the five mothers who had produced anti-NEP antibodies. The infants from four mothers presented at birth with renal failure, whereas all four children from mother IIM8 (Figure 3) had no overt manifestation of renal disease either at birth or at the most recent follow-up assessment. We found that this mother produced approximately 10 times less anti-NEP antibody than the others, and, perhaps more important, she produced only IgG4 subclass antibodies, whereas the four other mothers produced both IgG1 and IgG4 anti-NEP antibodies (9). The lack of renal manifestation in the neonates is not explained by deficient transplacental transfer of IgG4 because at birth, fetal and maternal IgG3 and IgG4 concentrations are normally equal, whereas IgG1 and IgG2 concentrations are higher and lower in the fetus than in the mother, respectively (56). A more plausible explanation is that IgG subclasses differ in their ability to induce cell injury because they interact differently with complement and Fc receptors (57). By comparison with IgG1, even aggregated IgG4 can weakly activate complement (58), a key mediator of proteinuria in MN as discussed above. Further studies are needed to establish the role of IgG4 in MN.

**Potential Role of the Blockade of NEP Enzymatic Activity by Nephritogenic Antibodies**

NEP (EC 3.4.24.11, neprilysin, enkephalinase, CD10, membrane metalloendopeptidase [MME]) is a 90- to 110-kD zinc-dependent metallopeptidase that is identical to the common acute lymphoblastic leukemia antigen (59,60). It is expressed in brain tissue; on polymorphonuclear leukocytes and lymphoid progenitor cells; and on epithelial cells within nonlymphoid organs such as the kidney, the liver, the breast, the lung, the prostate, and the placenta (61,62). In the kidney, NEP is found on BB, podocyte, and vascular smooth muscle cells (25,63). NEP is involved in the metabolism of a number of regulatory peptides, including natriuretic peptides, endothelin, bradykinin, enkephalin, and substance P, and plays an important role in turning off peptide-signaling events at the cell surface (64).

For evaluating a potential effect of anti-NEP antibodies on enzymatic activity, lysates of human podocytes were preincubated with maternal or paternal IgG. The NEP-specific activity
of podocyte lysates was dose-dependently inhibited by maternal but not paternal IgG. These findings suggest that some of the deleterious effects of anti-NEP antibody may be mediated by the blockade of NEP enzymatic activity. In the first case reported (8), the infant’s kidney biopsy specimen showed unusually severe arterial lesions without immune deposits and a collapse of glomerular capillary tufts that was suggestive of major renal ischemia during prenatal development. Because the mother’s antibodies inhibited NEP activity, their transplacental passage might increase concentrations of vasoconstrictor peptides, particularly endothelin, in the vascular wall and thus induce the proliferation of vascular smooth muscle cells. The anti-NEP antibodies might also induce podocyte alterations and proteinuria via their blocking enzyme activity, as previously shown after injection of an anti-aminopeptidase A monoclonal antibody in the mouse (19). However, given the size of subepithelial deposits and the intensity of C5b-9 staining, it is likely that podocyte alterations and increased permeability of the glomerular capillary wall resulted mostly from immune complex formation.

Alloimmunization: A Novel Mechanism of Renal Disease
Mechanisms of the Immunization against NEP in the Infant’s Mother

Because the first mother reported (8) had no apparent renal abnormalities despite high serum titers of anti-NEP antibody, we hypothesized that she might be deficient in NEP and analyzed NEP expression in granulocytes from both parents. Fluorescence-activated cell sorter analysis of the mother’s granulocytes showed no NEP at the cell membrane. Cell extracts prepared from maternal granulocytes failed to react with either monoclonal or polyclonal antibodies against NEP after Western blotting. 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 her miscarriage, given that a plasma sample obtained earlier did not show anti-NEP antibodies (8). At that time, the mother’s immune system was massively exposed to NEP antigen expressed by syncytiotrophoblasts and fetal cells.

Identification of Mutations in the Mothers’ NEP Gene

We found that the four other anti-NEP immunized mothers from the Dutch and Moroccan families were also NEP deficient, which led us to search for mutations in the MME gene for NEP (9). 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. We identified two truncating mutations in these families (Figure 3). The first mutation, located in exon 7, is a cytosine deletion at position 466 that results in a frameshift and premature termination codon at codon 169. The second mutation, located in exon 15, is a single-base nonsense mutation (1342C→T) that generates a stop codon at position 448. The Portuguese mother is a compound heterozygote who inherited one mutant allele from each parent, whereas the Dutch and Moroccan mothers were homozygous for the same deletion mutation 466delC and inherited the mutant allele from their heterozygous parents (Figure 3).

Theoretically, mutations in exon 7 and exon 15 lead to highly truncated proteins of 168 and 450 amino acids, respectively, devoid of enzymatic activity (the zinc-binding motif is encoded by exon 19). However, we failed to detect truncated proteins in the mothers’ granulocytes and urine samples (9). These findings indicate that the mutated MME gene is knocked out functionally probably because of decreased stability of the mutated mRNA or protein.

Despite the absence of NEP protein in the five mothers and in a male individual, these individuals, aged 16 to 42, were healthy (as were heterozygous family members except for the neonates who were born with MN). By contrast with MME null mice (65), they had normal BP, renal functional tests, and lymphocyte phenotype and function (9). The lack of apparent consequence of NEP deficiency can be partly explained by redundancy of the enzyme activity (66,67).

We thus have characterized a novel fetomaternal disease in which a genetic defect in the mother leads to the development of MN in her fetus. Currently, Rhesus incompatibility is the paradigm of fetomaternal diseases because of alloimmunization, and such 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 alloimmunization and transplacental transfer of nephritogenic antibodies. Along the same line, immunization against allovariants of proteins expressed by placental cells in the mother and glomerular cells in the fetus might cause neonatal renal disease. This pathophysiologic scenario might also occur in neonatal diseases that affect organs other than the kidney and the blood.

Do Anti-NEP Antibodies Play a Role in Adult Cases of MN?

We investigated the outcome of antenatal MN in the four infants who were born to NEP-immunized mothers. All infants showed a rapid improvement of renal failure and the nephrotic syndrome. However, children IV P1 and III M1 (Figure 3) showed persistent albuminuria. Patient III N1, now 20 yr old, is of particular clinical interest because of the postponed development of severe chronic renal failure with nephrotic-range proteinuria. Although we could not undertake a second kidney biopsy in the oldest patient, current renal manifestations are likely to result from an aged MN combined with the postponed consequences of immunologically mediated antenatal nephrosis. Deposition of IgG produced by infants to idiotypes or allotypes on the maternal IgG could contribute to later progression of the disease. These observations suggest that anti-NEP–induced antenatal renal disease might account for “idiopathic” MN or chronic renal failure detected during adolescence or early adulthood.

As yet, we have failed to find NEP in the subepithelial immune deposits in patients with “idiopathic” MN. This does not rule out a role for NEP in the disease, because the initiating
antigen may no longer be present in aged immune deposits. However, should anti-NEP antibody be produced, they would bind to NEP that is heavily expressed on granulocytes; therefore, they would not be available for their glomerular target antigen. Conversely, one can also hypothesize that NEP-anti-NEP immune complexes formed on the surface of granulocytes are shed in the serum, where they partially dissociate, allowing their components to traverse the basement membrane before they reassociate between the lamina rara externa and the slit diaphragms.

The implication of the NEP system should also be considered in patients who develop de novo MN after renal transplantation, because of possible alloimmunization of a NEP-deficient recipient against a NEP-expressing kidney graft. More generally, it may be worth searching for an alloimmunization process in MN that occurs in special clinical settings such as graft-versus-host disease (68).

In conclusion, the discovery of an alloimmunization against the podocyte antigen NEP in neonatal cases of MN sheds light on the pathomechanisms and molecular bases of MN. First, a common denominator seems to be that podocytes and their membrane-associated proteins have a pivotal role in the development of the disease by providing antigenic targets for circulating antibodies for in situ formation of glomerular deposits. Further studies should aim at searching for other target antigens on human podocytes. Second, the NEP system involves the activation of complement within immune deposits, which gives credence to the role of complement in human MN. Third, alloimmunization is a novel pathomechanism of MN. Other types of alloimmunization should be investigated in MN but also in other renal and nonrenal diseases, particularly those that affect the pediatric age.

Acknowledgments
This work was supported by grants from INSERM, University Paris 6, European Union Contract QLG1-CT-2002-01215, Amgen France, and a gift from Mrs. Halpin to the American branch of the Foundation of France.

We are indebted to Béatrice Mougenot for analysis of renal biopsies and to Vincent Guigonis, Georges Deschênes, and Albert Bensman (Armand-Trousseau hospital, Paris), Jeroen Nauta (Sophia Children’s hospital, Rotterdam, The Netherlands), Françoise Janssen and Thierry Schurmans (Brussels Free University, Belgium) for referring the patients. We are grateful to the members of the families who collaborated on this work; to Florence Coulet, Florent Soubrier (Tenon Hospital, Paris) and Mirjam van der Burg (Erasmus MC, Rotterdam, The Netherlands) for help in genetic studies; and to Christine Vial for assistance in the preparation of the manuscript.

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