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CASE REPORT

Downregulation of megalin, cubilin, ClC-5 and podocin in Fabry nephropathy: potential implications in the decreased effectiveness of enzyme replacement therapy

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Abstract
Fabry disease is an X-linked disorder due to mutations in α-galactosidase A, resulting in the accumulation of enzyme substrates and cell malfunction. Kidney involvement is frequent, affecting all native kidney cell types. Podocyte damage results in proteinuria and chronic kidney disease. End-stage kidney disease is the rule in middle-aged males and some females with the classic phenotype. In podocytes and kidney proximal tubular cells, megalin is one of the molecules involved in enzyme replacement therapy (ERT) cellular absorption. After podocyte damage, podocin concentration is decreased and contributes to progressive proteinuria. We report in a male and a female patient the decreased expression of megalin, cubilin, ClC-5 and podocin compared to controls and chronic kidney disease (CKD) biopsies. Moreover, the decrease in ClC-5, a molecule engaged in endosomal-lysosomal acidification, could also affect ERT. These findings may partially explain some of the dysfunctions described in Fabry nephropathy and could highlight possible alterations in the pharmacokinetics of the delivered enzyme.

Keywords Chronic kidney disease · CLC-5 · Cubilin · End-stage kidney disease · Fabry disease · Megalin · Podocin · Podocyte · Proteinuria · Synaptopodin

Introduction

Fabry disease is an X-linked disease due to mutations in α-galactosidase A, with an incidence that varies between 1 in 80,000 and 120,000 births. Kidney involvement is an important cause of morbidity and mortality in Fabry disease. Decreased glomerular podocyte mass secondary to podocyte detachment has been reported to be associated with proteinuria and to worse kidney outcomes in Fabry nephropathy (FN), mainly due to the accumulation of α-galactosidase substrates globotriaosylceramide (GB3) and lyso-globotriaosylceramide [1]. Proximal tubular cells are also affected in FN, resulting in decreased bicarbonate generation and solute malabsorption [2]. The normal integrity of both podocytes and proximal tubular cells is critical for filtered protein management and tubular reabsorption. At the tubular level protein endocytosis is a receptor-mediated process that involves the macromolecular complex composed of megalin, cubilin, amnionless, disabled-2 and ClC-5. The electrogenic chloride/proton exchanger ClC-5 is mainly expressed in proximal tubular cells but also in collecting duct α-intercalated cells, and in epithelial cells of the thick ascending limb of Henle's loop. It is localized in early endosomal and lysosomal intracellular membranes and cooperates with V-type H^+−ATPase in the endosomal acidification process [3]. In addition, ClC-5 plays a role in the recycling process of megalin and cubilin receptors from endosomal vesicles to the brush border, which is critical for normal endosomal function. Megalin, cubilin, and ClC-5 were demonstrated to also be expressed in human podocytes [4].

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We investigated the expression pattern of megalin and cubilin (involved in protein uptake) and ClC-5 in addition to podocin (a podocyte protein involved in slit-diaphragm integrity and normal function) and synaptopodin (a cytoplasmic podocyte marker) in kidney biopsies of a male and a female with classic FN, and in controls [with or without chronic kidney disease (CKD)]. Since the two Fabry patients had a concomitant pattern of focal and segmental glomerulosclerosis (FSGS), kidney biopsies with FSGS (a morphological pattern of injury with podocytic damage) were employed in our study as an additional control group to exclude CKD as one of the causes of the downregulation of the macromolecular complex.

Materials and methods

Patients

Patient characteristics are depicted in (Table 1). Two classic Fabry patients with mutation c.98A>G (D33G) are presented. Briefly, a 42-year-old male patient, stage IIIb CKD, proteinuria: 2.1 g/day and his 54-year-old sister, stage I CKD, proteinuria: 0.8 g/day due to FN underwent biopsy. The male has been receiving enzyme replacement therapy (ERT) for the last 6 years with agalsidase β (Fabrazyme, Genzyme Corporation) intravenously at 1 mg/kg body weight every fortnight, and circulating lyso-GB3 levels were 7.9 nmol/L. The female was not receiving ERT, and had lyso-GB3 levels of 6.6 nmol/L.

Two control groups were included (Table 1). The first group (CTRL) was composed of eight cortical sections obtained from nephrectomies performed for renal cancer (showing normal morphology and negative immunofluorescence) from patients with normal kidney function; the second one included four biopsies of patients with CKD showing a histological pattern of FSGS. These four patients had idiopathic FSGS. All biopsies were performed for diagnostic purposes and made available for immunolabeling studies after obtaining signed informed consent.

Control patients showed no proteinuria and normal kidney function, while FSGS patients showed a range of proteinuria from 4.07 to 12.69 g/day and were CKD stage III (Table 1).

The study was approved by Ethics for experimental studies of Azienda Ospedaliera of Padova protocol number 0027778 (29/5/2012) and by the Institutional Review Board of the Hospital Británico de Buenos Aires.

Immunohistochemistry

In order to detect ClC-5, podocin, and synaptopodin renal expression, immunohistochemistry (IHC) was conducted on formalin-fixed, paraffin-embedded sections using an indirect immunoperoxidase method. Specimens were treated as previously described [5] and incubated overnight with a rabbit polyclonal antibody against human ClC-5 (HPA000401 Sigma-Aldrich St Louis, MO, USA) diluted 1:200, with a rabbit polyclonal antibody against human podocin (H-130, sc-21009 Santa Cruz Biotechnology Santa Cruz, CA, USA) diluted 1:600, and with rabbit polyclonal antibody against human synaptopodin (Abcam 224,491 Cambridge, UK) diluted 1:1,000 in PBS at 4 °C in a humidified chamber. The specificity of the immunolabeling was confirmed by incubation without primary antibody or with nonimmune rabbit IgG (Sigma-Aldrich). Images were acquired using the Diaplan light microscope (Leitz, Wetzlar, Germany) with 20X/0.45 objective using a Micropublisher 5.0 RTV camera (QImaging, Surrey, Canada).

Table 1 Patient characteristics

	CTRL ($n=8$)	CKD ($n=4$)	Fabry ($n=1$)	Fabry ($n=1$)
Age (years)	55 (46–71)	36 (29–68)	46	54
Gender	5 M (62.5)	3 M (75)	Male	Female
sCr (mg/dL)	1.13 (0.94–1.44)	177 (1.45–2.11)	2.9	1
uProt (g/day)	N/A	9.59 (4.07–12.69)	2.1	0.8
Hypertension (%)	5/8 (62.5)	1/4 (25)	No	Yes
Diabetes (%)	1/8 (12.5)	0/4 (0)	No	No
eGFR ml/min	90 (77–101)	70 (70.8–61.3)	31	92
ACEi/ARB (%)	4/8 (50)	1/4 (25)	Yes	Yes
Immunosuppressants	No	2/4 (50)	No	No

Categorical variables are shown as number of subjects (%), continuous data are shown as median (Min–Max)

CTRL controls; *CKD* chronic kidney disease; *uProt* Proteinuria; *sCr* Serum creatinine; *eGFR* estimated glomerular filtration rate as per CKD-EPI; *ACEi/ARB* Angiotensin converting enzyme inhibitor/Angiotensin receptor blocker therapy

Immunofluorescence

To detect cubilin and megalin, immunofluorescence (IF) analyses were performed on serial sections of the same biopsies studied in IHC. Samples were treated as described elsewhere [5] permeabilizing with 0.4% Triton X-100 (Sigma–Aldrich) in PBS for 15 min at room temperature (RT), blocking with 10% normal donkey serum (Abcam) for 30 min at RT and then incubating with primary antibody sheep anti human cubilin (AF3700 R&D Discovery Systems Minneapolis, MN, USA) or rabbit anti human megalin (cod. LS-B105 LS-Bio Seattle, WA, USA) both diluted 1:100 in BSA 5% in PBS at 4 °C overnight. Sections were incubated with the appropriate fluorescent secondary antibody as previously described [6]. Nuclei were counterstained with DAPI diluted 1:1,000 in PBS for 5 min at RT. Negative controls were run by omitting the primary antibody. Images were acquired using a DMI6000CS-TCS SP8 fluorescence microscope (Leica Microystems, Wetzlar, Germany) with 20X/0.4 objective and analyzed using the LAS-AF software (Leica Microsystems).

LTA staining

To highlight proximal tubular brush border, Lotus tetragonolobus lectin (LTA) staining was performed. Kidney biopsies were treated according to the IF protocol. Following the permeabilization step, sections were incubated with LTA-FITC (Vector Laboratories, Burlingame CA, USA) diluted 1:100 in BSA 5% in PBS for 1 h at RT in a humidified chamber 5). Sections were then counterstained with DAPI diluted 1:1000 in PBS for 5 min at RT, mounted with Elvanol. LTA images were acquired using a DMI6000CS-TCS SP8 fluorescence microscope (Leica Microystems) with 20X/0.4 objective and analyzed using the LAS-AF software (Leica Microsystems).

Morphometric analysis

IF and IHC images were acquired with the same characteristic of time exposure, gain, and intensity. The IHC signal of ClC-5 and the IF signals of cubilin and megalin were quantified by morphometric analysis using the Image Pro-Plus software (Media Cybernetics Abingdon, United Kingdom). In each biopsy, cortical tubular interstitial tissue was separately evaluated from glomeruli. For ClC-5, only apical or subapical tubular staining was considered as positive. Quantities were expressed as the mean area covered by pixels (%).

Statistical analysis

To compare expression data among FN patients and the two control groups, Kruskal–Wallis test with Bonferroni's continuity correction was used. A p value < 0.05 was considered statistically significant. All analyses were conducted with R v.3.6.3 software [7].

Results

Glomerular expression

ClC-5, cubilin and megalin positive signals were observed in the glomeruli of CTRL [range 85–169 glomeruli (g)] and CKD biopsies (range 33–42 g). In particular, positivity was located in podocytes and parietal epithelial cells as previously reported [5, 8, 9] (Figs. 1a–c, 2a–c).

In FN, the podocyte expression of cubilin ($n = 15$ g) and megalin ($n = 14$ g) was highly decreased vs. CTRL (cubilin $n = 169$ g, megalin $n = 150$ g; $p < 0.001$ both) and CKD (cubilin $n = 40$ g, $p < 0.001$; megalin $n = 42$ g, $p < 0.01$) (Figs. 3b, c, 4b, c and Supplementary Fig. 1). ClC-5 was also downregulated in FN ($n = 23$ g) vs. CTRL ($n = 85$ g) and CKD ($n = 33$ g) (Figs. 3a, 4a) but it resulted significant only vs. CTRL ($p < 0.001$) (Supplementary Fig. 1). Moreover, a decrease in glomerular ClC-5 and cubilin was observed in CKD vs. CTRL ($p < 0.001$ both) (Supplementary Fig. 1).

Cytoplasmic podocyte marker synaptopodin was detected in all glomeruli of all analyzed biopsies (Figs. 1h, 2h, 3h, 4h) confirming podocyte presence, although severely damaged in the Fabry male patient. Staining for podocin (a slit diaphragm component) was almost absent in FN patients while a signal was detected in both CTRL and CKD biopsies, although less expressed in the latter (Figs. 1g, 2g, 3g, 4g).

Tubular expression

In FN proximal tubules we observed a high decrease of ClC-5 [$n = 58$ fields (f) vs. CTRL ($n = 93$ f) and CKD ($n = 62$ f) ($p < 0.001$ both)]. Moreover, a decrease in proximal tubular ClC-5 was observed in CKD vs. CTRL ($p < 0.001$). Megalin immunostaining was also downregulated in FN ($n = 27$ f) vs. CTRL ($n = 149$ f, $p < 0.001$) and CKD ($n = 54$ f, $p < 0.01$) (Figs. 1d, f 2d, f, 3d, f, 4d, f, Supplementary Fig. 1). Noteworthy, while in the female patient the ClC-5 apical signal was still present in a few tubules, this pattern was absent in the male and the positivity was mainly in the cytoplasm (Figs. 3d, 4d). In FN and in CKD proximal tubules ($n = 36$ f and $n = 63$ f respectively) we observed a decrease in cubilin expression compared to CTRL ($n = 154$ f) (Figs. 1e, 2e, 3e, 4e), although significant only for CKD ($p < 0.01$) (Supplementary Fig. 1). Megalin and cubilin proximal tubular signals disclosed only cytoplasmic expression in both FN patients (Figs. 3e, f, 4e, f).

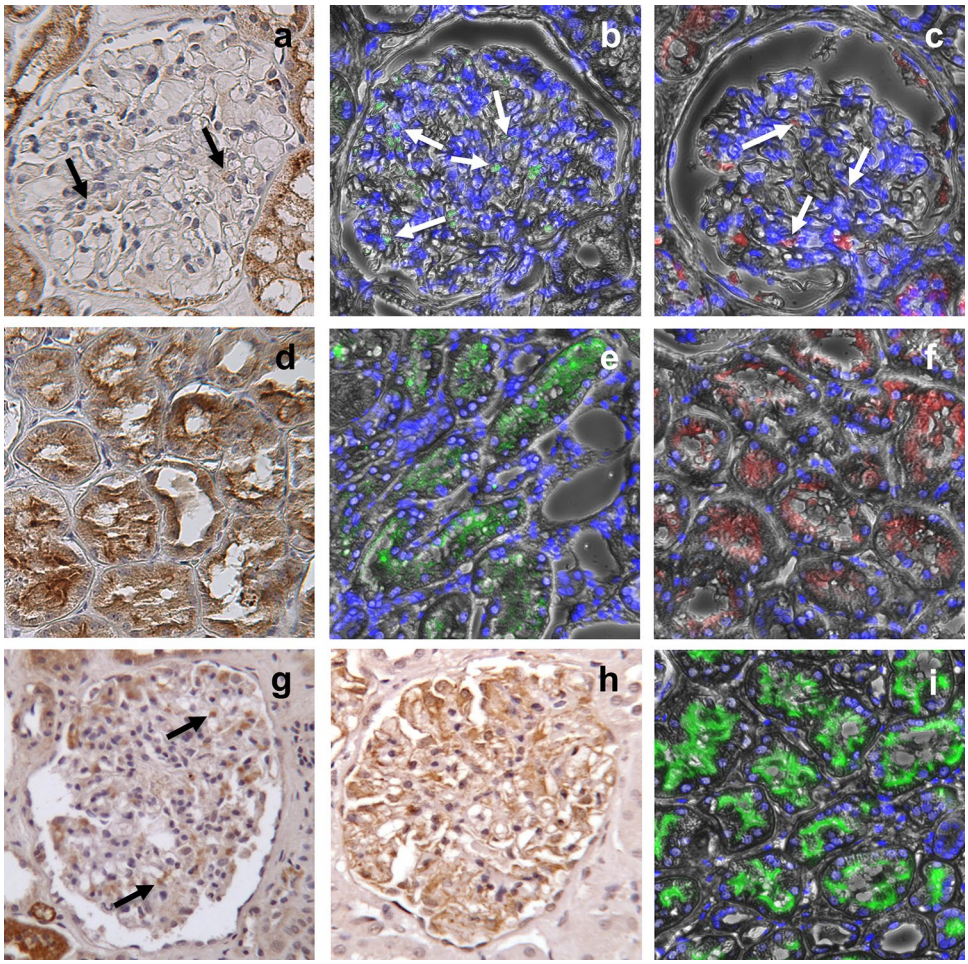

Fig. 1 Glomerular and proximal tubular expression pattern in CTRL patients. ClC-5 (**a**–**d**), cubilin (**b**–**e**), megalin (**c**–**f**), podocin (**g**), synaptopodin (**h**), LTA (**i**). Black and white arrows indicate positive cells. Brown: ClC-5, podocin or synaptopodin, green: cubilin or LTA, red: megalin, blue: DAPI

To evaluate whether proximal tubular cells retained their brush border, LTA staining was performed, and no differences were observed in FN patients compared to CTRL and CKD biopsies (Figs. 1i, 2i, 3i, 4i).

Discussion

In the present study, we encountered a significant decrease in podocyte megalin, cubilin and ClC-5 expression in FN patients compared to CTRL subjects, and of megalin and cubilin vs. CKD patients, while that of ClC-5 was lower but non-significant. However, ClC-5 was significantly lower in CKD individuals vs. CTRL group. Between FN siblings, all molecules were found to be lower in the male compared to the female, accounting for a more advanced stage of FN and a more aggressive disease pattern, as described in classic FN patients [1]. Podocyte and slit-diaphragm damage was higher in the male (Figs. 3, 4). The pattern of kidney proximal tubular expression of megalin and ClC-5 was similar to that found in the glomeruli, while cubilin was lower in FN and CKD patients, but only significantly different between the CTRL and CKD groups (Supplementary Fig. 1).

It has been reported that in FN distal tubules are mostly affected by GB3 cytoplasmic overload compared to proximal tubules [10]. However, our findings demonstrate that proximal tubules are functionally affected by the decrease of megalin, cubilin and ClC-5 (Figs. 3, 4). These proximal tubular derangements may have relevant consequences in FN. Due to the accumulation of GB3 and lyso-GB3 in lysosomes, there is a disruption of the endosomal-lysosomal structure and function in this disease. We hypothesized that this abnormality could affect the activity of ClC-5, a chloride channel Cl^-/H^+ antiporter mainly located in early endosomes where it is involved in the acidification process [3].

Moreover, since megalin and cubilin recycling from the apical brush border to the cytoplasm and back to the apical membrane of proximal tubular cells via vesicles requires ClC-5 action [11], it was conceivable to suppose that GB3 accumulation could also affect their expression.

In normal glomeruli, the expression of the three proteins was preserved, while in CKD patients due to FSGS,

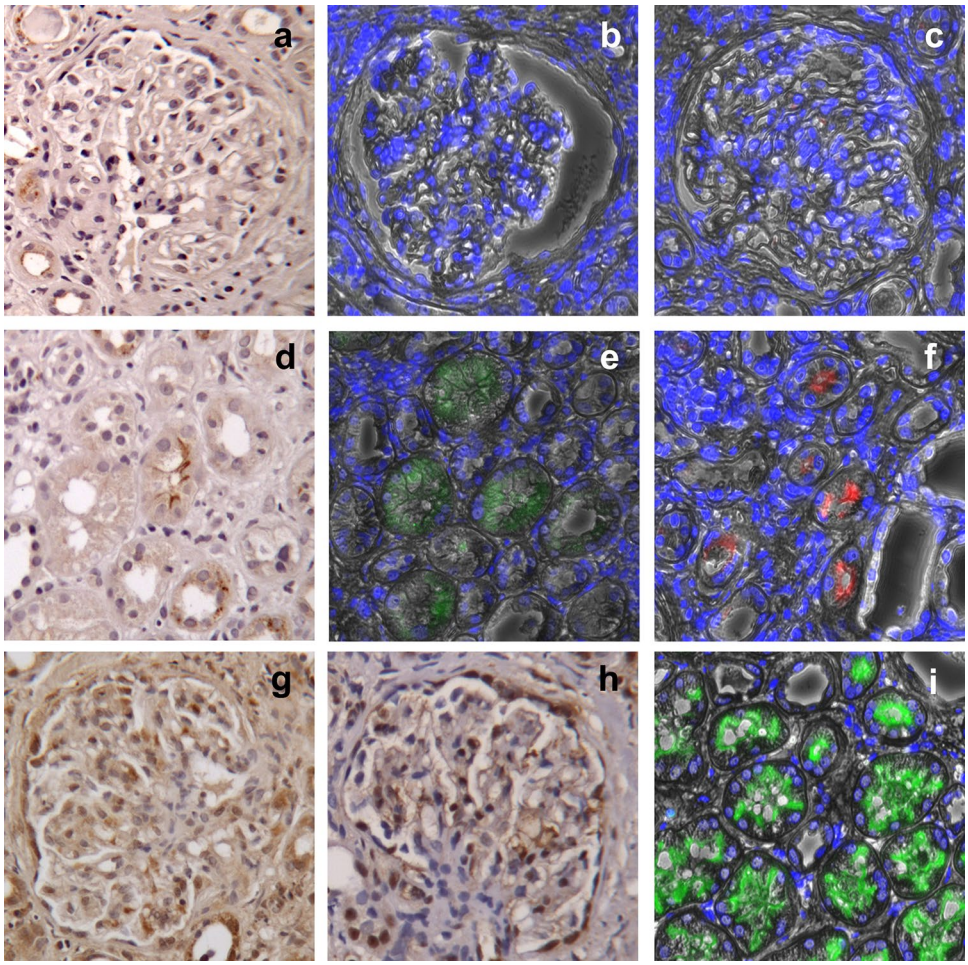

Fig. 2 Glomerular and proximal tubular expression pattern in CKD. ClC-5 (**a**–**d**), cubilin (**b**–**e**), megalin (**c**–**f**), podocin (**g**), synaptopodin (**h**), LTA (**i**). Brown: ClC-5, podocin or synaptopodin, green: cubilin or LTA, red: megalin, blue: DAPI

the expression was decreased but not like in FN patients, except for ClC-5, which was significantly lower only in CKD vs. CTRLs. Hence, we suggest that these findings may be due to the potential effects of GB3 accumulation in FN on the expression of these molecules. Finally, we also tested the podocyte mass with synaptopodin and podocin, which was severely decreased in both Fabry patients, particularly in the male. In order to demonstrate that megalin, cubilin and ClC-5 were altered in podocytes, we performed co-localization with synaptopodin, a better-preserved podocyte marker due to its cytoplasmic localization, as we previously published [12]. As expected, in normal glomeruli the staining for synaptopodin and podocin was preserved, while in FN patients the podocyte population was diminished. Interestingly, albeit in FSGS glomeruli synaptopodin displayed higher staining than podocin, podocin positive podocytes were higher than in Fabry subjects. This finding could be due to the fact that the degree of FSGS in the FN female was lower than that displayed by the male. However, the relative decrease in podocin to synaptopodin in FN patients may support previous findings reporting that the slit diaphragm is particularly damaged in FN, regardless of the FSGS pattern [13]. Finally, there is increasing evidence that demonstrates the presence of megalin in human podocytes [14]. Mice podocyte megalin appears to be involved in albumin reabsorption and its physiologic activity is counteracted by local angiotensin II, which causes transcytosis of albumin from the podocyte cytoplasm to the urinary space, resulting in albuminuria as well as sodium retention and edema formation [15, 16]. Renin angiotensin system blockade has been shown to be an effective therapy to reduce proteinuria in FN [17].

We demonstrated an important downregulation of ClC-5, cubilin and megalin in the two Fabry patients at different stages of the disease, compared to both control groups in glomerular and tubular compartments, suggesting an association between lysosomal GB3 accumulation and this macromolecular complex. With respect to cubilin, which is physiologically involved in tubular albumin reabsorption, the greater downregulation found in our CKD group may in part explain the higher degree of proteinuria encountered in primary FSGS subjects.

Recently, it has been reported that GB3 significantly influences the reabsorption of low-molecular weight proteins

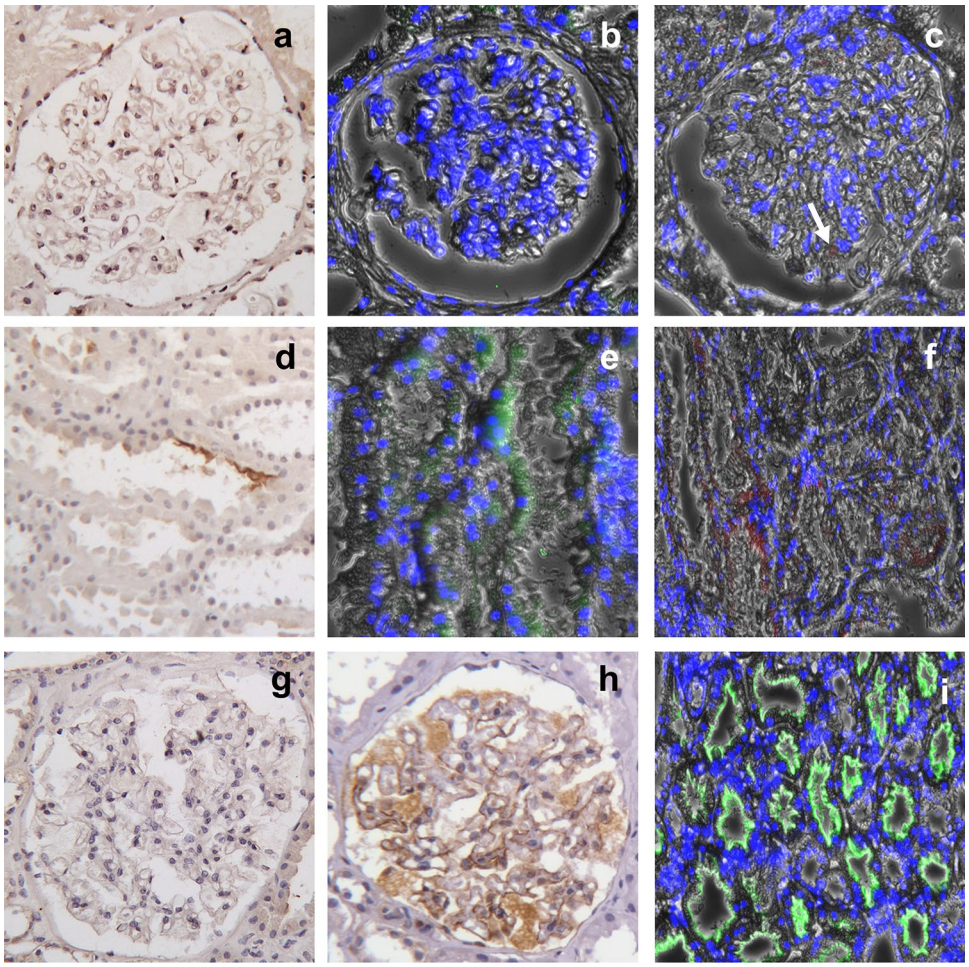

Fig. 3 Glomerular and proximal tubular expression pattern in female FN patient. ClC-5 (**a**–**d**), cubilin (**b**-–**e**), megalin (**c**–**f**), podocin (**g**), synaptopodin (**h**), LTA (**i**). Brown: ClC-5, podocin or synaptopodin, green: cubilin or LTA, red: megalin, blue: DAPI

in murine proximal tubules in vivo and in human renal proximal tubular cells in vitro [18]. It is well-known that the main actors in protein uptake by proximal tubular cells are megalin, cubilin and ClC-5 [3, 11]. In FN proximal tubules, we observed a downregulation of this system, whilst the remaining signal was mainly misplaced. In addition, the downregulation of this macromolecular complex is not related to morphological damage of the brush border of proximal tubular cells since LTA staining was maintained, further supporting our hypothesis of an accurate cross-talk between GB3 accumulation and this protein uptake machinery.

With respect to podocytes, it is worth mentioning that intrinsic renal cell injury can promote either cell proliferation, hypertrophy or podocyte detachment [19]. Like postmitotic cells, in general podocytes do not proliferate upon injury. Podocytes are severely damaged in FN. Podocyte hypertrophy is a well-described feature of FN [10].

Moreover, the irreversible loss of podocytes in the urine, a phenomenon denominated podocyturia, is also a well-described phenomenon in FN [20]. Since FN is an entity with proteinuria and heavy podocyturia, certain intracellular pathways may be involved in podocyte hypertrophy and megalin/cubilin activity. In this regard, Wilm's tumor-1 protein, which controls the expression of components of the cytoskeleton for establishing podocyte polarity, slit diaphragm structure, and attachment to the glomerular basement membrane, has been shown to co-localize with megalin and cubilin in Human FN podocytes [14, 21]. It is possible that this mechanism may be involved in the altered cytoplasmic distribution of megalin and cubilin in our two patients. Finally, in vitro studies in human podocytes with FN have demonstrated that upon lyso-GB3 stimulation, the NOTCH1 pathway (a mediator of podocyte injury) is also activated, causing podocyte inflammation and hypertrophy [22], likely interfering with megalin and cubilin activity.

The presence of synaptopodin and the decrease in podocin suggest that podocyte damage is mainly at the slit diaphragm level, probably leading to foot process effacement and secondary podocyturia [20]. Prabakaran et al. demonstrated in vitro that human podocytes and proximal tubular cells express megalin, sortilin and mannose-6-phospate receptor, which are endowed to internalize α-galactosidase A [14]. The mentioned decrease in megalin in FN patients may alter ERT efficacy.

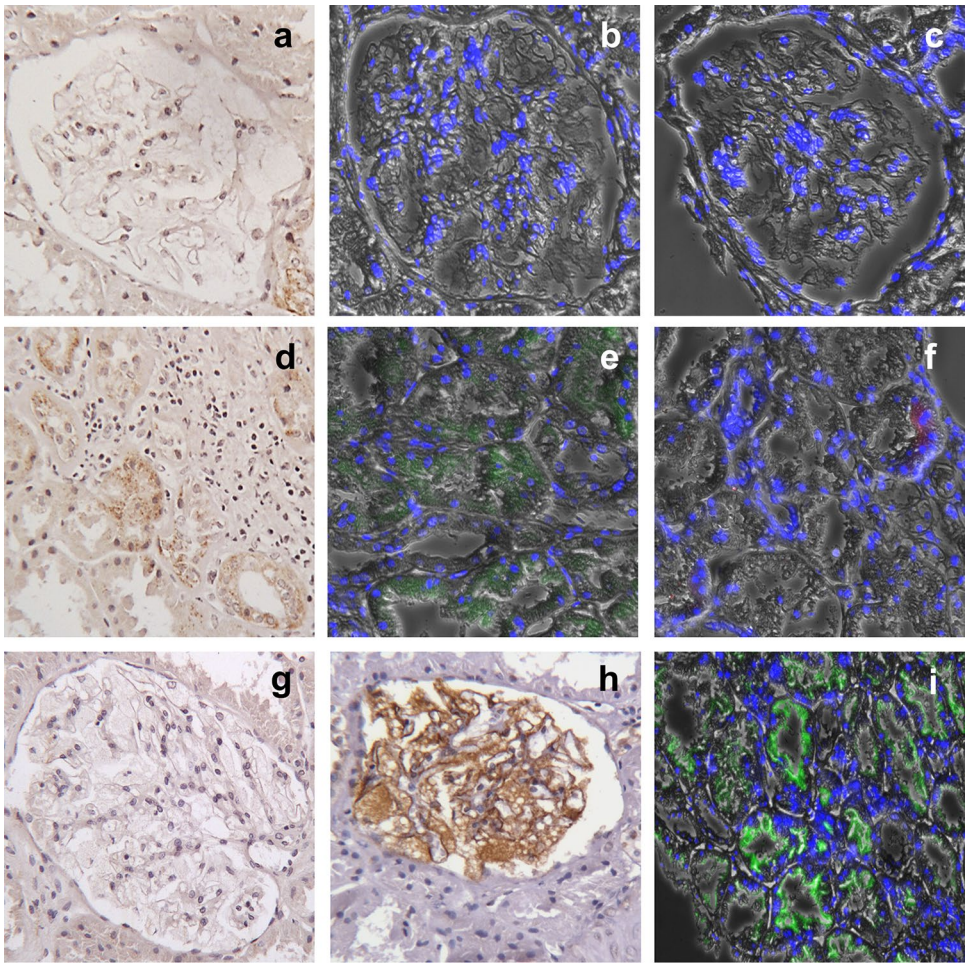

Fig. 4 Glomerular and proximal tubular expression pattern in male FN disease patient. ClC-5 (**a**–**d**), cubilin (**b**–**e**), megalin (**c**–**f**), podocin (**g**), synaptopodin (**h**), LTA (**i**). White arrow indicates megalin positive cells. Brown: ClC-5, podocin or synaptopodin, green: cubilin or LTA, red: megalin, blue: DAPI

The decrease in ClC-5 in podocytes and proximal tubular cells may also present important therapeutic consequences. After recombinant α-galactosidase A is uptaken by cells, it is stored in lysosomes, where a low pH is necessary for the enzyme activity. ClC-5 is critical in this process. Thus, besides a lower recombinant α-galactosidase A uptake by podocytes and proximal tubular cells, the activity of the enzyme may be decreased due to lower ClC-5 lysosomal content. In this regard, we speculate that a fast enzyme infusion and/or a reduced dose should be taken into consideration, particularly when patients do not respond as expected. To our knowledge, no reports on ClC-5 or cubilin involvement in ERT have been published.

One of the limitations of our work is that it is based on only two Fabry biopsies, but this is due to the fact that it is a rare disease. We believe that the inclusion of two control groups strengthens the findings of the reported research. Moreover, the findings of this report are based on immunohistochemistry and immunofluorescence, which are semi-quantitative. Properly quantitative studies need to be done to validate our preliminary and novel findings.

No robust conclusive statements can be drawn based solely on the histological findings encountered in our two Fabry patients. Real-time PCR and western blot analyses are required to support these findings. However, our novel data may suggest that in Fabry podocytes and proximal tubular cells, the recombinant α-galactosidase A altered cellular uptake due to the downregulation of megalin could lead to a potential decrease in the therapeutic efficacy. ClC-5 downregulation could also affect the activity and efficacy of the delivered enzyme. Studies with a higher number of patients are necessary to confirm our findings.

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Compliance with ethical standards

Conflict of interest Hernán Trimarchi received honoraria from Sanofi-Genzyme and Takeda-Shire. Other authors declare no conflicts of interest.

Ethical approval The present work was based on research made on human kidney biopsies and did not involve human participants and/or animals. The Institutional Review Boards of both institutions approved the present work.

Informed consent All patients signed informed consent.

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