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Mas-related G protein-coupled receptor type D deficiency promotes tubulointerstitial injury and fibrosis associated with proteinuria in male mice

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Abstract

Kidney diseases are non-communicable, progressive diseases with high morbidity and mortality rates worldwide. A key feature of disease progression is the development of tubulointerstitial injury accompanied by proteinuria, a process mediated in part by dysregulation of the Renin-Angiotensin System (RAS). Recently, a novel protective RAS axis consisting of alamandine (Ala) and its receptor, the Mas-related G protein-coupled receptor type D (MrgD), has been identified. While the Ala/MrgD pathway has been implicated in the cardiovascular regulation, its role in renal physiology remains unknown. In this study, we investigated whether basal MrgD deficiency affects the renal structure and function. Male 8-12-week-old C57Bl6/J wild-type (WT) and MrgD knockout (MrgD-KO) mice were used. MrgD-KO exhibited reduced water intake and urine output, increased tubular reabsorption of Na^+ and glucose, and marked proteinuria associated with increased fractional excretion of proteins. In addition, MrgD deficiency was associated with elevated urinary lactate dehydrogenase levels, increased urinary proteins: creatinine ratio, enhanced urinary γ-glutamyltransferase activity, and the presence of tubulointerstitial injury and fibrosis in the renal cortex. Collectively, these findings demonstrate that basal MrgD deficiency promotes tubular dysfunction and injury, thereby expanding current understanding of the role of novel RAS peptides in kidney disease.

KEYWORDS

alamandine, kidney, kidney disease, mas-related g protein-coupled receptor type D, proteinuria, renin-angiotensin system, tubulointerstitial injury

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1 | INTRODUCTION

Kidney disease is a progressive, chronic, non-communicable disease with high incidence and prevalence worldwide, imposing a substantial burden on healthcare systems in both developed and developing countries (Bello et al., 2024). A key feature common to multiple forms of kidney disease is the development of tubulointerstitial injury associated with tubular dysfunction and proteinuria (Baker & Cantley, 2025; Cortinovis et al., 2024). Importantly, these alterations are also major risk factors for maladaptive repair and for disease progression from acute kidney injury (AKI) to chronic kidney disease (CKD) (Zhang et al., 2024). Despite their clinical relevance, the pathophysiological mechanisms underlying these processes remain poorly understood. Elucidating these mechanisms is therefore essential for the development of strategies aimed at halting or even preventing kidney disease progression.

One potential mediator of these processes is the Renin-Angiotensin System (RAS). The RAS is a well-characterized peptide cascade and enzymatic network that regulates several physiological functions, including cardiovascular and renal function, body fluid homeostasis, and blood pressure control (Bader et al., 2024; Santos et al., 2018). Studies have shown that both the classical pathway [angiotensin II (Ang II)/type 1 Ang II receptor (AT1R)] and alternative, counter-regulatory pathway [angiotensin-(1–7) Ang-(1–7)/Mas receptor (MasR)] modulate kidney injury in AKI and CKD (Almutlaq et al., 2021; Brenner et al., 2001; He et al., 2010; Peruchetti et al., 2021; Sharma et al., 2019; Zhang et al., 2015). Although several reports indicates that increased circulating and renal levels of Ang-(1–7) attenuate kidney injury in AKI models (Abdel-Fattah et al., 2021; Pacheco et al., 2021; Yu et al., 2024; Zaman & Banday, 2022; Zhu, Xu, et al., 2021), the molecular mechanism underlying these protective effects remain largely unknown.

More recently, a new branch of the alternative RAS pathway involving the heptapeptide alamandine (Ala) has been identified (Lautner et al., 2013). Ala can be generated either by the hydrolysis of angiotensin A via angiotensin-converting enzyme 2 (ACE2) or through the direct decarboxylation of Ang-(1–7) by aspartate decarboxylase (Jha et al., 2020; Lautner et al., 2013). Ala binds to and activates the Mas-related G protein-coupled receptor type D (MrgD), thereby modulating cardiovascular function during physiological and pathological conditions (Jesus et al., 2020; Lautner et al., 2013; Oliveira et al., 2019). Previous studies have shown that MrgD-deficient mice develop cardiac hypertrophy and dysfunction, including dilated cardiomyopathy, as well as progressive alterations in retina, carotid arteries, and fat mass (Da Silva et al., 2017; Dapper et al., 2019; Oliveira et al., 2019; Zhu et al., 2020).

In the kidney, emerging evidence suggests that basal MrgD expression is widespread across human renal cell types, with potentially higher abundance in proximal tubule epithelial cells (PTECs) and distal tubule cells (Larrinaga et al., 2024; Wang et al., 2022). Moreover, several studies have demonstrated that Ala ameliorates renal inflammation, oxidative stress, and tissue injury in different AKI animal models (Gong et al., 2022; Hu et al., 2021; Soltani Hekmat et al., 2021; Songür et al., 2023; Zhu, Qiu, et al., 2021), supporting a protective role for the Ala/MrgD pathway in kidney disease. However, the impact of MrgD deficiency on the renal structure and function has not yet been directly investigated, and remains to be elucidated.

In the present work, we aimed to investigate the role of basal MrgD expression in kidney physiology. To this end, we used male C57Bl6/J wild-type (WT) and MrgD knockout (MrgD-KO) mice. Our findings highlight the importance of MrgD in renal biology and expand current understanding of the contribution of alternative RAS components to kidney function.

2 | METHODS

2.1 | Animals

MrgD-KO mice were obtained from the Mutant Mouse Regional Resource Center (National Institutes of Health, RRID: MMRRC_036050-UNC). Male C57BL/6 (wild-type, WT) and MrgD-KO (knockout, KO) mice aged 8–12 weeks were maintained and provided by the Animal Facility of the Biological Science Institute at the Federal University of Minas Gerais (UFMG). MrgD deficiency was confirmed by PCR genotyping using the following primers: MrgD-8, 5′-CATGAGATGCTCTATCCATTGGG-3′; rtTA1, 5′-GG AGAAACAGTCAAAGTGCG-3′; and MrgD-1, 5′-CTGCT CATAGTCAACATTTCTGC-3′. PCR reactions were performed using GoTaq® Green Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. The cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s (annealing), and 72°C for 45 s, with a final extension at 72°C for 5 min. The expected amplicon sizes were 981 bp for the WT allele and 516 bp for the KO allele. Positive controls for both the WT and KO alleles, as well as a negative control, were included in all reactions.

Animals were housed in an experimental animal facility with free access to filtered water and standard chow (Nuvilab CR-1, catalog number 100110067, Quintia SA, Colombo, PR, Brazil), under controlled temperature and humidity

conditions and a 12:12 h light–dark cycle. All animal care and experimental procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (National Research Council (U.S.), 2011) and approved by the Institutional Ethical Committee (CEUA-UFMG#5/2018). Briefly, mice were placed in metabolic cages for water intake measurements and 24 h urine collection. Subsequently, animals were euthanized under deep anesthesia by a single intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), followed by exsanguination. Blood was collected from the inferior vena cava using heparinized syringes and tubes, and plasma was obtained after centrifugation. Urine and plasma samples were used for subsequent renal function analyses. Kidneys were then perfused with heparinized saline and processed for histological and histomorphometric analyses.

2.2 | Assessment of renal function and kidney injury markers

Renal function was assessed as previously described (Farias et al., 2023; Fonseca et al., 2024). Briefly, the mice were housed in metabolic cages for a total of 72 h, with the first 48 h serving as acclimation and the final 24 h used for urine and fluid balance measurements, as depicted in Figure 1a. Water intake (mL/24 h) and urinary volume (mL/24 h) were used to calculate the fluid balance (%) using the following formula: fluid balance = (urinary volume/water intake) × 100.

To remove sediments, the 24-h urine samples were clarified by centrifugation at 8000×*g* at room temperature for 10 min, and the supernatant was collected. This procedure was repeated at least five times, and the final supernatant was used for biochemical analyses. Clarified urine and plasma samples were used to measure creatinine, Na^+, K^+, Cl^-, glucose, and total protein levels using commercially available colorimetric or enzymatic kits (catalog nos. 35, 124, 152, 115, 84, and 36, respectively; Labtest, Lagoa Santa, MG, Brazil), according to the manufacturer's instructions. Plasma and urinary glucose were measured using an end-point enzymatic assay (high sensitivity, limit of detection = 0.32 mg/dL) Labtest (catalog no. 84).

These parameters were used to calculate the renal clearance of each analyte (C_x) using the formula: C_x = (urinary flow × urinary concentration of *x*)/plasma concentration

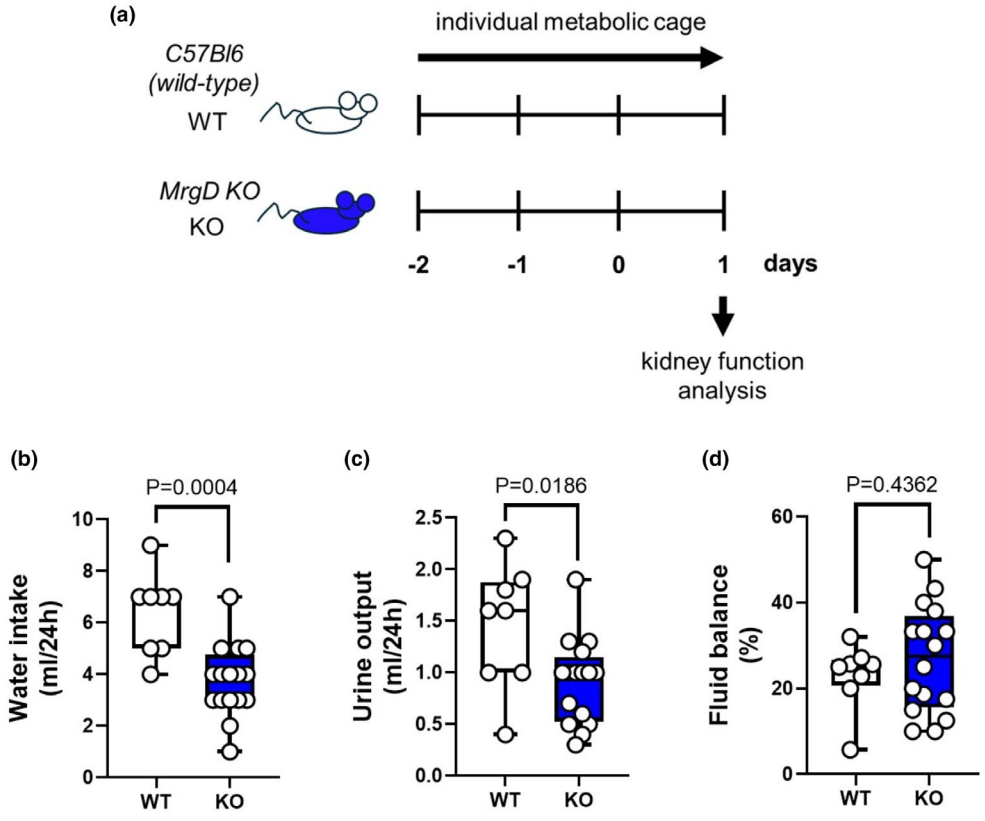


FIGURE 1 MrgD deficiency induces renal dysfunction. Male C57Bl6 (8–12 weeks old) wild-type (WT, $n = 8$) and MrgD knockout (KO, $n = 16$) mice were housed in individual metabolic cages for different experimental analyses as depicted in panel (a). Water intake (b), urine output (c), and fluid balance (d) were measured. Open circles represent individual animals. Data were expressed in median with interquartile range. Statistical analysis for panels B–D was performed using an unpaired Student's *t*-test.

of x, where urinary flow units is expressed in mL/min and concentrations in mg/dL. Creatinine clearance (C_{Cr}), plasma creatinine, and plasma urea were used as markers of glomerular function. Renal clearance values of electrolytes and organic solutes were normalized to C_{Cr} to calculate the fractional excretion (FE). FE values were used as markers of tubular function and to estimate the tubular transport of Na^+, K^+, Cl^-, glucose, and total proteins, as previously described (Farias et al., 2023; Peruchetti et al., 2019; Silva-Aguiar et al., 2023).

To assess kidney injury, urinary levels of lactate dehydrogenase (LDH, a marker of renal cell injury) and γ-glutamyl transferase (γ-GT, a specific marker of proximal tubule epithelial cell injury) were measured in clarified urine samples using enzymatic kits (catalog nos. 86 and 105, respectively; Labtest). In addition, protein excretion mass (proteinuria, mg/24 h) and the urinary protein: creatinine ratio (UP:Cr) were determined as markers of progressive kidney disease.

The selection of LDH and γ-GT as injury biomarkers was based on their reliability, well-optimized protocols in our laboratory, and their proven utility in detecting the generalized renal injury we hypothesized in this study. Although novel biomarkers such as neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) provide high specificity (Abousaad et al., 2025; Ahmed et al., 2025), the present experimental design prioritized a broad functional and histological characterization of MrgD-KO phenotype. The evaluation of more specific tubular injury markers will be addressed in future mechanistic studies.

2.3 | Histology and histomorphometric analysis

Histology and histomorphometric analyses were performed as previously described (Farias et al., 2023; Peres et al., 2023). Briefly, kidney samples were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 μm thickness. Sections were stained with: (1) periodic acid-Schiff (PAS) for the evaluation of glomerular and tubular structural parameters; or (2) SiriusRed (Direct Red 80, catalog no. 365548, Sigma-Aldrich, St. Louis, MO, USA) for the assessment of glomerular and tubular collagen deposition. One section per kidney was analyzed. Thirty images per section were acquired using systematic random sampling with a Zeiss microscope (AxioCam M2, Carl Zeiss, Jena, Germany). Quantitative analyses were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and Image Pro-Plus version 4.5.0.29 (Media Cybernetics, Rockville, MD, USA).

In PAS-stained sections, the following glomerular parameters were evaluated: glomerular cellularity (number of cell nuclei within the glomerular tuft normalized to tuft area); mesangial matrix expansion (PAS-positive areas normalized to tuft area); glomerular tuft area (normalized to total glomerular area); and Bowman's capsule space area (normalized to total glomerular area). Tubular parameters included the number of interstitial cells and number of proximal tubular epithelial cells (PTECs), identified as cortical epithelial cells with a brush border.

In SiriusRed-stained sections, glomerular and tubular collagen deposition were quantified by measuring the density of red-stained fibers and normalized to the glomerular or tubular area, respectively. Collagen accumulation was calculated using the following formula: collagen deposition (%) = (red-stained area/total glomerular tuft or tubular area) × 100. Perivascular areas were excluded from the analysis to ensure methodological accuracy. All image acquisition and histomorphometric analyses were performed in a blinded manner by L.B.F.O. and A.F.I. and were subsequently validated by an experienced renal histopathologist.

2.4 | Statistical analysis

All graphs and statistical analyses were performed using GraphPad Prism software version 8.0.2 (GraphPad Software, Inc., San Diego, CA, USA). Data are presented as medians with interquartile ranges. In the graphs, symbols represent the number of independent experiments (n). The normal distribution was analyzed using the Shapiro–Wilk test. Comparisons between two groups were performed using an unpaired Student's t-test for normally distributed data or the Mann–Whitney test for non-normally distributed data. Statistical significance was defined as $p < 0.05$. Correlation analyses were performed using Spearman's rank test for non-Gaussian distributions.

3 | RESULTS

3.1 | MrgD deficiency presents a reduction in renal function

To investigate the impact of basal MrgD expression on renal function and structure, male C57Bl6 wild-type (WT) or MrgD knockout (MrgD-KO) mice were used as depicted in Figure 1a. MrgD-KO mice exhibited a significant reduction in water intake (41%, $p = 0.0004$) and urinary volume (37%, $p = 0.0186$) compared with the WT group (Figure 1b,c). However, no significant differences in fluid balance were observed between the experimental groups (Figure 1d).

3.2 | MrgD deficiency does not alter the basal glomerular function and structure

Because impaired renal function may result from glomerular and/or tubular alterations (Cortinovis et al., 2024; Vallon, 2016; Woroniecki & Schnaper, 2009), we first evaluated glomerular function and structure in MrgD-deficient mice. Despite the reduced urinary flow observed in the MrgD-KO group, no significant differences were detected in urinary and plasma creatinine concentrations, creatinine clearance (C_{Cr}) [a surrogate marker of glomerular filtration rate (GFR)] or plasma urea levels between groups (Figure 2a–e).

Structural analyses of PAS-stained kidney sections revealed no significant differences in total glomerular area, Bowman's capsule space area, glomerular cellularity, or mesangial matrix density (PAS-positive area per tuft) between WT and MrgD-KO mice (Figure 3a–e). Consistently, SiriusRed staining showed no differences in glomerular collagen deposition between groups (Figure 3f,g). Collectively, these findings indicate that basal MrgD deficiency did not affect glomerular function and structure under basal conditions.

3.3 | MrgD deficiency presents increased tubular Na^+ and glucose reabsorption

We next investigated the role of MrgD in tubular transport. No significant differences were observed in plasma electrolytes (Na^+, K^+, and Cl^-) or glucose between groups (Figure 4a–d). In contrast, urinary excretion analyses revealed a significant reduction in urinary Na^+ excretion (60%, $p=0.0212$) and glucose excretion (55%, $p=0.0072$) in the MrgD-KO group compared with the WT group (Figure 4e,h). No differences were observed in urinary K^+ or Cl^- excretion (Figure 4f,g).

Consistent with these findings, MrgD-KO mice exhibited reduced C_{Na+} (59%, $p=0.0218$, Figure 4i) and $C_{glucose}$ (64%, $p=0.0029$, Figure 4l), as well as decreased FE_{Na+} (52%, $p=0.0374$, Figure 4m) and $FE_{glucose}$ (62%, $p=0.0085$, Figure 4p). In contrast, no significant changes were observed in C_{K+} and FE_{K+} (Figure 4j,n), as well as C_{Cl-} and FE_{Cl-} (Figure 4k,o).

Moreover, positive Spearman's correlations were observed between urinary Na^+ levels and FE_{Na+} ($r=0.7777$; $p<0.0001$; Figure 4q) and between urinary glucose levels and $FE_{glucose}$ ($r=0.8089$; $p<0.0001$; Figure 4r). Together,

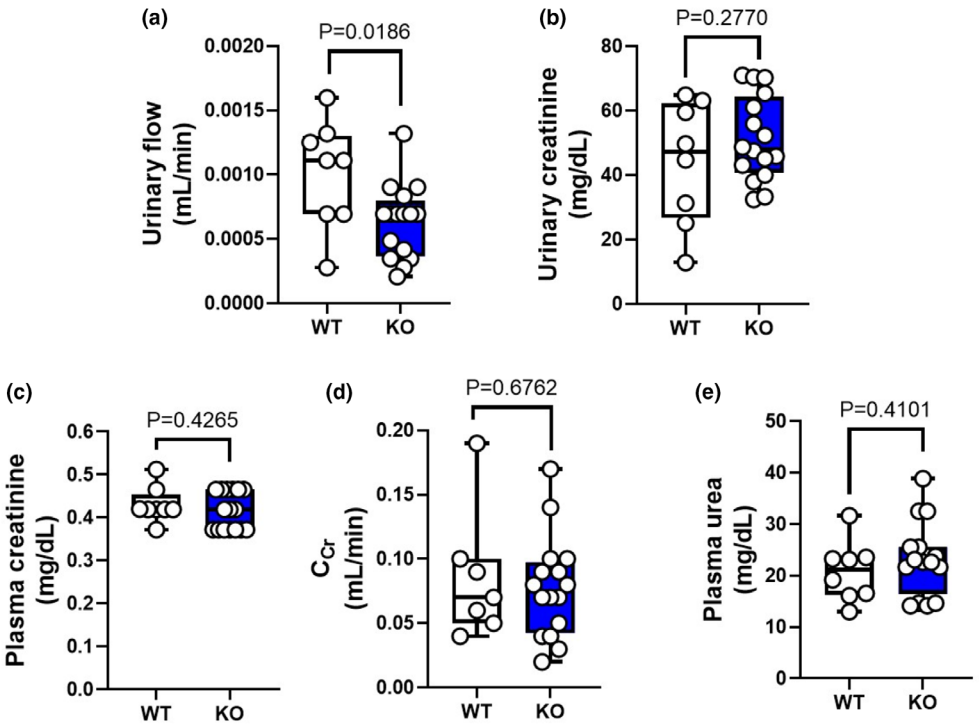


FIGURE 2 MrgD deficiency did not change glomerular function. Male C57Bl6 (8–12 weeks old) wild-type (WT, $n=8$) and MrgD knockout (KO, $n=16$) mice were housed in individual metabolic cages for different experimental analyses as depicted in Figure 1a. Urinary flow expressed in mL/min (a), urinary creatinine concentration (b), plasma creatinine concentration (c), creatinine clearance (C_{Cr}, d), and plasma urea concentration (e) were measured. Open circles represent individual animals. Data were expressed in median with interquartile range. Statistical analysis for panels a, b, d, and e was performed using an unpaired Student's *t*-test. Statistical analysis for panel c was performed using a Mann–Whitney test.

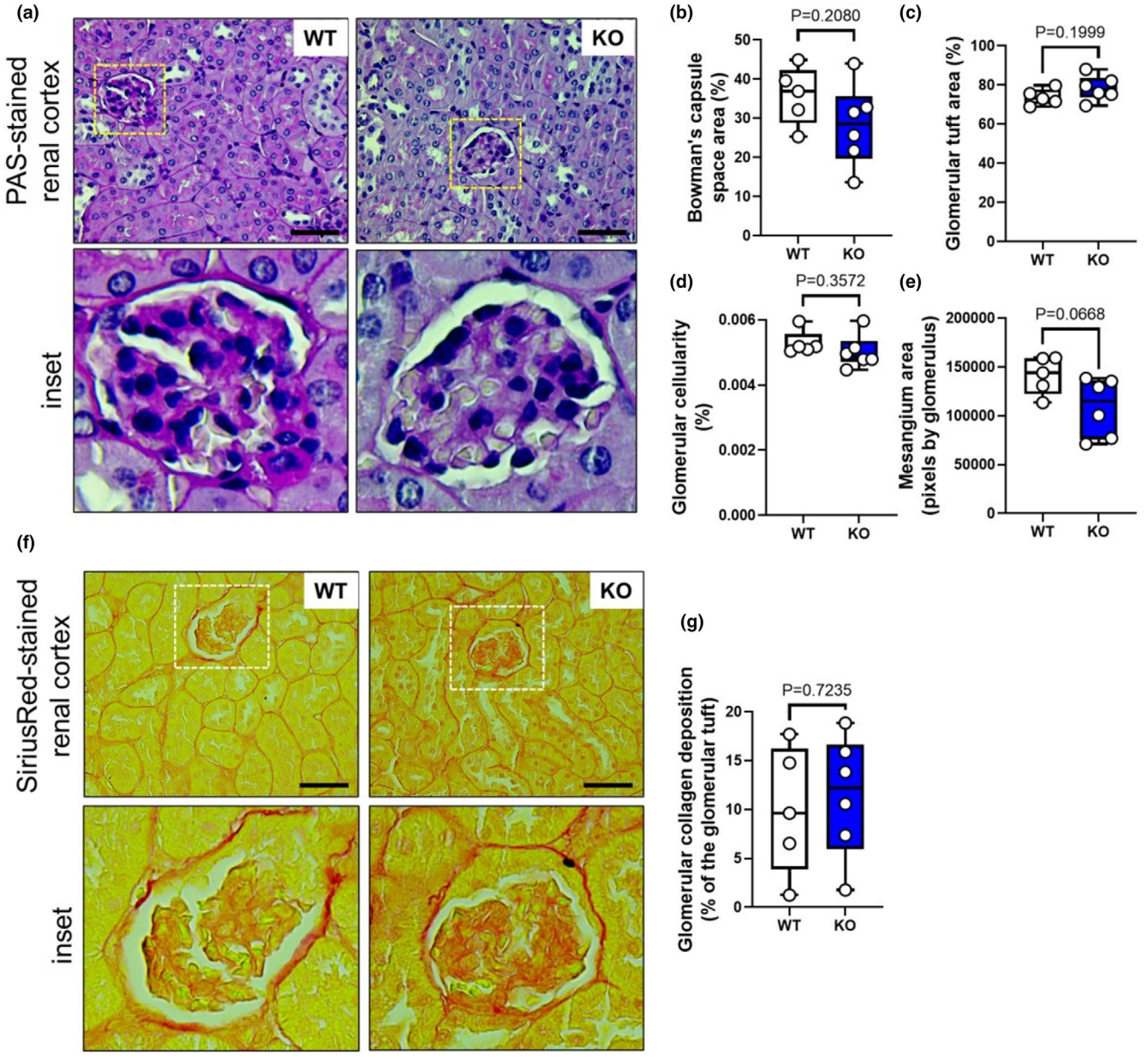
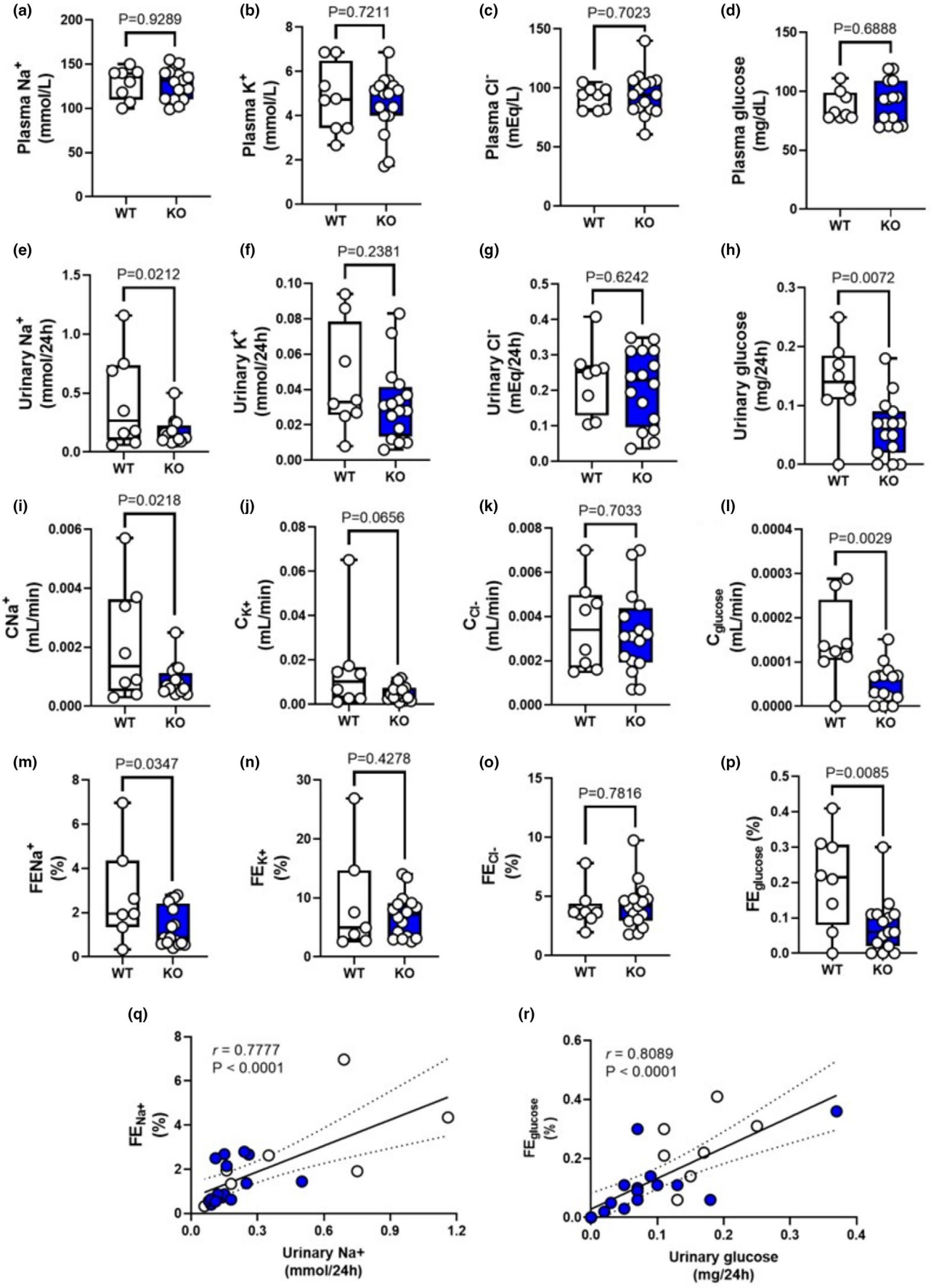


FIGURE 3 MrgD deficiency did not change glomerular structure. Male C57Bl6 (8–12 weeks old) wild-type (WT, $n=5$) and MrgD knockout (KO, $n=6$) mice were housed in individual metabolic cages for different experimental analyses as depicted in Figure 1a. (a) Representative images of micrographs from kidney cortex slices stained with Periodic acid-Schiff (PAS) reagent (scale bar = 50 μm). The glomeruli analyzed are shown in the inset presented in the lower panels. Based on these images, Bowman's capsule space area (b), glomerular tuft area (c), glomerular cellularity (d), and mesangium area (e) were measured. (f) Representative images of micrographs from kidney cortex slices stained with SiriusRed (scale bar = 50 μm). (g) Quantification of intensity related to red collagen fibers. Open circles represent individual animals. Data were expressed in median with interquartile range. Statistical analysis for panels b, c, e, and g was performed using an unpaired Student's *t*-test. Statistical analysis for panel D was performed using a Mann–Whitney test.

FIGURE 4 MrgD deficiency promoted increased tubular Na^+-glucose reabsorption. Male C57Bl6 (8–12 weeks old) wild-type (WT, $n=8$) and MrgD knockout (KO, $n=16$) mice were housed in individual metabolic cages for different experimental analyses as depicted in Figure 1a. Plasma concentrations of Na^+ (a), K^+ (b), Cl^- (c), and glucose (d) were measured. Urinary mass of Na^+ (d), K^+ (e), Cl^- (f), and glucose (g) in 24 h were measured. (i-l) Renal clearance (*C*) of electrolytes and glucose. (i) C_{Na+}, (j) C_{K+}, (k) C_{Cl-}, and (l) $C_{glucose}$. (m-p) Fractional excretion (FE) of electrolytes and glucose. (m) FE_{Na+}, (n) FE_{K+}, (o) FE_{Cl-}, and (p) $FE_{glucose}$. Open circles represent individual animals. Data were expressed in median with interquartile range. Statistical analysis for panels b, c, d, g, and h was performed using an unpaired Student's *t*-test. Statistical analysis for panels a, e, f, i, j, k, and l was performed using a Mann–Whitney test. (q) Positive Spearman's correlation between urinary Na^+ and FE_{Na+} ($r=0.7777$; $p<0.0001$). (r) Positive Spearman's correlation between urinary glucose and $FE_{glucose}$ ($r=0.8089$; $p<0.0001$).


(a)
Plasma Na+ (mmol/L)
P=0.9289
(b)
Plasma K+ (mmol/L)
P=0.7211
(c)
Plasma Cl- (mEq/L)
P=0.7023
(d)
Plasma glucose (mg/dL)
P=0.6888
(e)
Urinary Na+ (mmol/24h)
P=0.0212
(f)
Urinary K+ (mmol/24h)
P=0.2381
(g)
Urinary Cl- (mEq/24h)
P=0.6242
(h)
Urinary glucose (mg/24h)
P=0.0072
(i)
CNa+ (mL/min)
P=0.0218
(j)
CK+ (mL/min)
P=0.0656
(k)
CCl- (mL/min)
P=0.7033
(l)
Cglucose (mL/min)
P=0.0029
(m)
FENa+ (%)
P=0.0347
(n)
FEK+ (%)
P=0.4278
(o)
FECl- (%)
P=0.7816
(p)
FEglucose (%)
P=0.0085
WT
KO
(q)
r = 0.7777
P < 0.0001
FENa+ (%)
Urinary Na+ (mmol/24h)
(r)
r = 0.8089
P < 0.0001
FEglucose (%)
Urinary glucose (mg/24h)

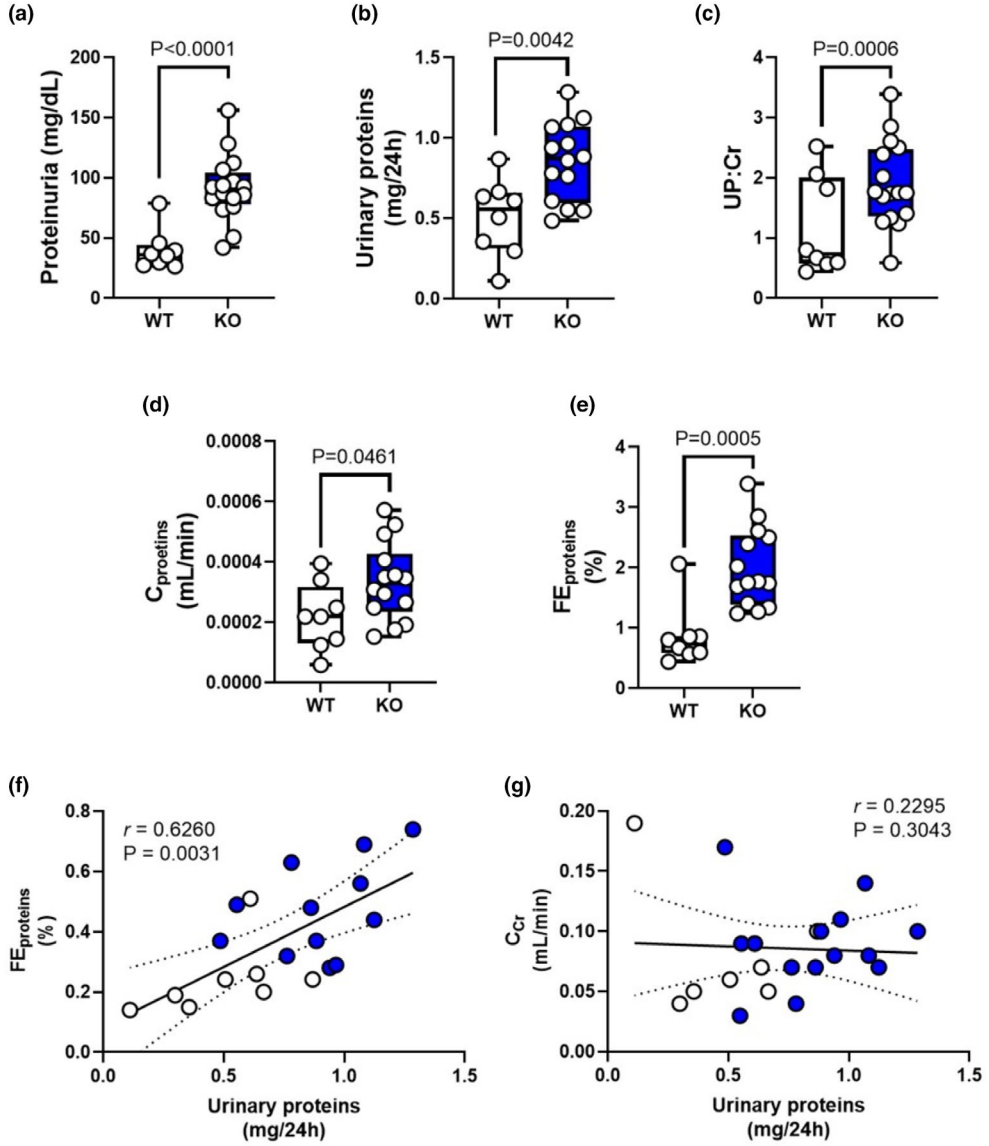


FIGURE 5 MrgD deficiency induced the development of tubular proteinuria. Male C57Bl6 (8–12 weeks old) wild-type (WT, $n=8$) and MrgD knockout (KO, $n=16$) mice were housed in individual metabolic cages for different experimental analyses as depicted in Figure 1a. Urinary protein concentration (a), urinary protein mass excretion (mg/24 h, b), urinary proteins: creatinine ratio (UP:Cr, c), renal clearance of proteins ($C_{proteins}$, d), and fractional excretion of proteins ($FE_{proteins}$, e) were measured. Open circles represent individual animals. Data were expressed in median with interquartile range. Statistical analysis for panels b and d was performed using an unpaired Student's *t*-test. Statistical analysis for panels a, c, and e was performed using a Mann–Whitney test. (f) Positive Spearman's correlation between urinary proteins and $FE_{proteins}$ ($r=0.6260$; $p=0.0031$). (g) Spearman's correlation between urinary proteins and C_{Cr} ($r=0.2295$; $p=0.3043$).

these data indicate that basal MrgD deficiency enhances tubular reabsorption of Na^+ and glucose.

3.4 | MrgD deficiency promotes tubular proteinuria

Because altered glucose handling is known to contribute to tubular proteinuria (Farias et al., 2023; Silva-Aguiar et al., 2023), we next evaluated renal protein handling. MrgD-KO mice showed a marked increase in proteinuria [urinary proteins concentration (2.28-fold; $p<0.0001$) and urinary protein mass excretion (1.7-fold; $p=0.0042$)] and in UPCr (urinary protein: creatinine ratio; 2.33-fold; $p=0.0006$), a well-known kidney injury marker, compared with the WT group (Figure 5a–c).

Additionally, MrgD-KO mice exhibited increased $C_{proteins}$ (1.53-fold; $p=0.0461$) and $FE_{proteins}$ (1.98-fold; $p=0.0005$) (Figure 5d,e). We found a positive Spearman's correlation between proteinuria and $FE_{proteins}$ ($r=0.6260$;

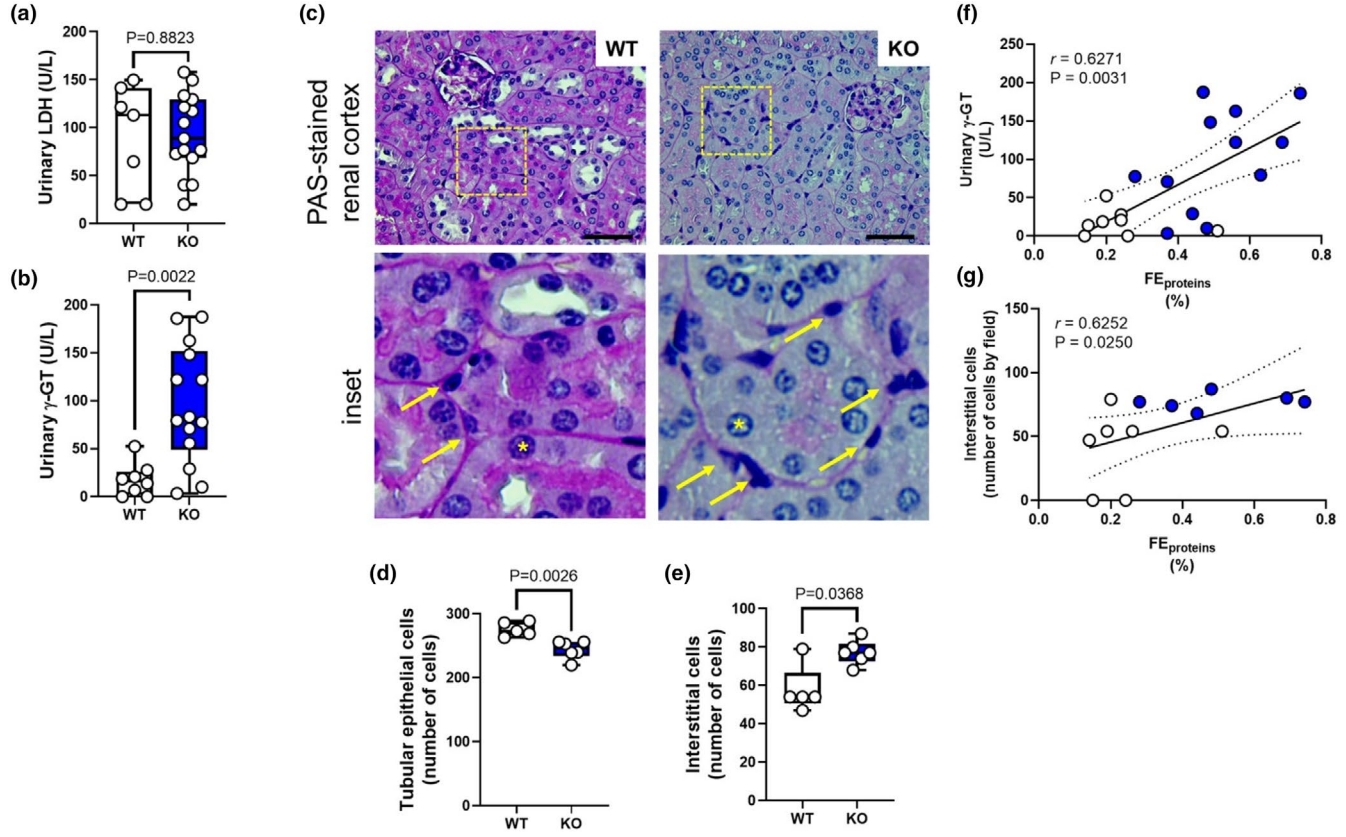


FIGURE 6 MrgD deficiency develops tubulointerstitial injury. Male C57Bl6 (8–12 weeks old) wild-type (WT, $n=8$) and MrgD knockout (KO, $n=16$) mice were housed in individual metabolic cages for different experimental analyses as depicted in Figure 1a. Urinary lactate dehydrogenase (LDH, a) and urinary γ-glutamyltransferase (γ-GT, b) were measured. Open circles represent individual animals. Data were expressed in median with interquartile range. (c) Representative images of micrographs from kidney cortex slices stained with periodic acid-Schiff (PAS) reagent (scale bar = 50 μm). Asterisk indicates the proximal tubule epithelial cell nucleus, while arrows indicate cortical interstitial cells. The tubular structures analyzed are shown in the inset presented in the lower panels. Based on these images, the number of tubular epithelial cells (d, $n=5$–6) and the number of interstitial cells (e, $n=5$–6) were measured. Statistical analysis for panels a, b, and d was performed using an unpaired Student's *t*-test. Statistical analysis for panel E was performed using a Mann–Whitney test. (f) Positive Spearman's correlation between $FE_{proteins}$ and γ-GT ($r=0.6271$; $p=0.0031$). (g) Positive Spearman's correlation between $FE_{proteins}$ and interstitial cells ($r=0.6252$; $p=0.0250$).

$p=0.0031$; Figure 5f), but not between proteinuria and C_{Cr} levels ($r=0.2295$; $p=0.3043$; Figure 5g). These findings indicate that MrgD deficiency leads to proteinuria probably due to impaired tubular protein reabsorption rather than glomerular dysfunction.

3.5 | MrgD deficiency promotes tubulointerstitial injury and fibrosis

Because tubular proteinuria is closely associated with tubulointerstitial injury and fibrosis in kidney disease (Farias et al., 2023; Peruchetti et al., 2020; Silva-Aguiar et al., 2022, 2023), we next evaluated markers of tubular injury and structural alterations. Urinary γ-glutamyltransferase (γ-GT), a specific marker of proximal tubule epithelial cell (PTEC) injury, was markedly increased in MrgD-KO mice (5.45-fold; $p=0.0022$), whereas urinary lactate dehydrogenase (LDH) levels were not significantly altered (Figure 6a,b).

Histological analysis of PAS-stained kidney sections revealed a significant reduction in tubular epithelial cell number (12%, $p=0.0026$) accompanied by an increased number of interstitial cells (1.34-fold, $p=0.0368$), indicative of renal inflammation, in the MrgD-KO group compared with the WT group (Figure 6c–e). In addition, we observed a positive Spearman's correlation between $FE_{proteins}$ and urinary γ-GT levels ($r=0.6271$; $p=0.0031$; Figure 6f) or interstitial cell number ($r=0.6252$; $p=0.0250$; Figure 6g).

Finally, SiriusRed staining demonstrated a significant increase in tubulointerstitial collagen deposition in MrgD-KO mice (1.36-fold, $p<0.0001$; Figure 7a,b). Moreover, we found a positive Spearman's correlation between $FE_{proteins}$ and tubular collagen deposition ($r=0.8091$; $p=0.0039$; Figure 7c).

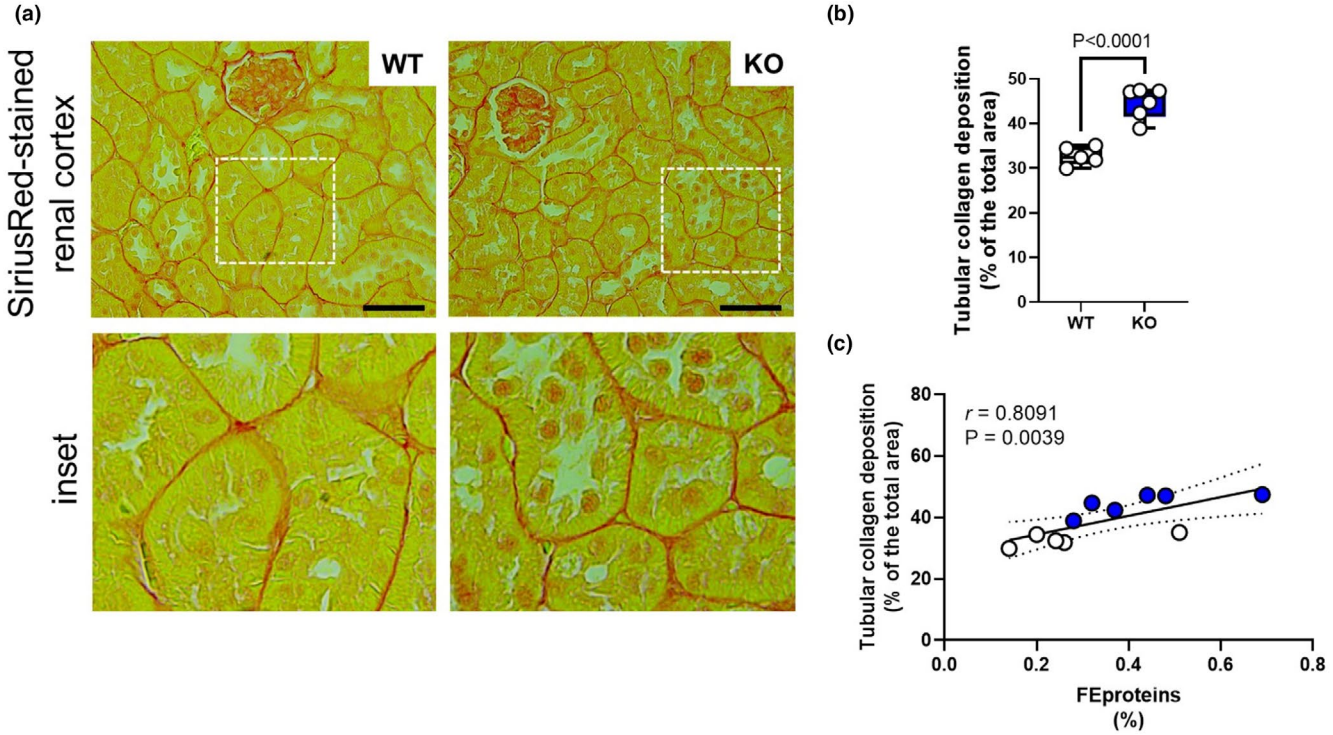


FIGURE 7 MrgD deficiency develops tubulointerstitial fibrosis. Male C57Bl6 (8–12 weeks old) wild-type (WT, $n=5$) and MrgD knockout (KO, $n=6$) mice were housed in individual metabolic cages for different experimental analyses as depicted in Figure 1a. (a) Representative images of micrographs from kidney cortex slices stained with SiriusRed (scale bar = 50 μm). The tubular structures analyzed are shown in the inset presented in the lower panels. (b) Quantification of intensity related to red collagen fibers. Open circles represent individual animals. Data were expressed in median with interquartile range. Statistical analysis for panel B was performed using an unpaired Student's *t*-test. (c) Positive Spearman's correlation between $FE_{proteins}$ and tubular collagen deposition ($r=0.8091$; $p=0.0039$).

4 | DISCUSSION

In this present study, we demonstrated that basal MrgD deficiency promoted increased tubular Na^+ and glucose reabsorption, which is associated with impaired renal protein reabsorption and consequently the development of tubulointerstitial injury and fibrosis. Together, these findings indicate that these renal alterations induced by basal MrgD deficiency may disrupt the balance of RAS, representing a potential risk factor for the development of AKI and/or CKD.

We observed significant reduction in urine output in MrgD deficiency. Songür et al. (Songür et al., 2023), using male Sprague–Dawley rats, reported that 20 h after intravenous injection of Ala (500 μg/2 mL/kg) did not change 30-min ureter output in the kidney perfusion system. However, Soltani Hekmat et al. (Soltani Hekmat et al., 2021) showed that treatment of male Sprague–Dawley rats with Ala (50 μg/kg/day) for 42 days promoted an increase in urine output. In agreement with these observations, our findings demonstrate that basal MrgD deficiency in mice significantly reduces urine output, supporting the notion that the Ala/MrgD pathway plays an important role in the maintenance of basal renal function.

The reduction of urine output observed in MrgD-deficient mice could result from alterations in glomerular and/or tubular function. However, we found no significant changes in glomerular structure and function, indicating that the Ala/MrgD pathway does not appear to be involved in basal glomerular regulation. This interpretation is consistent with previous studies showing that Ala does not alter basal blood pressure or glomerular function markers (plasma creatinine, BUN, and CCr) in healthy rats (Gong et al., 2022; Soltani Hekmat et al., 2021).

In contrast, we observed reduced urinary Na^+ and glucose excretion, accompanied by significant decreases in FE_{Na+} and $FE_{glucose}$, indicating that basal MrgD deficiency enhances tubular Na^+ and glucose reabsorption. Supporting this interpretation, Ala infusion restored the urinary Na^+ excretion impaired by renal ischemia/reperfusion in rats (Zhu, Qiu, et al., 2021). Because PTECs are responsible for bulk glucose reabsorption via sodium/glucose co-transporters (SGLTs)-dependent mechanism (Vallon, 2020), it may indicate that higher tubular Na^+ and glucose reabsorption induced by MrgD deficiency not only provides the electrochemical energy for increased bulk fluid reabsorption and fluid retention as well as it may also contribute to the impairment of other PTECs'

functions. In agreement with this view, both in vitro and in vivo studies have demonstrated that excessive SGLT-dependent high glucose influx in PTECs promoyes tubular injuries and dysfunction (Farias et al., 2023; Peruchetti et al., 2018; Silva-Aguiar et al., 2023).

Tubular dysfunction is commonly associated with the development of tubular proteinuria in multiple kidney diseases (Baker & Cantley, 2025; Cortinovis et al., 2024; Peruchetti et al., 2020; Silva-Aguiar et al., 2022). In line with this, we observed increased protein excretion and elevated $FE_{proteins}$ in MrgD-deficient mice, indicating impaired tubular protein reabsorption. In addition, no correlation was found between urinary proteins and C_{Cr} levels, reinforcing a tubular rather than glomerular origin. In agreement with this view, different studies showed that Ala infusion ameliorated proteinuria in different experimental models of AKI (Gong et al., 2022; Soltani Hekmat et al., 2021; Zhu, Qiu, et al., 2021).

Previous studies have demonstrated that tubular proteinuria resulting from impaired PTEC endocytic machinery is linked to modulation of tubular Na^+-dependent active transporters (Peruchetti et al., 2011, 2019; Silva-Aguiar et al., 2023; Teixeira et al., 2020). Thus, it is plausible that increased Na^+ and glucose reabsorption induced by MrgD deficiency contributes to defective tubular protein reabsorption and development of proteinuria. Supporting this hypothesis, previous studies have shown that SGLT-dependent high glucose influx into PTECs reduced albumin endocytosis, leading to proteinuria of tubular origin (Farias et al., 2023; Peruchetti et al., 2018; Silva-Aguiar et al., 2023; Tojo et al., 2001).

We also observed the development of tubulointerstitial injury and fibrosis in the renal cortex of MrgD-deficient mice. Consistently, several studies have demonstrated that Ala treatment ameliorated oxidative stress, apoptosis, inflammation, and/or fibrosis in ischemic or septic AKI models as well as in hypertensive Dahl salt-sensitive rats (Gong et al., 2022; Songür et al., 2023; Zhu, Qiu, et al., 2021). These findings support the concept that basal MrgD signaling is required to maintain homeostasis in the renal tubulointerstitial compartment. However, the link between MrgD deficiency and the development of tubulointerstitial injury and fibrosis is still an open matter.

One potential link is the strict correlation between tubular proteinuria and tubulointerstitial injury and fibrosis across different kidney diseases (Baker & Cantley, 2025; Cortinovis et al., 2024; Ferenbach & Bonventre, 2015; Larsen et al., 2018; Peruchetti et al., 2020; Silva-Aguiar et al., 2022; Venkatachalam et al., 2015). In our study, basal MrgD deficiency increased urinary γ-GT, a marker of PTECs injury, and promoted tubulointerstitial injury and fibrosis in the renal cortex. All these findings were positively correlated with proteinuria. In agreement, multiple studies have demonstrated that dysfunction in PTEC endocytic machinery strongly correlates with tubulointerstitial injury in both subclinical and established kidney disease animal models (de Souza Barcelos et al., 2024; Kulkarni & Hussain, 2025; Larsen et al., 2018; Peruchetti et al., 2020, 2021; Rodrigues et al., 2024; Silva-Aguiar et al., 2022). Thus, it is plausible that impaired renal protein reabsorption induced by MrgD deficiency mediates the development of tubulointerstitial injury and fibrosis.

An important unresolved question concerns the mechanism underlying the impairment of renal protein reabsorption induced by MrgD deficiency. Previous studies have shown that the activation of the Ang II/AT1R pathway promotes tubular proteinuria by disrupting PTEC protein reabsorption (Afonso et al., 2024; Peruchetti et al., 2021; Tojo et al., 2003). Moreover, Ang II promoted increased SGLT2 mRNA and protein expression in the HK-2 cell line and Ang II-infused mice or angiotensinogen (Agt) overexpressed mice (Miyata et al., 2021). Based on this evidence, it is conceivable that the inhibition of the Ala/MrgD pathway can facilitate the activation of the Ang II/AT1R pathway on PTECs, thereby impairing renal protein reabsorption and promoting tubulointerstitial injury. Alternatively, Ala metabolism into alamandine-(1–5) (Souza-Silva et al., 2025), which may still retain biological activity in the kidney, could contribute to these effects. Whether Ang II, alamandine-(1–5) or both mediates the renal effects of basal MrgD deficiency remains to be determined.

The long-term impact of tubular injury induced by MrgD deficiency is currently unknown. Pre-existing proteinuria, renal injury, and fibrosis are well-known risk factors for AKI or CKD development and for AKI-CKD transition (Baker & Cantley, 2025; Ferenbach & Bonventre, 2015; Venkatachalam et al., 2015). Furthermore, inhibition of the protective RAS axis may favor the overactivation of the classical axis of RAS under both physiological and pathological conditions (Khajehpour & Aghazadeh-Habashi, 2021; Santos et al., 2018). Dysregulation of RAS balance has been consistently reported in AKI and CKD onset and progression (Almutlaq et al., 2021; He et al., 2010; Sharma et al., 2019; Zhang et al., 2015). In this context, the structural and functional alterations induced by MrgD deficiency may predispose the kidney to injury or exacerbate pre-existing renal dysfunction. Future experiments to determine Ala or MrgD fragments in blood and urine could help to monitor this potential risk factor and develop possible new therapeutic strategies to avoid the progression of kidney disease.

Our study has some limitations. First, we identified increased $FE_{proteins}$ in MrgD-KO mice, suggesting impaired renal protein reabsorption. Although this is a reliable indicator of this process, confirmation will require direct

assessment of albumin uptake by renal epithelial cells or evaluation of the expression of receptors involved in protein reabsorption. Second, we observed an increased number of interstitial cells in the renal parenchyma of MrgD-KO mice, indicating renal inflammation. However, confirmation of this finding will require identification of the phenotype of these cells, such as macrophages, fibroblasts, or other specific cell types. Third, we observed a decrease in $FE_{glucose}$ in MrgD-KO mice, suggesting increased tubular glucose transport by PTECs. To confirm this hypothesis, it will be necessary to assess potential changes in insulin secretion, peripheral glucose uptake, and/or SGLT-mediated transport. These limitations will be addressed in the future in more detailed studies.

In conclusion, our results, together with existing literature, support a model in which basal MrgD deficiency increases tubular Na^+ and glucose reabsorption, leading to impaired tubular protein reabsorption, proteinuria of tubular origin, and the development of tubulointerstitial injury and fibrosis. These findings expand current understanding of the physiological and pathological roles of the protective branch of RAS peptides in the renal function.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

The data supporting these results in the paper are available from the corresponding author upon request.

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