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Atypical focal segmental glomerulosclerosis associated with a new *PODXL* nonsense variant

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Abstract

Background: Podocalyxin (PODXL) is a highly sialylated adhesion glycoprotein that plays an important role in podocyte's physiology. Recently, missense and nonsense dominant variants in the *PODXL* gene have been associated with focal segmental glomerulosclerosis (FSGS), a leading cause of nephrotic syndrome and kidney failure. Their histologic description, however, was superficial or absent.

Methods: We performed exome sequencing on a three-generation family affected by an atypical glomerular nephropathy and characterized the disease by light and electron microscopy.

Results: The disease was characterized by FSGS features and glomerular basement membrane duplication. Six family members displayed chronic proteinuria, ranging from mild manifestations without renal failure, to severe forms with end-stage renal disease. Exome sequencing of affected twin sisters, their affected mother, healthy father, and healthy maternal uncle revealed a new nonsense variant cosegregating with the disease (c.1453C>T, NM_001018111) in the *PODXL* gene, which is known to be expressed in the kidney and to cause nephropathy when mutated. The variant is predicted to lead to a premature stop codon (p.Q485*) that results in the loss of the intracytoplasmic tail of the protein.

Conclusion: This is the first description of a peculiar association combining a *PODXL* stop-gain variant and both FSGS and membranoproliferative glomerulonephritis features, described by light and electron microscopy.

KEYWORDS

exome sequencing, focal segmental glomerulosclerosis, glomerular basement membrane duplication, membranoproliferative glomerulonephritis, podocalyxin, *PODXL*

1 | INTRODUCTION

Familial glomerular nephropathies are characterized by proteinuria and may lead to nephrotic syndrome and end-stage renal failure. Since the 1990s and the generalization of genetic analyses, there have been numerous discoveries of variants causing familial glomerular nephropathies (Devuyst et al., 2014). The variants often affect podocytic proteins or less frequently proteins involved in the regulation of the alternative complement pathway (Mele et al., 2015). Among the podocytic proteins, the *PODXL*-encoded podocalyxin has been recently associated to both recessive (compound heterozygous variants) and dominant forms of familial nephropathies (OMIM#602632). These include (a) a new syndromic nephrotic syndrome in a newborn child with a missense (p.M1I) and a nonsense variant (p.W341*), inherited from the father and the mother, respectively (Kang et al., 2017), (b) two independent autosomal dominant focal segmental glomerulosclerosis (FSGS) cases with novel heterozygous missense and nonsense variants (p.L474R and p.S378*) (Barua et al., 2014; Lin et al., 2019), and (c) a patient with proteinuria and renal insufficiency with a heterozygous nonsense variant (p.R326*) (Lin et al., 2019).

Podocalyxin (PODXL) is a transmembrane podocytic sialoprotein which imparts negative charges to the podocyte and is thought to contribute to maintaining the aperture of the slit diaphragm (Nielsen & McNagny, 2009b). PODXL is part of the CD34 family, which also includes the endoglycan and the CD34 protein. It is strongly expressed by kidney podocytes and endothelial cells among others (Horrillo et al., 2016). The intracellular portion of PODXL interacts with ezrin, as well as with the Na(+)/H(+)-exchanger regulatory factor 1 and 2 (NHERF1 and NHERF2), involved in protein trafficking, ion transport, and signaling. Ezrin serves as an intermediate between PODXL and the actin cytoskeleton (Takeda et al., 2001). The actin cytoskeleton contributes to the structural integrity of the podocyte by involving RhoA, in particular by redistributing the actin filaments toward the apical membrane (Schmieder et al., 2004). Possible pathological disruption of the actin cytoskeleton mediated by PODXL highlights the potential importance of this protein in nephrotic syndromes (Schmieder et al., 2004).

Animal and in vitro models also confirmed that variants affecting *PODXL* are deleterious. Inactivation of *PODXL* in mice results in renal impairment and $PODXL^{-/-}$ mice are anuric, die in the neonatal period and have severe renal abnormalities, including absence of podocytic slit diaphragm and pedicels. Some mice are edematous at birth and others have omphalocele (Doyonnas et al., 2001). Mice with a selective knockout of *PODXL* in endothelial cells are viable but die spontaneously at 3 months. They present an inflammatory infiltrate within the vessel walls, subendothelial edema, and a significant increase of cellularity in renal glomeruli and increased permeability to dextrans (Horrillo et al., 2016). Likewise, kidney cells derived from human pluripotent stem cell-derived knockout cells for *PODXL* were shown to have junctional organization defects in podocyte-like cells (Freedman et al., 2015).

Here, we report a family in which several members displayed proteinuric nephropathy and variable chronic kidney failure affecting three generations, ranging from no kidney failure to end-stage renal failure and death during childhood. Histology shows an unusual frontier form of FSGS and MPGN. Through familial exome sequencing, we show that the disease is most probably caused by a heterozygous nonsense variant in the *PODXL* gene.

2 | SUBJECTS AND METHODS

2.1 | Exome sequencing

DNA was extracted from saliva and/or blood and purified according to standard protocols. Exome Sequencing of the two affected sisters (III2 and III3), their affected mother (II4), their healthy father (II3), and maternal uncle (II5) was performed as previously described (Molitor et al., 2019). Briefly, after library preparation with the TruSeq Exome Kit (Illumina, San Diego, CA, USA), 2 × 75 bp paired-end sequencing was performed on a NextSeq500 instrument (Illumina, San Diego, CA, USA). Copy Number Variations (CNVs) were identified with the Conifer software (Krumm et al., 2012). Other variants were called using GATK (DePristo et al., 2011). Only variants covered by more than 10 variant reads were considered. Annotation was performed

with the KGGSeq software package (Li et al., 2012). We focused only on protein altering variants (missense, nonsense, splice-site variants, and coding indels) absent in the 1000 Genomes Project, Exome Aggregation Consortium (ExAC), gnomAD, and an in-house database including ~1000 exomes. To identify potential causal variants, we focused on dominantly inherited variants, that is, those common to patients III2, III3 (daughters), and II4 (mother), and absent in individuals II3 (father) and II5 (uncle). Other modes of transmission were excluded beforehand.

2.2 | Sanger sequencing

The candidate variant was confirmed by capillary Sanger sequencing in all available family members. The following primers were used for confirmation of the variant: *PODXL*-F1: 5′-ATGTGTACGAGCGGCTGAAGGA-3′. *PODXL*-R1: 5′-AGGAACCTGATGCCCAAAGAGCT-3′. Briefly, a 1019 bp fragment was amplified using the Expand Long Template PCR System (Roche Diagnostics Corporation, Indianapolis, IN, USA), following manufacturer's recommendations. After purification with the Exostar® kit (GE Healthcare Life Sciences, Velizy-Villacoublay, France), the PCR product was sequenced bidirectionally using the same amplification primers and the Big Dye Terminator Kit v3.1 (Thermo Fisher Scientific, Waltham, MA, USA). Sequence reactions were run on an ABI PRISM 3730*xl* sequencer (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed on the SeqScape software (Thermo Fisher Scientific, Waltham, MA, USA).

2.3 | Renal pathology

Part of the renal biopsy tissue was fixed in Formalin Acetic Acid Alcohol (FAA), embedded in paraffin, sectioned in 3 μm-thick layers and stained with hematoxylin-eosin, Masson's trichrome, silver, and periodic acid Schiff for examination by light microscopy. Part of the remaining tissue was frozen for immunohistology and another part was fixed in glutaraldehyde for electron microscopy. Immunohistochemistry was performed using antibodies directed against C1q, C3, IgA, IgG, IgM, kappa, and lambda light chains of immunoglobulins using standard protocols. Immunohistochemistry for PODXL was performed according to the manufacturer's protocol using polyclonal rabbit antibodies (Sigma-Aldrich product reference HPA002110). Semiquantitative analysis of staining was performed using Image-Pro Plus 7 (Media Cybernetics, Inc. Rockville, MD, USA). Ultrathin sections were prepared for electron microscopy studies and were examined using a JEOL JEM-1010 electron microscope (JEOL, Tokyo, Japan).

3 | RESULTS

This is a non-consanguineous family of Caucasian origin (Figure 1a). The major clinical and biological characteristics of affected members are presented in Table 1.

3.1 | Clinical and biological description of patients

3.1.1 | Patient I1

The grandfather (I1) presented with low-grade proteinuria diagnosed in a nephrological setting around 1950–1960 when he was in his thirties. Proteinuria was confirmed on numerous occasions over a period of nearly four decades and was always reported as non-nephrotic. He did not develop renal failure and died of extrarenal causes at 76 years of age. An exploration of the circulating complement levels was carried out in the context of the family study and did not show low complement levels. His wife (I2) had no documented nephropathy and died of breast cancer at age 68.

3.1.2 | Patient II1

Patient II1 developed nephrotic syndrome in the 1960s and died at the age of 10. A renal biopsy was performed before her death and the slides were retrospectively interpreted as membranoproliferative glomerulonephritis (MPGN) in the 1970s when her siblings (II2 and II4) became ill.

3.1.3 | Patient II2

Patient II2 presented with glomerular proteinuria in childhood. Renal biopsy was reported to show MPGN. He showed no serum complement consumption. He had end-stage renal disease by the age of 20, underwent renal transplantation twice, had a cerebral stroke at age 51 and died while on hemodialysis 3 years later. The cause of graft dysfunction is unknown.

3.1.4 | Patient II4

Patient II4's proteinuria was first diagnosed at the age of 10 years. Renal biopsy was performed rapidly thereafter and reportedly showed MPGN. She developed nephrotic syndrome during adolescence and end-stage renal failure at age 19. She was on hemodialysis for 5 years and then received a kidney transplant at age 24. This patient had moderate macrothrombocytopenia, around $120–140.10^9$ platelets/liter with a high mean
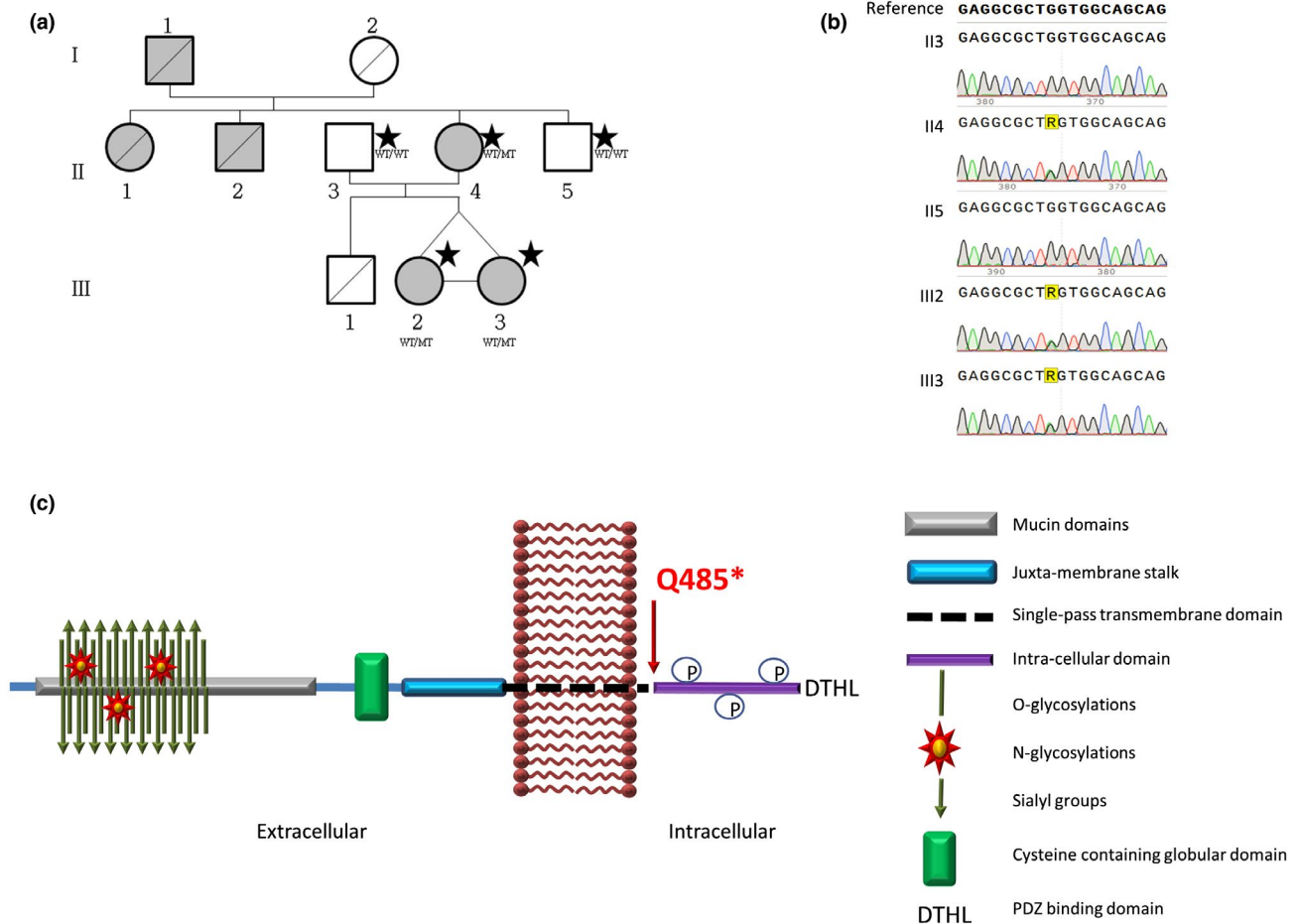
FIGURE 1 Pedigree and validation of the *PODXL* variant. (a) Family tree. Gray symbols indicate affected individuals; stars indicate subjects who underwent exome sequencing. The genotype of the *PODXL* gene is indicated as wild type (WT) and mutated (MT). (b) Sanger sequencing and intrafamilial segregation pattern of the c.1453C>T *PODXL* variant. (c) Secondary structure of the protein with localization of the p.Q485* variant (adapted from (Nielsen & McNagny, 2009a))

platelet volume around 12–14 fL. She showed platelet anisocytosis with an immature platelet fraction of 15%, (normal<4.5%) and a tendency for the formation of platelet aggregates on the slide, but without hemorrhagic disorder. These platelet abnormalities were first documented before kidney transplantation and have remained ever since. No major platelet functional defect was observed by light transmission aggregometry testing. GPIIb-IIIa, GPIb-V-IX, and p-selectin exposure after TRAP6 stimulation were normal, hence, excluding platelet functional defects such as Glanzmann thrombasthenia, Bernard–Soulier syndrome, and gray platelet syndrome (Figure S1).

Shortly after transplantation the patient had a child (III1) who died on the 5th day of life, from congenital left ventricular hypoplasia. She then had a pregnancy complicated with gestational hypertension but without recurrence of proteinuria on her kidney transplant and resulting in the birth of twin girls that were confirmed to be monozygotic after exome sequencing in the present study.

Allograft biopsy performed 11 years after transplantation on the occasion of a discrete degradation of the allograft

function did not show any sign of MPGN, nor chronic thrombotic microangiopathy, in particular no double contours or deposits on immunofluorescence. There was no recurrence of proteinuria. The renal allograft is functional up to now.

A series of specialized immunological examinations were performed due to an occasional decrease in serum complement C4 levels. Because familial MPGN was suspected, familial atypical hemolytic uremic syndrome and other pathologies affecting the alternative pathway of the complement had to be ruled out. Haptoglobin was not decreased, factor B was within the normal range and the sequencing of complement factors B, I, H, MCP, and CFHR5 revealed no abnormalities.

3.1.5 | Patients III2 and III3

The monozygous twin sisters III2 and III3 had both proteinuria and intermittent hematuria in their first decade of life. The clinical course was initially similar in both twins. None

TABLE 1 Clinical and biological characteristics of affected family members

	I1	II1	II2	II3	II4	III2	III3
Renal phenotype							
Proteinuria	Yes	Yes	Yes	Yes	Yes >3 g/day before ESRD, nephrotic syndrome	1.5–4 g/day at age 18, nephrotic syndrome	1.5–4 g/day at age 18, nephrotic syndrome
Serum creatinine (µmol/L)/ eGFR MDRD (ml/min/1.73 m²)	« Normal »	ESRD and death during childhood	ESRD at end of adolescence		ESRD in third decade	71/>90 at age 18, ESRD at age 25	65/>90 at age 18, 117/56 at age 25
Hematuria	No	NA	NA	NA	Low or none before transplantation	Low or none	Low or none
Renal ultrasound/morphology	NA	NA	NA	NA	Unknown before transplantation	fused kidney duplication	Normal
Renal histology	No biopsy was performed	Reported MPGN	Reported MPGN	Reported MPGN	Reported MPGN	Atypical FSGS: presence of double contour	Atypical FSGS: presence of double contour
Complement pathway	No hypocomplementemia	NA	No hypocomplementemia	NA	Normal before transplantation, on occasion C4 levels close to lower limit (0.14, 0.15, & 0.18 g/L)	Total complement: between 55 and 76 U/ml, C3 and C4 levels normal	On occasion C4 levels close to lower limit (0.14 & 0.19 g/L); total complement levels are normal

Abbreviations: eGFR MDRD, estimated Glomerular Filtration Rate by the MDRD (Modification of Diet in Renal Disease) equation; ESRD, end-stage renal disease; FSGS, focal segmental glomerulosclerosis; MPGN, membranoproliferative glomerulonephritis; NA, not available.

of the twins had failure to thrive. Both had proteinuria and hypertension and were treated with Labetalol at 12 years of age and Enalapril 6 months later. At the age of 12, just before Enalapril was started, proteinuria was 65–110 and 35–100 mg/kg/day in III2 and III3, respectively. At this time serum albumin was 38 and 37 g/L in III2 and III3, respectively. The hemolytic complement activity was repeatedly assessed and was normal in both sisters, serum C3 was also normal and C4 at the lower limits of the normal range.

The twins are presently 25 years old. Until 2019, proteinuria in both of them fluctuated between 1.5 and 4 g/day under the combination of Irbesartan 150 mg/day and Enalapril 40 mg/day. In 2019, individual III2 presented a sharp increase in proteinuria (>10 g/day) with nephrotic syndrome and rapid decline of the estimated Glomerular Filtration Rate (eGFR) and had to be treated by peritoneal dialysis. Meanwhile proteinuria remained at 1.5 g/day for III3. At the latest exam, the eGFR of III3 was 65 ml/min/1.73 m² according to MDRD (Modification of Diet in Renal Disease (Levey et al., 2006)). The hemogram was normal.

3.2 | Renal histology of patients III2 and III3

A renal biopsy was performed in both twins at the age of 18, with almost identical results in both, except for the percentage of sclerotic glomeruli and minor changes in immunofluorescence deposition intensity. Immunohistochemistry showed moderately abundant granular deposits in mesangial and parietal areas as well as in sclerotic glomeruli. Deposits were positive for C3 (Figure 2a), IgM and kappa and lambda light chains and in scarce amounts with a lower intensity for C1q. Light microscopy analyses showed that one-third of the glomeruli were sclerotic. There was slight hypercellularity of the mesangial axes embedded in mesangial matrix expansion, either segmental or global, without lobulation. Numerous adhesions between the glomerular tuft and Bowman's capsule (synechia) and hyaline deposits were observed, sometimes obstructing the lumen of glomerular capillaries. Upon silver staining, the walls of the capillary loops and basement membranes were thickened. Double contours were evident and prominent (Figure 2b,c). There was no extracapillary proliferation but extensive interstitial fibrosis and a large diffuse lymphoplasmocytic inflammatory infiltrate, occasionally arranged in clusters. Upon light microscopy, histological lesions were those of FSGS with additional double contours (Figure 2d).

Electron microscopy revealed early lesions that consisted of detachment of endothelial cells and podocytes from the basement membrane (Figure 3). Moreover subendothelial and subepithelial multilayered basement membranes were conspicuous, resulting in double or even triple contours of the glomerular capillary wall. There were no osmiophilic
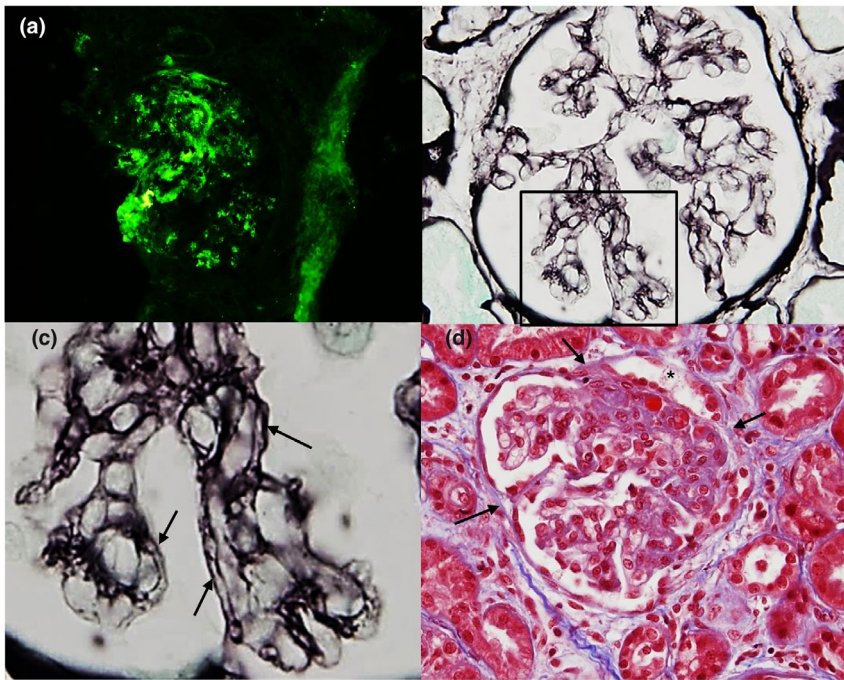
FIGURE 2 Renal histology of patient III2. (a) Immunofluorescence labeling (C3 × 400) showing the presence of intense mesangial deposits. (b) Silver staining (periodic acid—silver methenamine, × 400). (c) Magnification of the squared region of panel b showing double contours indicated by arrows. (d) FSGS features with synechiae are indicated by arrows. Mesangial sclerosis, hyaline deposits, and pseudotubular appearance (indicated by *)
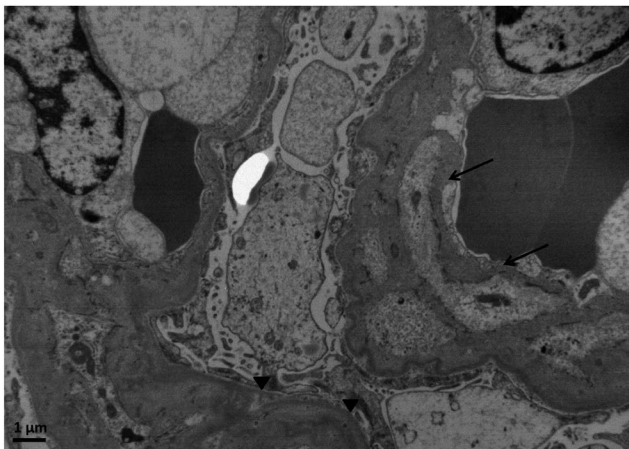

FIGURE 3 Transmission electron microscopy of the kidney biopsy of subject III3. Original magnification ×5000, focus on glomerular basement membrane in a glomerulus devoid of FSGS lesions. We notice complex rearrangements of the basement membrane. Arrows show double and even triple contours. Podocytes and endothelial cells are detached from the basement membrane

deposits in these areas but clear spaces or mesangial cell interpositions. The pathology's conclusion was a frontier form of FSGS and MPGN.

3.3 | Identification of a new *PODXL* variant by exome sequencing

Exome sequencing was performed in the parents (healthy father II3 and affected mother II4), the healthy maternal uncle (II5) and the affected twin sisters III2 and III3 (that were not known

to be monozygotic at this stage) in order to identify variants segregating with this familial disease. After data filtering (see materials and methods), four candidate variants segregated with the disease. These variants are located in the following genes: *KIF1B*, *ZC3H6*, *PODXL*, and *ETHE1* (Table S1). Among those, only *PODXL* is described to be strongly expressed in glomeruli, according to the Human Protein Atlas database (Thul et al., 2017; Uhlen et al., 2015). The *PODXL* variant is a heterozygous c.1453C>T single nucleotide variant that is shared by all affected members (Figure 1b) and is absent in healthy members, as well as in all major variant databases (ExAc, gnomAD, 1000 genomes). This variant causes a stop codon at the beginning of the intracellular portion of the protein (p.Q485*, NM_005397). The stop codon is, therefore, predicted to lead to a truncated protein without intracellular domain (Figure 1c). No rare variant segregating with the disease was found in the known MPGN-associated genes *CFH*, *CFHR5*, and *DGKE*.

3.4 | Immunostaining of PODXL

Immunostaining using anti-PODXL antibodies was performed in the affected twins III2 and III3 and in a healthy control (Figure 4). No obvious differences were observed between affected and unaffected individuals by semiquantitative analysis (Figure S2).

4 | DISCUSSION

Here, we report a three-generation family with a dominant form of early onset glomerular disease associating

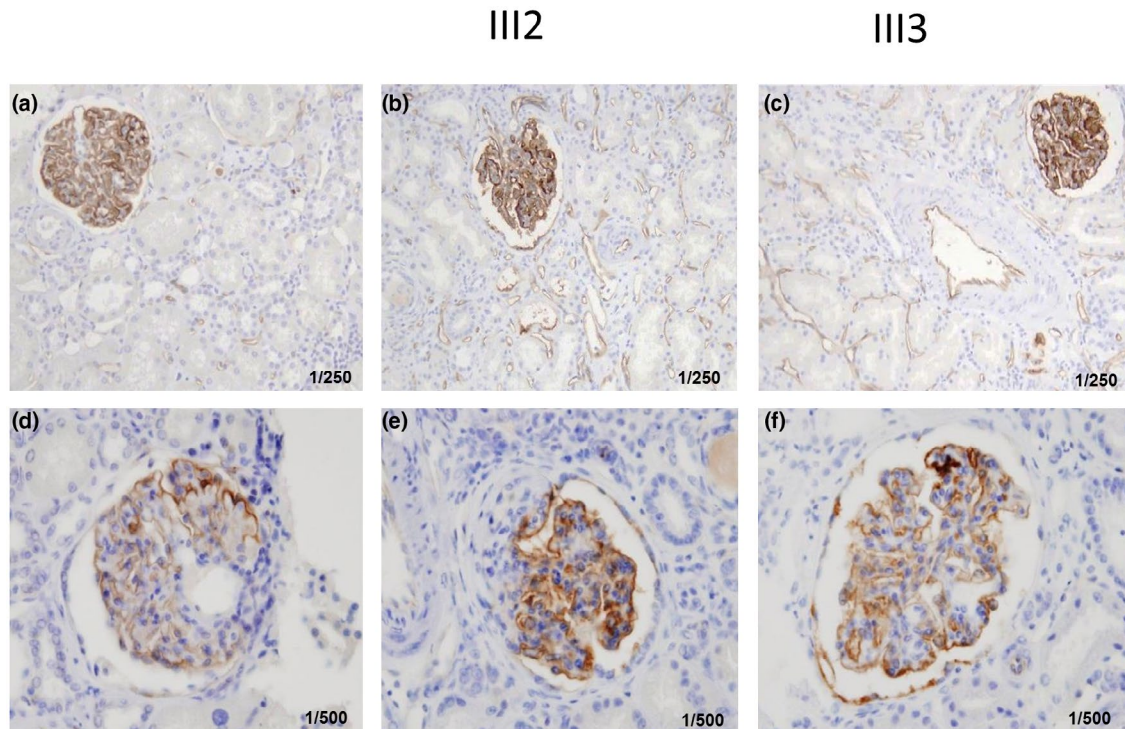III2   III3

FIGURE 4 Immunoperoxidase staining of PODXL in patients III2 and III3. A healthy control is represented in a and d. Patient III2 is shown in b and e, and patient III3 in c and f. Anti-PODXL antibodies were used at a concentration of 1/250 (a–c) and 1/500 (d–f)

histological features of atypical FSGS, double contours of the glomerular basement membrane, and proteinuria of variable intensity. We show that a new nonsense variant in the *PODXL* gene coding for podocalyxin is the likely cause of this atypical disease presentation.

Previously reported cases with *PODXL* variants confirm the role of this protein in kidney diseases. The total absence of the protein caused by compound heterozygous variants in a newborn baby with an omphalocele and nephrotic syndrome was reported to be fatal at 130 days of life (Kang et al., 2017). Copelovitch et al. reported a FSGS family in which exome sequencing revealed a heterozygous *PODXL* p.L442R variant segregating with the disease (Barua et al., 2014; Copelovitch et al., 2007). Although the clinical course of the disease was similar to the one of the present family, no double contours were reported after silver staining and the peculiar aspect of the basement membrane under electron microscopy was not shown. Very recently two other pedigrees with deleterious rare variants affecting *PODXL* were reported by Lin et al. (2019). Finally, heterozygous loss-of-function variants in *PODXL* were found in adult-onset proteinuric renal disease in pedigrees of Chinese and British-Indian origins, respectively (Refaeli et al., 2019). Both PODXL protein abundance and total mRNA were decreased in circulating blood cells of patients with end-stage renal disease in these families, as compared to controls. By blocking the nonsense-mediated mRNA decay mechanism in vitro, the authors observed increased *PODXL* mRNA and protein levels, suggesting that this mRNA degradation pathway is responsible for PODXL insufficiency and associated podocyte dysfunction caused by defects in motility and cytoskeletal organization (Refaeli et al., 2019).

Similarly to the previously published cases, we report considerable heterogeneity in disease onset, intensity of proteinuria, and consequences in terms of renal failure. A specific finding in our family is the prominent double contours of the glomerular basement membrane as shown by light and electron microscopy. In addition, podocytes and endothelial cells tend to detach from the basement membrane, suggesting that adhesion is impaired. Because PODXL is expressed in both podocytes and endothelial cells (Horvat et al., 1986), we hypothesize that the double contours could be consequences of the variant affecting the *PODXL* gene in this pedigree.

The *PODXL* variant is predicted to cause the loss of the main part of the cytoplasmic tail of the protein (Figure 1c). This highly conserved domain is known to contribute to the unique organization of the podocyte mediated by its binding to NHERF2, which in turn interacts with the protein ezrin (Takeda et al., 2001). The trimeric complex PODXL/NHERF2/ezrin is central in the maintenance of the glomerular podocyte structure as it directly interacts with the actin cytoskeleton. Loss of this interaction may, therefore, be the underlying mechanism of the disease pathophysiology in the family.

Abundance of PODXL in glomeruli was assessed semi-quantitatively using immunostaining with a polyclonal anti-PODXL antibody exclusively directed against the

extracellular portion of the protein. Glomerular staining was similar in affected patients and healthy controls. The p.Q485* variant, however, is thought to affect the intracellular portion of the protein. Whether transcription of the mutated gene to mRNA and subsequent translation to a truncated protein takes place at all is debated (Barua et al., 2014; Refaeli et al., 2019). Moreover, because our patients are heterozygous for wild-type PODXL, they still express the unaffected form of the protein.

Several elements hint toward a possible impairment of the vasculature in the patients. Patient III1 presented a congenital left ventricular hypoplasia causing neonatal death and II4 has a mitral valve prolapse discovered at the age of 20 years. Although the *PODXL* knockout mouse do not present these precise malformations (Doyonnas et al., 2001) selective *PODXL* knockout in the central nervous system was reported to lead to abnormal carotid artery morphology, and vascular permeability abnormalities (Nowakowski et al., 2010).

In conclusion, we report the first family with a frontier form of glomerular disease associating prominent double contours of the glomerular basement membrane and FSGS features segregating with a nonsense *PODXL* variant predicted to cause loss of the nearly entire intracellular part of the protein. Clinically, patients have nephrotic syndrome and end-stage renal failure and without relapse after kidney graft. This report expands the phenotypic spectrum associated to *PODXL* variants and highlights the importance to screen the *PODXL* gene for deleterious variants in similar forms associating double contours and FSGS.

ACKNOWLEDGMENTS

ETHICAL COMPLIANCE

We obtained written informed consent from all participants to collect and sequence DNA for research purposes. The research protocol was approved by the institutional review board of Strasbourg University Hospitals under the reference CPP-EST DC-2013-1990.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

David Marx, Jérôme Olagne, Arnaud Dupuis, Anne Molitor, and Raphael Carapito carried out experiments. Raphael Carapito and Anne Molitor performed computational analysis of the data. David Marx, Sophie Caillard, Bruno Moulin, and Thierry Hannedouche recruited the patients and carried out clinical phenotyping. Guy Touchard and Arnaud Dupuis analyzed microscopy results. David Marx, Sophie Caillard, Bruno Moulin, Thierry Hannedouche, Arnaud Dupuis, Christian Gachet, Anne Molitor, Seiamak Bahram, and Raphael Carapito analyzed and interpreted the data. David Marx, Sophie Caillard, Seiamak Bahram, and Raphael Carapito conceived and initiated the project. David Marx and Raphael Carapito wrote the manuscript, with input from all other authors.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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