

# Protein endocytosis dysfunction in kidney biopsies of patients with diabetic nephropathy and minimal change disease: a pilot study on CIC-5 and the megalin receptor

Dorella Del Prete<sup>1,†</sup>, Monica Ceol<sup>1†</sup>, Elena Sgrò<sup>1</sup>, Claudia Maria Radu<sup>2</sup>, Giovanna Priante<sup>1</sup>, Francesca Katiana Martino<sup>1</sup>, Federica Stefanelli<sup>1</sup>, Aleandro Iotti<sup>3</sup>, Pablo Young<sup>4</sup>, Federico Nalesso<sup>1</sup>, and Hernan Trimarchi<sup>5</sup>

<sup>1</sup>Kidney Histomorphology and Molecular Biology Laboratory, Nephrology Unit, Department of Medicine—DIMED, University of Padua, Padua, Italy

<sup>2</sup>Division of Thrombotic and Hemorrhagic Disease, Department of Medicine—DIMED, University of Padua, Padua, Italy

<sup>3</sup>Pathology Service, Hospital Británico de Buenos Aires, Buenos Aires, Argentina

<sup>4</sup>Internal Medicine Service, Hospital Británico de Buenos Aires, Buenos Aires, Argentina

<sup>5</sup>Nephrology Service, Hospital Británico de Buenos Aires, Buenos Aires, Argentina

\*Corresponding author: Del Prete Dorella, MD, PhD, Nephrology Unit, Department of Medicine—DIMED, University of Padua, Via Giustiniani 2, Padua 35023, Italy ([dorella.delprete@unipd.it](mailto:dorella.delprete@unipd.it)).

†Del Prete D and Ceol M contributed equally to this work.

## Abstract

**Background** Diabetic nephropathy (DN) is a major cause of chronic kidney disease. Proteinuria is a hallmark of DN. An active receptor-mediated pathway involving megalin and cubilin receptors and the Cl<sup>−</sup>/H<sup>+</sup> antiporter CIC-5 is responsible for filtered albumin uptake in the proximal tubular epithelium. The aim of our pilot study was to analyze the patterns of megalin and CIC-5 expression in the kidney biopsies of patients with DN, minimal change disease (MCD), and controls, and their correlations with kidney dysfunction.

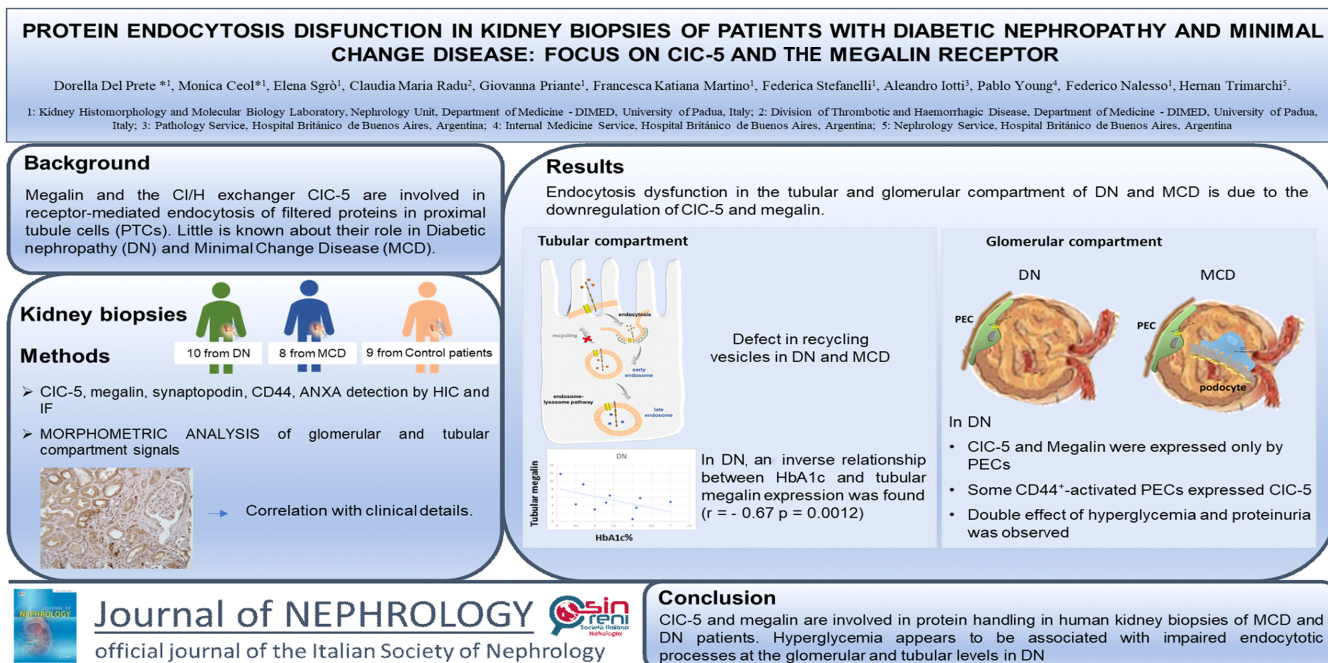
**Methods** Kidney biopsies of 10 DN and 8 MCD patients, and 9 controls (CTRs) were analyzed. Immunohistochemical and immunofluorescence staining, and morphometric analysis were performed to quantify megalin, CIC-5 and synaptopodin signals.

**Results** The CIC-5 glomerular signal was significantly reduced in DN and MCD compared with controls. In MCD, CIC-5 was detected in podocytes and parietal cells, while in DN it was detected only in parietal cells and colocalized with CD44 in hypertrophic parietal cells (ANXA3+). Megalin was also significantly reduced in DN compared with controls, and the correlation between CIC-5 and megalin was lost at the glomerular level. At the tubular level, the CIC-5 signal was reduced in DN and MCD compared with controls. In both glomerulopathies, tubular CIC-5 and megalin expression correlated with proteinuria and kidney function. In DN, glycosylated hemoglobin inversely correlated with tubular megalin expression.

**Conclusion** We found impairment of the endocytic process in DN and MCD patients. In patients with DN, hyperglycemia appeared to be associated with an altered endocytic process at the glomerular and tubular levels.

**Keywords** CIC-5, diabetic nephropathy, kidney biopsies, megalin, MCD

## Graphical abstract



## Introduction

Diabetic nephropathy (DN) is a serious, long-term complication in diabetic patients, causing damage to both the glomerular and tubular interstitial compartments. Among the many structural derangements and alterations, podocyte dedifferentiation and epithelial-mesenchymal transition are key drivers that lead to irreversible parenchymal damage and renal fibrosis, eventually resulting in kidney failure.<sup>1</sup> The earliest clinical biomarker of kidney damage identified to date is microalbuminuria (>30 mg/day), which progresses to macroalbuminuria (>300 mg/day), leading to a decline in the glomerular filtration rate (GFR). Proteinuria level is currently the best surrogate marker of kidney prognosis.<sup>2</sup> In this respect, albuminuria is usually used as a marker of glomerular injury in glomerular diseases, and has been recognized as an independent risk factor for both renal and cardiovascular disease, particularly in diabetic patients.<sup>3</sup>

Proteinuria is a hallmark of DN and develops as a result of increased permeability of the glomerular capillary wall and/or decreased proximal tubule endocytosis of filtered proteins. DN is also viewed as a glomerular disease caused by hyperfiltration in response to high glucose levels, among other variables, although tubulointerstitial injury is also an important factor in the progression of kidney disease.<sup>4</sup> Proximal tubular cells can reabsorb substantial amounts of the filtered albumin through an active receptor-mediated pathway that involves the megalin and cubilin receptors and the Cl<sup>-</sup>/H<sup>+</sup> antiporter CIC-5. The endocytic process at the tubular level is well known, but little is known about its involvement in DN in humans. To our knowledge, the only data reported in the literature concern studies in animal models.<sup>5</sup> The aim of our study was to analyze the interconnection between the expression of megalin and CIC-5, and the consequent kidney damage in both the glomerular and tubular compartments of human

diabetic kidney tissue. In order to assess any potential effect of hyperglycemia on the expression of megalin and CIC-5, we also analyzed kidney biopsies from patients with minimal change disease (MCD), who presented matched proteinuria levels non-different to those of DN patients, and controls.

## Methods

### Patients

Serial sections of human kidney biopsies from 10 patients with DN and 8 patients with MCD were analyzed for immunolabeling studies. Nine control human kidney biopsies containing cortex areas obtained from nephrectomies for renal cancer (sites remote from the tumor-bearing renal tissue) with normal morphology and negative immunofluorescence were also analyzed. Patients' clinical characteristics are reported in Table 1. All patients in the MCD and DN groups were on renin-angiotensin-aldosterone system (RAAS) inhibitors. This pilot study was approved by Padua University Hospital's Ethical Committee, protocol 0007452 (February 1st, 2018) and by the Institutional Review Board of the Hospital Británico de Buenos Aires. All patients gave informed consent.

All kidney biopsies from controls and of patients with proteinuria due to DN or MCD were immunostained for Annexin A3 (ANXA3), megalin, CIC-5, and synaptopodin. The latter molecule was used to identify podocytes. Serial sections from the biopsies of selected patients showing parietal epithelial cells with a hypertrophic appearance were characterized for CD44 and ANXA3 expression.

## Immunohistochemistry

Immunohistochemistry was conducted on formalin-fixed, paraffin-embedded sections using an indirect immunoperoxidase

method. Specimens were treated as previously described.<sup>6,7</sup> Antigens were retrieved by heating the sections in a microwave oven (in 5 mM citrate buffer solution, pH 6.0, at 500 W for 5 min, 250 W for 5 min, and 500 W for 5 min) and incubating overnight at 4 °C in a humidified chamber with rabbit polyclonal anti-human CIC-5 antibody (Code HPA000401, Atlas Antibodies, Stockholm, Sweden) diluted 1:100 in phosphate buffered saline, rabbit polyclonal anti-megalin antibody (Code HPA005980, Atlas Antibodies, Stockholm, Sweden) diluted 1:150 in phosphate buffered saline, and with rabbit polyclonal antibody against human synaptopodin<sup>8</sup> (Abcam 224491 Cambridge, UK) diluted 1:1000 in phosphate buffered saline. The sections were then rinsed and treated with Polyview<sup>®</sup> Plus HRP anti-rabbit (ENZO Life Science, Switzerland) in a humidified chamber at room temperature for 1 h. The signal was visualized with 3,3'-diaminobenzidine-tetrachloride (DAB; DAKO Corporation, Carpinteria, CA, USA), and the sections were counterstained with hematoxylin.

Immunolabeling specificity was confirmed by incubating the samples without any primary antibody. Images were acquired with a Micropublisher 5.0 RTV camera (Teledyne QImaging, Surrey, BC, Canada) on top of a Diaplan light microscope (Leitz, Como, Italy) with a 20X/0.45 lens.

Immunofluorescence

Immunofluorescence analyses were performed on serial paraffin-embedded sections of the kidney biopsies. Samples were treated as previously described<sup>9</sup> and incubated overnight at 4 °C in a humidified chamber with specific primary antibodies diluted in phosphate buffered saline, 5% BSA: mouse anti-human CD44 (clone HI44a; Code 44PU2-O1MG, Immunostep) diluted 1:20; rabbit polyclonal anti-human ANXA3 (Code HPA013398 Sigma-Aldrich) diluted 1:500. Alexa Fluor 488 goat anti-mouse IgG (1:1000; ThermoFisher Scientific, Rockford, USA), and Alexa Fluor 594 goat anti-rabbit (H + L) (1:500; ThermoFisher Scientific, Rockford, USA) were subsequently used as secondary antibodies. Nuclei were counterstained with DRAQ5 (ThermoFisher Scientific, Rockford, USA). Negative controls were performed by omitting the primary antibodies.

Signal acquisition

All sections were stained and analyzed simultaneously to exclude artifacts due to variable decay of the fluorochrome. Images were

acquired with a DMI6000CS microscope and analyzed with differential interference contrast and fluorescence (Leica Microsystems) using 40X/0.6 and 63X/1.4 dry and oil immersion lenses, respectively. Samples were acquired with a DFC7000T camera and analyzed using the Leica Application Suite X (LAS X) software (Leica Microsystems).

Morphometric analysis

Immunohistochemistry images were acquired with the same time exposure, gain, and intensity. The signal was acquired for all fields and by selecting all glomeruli from each biopsy: 69 glomeruli for CIC-5, 51 for megalin, and 53 for synaptopodin in DN; 78 glomeruli for CIC-5, 59 for megalin, and 52 for synaptopodin in MCD; 100 glomeruli for CIC-5, 78 for megalin, and 85 for synaptopodin in controls. The immunohistochemical signals of CIC-5, megalin, and synaptopodin were quantified by morphometric analysis using the Image Pro-Plus software (Media Cybernetics, Abingdon, United Kingdom) at 200X magnification. For each biopsy, cortical tubular interstitial tissue was evaluated separately from the glomeruli. Thresholds for positivity were defined by selecting 10 tubular fields and 10 glomerular fields for the five different control biopsies, and incubating the samples without any primary antibody for each secondary antibody. For CIC-5 and megalin, only apical or subapical tubular staining was considered positive. Quantities were expressed as the mean area covered by pixels (%).

Clinical data

The main patient characteristics and clinical data are reported in Table 1. Patients with absence of cardiovascular disease, chronic kidney disease as per historical and laboratory data, and diabetes mellitus were included in the control group. Diabetic and MCD patients were taking the following blood pressure medications: ACEIs (angiotensin-converting enzyme inhibitors), ARBs (angiotensin receptor blockers), beta-blockers, calcium channel blockers, and alpha-1 antagonists. All DN and MCD patients were on RASi (renin-angiotensin system inhibition). Rosuvastatin and atorvastatin were administered for dyslipidemia. Three of the controls were on RASi.

Insulin, metformin, gliclazide, vidagliptine, and linagliptine were prescribed for glycemic control. The glycated hemoglobin (HbA1c)

Table 1 Patients' clinical characteristics.

|                                 | CTR (n = 9)      | DN (n = 10)      | MCD (n = 8)       |
|---------------------------------|------------------|------------------|-------------------|
| Age (years)                     | 55 (46–71)       | 63 (47–78)       | 48 (17–83)        |
| Gender (male, M)                | 7                | 7                | 4                 |
| sCreat mg/dL                    | 1.13 (0.93–1.44) | 1.65 (1.26–3.39) | 0.91 (0.43–2.42)  |
| uProt g/day                     | NA               | 5.7 (0.98–13.59) | 7.40 (2.18–14.78) |
| HbA1c%                          | NA               | 5.35 (4.1–7)     | NA                |
| Systolic Blood Pressure (mmHg)  | 128 (114–146)    | 138 (132–158)    | 134 (130–150)     |
| Diastolic Blood Pressure (mmHg) | 82 (72–94)       | 94 (84–98)       | 88 (82–92)        |
| Serum cholesterol (mg/dL)       | 194 (182–210)    | 224 (174–253)    | 228 (180–266)     |

Data are reported as medians and ranges (min-max). CTR: controls; DN: diabetic nephropathy; M: male; MCD: minimal change disease; sCreat: serum creatinine; uProt: urinary protein excretion; HbA1c: glycated hemoglobin; NA: not available.

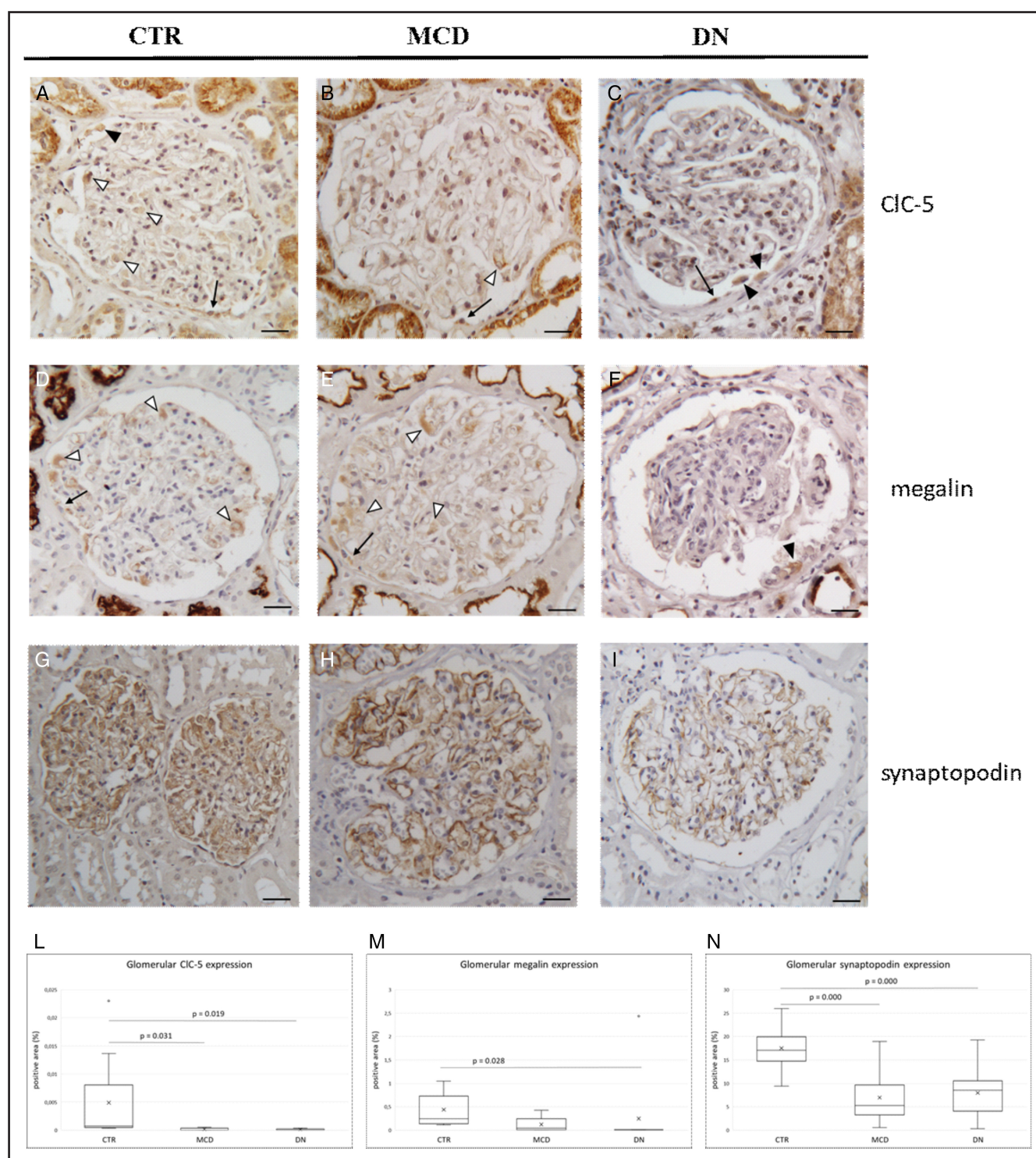


**Figure 1 CIC-5, megalin, and synaptopodin expressions in the glomerular compartment.** Representative CIC-5 (A, B, C), megalin (D, E, F) and synaptopodin (G, H, I) immunohistochemical signals (brown DAB) obtained by the indirect immunoperoxidase method in control (CTR), minimal change disease (MCD) and diabetic nephropathy (DN) biopsies. Rabbit polyclonal anti-human CIC-5 antibody (Code HPA000401), rabbit polyclonal anti-megalín antibody (Code HPA005980) and rabbit polyclonal antibody anti-human synaptopodin (Abcam 224491). CIC-5 and megalín signals were detected in podocytes (white arrowheads), flat parietal cells (PECs) (black arrows) and hypertrophic PECs (black arrowheads) in CTRs (A, D) and MCD (B, E), while in DN (C, F) the biopsies revealed CIC-5 and megalín signals only in PECs, both flat (black arrows) and hypertrophic (black arrowheads). Nuclei were counterstained with Mayer's hematoxylin (blue image). Magnification 20X/0.45. Scale bar 10  $\mu$ m. CIC-5, megalín and synaptopodin protein quantification in CTR, MCD and DN biopsies by morphometric analysis: CIC-5 expression was significantly reduced in MCD and DN compared with CTR (J); megalín deposition was significantly reduced in DN compared with CTR (K); synaptopodin deposition was significantly reduced in MCD and DN compared with CTR (L).

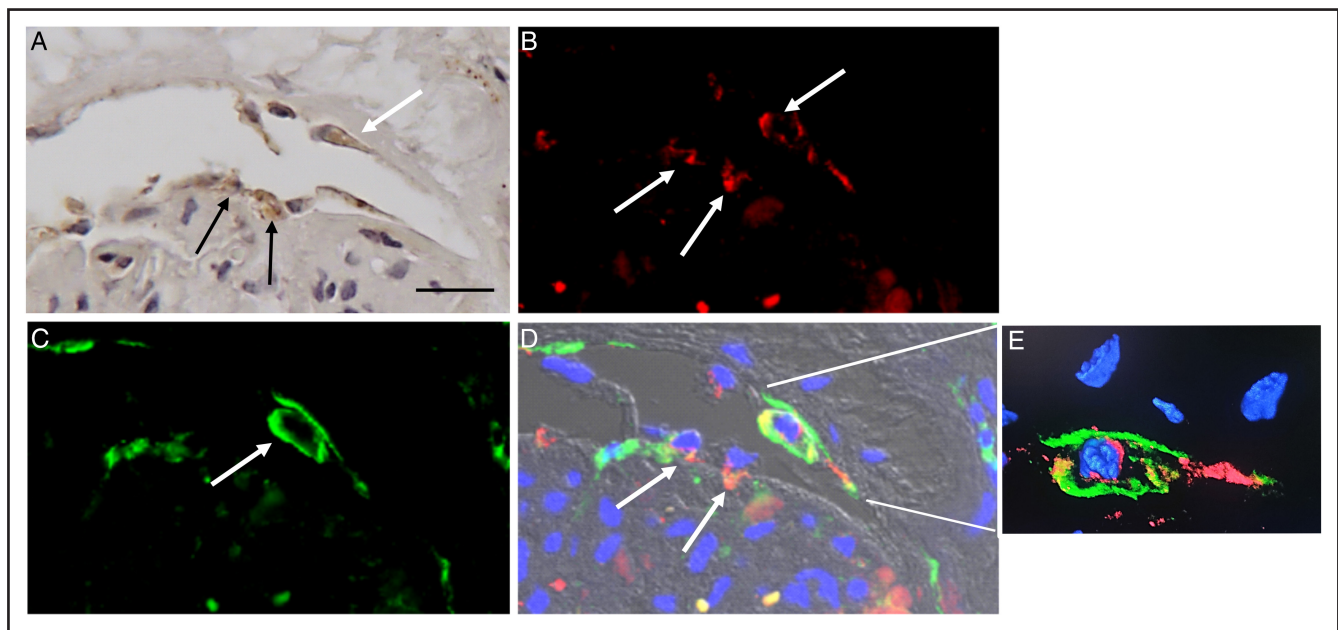


**Figure 2 Phenotypic characterization of parietal cells in diabetic nephropathy kidney biopsies.** Representative images of serial sections of glomerulus with hypertrophic parietal cells (PECs). Indirect immunohistochemistry staining showing a positive signal for CIC-5 (A) in hypertrophic PECs (white arrow), and in some PECs detected in the glomerular tuft (black arrows) (brown: DAB). Rabbit polyclonal anti-human CIC-5 antibody (Code HPA000401), magnification 20X/0.45. Indirect immunofluorescence (IF) staining of ANXA3+ (red: CFL647) (B), CD44 (green: Alexa Fluor 488) (C), and their co-localization in PECs (yellow/orange) (D) magnification 40X/0.6, (E) magnification 63X/1.4. Rabbit polyclonal anti-human ANXA3 (Code HPA013398) and mouse anti-human CD44 (clone HI44a). Nuclei were counterstained with DR (IF images, blue), and Mayer's hematoxylin (immunohistochemical image, blue). Scale bar 10  $\mu$ m.

levels reported in this study were obtained from blood samples taken on the same day as the kidney biopsy, in accordance with local site policies. We were confident that the measurements obtained on the biopsy date would be representative of metabolic control of diabetes to be assessed with the biopsy results.

With regard to the controls, we analyzed normal kidney tissue (outside the tumor borders) of patients with normal kidney function and no proteinuria detected on routine spot urine samples. We consider this to be a practical and ethical way to study normal kidney tissue, which would otherwise have been impossible to obtain.

## Statistical analysis

Statistical analysis was performed using non-parametric tests, and interquartile ranges and medians. We used the Kruskal-Wallis H-test and the Mann-Whitney U-test for multiple comparisons to assess differences among groups. Correlations were evaluated using the Spearman rho correlation coefficient with Bonferroni correction. Statistical significance was assessed using the R software v.4.1.040.<sup>10</sup> Results were considered significant at a *P* value less than 0.05.

## Results

We analyzed 27 kidney biopsies from patients with MCD (*n*=8) and DN (*n*=10), and controls (*n*=9). Clinical parameters are reported in Table 1. All DN biopsies were classified according to the Research Committee of the Renal Pathology Society guidelines.<sup>11</sup> The glomerular compartments of the DN biopsies were

classified as Class IIa (3 patients; HbA1c 5.5%; urinary protein excretion [uProt] 2.87 g/day), Class IIb (3 patients; HbA1c 5.1%; uProt 4.71 g/day), Class III (3 patients; HbA1c 5.83%; uProt 9.54 g/day), or Class IV (1 patient; HbA1c 5%; uProt 2.5 g/day).

CIC-5, megalin, and synaptopodin expressions were detected in all biopsies (Figure 1A-I), and were quantified (Figure 1J-L).

CIC-5 and megalin expressions in the glomerular compartment of the DN biopsies were significantly downregulated compared with control glomeruli (*P*=.019 and *P*=.028, respectively) (Figure 1J and K), while in the MCD biopsies only the CIC-5 signal was significantly reduced in the glomerular compartment compared with the control group (*P*=.031) (Figure 1J).

In controls and MCD, both podocytes and parietal cells were involved in the expression of CIC-5 and megalin (Figure 1A, B, D, E), although in MCD the CIC-5 signal was less expressed in podocytes. In the DN biopsies, the CIC-5 and megalin signals were detected exclusively in parietal cells (Figure 1C, F). The cytoplasmic podocyte marker synaptopodin was detected in all the glomeruli of all the biopsies analyzed (Figure 1G, H, I), and its expression was significantly reduced in DN and MCD compared with controls (*P*<.000 and *P*<.000, respectively) (Figure 1L).

Besides the classic flat parietal cells, some parietal cells, mainly in the DN biopsies, had a hypertrophic appearance and were larger with bulging cytoplasm. The CIC-5 signal was detected in both the flat and hypertrophic ANXA3+ parietal cells in all the biopsies analyzed. Phenotypic characterization of the hypertrophic parietal cells in the DN kidney biopsies showed the positive signal of the pro-sclerotic marker CD44 to be colocalized with ANXA 3 and CIC-5 (Figure 2A, B, C, D, E). Some parietal cells (ANXA 3+) were also detected in the glomerular tuft of DN sections (Figure 2B, D).

The CIC-5 signal at the brush border in the proximal tubular compartment of MCD and DN biopsies (Figure 3B, C) was significantly downregulated compared with the CIC-5 signal in controls ( $P < .005$  and  $P < .002$ , respectively) (Figure 3J). In MCD and DN, the decrease in the CIC-5 specific signal at the brush border was accompanied by a diffuse cytoplasmic signal in the form of droplets in the basolateral region (Figure 3E, F). The megalin signal

was downregulated in both MCD and DN compared with control patients (Figure 3G, H, I), albeit not significantly ( $P < .07$  and  $P < .06$ , respectively) (Figure 3K).

Finally, in both glomerulopathies, proximal tubular CIC-5 and megalin expressions correlated with proteinuria and kidney function. Correlations between the expressions of CIC-5 and megalin in both the glomerular and tubular compartments were observed

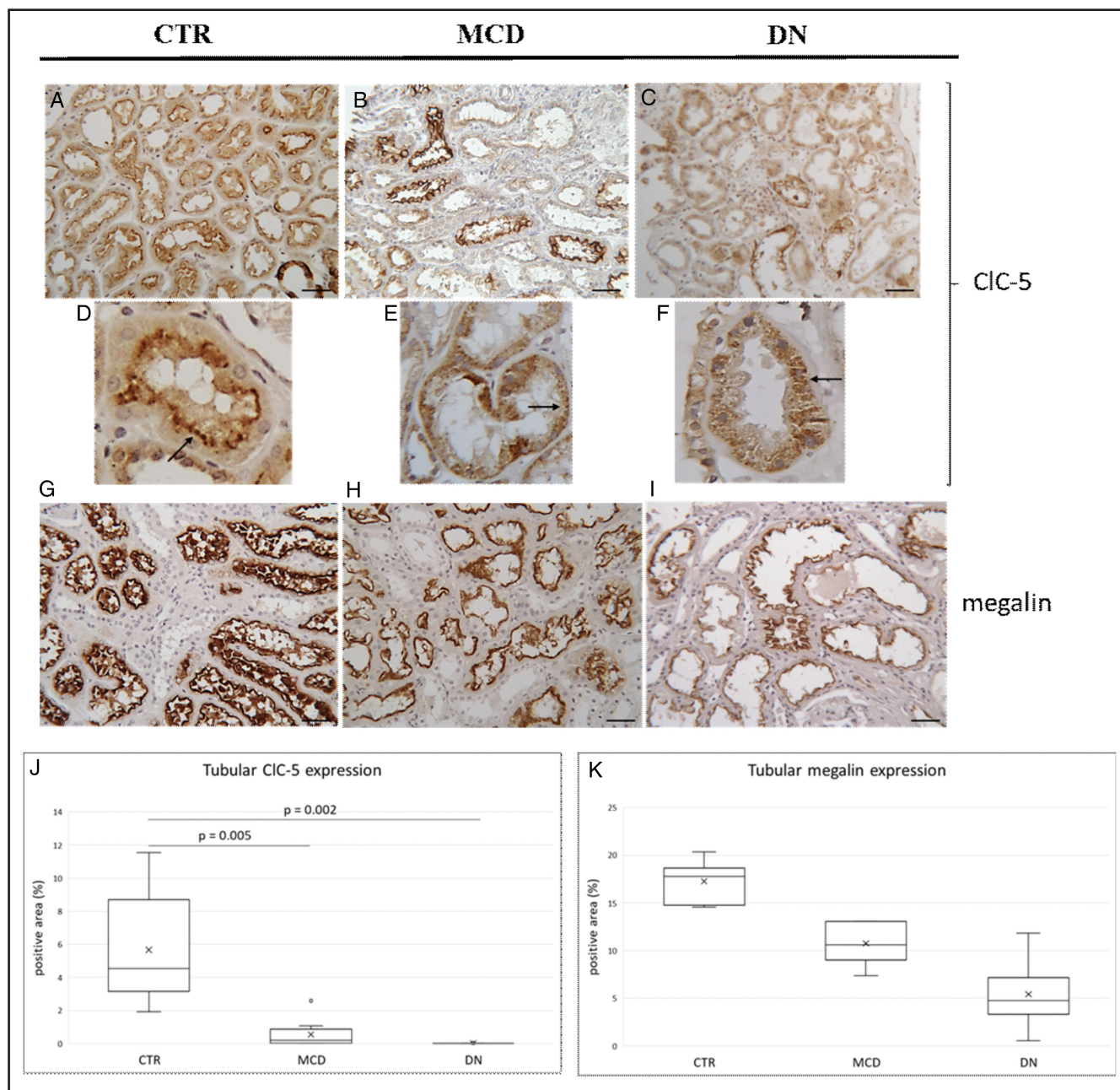


**Figure 3 CIC-5 and megalin expressions in the tubular compartment.** Representative immunohistochemical CIC-5 and megalin signals (brown: DAB) in controls (CTR) (A, D, G), minimal change disease (MCD) (B, E, H) and diabetic nephropathy (DN) (C, F, I). In CTR (A, D) the CIC-5 signal was normally expressed at the brush border in all tubules, while in MCD and DN the reduction in the CIC-5 specific signal at the brush border was accompanied by a diffuse cytoplasmic signal in the form of droplets in the basolateral region (E, F) (black arrow). The megalin signal (brown: DAB) was normally expressed at the brush border in all tubules in CTR (G), MCD (H), and DN (I). Magnification 20X/0.45 (A, B, C, G, H, I), 40X/0.45 (D, E, F). Nuclei were counterstained with Mayer's hematoxylin (blue image). Scale bar 10  $\mu$ m. CIC-5 (J) and megalin (K) protein quantification in CTR, MCD and DN biopsies by morphometric analysis evaluated exclusively at the brush border. The CIC-5 signal was significantly downregulated in MCD and DN compared with CTR, while the megalin signal was downregulated in MCD and DN compared with CTR, albeit not significantly ( $P < .07$  and  $P < .06$ , respectively).

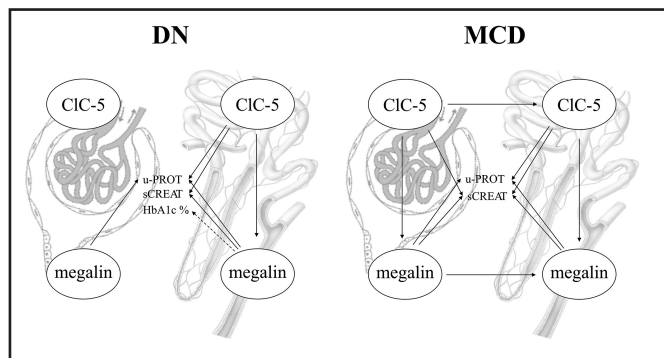


**Figure 4** Graphical representation of the Spearman correlations between CIC-5, megalin, and clinical parameters in the glomerular and tubular compartments of minimal change disease (MCD) and diabetic nephropathy (DN) patients. Correlations between CIC-5 and megalin expressions in both the glomerular and tubular compartments were observed in MCD. In DN, CIC-5 and megalin correlated only in the tubular compartment, and megalin correlated with urinary protein excretion (uProt) in the glomerular compartment. The correlation between CIC-5 expression and kidney function was lost at the glomerular level in DN. The inverse correlation between megalin and glycated hemoglobin (HbA1c) is represented by the dotted line.

in MCD. This pattern of expression was lost in DN (Figure 4). The correlation between CIC-5 expression and kidney function was also lost at the glomerular level in DN (Figure 4). Table S1 reports the correlations between CIC-5, megalin, and clinical parameters in the glomerular and tubular compartments of MCD and DN patients.

## Discussion

The pathophysiology of DN is a complex process involving numerous factors that lead to progressive kidney damage in

the glomerular, vascular and tubulointerstitial compartments.<sup>12</sup> Proteinuria, usually used as a marker of kidney injury in various nephropathies, including diabetes, is the result of increased permeability of the glomerular capillary wall and/or decreased proximal tubule endocytosis, which may be due to initial hyperfiltration, glomerulosclerosis and/or podocytopathy. In our study, we explored the expression patterns of CIC-5 and the megalin receptor, normally involved in the endocytosis process of albumin, in kidney biopsies of patients with DN and MCD and in controls. We observed a reduction in CIC-5 and megalin expression at the glomerular level in both types of glomerulonephritis with similar levels of proteinuria. Previously, we demonstrated that podocytes were able to perform endocytosis via a receptor-mediated mechanism in human kidney biopsies<sup>6</sup> and *in vitro* in an immortalized human podocyte cell line<sup>9</sup>. This suggests that podocytes may play a role in protein endocytosis in proteinuric diseases, likely related to an effect that counteracts the larger amount of filtered proteins. The downregulation of CIC-5 and megalin in the glomerular compartment in DN and MCD biopsies may be explained by the presence of podocyte damage, as evidenced by the significant reduction in the synaptopodin signal compared with controls.

We also identified the involvement of different cells in the expression of CIC-5 and megalin at the glomerular level among the groups. While in the control group, and to a lesser extent in MCD, both podocytes and parietal cells were positive for CIC-5 and megalin, in DN only parietal cells appeared to be involved in CIC-5 and megalin expression, a pattern that may be due not only to proteinuria but also to the direct physical toxic effect of hyperglycemia and advanced glycosylated end products on podocytes.<sup>13</sup> Understanding the mechanisms involved in protein handling in podocytes and parietal cells is important not only to understand the pathogenesis of proteinuria, but also to identify novel drug targets. In this respect, receptor-mediated endocytosis is an important mechanism involved in the physiology of several

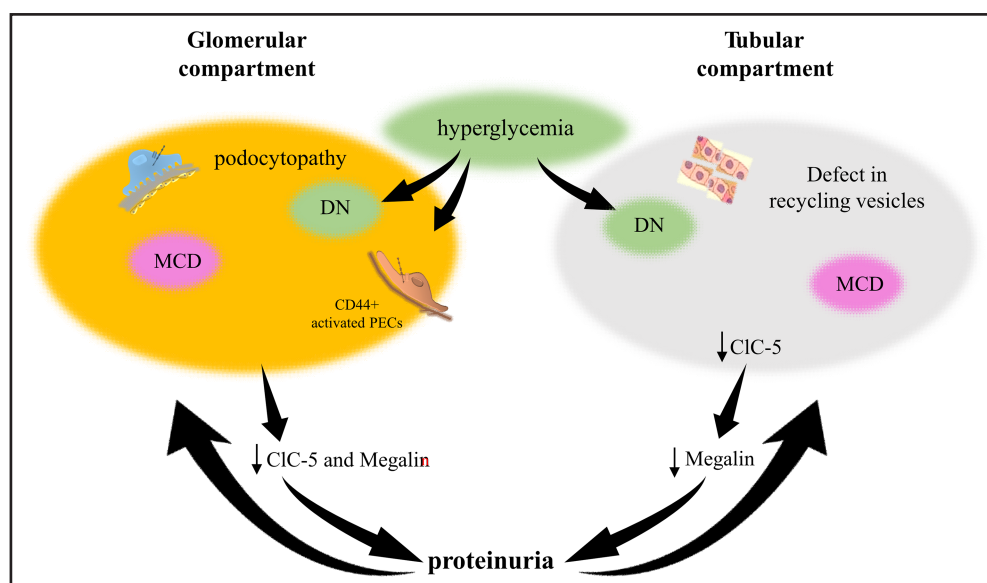


**Figure 5** Schematic diagram summarizing the mechanisms involved in CIC-5 and megalin dysfunction in diabetic nephropathy and minimal change disease. Podocyte injury in minimal change disease (MCD) and in diabetic nephropathy (DN) leads to downregulation of the protein involved in the receptor-mediated endocytic process and to proteinuria. In DN, the dual insult of hyperglycemia and proteinuria leads to podocyte loss, parietal cell activation and fibrosis in the glomerular compartment, and a defect in the recycling vesicles in the tubular compartment.

cell types, and numerous proteins and signal transduction molecules are needed to guarantee this function.<sup>14</sup> The involvement of parietal cells in glomerular cell damage and regeneration has been highlighted in several glomerular diseases, which could be explained as an attempt to mitigate the loss of podocytes, and also to respond to the expansion of the mesangial matrix upon insult.<sup>15</sup> Our results confirmed this observation, as demonstrated by the presence of parietal cells in the glomerular tuft. Moreover, the detection of CD44+ parietal cells activated in both Bowman's space and glomerular tuft in DN is consistent with previous reports addressing this migration process of parietal cells expressing CD44.<sup>16</sup> Interestingly, we found colocalization of CD44 and CIC-5 in parietal cells. Recently, a novel mechanism has been described linking CIC-5 with renal fibrosis through regulation of the synthesis and degradation of collagen via the  $\beta$ -catenin pathway and endosomal acidification.<sup>17</sup>

Dysfunctional tubular epithelial cells induced by high glucose and protein handling overload are commonly observed in the kidney tissue of DN patients, which may be caused by mitochondrial damage and apoptosis.<sup>18</sup> It is well known that the role of albumin reabsorption in proximal tubular cells is an important factor in the genesis of albuminuria observed in the early stage of diabetes, which can also lead to the epithelial-to-mesenchymal transition of tubular cells to myofibroblasts, ultimately contributing to interstitial fibrosis and tubular atrophy. Evidence in human and animal models has shown that a decrease in megalin expression could be the key mechanism in this process.<sup>13,19,20</sup> According to Figueira et al., endocytosis dysfunction in the tubular compartment of DN rats was due to the downregulation of CIC-5 and megalin.<sup>5</sup>

In our study, the downregulation of CIC-5 and megalin in the tubular compartment suggests a dysfunction in the critical components of the endocytic apparatus, which alters the handling of the filtered overload of proteins in MCD and DN. Despite our small sample size, which did not allow us to distinguish between the roles of proteinuria and hyperglycemia, we have shown that the expressions of both CIC-5 and megalin were lower in DN than in MCD patients, which may be due to the fact that the former are subjected to the dual insult of hyperglycemia and proteinuria (Figure 5). To our knowledge, this is the first time that CIC-5 and megalin have been analyzed in human MCD and DN kidney biopsies. The decrease in the CIC-5 signal at the brush border, accompanied by diffuse cytoplasmic droplets in the basolateral region in both MCD and DN, could be explained by a defect in the recycling vesicles.<sup>20,21</sup>

Under normal conditions, CIC-5 and megalin work in a coordinated manner,<sup>22,23</sup> which is maintained in MCD, but is lost in DN at the glomerular level. Given that the two glomerulopathies have similar levels of proteinuria, we can hypothesize that the toxic effect of hyperglycemia on the expressions of CIC-5 and megalin is greater in the glomerular compartment, particularly in podocytes, as these diabetic patients did not present glycosuria.

The importance of the involvement of megalin in the pathogenesis of diabetic nephropathy is highlighted by the inverse relationship between glycosylated hemoglobin levels and the expression of this receptor, which suggests that better clinical control of diabetic disease may help maintain a greater expression of megalin.

The main limitation of our study is the small cohort size. However, despite the small number of cases analyzed, our results

provide important new insights into the pathogenic mechanisms involved in diabetic nephropathy. A further limitation is that we did not characterize the type of vesicle containing CIC-5, assuming there is a disruption of endo-lysosomal trafficking in these nephropathies. The subcellular localization of certain Rab proteins (Rab 4/Rab11 for recycling endosomes, Rab 5 for early endosomes, and Rab 7/Rab9 for late endosomes) in these nephropathies may contribute to a better understanding of this complicated system in these clinical settings.<sup>14,22</sup>

In conclusion, our study highlights the involvement of CIC-5 and megalin in human kidney biopsies of MCD and DN patients. We have demonstrated endocytosis dysfunction with some differences between the two glomerulopathies. We have also shown that in DN, hyperglycemia affects the expression of CIC-5 in terms of its different localization in the glomerular and tubular compartments. Further investigation is needed to understand why CIC-5 cannot reach the tubular sub-apical compartment yet is retained within the cytoplasmic vesicles.

## Acknowledgements

This work would have not been possible without the collaboration of patients.

## Author contributions

Conceptualization and writing original draft: DPD and CM. Writing-review and editing: TH and NF. Methodology: CM, SE and RC performed the immunohistochemical and immunofluorescence analyses; DPD and CM performed morphometric analysis. SF and YP supervised the clinical data. MFK and PG carried out the statistical analysis. IA carried out the histopathological evaluation. Project administration: DPD. Supervision: DPD, CM and TH. Funding acquisition: DPD.

All authors read and approved the final manuscript.

## Conflicts of interest

All the authors declare no competing interests.

## Funding

The study was supported by Grant n° DOR2322480 from the University of Padua, awarded to D.D.P.

## Data availability

All the data generated and analyzed during this study are available from the corresponding author on reasonable request.

## Ethical disclosure

The present work was based on our research on human kidney biopsies and did not involve any human participants and/or animals. The study was conducted according to the guidelines of the Declaration of Helsinki. The study protocol was reviewed and approved by Padua University Hospital's Ethical Committee, protocol number [0007452]. The Institutional Review Board of the Hospital Británico de Buenos Aires also approved the present work (21/11/2017).

## Informed consent

All patients gave written informed consent.

## Supplementary material

Supplementary material is available at *Journal of Nephrology* online.

## References

- Shiwan H, Xing H, Yu W, *et al.* Crosstalk among podocytes, glomerular endothelial cells and mesangial cells in diabetic kidney disease: an updated review. *Cell Commun Signal.* 2024;22:136. <https://doi.org/10.1186/s12964-024-01502-3>
- Rocco MV, Berns JS. KDOQI in the era of global guidelines. *Am J Kidney Dis.* 2009;54:781–787. <https://doi.org/10.1053/j.ajkd.2009.08.001>
- Van der Velde M, Matsushita K, Coresh J, *et al.* Lower estimated glomerular filtration rate and higher albuminuria are associated with all-cause and cardiovascular mortality. A collaborative meta-analysis of high-risk population cohorts. *Kidney Int.* 2011;79:1341–1352. <https://doi.org/10.1038/ki.2010.536>
- Eriguchi M, Lin M, Yamashita M, *et al.* Renal tubular ACE-mediated tubular injury is the major contributor to microalbuminuria in early diabetic nephropathy. *Am J Physiol Renal Physiol.* 2018;314:F531–F542. <https://doi.org/10.1152/ajprenal.00523.2017>
- Figueira MF, Castiglione RC, de Lemos Barbosa CM, *et al.* Diabetic rats present higher urinary loss of proteins and lower renal expression of megalin, cubilin, CIC-5, and CFTR. *Physiol Rep.* 2017;5:e13335. <https://doi.org/10.14814/phy2.13335>
- Ceol M, Tiralongo E, Baelde HJ, *et al.* Involvement of the tubular CIC-type exchanger CIC-5 in glomeruli of human proteinuric nephropathies. *PLoS One.* 2012;7:e45605. <https://doi.org/10.1371/journal.pone.0045605>
- Gianesello L, Ceol M, Bertoldi L, *et al.* Genetic analyses in Dent disease and functional characterization of CLCN5 mutations in patients' kidney biopsies. *Int J Mol Sci.* 2020;21:516. <https://doi.org/10.3390/ijms21020516>
- Trimarchi H, Ceol M, Gianesello L, *et al.* Downregulation of megalin, cubilin, CIC-5 and podocin in Fabry nephropathy: potential implications in the decreased effectiveness of enzyme replacement therapy. *J Nephrol.* 2021;34:1307–1314. <https://doi.org/10.1007/s40620-020-00835-9>
- Gianesello L, Priante G, Ceol M, *et al.* Albumin uptake in human podocytes: a possible role for the cubilin-amnionless (CUBAM) complex. *Sci Rep.* 2017;7:13705. <https://doi.org/10.1038/s41598-017-13789-z>
- R Core Team-European Environment Agency. 2020. <https://www.eea.europa.eu/data-and-maps/indicators/oxygen-consuming-substances-in-riverse/r-development-core-team-2006>.
- Tervaert TW, Mooyaart AL, Amann K, *et al.* Pathologic classification of diabetic nephropathy. *J Am Soc Nephrol.* 2010;21:556–563. <https://doi.org/10.1681/ASN.2010010010>
- Afonso LG, Silva-Aguiar RP, Teixeira DE, *et al.* The angiotensin II/type 1 angiotensin II receptor pathway is implicated in the dysfunction of albumin endocytosis in renal proximal tubule epithelial cells induced by high glucose levels. *Biochim Biophys Acta Gen Subj.* 2024;1868:130684. <https://doi.org/10.1016/j.bbagen.2024.130684>
- Tian D, Shi X, Zhao Y, *et al.* The effect of AI adenosine receptor in diabetic megalin loss with caspase-1/IL 18 signaling. *Diabetes Metab Syndr Obes.* 2019;12:1583–1596. <https://doi.org/10.2147/DMSO.S215531>
- Molitoris BA, Sandoval RM, Yadav SPS, Wagner MC. Albumin uptake and processing by the proximal tubule: physiological, pathological, and therapeutic implications. *Physiol Rev.* 2022;102:1625–1667. <https://doi.org/10.1152/physrev.00014.2021>
- Xueying Z, Xiaoming C, Ashmeer C, *et al.* Albumin induced CD44 expression. *J Cell Physiol.* 2019;234:7224–7235. <https://doi.org/10.1002/jcp.27477>
- Eymael J, Sharma S, Loeven MA, *et al.* CD44 is required for the pathogenesis of experimental crescentic glomerulonephritis and collapsing focal segmental glomerulosclerosis. *Kidney Int.* 2018;93:626–642. <https://doi.org/10.1016/j.kint.2017.09.020>
- Durán M, Ariceta G, Semidey ME, *et al.* Renal antiporter CIC-5 regulates collagen I/IV through the  $\beta$ -catenin pathway and lysosomal degradation. *Life Sci Alliance.* 2024;7:e202302444. <https://doi.org/10.26508/lsa.202302444>
- Xu D, Moru P, Liao K, *et al.* High glucose-induced senescence contributes to tubular epithelial cell damage in diabetic nephropathy. *Exp Gerontol.* 2024;197:112609. <https://doi.org/10.1016/j.exger.2024.112609>
- Zhou L, Liu F, Huang XR, *et al.* Amelioration of albuminuria in ROCK1 knockout mice with streptozotocin-induced diabetic kidney disease. *Am J Nephrol.* 2011;34:468–475. <https://doi.org/10.1159/000332040>
- Saito A, Takeda T, Hama H, *et al.* Role of megalin, a proximal tubular endocytic receptor, in the pathogenesis of diabetic and metabolic syndrome-related nephropathies: protein metabolic overload hypothesis. *Nephrology.* 2005;19:S26–S31. <https://doi.org/10.1111/j.1440-1797.2005.00453.x>
- Hall AM. Protein handling in kidney tubules. *Nat Rev Nephrol.* 2025;21:241–252. doi: [10.1038/s41581-024-00914-1](https://doi.org/10.1038/s41581-024-00914-1)
- Christensen EL, Devust O, Dom G, *et al.* Loss of chloride channel CIC-5 impairs endocytosis by defective trafficking of megalin and cubilin in kidney proximal tubules. *Proc Natl Acad Sci U S A.* 2003;100:8472–8477. <https://doi.org/10.1073/pnas.1432873100>
- De S, Kuwahara S, Saito A. The endocytic receptor megalin and its associated proteins in proximal tubule epithelial cells. *Membranes (Basel).* 2014;4:333–355. <https://doi.org/10.3390/membranes4030333>