POLYCYSTIN-1 DYSFUNCTION IMPAIRS ELECTROLYTE AND WATER HANDLING 
IN A RENAL PRE-CYSTIC MOUSE MODEL FOR ADPKD

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Short Title: Renal electrolyte handling in a pre-cystic ADPKD model

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ABSTRACT

The *PKD1* gene encodes polycystin-1 (PC1), a mechanosensor triggering intracellular responses upon urinary flow sensing in kidney tubular cells. Mutations in *PKD1* lead to autosomal dominant polycystic kidney disease (ADPKD). The involvement of PC1 in renal electrolyte handling remains unknown since renal electrolyte physiology in ADPKD patients has only been characterized in cystic ADPKD. We thus studied the renal electrolyte handling in inducible kidney-specific *Pkd1* knockout (iKsp-*Pkd1*+/−) mice manifesting a pre-cystic phenotype. Serum and urinary electrolyte determinations indicated that iKsp-*Pkd1*+/− mice display reduced serum levels of magnesium (Mg²⁺), calcium (Ca²⁺), sodium (Na⁺) and phosphate (P_i) compared with control (*Pkd1*+/+) mice; and renal Mg²⁺, Ca²⁺ and P_i wasting. In agreement with these electrolyte disturbances, downregulation of key genes for electrolyte reabsorption in the thick ascending limb of Henle’s loop (TAL, *Cldn16, Kcnj1* and *Slc12a1*), distal convoluted tubule (DCT, *Trpm6* and *Slc12a3*) and connecting tubule (CNT, *Calb1, Slc8a1, Atp2b4*) was observed in kidneys of iKsp-*Pkd1*+/− mice compared with controls. Similarly, decreased renal gene expression of markers for TAL (*Umod*) and DCT (*Pvalb*) was observed in iKsp-*Pkd1*+/− mice. Conversely, mRNA expression levels in kidney of genes encoding solute and water transporters in the proximal tubule (*Abcg2* and *Slc34a1*) and collecting duct (*Aqp2, Scnn1a* and *Scnn1b*) remained comparable between control and iKsp-*Pkd1*+/− mice, though a water reabsorption defect was observed in iKsp-*Pkd1*+/− mice. In conclusion, our data indicate that PC1 is involved in renal Mg²⁺, Ca²⁺ and water handling, and its dysfunction resulting in a systemic electrolyte imbalance characterized by low serum electrolyte concentrations.

Keywords: PC1, *Pkd1*, ADPKD, pre-cystic, electrolyte imbalance
INTRODUCTION

The primary function of the kidneys is the removal of waste products from our metabolism. This process accounts for the challenge of filtering an average of 180 liters of blood daily. Upon filtration, the kidney reabsorbs 95% of the electrolytes contained in the filtrate. Consequently, a minor loss of kidney function yields disturbed plasma concentrations due to excessive urinary electrolyte excretion or absorption. This dysregulation of the electrolyte balance results in renal and extrarenal disorders including hypertension, renal stone formation and development of cardiovascular calcifications (8, 14, 21).

In the nephron, consecutive epithelial segments, i.e. the proximal tubule (PT), the thick ascending limb of Henle’s loop (TAL), the distal convoluted tubule (DCT), the connecting tubule (CNT) and the collecting duct (CD), maintain electrolyte balance through passive and/or active regulation of electrolyte reabsorption. Renal electrolyte handling is accomplished through the interplay of various tight junction proteins and ion channels and transporters expressed alongside the nephron (2, 19, 31, 41, 49). It is largely unknown how the activity of these channels and transporters is regulated. One of the factors that may comprise this regulation is the variable urinary flow in the nephron tubules. After all, renal electrolyte transport needs to be adjusted to the reabsorption demands that are dictated by the variable urinary flow in order to maintain electrolyte balance. In this context, it appears that tubular variable urinary flow is sensed by primary cilia, which are expressed in almost all epithelial cells within the kidney (9).

The protein polycystin-1 (PC1), located at the cellular apical plasma membrane and in primary cilia (protruding from the apical surface of renal tubular cells), is suggested to act as a mechanosensory molecule for urinary flow (24, 33, 45, 56).

The gene *PKD1* encodes PC1 and is involved in the regulation of various signaling pathways important for the maintenance and differentiation of kidney tubular epithelial cells (5). Mutations in *PKD1* lead to autosomal dominant polycystic kidney disease (ADPKD), which is one of the most common inherited renal diseases accounting for 7 to 10% of all patients on
renal replacement therapy (16, 38). ADPKD is characterized by increased cell proliferation, fluid accumulation and altered extracellular matrix synthesis, resulting in cyst formation and eventually in end-stage renal disease (ESRD). In advanced ADPKD, hypertension is common and glomerular filtration rate (GFR) is reduced (6, 48). Electrolyte disturbances in ADPKD are described in literature, but these reports are mostly restricted to cystic ADPKD (4, 11, 13, 34, 39, 40, 43, 44, 47, 51, 55, 57, 58). When electrolyte imbalances are detected in cystic ADPKD, it is not possible to discern whether these disturbances are caused by dysfunctional PC1 or by cyst formation or defects in GFR, which dramatically impair renal fluid flow and blood filtration, respectively. In \textit{Pkd1}\textsuperscript{+/−} mice, urinary wasting of Na\textsuperscript{+}, and reduced urinary Ca\textsuperscript{2+} excretion and serum Na\textsuperscript{+} levels have been reported (1). However, \textit{Pkd1}\textsuperscript{+/−} mice are not adequate to disclose PC1 function since one \textit{Pkd1} allele still translates into a functional PC1 protein, while \textit{Pkd1}\textsuperscript{−/−} mice die prematurely. Therefore, use of kidney-specific \textit{Pkd1}\textsuperscript{+/−} mice, which are viable (27) and in a stage preceding cyst formation (pre-cystic), is key to elucidate the involvement of PC1 in renal electrolyte handling. Identification of putative electrolyte disturbances in kidney-specific \textit{Pkd1}\textsuperscript{+/−} mice can be of paramount relevance to fully characterize the function of PC1 and thus delineate the physiological consequences of sensing urinary flow along the nephron.

The aim of this study was, therefore, to study the function of PC1 in renal electrolyte handling in relation to pre-cystic ADPKD by using an inducible kidney-specific \textit{Pkd1}\textsuperscript{+/−} mouse model.

METHODS

\textit{Animal Procedures}

Inducible kidney-specific \textit{Pkd1} knockout mice (\textit{iKsp-Pkd1}\textsuperscript{lox/lox}) were used during experimentation. In this mouse model, the \textit{Pkd1}\textsuperscript{lox/lox} allele has Lox-P sites flanking exons 2-11.
Tamoxifen was orally administered to iKsp-Pkd1\textsuperscript{lox/lox} mice on postnatal days 18, 19 and 20 (PN18) to induce a kidney specific knockout of Pkd1 (iKsp-Pkd1\textsuperscript{-/-}) and thus model ADPKD (27, 28). For experimentation, 8 male mice (obtained from 3 litters) received tamoxifen (iKsp-Pkd1\textsuperscript{-/-}) and 7 male mice (obtained from 3 litters) received no treatment (control). Only male mice were used in order to exclude sex as a factor influencing electrolyte handling since estrogen can influence Mg\textsuperscript{2+} absorption rates (8). At PN18 + 22 days and at PN18 + 29 days, mice were placed in metabolic cages for 24hrs to collect urine and faeces. Subsequently, body weight, faeces weight, urinary volume, food and water intake were assessed. Next, mice were anesthetized using isoflurane, and blood was collected via eye extraction. Finally, mice were sacrificed by cervical dislocation. Serum was obtained from the blood by centrifugation. Kidneys were extracted and weighed, and different segments of the intestine were collected in liquid nitrogen and stored at -80°C for mRNA and protein isolation. Part of the kidney was fixed in 4% (v/v) formalin before imbedding in paraffin for immunohistochemistry. Urine and faeces were stored at -20°C for assessment of the electrolyte content. The local animal experimental committee of the Leiden University Medical Center and the Commission Biotechnology in Animals of the Dutch Ministry of Agriculture approved the animal procedures performed.

Analytical Procedures

Serum, urinary and faecal electrolyte content was measured using inductively coupled plasma mass spectrometry (ICP-MS, ppb, for Mg\textsuperscript{2+}, Ca\textsuperscript{2+}, Na\textsuperscript{+} and K\textsuperscript{+}), a chloride autoanalyzer (ppb, for Cl\textsuperscript{-}), and inductively coupled plasma optic emission spectrometry (ICP-OES, ppm, for total phosphorus (as a measurement of inorganic phosphate, P\textsubscript{i})). Samples were prepared by dissolving 20μl of serum or urine in 50μl nitric acid (HNO\textsubscript{3}) and further diluted in 5ml MQ water. Faeces were incubated in 10ml HNO\textsubscript{3} at 50°C for 1hr. Next, total faeces samples were diluted with 10ml MQ water, homogenized by shaking, and 100μl of sample was further diluted with 5ml MQ. Diluted samples were then analyzed for electrolyte content. In addition, blood urea nitrogen
(BUN, mg/dl) was analyzed in the serum. Serum glucose (mmol/L) was analyzed using a glucose liquicolor kit (HUMAN GmbH, Germany). Osmolality (mOsm/kg) was assessed in the urine and serum using an osmometer (Osmometer Model 3320, Advanced Instruments Inc, MA, USA). Furthermore, the calculated serum osmolarity was determined using the following formula: 2 x serum[Na⁺] + serum[glucose] + [BUN] (52). Non-acetylated cAMP (nmol/24-hrs) was analyzed in the urine using a nonradioactive enzyme immunoassay kit (Cayman Chemical, MI, USA). The weight of both kidneys (2KW) was compared to the total body weight (BW) in order to determine the 2KW/BW ratios (%) for each mouse.

Histology & Cystic Index

Formalin fixed kidneys were embedded in paraffin and sections (4μm) were prepared. Sections were stained with periodic-acid Schiff (PAS) and hematoxylin and eosin (HE) using standard procedures. PAS and HE stainings were analyzed in order to examine features such as tubular dilation and/or cyst formation. The cystic index of kidneys from control and iKsp-Pkd1-/- mice was defined as the percent of lumen area over the total image area and assessed from total scans of hematoxylin and eosin-stained kidney sections (Figure 1). The stained lumen content of larger dilations and/or potential small cysts was removed from the images using Photoshop CC 2017 (Adobe Systems, CA, USA). Cystic index, using the ratio of total renal area plus lumen and total renal area minus lumen was determined by ImageJ software (National Institute of Health, MA, USA).

Immunohistochemistry

Specific nephron segments were distinguished by immunofluorescence using segment specific primary antibodies, namely rat anti-breast cancer resistance protein (BCRP) for the PT (1:250 in Tris-NaCl-blocking buffer (TNB), Kamiya Biomedical Company, WA, USA), sheep anti-Tamm-Horsfall protein (THF) for the TAL (1:200 in TNB, Biotrend, Germany), rabbit anti-NCC for the
DCT (1:200 in TNB, Millipore, MA, USA), guinea pig anti-TRPV5 for the CNT (1:2000 in TNB) (20) and rabbit anti-Aquaporin-2 (AQP2) for the CD (1:100 in TNB, Millipore, MA, USA). Sections were deparaffinized in xylene and subjected to heat-mediated antigen retrieval in citrate buffer (pH 6.0, Sigma-Aldrich, MI, USA) for 15min. Sections were incubated in 0.1% (v/v) PBS-Triton for 15min for permeabilization. Sections with staining for BCRP, THF, NCC and AQP2 were blocked for 30min in TNB and incubated with primary antibodies overnight. Next, sections were washed with Tris-NaCl (TN-Tween) buffer and incubated with secondary antibodies for 1hr in dark at room temperature: goat anti-rat Cy5 (1:100 in TNB, for BCRP, Jackson ImmunoResearch, PA, USA), goat anti-sheep Alexa594 (1:300 in TNB, for THF, Molecular Probes, OR, USA) and goat anti-rabbit Alexa594 (1:300 in TNB, for NCC and AQP2, Molecular Probes, OR, USA). Finally, sections were washed with TN buffer and mounted (DAPI Fluoromount-G, SouthernBiotech, AL, USA). For anti-TRPV5, after permeabilization, sections were blocked with 0.3% (v/v) H$_2$O$_2$ for 30min for endogenous peroxidase activity. Next, sections were blocked with a few droplets of endogenous Avidin and Biotin (Vector Laboratories, CA, USA) for 15min each. Subsequently, sections were blocked using TNB for 30min and incubated with primary antibody overnight. Next, sections were washed with TN-Tween buffer and incubated with secondary antibody for 1hr in dark at room temperature: goat anti-guinea pig Biotin SP (1:2000 in TNB, Jackson ImmunoResearch, PA, USA). Subsequently, sections were incubated in strep-HP (1:100 in TNB, PerkinElmer, MA, USA) for 30min followed by fluorescein tyramide (1:50 in amplification diluent, PerkinElmer, MA, USA) for 7min. Finally, sections were mounted (DAPI Fluoromount-G, SouthernBiotech, AL, USA) and analyzed with a fluorescence microscope (Axio Imager 2, Zeiss, Germany).

Quantitative Real-Time PCR

Tissue RNA was extracted using TriZol/chloroform extraction (Invitrogen, CA, USA). After DNase treatment (Promega, WI, USA), cDNA was synthesized using Molony-Murine Leukemia...
Virus-Reverse Transcriptase (Invitrogen, CA, USA) as previously described (18). The cDNA was mixed with Power SYBR green PCR master mix (Applied Biosystems, CA, USA) and with primers (400nM) for the gene of interest as previously described (3). The expression of the following genes was assessed via RTqPCR (7min at 95°C, 40 cycles of 15 sec at 95°C and 1 min at 60°C) in the kidney; Abcg2, Atp2b4, Aqp2, Calb1, Cldn16, Cldn19, Cnnm2, Kcnj1, Kim-1, Prom1, Prom2, Pvalb, Scnn1a, Scnn1b, Slc8a1, Slc12a1, Slc12a3, Slc34a1, Slc41a3, Trpm6, Trpm7, Trpv5 and Umod (Table 1). In the intestine, the expression of the following genes was assessed: Atp2b4, Cnnm4, Trpm6 and Trpv6. As a reference gene, Gapdh was used, and negative controls (samples where the reverse transcriptase was omitted during cDNA synthesis, and non-template samples) were taken along with each gene. The relative gene expression was analyzed using the Livak method ($2^{-\Delta\Delta Ct}$).

**Statistical Analyses**

Differences between groups were assessed using an unpaired Student's t-test. All data were expressed as mean ± SEM. Statistical significance was accepted at $P < 0.05$. Statistical analyses were performed using GraphPad Prism 6 (GraphPad, San Diego, CA, USA).

**RESULTS**

**Pre-cystic kidneys of iKsp-Pkd1−/− mice do not manifest tubular dilation in TAL, DCT and CNT**

Normal renal histology was observed in the kidneys of mice treated without tamoxifen (controls) by Periodic acid-Schiff (PAS) staining, whereas tamoxifen-treated mice (kidney specific Pkd1−/− (iKsp-Pkd1−/−) mice) displayed mild dilated tubules in the cortex, outer and inner medulla at PN18 + 29 days (Figure 1, 2A). In detail, after immunofluorescent staining for specific nephron
segments, only mild tubular dilation, restricted to the PT and CD, was observed. Importantly, no
tubular dilation was observed in TAL, DCT and CNT (Figure 2B). Remarkably, at this pre-cystic
stage, *Kim-1* (Kidney injury molecule-1) mRNA expression was significantly increased \((P < 0.05)\), whereas the Blood Urea Nitrogen (BUN) levels were not altered between control and
iKsp-*Pkd1*\(^{−/−}\) mice (Figure 2C-D). Furthermore, a significantly increased 2KW/BW ratio \((1.3 \pm 0.1\% \text{ and } 1.6 \pm 0.1\% \text{ for control versus iKsp-}Pkd1^{−/−}\text{ mice, respectively, } P < 0.05)\) and cystic
index \((1.8 \pm 0.2\% \text{ and } 3.6 \pm 0.4\% \text{ for control versus iKsp-}Pkd1^{−/−}\text{ mice, respectively, } P < 0.05)\)
was observed (Figure 2E-F), indicative of enlargement of the kidneys due to the mild tubular
dilations seen in the PT and CD.

Pre-cystic iKsp-*Pkd1*\(^{−/−}\) mice display disturbances in renal electrolyte and water handling

Serum and 24-hrs urine were collected to characterize the renal electrolyte and water handling
in iKsp-*Pkd1*\(^{−/−}\) mice with pre-cystic kidneys, and in control mice. In detail, at PN18 + 22 days,
urinary wasting of Ca\(^{2+}\) and Mg\(^{2+}\) was observed \((P < 0.05)\) (Table 2); however, this effect was
not observed at PN18 + 29 days (Table 2). Conversely, analysis at PN18 + 29 days showed that
iKsp-*Pkd1*\(^{−/−}\) mice exhibited lower serum Ca\(^{2+}\), Mg\(^{2+}\), Na\(^{+}\) and P\(_{\text{i}}\) levels \((P < 0.05)\) and a renal P\(_{\text{i}}\)
leakage \((P < 0.05)\) (Table 2). A non-statistically significant increase in urinary volume was
observed in iKsp-*Pkd1*\(^{−/−}\) mice as compared to controls \((P = 0.23 \text{ and } P = 0.08 \text{ for PN18 + 22
days and PN18 + 29 days, respectively). No changes in urine osmolality and cAMP levels at
PN18 + 22 days were observed. However, at PN18 + 29 days, urine osmolality was significantly
lower \((P < 0.05)\) in iKsp-*Pkd1*\(^{−/−}\) versus control mice. At this time point, urinary cAMP was
significantly higher \((P < 0.05)\) in iKsp-*Pkd1*\(^{−/−}\) mice as compared to controls (Table 2), indicating
an activation of the arginine vasopressin (AVP)-cAMP-AQP2 axis. Significant changes in serum
glucose were not observed between iKsp-*Pkd1*\(^{−/−}\) and control mice at PN18 + 29 days. Serum
osmolality was similar between iKsp-*Pkd1*\(^{−/−}\) and control mice at PN18 + 29 days. The calculated
serum osmolarity was significantly lower in iKsp-Pkd1−/− mice as compared to controls. Furthermore, control and iKsp-Pkd1−/− mice had a comparable food and water intake (Table 2).

**Decreased expression of key genes for electrolyte reabsorption in TAL, DCT and CNT**

To assess whether the electrolyte imbalances in iKsp-Pkd1−/− mice resulted from aberrant gene expression, the mRNA expression of key genes relevant for electrolyte handling in the kidney were examined. At PN18 + 29 days, downregulation of the mRNA levels in whole kidney of several key genes for electrolyte reabsorption in TAL, DCT and CNT was observed in iKsp-Pkd1−/− mice compared to control mice. In TAL, the expression of Cldn16 (Claudin16), Kcnj1 (ROMK) and Slc12a1 (NKCC2) was decreased (P < 0.05) (Figure 3B). In DCT, reduced expression of Trpm6 (TRPM6) and Slc12a3 (NCC) was observed (P < 0.05) (Figure 3C). The expression of Calb1 (Calbindin1), Slc8a1 (NCX1) and Atp2b4 (PMCA4) was downregulated in the CNT (P < 0.05) (Figure 3D). Genes encoding channels and transporters in the PT (Abcg2 and Slc34a1) and CD (Aqp2, Scnn1a and Scnn1b) were not affected (Figure 3A, 3E). Gene expression of Trpm7 (TRPM7), a gene ubiquitously expressed along the nephron, was similar in iKsp-Pkd1−/− and control mice (Figure 3F).

**Decreased gene expression of renal segment markers in pre-cystic iKsp-Pkd1−/− mice**

The expression of Umod (Uromodulin), a marker of the TAL (46), and Pvalb (Parvalbumin), a marker of the DCT (36), was downregulated in iKsp-Pkd1−/− mice compared to control mice (P < 0.05) (Figure 4A). Furthermore, decreased expression of Prom2 (Prominin-2), a marker of TAL, DCT, CNT and CD was also observed iKsp-Pkd1−/− mice compared to control mice (P < 0.05), whereas Prom1 (Prominin-1) expression, a marker of the PT (23), was similarly expressed in the kidneys of control and iKsp-Pkd1−/− mice (Figure 4B).
Compensation of the renal electrolyte disturbances in the intestine

In order to disclose extra-renal mechanisms compensating for the electrolyte imbalances elicited by knocking out *Pkd1* in the mouse kidney, we assessed the mRNA expression of genes relevant for electrolyte handling in the intestine. Interestingly, *Trpv6* (TRPV6) expression was increased in the duodenum (*P* < 0.05) of iKsp-*Pkd1*−/− mice as compared to controls (Figure 5A), whereas in colon, *Trpm6* expression was decreased (*P* < 0.05). In duodenum and caecum, no changes in *Trpm6* expression were observed. Furthermore, in colon and caecum, no changes in gene expression were observed between iKsp-*Pkd1*−/− and control mice for *Cnnm4*, *Trpv6* and *Atp2b4* (Figure 5B, 5C).

DISCUSSION

This study is the first characterization of the renal electrolyte and water handling in a model of ADPKD during the renal pre-cystic phase. We show that the knockout of PC1 in the mouse kidney leads to decreased serum Mg^{2+}, Ca^{2+}, Na^{+} and P_{i} levels; and urinary wasting of Mg^{2+} and Ca^{2+} during the pre-cystic stage, illustrating the role of PC1 in renal Mg^{2+} and Ca^{2+} handling. In addition, our data support the involvement of PC1 in the regulation of water reabsorption in the kidney. The Mg^{2+} and Ca^{2+} imbalances elicited by dysfunctional PC1 were likely caused by a decrease in the expression of key genes for the reabsorption of Mg^{2+} and Ca^{2+} in TAL, DCT and CNT of the nephron.

By characterizing the renal electrolyte and water handling, and its influence on serum electrolyte levels, in the renal pre-cystic stage of iKsp-*Pkd1*−/− mice, information about the early stages of development of ADPKD is provided. Most studies using models for ADPKD have only investigated renal cystic stages, and thus, later stages to the pre-cystic phase. The mice used in our study clearly show a renal pre-cystic phenotype. This is supported by the low 2KW/BW ratios, the low cystic index, and the absence of cysts in the PAS-stained kidney sections of


iKsp-\(Pkd1\)/ mice showed renal Mg\(^{2+}\) and Ca\(^{2+}\) wasting at PN18 + 22 days, pointing to a role of PC1 in the reabsorption of Mg\(^{2+}\) and Ca\(^{2+}\) in the kidney. This Mg\(^{2+}\) and Ca\(^{2+}\) leak in the kidney of iKsp-\(Pkd1\)/ mice was manifested as reduced serum Mg\(^{2+}\) and Ca\(^{2+}\) levels as compared with control mice at a later time point of PN18 + 29 days. Changes in urinary electrolyte excretion precede changes in serum electrolyte concentrations (12). Thus, the renal Mg\(^{2+}\) and Ca\(^{2+}\) leak detected in iKsp-\(Pkd1\)/ mice compared to control mice at PN18 + 22 days illustrates evolving systemic (serum) Mg\(^{2+}\) and Ca\(^{2+}\) disturbances, which become apparent at PN18 + 29 days. The comparable Mg\(^{2+}\) and Ca\(^{2+}\) excretion between control and iKsp-\(Pkd1\)/ mice at PN18 + 29 days illustrate further the inability of the kidneys at this time point to restore the serum electrolyte balance by increasing Mg\(^{2+}\) and Ca\(^{2+}\) reabsorption. These data are consistent with adult Slc41a3\(^{+}\) and Trpm6\(^{+}\) mice of 8-12 weeks, that display lower serum Mg\(^{2+}\) concentrations and a comparable urinary Mg\(^{2+}\) excretion compared with control (Slc41a3\(^{+}\) and Trpm6\(^{+}\), respectively) mice (7, 54).

In addition to renal Mg\(^{2+}\) and Ca\(^{2+}\) wasting, urinary Pi excretion was increased in iKsp-\(Pkd1\)/ mice compared to control mice at PN18 + 29 days. This finding relates PC1 function to the control of renal Pi excretion in addition to regulating renal Mg\(^{2+}\) and Ca\(^{2+}\) handling.

In agreement with the decreased Na\(^{+}\) levels in serum found in our iKsp-\(Pkd1\)/ mice compared with control mice, haploinsufficient \(Pkd1\) mice that do not develop cysts, had lower serum Na\(^{+}\) levels than \(Pkd1^{+/+}\) mice (1). A decreased serum Na\(^{+}\) concentration relates to an excess of water in the blood (32) or a renal salt wasting resulting in hypovolaemia (30). However, control and iKsp-\(Pkd1\)/ mice had a similar serum osmolality (PN18 + 29 days), though the calculated serum osmolarity was lower in iKsp-\(Pkd1\)/ mice. Control and iKsp-\(Pkd1\)/ mice displayed a comparable water intake and urine output, not indicating water overload or
hypovolaemia, respectively. Thus, the origin of the lower levels of Na\(^+\) in the serum of iKsp-
\textit{Pkd1}\(^{-/-}\) mice compared with controls remains elusive.

In contrast with serum osmolality, urine osmolality was significantly decreased at PN18 +
29 days in iKsp-\textit{Pkd1}\(^{-/-}\) mice as compared to controls. Taking into account the increase in urine
production between iKsp-\textit{Pkd1}\(^{-/-}\) \textit{versus} control mice (though not statistically significant) (Table
2), these data clearly indicate an inability of the kidneys of iKsp-\textit{Pkd1}\(^{-/-}\) mice to concentrate ions
in urine. This is supported by increased urinary cAMP levels in iKsp-\textit{Pkd1}\(^{-/-}\) mice, which
indicates a compensatory response to the decreased water reabsorption by activation of the
AVP-cAMP-AQP2 axis (42).

Importantly, BUN, a common marker for kidney function, remained unchanged in \textit{Pkd1}\(^{-/-}\)
mice, indicating that the disturbances in Mg\(^{2+}\), Ca\(^{2+}\) and Na\(^{+}\) balance observed are not caused
by defects in glomerular filtration. However, an increase in the expression of \textit{Kim-1} in the pre-
cystic kidneys of iKsp-\textit{Pkd1}\(^{-/-}\) mice was observed as compared with control mice. These findings
point to mild tubular injury as a result of \textit{Pkd1} gene disruption. \textit{Kim-1} encodes a membrane
protein, which is up-regulated in proliferating and dedifferentiated tubular cells after renal
ischemia (25). \textit{Kim-1} is postulated to be a potential biomarker for ADPKD progression (15, 39).
Our data further support this notion, especially when considering ADPKD in a pre-cystic stage.

The underlying cause of the impaired renal Mg\(^{2+}\) and Ca\(^{2+}\) handling observed in iKsp-
\textit{Pkd1}\(^{-/-}\) mice is likely the decreased renal gene expression of \textit{Cldn16}, \textit{Kcnj1} and \textit{Slc12a1}, key
genes for paracellular Mg\(^{2+}\) and Ca\(^{2+}\) transport in the TAL; of \textit{Trpm6}, \textit{Slc12a3} and \textit{Cnnm2},
relevant genes for transcellular Mg\(^{2+}\) reabsorption in the DCT; and \textit{Calb1}, \textit{Slc8a1} and \textit{Atp2b4},
genes coding the players that facilitate transcellular Ca\(^{2+}\) reabsorption in the CNT. Some of
these genes, i.e. \textit{Cldn16}, \textit{Slc12a1} and \textit{Slc12a3}, encode proteins that are also involved in Na\(^{+}\)
reabsorption and thus might evoke aberrant renal Na\(^{+}\) transport in iKsp-\textit{Pkd1}\(^{-/-}\) mice. Therefore,
renal PC1 dysfunction seems to predominantly affect the TAL, DCT and CNT of the nephron,
eliciting aberrant gene expression of regulators of Mg\(^{2+}\), Ca\(^{2+}\) and Na\(^{+}\) transport in these
segments. In contrast with serum Na\(^+\) levels, the concentration of Ca\(^{2+}\) and Mg\(^{2+}\) in serum is influenced by renal Mg\(^{2+}\) and Ca\(^{2+}\) transport (8). Thus, the decreased expression of genes relevant for Mg\(^{2+}\) and Ca\(^{2+}\) in the TAL, DCT and CNT, can explain the lower serum Mg\(^{2+}\) and Ca\(^{2+}\) concentrations observed in iKsp-Pkd1\(^{-/-}\) mice compared to controls. In addition, a compensatory mechanism for the renal Ca\(^{2+}\) leak was detected in the duodenum of iKsp-Pkd1\(^{-/-}\) mice as an increased mRNA expression of Trpv6 was observed in this segment of the intestine in comparison with control mice. The same phenomenon was observed in wild-type mice on a low Ca\(^{2+}\) diet in a previous study (53).

Conspicuously, in correlation with the decreased expression of key genes for electrolyte reabsorption in the kidney, a lower gene expression of TAL (Umod) and DCT (Pvalb) segment markers was observed in iKsp-Pkd1\(^{-/-}\) compared to control mice, pointing to a potential remodeling of TAL and DCT segments evoked by renal PC1 dysfunction. This finding is supported by the decreased expression of Prom2, a marker for TAL, DCT, CNT and CD, whereas the expression of Prom1, a marker for PT, was not decreased in iKsp-Pkd1\(^{-/-}\) mice when compared to control mice. While remodeling events, eventually leading to cyst formation, are clearly intertwined with ADPKD (5, 38), this study is the first to show that remodeling due to PC1 dysfunction in a pre-cystic context results in broad electrolyte imbalances. The association of the electrolyte imbalances in iKsp-Pkd1\(^{-/-}\) mice with remodeling events in the kidney is congruent with the de-differentiation and persistent cell proliferation already reported for altered PC1 expression in kidneys (22, 29).

In conclusion, we have demonstrated that dysfunction of PC1 impairs renal Mg\(^{2+}\), Ca\(^{2+}\) and water reabsorption in pre-cystic kidneys leading to serum Mg\(^{2+}\) and Ca\(^{2+}\) levels. These electrolyte disturbances preceding cyst formation observed in our model provide novel insights into PC1 function (Table 3). More research is required to disclose whether the electrolyte disturbances shown in this study might serve as early biomarkers of disease progression in...
ADPKD and/or might aid the development of treatment options in this early stage of the disease.

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DISCLOSURES
The authors declare no conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS
E.V., R.B., D.P., and F.A. conceived and designed the research reported here; E.V., S.M., W.L., C.B., and K.V. performed the experiments; E.V., S.M., W.L., C.B., R.B., D.P., and F.A. analyzed the data; E.V., S.M., W.L., J.H., R.B., D.P., and F.A. interpreted the results of experiments; E.V., and S.M. prepared figures; E.V. drafted the manuscript; E.V., S.M., R.B., D.P., and F.A. edited and revised the manuscript.
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**FIGURE LEGENDS**

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**Figure 1.** Illustrative examples of the images used for the calculation of the cystic index in iKsp-*Pkd1*−/− and control kidneys. The cystic index of kidneys from control and iKsp-*Pkd1*−/− mice were assessed from total scans of hematoxylin and eosin-stained kidneys sections (A, D). The area of the total kidney minus the stained lumen area was calculated (C, F) and subtracted from the total renal plus lumen area (B, E).
Figure 2. Kidneys of iKsp-Pkd1−/− mice display a pre-cystic phenotype at PN18 + 29 days. (A, B and C) iKsp-Pkd1lox/lox mice untreated (control) or treated (kidney specific Pkd1−/−) with tamoxifen on post natal days 18, 19 and 20 (PN18) and sacrificed 29 days later (PN18 + 29 days). (A) Periodic acid-Schiff (PAS) staining indicating normal renal histology in mice without tamoxifen treatment (control) and mild tubular dilation 29 days after tamoxifen treatment (Pkd1−/−). Mild tubular dilation is observed in the cortex, outer and inner medulla. (B) Mild tubular dilation was observed predominantly in the PT (anti-BCRP, green) and CD (anti-AQP2, red). No significant tubular dilation was observed in the TAL (anti-THF, red), DCT (anti-NCC, red) and CNT (anti-TRPV5, green). (C) Increased Kim-1 mRNA expression observed in pre-cystic kidneys of Pkd1−/− mice. (D) Blood Urea Nitrogen (BUN) levels displayed as mg/dL, no significant differences were observed. (E) Ratio of the kidney weight to body weight expressed as a percentage (2KW/BW %) and (F) the calculated cystic index showing the percentage of dilated/cystic area, values are presented as means ± SEM (n = 7-8), *P < 0.05 is considered statistically significant.

Figure 3. Renal expression of transporters relevant for electrolyte reabsorption. (A-F) iKsp-Pkd1lox/lox mice were either untreated (control, white bars) or treated (kidney specific Pkd1−/−, black bars) with tamoxifen on post natal days 18, 19 and 20 (PN18) and sacrificed 29 days later (PN18 + 29 days). (A) Relative mRNA expression of genes enriched in the PT. The genes assessed were Abcg2 (encoding BCRP) and Slc34a1 (encoding NaPi-2a). (B) Relative mRNA expression of genes enriched in the TAL. Genes measured were Cldn19, Cldn16, Kcnj1 (encoding ROMK) and Slc12a1 (encoding NKCC2). (C) Relative mRNA expression of genes enriched in the DCT. Genes measured were Trpm6, Slc12a3 (encoding NCC), Cnnm2 and Slc41a3. (D) Relative mRNA expression of genes enriched in the CNT. Genes measured were Trpv5, Calb1 (encoding calbinin-D28K), Slc8a1 (encoding NCX1) and Atp2b4 (encoding...
PMCA4A). (E) Relative expression to controls of genes enriched in the CD. Genes measured were Aqp2, Scnn1a (encoding ENaCα) and Scnn1b (encoding ENaCβ). (F) Relative mRNA expression of Trpm7 (ubiquitous expressed along the nephron). (A-F) mRNA levels were assessed by RTqPCR and normalized against the reference gene Gapdh. Gene expression data were calculated using the Livak method \((2^{-\Delta\Delta Ct})\), and they represent the mean fold difference (mean ± SEM, n = 7-8) from the calibrator group (control mice). *P < 0.05 is considered statistically significant.

Figure 4. Decreased gene expression of markers for TAL and DCT. (A-B) iKsp-\(Pkd1^{lox/lox}\) mice were either untreated (control, white bars) or treated (kidney specific \(Pkd1^{-/-}\), black bars) with tamoxifen on post natal day 18, 19 and 20 (PN18) and sacrificed 29 days later (PN18 + 29 days). (A) Relative mRNA expression of genes encoding specific renal segment markers, namely Umod (encoding Uromodulin) for the TAL and Pvalb (encoding Parvalbumin) for the DCT. (B) Relative mRNA expression of genes encoding for a specific marker of the PT, namely Prom1 (encoding Prominin-1) and Prom2 (encoding Prominin-2), a marker for distal tubules. mRNA expression levels were assessed by RTqPCR and normalized against the reference gene Gapdh. Gene expression data were calculated using the Livak method \((2^{-\Delta\Delta Ct})\), and they represent the mean fold difference (mean ± SEM, n = 7-8) from the calibrator group (control mice). *P < 0.05 is considered statistically significant.

Figure 5. Intestinal expression of transporters relevant for electrolyte reabsorption. (A-C) iKsp-\(Pkd1^{lox/lox}\) mice were either untreated (control, white bars) or treated (kidney specific \(Pkd1^{-/-}\), black bars) with tamoxifen on post natal day 18, 19 and 20 (PN18) and sacrificed 29 days later (PN18 + 29 days). Relative mRNA expression of key genes for Ca\(^{2+}\) and Mg\(^{2+}\) absorption in the
duodenum (A), caecum (B) and colon (C). Genes assessed were *Trpm6*, *Cnnm4*, *Trpv6* and *
Atp2b4* (encoding PMCA4A). mRNA levels were assessed by RTqPCR and normalized against
the reference gene *Gapdh*. Gene expression data were calculated using the Livak method (
$2^{-\Delta\Delta Ct}$), and they represent the mean fold difference (mean ± SEM, n = 7-8) from the calibrator
group (control mice). *P < 0.05 is considered statistically significant.
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<td>2.09 ± 0.04 (8)*</td>
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<td>P&lt;sub&gt;i&lt;/sub&gt;</td>
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<td>Urine (μmol/24-hrs)</td>
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<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
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<td>Cl&lt;sup&gt;−&lt;/sup&gt;</td>
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<td>306 ± 71 (7)</td>
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<td>27 ± 5 (7)</td>
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<td>Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td>123 ± 24 (7)</td>
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<td>K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>159 ± 31 (4)</td>
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<td>Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>205 ± 3 (4)</td>
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<td>Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td>118 ± 11 (4)</td>
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<td>279 ± 4 (4)</td>
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<td>Urinary volume (mL)</td>
<td>0.66 ± 0.14 (7)</td>
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<td>Urine osmolality (mOsm/kg)</td>
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<td>Serum osmolality (mOsm/kg)</td>
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<td>Calculated serum osmolarity (mOsm/L)</td>
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<td>BUN (mg/dL)</td>
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<td>Serum glucose (mmol/L)</td>
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<td>Urinary cAMP (nmol/24-hrs)</td>
<td>52 ± 7 (7)</td>
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<td>Bodyweight (g)</td>
<td>18.9 ± 0.9 (7)</td>
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<td>2KW/BW (%)</td>
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<td>Cystic index (%)</td>
<td>1.8 ± 0.2 (7)</td>
<td>3.6 ± 0.4 (8)*</td>
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<td>Water intake (mL)</td>
<td>4.1 ± 0.7 (7)</td>
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Values are presented as the mean ± SEM. The number of animals per group (n) is given. Significant differences are indicated with an asterisk (*).
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- = not reported; ↔ = no difference in electrolyte content; ↓ = decreased electrolyte content; ↑ = increased electrolyte content

\(^{A}\) = values from PN18 + 29 days; \(^{B}\) = values from PN18 + 22 day

Pavik et al. (40)

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Nishiura et al. (34)

Ferraz et al. (11)

Ferraz et al. (11)

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Fonseca et al. (13)

Fonseca et al. (13)

Bastos et al. (4)

Present study