Cubilin and amnionless protein are novel target antigens in anti–brush border antibody disease

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The kidney proximal tubule reabsorbs large amounts of low-molecular-weight proteins, albumin, electrolytes, and solutes from the glomerular filtrate to the systemic circulation. Alteration of the specialized transport systems that operate in the brush border of proximal tubule cells leads to urinary loss of these molecules and compromises homeostasis.[1,S5]

Anti–brush border antibody (ABBA) disease is a rare acquired disorder characterized by tubular injury, immune complex deposition along tubular basement membranes (TBMs), and circulating autoantibodies against proximal tubule brush borders.[2–5] The multiligand receptor megalin, also known as low-density lipoprotein receptor-related protein 2 (LRP2), was previously identified as the autoantigen in most cases of ABBA, and the disease was referred to as anti-LRP2 nephropathy.[5] Patients with anti-LRP2 nephropathy typically present with acute kidney disease and subnephrotic range proteinuria, and half of them progress to kidney failure.[5] Since the initial discovery of LRP2 as target antigen in a series of 10 patients, some additional cases have been reported, sometimes in association with other kidney disorders, such as minimal change disease and lupus nephritis.[S6,S7] Thus far, LRP2 was the only antigen described in ABBA disease. Herein, we investigated a patient with ABBA disease unrelated to LRP2, and combined immunoprecipitation, mass spectrometry, confocal imaging, and Western blot analysis to discover the identity of novel antigens in the proximal tubule brush border.

RESULTS

A 75-year-old European female patient with hypertension, type 2 diabetes, and stage G3/A1 chronic kidney disease was referred for rapid decline in kidney function, with an increase in serum creatinine from 1.3 to 2.8 mg/dl, corresponding to a decrease in glomerular filtration rate estimated by the Chronic Kidney Disease Epidemiology Collaboration equation from 41 to 16 ml/min per 1.73 m^2 over 10 months (Supplementary Figure S1 and Supplementary Table S1). Medications included olmesartan, bisoprolol, and simvastatin. There was no family history of kidney, bowel, or autoimmune disease. On physical examination, blood pressure was 135/79 mm Hg, and there was no edema. Urinalysis showed a bland urinary sediment and new-onset proteinuria (urine protein-to-creatinine ratio, 0.7 g/g;

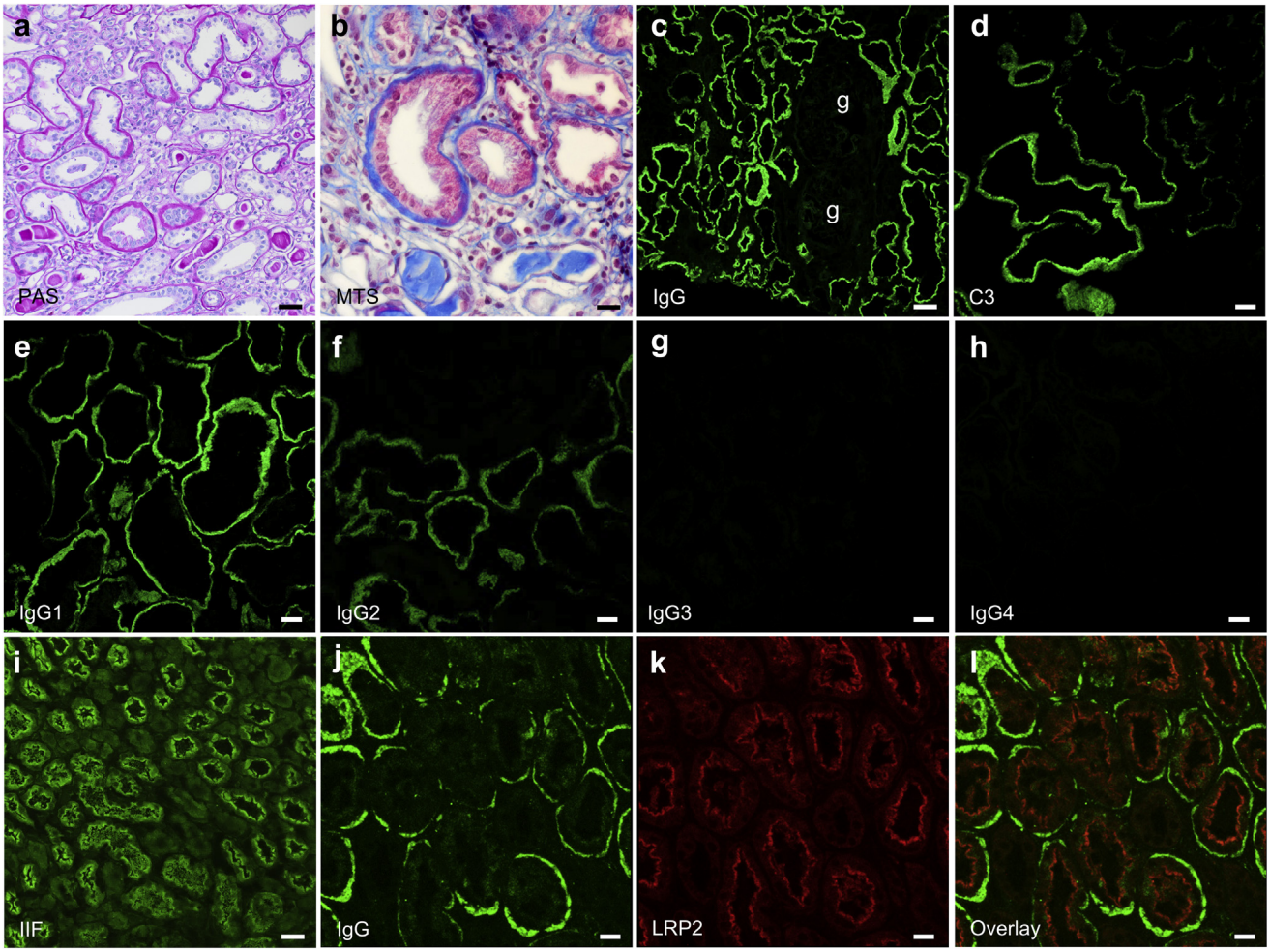

Figure 1 | Anti–brush border antibody disease not mediated by low-density lipoprotein receptor-related protein 2 (LRP2). (**a**) Light microscopy, demonstrating tubular injury characterized by loss of brush borders and thickened tubular basement membranes (TBMs) (periodic acid–Schiff [PAS], original magnification ×200), bar = 50 μm. (**b**) Light microscopy, showing fuchsinophilic deposits and thickened TBMs (Masson-trichrome stain [MTS], original magnification ×600), bar = 20 μm. (**c**) Immunofluorescence microscopy, demonstrating granular IgG positivity along TBMs with absence of staining within glomeruli (*g*; original magnification ×400), bar = 50 μm. (**d**) Immunofluorescence microscopy, showing granular C3 staining along TBMs (original magnification ×400), bar = 20 μm. (**e–h**) Representative immunofluorescence images, showing the presence of IgG1/IgG2 and absence of IgG3/IgG4 subclasses within granular TBM deposits (original magnification ×400), bar = 20 μm. (**i**) Indirect immunofluorescence (IIF), showing seroreactivity against proximal tubular brush borders of normal kidney tissue. (**j–l**) Confocal microscopy, demonstrating a lack of colocalization with IgG (fluorescein isothiocyanate; **j**) and with LRP2 (red: rhodamine red X; **k**) in TBM deposits; overlay image (**l**) (original magnification ×400), bar for all images = 20 μm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

normal, <0.2 g/g), composed of albumin, transferrin, and vitamin D–binding protein (Supplementary Figure S1 and Supplementary Table S1). Routine assessment of low-molecular-weight proteinuria and albuminuria, performed 3 years earlier, was negative. Serum levels of phosphate, uric acid, bicarbonates, potassium, and cobalamin were within normal ranges, and there was no anemia or glucosuria (Supplementary Table S1). There were no circulating antibodies to hepatitis B and C viruses or against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and SARS-CoV-2 reverse transcription–polymerase chain reaction on nasopharyngeal swab was negative. Serologic tests showed the presence of antinuclear antibodies (1:640), without anti–double-stranded DNA antibodies; complement C3 and C4 levels were normal; and serum IgG4 level was mildly elevated (0.96 g/l; normal, <0.86 g/l; Supplementary Table S1). There was no evidence of monoclonal gammopathy, systemic autoimmune disease, or known exposure to environmental toxins. Ultrasound showed small-sized kidneys (long axis, 78 and 73 mm) and absence of urinary tract obstruction. A kidney biopsy was performed.

The kidney biopsy specimen included 14 glomeruli, of which 7 were globally sclerotic. Light microscopy showed a pattern of protracted tubular injury, with tubular dilation, brush border loss, and severely thickened TBMs (Figure 1a–c and Supplementary Figure S2). Nonsclerotic glomeruli were unremarkable, and the interstitium focally showed mild inflammation and moderate interstitial fibrosis and tubular

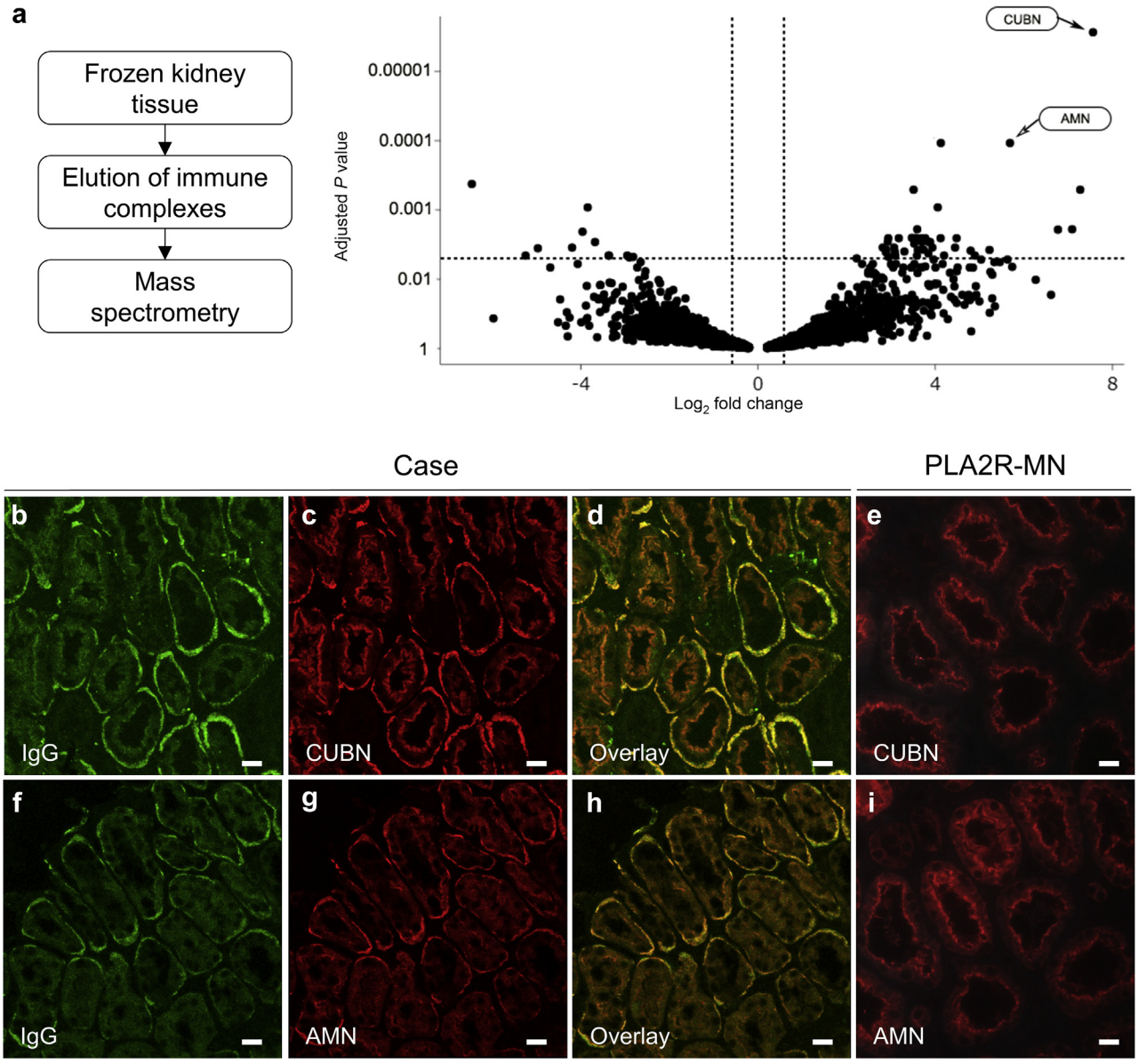

Figure 2 | Identification of cubilin (CUBN) and amnionless protein (AMN) as novel target antigens in anti–brush border antibody disease. (**a**) Volcano plot, comparing mass spectrometry (MS) of Ig eluted and captured from the patient's kidney biopsy compared with biopsy specimen from 8 patients with anti-LRP2 nephropathy. CUBN and AMN were identified as the proteins with the highest combination of adjusted *P* value and fold change. Dashed lines depict heuristic cutoffs for fold change and adjusted *P* value. Each dot on the volcano plot represents a protein detected by MS. (**b–d**) Confocal microscopy, showing colocalization of IgG (green: fluorescein isothiocyanate [FITC]; **b**) with CUBN (red: rhodamine red X; **c**) in tubular basement membrane (TBM) deposits from the case; overlay image shown (**d**) (original magnification ×400), bar for all images = 20 μm. (**e**) Representative immunofluorescence image, showing the absence of CUBN in TBM of a patient with PLA2R-associated membranous nephropathy (original magnification ×200), bar = 50 μm. (**f–h**) Confocal microscopy, showing colocalization of IgG (green: FITC; **f**) with AMN (red: rhodamine red X; **g**) in TBM deposits from the case; overlay image shown (**h**) (original magnification ×400), bar for all images = 20 μm. (**i**) Representative immunofluorescence image, showing the absence of AMN in TBM of a patient with PLA2R-associated membranous nephropathy (PLA2R-MN) (original magnification ×200), bar = 50 μm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

atrophy (26%–50%). Immunofluorescence studies showed widespread, circumferential, granular deposits along TBM of the proximal tubule, staining brightly (3+) for IgG and C3 (Figure 1c and d), without κ or λ light chain restriction. The TBM deposits stained with IgG1 (3+) and IgG2 (2+), but not with IgG3 or IgG4 (Figure 1e–h and Supplementary Figure S3). Of note, there was no immunofluorescent staining along glomerular capillary loops or Bowman's capsule (Figure 1c).

The presence of tubular injury and IgG-containing immune complexes along TBM, without any evidence for lupus nephritis or IgG4-related disease, was suggestive of ABBA disease. The patient's serum was tested using indirect immunofluorescence on normal kidney sections and showed

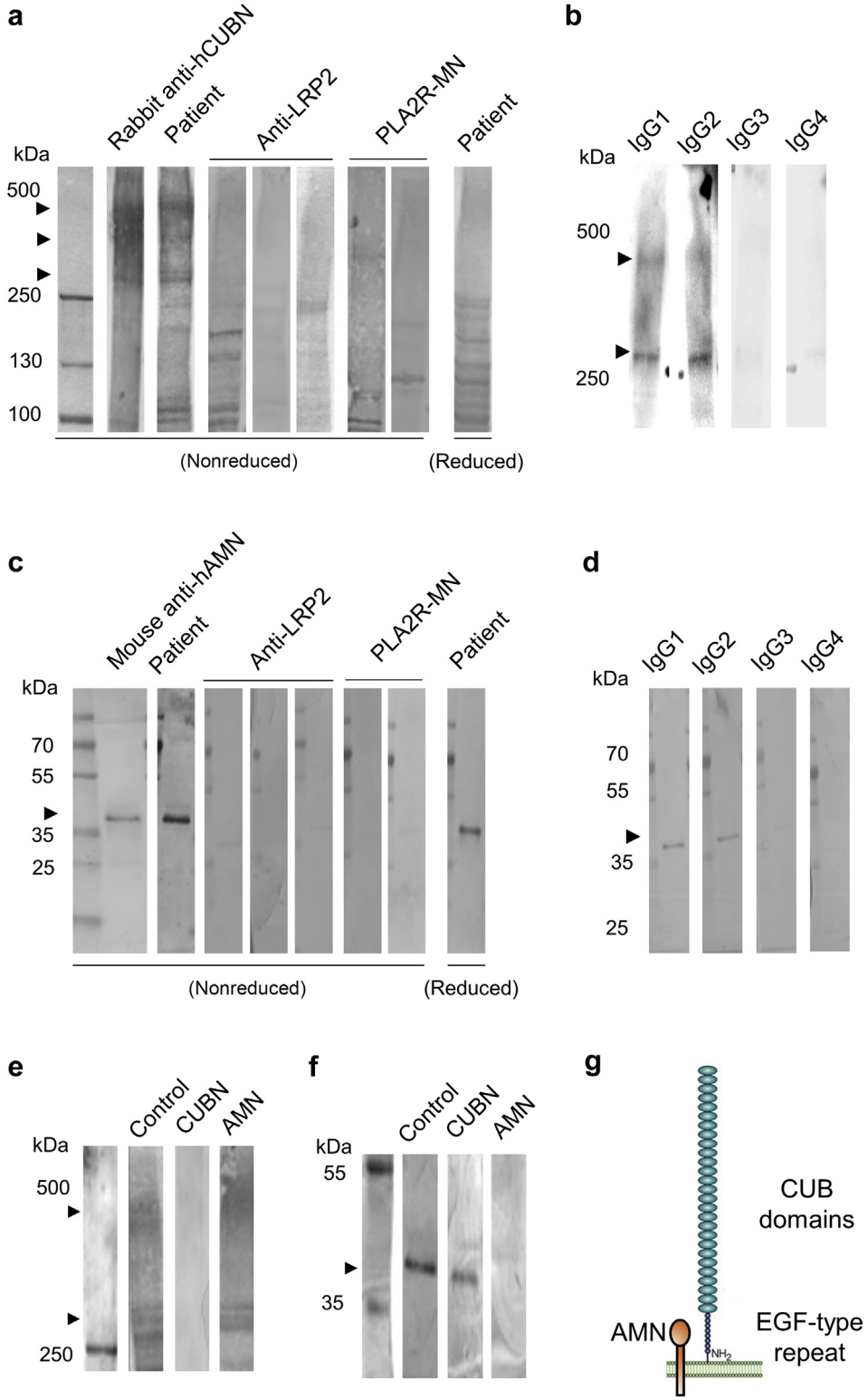

Figure 3 | Detection of circulating anti–cubilin (CUBN) and anti–amnionless protein (AMN) autoantibodies by Western blot analysis. (**a**) Under nonreducing conditions, CUBN was detected in human CUBN overexpression cell lysate using rabbit anti-human CUBN (hCUBN) antibody as a smear of high molecular weight. The same smear with 3 major components, indicated by arrows, was recognized by serum from the index patient but not by sera from other patients, including those with anti–low-density lipoprotein receptor-related protein 2 (LRP2) nephropathy. Under reducing conditions, reactivity against CUBN was lost, suggesting the patient's autoantibodies recognize conformation-dependent epitopes. (**b**) Western blotting shows that anti-CUBN autoantibodies are mainly carried by the IgG1 and IgG2 subclasses. (**c**) Under nonreducing conditions, recombinant human AMN was detected using mouse anti-human AMN (hAMN) antibody as a band at around 37 kDa. The same band was recognized by serum from the index patient but not by sera from controls. Under reducing conditions, (continued)

reactivity (1:100) against brush borders (Figure 1i). On confocal microscopy of kidney biopsy sections, LRP2 expression was restricted to the brush borders and did not colocalize with IgG immune complexes in TBM (Figure 1j–l), suggesting that circulating autoantibodies target other antigens in the proximal tubule brush border.

To identify the unknown antigens, we performed proteomic analysis of immune complexes eluted from the patient's frozen kidney biopsy tissue. Mass spectrometry of Ig captured from this biopsy identified cubilin (CUBN) and amnionless protein (AMN) as the most enriched proteins within immune complexes, compared with anti-LRP2 nephropathy samples (Figure 2a and Supplementary Figures S4 and S5). Confocal imaging showed an accumulation of CUBN and AMN in TBMs, where they colocalized with IgG deposits (Figure 2b–d and f–h), in the absence of cross-reactivity with primary antibodies (Supplementary Figure S6). Staining of TBM for CUBN or AMN was negative in biopsies from patients with other immune complex–mediated kidney diseases, including phospholipase A2 receptor (PLA2R)–associated membranous nephropathy (Figure 2e and i).

To assess the reactivity of circulating ABBA against CUBN and AMN in our patient, we performed immunoblots using human CUBN overexpression lysate or recombinant human AMN (Figure 3a–f). Serum from the patient revealed a band at about 500 kDa, the expected molecular weight for CUBN (460 kDa), and a second band at about 250 kDa, most likely a proteolytic product, that both comigrated with the bands identified by the control anti-CUBN rabbit serum under nonreducing conditions (Figure 3a). Reactivity was lost under reducing conditions, suggesting that CUBN epitopes identified by the autoantibodies were conformational. Serum from the patient also reacted against recombinant AMN (37 kDa), both under nonreducing and reducing conditions (Figure 3c). IgG subclass analysis revealed that reactivity against CUBN and AMN was only carried by IgG1 and IgG2, the same Ig subclasses detected along TBMs in the kidney biopsy specimen (Figure 3b–d). Control sera from patients with anti-LRP2 nephropathy and PLA2R-associated membranous nephropathy did not show any reactivity with the identified proteins (Figure 3a and b). There was no cross-reactivity between anti-CUBN and anti-AMN antibodies (Figure 3e and f).

DISCUSSION

Our combined clinical, pathologic, and proteomic data identified CUBN and AMN as new target antigens in ABBA disease, a rare autoimmune disorder causing proximal tubule dysfunction and kidney failure.

The 460-kDa protein CUBN and 45-kDa transmembrane AMN form an endocytic receptor complex (Figure 3g) essential for protein (e.g., albumin and transferrin) reabsorption in the kidney proximal tubule.[6,S8] CUBN was first reported as a 280-kDa protein restricted to the coated pits of the renal brush border and the epithelial cells of the yolk sac in the rat.[7] CUBN has been suggested to be an important mediator of albumin reabsorption, whereas LRP2 facilitates cellular internalization of the CUBN-albumin complex.[S9] Mutations in the *CUBN* gene cause Imerslund-Gräsbeck syndrome or megaloblastic anemia 1 (Online Mendelian Inheritance in Man number 261100), a rare inherited disorder characterized by proteinuria and malabsorption of vitamin B12,[8] and biallelic variants are associated with chronic isolated proteinuria.[S10] In our patient, new-onset albuminuria along with the presence of transferrin and vitamin D binding protein in the urine were consistent with impaired CUBN-AMN complex function in the kidney proximal tubule. Although the CUBN-AMN complex is also expressed in the intestinal epithelium, where it acts as a receptor for intrinsic factor–vitamin B12 complexes,[6] serum cobalamin levels were repeatedly normal in our patient. The reason for autoantibody formation against both proteins in this patient is unknown, although it is possible their interaction in the proximal tubular brush border could provide opportunity for epitope spreading. This hypothesis is supported by the lack of cross-reactivity between anti-CUBN and anti-AMN antibodies in cross-inhibition experiments. However, the first target of immunization and the mechanism of spreading remain to be elucidated.

Clinical presentation of our patient was similar to that reported in patients with anti-LRP2 nephropathy, including older age, rapid decline in kidney function, and tubular proteinuria. However, some pathologic findings differed, including absence of IgG4 subclass deposition and negative IgG staining on glomerular basement membranes and Bowman's capsule, which were observed in 10 of 10 (100%), 9 of 10 (90%), and 10 of 10 (100%) cases of anti-LRP2 nephropathy, respectively.[5] Predominance of IgG1 subclass in our case may indicate a different pathophysiology because these antibodies activate the classic complement pathway more efficiently and vary in Fc receptor binding compared with IgG4.[S11] Although the timing of appearance of circulating autoantibodies against CUBN and AMN is unknown, the stability of estimated glomerular filtration rate and the lack of albuminuria 3 years before the diagnosis suggest that preexisting CKD might not be related to the autoimmune disease. Unfortunately, no residual serum was available for longitudinal testing. The demonstration of a pathogenic role

Figure 3 | (continued) reactivity against AMN was conserved. (**d**) Anti-AMN autoantibodies are carried by the IgG1 and IgG2 subclasses. (**e,f**) Cross-inhibition experiments under nonreducing conditions of patient's antibody binding to CUBN overexpression cell lysate (**e**) or recombinant AMN (**f**) after serum preincubation in the absence (control) or presence of CUBN or AMN as inhibitors. In (**e**), reactivity is inhibited by CUBN but not by AMN, whereas in (**f**), the opposite is observed. (**g**) Schematic representation of CUBN and AMN. CUB, complement C1r/C1s, Uegf (epidermal growth factor–related sea urchin protein), and bone morphogenic protein 1; EGF, epidermal growth factor; PLA2R-MN, PLA2R-associated membranous nephropathy.

for autoantibodies against the brush border suggests that immunosuppressive drugs directed against B cells may help in improving outcome in patients with ABBA disease.[4,9,S12] In our patient, no immunosuppressive treatment was initiated because of the presence of chronic irreversible lesions in the kidney parenchyma, the ongoing pandemic caused by SARS-CoV-2, and delayed diagnosis. Twelve months after kidney biopsy, the estimated glomerular filtration rate had decreased to 11 ml/min per 1.73 m^2 and the patient was being prepared to start hemodialysis.

In summary, CUBN and AMN are novel antigens involved in ABBA disease that need to be considered in patients with rapid decline in kidney function, tubular injury, and IgG-containing immune complexes in the TBM. In the absence of systemic lupus erythematosus and IgG4-related disease, indirect immunofluorescence should test for the presence of ABBA, and confocal imaging on kidney biopsy is useful to assess the distribution and colocalization of LRP2, CUBN, and AMN with immune deposits in TBM. Given the identification of these novel autoantigens, we propose that ABBA disease represents a morphologic pattern rather than a single disease entity.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Methods.
Figure S1. Longitudinal changes in kidney function and characterization of proteinuria.
Figure S2. Additional light microscopy images.
Figure S3. Immunofluorescence staining for IgG subclasses in control patients.
Figure S4. Mass spectrometry coverage for cubilin (CUBN) and amnionless protein (AMN).
Figure S5. Peptide heat maps for cubilin (CUBN) and amnionless protein (AMN).
Figure S6. Confocal microscopy images of IgG and cubilin (CUBN) or amnionless protein (AMN) immunofluorescence staining in control patients.
Table S1. Laboratory (Lab) tests at the time of kidney biopsy.

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