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# High citrate diet delays progression of renal insufficiency in the ClC-5 knockout mouse model of Dent's disease

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**High citrate diet delays progression of renal insufficiency in the ClC-5 knockout mouse model of Dent's disease.**

*Background.* Dent's disease, an X-linked renal tubular disorder, is characterized by low-molecular-weight proteinuria, hypercalciuria, nephrocalcinosis, nephrolithiasis, and progressive renal failure. Dent's disease results from mutations of the voltage-gated chloride channel CLC-5.

*Methods.* We studied the effect of zero and high citrate diet on renal function of ClC-5 knockout mice and wild-type mice. The mice were placed in metabolic cages from which the urine was collected. Mice were sacrificed to obtain serum and tissues for analysis.

*Results.* ClC-5 knockout mice fed zero or high citrate diet had significantly increased urinary calcium excretion compared with wild-type mice fed the same diets. Nine-month-old ClC-5 knockout mice on a zero citrate diet had significantly decreased glomerular filtration rate (GFR), whereas 9-month-old ClC-5 knockout mice on a high citrate diet had normal renal function. ClC-5 knockout mice fed a zero citrate diet had significantly increased tubular atrophy, interstitial fibrosis, cystic changes, and nephrocalcinosis compared to ClC-5 knockout mice fed a high citrate diet. Transforming growth factor-β1 (TGF-β1) was significantly increased in 9-month-old ClC-5 knockout mice on zero citrate diet compared to 9-month-old wild-type mice on the same diet.

*Conclusion.* High citrate diet preserved renal function and delayed progression of renal disease in ClC-5 knockout mice even in the apparent absence of stone formation. We conclude from this that long-term control of hypercalciuria is an important factor in preventing renal failure in these mice.

Dent's disease is an X-linked renal tubular disorder with features of Fanconi syndrome, including low-molecular-weight proteinuria, phosphaturia, aminoaciduria, glycosuria, renal potassium-wasting, uricosuria, and impaired urinary acidification [1]. The disease is also characterized by hypercalciuria, nephrocalcinosis, and kidney stones. In addition one third of the patients present with rickets [2]. Dent's disease is caused by mutations of the *CLCN5* gene [3]. *CLCN5* encodes CLC-5, a member of the CLC family of voltage-gated chloride channels [4] which conducts outwardly rectifying chloride currents that are activated by strong depolarizing voltages [5]. Mutations of CLC-5 that are found in Dent's disease patients abolish or markedly reduce these chloride currents [6, 7]. The highest expression of CLC-5 is in the nephron, where it is localized to the proximal tubule, the thick ascending limb, and the α-intercalated cells of the collecting duct [8–10]. In the proximal tubule, CLC-5 is located in the early endosomes that form part of the receptor-mediated endocytic pathway [8–10]. The megalin receptor-mediated endocytic pathway, reabsorbs low-molecular-weight proteins less than 70 kD such as $\beta_2$-microglobulin and the vitamin D binding protein that are freely filtered through the glomerulus of the kidney [11, 12]. This pathway is defective in Dent's disease.

Of all patients with various types of nephrolithiasis including Dent's disease, hypercalciuria is present in more than half [13, 14]. With increased urinary calcium excretion, the ionic activity of calcium increases and urine becomes saturated with stone-forming calcium salts which tend to crystallize [15, 16]. Besides hypercalciuria, hypocitraturia is another very common metabolic risk factor for stone formation in adult and pediatric populations [17]. Citrate decreases ionized calcium in the urine by complexing calcium [18, 19], and citrate also has a direct inhibitory effect against crystallization of calcium oxalate and calcium phosphate [18, 19]. Potassium citrate is one of the drugs used for prevention of recurrent calcium oxalate nephrolithiasis [20, 21].

**Key words:** chloride channel, hypercalciuria, nephrocalcinosis, renal insufficiency, Dent's disease, citrate.

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Previous studies [22, 23] and preliminary data have established that the ClC-5 knockout mouse model we have developed has most of the aspects of human Dent's disease. These mice, lacking the ClC-5 channel, develop polyuria, low-molecular-weight proteinuria, aminoaciduria, glycosuria, and hypercalciuria. The daily urinary excretion of calcium is on average about twofold higher in ClC-5 knockout mice, an increase that is similar to that observed in patients with Dent's disease [2, 24, 25]. Experiments in ClC-5 knockout proximal tubules evaluating the uptake of various markers of endocytosis such as $\beta_2$-microglobulin [12] have shown that there is decreased receptor-mediated uptake of low-molecular-weight proteins, causing the low-molecular-weight proteinuria. Dysfunction of ClC-5 channels leads to defective endocytosis [8] but the actual reason for this is not clear. It has been hypothesized that the loss of ClC-5 leads to defective acidification of the endocytic compartment [22, 26]. Similarly, horseradish peroxidase, used as a marker of fluid phase uptake, showed little uptake in ClC-5 knockout mice [22].

We now show that aging ClC-5 knockout mice have other attributes of Dent's disease, including nephrocalcinosis, progressive renal disease, progressive fibrosis, and elevation of transforming growth factor-β1 (TGF-β1), a renal fibrosis marker. We hypothesized that removal of citrate from the normal high citrate chow would accelerate the renal disease. Comparing wt and ClC-5 knockout mice for biochemical markers of renal function such as blood urea nitrogen (BUN) and creatinine, glomerular filtration rate (GFR), and histologic markers such as interstitial inflammation, tubular atrophy, interstitial fibrosis, and nephrocalcinosis show that removal of citrate from the diet accelerates renal insufficiency in ClC-5 knockout mice. Conversely, high citrate diet preserves renal function and delays progression of renal disease in this model.

## METHODS

### Animals, treatments, and diets

All animal use complied with the guiding principles of the Institutional Animal Care and Use Committee, and the protocols for this work were approved by this committee. All mice had unlimited access to water and were fed a normal laboratory chow from weaning. This chow contained 0.27% citrate, 0.95% calcium, 0.67% phosphorus, 0.27% sodium, 0.66% potassium, with a caloric value of 4.0 kcal/g, total protein of 23%, and a fiber content of 5.3% (5P07) (LabDiet, Baltimore, MD, USA). This diet provides a citrate amount that is equivalent to high-dose human potassium citrate therapy (40 to 100 mEq/day) [21]. Mice fed this diet are designated high citrate mice. A second group of mice was given a diet without citrate. This diet is referred to as zero citrate diet. This diet contained 0.6% calcium, 0.4% phosphorous, 0.1% sodium, 0.64% potassium, a caloric value of 3.9 kcal/g, with a protein and fiber content of 20% and 4%, respectively (TD 00640) (Harlan Teklad, Madison, WI, USA). Mice fed this zero citrate diet are designated as zero citrate mice. Wild-type and ClC-5 knockout zero citrate mice were sacrificed at the age of 6 months (after being on zero citrate diet for 4 months) or were sacrificed at the age of 9 months (after being on zero citrate diet for 7 months).

Control groups of wild-type and ClC-5 knockout mice fed a high citrate diet were followed for 6 months, 9 months, 12 months, and 17 months. All mice were placed in metabolic cages for urine collection, and then sacrificed to obtain serum and kidneys for histology and protein isolation. At the ages of 2, 4, and 6 months ClC-5 knockout and wild-type mice were placed in metabolic cages. The 24-hour urine was collected to determine whether the ClC-5 knockout mice remained hypercalciuric over time with respect to wild-type mice.

### Urine and serum parameters

Urinary creatinine, urea, calcium, sodium, and potassium, as well as BUN, serum creatinine, and calcium were measured by Animal Diagnostic Laboratory (Baltimore, MD, USA) or Antech Diagnostics (Lake Success, NY, USA). Urinary pH was measured with a pH meter 220 (Corning, Corning, NY, USA). Urinary citrate was measured with a citric acid test (R-Biopharm, Darmstadt, Germany).

Fractional calcium excretion was calculated using the following formula:

$$100^* (\text{UCa}^* \text{SCr})/(\text{SCa}^* \text{UCr})$$

where UCa is urinary calcium, SCr is serum creatinine, SCa is serum calcium and UCr is urinary creatinine.

Estimated GFR was calculated by the following formula:

$$[(\text{UCr}^* \text{Uv}/\text{SCr}/\text{Bm}/24\text{hours}) + (\text{Uu}^* \text{Uv}/\text{BUN}/\text{Bm}/24\text{hours})]/2$$

where UCr is urinary creatinine, Uv is urinary volume, SCr is serum creatinine, Bm is body mass, and Uu is urinary urea.

### Western blotting

One kidney from each mouse was homogenized at 4°C with a polytron PT10 (Kinematica AG, Switzerland) in radioimmunoprecipitation assay (RIPA) buffer [1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) in phosphate-buffered saline (PBS)] containing Complete™ protease inhibitor (Roche Molecular Biochemicals, Mannheim, Germany). The homogenate was lysed on ice for 1 hour and centrifuged at 5000 × *g* for 20 minutes. The protein in the supernatant was quantified with BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and stored at −80°C.

The cell lysates were mixed with Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) and incubated at room temperature for 60 minutes. After separation on 12% SDS-polyacrylamide gel electrophoresis (PAGE), proteins were transferred to polyvinylidine difluoride (PVDF) membranes (Bio-Rad) in a Tris/glycine transfer buffer (Bio-Rad) containing 10% methanol and 0.007% SDS. Membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 1 hour at room temperature and then were incubated overnight with a mouse monoclonal anti-TGF-β1 primary antibody (R&D Systems, Minneapolis, MN, USA) (1:50,000 dilution). The membranes were washed three times for 10 minutes with TBST and incubated with horseradish peroxidase–conjugated sheep antimouse antibody (Amersham Biosciences, Piscataway, NJ, USA) (1:3000 dilution) for 1 hour at room temperature. After washing as above, blots were visualized by chemiluminescence (Western Lightning, Perkin Elmer Life Sciences, Boston, MA, USA). Membranes were stripped by washing in 100 mL of 2% SDS buffer containing 62.5 mmol/L Tris, pH 6.8, and 684 μL of 2-mercaptoethanol at 50°C for 20 minutes, then washed for 10 minutes with TBST. Finally, a 60-minute block, the membranes were reprobed with a mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibody (1:50,000) (US Biological, Swampscott, MA, USA). Subsequent incubation with secondary antibody and detection was as described above. Pixels on blots from eight ClC-5 knockout and six wild-type mice were measured using Scion Image for morphometric analysis.

### Kidney histology

Kidneys were quickly excised, stripped of the renal capsule, sagitally sectioned, and fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer for 6 hours, and then paraffin embedded and sectioned. Sections were deparaffinized with xylene and rehydrated with descending ethanol into PBS, then stained with trichrome, hematoxylin and eosin, Von Kossa, or periodic acid-Schiff (PAS). Sections were read by a renal pathologist blinded to animal group and were graded for acute tubular injury, largely cell swelling and vacuolization, interstitial inflammation, tubular atrophy, interstitial fibrosis, increases in glomerular mesangial matrix, and dilated tubules with or without cystic changes using a scale of 0 to 3. The following criteria were used to grade pathologic lesions: 0, pathologic lesions were present in up to 5% of the cortex; 1, pathologic lesions involving 6% to 25% of the cortical area; 2, pathologic lesions involving 26% to 50% of the cortical area; and 3, pathologic lesions involving more than 50% of the cortical area. The same criteria were used to read nephrocalcinosis in the medullary area. A total of three to four histologic sections were analyzed for each mouse included in Table 2. The numbers of animals are in parenthesis.

### Statistical analysis

Data are expressed as mean ± SE ($N$ = number of animals). Comparisons within the same mouse genotype were assessed using analysis of variance (ANOVA) considering the different treatments as factors, and $P$ values less than 0.05 were considered significant. Comparisons between ClC-5 knockout and wild-type mice were assessed by using unpaired Student $t$ test, and $P$ values less than 0.05 were considered significant.

## RESULTS

### Urinary calcium excretion

Two month old ClC-5 knockout mice and wt mice were fed a defined high citrate diet with 0.95% calcium. Compared with wild-type mice on this diet, ClC-5 knockout mice had an average urinary calcium excretion that was significantly and consistently elevated (Fig. 1A and B) over a period of 4 months. When the mice were placed on the zero citrate diet, the ClC-5 knockout mice remained hypercalciuric compared to wild-type mice. This finding is consistent with our previous studies that showed persistent hypercalciuria of the ClC-5 knockout mice when dietary calcium was altered from 0.95% to 0.02% [22, 23].

To study the effect of aging on dietary calcium excretion, we evaluated both ClC-5 knockout and wild-type mice for 6, 9, 12, or 17 months on either high citrate or zero citrate diets. Again, on high citrate diet, ClC-5 knockout mice remained hypercalciuric compared to wt mice (Table 1). In addition, complete removal of citrate from the diet significantly increased urinary calcium excretion in both ClC-5 knockout mice and wild-type mice, but the ClC-5 knockout mice remained hypercalciuric compared to control wild-type mice (Table 1).

### Urinary sodium and potassium excretion

Compared with wild-type mice fed a high citrate diet, ClC-5 knockout mice showed significantly elevated urinary sodium excretion at 6, 9, and 12 months (Table 2). Significant elevation of urinary sodium was observed also in ClC-5 knockout mice fed zero citrate diet, compared to 6-month-old wild-type mice fed the same diet (Table 1). ClC-5 knockout mice and wild-type mice fed either a high citrate or zero citrate diet showed no significant difference in urinary potassium excretion (Table 1). There were insufficient samples to measure urinary sodium and potassium in 9-month-old mice on zero citrate diet and in 17-month-old mice on high citrate diet.

**Table 1.** Summary of biochemical markers of renal function

| | 6 months old | | | |
|---|---|---|---|---|
| | High citrate diet | | Zero citrate diet | |
| | Wild-type | Knockout | Wild-type | Knockout |
| Blood urea nitrogen *mg/dL* | 31.5 ± 1.18 ($N = 6$) | 31 ± 2.45 ($N = 9$) NS | 26.3 ± 0.9 ($N = 12$) | 40.2 ± 2.5 ($N = 11$)[a] |
| Serum creatinie *mg/dL* | 0.33 ± 0.01 ($N = 6$) | 0.37 ± 0.02 ($N = 9$) NS | 0.31 ± 0.01 ($N = 12$) | 0.35 ± 0.03 ($N = 11$) NS |
| Glomerular filtration rate | 0.19 ± 0.03 ($N = 4$) | 0.19 ± 0.03 ($N = 9$) NS | 0.25 ± 0.02 (11) | 0.24 ± 0.02 ($N = 11$) NS |
| Urinary calcium excretion *mg/24 hours* | 0.06 ± 0.01 ($N = 6$) | 0.18 ± 0.02 ($N = 9$)[a] | 0.18 ± 0.02 ($N = 11$) | 0.33 ± 0.04 ($N = 11$)[a] |
| Urinary calcium/urinary creatinine ratio | 0.12 ± 0.02 ($N = 6$) | 0.26 ± 0.02 ($N = 9$)[a] | 0.32 ± 0.03 ($N = 11$) | 0.41 ± 0.03 ($N = 11$)[b] |
| Fractional calcium excretion | 0.45 ± 0.06 ($N = 4$) | 0.82 ± 0.09 ($N = 9$)[b] | 1.21 ± 0.1 ($N = 11$) | 1.7 ± 0.21 ($N = 11$)[b] |
| Urinary sodium/urinary creatinine ratio | 1.45 ± 0.3 ($N = 5$) | 2.52 ± 0.28 ($N = 5$)[b] | 1.63 ± 0.22 ($N = 6$) | 5.67 ± 1.18 ($N = 6$)[a] |
| Urinary potassium/urinary creatinine ratio | 5.06 ± 0.37 ($N = 5$) | 4.68 ± 0.67 ($N = 5$) NS | 5.44 ± 1.07 ($N = 6$) | 5.56 ± 0.44 ($N = 6$) NS |
| Urinary pH | 6.79 ± 0.25 ($N = 5$) | 6.06 ± 0.07 ($N = 5$)[b] | 5.94 ± 0.06 ($N = 6$) | 6.03 ± 0.07 ($N = 6$) NS |
| Urinary citrate/urinary creatinine ratio | 0.43 ± 0.06 ($N = 5$) | 0.75 ± 0.27 ($N = 5$) NS | 0.007 ± 0.002 ($N = 4$) | 0.014 ± 0.002 ($N = 6$) NS |

| | 9 months old | | | |
|---|---|---|---|---|
| | High citrate diet | | Zero citrate diet | |
| | Wild-type | Knockout | Wild-type | Knockout |
| Blood urea nitrogen *mg/dL* | 35.4 ± 1.86 ($N = 5$) | 34.8 ± 1.92 ($N = 6$) NS | 31 ± 2.18 ($N = 6$) | 52.4 ± 8.33 ($N = 9$)[b] |
| Serum creatinine *mg/dL* | 0.32 ± 0.02 ($N = 5$) | 0.37 ± 0.02 ($N = 6$) NS | 0.36 ± 0.04 ($N = 6$) | 0.57 ± 0.09 ($N = 9$) NS |
| Glomerular filtration rate | 0.23 ± 0.04 ($N = 5$) | 0.27 ± 0.03 ($N = 6$) NS | 0.25 ± 0.03 ($N = 6$) | 0.13 ± 0.02 ($N = 9$)[a] |
| Urinary calcium excretion *mg/24 hours* | 0.06 ± 0.01 ($N = 5$) | 0.35 ± 0.03 ($N = 6$)[a] | 0.08 ± 0.02 ($N = 6$) | 0.18 ± 0.03 ($N = 9$)[b] |
| Urinary calcium/urinary creatinine ratio | 0.11 ± 0.01 ($N = 5$) | 0.34 ± 0.02 ($N = 6$)[a] | 0.23 ± 0.01 ($N = 6$) | 0.32 ± 0.03 ($N = 9$)[b] |
| Fractional calcium excretion | 0.36 ± 0.05 ($N = 5$) | 1.27 ± 0.12 ($N = 6$)[a] | 0.85 ± 0.13 ($N = 6$) | 1.34 ± 0.15 ($N = 9$)[b] |
| Urinary sodium/urinary creatinine ratio | 1.49 ± 0.12 ($N = 5$) | 4.19 ± 0.75 ($N = 6$)[b] | | |
| Urinary potassium/urinary creatinine ratio | 6.7 ± 0.19 ($N = 5$) | 5.98 ± 0.18 ($N = 6$)[b] | | |
| Urinary pH | 7.97 ± 0.25 ($N = 5$) | 6.65 ± 0.15 ($N = 6$)[a] | | |
| Urinary citrate/urinary creatinine ratio | 1.62 ± 0.2 ($N = 5$) | 1.65 ± 0.17 ($N = 6$) NS | | |

| | 12 months old High citrate diet | |
|---|---|---|
| | Wild-type | Knockout |
| Blood urea nitrogen *mg/dL* | 30.4 ± 1.34 ($N = 6$) | 50.4 ± 6.88 ($N = 9$)[b] |
| Serum creatinine *mg/dL* | 0.33 ± 0.02 ($N = 6$) | 0.38 ± 0.02 ($N = 9$) NS |
| Glomerular filtration rate | 0.33 ± 0.05 ($N = 6$) | 0.26 ± 0.03 ($N = 9$) NS |
| Urinary calcium excretion *mg/24 hours* | 0.21 ± 0.04 ($N = 6$) | 0.48 ± 0.06 ($N = 9$)[a] |
| Urinary calcium/urinary creatinine ratio | 0.21 ± 0.02 ($N = 6$) | 0.45 ± 0.16 ($N = 9$)[a] |
| Fractional calcium excretion | 0.72 ± 0.06 ($N = 6$) | 1.6 ± 0.2 ($N = 9$)[a] |
| Urinary sodium/urinary creatinine ratio | 1.59 ± 0.19 ($N = 6$) | 4.41 ± 0.71 ($N = 6$)[a] |
| Urinary potassium/urinary creatinine ratio | 5.61 ± 0.23 ($N = 6$) | 5.45 ± 0.46 ($N = 6$) NS |
| Urinary pH | 6.55 ± 0.14 ($N = 6$) | 5.98 ± 0.14 ($N = 6$)[b] |
| Urinary citrate/urinary creatinine ratio | 1.29 ± 0.1 ($N = 6$) | 1.52 ± 0.2 ($N = 6$) NS |

| | 17 months old High citrate diet | |
|---|---|---|
| | Wild-type | Knockout |
| Blood urea nitrogen *mg/dL* | 25.3 ± 1.67 ($N = 6$) | 98.8 ± 14.27 ($N = 6$)[a] |
| Serum creatinine *mg/dL* | 0.38 ± 0.03 ($N = 6$) | 0.85 ± 0.15 ($N = 6$)[b] |
| Glomerular filtration rate | 0.17 ± 0.04 ($N = 5$) | 0.07 ± 0.02 ($N = 6$)[b] |
| Urinary calcium excretion *mg/24 hours* | 0.14 ± 0.02 ($N = 5$) | 0.42 ± 0.14 ($N = 6$)[b] |
| Urinary calcium/urinary creatinine ratio | 0.27 ± 0.01 ($N = 5$) | 0.56 ± 0.14 ($N = 6$)[b] |
| Fractional calcium excretion | 1.16 ± 0.1 ($N = 5$) | 4.78 ± 1.6 ($N = 6$)[b] |

ClC-5 knockout high citrate mice at the ages of 6 and 9 months have the same blood urea nitrogen, serum creatinine, and glomerular filtration rate as 6- or 9-month-old wild-type high citrate mice, respectively (columns 1 and 2). ClC-5 knockout zero citrate mice have significantly increased blood urea nitrogen compared to wild-type zero citrate mice at the ages of 6 and 9 months and significantly decreased glomerular filtration rate compared to wild-type zero citrate mice at the age of 9 months (columns 3 and 4). Twelve-month-old ClC-5 knockout high citrate mice had significantly elevated blood urea nitrogen compared with 12-month-old wild-type high citrate mice (columns 1 and 2). Seventeen-month-old ClC-5 knockout high citrate mice had significantly elevated blood urea nitrogen and serum creatinine compared with 17-month-old wild-type high citrate mice (columns 1 and 2). Twenty-four–hour urinary calcium excretion, urinary calcium/creatinine ratio, and fractional calcium excretion were elevated in ClC-5 knockout mice compared with matched wild-type mice at the ages of 6, 9, 12, and 17 months on high citrate diet (columns 1 and 2) and at the ages of 6 and 9 months on zero citrate diet (columns 3 and 4). Urinary sodium/urinary creatinine ratio was significantly elevated in ClC-5 knockout mice compared with matched wild-type mice at the ages of 6, 9 and 12 months on high citrate diet (columns 1 and 2) and at the age of 6 months on zero citrate diet (columns 3 and 4). ClC-5 knockout mice and wild-type mice fed either a high citrate or zero citrate diet showed no significant difference in urinary potassium excretion, expressed as urinary potassium/urinary creatinine ratio (columns 1, 2, 3, and 4). Urinary pH was significantly elevated in wild-type high citrate mice compared with matched ClC-5 knockout high citrate mice at the ages of 6, 9, and 12 months (columns 1 and 2). Six-month-old ClC-5 knockout mice and wild-type mice fed zero citrate diet showed no significant difference in urinary pH (columns 3 and 4). ClC-5 knockout mice and wild-type mice fed either a high citrate or zero citrate diet showed no significant difference in urinary citrate excretion, expressed as urinary citrate/urinary creatinine ratio (columns 1, 2, 3, and 4).

[a] $P < 0.01$; [b] $P < 0.05$; NS $P > 0.05$ (unpaired $t$ test), ClC-5 knockout vs. wild-type on the same diet.

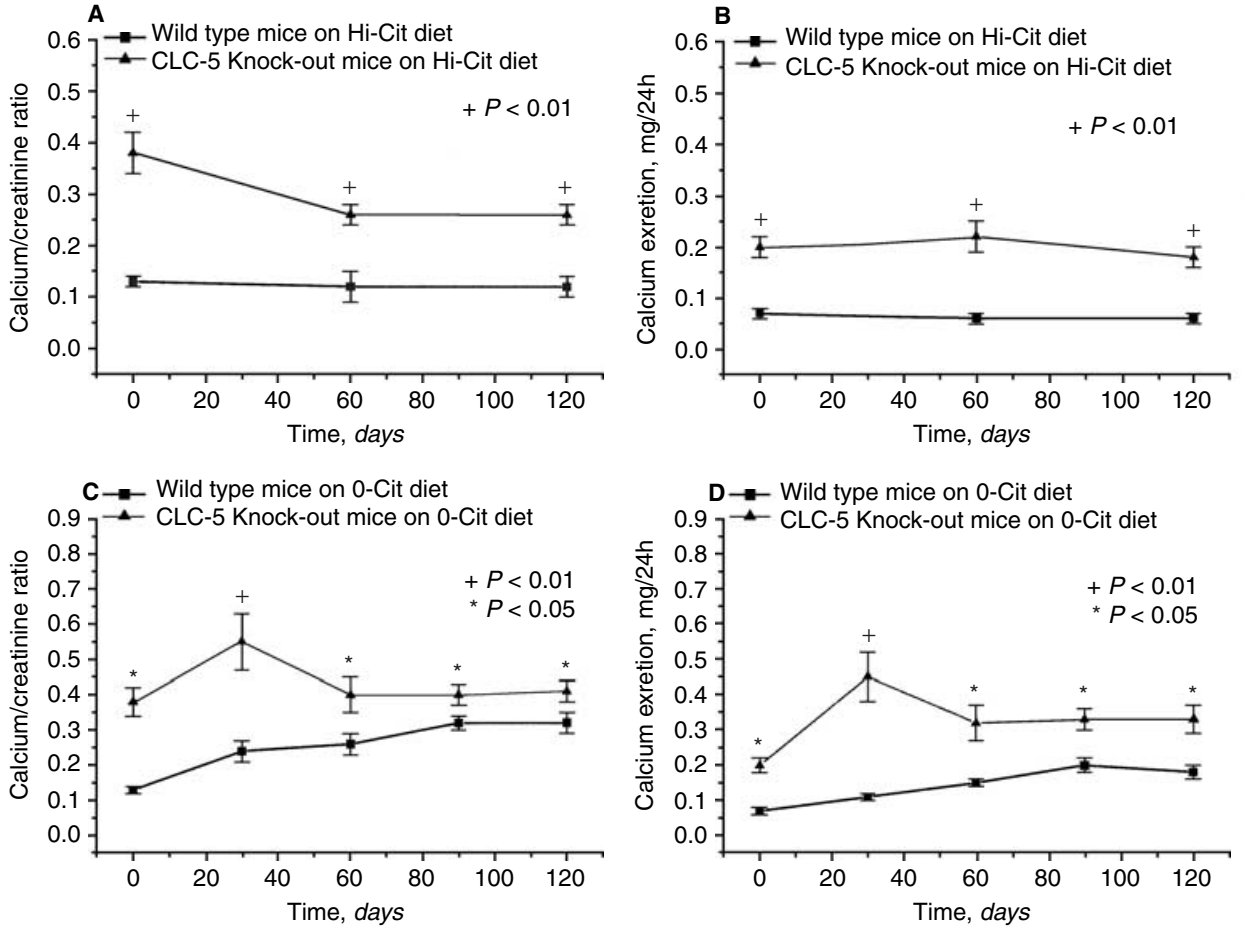


**Fig. 1. Urinary calcium excretion of ClC-5 knockout and wild-type mice on high citrate (Hi-Cit) and zero citrate (0-Cit) diets.** ClC-5 knockout mice fed a high citrate diet had significantly elevated urinary calcium excretion compared to wild-type mice on the same diet on days 0, 60, and 120 (*A* and *B*). ClC-5 knockout mice fed a zero citrate diet had significantly elevated urinary calcium excretion compared to wild-type mice on days 0, 30, 60, 90, and 120 (*C* and *D*). Shown are means ± SE. Urinary calcium/creatinine ratio and 24-hour calcium excretion at the beginning of experiment when mice were fed high citrate diet is shown at days 0 to 120 (A and B). Urinary calcium/creatinine ratio and 24-hour calcium excretion of ClC-5 knockout and wild-type mice fed zero citrate diet is shown as days 0 to 120 (C and D). Time course of urinary calcium/creatinine ratio and 24-hour calcium excretion by ClC-5 knockout and wild-type mice fed high citrate diet (days 0 to 120) (A and B). Points labeled + or * were days when ClC-5 knockout mice had values significantly elevated ($P < 0.01$) or ($P < 0.05$) compared to wild-type mice.

### Urinary pH

The 6-, 9-, and 12-month-old ClC-5 knockout mice fed a high citrate diet had significantly increased urinary pH compared to 6-, 9-, and 12-month-old wild-type mice fed the same diet (Table 1). Six-month-old ClC-5 knockout mice and wild-type mice on zero citrate diet showed no significant difference in urinary pH (Table 1). Again there were insufficient samples to measure urinary pH in 9-month-old mice on zero citrate diet and in 17-month-old mice on high citrate diet.

### Urinary citrate excretion

ClC-5 knockout mice and wild-type mice fed a high citrate or zero citrate diet showed no significant difference in urinary citrate excretion (Table 2). Nine-month-old mice on zero citrate diet and in 17-month-old mice on high citrate diet were not measured.

### Serum BUN, creatinine, and GFR

To evaluate renal function in response to changes in dietary citrate, BUN, and creatinine were measured and GFR was calculated. Six- and 9-month-old ClC-5 knockout mice and wild-type mice fed a high citrate diet showed no significant differences in serum creatinine, BUN, or GFR (Table 1). Differences were observed after 12 months on a high citrate diet, when ClC-5 knockout mice had significantly increased serum BUN and a lowered, but not significantly different, GFR compared with 12-month-old wild-type high citrate mice (Table 1). At

**Table 2.** Summary of renal histopathologic changes seen ClC-5 knockout and wild-type mice fed a high citrate or zero citrate diet

| | Acute tubular injury | Interstitial inflammation | Tubular atrophy | Fibrosis | Increase in mesangial matrix | Tubular dilatations | Calcinosis |
|---|---|---|---|---|---|---|---|
| Zero citrate diet (6 mo) | | | | | | | |
| Wild-type (6) | 0.83 ± 0.17 | 0 | | 0 | 0 | 0 | 0 |
| Knockout (6) | 1 | 0.83 ± 0.11 | 0 | 0.67 ± .11 | 0.17 ± 0.17 | 0.58 ± 0.08 | 0 |
| *P* value | NS | 0.00001 | 0.0001 | 0.0001 | NS | 0.00004 | NS |
| ANOVA (6 mo) | 5.00 (E14) | 0.073, NS | 0.022 | 0.007 | NS | 0.038 | NS |
| ANOVA (9 mo) | 0.0001 | NS | NS | 0.007 | NS | NS | NS |
| ANOVA (12 mo) | NS | NS | NS | NS | NS | NS | NS |
| ANOVA (17 mo) | NS | NS | NS | NS | NS | NS | NS |
| Zero citrate diet (9 mo) | | | | | | | |
| Wild-type (6) | 1.5 ± 0.34 | 0.75 ± 0.28 | 0.33 ± 0.21 | 0.25 ± 0.17 | 0 | 0.33 ± 0.17 | 0 |
| Knockout (8) | 0.88 ± 0.08 | 2.13 ± 0.5 | 1.13 ± 0.23 | 1.13 ± 0.26 | 0.44 ± 0.29 | 0.75 ± 0.23 | 0.88 ± 0.27 |
| *P* value | NS | 0.038 | 0.03 | 0.024 | NS | NS | 0.018 |
| ANOVA (9 mo) | 0.001 | 0.065, NS | 0.05 | 0.011 | NS | NS | 0.036 |
| ANOVA (12 mo) | NS | 0.02 | 0.034 | 0.05 | NS | NS | 0.036 |
| ANOVA (17 mo) | NS | NS | NS | NS | NS | NS | NS |
| High citrate diet (6 mo) | | | | | | | |
| Wild-type (6) | 0.5 ± 0.26 | 0.33 ± 0.11 | 0 | 0 | 0 | 0 | 0 |
| Knockout (6) | 0 | 0.5 ± 0.13 | 0.25 ± 0.11 | 0.17 ± 0.11 | 0 | 0.25 ± 0.11 | 0 |
| *P* value | NS | NS | 0.049 | NS | NS | 0.049 | NS |
| High citrate diet (9 mo) | | | | | | | |
| Wild-type (6) | 0.42 ± 0.2 | 0.33 ± 0.11 | 0 | 0 | 0 | 0 | 0 |
| Knockout (6) | 0.33 ± 0.11 | 0.92 ± 0.15 | 0.5 ± 0.13 | 0.17 ± 0.11 | 0 | 0.25 ± 0.17 | 0.08 ± 0.08 |
| *P* value | NS | 0.011 | 0.003 | NS | NS | NS | NS |
| High citrate diet (12 mo) | | | | | | | |
| Wild-type (6) | 0.6 ± 0.24 | 0 | 0 | 0 | 0 | 0.5 | 0 |
| Knockout (6) | 0.58 ± 0.17 | 0.5 ± 0.16 | 0.42 ± 0.13 | 0.5 ± 0.19 | 0 | 0.67 ± 0.29 | 0.08 ± 0.07 |
| *P* value | NS | 0.021 | 0.022 | 0.043 | NS | NS | 0.036 |
| High citrate diet (17 mo) | | | | | | | |
| Wild-type (6) | 1.17 ± 0.38 | 0.5 ± 0.13 | 0.17 ± 0.11 | 0.17 ± 0.11 | 0 | 0 | 0 |
| Knockout (6) | 1 ± 0.29 | 1.64 ± 0.43 | 1.21 ± 0.41 | 1.21 ± 0.41 | 0.43 ± 0.3 | 1.36 ± 0.39 | 0.5 ± 0.29 |
| *P* value | NS | 0.038 | 0.04 | 0.04 | NS | 0.013 | NS |

Sections read by a renal pathologist blinded to animal group were graded 0 to 3 for acute tubular injury, interstitial inflammation, tubular atrophy, interstitial fibrosis, increases in mesangial matrix, and cystic changes. The following criteria were used to grade pathologic lesions: 0, pathologic lesions were present in up to 5% of the cortex; 1, pathologic lesions involving 6% to 25% of the cortical area; 2, pathologic lesions involving 26% to 50% of the cortical area; and 3, pathologic lesions involving more than 50% of the cortical area. The same criteria were used to read nephrocalcinosis in the medullary area. Unpaired *t* tests were used to compare ClC-5 knockout vs. wild-type and to determine *P* value. Analysis of variance (ANOVA) was used to compare 6-month-old ClC-5 knockout mice on zero citrate diet vs. 6-, 9-, 12-, or 17-month-old mice on high citrate diet and 9-month-old ClC-5 knockout mice on zero citrate diet vs. 9-, 12-, or 17-month old ClC-5 knockout mice on high citrate diet, where mouse age is designated in months (mo). NS represents $P > 0.05$. The number of kidney analyzed appears as values in the parentheses.

17 months of age ClC-5 knockout mice had significant increases in serum BUN ($P < 0.01$) and serum creatinine ($P < 0.05$), and showed significant decrease in GFR ($P < 0.05$) (Table 1) compared to 17-month-old wild-type mice. Some 17-month-old ClC-5 knockout mice had elevated GFR, whereas some mice had the same GFR as wild-type mice. This finding is consistent with the irregular patient to patient progression of renal failure in Dent's disease [27].

Renal function assessment showed clear differences when mice were fed a zero citrate diet. Both 6- and 9-month-old ClC-5 knockout mice on a zero citrate diet had significantly elevated serum BUN (Table 1) ($P < 0.01$ and $P < 0.05$), compared to wild-type mice (Table 2). GFR was significantly decreased in 9-month-old ClC-5 knockout mice on a zero citrate diet, compared to age-matched wild-type mice (Table 1). ClC-5 knockout mice showed a trend for deterioration of renal function at 6 months (similar to 12-month-old ClC-5 knockout high citrate mice), manifested by significantly increased serum BUN. A more advanced renal disease in 9-month-old ClC-5 knockout zero citrate mice was manifested by significantly increased serum BUN and significantly decreased GFR (Table 1). The most dramatic effect occurred in 9-month-old ClC-5 knockout mice that had a renal disease defined by increases in BUN and creatinine, and decreases in GFR that were comparable to those seen in 17-month-old high citrate mice.

In summary, biologic markers of renal function show a pattern of loss of renal function in ClC-5 knockout mice fed a zero citrate or high citrate diet. At the age of 6 months on a zero citrate diet or 12 months on a high citrate diet ClC-5 knockout mice had very early signs of renal disease. At 9 months on a zero citrate diet or 17 months on a high citrate diet ClC-5 knockout mice had renal insufficiency. These data suggest that a high citrate diet preserved renal function and delayed the onset of renal insufficiency in ClC-5 knockout mice. The renal function of wild-type mice was not affected by citrate.

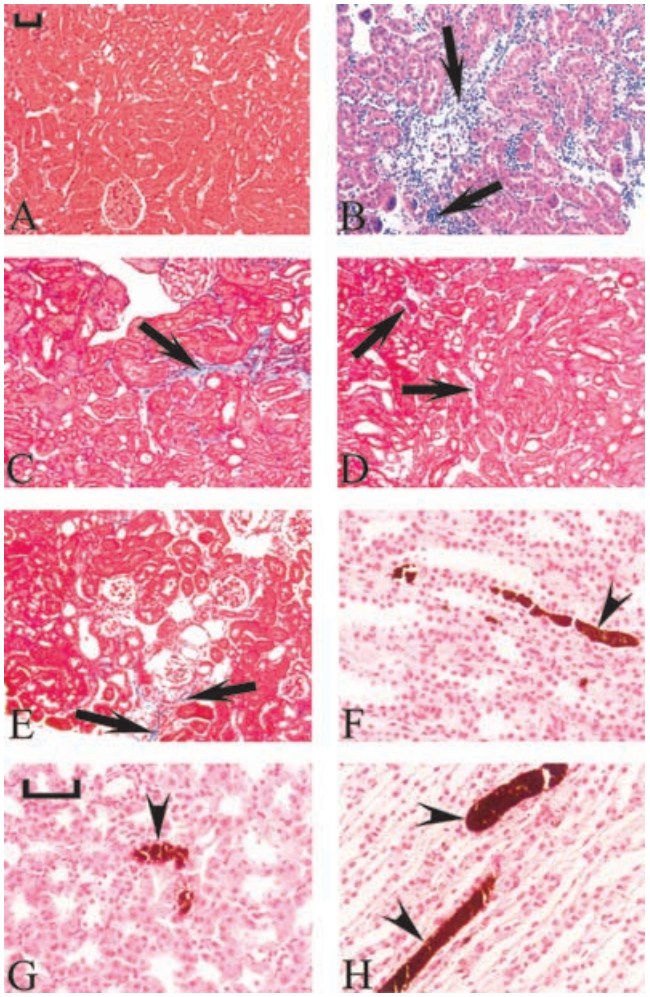


**Fig. 2. Representative histological stains of kidneys from ClC-5 knockout mice on high citrate or zero citrate diets.** (*A*) The cortex of a 9-month-old ClC-5 knockout high citrate mouse stained with trichrome blue, shows well-preserved tubular structures and absence of fibrosis (lack of blue collagen staining), whereas (B) that of a 9-month-old ClC-5 knockout zero citrate mouse shows lymphocytic or lymphoplasmacytic infiltrate (arrows). (*C* and *D*) Trichrome blue staining of the cortex of a 9-month-old ClC-5 knockout zero citrate mouse. (*E*) The cortex of a 17-month-old ClC-5 knockout high citrate mouse stained with Trichrome blue. (C to E) Interstitial fibrosis is marked by arrows. (*F* and *G*) Von Kossa staining indicates that the inner medulla of a 9-month-old ClC-5 knockout zero citrate mouse and (*H*) that of a 17-month-old ClC-5 knockout high citrate mouse show intratubular calcium deposits (arrowheads). Bars represent 50 μm (A and G) [magnification 10× (A to E) and 20× (F to H)].

## Histologic data

We also performed a histologic analysis of the tissue in order to evaluate renal function. Nine-month-old ClC-5 knockout high citrate mice had an overall normal kidney architecture (Fig. 2A). ClC-5 knockout zero citrate or high citrate mice showed levels of acute tubular injury comparable to age- and diet-matched wild-type mice (Table 1). Six-month-old ClC-5 knockout zero citrate mice had a significant increase in acute tubular injury compared to 6- or 9-month-old ClC-5 knockout high citrate mice (Table 2). Nine-month-old ClC-5 knockout zero citrate mice had a significant increase in acute tubular injury compared to 9-month-old ClC-5 knockout high citrate mice (Table 2). These data imply that zero citrate significantly increased acute tubular injury.

On a high citrate diet, 9-, 12-, and 17-month-old ClC-5 knockout mice had significantly increased interstitial inflammation compared with their wild-type counterparts (Table 2). Six- and 9-month-old ClC-5 knockout mice fed a zero citrate diet showed significantly increased interstitial inflammation compared to wild-type zero citrate mice (Table 2). A representative kidney section of a 9-month-old ClC-5 knockout zero citrate mouse (Fig. 2B) shows lymphocytic and lymphoplasmacytic infiltration.

Tubular atrophy was significantly increased in 6- and 9-month-old ClC-5 knockout zero citrate mice compared to 6- and 9-month-old wild-type zero citrate mice (Table 2). Six-month-old ClC-5 knockout zero citrate mice had a significant increase in tubular atrophy compared to age-matched ClC-5 knockout high citrate mice (Table 2). Nine-month-old ClC-5 knockout zero citrate mice had a significant increase in tubular atrophy compared to 9- or 12-month-old ClC-5 knockout high citrate mice (Table 2). This level of atrophy was comparable to that found in 17-month-old ClC-5 knockout high citrate mice (Table 2). Cortical sections from 9-month-old ClC-5 knockout high citrate mice showed well-preserved tubular structures (Fig. 2A), whereas those from 9-month-old ClC-5 knockout zero citrate mice and 17-month-old ClC-5 knockout high citrate mice have tubular atrophy (Fig. 2C, D, and E, arrowheads, respectively).

Six- and 9-month-old ClC-5 knockout zero citrate mice had significantly increased interstitial fibrosis, compared to age-matched wild-type zero citrate mice (Table 2). Six-month-old ClC-5 knockout zero citrate mice had a significant increase in interstitial fibrosis compared with 6- and 9-month-old ClC-5 knockout high citrate mice (Table 2). Nine-month-old ClC-5 knockout zero citrate mice had a significant increase in interstitial fibrosis compared with 9- and 12-month-old ClC-5 knockout high citrate mice (Table 2). The fibrosis in these mice was comparable to that seen in the 17-month-old ClC-5 knockout high citrate mice. Representative kidney sections of 9-month-old ClC-5 knockout zero citrate mice (Fig. 2C and D) and 17-month-old ClC-5 knockout high citrate mice (Fig. 2E) show fibrosis (blue collagen staining), whereas a 9-month-old ClC-5 knockout high citrate mice did not show fibrosis (lack of blue collagen staining) (Fig. 2A). In conclusion, zero citrate diet significantly increased interstitial fibrosis in 6- and 9-month-old ClC-5 knockout mice.

Six- and 9-month-old ClC-5 knockout zero citrate mice and 17-month-old ClC-5 knockout high citrate mice did not have a significant increase in mesangial matrix compared with matched wild-type mice (Table 2). Mesangial

matrix in 6- or 9-month-old ClC-5 knockout zero citrate mice was comparable to 6-, 9-, 12-, or 17-month-old ClC-5 knockout high citrate mice (Table 2). These data suggest that mesangial matrix in ClC-5 knockout mice was not affected by dietary citrate.

Six-month-old ClC-5 knockout zero citrate mice had a significant increase in tubular dilation and cystic changes compared to 6-month-old wild-type zero citrate mice or 6-month-old ClC-5 knockout high citrate mice, whereas 9-month-old ClC-5 knockout zero citrate mice did not show a significant increase in tubular atrophy and cystic changes compared to 9-month-old wild-type zero citrate mice or 9-month-old ClC-5 knockout high citrate mice (Table 2). Seventeen-month-old ClC-5 knockout high citrate mice had a significant increase in tubular atrophy and cystic changes compared to 17-month-old wild-type high citrate mice (Table 2). These data suggest that dietary citrate had no effect on tubular dilation and cystic changes.

Renal medullary calcinosis was significantly increased in 9-month-old ClC-5 knockout zero citrate mice compared to 9-month-old wild-type zero citrate mice (Table 2). Four kidneys from 9-month-old ClC-5 knockout zero citrate mice stained using the Von Kossa method were examined, three mice had mild nephrocalcinosis and one mouse had moderate to severe nephrocalcinosis. Two representative sections are shown in Figure 2G and H. These calcifications are similar to those described by Moulin et al [28] in Dent patients. Nine- and 12-month-old ClC-5 knockout high citrate mice showed significantly diminished calcinosis compared to 9-month-old ClC-5 knockout zero citrate mice. Six kidneys from 9-month-old ClC-5 knockout high citrate mice were examined and only one had mild nephrocalcinosis. In the 12-month-old group, one out of six ClC-5 knockout high citrate mice had mild to moderate nephrocalcinosis. There was a moderate to severe nephrocalcinosis in one mouse and mild nephrocalcinosis in two out of six of the 17-month-old high citrate mice. A representative section is shown in Figure 2H. Note that the amount of calcinosis in 17-month-old ClC-5 knockout mice on a high citrate diet was comparable to 9-month-old ClC-5 knockout zero citrate mice. In conclusion, the zero citrate diet was associated with significantly increased nephrocalcinosis.

### TGF-β1

Nine-month-old ClC-5 knockout zero citrate mice ($N = 5$) had much greater TGF-β1 in total kidney lysates compared with 9-month-old wild-type zero citrate mice ($N = 4$) (Fig. 3, Western blot). In a comparison of six wild-type kidneys and eight ClC-5 knockout kidneys the TGF-β1 was significantly increased in ClC-5 knockout mice (Fig. 3C). On overexposure of the blots, 6-, 9-, and 12-month-old ClC-5 knockout high citrate mice and 6-month-old ClC-5 knockout zero citrate mice revealed barely detectable levels of TGF-β1 (data not shown).

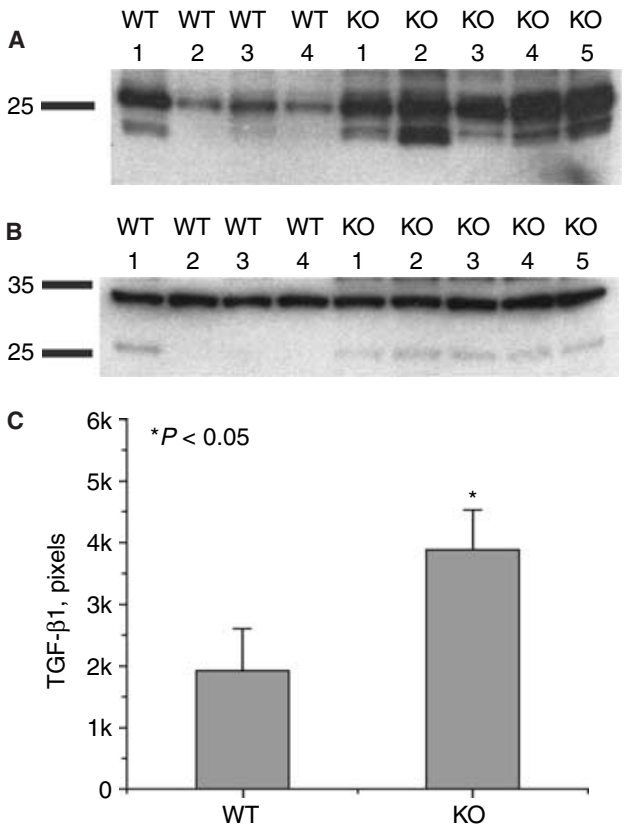


**Fig. 3. ClC-5 knockout (KO) mice have elevated transforming growth factor-β1 (TGF-β1).** Western blot analysis of TGF-β1 (*A*) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*B*) from kidneys of 9-month-old ClC-5 knockout and wild-type (WT) zero citrate mice. (A) TGF-β1 was greatly elevated in five out of five ClC-5 knockout mice and somewhat elevated in one out of four wild-type mice. GAPDH was used as an internal control and its expression was nearly identical in ClC-5 knockout and wild-type mice (B). (*C*) Comparison of the quantifications of the TGF-β1 signals expressed as average pixels and standard errors, from eight ClC-5 knockout and six wild-type mice, $^*P < 0.05$ (unpaired *t* test).

## DISCUSSION

In the present study, we found that ClC-5 knockout zero citrate mice develop renal insufficiency by the age of 9 months, whereas the development of a similar stage of renal insufficiency in ClC-5 knockout high citrate mice was delayed to about 12 to 17 months. High citrate diet preserves renal function, as measured by many parameters, including histologic changes, cytokines, and urine chemistry. The high citrate diet appears to slow the progression to advanced stages of renal disease in ClC-5 knockout mice, even in the absence of stone formation.

Clinical features of Dent's disease, caused by mutations in CLC-5, include hypercalciuria,

low-molecular-weight proteinuria, nephrocalcinosis, nephrolithiasis, and renal failure. Almost all of the males affected with Dent's disease present with hypercalciuria [2, 27]. The ClC-5 knockout mouse produced at Johns Hopkins University has hypercalciuria at ages 6 weeks to 17 months, on both normal calcium [22] and low calcium diets [23], whether 24-hour calcium excretion, calcium creatinine ratio, or fractional excretion of calcium are evaluated. We now show that ClC-5 knockout mice remain hypercalciuric compared to wild-type mice on zero citrate diet and that this hypercalciuria persists over many months. This finding is quite different from the outcome found in the Jentsch mouse model that does not have hypercalciuria [26]. Moreover, that mouse model did not have calcifications, which could be explained by lack of hypercalciuria [5]. The reasons for the lack of hypercalciuria in the Jentsch mouse model are not known. However, because the Hopkins ClC-5 knockout mouse model has elevated 1α,25 dihydroxyvitamin $D_3$, it is possible that a small amount of intestinal calcium absorption enables the hyperexcretion of calcium.

Almost half of the Dent's disease patients have stones [27, 29] and about two thirds of patients have variable degrees of medullary nephrocalcinosis [2, 24, 27, 30]. Hypercalciuria and hypocitraturia are two major metabolic risk factors contributing to the generalized condition of nephrolithiasis. Citrate has an inhibitory effect against the crystallization of stone-forming calcium salts [18] by binding to calcium and lowering ionic calcium in the urine [19]. Dent's disease patients have been reported to have normal urinary citrate, except those with reduced renal function who have hypocitraturia [2]. In the present study, nephrocalcinosis (calcium deposits identified by Von Kossa staining) was significantly increased in 9-month-old ClC-5 knockout zero citrate mice compared with age-matched wild-type mice, and the calcinosis of the 9-month-old ClC-5 knockout mice was comparable to that of 17-month-old ClC-5 knockout high citrate mice. We did not see evidence of stones in the ClC-5 knockout mice for several possible reasons. First, the stones could be too small to be detected by the conventional magnetic resonance imaging (MRI) scans to which we had access (data not shown). Second, the mice could form fewer stones than humans, and/or they may need to be monitored over a longer time period.

Recurrent renal stones and nephrocalcinosis can be managed with increased volume intake, dietary modification, and thiazide and potassium citrate in the case of hypocitraturia. Thiazide might reduce recurrent nephrolithiasis in Dent's disease patients by decreasing urinary calcium excretion [31], but it is not known whether thiazide would have any long-term benefit in protecting renal function in these patients. A recent study did show a reduction in calcium excretion with thiazide in Dent's disease patients, but this study was performed over a short time period so changes in renal pathology were not addressed.

Renal fibrogenesis leads to end-stage renal failure in a number of renal diseases, including obstructive nephropathy [32]. In the initial phase, mononuclear cells infiltrate the interstitium. This leads to the activation and proliferation of fibroblasts that in turn are thought to increase the interstitial matrix [33]. Proximal tubular cells become hypertrophied in order to compensate for the functional loss. This is followed by tubular atrophy [33]. Excessive deposition of matrix results in destruction of kidney structure and decreased renal function [34]. Dent's disease patients have inflammatory changes, tubular atrophy, interstitial fibrosis, and glomerulosclerosis [2, 24, 35]. Wrong, Norden, and Feest [2] also described numerous small cysts in one third of the Dent's disease patients. Interstitial inflammation, tubular atrophy, and interstitial fibrosis were significantly increased in 6- and 9-month-old ClC-5 knockout zero citrate mice compared to age- and diet-matched wild-type mice. There was a significantly increased interstitial inflammation in 9-month-old ClC-5 knockout zero citrate mice compared to 6-month-old ClC-5 knockout zero citrate mice (data not shown). Interstitial fibrosis was noted to be minor to moderate in 6-month-old ClC-5 knockout zero citrate mice and comparable to 12-month-old ClC-5 knockout high citrate mice. In 9-month-old ClC-5 knockout zero citrate mice interstitial fibrosis and tubular atrophy were mild to moderate and comparable to 17-month-old ClC-5 knockout high citrate mice. Although some ClC-5 knockout high citrate mice had minor to mild tubular atrophy and/or interstitial fibrosis at 9 or 12 months, these histologic changes were significantly decreased compared to 9-month-old ClC-5 knockout zero citrate mice. These findings demonstrate that the interstitial fibrosis and tubular atrophy can be delayed in ClC-5 knockout mice fed a high citrate diet on a long-term basis.

Increased levels of TGF-β1, a fibrogenic cytokine, both stimulates the synthesis of extracellular matrix protein [36] and chemoatractants, as well as activates fibroblasts [37, 38], leading to tubular atrophy, tubulointerstitial fibrosis, and glomerulosclerosis [39]. Tubular obstruction leads to increases in TGF-β1 [36]. Proximal tubular cells may contribute to the progression of renal disease by synthesizing TGF-β1 [40–42]. TGF-β1 was increased in kidneys of 9-month-old ClC-5 knockout zero citrate mice compared to kidneys of 9-month-old wild-type zero citrate mice. In 9- and 12-month-old ClC-5 knockout and wild-type high citrate mice, TGF-β1 was undetectable. Although TGF-β1 is elevated in a number of progressive renal diseases [43], it appears that in ClC-5 knockout mice the activation of TGF-β1 cascade is most closely related to calcinosis and interstitial fibrosis.

Wrong, Norden, and Feest [2] found that 9 out of 11 of his Dent's disease patients had varying degrees of renal

failure at the age of 47 ± 13 years, whereas Frymoyer et al [24] found some degree of renal failure in seven out of eight patients between the age of 9 and 54 years. The average mouse life span is 24 ± 6 months. The ClC-5 knockout zero citrate mice had renal insufficiency at 9 months, whereas the ClC-5 knockout high citrate mice had renal insufficiency at 17 months, each group showing increased serum creatinine and BUN, and decreased GFR. Some ClC-5 knockout mice of the zero citrate cohort showed the first signs of renal disease with increased BUN at 6 months of age, but it was only at 12 months of age that some ClC-5 knockout high citrate mice showed increased BUN. Furthermore, ClC-5 knockout zero citrate mice develop signs of renal insufficiency with increased serum creatinine, increased BUN, and decreased GFR at 9 months age, whereas ClC-5 knockout high citrate mice did not develop similar signs until 17 months. Dietary citrate appears to delay the progression of renal disease, significantly preserving renal function in ClC-5 knockout mice compared to animals on a zero citrate diet.

The present study documents the progression of renal disease in the Johns Hopkins ClC-5 knockout mouse model. Dietary citrate apparently prolongs renal function in these mice despite the absence of stones and rather sporadic evidence of nephrocalcinosis. Maintenance of this mouse on zero citrate diet enables further studies of development of renal disease and the identification of new markers of impending renal function loss. If the results of the present study on a mouse model can be applied to human renal function in Dent's disease patients, the use of long-term dietary citrate supplementation would possibly delay progression of the renal disease.

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