Ketogenic Diet and Progression of Kidney Disease in Animal Models of Nephropathic Cystinosis

Francesco Bellomo[1], *Sara Pugliese*[1], *Sara Cairoli*[2], *Patrick Krohn*[3], *Cristiano De Stefanis*[4], *Roberto Raso*[1], *Laura Rita Rega*[1], *Anna Taranta*[1], *Ester De Leo*[1], *Andrea Ciolfi*[5], *Nicolò Cicolani*[6], *Stefania Petrini*[6], *Alessandro Luciani*[3], *Bianca Maria Goffredo*[2], *Ottavia Porzio*[7], *Olivier Devuyst*[3], *Carlo Dionisi-Vici*[2], and *Francesco Emma*[1,8]

Key Points

- Ketogenic diet can change the metabolism in the body and helped restore the function of altered pathways in nephropathic cystinosis.
- Ketogenic diet had significant benefits for preventing kidney damage, even when initiated after the onset of kidney impairment.
- Ketogenic diet may provide a partial therapeutic alternative in countries where cysteamine therapy is too expensive.

Abstract

Background Nephropathic cystinosis is a rare inherited lysosomal storage disorder caused by mutations in the *CTNS* gene that encodes for cystinosin, a lysosomal cystine/H^+ symporter. From the standpoint of the kidneys, patients develop early-onset renal Fanconi syndrome and progressive CKD. Current therapy with cysteamine delays but does not prevent kidney failure and has significant side effects that limit adherence and reduce the quality of life of patients.

Methods We have tested biochemically and histologically the effects of ketogenic diet on kidney disease of two animal models of nephropathic cystinosis.

Results When $Ctns^{-/-}$ mice were fed with ketogenic diet from 3 to 12 months of age, we observed significant nearly complete prevention of Fanconi syndrome, including low molecular weight proteinuria, glycosuria, and polyuria. Compared with wild-type animals, BUN at 12 months was higher in cystinotic mice fed with standard diet ($P < 0.001$), but not with ketogenic diet. At sacrifice, kidneys of knockout mice fed with ketogenic diet appeared macroscopically similar to those of wild-type animals, which was reflected microscopically by a significant reduction of interstitial cell infiltration (CD3 and CD68 positive cells, $P < 0.01$), of interstitial fibrosis (Masson and α-smooth muscle actin staining, $P < 0.001$), and of apoptosis (cleaved caspase-3 levels; $P < 0.001$), and by indirect evidence of restoration of a normal autophagic flux (SQSTM1/p62 and LC3-II expression, $P < 0.05$). Beneficial effects of ketogenic diet on tubular function were also observed after mice were fed with this ketogenic diet from the age of 6 months to the age of 15 months, after they had developed proximal tubular dysfunction. Although slightly less pronounced, these results were replicated in $Ctns^{-/-}$ rats fed with ketogenic diet from 2 to 8 months of life.

Conclusions These results indicate significant mitigation of the kidney phenotype in cystinotic animals fed with ketogenic diet.

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Introduction

Ketogenic diet is most commonly used as a dietary approach for treating adult obese patients. In pediatrics, ketogenic diet was initially used in the 1990s for treating children with severe drug-resistant epilepsy.[1] Thereafter, indications of ketogenic diet have extended to other conditions, in particular to metabolic diseases. Currently, ketogenic diet is a proven therapy for treating drug-resistant epilepsy in children,[2] glucose transporter type 1 deficiency,[3] and pyruvate dehydrogenase complex

[1]Laboratory of Nephrology, Bambino Gesù Children's Hospital IRCCS, Rome, Italy
[2]Division of Metabolic Diseases and Drug Biology, Bambino Gesù Children's Hospital IRCCS, Rome, Italy
[3]Institute of Physiology, University of Zurich, Zurich, Switzerland
[4]Core Facilities, Bambino Gesù Children's Hospital IRCCS, Rome, Italy
[5]Molecular Genetics and Functional Genomics, Bambino Gesù Children's Hospital IRCCS, Rome, Italy
[6]Confocal Microscopy Core Facility, Bambino Gesù Children's Hospital IRCCS, Rome, Italy
[7]Clinical Biochemistry Laboratory, Bambino Gesù Children's Hospital IRCCS, Rome, Italy
[8]Division of Nephrology, Bambino Gesù Children's Hospital IRCCS, Rome, Italy

Correspondence: Dr. Francesco Bellomo, email: francesco.bellomo@opbg.net

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deficiency.[4] Other indications have been proposed and are being tested, including Parkinson disease, Alzheimer disease, multiple sclerosis, migraine, and cancer.[5,6] The mechanisms of action of ketogenic diet in various conditions have not been fully elucidated. Several lines of evidence support the role of ketogenic diet in reducing oxidative stress, preventing mitochondria damage, and mitigating tissue inflammation and fibrosis.[7–10] These features have been reported in nephropathic cystinosis, a rare lysosomal storage disorder.[11–16] On these bases, we have tested ketogenic diet in *Ctns*$^{-/-}$ mice and rats.

Nephropathic cystinosis is caused by mutations in the *CTNS* gene that encodes for cystinosin, a lysosomal cystine/H^+ symporter.[17] In its most frequent and severe form termed nephropathic cystinosis (OMIM: 219800), patients present with early-onset renal Fanconi syndrome and develop with time a multisystem disease as a consequence of the accumulation of cystine and cystine crystals in nearly all organs.[18,19] The renal Fanconi syndrome corresponds to a global dysfunction of proximal tubular cells resulting in polyuria, urinary losses of low molecular weight (LMW) proteins, metabolic acidosis, glycosuria, hyperphosphaturia, and aminoaciduria. Before the introduction of cysteamine therapy that allows clearance of cystine from lysosomes, patients with nephropathic cystinosis developed progressive CKD leading to kidney failure around age 10 years.[18,19] With cysteamine, the age of need for dialysis is postponed by approximately 8–10 years.[19,20] Once established, the Fanconi syndrome does not respond to cysteamine.[21] Recently, positive effects have been reported only when cysteamine was started in the first 2 months of life.[22]

In this study, we have investigated the effects of ketogenic diet in two murine models of nephropathic cystinosis.

Methods

Animals and Experimental Diets

Ctns knockout (KO) mice were kindly provided by Prof. Corinne Antignac.[23] Sprague–Dawley rats *Ctns* KO rats were produced in by CRISPR-Cas9 technology.[24]

Animals were fed with a standard diet (diet code: 4RF21, Mucedola Srl, Settimo Milanese, Italy) or with a ketogenic diet (diet code: TD.96355, Teklad Diets, Envigo).

Animal care and experimental procedures were conducted following the European 2010/63/EU guidelines on the protection of animals used for scientific purposes and were authorized by the Italian Ministry of Health.

Experiments were performed using female animals that accumulate more cystine in the kidneys compared with males.[25]

Body mass index (BMI) was calculated as the weight/body surface area (kg/m^2) ratio. The body surface area was calculated with the following formula: $0.007184 \times \text{length}^{0.725} \times \text{weight}^{0.425}$, where length is in cm and weight in kg. Venous blood was collected from the tail vein to measure β-hydroxybutyrate using a ketone body tester (On Call GK Dual, Swiss Point of Care).

Assessment of Kidney Function

Animals were acclimatized in metabolic cages for 24 hours with *ad libitum* access to food and drinking water. Urine was collected during the following 24 hours in the presence of 0.1% sodium azide and 1× protease inhibitors (Thermo Scientific). Body weight and length were recorded; plasma was collected before entering metabolic cages. Urine and plasma analyses were performed by the veterinary laboratory Appia lab Srl (Rome, Italy). Urinary levels of low-molecular weight Clara cell protein 16 (CC16) and β2-microglobulin were measured by ELISA according to the manufacturer's instructions (Cloud-Clone Corp. CCC).

Histology

Immunohistochemistry was performed on 3-μm thick sections obtained from formalin-fixed tissue embedded in paraffin. After dewaxing and rehydrating, heat-induced epitope retrieval was performed by boiling slides in the presence of EDTA (Dako, Glostrup, Denmark) at pH=9. Slides were blocked in 5% BSA for 1 hour. Sections were then incubated overnight at 4°C with proper antibodies diluted at 1:100. Specifically, we used rabbit polyclonal anti-CD3 antibodies (ab5690, Abcam, United Kingdom), rabbit polyclonal anti-CD68 antibodies (PA5-78996, Thermo Fisher Scientific), or rabbit polyclonal anti-α-smooth muscle actin (GTX100034, GeneTex). Detection of primary antibody was performed using appropriate secondary biotinylated antibodies (Dako, Carpinteria) and the 3,3′-diaminobenzidine tetrahydrochloride kit peroxidase (Dako) with counterstaining with Gill's hematoxylin (Dako). Immunofluorescent staining was performed by using Lotus Tetragonolobus Lectin, Biotinylated (B-1325, Vector Laboratories) diluted at 1:100, and Phycoerythrin Streptavidin 1:200 (554061, BD Biosciences).

Microscopy Imaging

Samples were acquired at the NanoZoomer S60 (Hamamatsu, Shizuoka, Japan) digital slide scanner platform, equipped with an Olympus 20×/0.75 PlanSAPO objective, a fluorescence imaging module equipped with a L11600 mercury lamp (Hamamatsu) and a linear ORCA-Flash 4.0 digital CMOS camera (Hamamatsu). Confocal microscopy was performed on a laser-scanning confocal microscope (FV3000, EVIDENT-Olympus, Hamburg, Germany) equipped with 405, 488, and 561 nm diode lasers, two photomultiplier tubes, and two gallium arsenide phosphide cathodes detectors. Sequential confocal images were acquired using extended apochromat 20×/0.80 dry objective and 60×/1.42 oil immersion objective (EVIDENT-Olympus), with a 1024×1024 format and 8 μs/pixel scan speed. Fluorochromes unmixing was performed by the acquisition of an automated-sequential collection of multichannel images to reduce spectral crosstalk between channels. Analysis was performed with the ImageJ 1.53t software (https://imagej.net/ij/).

Western Blotting

Kidney cortex tissue was homogenized with a disperser (Z722359, IKA-Werke, Staufen im Breisgau, Germany) in the RIPA lysis buffer (R0278, Sigma-Aldrich) in the presence of phosphatase (04906845001, PhosSTOP Sigma- Aldrich) and protease inhibitors (1836153001, Roche, Basel, Switzerland). After sonication and centrifugation at 20,000×g for 10 minutes at 4°C, samples were dissolved in Laemmli sample buffer to load approximately 20 μg of

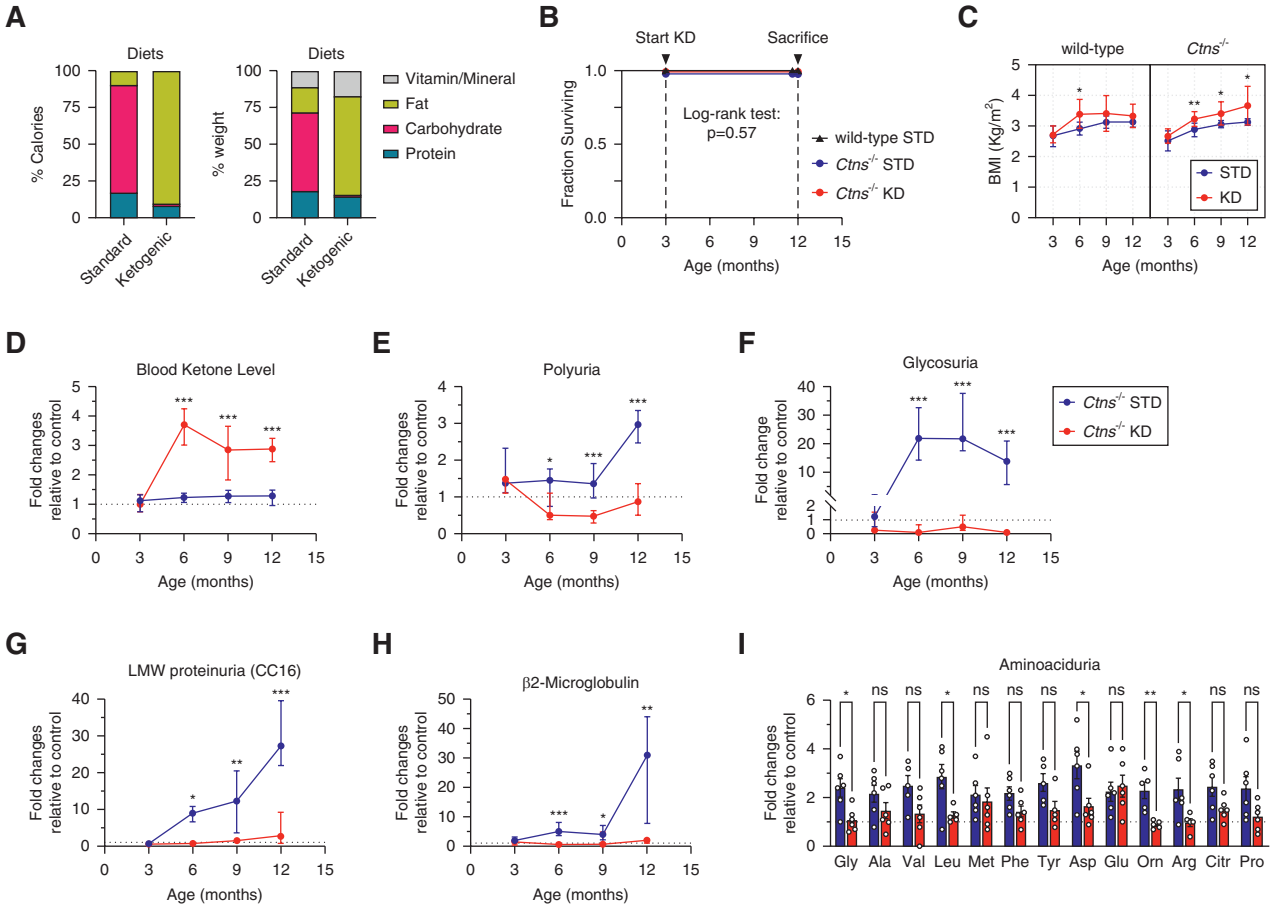

Figure 1. Preventive effect of ketogenic diet on the decline of kidney function in *Ctns*$^{-/-}$ mice. (A) The major components of the standard diet (4RF21, Mucedola Srl) and of the ketogenic diet (TD.96355, Teklad Diets) used in all experiments are shown as relative energy supply (left panel and in percent of weight composition (right panel). (B) No animal died during the duration of the study. (C) The BMI was significantly increased at certain ages in mice fed with the ketogenic diet. (D) Blood ketone levels were measured every 3 months by test strip on blood from tail. (E) Every 3 months, 24-hour urine was collected in metabolic cage, and it was calculated by comparing with WT mice fed with standard diet, the relative amount of 24-hour urine volume; (F) the relative level of glucose/creatinine ratio; (G) the relative level of CC16/creatinine ratio; (H) the relative level of β-2-microglobulin/creatinine ratio. (I) Relative level of aminoaciduria was assayed in 12-month-old mice. After tests for normal distribution, data in (C–H) were represented as median±interquartile range, and statistical differences between *Ctns*$^{-/-}$ standard diet and *Ctns*$^{-/-}$ ketogenic diet groups (n=8) were calculated by the Mann–Whitney test. Histogram of (I) was represented as mean±SEM, and statistical differences between groups (n=6) were calculated by unpaired t test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Ala, alanine; Arg, arginine; Asp, aspartic acid; BMI, body mass index; CC16, Clara cell protein 16; Citr, citrulline; Glu, glutamic acid; Gly, glycine; KD, ketogenic diet; Leu, leucine; Met, methionine; Orn, ornithine; Phe, phenylalanine; Pro, proline; STD, standard diet; Tyr, tyrosine; Val, valine; WT, wild type.

proteins per lane. After separation by SDS-PAGE in reducing conditions, blotting onto PVDF membranes, and blocking with 5% nonfat milk (1706404, Bio-Rad, Hercules, CA) diluted in PBS, membranes were incubated overnight at 4°C with primary antibodies at 1:200 dilution, including rabbit anti-p62/SQSTM1 antibodies (PM045, MBL Life Sciences; Japan) or rabbit anti-LCN2/Megalin antibodies (ab63929, Abcam). After incubation with peroxidase-labeled secondary antibodies, immunoblots were visualized with enhanced chemiluminescence (WBKLS0050, Millipore, Life technologies). Quantitative analyses were performed by scanning blots and measuring relative densities of each band normalized to mouse monoclonal β-actin (A2228, Sigma-Aldrich; dilution 1:10,000) using the ImageJ 1.53t software (https://imagej.net/ij/).

Cystine Measurements

Renal cystine content was assayed as previously described.[26] Briefly, dissected tissues were homogenized in 10 mM *N*-ethylmaleimide (E3876, Sigma-Aldrich) and treated with 10% 5-sulfosalicylic acid (S2130, Sigma-Aldrich) in equal parts to precipitate the protein fraction. The supernatant was extracted in acetonitrile and analyzed by ultra-high-performance liquid chromatography using the Agilent 1290 Infinity II 6470 instrumentation (Agilent Technologies), equipped with a mass spectrometer using ESI-JET-STREAM source operating in the positive ion (ESI+) mode.

RNA-Seq, Library Construction, and Data Processing

Total RNA was extracted from kidney cortex of 3-month-old mice that had been fed with standard or with ketogenic diet

Table 1. Plasma values in mice at 12 months

Parameters	WT STD	WT KD	*Ctns*$^{-/-}$ STD	*Ctns*$^{-/-}$ KD
AST, IU/L	199±77	209±100	228±67	189±25
ALT, IU/L	46±29	31±22	45±15	27±8
Creatinine, mg/dl	0.52±0.23	0.41±0.27	0.44±0.29	0.34±0.24
CPK, IU/L	588±253	655±553	1242±152[a]	308±186[b]
BUN, mg/dl	22±2	14±5	37±8[c]	13±7[b]
Glucose, mg/dl	160±10	179±41	153±27	139±29
Cholesterol, mg/dl	63±12	83±13	62±14	83±10
Triglycerides, mg/dl	97±17	113±18	102±18	119±21

Data are indicated as mean±SD of eight animals per group. Statistical comparison between groups was determined by one-way ANOVA corrected with the Tukey multiple comparisons test. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CPK, creatinine phosphokinase; KD, ketogenic diet; STD, standard diet; WT, wild type.
[a]*Ctns*$^{-/-}$ standard diet versus wild-type standard diet, $P < 0.01$.
[b]*Ctns*$^{-/-}$ ketogenic diet versus *Ctns*$^{-/-}$ standard diet, $P < 0.001$.
[c]*Ctns*$^{-/-}$ standard diet versus wild-type standard diet, $P < 0.001$.

for 1 month. Library construction and sequencing services were performed at BGI Genomics (Shenzhen, China) (see Supplemental Methods). The RNA-Seq data of this publication are available through NIH's archive of high-throughput sequencing data Sequence Read Archive, with BioProject accession number: PRJNA1112991.

Statistics

All statistical analyses were performed using the GraphPad Prism Software version 10.1.2 (324).

Normal distribution was assessed by the Kolmogorov–Smirnov normality test and the Shapiro–Wilk normality test. Nonparametric data were expressed as median±interquartile range and were analyzed using the Kruskal–Wallis test with Dunn multiple comparison correction. Pairwise comparisons were evaluated by the Mann–Whitney *U* test.

Normal distributed data were expressed as mean±SEM and were compared by one-way ANOVA followed by Bonferroni multiple comparison test, when applicable.

Survival analysis was performed with the Kaplan–Meier method and assessed by the log-rank test.

All *P* values were considered significant at the < 0.05 level.

Results

Mice were treated with standard or ketogenic diet from age 3 to 12 months. All experiments were performed on eight animals per group. The energy content of the ketogenic diet used for the study derived from fat by 90.5% and carbohydrate by 0.6% (Figure 1A).

We observed no premature deaths in all treatment groups (Figure 1B). The average BMI of mice fed with ketogenic diet, in particular of KO mice, was 11%–17% higher ($P < 0.05$) during the study period compared with mice fed with standard diet (Figure 1C). As expected, serum ketone levels increased in all animals fed with ketogenic diet (Figure 1D).

Table 1 shows the results of blood tests performed at age 12 months, after sacrifice. No significant abnormalities were observed in liver function tests. Compared with wild-type (WT) animals, KO mice fed with standard diet developed biochemical evidence of myopathy as documented by higher creatinine phosphokinase levels (1242±152 versus 588±253 IU/L, $P = 0.002$), which were normalized if the same animals had been fed with a ketogenic diet (308±186 IU/L, $P < 0.001$).

From the kidney standpoint, serum creatinine remained within the normal range (0.3–1.0 mg/dl) in all animals. Under standard diet, BUN levels increased significantly in KO mice compared with WT animals (WT versus KO: 22±2 versus 37±8 mg/dl, $P < 0.001$). Conversely, under ketogenic diet, BUN levels remained unchanged also in KO animals (WT versus KO: 14±5 versus 13±7, $P = 0.74$). As shown in Figure 1, E–I, *Ctns*$^{-/-}$ mice fed with standard diet developed from age 6 months significant polyuria, glycosuria, LMW proteinuria (*i.e.*, CC16 and β2-microglobulin urinary excretion), and aminoaciduria. These were almost completely prevented in animals fed with ketogenic diet.

After sacrifice, kidney samples were collected and analyzed. As shown in Figure 2, kidneys harvested from KO animals fed with standard diet appeared smaller and pale, while those harvested from KO animals fed with ketogenic diet had a normal macroscopic appearance (Figure 2, A–C). At the microscopic levels, kidneys of KO mice fed with standard diet showed significant lymphocyte (CD3-positive cells) and macrophage (CD68-positive cells) infiltrates (Figure 2, D and E) and extensive areas of interstitial fibrosis (Figure 2, F and G), all of which were preserved in mice fed with ketogenic diet. The area covered by proximal tubules was preserved in KO animals fed with ketogenic diet, as well as the overall expression of megalin (Figure 3, A–C). By Western blotting, megalin expression was decreased in kidney cortex homogenates obtained from KO mice fed with standard diet (Figure 3D). By confocal microscopy, megalin expression at the brush border level was preserved in these same animals (Supplemental Figure 1), indicating that the decreased expression of megalin observed by Western blotting was primarily because of the loss of proximal tubules, rather than to a decrease in megalin expression in the remaining tubular cells. These changes were prevented by ketogenic diet. Increased apoptosis and impaired autophagic flux have been well documented in this model of

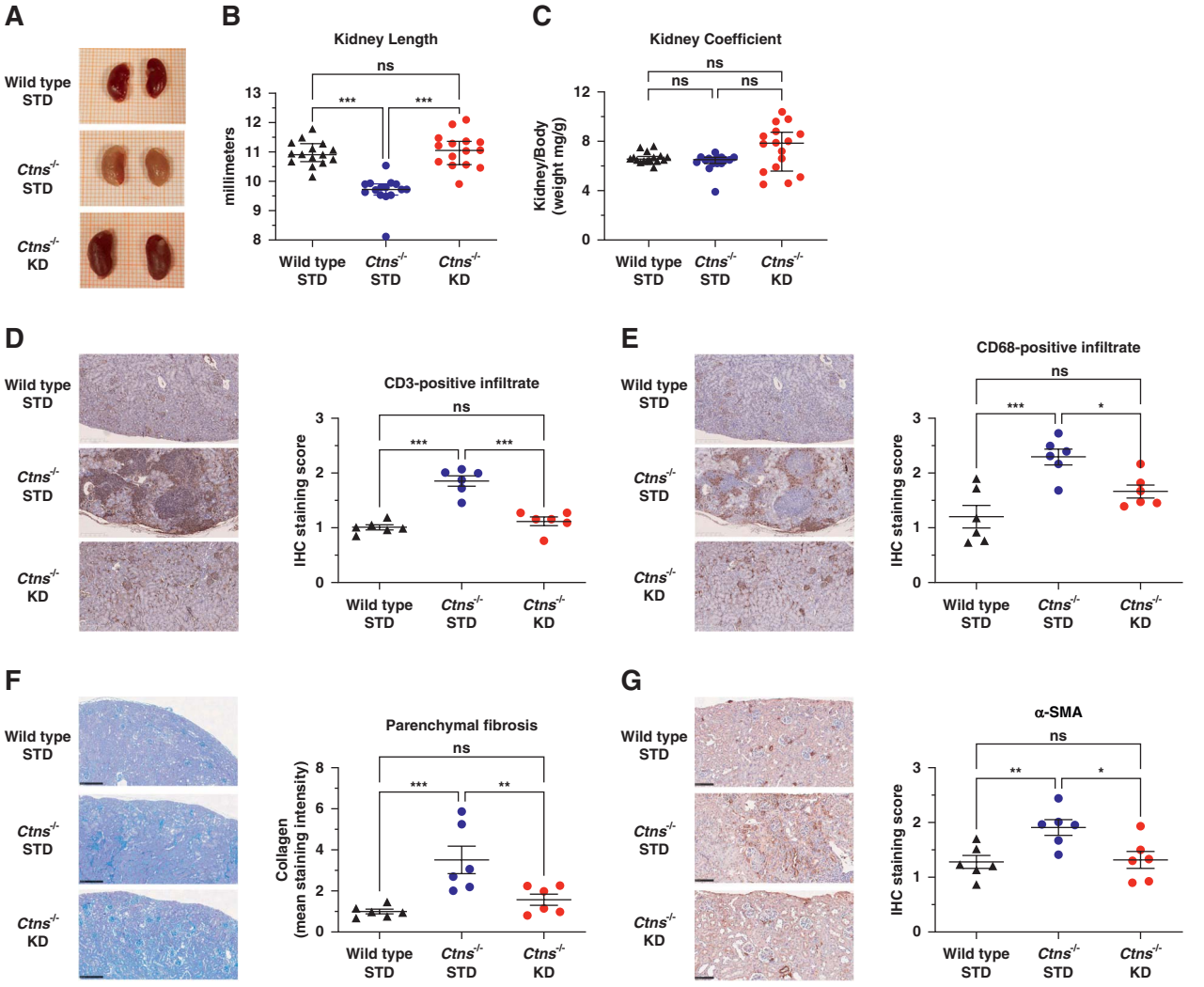

Figure 2. Morpho-histological outcome on kidney of *Ctns*$^{-/-}$ mice fed with ketogenic diet. Kidney explanted in 12-months-old mice, after 9 months of diet, were analyzed for their morphological aspect and morphometric parameters. (A) Representative images of kidneys from WT mice fed with standard diet and *Ctns*$^{-/-}$ fed with ketogenic diet shows macroscopic morphological differences between groups. (B) Pulled left and right kidneys were evaluated for their length and (C) their weight normalized for animal body weight; statistical differences between groups (n=16) were calculated by Mann–Whitney test. (D and E) Inflammation was assessed by IHC staining and analysis on deconvolved images by evaluating CD3-positive lymphocytic infiltrates and CD-68-positive macrophagic infiltrates. (F and G) Kidney fibrosis was evaluated by Masson's trichrome stain for collagen accumulation and by IHC staining with α-SMA for activated fibrogenic cells content. Differences between groups (n=6) were calculated by ANOVA corrected by using Bonferroni statistical hypothesis testing, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. α-SMA, alpha smooth muscle actin; IHC, immunohistochemistry.

nephropathic cystinosis. Consistently, we observed increased levels of cleaved caspase-3 expression in KO animals, which were normalized if these were fed with ketogenic diet (Figure 4A). Similarly, we indirectly observed preservation of the autophagic flux, as shown by the normalization of SQSTM1/p62 levels and LC3-II (Figure 4B). As expected, cystine accumulated in kidneys harvested from KO mice, but significantly less in those fed with ketogenic diet (Figure 4C). Furthermore, proximal tubules of *Ctns*$^{-/-}$ mice appeared more dilated compared with WT, but this morphological aspect was corrected by the ketogenic diet (Figure 4D).

In the vast majority of cases, the diagnosis of nephropathic cystinosis is made in humans after they develop symptoms related to the Fanconi syndrome, and treatment is started only after proximal tubular dysfunction has developed. We have therefore tested if ketogenic diet maintains its beneficial effects if started after the proximal tubulopathy has developed. To this end, we have treated KO mice (n=8 per group) with ketogenic diet from the age of 6 months until the age of 15 months. Three animals died in each group between the ages of 12 and 15 months (Figure 5A), and BMI is essentially unchanged (Figure 5B). Blood ketone levels of animals fed with standard diet tended to increase with age. Animals fed with ketogenic diet showed significant increase in ketonemia during the first 12 months of life, after which it dropped almost to normal levels (Figure 5C). Polyuria was prevented, glycosuria

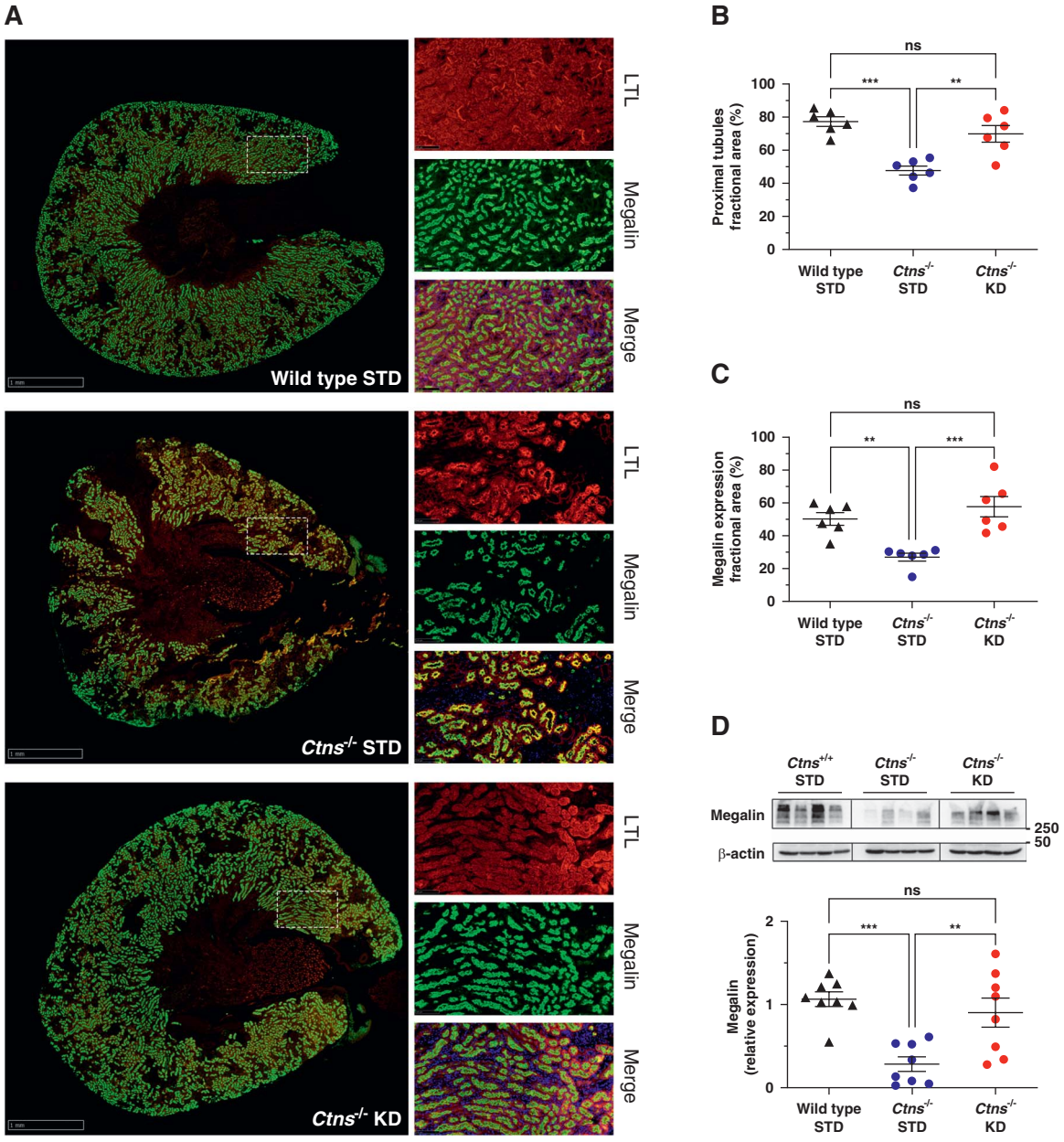

Figure 3. Ketogenic diet preserves renal proximal tubules in *Ctns*$^{-/-}$ mice. (A) Transversal kidney sections were stained with LTL in red, used to label the brush border of the proximal tubules, and megalin in green. Representative images, at 2× magnification scale bar=1 mm, macroscopically show large areas of proximal tubular injury in *Ctns*$^{-/-}$ mice fed with standard diet, whereas the aspect of *Ctns*$^{-/-}$ mice fed with ketogenic diet was comparable with WT mice. Quantitative evaluation was assessed with ImageJ software by calculating (B) the fractional area of the proximal tubules and (C) the fractional area of megalin in five microscopic fields (20×) for each kidney section. (D) Levels of megalin expression was also estimated by Western blotting in kidney cortex homogenates. Differences between groups were calculated by ANOVA corrected by using Bonferroni statistical hypothesis testing, $^{**}P < 0.01$; $^{***}P < 0.001$. LTL, *Lotus tetragonolobus* lectin.

decreased rapidly, and LMW proteinuria was stabilized after the initiation of ketogenic diet (Figure 5, D–F). Parenchymal fibrosis and inflammatory infiltrates tended to be mitigated, although the results did not reach statistical significance (Figure 5, G–J). These latter evaluations were possibly underpowered because animals died.

To identify pathways involved in the rescue of the kidney phenotype of *Ctns*$^{-/-}$ mice, we performed comparative transcriptomic analyses on kidney cortex harvested from 3-month-old mice that had been fed with ketogenic diet for 1 month (n=5 per group). At age 3 months, KO mice have no urinary evidence of proximal tubulopathy. RNA-seq data are therefore more likely to identify primary process rather than changes secondary to chronic lesions. As shown in Figure 6A, the analysis of differentially expressed genes (DEGs) revealed that *Ctns*$^{-/-}$ mice fed with standard diet showed an upregulation of 135 genes and a downregulation of 261 genes compared with WT animals fed with the same

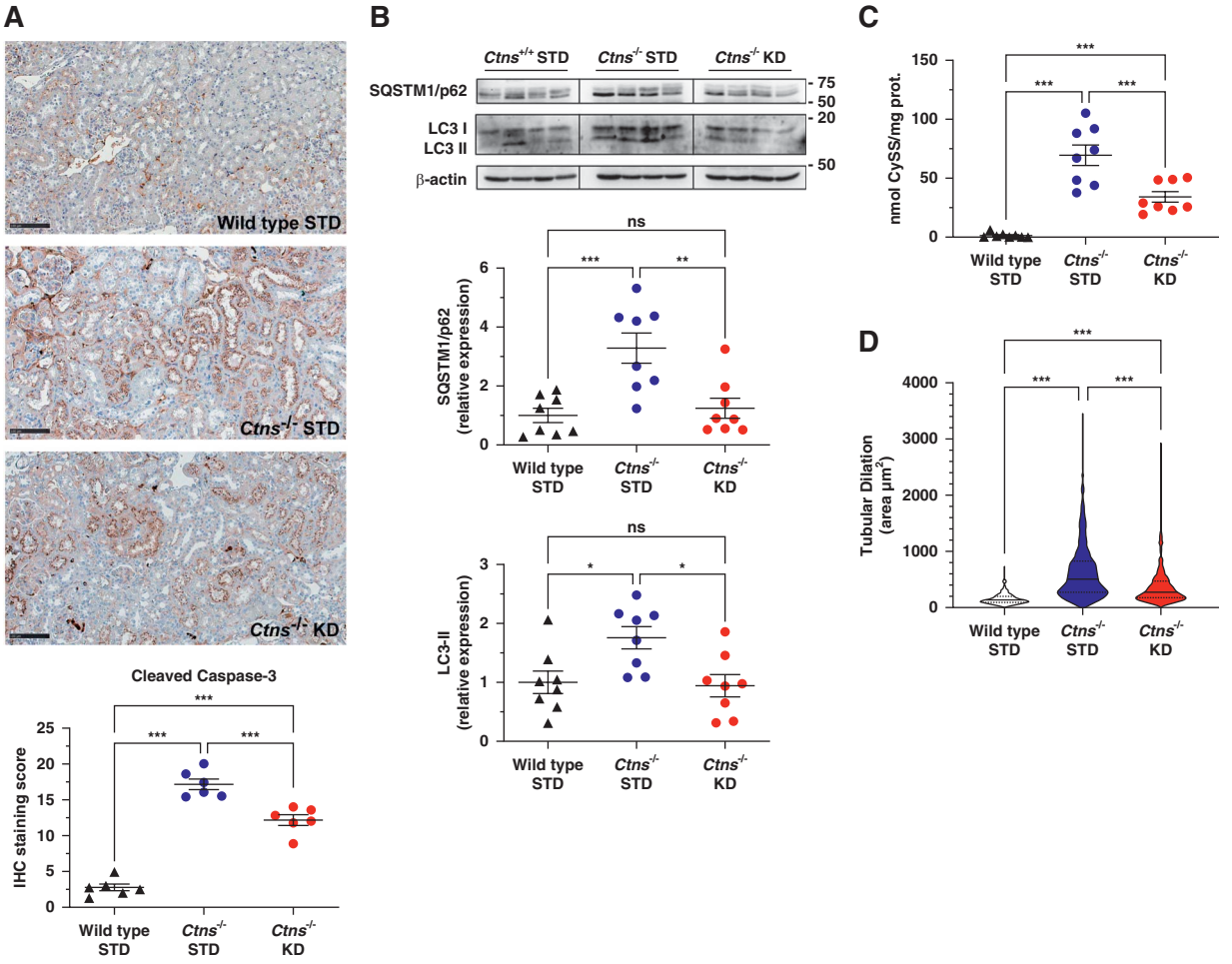

Figure 4. Ketogenic diet ameliorates cellular phenotype in kidneys of *Ctns*$^{-/-}$ mice. (A) Kidney cortex samples obtained from 12-month-old mice of different groups were immunoassayed for cleaved caspase-3 to evaluate apoptosis. (B) Kidney cortex homogenates were analyzed by Western blotting for SQSTM1/p62 expression and for LC3 expression in its cytosolic form (LC3-I) and LC3-phosphatidylethanolamine conjugate form (LC3-II), all normalized to the β-actin expression (the images show representative samples; each lane corresponds to a different animal). (C) Analogously, cystine levels were measured on snap-frozen samples from kidney cortex. (D) Tubular dilation was evaluated as tubular area measured in kidney sections of four mice for each experimental condition (about 70 tubules/animal). Dot plots in (A–C) were represented as mean±SEM, and statistical differences between groups (n=8) were calculated by ANOVA corrected by using Bonferroni statistical hypothesis testing. Statistics for violin plot in (D) was calculated with the Kruskal–Wallis test and corrected with the Dunn test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

diet. Overall, 476 and 212 genes were significantly upregulated and downregulated by ketogenic diet in *Ctns*$^{-/-}$ mice, respectively (Figure 6B). Of these, 144 genes (listed in Supplemental Table 1) were abnormally expressed in KO animals compared with WT animals (Figure 6, C and D). At the cellular level, gene ontology analysis showed that ketogenic diet significantly ameliorated the expression of mitochondria, peroxisome, and endoplasmic reticulum genes that were dysregulated in KO animals fed with standard diet (Figure 6E). In biological processes, gene ontology analysis showed that ketogenic diet restored mainly lipid metabolism processes that were altered in KO animals and other processes that are directly or indirectly associated with lipid metabolism (Figure 6F). Analysis of the Kyoto Encyclopedia of Genes and Genomes database showed that ketogenic diet significantly affected peroxisomal, fatty acid, and endoplasmic reticulum pathways (Figure 6G). Additional analyses of the effects of ketogenic diet on gene expression in the kidney cortex of WT and *Ctns*$^{-/-}$ mice are provided in Supplemental Figure 2.

Finally, we have validated findings in mice in our recently developed rat model of cystinosis. Compared with mice, kidney lesions develop earlier in rats. Animals were fed from the age of 2 months with a ketogenic diet and were sacrificed at age 8 months. As expected, ketones increased under ketogenic diet, but unlike what we observed in mice, the BMI was not modified (Supplemental Figure 3, A and B). As shown in Supplemental Figure 3, C–F, feeding rats with ketogenic diet significantly prevented polyuria and glycosuria and decreased LMW proteinuria, although not as completely as what we observed in mice. Similar to mice, we observed at the parenchymal level a significant reduction of inflammatory infiltrates and kidney fibrosis (Supplemental Figure 3, G–J).

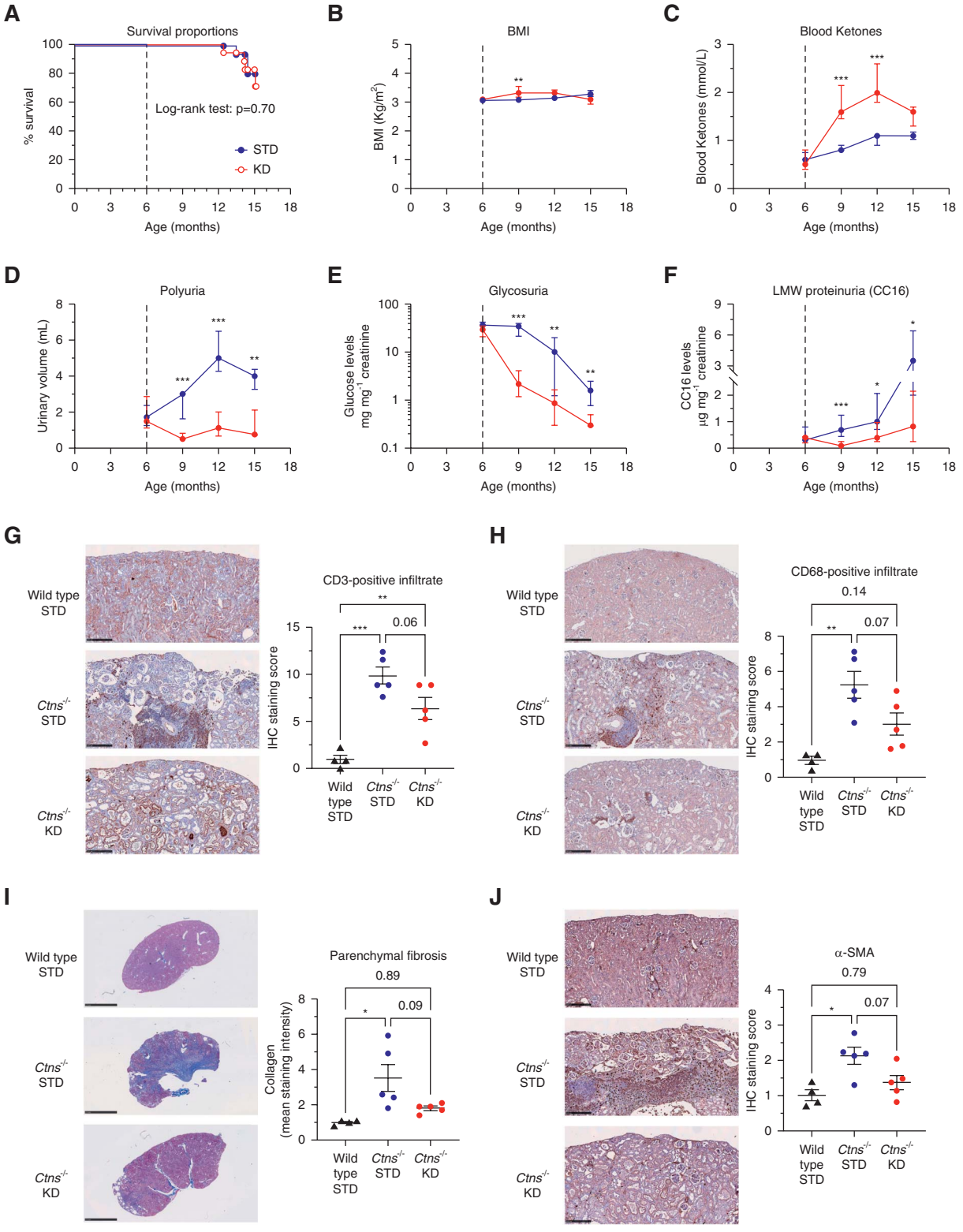

Figure 5. Therapeutic effect of ketogenic diet in *Ctns*$^{-/-}$ mice with an intermediate stage of disease. To show a therapeutic achievement of ketogenic diet, *Ctns*$^{-/-}$ mice was split in two groups (n=9) fed with standard diet (blue line) or ketogenic diet (red line) starting from 6 months of life (dotted line). (A) The survival proportion was not affected, other than natural decline after 12 months of life as well as (B) the BMI, which was substantially unchanged. (C) Blood ketones significantly increased during first 6 months of ketogenic diet as expected, however *Ctns*$^{-/-}$ mice fed with standard diet as they grew older tended to increase blood ketones. Every 3 months, 24-hour urine was collected in metabolic cage, and (D) polyuria, (E) glycosuria, and (F) LMW proteinuria as CC16/creatinine have been evaluated. Data are represented as median±interquartile range, and statistical differences between groups were calculated by the

Figure 5. *Continued.* Mann–Whitney test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. At sacrifice, kidneys from 15-month-old mice were collected and analyzed to evaluate inflammation with (G) CD3 immunostaining and (H) CD68 immunostaining and fibrosis with (I) Masson trichrome staining and (J) α-SMA immunostaining. Histological data are represented as mean±SEM, and differences between groups (n=5) were calculated by ANOVA corrected by using Bonferroni statistical hypothesis testing. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Bar=250 μm in (G), (H), and (J). Bar=2.5 mm in (I). LMW, low molecular weight.

Discussion

In the present work, we have used two different preclinical models of nephropathic cystinosis and have shown that feeding mice or rats with a ketogenic diet prevented or delayed the development of overt kidney disease.

Since the 1980s, treatment with cysteamine, which allows clearance of cystine from lysosomes, has significantly improved the prognosis of nephropathic cystinosis, allowing patients to live well into adulthood. Nonetheless, nephropathic cystinosis remains a severe disease. With cysteamine, which has significant side effects, kidney failure is postponed only by 8–10 years, and most patients develop in time extrarenal symptoms.

The cystinosin protein has a pleiotropic role, acting as a lysosomal cystine transporter and interacting with multiple proteins.[27–29] Using different *in vitro* and *in vivo* models, several laboratories, including ours, have documented in recent years abnormal mammalian target of rapamycin complex 1 signaling, altered macro-mediated and chaperon-mediated autophagy, impaired cellular trafficking, cell oxidation, mitochondrial damage, increased apoptosis, cell dedifferentiation, and increased inflammation in cystinosis.[11–14,30–41] Among these processes, cell oxidation and inflammation probably play key roles in the final steps of cell and tissue damage. Several of the above-mentioned pathways do not respond well to cysteamine and could be amendable to treatment with ketogenic diet.[14,42–45]

In human medicine, ketogenic diet has been introduced in the 1990s to treat severe forms of childhood epilepsy and has gained popularity in recent years for treating several conditions, including lysosomal storage diseases.[46–49] The exact mechanisms of action of ketogenic diet are not completely understood. Ketogenic diet has been shown to reduce oxidative stresses, to mitigate inflammation and fibrosis, to exert an anaplerotic effect by increasing the availability of energy substrates to the Krebs cycle, and to modulate favorably epigenetic changes.[50–55] Inhibition of apoptosis and mitigation of mitochondrial damage has been documented in rat models of traumatic brain injury fed with a ketogenic diet.[56] *In vitro*, the ketone body β-hydroxybutyrate has been shown to promote intestinal cell differentiation by direct or indirect interaction with the mammalian target of rapamycin complex 1 system and to stimulate the autophagic flux in cortical cultured neurons.[57,58] Relevant to the present work, prolonged ketogenic diet has been shown to delay cardiac aging in mice by reducing oxidative stress, by improving mitochondrial function, and by stimulating the autophagic flux.[59] Ketogenic diet also attenuates oxidative damage and inflammation in murine models of renal ischemia–reperfusion injury.[8]

On these bases, we have hypothesized that feeding cystinotic mice and rats with a ketogenic diet could correct cell processes that do not respond well to cysteamine and may represent a complementary approach for treating patients with nephropathic cystinosis. We observed that ketogenic diet induced a remarkable functional and structural protection against the development of kidney disease. Moreover, this positive effect was retained when animals were treated after proximal tubular dysfunction had developed. In mice, preemptive therapy nearly completely abolished kidney lesions; results in rats were slightly less pronounced.

One of the hallmarks of Fanconi syndrome is disruption of the receptor-mediated endocytic mechanisms that allow LMW proteins reabsorption by tubular cells.[60] Accordingly, we observed a marked reduction in urinary excretion of CC16 and β2-microglobulin in animals fed with a ketogenic diet. At the histological level, we observed a remarkable preservation of proximal tubular structures, associated with reduced apoptosis and indirect evidence of improved autophagy. Since these latter processes are ubiquitous, ketogenic diet may also positively affect lesions in other organs that are progressively damaged in patients with nephropathic cystinosis. SQSTM1/p62 and LC3 are key players in the autophagic process, and their upstream accumulation may be used to indirectly assess the integrity of the autophagic flux.[61] Consistently, we observed in KO mice increased expression of SQSTM1/p62 and LC3-II, which was restored after feeding animals with ketogenic diet.

Inflammation plays an important role in the progression of kidney diseases, including cystinosis, as indicated by studies in *Ctns*$^{-/-}$ mice that have shown positive effects of anti-inflammatory approaches.[15,28,62–65] We also observed a marked reduction in renal inflammatory cell infiltration in animals fed with ketogenic diet. Several mechanisms may have mediated the anti-inflammatory effects of ketogenic diet. Notably, β-hydroxybutyrate has been shown to inhibit NOD-like receptor family pyrin domain containing 3,[66] which is activated in cystinotic cells.[67] Ketogenic diet could have also acted through its inhibitory activity on the renin-angiotensin system,[68] which plays a pivotal role in promoting kidney fibrosis.[69]

Since ketogenic diet affects directly or indirectly multiple metabolic pathways, its mechanisms of action are inevitably complex and may vary in different organs. We have begun studying these effects in the kidney cortex through a transcriptomic approach. Our results indicate significant changes in the expression of genes involved in lipid metabolism, peroxisomal and mitochondrial function, and pathways linked to the endoplasmic reticulum, among others. These data represent the starting point for future studies.

Ketogenic diet could also have produced beneficial effects by lowering renal cystine accumulation. Previous studies using the same *Ctns*$^{-/-}$ mouse model have shown that cystine crystals promote tubular cell damage.[70] Currently, cysteamine is the standard of care for cystinosis. In *Ctns*$^{-/-}$

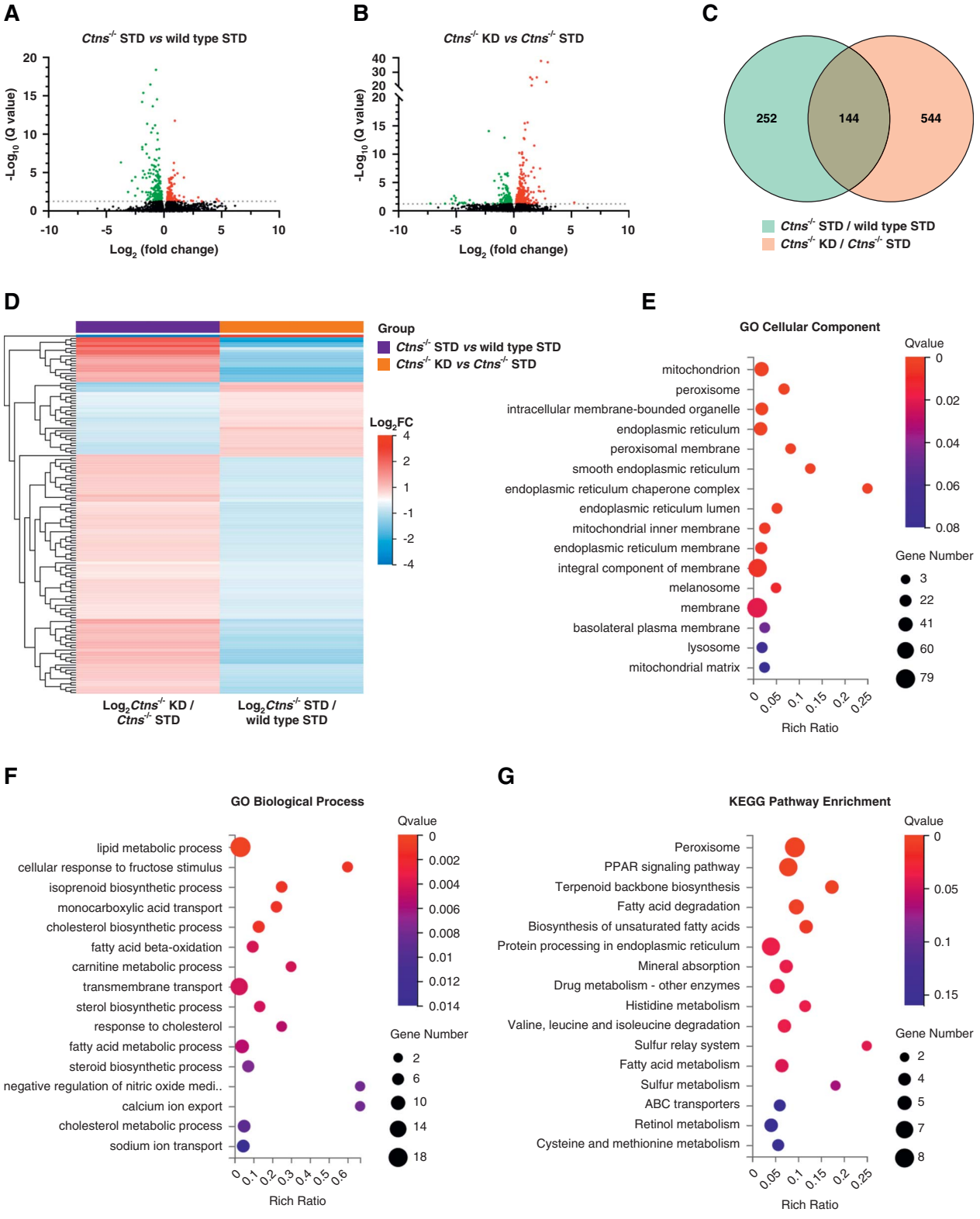

Figure 6. RNA-seq analysis of cortical kidney from 3-month-old mice. RNA-seq was performed on kidney cortex harvested from 3-month-old mice that had been fed with ketogenic diet for 1 month and compared them with those fed with standard diet (n=5 per group). (A) The analysis of differentially expressed genes (DEGs) revealed by a Volcano plot that *Ctns*$^{-/-}$ mice fed with standard diet showed upregulation of 135 genes (Q value ≤0.05, log2 [fold change] ≥0.2, and a downregulation of 261 genes (Q value ≤0.05, log2 [fold change] ≤−0.2) compared with WT animals fed with the same diet. (B) Analysis of DEGs in *Ctns*$^{-/-}$ mice fed with ketogenic diet showed upregulation of 476 genes (Q value ≤0.05, log2 [fold change] ≥0.2, and a downregulation of 212 genes (Q value ≤0.05, log2 [fold change] ≤−0.2) compared with animals fed with standard diet. (C) Comparison between WT and *Ctns*$^{-/-}$ mice on a standard diet, and

Figure 6. *Continued.* Ctns$^{-/-}$ mice on a ketogenic diet versus a standard diet, revealed significant shifts in gene expression. This analysis identified 144 DEGs abnormally expressed in *Ctns*$^{-/-}$ animals, whose expression was modified by the ketogenic diet (detailed in Supplemental Table 1). (D) These data are further illustrated in a differential cluster heat map. (E–G) Analyses of cell processes and metabolic pathways using the GO and the KEGG pathway databases identified abnormally expressed genes that were potentially corrected by the ketogenic diet (see text for details). The size of the dots represents the number of genes in different items, and the rich factor is the proportion of DEGs in each item. GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

mice, we observed that cysteamine at a dose of 400 mg/kg per day administered from the age of 2 months prevented nearly completely the development of LMW proteinuria and decreased urine output by 55% at age 12 months compared with untreated animals.[26] Glycosuria in this mouse model has a biphasic evolution as shown in Figure 1F, with an initial increase that peaks around age 6–9 months and decreases afterward. When mice were treated with cysteamine, the peak of glycosuria was abolished, but residual glycosuria could be appreciated at age 12 months.[26] At sacrifice, at the age of 16 months, renal cystine content was completely normalized under cysteamine therapy, lysosomal distribution in the kidney cortex was markedly improved, and atrophy of proximal tubules was significantly prevented.[26] Although these experiments were performed using the same *Ctns*$^{-/-}$ mouse model, direct comparison between ketogenic diet and cysteamine is not possible because we did not include a parallel group treated with cysteamine. This is a limitation of the present study. Both treatments significantly prevent the development of Fanconi syndrome and of kidney parenchyma lesions in *Ctns*$^{-/-}$ mice, while cysteamine prevents more efficiently kidney cystine accumulation.

Interestingly, we have observed that genistein, a flavonoid molecule, protects kidney parenchyma in *Ctns*$^{-/-}$ mice, possibly through stimulation of lysosomal exocytosis, which is dependent on the activation of several interconnected pathways, including activation of the transcription factor EB, activation of autophagy, and inhibition of mammalian target of rapamycin.[26] Ketogenic diet may have lowered renal cystine through similar effects. Of note, the protein content of the ketogenic diet used in this study was 15.3%, as opposed to 18.5% in the standard diet. However, it is unlikely that these differences have affected renal cystine content because cystine accumulation in proximal tubular cells depends primarily on endocytic processes that allow the reabsorption of LMW proteins.[71]

Finally, sodium-glucose cotransporter-2 (SGLT2) inhibitors have emerged in recent years as potent drugs to slow the progression of CKD.[72] Interestingly, SGLT2 inhibitors can induce ketoacidosis in patients with diabetes mellitus,[73] raising the possibility that ketogenic diet and SGLT2 inhibitors may act on similar pathways.

Proximal tubulopathy in humans with nephropathic cystinosis is more severe and develops proportionally earlier than in mice and rats. This limits the interpretation of data obtained in murine models and represents a limitation of this study. However, we observed beneficial effects even when animals were fed with ketogenic diet after overt kidney disease had developed, which mimics better the human condition. Other limitations include the fact that BUN is particularly variable in the C57/BL6 mouse strain, which limits the evaluation of kidney function.

Taken together, this study shows that ketogenic diet has significant protective effects on the kidney disease of murine models of cystinosis and may represent a complementary approach to cysteamine. In future studies, it will be important to assess the benefits of combining treatments. Because both high doses of cysteamine and ketogenic diet nearly completely abolish the kidney phenotype in mice,[26] studies will need to use low doses of cysteamine and only partial ketogenic diets, such as the modified Atkin diet, which is increasingly used in human medicine.

Disclosures

Disclosure forms, as provided by each author, are available with the online version of the article at http://links.lww.com/JSN/E772.

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Author Contributions

Conceptualization: Francesco Bellomo, Francesco Emma.
Data curation: Ester De Leo, Sara Pugliese, Laura Rita Rega, Anna Taranta.
Formal analysis: Francesco Bellomo, Sara Cairoli, Andrea Ciolfi, Ester De Leo, Patrick Krohn, Sara Pugliese.
Funding acquisition: Francesco Bellomo, Francesco Emma.
Investigation: Francesco Bellomo, Sara Cairoli, Nicolò Cicolani, Cristiano De Stefanis, Patrick Krohn, Sara Pugliese, Roberto Raso, Laura Rita Rega, Anna Taranta.
Methodology: Bianca Maria Goffredo, Stefania Petrini, Ottavia Porzio.
Project administration: Francesco Bellomo, Francesco Emma.
Resources: Nicolò Cicolani, Cristiano De Stefanis, Sara Pugliese, Roberto Raso.

Supervision: Francesco Bellomo, Olivier Devuyst, Carlo Dionisi-Vici, Francesco Emma, Alessandro Luciani, Ottavia Porzio.
Validation: Francesco Bellomo, Ester De Leo, Alessandro Luciani.
Visualization: Francesco Bellomo.
Writing – original draft: Francesco Bellomo, Francesco Emma.
Writing – review & editing: Francesco Bellomo, Ester De Leo, Olivier Devuyst, Carlo Dionisi-Vici, Francesco Emma, Alessandro Luciani, Stefania Petrini, Laura Rita Rega, Anna Taranta.

Data Sharing Statement

The RNA-Seq data of this publication are available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1112991. Data provided in SRA public archive have been released for free and open accessibility.

Supplemental Material

This article contains the following supplemental material online at http://links.lww.com/JSN/E771.

Supplemental Methods. RNA-Seq methodology.

Supplemental Table 1. Differentially expressed genes (DEGs) in mice at sacrifice (age 3 months).

Supplemental Figure 1. Megalin keeps its localization on the brush border of proximal tubules.

Supplemental Figure 2. Common effect of ketogenic diet in wild type and *Ctns*$^{-/-}$ mice.

Supplemental Figure 3. Effect of ketogenic diet in kidneys of *Ctns*$^{-/-}$ rats.

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