Beyond the tubule: Pathologic variants of LRP2, encoding the megalin receptor, result in glomerular loss and early progressive chronic kidney disease

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ABSTRACT

Pathogenic variants in the LRP2 gene, encoding the multiligand receptor megalin, cause a rare autosomal recessive syndrome: Donnai-Barrow/Facio-Oculo-Acoustic-Renal (DB/FOAR). Due to the rarity of the syndrome, the long-term consequences of the tubulopathy on human renal health have been difficult to ascertain and the human clinical condition has hitherto been characterized as a benign tubular condition with asymptomatic low-molecular-weight proteinuria.

We investigated renal function and morphology in a murine model of DB/FOAR syndrome and in DB/FOAR patients. We analyzed glomerular filtration rate (GFR) in mice by FITC-inulin clearance and clinically characterized six families including nine DB/FOAR patients and nine family members. Urine samples from patients were analyzed by western blotting and biopsy material by histology. In the mouse model, we used histologic methods to assess nephrogenesis and post-natal renal structure, and contrast-enhanced magnetic resonance imaging to assess glomerular number.

In megalin deficient mice, we found a lower GFR and an increase in the abundance of injury markers such as kidney injury molecule-1 and NAGase. Renal injury was validated in patients, who presented with increased urinary kidney injury molecule-1, classical markers of chronic kidney disease and glomerular proteinuria early in life. The megalin deficient mice had normal nephrogenesis, but they had 19% fewer nephrons in early adulthood and an increased fraction of nephrons with disconnected glomerulotubular junction.

In conclusion, megalin dysfunction as present in DB/FOAR syndrome confers an increased risk of progression into chronic kidney disease.
The autosomal recessive Donnai-Barrow/Facio-Oculo-Acoustico-Renal (DB/FOAR) syndrome is caused by pathogenic variants in the \textit{LRP2} gene, encoding the receptor megalin (20). Megalin is a 600 kDa endocytic receptor present in low amounts in podocytes and abundant at the apical membrane of the renal proximal tubule. It is also located in many extrarenal epithelia such as the eye, choroid plexus, ear, and embryonic tissues, including the neuroectoderm (11, 13, 14, 22, 31, 33, 39, 40, 42, 48, 49). Consequently, patients with pathogenic variants in \textit{LRP2} exhibit a multifaceted phenotype including hypertelorism, anomalies of corpus callosum and high myopia (20). However, reports of patients with only few of the classical DB/FOAR symptoms and pathogenic variants in \textit{LRP2} have been published (2, 34, 44), suggesting a broader phenotype and potentially a higher prevalence of the disease.

The well described, clinical renal phenotype of DB/FOAR patients is a tubular defect resulting in low-molecular-weight proteinuria (20, 41), consistent with the known role of megalin as a multiligand receptor. Megalin, in concert with the receptor cubilin, reabsorbs virtually all filtered proteins from urinary loss by endocytosis in the proximal tubule (12). Patients with \textit{LRP2} pathogenic variants experience urinary loss of vitamin D binding protein (VDBP), retinol binding protein (RBP) and albumin (1, 27, 41, 46, 47), similar to mouse models with megalin deletion in the kidney (27, 46). Interestingly, urinary loss of low-molecular weight proteins is reported in DB/FOAR patients no matter of the severity of the disease, and a glomerular phenotype has been observed in a few patients (24, 35, 38). Our aim was to establish if the renal phenotype of this classical tubular disease increases the risk of a glomerular dysfunction and renal decline.

In this study, we show kidney injury and a decline in function, in a mouse model with embryonic kidney-specific deletion of megalin mimicking the human phenotype of DB/FOAR syndrome. We provide evidence that DB/FOAR patients develop glomerular proteinuria and chronic kidney disease (CKD) early in life. In the mouse model, we observe that the duration of nephrogenesis is unaffected, but that megalin deficiency results in nephron loss.
and abnormalities in the glomerulotubular junction in early adulthood. Our data suggest that megalin is not only an important tubular receptor, but that it is also required for glomerular health.
METHODS

Patients and families

Nine patients (3 months to 35 years of age) from six families were included in the study and family members were included if available (Supplementary material includes a description of each family). Each patient is identified by a two-number label e.g. 1-1, where the first stands for the family and the second for the individual. Family members are also designated “carriers” and presented with the family number and a letter (e.g. 2a). Each individual was coupled to a symbol making it possible to see the location of their pathogenic variant in the protein and their urinary protein excretion in Figure 2.

Urinary protein excretion

Spot urine samples collected from the patients were combined with a protease inhibitor cocktail (Complete; Roche, Denmark) and stored at -80°C. Urinary protein excretion was compared with urinary protein excretion in 5-9 age-matched healthy individuals. A urinary volume corresponding to four μg of creatinine (corresponding to 35 nmol) was analyzed using SDS-PAGE and transferred to an Immobilon™-FL PVDF transfer membrane (Millipore, Copenhagen, Denmark) using the iBlot™ Dry Blotting System (Invitrogen, Taastrup, Denmark). Membranes were subsequently blocked and incubated with primary and fluorophore-coupled secondary antibodies according to manufacturer (LI-COR Biosciences). Proteins were detected using the Odyssey™ infrared imager (LI-COR Biosciences, Cambridge, United Kingdom). Urinary albumin, creatinine and protein were additionally measured in certified biochemical laboratories in the specific countries.

Immunohistochemistry

Renal tissue samples collected from patients for diagnostic purposes were fixed and embedded in paraffin for routine pathology. For light microscope immunohistochemistry, sections from the patients and controls were prepared as previously described (45). Sections were incubated with a primary antibody in 0.01 M PBS, 0.1% BSA and 0.02 M NaN₃, followed by incubation with HRP-conjugated secondary antibody. Peroxidase labelling was visualized by incubation with
diaminobenzidine and 0.03% H₂O₂ for 10 min. Sections were counterstained with Meier’s
haematoxylin stain and examined in a Leica DMR microscope equipped with a Leica DFC320
camera. Images were transferred by a Leica TFC Twain 6.1.0 program and processed using
Adobe Photoshop 8.0. Fluorescence microscopy was performed by standard methods.

**Antibodies**

Primary antibodies: rabbit anti vitamin D-binding protein (A0021), rabbit anti transferrin (A0061),
rabbit anti albumin (A0001), rabbit anti retinol-binding protein (A0040), rabbit anti β2-
microglobulin (A0072), rabbit anti human IgG (A423) were all polyclonal anti-human antibodies
(Dako, Glostrup, Denmark). Goat anti KIM-1 (TIM-1, R&D, USA); goat anti mouse cystatin C
(R&D, USA); Biotinylated *Lotus tetragonolobus* (*Lotus*) lectin (Vector Labs, B-1325, USA);
rabbit anti-horse spleen ferritin antibody (Sigma Aldrich, MO, USA, F6136) and mouse anti-
synaptopodin antibody (Santa Cruz Biotechnology SC-21537). Rabbit anti rat cubilin (26); rabbit
anti human megalin (31) (kindly provided by Dr. S.K. Moestrup); and sheep anti rat megalin
(kindly provided by Dr. P Verroust). Secondary antibodies: IRDye®- (LI-COR), Alexa Fluor®-
(Invitrogen), and HRP-conjugated (Dako, Denmark).

**Animals**

Animal experiments and breeding were approved by the Danish Animal Experiments
Inspectorate and performed in the animal facility of Department of Biomedicine, Aarhus
University, Denmark. The study adheres to the NIH Guide for the Care and Use of Laboratory
animals. Female mice with homozygous conditional inactivation of the *Lrp2* gene in the kidney
were generated by breeding Tg(Wnt4-Cre)129SvE-F Tac IK or Tg(Wnt4-Cre)C57BL/6JTac
transgenic mice with mice bearing a loxP-flanked Lrp2 allele (Lrp2tm1Tew) to create embryonic
kidney specific megalin knockout (KO) mice both on a pure C57BL/6JTac – and a pure 129SvE-
F Tac IK background. Embryonic Wnt4 expression occurs in tubular cells and podocytes (36,
46). An outline of the number of animals and strain used in each experiment is given in
Supplementary Table 1. Cre-negative littermates served as controls in all experiments. The KO
degree was determined by RT-q-PCR, but when both kidneys were used for other analyses,
the KO degree was determined by immunohistochemical analyses. In general, we observed 80-95% deletion of megalin, but five animals included in the structural analyses had KO degrees in the range 40-80%.

**Mouse kidney function analyses**

GFR were investigated by the method of Rieg et al. (32), using intravenous injections of FITC-inulin per gram bodyweight and measurement of plasma clearance of 60-98 days old mice. The weight of the mice did not differ significantly. Plasma and urine creatinine were determined according to standard procedures (Siemens Diagnostics® Clinical Methods for ADVIA 1800) (Jaffe, 74016). Analyses were performed using an autoanalyzer, ADVIA 1800 Chemistry System (Siemens Medical Solutions, Tarrytown, NY 10591, USA). Relative abundance of KIM-1 in urine was measured by Western blotting according to creatinine. N-acetyl-b-D-glucosaminidase (NAGase) activity in urine was detected by an end point fluorometric method, according to Larsen et al. (25).

**Cationic ferritin-enhanced magnetic resonance imaging (CFE-MRI)**

To label the glomeruli for MRI detection, the mice received 5.75 mg/100 g body weight of horse spleen cationic ferritin (CF) (Sigma Aldrich F7879, MO) at the age of 50-70 days (4, 8). The CF was administered in 2 equal retro-orbital injections separated by 90 minutes under isoflurane anesthesia (4). All animals were euthanized 90 minutes after the last injection of CF followed by retro-aortic perfusion of Hanks buffered salt solution, followed by perfusion with 2% PFA in 0.1 M cacodylate buffer, pH 7.4. One kidney was stored whole in 2% glutaraldehyde/0.1 mol/L cacodylate solution for CFE-MRI, the other was post-fixed with formalin for paraffin embedment. The intact kidney was placed in a customized holder with the capacity to image 8 kidneys using a Bruker quadrature RF probe (inner diameter=30 mm). Imaging was performed on a Bruker 7T/30 MRI (Bruker, Co., Billerica, MA, USA) with Siemens software for acquisition and reconstruction (Siemens, Munich, Germany) at the University of Virginia (sequence parameters: 3D T2*-weighted scan: TE/TR: 20/80 ms, slice thickness: 60 µm, 640x640). The CF-labeled glomeruli appear as dark spots in the cortex of gradient-echo MR images. This
appearance is consistent with previous publications in mouse (4), rat (7), rabbit (9) and ex vivo human (6) MRI, where CF accumulates in the glomerular basement membrane (Supplementary material Figure S1).

**Image processing for number and volume of glomeruli $N_{\text{glom}}$ and $V_{\text{glom}}$**

As in previous publications (4), the images were manually segmented to separate each kidney from the remaining eight and to remove the medullary region. This allows for the measurement of cortical and medullary volumes. The resolution was increased by linear interpolation to 19.53 x 19.53 x 20 mm using Amira (FEI, Bordeaux, France) software. 3D raw data of the segmented kidney were processed in Matlab (The Mathworks, Inc. Nantick, MA, USA) to create two sets of 2D images along the x and y axes. MIPAR was used to manually adjust contrast (low level, high level, and $\gamma$ values of 2, 128, and 1.5 respectively). Kidney images were processed using an adaptive thresholding in MIPAR with a threshold of 50 percent and a window size of 15 pixels. Segmented glomeruli smaller than four voxels were rejected. The glomerular images and segmented medulla regions were analyzed with custom MATLAB scripts to obtain number of glomeruli ($N_{\text{glom}}$) and apparent volume of glomeruli ($aV_{\text{glom}}$) as previously described (4).

**Validation of CF labeling**

Immunofluorescence was performed on formalin fixed kidney tissue from the contralateral kidney that underwent MRI to confirm targeted CF labeling in the glomeruli (Supplementary material Figure S1). Kidney samples were embedded in paraffin, sectioned at four microns thick, rehydrated using Histoclear (National diagnostics, GA) and graded ethanol dilutions. Antigen retrieval was accomplished by boiling the slides in a 10 mM citrate buffer. The tissue was blocked using normal donkey serum 1:10 in 5% of BSA in PBS for 1 hour in a humidity chamber. The sections were incubated with the following primary antibodies overnight (1) rabbit anti-horse spleen ferritin antibody (concentration -1:100, Sigma Aldrich, St. Louis, MO, USA, F6136) and (2) mouse anti-synaptopodin antibody to highlight the podocytes (concentration -1:200). Secondary antibodies were applied for 2 hours at room temperature: donkey anti-rabbit Alexa 594 (1:200, Life Technologies) and donkey anti-mouse Alexa 488 (1:250, Life
To stain the nuclei, 4’,6-diamidino-2-phenylindole (DAPI) was applied and each slide was rehydrated. Images were obtained using Microscope Leica Microsystems CMS GmbH.

**Histologic assessment**

Kidney samples from the 50-70 day group were prepared by standard techniques, embedded in paraffin and sectioned. The presence of *Lotus* lectin (Vector Laboratories) was identified in by treating sections with proteinase K enzymatic digestion followed by biotinylated *Lotus* lectin (1:50 dilution) and the ABC-DAB reaction was induced. Quantitation of the proximal tubules was accomplished by analyzing the DAB reaction within each image (ImagePro Plus 5.1, Media Cybernetics, Silver Springs, MD). Renal cortical volume fraction of proximal tubules was measured using a stereologic approach as described in previous publications (18). Ten fields were photographed at 20x magnification in the subcapsular region and the DAB reaction product was expressed as a percent area value (volume fraction \(V_v\)). As *Lotus* lectin staining is specific to the mature proximal tubular cells and papillary collecting duct, the identification of the *Lotus* positive area is useful for quantifying the preservation of the renal cortex and the lack of staining present in Bowman’s capsule represents disruption of the glomerulotubular connection (“atubular glomeruli”). In essence, the latter fraction includes both real atubular glomeruli and glomeruli assessed at a plane where the junction is not visible. Comparison of the *Lotus* lectin negative glomeruli in the two groups indicates whether there is an increase in real atubular glomeruli in either group. This method has been extensively validated in serial sections (18).

**Neonatal cohort**

It is not feasible to do CFE-MRI on neonatal mouse kidneys, therefore the kidneys were prepared and stained with Periodic Acid Schiffs (PAS) to identify glomeruli. Mature glomeruli were counted in a mid-sagittal section at the completion of nephrogenesis, postnatal day four. Using Amira Software (FEI, Bordeaux, France), a 3D visualization program, the cortical area was determined from each sample. Glomerular density was defined as mature...
glomeruli/cortical area. To determine duration of the nephrogenic zone, kidney sections were stained with *Lotus* lectin to identify the presence of a nephrogenic zone. Cessation of the nephrogenic zone was defined by a lack of cap mesenchyme and the presence of *Lotus* lectin stained cells just under the capsule.

**Study approval**

The study was performed according to the Declaration of Helsinki and approved by the National Ethical Review Boards. Informed consent was obtained from all participants.

**Statistics**

For analyses of urine content in patients, a one-way ANOVA was used if the populations were normally distributed evaluated by a D’Agostino & Pearson normally test and had variance homogeneity. The number of patients in the DB/FOAR group was too small to analyze for normal distribution. If the groups were not normally distributed or did not have a positive test for variance homogeneity, a non-parametric Kruskal-Wallis test with a Dunn’s test for multiple comparisons was performed. When two groups were compared, a Students t-test was performed in case of normal distribution and a Mann-Whitney test was performed otherwise.
RESULTS

Renal injury and decline of renal function in murine megalin deficiency.

We investigated the role of megalin on renal function in a mouse model of DB/FOAR syndrome. The model is obtained by embryonic kidney-specific knockout of megalin (megalin KO) (36, 46). In the adult mice, we found a reduced kidney function evidenced by decreased GFR (mean ± SEM KO: 287 ± 25 μl/min vs. wildtypes (WT): 368 ± 26 μl/min, p=0.04) and slightly increased plasma creatinine (mean ± SEM KO: 14.4 ± 1.2 μmol/l vs. WT: 11.2 ± 0.8 μmol/l, p=0.04) (Figure 1, A and B). At this young age, the renal parenchyma from some megalin KO mice revealed areas lacking proximal tubules (Figure 1C). In megalin KO mice, we have previously reported urinary excretion of kidney injury markers such as cystatin C (30). As these are megalin ligands we also investigated injury markers in the urine that are not megalin ligands, which were also elevated in the megalin KO mice as compared to WT including N-acetyl-beta-D-glucosaminidase (NAGase: mean ± SEM KO: 0.08 ± 0.013 U/μmol creatinine vs. WT: 0.005 ± 0.0002 U/μmol creatinine, p<0.001) and kidney injury molecule-1 (KIM-1: mean ± SEM KO: 10.8 x 10^6 ± 1.2 x 10^6 AU/creatinine vs. WT: 1.5 x 10^6 ± 0.3 x 10^6 AU/creatinine, p<0.016) (Figure 1, D and E). These data suggest that megalin deficiency results in renal injury.

DB/FOAR patients demonstrate renal decline

To investigate renal status in the human counterpart of our mouse model, we examined six families with pathogenic variants in the megalin encoding gene, LRP2. Four of the six families were newly identified (Table 1, Families 1, 2, 5 and 6). All patients are homozygous for the pathogenic variant indicated in Table 1 and depicted in Figure 2A, whereas carriers are heterozygous for the variant. Clinical data from the patients showed urine protein-creatinine (UP/C) levels in the range 166-704 mg/mmol (normal <15 mg/mmol), albumin-creatinine (UA/C) levels ranging from 7–33 mg/mmol (normal <3 mg/mmol) indicating a glomerular leak and four patients had low GFR (Table 1). Analyses of patient urines showed elevated excretion of classical megalin and cubilin ligands such as RBP (megalin ligand), transferrin (cubilin ligand),...
albumin (shared ligand), cystatin C (megalin ligand) and β2-microglobulin (megalin ligand), as compared to controls and carriers (Figure 2B and Supplementary material Figure S2). In addition to low-molecular-weight ligands, we also detected a significantly increased excretion of intact (150 kDa) IgG in all patient urines, indicating an effect on the glomerular filtration barrier (Figure 2B). Thus, in addition to low-molecular-weight proteinuria the patients also demonstrated proteinuria of glomerular origin. Furthermore, elevated presence of kidney injury marker-1 (KIM-1) in all DB/FOAR patients and in three of the carriers from Family 2 (Figure 2B), suggesting renal injury and glomerular dysfunction.

All patients showed a characteristic urinary protein profile, which was different from carriers and controls (Supplementary material Figure S3). As expected, DB/FOAR patients from Families 2, 3, 4 and 6 had almost no full-length megalin excretion in the urine, consistent with the absence of megalin protein products. Surprisingly, urinary full-length megalin was also virtually absent in urines from the carriers (Figure 2B).

Analyses of biopsy material (available from Families 1, 4 and 6), showed no brush border immunoreactivity for megalin in Families 4 (41) or 6 (Figure 3A), which has also previously been shown in family 3 (15). Surprisingly, the two index patients from Family 1 had reduced, normally localized megalin (Figure 3A). Consistent with the remnant presence of the receptor in patients from Family 1, we detected uptake of ligands like RBP and albumin (Figure 3A), which was not present in Families 4 (41) or 6 (Figure 3A). Despite the presence of immunodetectable ligands in patients from Family 1, they also presented with proteinuria (Table 1; unfortunately, urine was inaccessible for further analyses). The presence of proteinuria could be caused by a combination of suboptimal reabsorption (compatible with reduced megalin) and increased glomerular protein leakage. PAS staining of kidney biopsy material from Patient 1-1 revealed chronic changes including focal glomerulosclerosis, interstitial fibrosis, inflammation and tubular atrophy (Figure 3B). In contrast, Patient 1-2 had fairly well-preserved renal parenchyma (Figure 3B), but the glomeruli from both Patients of Family 1 showed signs of advanced renal disease. Some glomeruli were sclerotic, whereas others appeared normal.
Immunofluorescence revealed immunoglobulin A deposits in all glomeruli investigated in Patient 1-2 and a more focal pattern in Patient 1-1 (Figure 3B). In summary, megalin dysfunction in DB/FOAR patients is associated with proteinuric CKD with glomerular and tubulointerstitial histological lesions.

**Nephrogenesis is normal in the megalin deficient kidney.**

Megalin is present early in nephrogenesis, which makes it possible that the fully functioning receptor is needed for proper regulation of kidney development through binding and clearing of regulating proteins such as sonic hedgehog (28, 29). To investigate the mechanism underlying renal decline, we investigated central parameters in nephrogenesis. We examined a cohort of neonatal mice (n=3/group from postnatal (PN) days 0-4) and found that cessation of the nephrogenic zone occurred on postnatal day four in both the megalin KO and WT groups as evidenced by a lack of cap mesenchyme and the presence of *Lotus* lectin stained cells just under the capsule (Figure 4A). Furthermore, we found that there was no difference in glomerular density at postnatal day four between the KO and WT mice (mean ± SEM KO: 1.3 ± 0.1/μm² x10³ vs. WT: 1.2 ± 0.27/μm² x10³, p=0.40) (Figure 4B) indicating apparent normal nephrogenesis in megalin KO mice.

**Nephron loss and disruption of the glomerulotubular junction in megalin deficiency.**

To assess if renal injury resulted in nephron loss in adulthood, we applied CFE-MRI (4, 6-8) to determine number (N_{glom}) and size (aV_{glom}) of the glomeruli in the adult kidney. We found the megalin KO group had significantly fewer glomeruli than the WT (mean ± SEM KO: 9702 ± 219; WT: 12056 ± 427, p<0.001, Figure 5A) at 50-70 days. At one year of age, the deposition of cationic ferritin in glomeruli of KO mice was virtually absent, whereas in WT the deposition appeared normal (Supplementary Figure S1) indicating a change of charge or size selectivity of the filtration barrier of megalin deficient mice. No difference in glomerular volume was observed by MRI (apparent glomerular volume (aV_{glom})) between the megalin KO and WT groups (mean ± SEM KO: 3.3 ± 0.1 mm³ x10⁻⁴; WT: 3.1 ± 0.2 mm³ x10⁻⁴, p=0.62, Figure 5B), and the intrarenal distribution of V_{glom} was unchanged (Figure 5C), indicating there was not a
population of small and large glomeruli in either group. The proximal tubule fraction was lower in the megalin KO group compared to WT measured as the area *Lotus*-positive cells in the subcapsular region (mean ± SEM KO: 42 ± 1.2% vs. WT: 46 ± 1.4%, p=0.03, Figure 5D). This is supported by MRI analyses showing smaller kidney volume in megalin KO (mean ± SEM KO: 1.1 x 10^{11} ± 5.5 x 10^9 \mu m^3 vs. WT: 1.3 x 10^{11} ± 4.7 x 10^9 \mu m^3, p=0.006, Figure 5E), smaller cortical volume in the megalin KO group (mean ± SEM KO: 6.2 x 10^{10} ± 3.1 x 10^9 \mu m^3 vs. WT: 7.7 x 10^{10} ± 2.7 x 10^9 \mu m^3, p=0.002, Figure 5F), but no change in medullary volume between the groups (mean ± SEM KO: 4.8 x 10^{10} ± 3.1 x 10^9 \mu m^3 vs. WT: 4.9 x 10^{10} ± 2.9 x 10^9 \mu m^3, p=0.8, Figure 5G). In the 50-70 days group, the health of the glomerulotubular junction was compromised; the fraction of *Lotus* negative glomeruli (true atubular glomeruli + glomeruli cut at a plane not assessing the junction) was greater in the KO than in WT (mean ± SEM KO: 32 ± 2.6%; WT: 25 ± 1.5%, p=0.048, Figure 5H). Our findings demonstrate that in a WT mouse *Lotus* lectin is not detectable in Bowman’s capsule in approximately 25% of the glomeruli, secondary to the direction of the sectioned tissue. However, in the megalin KO mice, the percentage of *Lotus* negative glomeruli is 7% higher than the WT, reflecting a population of atubular glomeruli. Thus, at 8-10 weeks of age the megalin KO mice have lost approximately 19% of their glomeruli and another 7% have an abnormal glomerulotubular junction which will likely result in their loss with time. Taken together these analyses suggest that glomerulotubular disconnection and nephron loss are a result of megalin dysfunction.
In this study, we aimed to establish if megalin deficiency or dysfunction plays a role in progressive kidney disease. We found that renal function was affected by megalin dysfunction both in our mouse model and human subjects (figure 6). The absence of megalin in mice resulted in renal decline, disruption of the glomerulotubular junction and nephron loss early in adulthood without an overt effect on nephrogenesis. To validate the renal decline observed in our mouse model in humans, we included six families with pathogenic variants in LRP2, to investigate the impact of these variants on renal health. All patients presented with tubular dysfunction, which was apparent by the urinary loss of megalin-cubilin ligands, consistent with megalin dysfunction. In addition, the patients experienced urinary loss of high-molecular-weight proteins like immunoglobulins and transferrin indicative of an affected glomerular filtration barrier. Supportive of a glomerular component of the proteinuria, the patients had urinary protein and albumin excretion which was higher than that of low-molecular-weight proteinuria. Furthermore, several patients had a low GFR (23) and a clinical diagnosis of CKD. In addition, all patients had elevated levels of urinary KIM-1. Urinary KIM-1 has been correlated with inflammation and proximal tubule injury, and has been shown to be a biomarker of renal injury and risk of CKD (37). All together our data strongly suggest that megalin dysfunction poses an increased risk of renal decline involving both the tubules and glomeruli. Our study is in line with a genome-wide association study showing that single nucleotide polymorphisms in LRP2 are associated with low GFR (10) and we speculate that milder forms of the disease might contribute to the population of patients with CKD without known aetiology. As megalin is present both in podocytes and tubules, future work will focus on the role of megalin in the glomeruli along with ligand loss as potential contributors to kidney health and their role in CKD progression.

Currently, the role of megalin in podocytes is not entirely clarified. Megalin appears to have endocytic function. It was originally described as the Heyman nephritis antigen in rats (21, 22, 31), but it has not been demonstrated to be involved in human nephritis. In Family 1
immunofluorescence revealed immunoglobulin A deposits; in Patient 1-1 all investigated glomeruli were affected, whereas in Patient 1-2 a more of a focal pattern was present. We speculate that the remnant immunoreactive megalin product may be antigenic in this family. Besides this glomerular change in this family, our patient data demonstrated that the filtration barrier was affected indicating a role of megalin in podocyte health. Significant glomerular changes were also present in our mouse model, where at one year of age the deposition of cationic ferritin in KO was almost absent as compared to controls. As CFE deposition requires a negatively charged filtration barrier and a barrier which retains it, this indicates that the filtration barrier is changed either with regards to charge or size selectivity, which points at a role of megalin in the maintenance of podocyte function. Thus, the lack of megalin in podocytes could potentially contribute to renal decline and the loss of the glomerulotubular junction.

To clarify the underlying mechanism of kidney disease, as we know that megalin is present throughout nephrogenesis (3, 33), we used a mouse model with embryonic kidney-specific megalin KO to investigate if the absence of the receptor influences nephron formation (46). We did not find any significant changes in nephrogenesis or early postnatal glomerular density, but a significant impact on renal structure, renal function and nephron abundance in early adulthood. Thus, our data suggest that nephron survival postnatally is affected in the megalin deficient state, which is in line with an earlier report of increased apoptotic cell numbers in megalin-negative cells observed by Theilig et al. in a mosaic megalin KO model (43). Further work is needed to differentiate the direct effect of the loss of megalin ligands in the urine versus the lack of the megalin receptor per se. Deficiency of megalin ligands, including the lack of uptake of antiapoptotic proteins such as survivin (19), may play a role in maintenance of the glomerulotubular connection and nephron survival. Recently, it has been shown that albumin loss as the consequence of cubilin variants does not cause kidney disease (5), indicating that the loss of albumin and potentially other cubilin ligands (which are much fewer that megalin ligands) does not affect renal health in humans (30). Thus, further work is necessary to determine if replacement of some specific megalin ligands could improve overall renal health.
or if also other yet unknown functions of megalin in both podocytes and the tubules could play a role.

The presence, although in low levels, of immunoreactive megalin and ligands in the proximal tubule of patients 1-1 and 1-2 was rather unexpected. The existence of DB/FOAR patients with megalin expression has also been reported by Kantarci et al. (20), supporting that DB/FOAR syndrome can develop despite the presence of an immunoreactive protein product. The pathogenic variant in Family 1 interferes with the YWTD repeat in an LDL class B domain changing Y into H. In the LDL receptor these repeats are involved in pH-dependent release of ligands in the endosomal compartment (16). It is therefore possible that the variant results in a protein product, but restricts ligand dissociation leading to (i) recycling of the whole ligand – receptor complex and (ii) a disturbed endocytic process. Recently, Flemming et al. (17) demonstrated that the pathogenic variant present in Family 6 interferes with receptor-ligand dissociation and causes aberrant trafficking of megalin for lysosomal degradation. A similar mechanism could play a role in the reduced megalin abundance we observed in Family 1. We cannot exclude that the remnant receptor expression in Family 1 mediates endocytosis, but that this is insufficient to avoid protein leakage into the urine combined with the presence of an affected filtration barrier leading to increased filtration.

In conclusion, our study shows pathogenic variants in \( LRP2 \) as an aetiology for early onset of CKD, which could also include patients without the advanced DB/FOAR phenotype pointing to awareness of this as a cause of CKD without a clear aetiology. We document that megalin dysfunction is associated with proximal tubular and glomerular dysfunction, disruption of the glomerulotubular junction with subsequent nephron loss, which most likely contributes to the development or progression of CKD.
AUTHOR CONTRIBUTIONS

JC, WT, SR, EIC, AC, RN: designed, and conducted experiments; collected, analyzed and interpreted data; generated the figures and co-wrote the manuscript. GD, LT, FE, FJ, JPO, LT, CF, TS: provided human material. TS, EIC AC, SN, KB, FH, SM: analyzed, collected and interpreted data, and edited the manuscript. All authors approved the manuscript.
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CONFLICT OF INTERESTS

The authors have declared that no conflict of interest exists.


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Figure legends

Figure 1.
Renal function in megalin KO- and WT mice at 60 – 98 days. (A) GFR measured by FITC inulin clearance. (B) Plasma creatinine. (C) Representative micrograph showing a megalin KO mouse section stained with *Lotus* lectin illustrating normal proximal tubules sometimes interspaced by areas lacking proximal tubules (arrow). Scale bars 50 μm. (D) Urinary NAGase excretion. (E) Urinary KIM-1 excretion. The number of datapoints equals n.

Figure 2.
Localization of pathogenic variants in megalin and urinary profile of DB/FOAR patients and carriers compared to controls. (A) Graphic depiction of megalin with domain organization and identified pathogenic variants. (B) Urinary profile of the following proteins: RBP, albumin, transferrin, β2-microglobulin, megalin, KIM-1, IgG and cystatin C. Values for each individual are showed by the symbol from Table 1 and the horizontal line indicates the mean +/- SEM. The number of datapoints equals n.

Figure 3.
Proximal tubule endocytic function and renal morphology in DB/FOAR patients and controls. (A) Immunohistochemical staining for proximal tubule receptors and ligands in DB/FOAR patients and controls. Scale bars 50 μm. (B) PAS staining and immunofluorescence analysis of IgA of biopsy material from Patient 1-1 and 1-2. Patient 1-1 displays inflammation (arrowhead), hypertrophic tubules (white arrow), tubular atrophy, and sclerotic glomeruli (black arrow). Patient 1-2 displays a more well-preserved parenchyma. Scale bars: 200 μm.

Figure 4.
Nephrogenesis in megalin KO- and WT mice at postnatal day four. (A) Micrograph of kidney sections from megalin KO and WT stained with *Lotus* lectin to identify the presence of the nephrogenic zone. Scale bars 200 μm. (B) Glomerular density at postnatal day four.

**Figure 5.**

Analyses of renal structure in megalin KO- and WT mice at 50-70 days. (A) Number of glomeruli. (B) Volume of glomeruli. (C) Distribution in percent of glomeruli volumes. (D) proximal tubule fraction in percent. (E) Total kidney volume. (F) Cortical volume. (G) Medullary volume. (H) Fraction of atubular glomeruli. In figure C, n=5 animals in each group encompassing 9-12,000 glomeruli. In all other figures n=the number of datapoints. Values for each individual mouse are showed by a symbol and the horizontal line indicates the mean +/- SEM.

**Figure 6.**

Schematic summary of the renal findings early in life of DB/FOAR patients and mice. In patients low GFR was detected, whereas urinary excretion of protein (UPC), albumin (UAC), high molecular weight proteins (Ig) and kidney injury marker, KIM-1 were elevated. In megalin KO mice we also found a lower GFR, elevated urinary excretion of kidney injury molecules (KIM-1 and NAG’ase), fewer glomeruli and increased number of atubular glomeruli (ATG).
Figure 1

(A) GFR (μl/min) comparison between WT and KO, p=0.04

(B) Plasma creatinine (μmol/l) comparison between WT and KO, p=0.04

(C) Photograph showing kidney tissue with cellular detail.

(D) NAGase Units/μmol creatinine comparison between WT and KO, p<0.001

(E) Kim-1 AU/creatinine comparison between WT and KO, p=0.016
Figure 2

**A**

- Complement-type repeat (LDL class A)
- Spacer region containing YWTD (LDL class B)
- EGF-type repeat
- Transmembrane domain
- Cytoplasmic domain
- Growth factor repeat

**B**

- Urine RBP/creatinine
- Urine albumin/creatinine
- Urine transferrin/creatinine
- Urine β2-microglobulin/creatinine
- Urine megalin/creatinine
- Urine KIM-1/creatinine
- Urine IgG (150 kDa)/creatinine
- Urine cystatin C/creatinine

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<td>Urine albumin/creatinine</td>
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<td>Urine transferrin/creatinine</td>
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<td>Urine megalin/creatinine</td>
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<tr>
<td>Urine KIM-1/creatinine</td>
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<tr>
<td>Urine IgG (150 kDa)/creatinine</td>
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<tr>
<td>Urine cystatin C/creatinine</td>
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**p-values**

- p=0.01
- p=0.007
- p=0.03
- p=0.002
- p=0.004
- p=0.001
- p<0.001
- p=0.02
- p=0.007
- p=0.001
- p=0.007
- p<0.001
- p=0.02
- p<0.001
Figure 3
Figure 6
Beyond the tubule: Pathologic variants of \textit{LRP2}, encoding the megalin receptor, result in glomerular loss and early progressive chronic kidney disease.

**OUTCOMES**

- DB patients
- Mouse model of DB syndrome

**CONCLUSION:** Pathogenic variants in \textit{LRP2} increase the risk of early onset of CKD. Disconnection of the glomerulo-tubular junction and nephron loss might be part of the underlying mechanism.
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<th>Patient</th>
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<th>Gender</th>
<th>Age</th>
<th>Ethnicity</th>
<th>Pathogenic variant (homozygous)</th>
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<th>UP/Crea. mg/mmol (ref: &lt;15 mg/mmol)</th>
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<tr>
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<td>■</td>
<td>F</td>
<td>12 Y</td>
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<td>c.7564T&gt;C p.(Y2522H) &amp; c.12623C&gt;A p.(P4208H)</td>
<td>33</td>
<td>225</td>
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<tr>
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Table 1. Biochemical - and genotype data of DB/FOAR patients and heterozygous carriers.