A Combination of β-Hydroxybutyrate and Citrate Ameliorates Disease Progression in a Rat Model of Polycystic Kidney Disease

Jacob A. Torres, Nickolas Holznecht, David A. Asplund,
Tselmeg Amarlkhagva, Bradley Kroes, Juliette Rebello, Shagun Agrawal,
Thomas Weimbs

Department of Molecular, Cellular, and Developmental Biology, University of California,
Santa Barbara, Santa Barbara, CA 93106-9625, USA

Corresponding author: Thomas Weimbs, Email: weimbs@ucsb.edu
Phone #:1 (805) 893-4144
Address: Department of Molecular, Cellular & Developmental Biology
University of California Santa Barbara
Santa Barbara, California 93106-9625

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Abstract

Our research has shown that interventions producing a state of ketosis are highly effective in rat, mouse, and cat models of polycystic kidney disease (PKD), preventing and partially reversing cyst growth and disease progression. The ketone ß-hydroxybutyrate (BHB) appears to underlie this effect. Additionally, we have demonstrated that naturally formed microcrystals within kidney tubules trigger a renoprotective response that facilitates tubular obstruction clearance in healthy animals but, alternatively, leads to cyst formation in PKD. The administration of citrate prevents microcrystal formation and slows PKD progression. Juvenile Cy/+ rats, a non-orthologous PKD model, were supplemented from 3 to 8 weeks of age with water containing titrated BHB, citrate, or in combination to find minimal effective and optimal dosages, respectively. Adult rats were given a reduced BHB/Citrate combination or equimolar control K/NaCl salts from 8 to 12 weeks of age. Additionally, adult rats were placed in metabolic cages following BHB, citrate, and BHB/Citrate administration to determine the impact on mineral, creatinine, and citrate excretion. BHB or citrate alone effectively ameliorates disease progression in juvenile rats, decreasing markers of cystic disease and, in combination, producing a synergistic effect. BHB/Citrate leads to partial disease regression in adult rats with established cystic disease, inhibiting cyst formation and kidney injury. BHB/Citrate confers benefits via multiple mechanisms, increases creatinine and citrate excretion, and normalizes mineral excretion. BHB and citrate are widely available and generally recognized as safe compounds and, in combination, exhibit high promise for supporting kidney health in polycystic kidney disease.
New and Noteworthy

Combining BHB and citrate effectively slows and prevents cyst formation and expansion in young Cy/+ rats using less BHB and citrate than when used alone, demonstrating synergy. In adult rats, the combination causes a partial reversal of existing disease, reducing cyst number and cystic area, preserving glomerular health, and decreasing markers of kidney injury. Our results suggest a safe and feasible strategy for supporting kidney health in PKD using a combination of BHB and citrate.
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Financial Disclosures
T.W. and J.A.T are inventors on issued and pending patents filed by UCSB related to PKD; T.W. is a shareholder and president, J.A.T. a shareholder and employee of Santa Barbara Nutrients, Inc.; T.W. was on the scientific advisory board of Chinook Therapeutics, has received research funding from Chinook Therapeutics and speaker fees from Otsuka.
Author Contributions

JAT Conceived and designed research, performed experiments, analyzed data, interpreted results of experiments, prepared figures, drafted manuscript, edited and revised manuscript, and approved the final version of the manuscript. NH performed experiments, analyzed data, interpreted the results of experiments, edited and revised the manuscript, and approved the final version of the manuscript. DAA performed experiments and analyzed data. TA performed experiments and analyzed data. BK performed experiments and analyzed data. JR performed experiments and analyzed data. SA performed experiments and analyzed data. TW conceived and designed research, edited and revised the manuscript, and approved the final version of the manuscript.
Introduction

Autosomal-dominant polycystic kidney disease (ADPKD), caused by mutations in the
PKD1 or PKD2 genes, is the most common life-threatening monogenic disease affecting
at least 1:1000 individuals worldwide (1). Currently, the only approved medication to slow
ADPKD progression, tolvaptan, is not readily available or desirable for many individuals
due to cost (2), eligibility restrictions, side effects, and toxicities (3). Identifying more
effective, accessible, affordable, and safe alternative treatment options is therefore of
great interest.

ADPKD causes the formation of large fluid-filled cysts that progressively replace
healthy kidney tissue, causing kidney function decline. Complications of ADPKD include
an increased risk of developing kidney stones (4, 5). Kidney stones grow from tubular
microcrystals that precipitate within supersaturated fluids. Genetic ablation of Pkd1 or
Pkd2 in adult rodents does not, in itself, result in rapid polycystic kidney disease but can
be accelerated following kidney insults (6, 7). Our lab previously showed that tubular
calcium oxalate and calcium phosphate microcrystals can trigger cystogenesis and
accelerate disease progression in a PKD rat model (Anks6 mutant, Cy/+ rats) (5). Citrate
in the tubule lumen is an essential natural defense against the formation of calcium-
derived microcrystals acting as a calcium chelator. Increasing citrate intake from food
sources, supplements, or prescription drugs is a common strategy to facilitate the
suppression of microcrystal formation in recurrent kidney stone formers (8–10). Tanner et
al. previously demonstrated that administering potassium citrate to the Cy/+ rat effectively
ameliorated PKD progression (11–13), although not recognizing that the mechanism may
involve the removal of tubular microcrystals. We demonstrated that oral citrate administered in drinking water effectively eliminates microcrystals in PKD kidneys (5).

The Cy/+ rat is a non-orthologous model of ADPKD where male rats rapidly develop proximal tubule-derived cysts until eight weeks of age and then exhibit slow kidney function decline until six months. Female rats develop a less severe phenotype, survive past one year of age, show slower renal function decline, and are resistant to the formation of microcrystals (5, 14). We chose to use male Cy/+ rats for this study primarily for their ability to form kidney microcrystals that citrate supplementation can remove.

Additionally, mutations in Pkd1/Pkd2 alter cellular metabolism, shifting cells towards a glycolytic phenotype with impaired fatty acid oxidation (15, 16) accompanied by alterations in mitochondrial structure and function, increased oxidative stress, and altered gene expression (15, 17–19). Addressing this metabolic switch, we previously reported that dietary interventions that induce a state of ketosis – including time-restricted feeding (TRF), fasting, and ketogenic diet – strongly inhibit cyst growth in rodent and feline models of PKD (20). We further reported that the supplementation in drinking water with the significant ketone produced by the liver during ketosis, β-hydroxybutyrate (BHB), mimicked the beneficial effects of dietary ketosis (20). A recent randomized, controlled clinical trial based on these preclinical results also suggested that a ketogenic diet improves renal function in individuals with ADPKD (21).

In this study, we test if a combination of BHB and citrate may provide enhanced benefit in the Cy/+ rat to a greater extent than when either BHB or citrate are supplemented alone. We hypothesized that BHB and citrate may help prevent disease progression by acting on distinct mechanisms of disease progression: mimicking the
effects of ketosis and preventing injury from tubule microcrystals, respectively. To test
this hypothesis, we supplemented titrated amounts of BHB and citrate to determine their
minimal effective dose and then combined them. BHB and citrate possess a strong safety
profile, are natural products of metabolism, and are inexpensive. If effective, BHB and
citrate would provide an accessible and safe option for those affected by ADPKD. Our
results indicate that BHB and citrate, at reduced doses, may act synergistically to prevent
disease progression in juvenile rats and ameliorate established kidney disease in adult
rats, reducing cystic area, preventing cystogenesis, kidney injury, increasing citrate
excretion, and maintaining glomerular health.
Materials and Methods

Animal Studies:

The Hannover Sprague Dawley (Cy/+) rat was used for all experiments (14). All rats were housed in the animal resource center at the University of California Santa Barbara using a 12-hour light/dark cycle with ad libitum access to food, water, and enrichment. All animal studies were performed with the approval of the University of California Santa Barbara Institutional Animal Care and Use Committee. Rats were weaned at postnatal day 21, separated by sex, group-housed, and randomly assorted. Experimental replicates from different litters were used for each treatment and analysis. Sample sizes were determined based on previous experimental observations using this model. Food and water intake were measured throughout all experiments, and rat weights were measured weekly. β-hydroxybutyrate was measured using a blood meter (Precision Xtra; Abbott) and glucometer (Contour Next EZ; Bayer) before rats were anesthetized using a combination of 200mg:20mg/kg ketamine:xylazine followed by cervical dislocation before tissue removal. Tissue samples were snap-frozen in liquid nitrogen following removal for later analysis. Serum samples were collected by cardiac puncture, transferred to a Microtainer tube (Cat# B-D365967; BD), separated by centrifugation, and snap-frozen in liquid nitrogen. During the experimental period, the lead researcher was responsible for sorting animals into experimental cages. Both PKD and wild-type rats were housed together, resulting in a semi-blinded experiment in which researchers involved in feeding and watering animals were blinded to the specific genotypes of animals within treatment groups during the experimental period. A detailed description of the animals used for each experiment is provided in the Animal Use Table with the supplemental figures.
BHB- An unflavored sodium/potassium salt of D/L-β-hydroxybutyrate (KetoForce; Ketosports) was added to water ad libitum for all BHB studies with a concentration of 4.2% and then halved to 2.1% and 1% for titration studies. These concentrations were rounded off and labeled 160mM, 80mM, and 40mM, respectively. We have previously tested a comparable salt control to our 160mM dose in Torres et al. (20). We showed that salt alone did not have an effect and, therefore, could not justify a repeat of that experiment in this study.

Citrate- A combination of tripotassium citrate (CAS# 6100-05-6; Sigma-Aldrich) and citric acid (CAS# 77-92-9; Sigma-Aldrich) were used to make citrate solutions as described previously (11). The concentrations of 55 mmol/L tripotassium citrate/67 mmol/L citric acid, 27.5 mmol/L tripotassium citrate/33.5 mmol/L citric acid, and 13.75 mmol/L tripotassium citrate/16.75 mmol/L citric acid were rounded off and labeled as 120mM, 60mM, and 30mM respectively.

BHB and citrate- BHB and citrate were made by combining the described mixtures above in their respective amounts indicated by their millimolar concentration. Adult rats received a 1% BHB and 27.5mM tripotassium citrate/33.5mM citric acid solution (40/60) and a solution containing 2.1% BHB 13.75mM tripotassium citrate/16.75mM citric acid (80/30). As a control, rats were supplemented with a salt solution of potassium/sodium chloride lacking BHB and citrate of 22mM sodium/98mM potassium (40/60 Salt) or 46.5mM sodium/73.5mM potassium (80/30 Salt), respectively.
Metabolic Cage Experiments—To analyze urine composition, adult rats aged 10-12 weeks were housed in metabolic cages (Product# 526715; Tecniplast) using a chiller (Product# 72906; Tecniplast) to preserve urine samples during collection. Rats were given ad libitum food and water access during the experiment. Collection occurred on T=0 to establish a 24-hour baseline followed by 4.2% BHB administration in water for three days, followed by a 24-hour collection period. Rats were then returned to their home cage and given plain water for a three-day "washout" period before receiving 120mM citrate in water for three days and subsequent 24-hour urine collection, followed by an additional three-day "washout" period and administration of 2.1% BHB/13.75 mmol/L tripotassium citrate/16.75 mmol/L citric acid solution for three days, followed by a 24-hour urine collection.

Glomerular Health Scoring—Kidney sections were stained for podocin, and all glomeruli were scored using the following rubric:

0: No obvious morphological changes; normal
1: Morphological change, e.g., changes in shape and structure
2: Morphological changes, as well as decreased filling of glomeruli space, increase in distance between Bowman's capsule and podocin

Creatinine—Serum creatinine was calculated using a QuantiChrom creatine assay kit (Cat# DICT-500; BioAssay Systems).
Mitochondrial DNA qPCR:

Mitochondrial DNA quantification was previously described by Zhang et al (22). DNA was extracted from 25mg of frozen tissue using a Zymo-spin TM IICR column mini-prep (Cat# D4068; Zymo Research) purified using sodium acetate, ethanol precipitated, washed with 70% ethanol, air-dried and resuspended in sterile TE buffer then diluted to 3ng/µl and subjected to qPCR (Cat# 6020; Promega).

Primers used:

<table>
<thead>
<tr>
<th>Primers</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>195bp product Rat Clusterin (TRPM-2)</strong></td>
<td></td>
</tr>
<tr>
<td>Forward 5’</td>
<td>GGTGTACTTGAGCAGAGCGCTATAAAT</td>
</tr>
<tr>
<td>Reverse 5’</td>
<td>CACTTACCCACGGCAGCTCTCTAC</td>
</tr>
<tr>
<td><strong>235bp product Rat Mitochondria Cytochrome B</strong></td>
<td></td>
</tr>
<tr>
<td>Forward 5’</td>
<td>CCTCCCATTCATTATCGGCCCTTGC</td>
</tr>
<tr>
<td>Reverse 5’</td>
<td>GTCTGGGTCTCCTAGTAGGTCTGGGAA</td>
</tr>
</tbody>
</table>

PCR conditions were as follows: 1 Cycle of 94°C for 5 minutes, 35 Cycles of: 94°C for 15 secs, 63°C for 45 secs, 72°C for 60 secs, then 1 cycle of 94°C for 60 secs, 1 cycle of 72°C for 30 secs, and 1 cycle of 95°C for 30 secs.

The mitochondrial number was determined by obtaining C_T values for mitochondrial and nuclear DNA and then calculating ΔC_T=(nucDNA C_T - mtDNA C_T). The relative mitochondrial number was calculated using (mitochondrial number = 2x2^ΔC_T).

Statistics:
When appropriate, an unpaired and paired two-tailed t-test was used to compare differences between groups. One-way ANOVA followed by ad hoc Tukey’s test was used for multiple comparisons. Statistical analysis was performed using Prism 8 (GraphPad). Individuals were used as the experimental unit. Cage cohorts were quantified for food and water intake analysis. For analyses that employ fewer than an entire group of experimental animals, animals were chosen randomly for inclusion. Multiple litters were used for each experimental condition.

**Ultra-High Pressure Liquid Chromatography Mass Spectrometry:**

L and D BHB isomers were measured in serum using Ultra-High-Pressure Liquid Chromatography Mass Spectrometry (UHPLC-MS) following derivatization to separate isomers. The complete methods are outlined in the supplemental information.

**ICP-OES Instrument and Parameters**

An Agilent 5800 Inductively Coupled Plasma-Optical Emission Spectrometer was used for the analysis of minerals in urine. The complete methods are outlined in the supplemental information.

**Antibodies:**

Primary antibodies:

Rabbit α-Alpha Smooth Muscle Actin (Abcam Cat# ab5694, RRID:AB_2223021) Diluted 1:200 for immunofluorescence.
Mouse α-Ki67, Clone B56 (RUO), (BD Biosciences Cat# 550609, RRID:AB_393778) Diluted 1:200 for immunofluorescence.

Rabbit α-PGC1α, (Novus Cat# NBP1-04676, RRID:AB_1522118) Diluted 1:1000 for Western blot.

Rabbit α-Podocin (Thermo Fisher Scientific Cat# PA5-79757, RRID:AB_2746872) Diluted 1:200 for immunofluorescence.

Rabbit α-p44/p44 MAPK-Erk ½ (137F5) (Cell Signaling Technology Cat# 4695, RRID:AB_390779) Diluted 1:1000 for Western blot.

Rabbit α-p44/p44 MAPK-P-(Thr202/Tyr204)-Erk ½ (D13.14.4E) (Cell Signaling Technology Cat# 4370, RRID:AB_2315112) Diluted 1:1000 for Western blot.

Rabbit α-pY STAT3 (Tyr705) (D3A7) (Cell Signaling Technology Cat# 9145, RRID:AB_2491009) Diluted 1:1000 for Western blot.

Mouse α-total STAT3 (124H6) (Cell Signaling Technology Cat# 9139, RRID:AB_331757) Diluted 1:1000 for Western blot.

Mouse α-ACTIN (Sigma-Aldrich Cat# A5441, RRID:AB_476744) Diluted 1:10000 for Western blot.

Goat α-KIM-1 (R and D Systems Cat# AF3689, RRID:AB_2116557) Diluted 1:1000 for Western blot.

Secondary antibodies:

Goat α-rabbit AlexFluor 594 (Thermo Fisher Scientific Cat# A-11012, RRID:AB_2534079) Diluted 1:1000 for immunofluorescence.

Western Blotting:
Approximately 10mg of snap-frozen tissue samples were lysed in 200µL of SDS lysis buffer (4% SDS, 100mM Tris HCl pH 6.8, 20% glycerol, 1:1000 protease inhibitor cocktail (pepsstatin E110, leupeptin E18, antipain E13; Chemicon, Benzmidine; Sigma-Aldrich, Traysol; Bayer) and 1:100 phosphatase inhibitor cocktail 2 & 3 (Cat# P5726 and P0044; Sigma-Aldrich) followed by heating at 100°C. 25µg of protein lysate was subjected to SDS-PAGE using 10% acrylamide gels, transferred onto nitrocellulose membrane, incubated overnight with primary antibodies, washed, incubated with secondary antibody, washed, then imaged using an Azure 600 (Azure Biosystems). Western blot images were quantified via densitometry using FIJI (ImageJ) (23).

Microscopy:
All control and experimental samples were imaged in the same session for use in figures and quantification. Brightfield images were white-balanced and altered to normalize color using Photoshop (Adobe). The contrast and brightness of control and experimental images were adjusted similarly within the figures.

Immunofluorescence:
Deparaffinized slides were subjected to pressure cooker antigen retrieval with 10mM sodium citrate pH 6.0, then blocked (1% BSA, 0.1% TX-100, 0.1% fish skin gelatin in
TBST) in a humid chamber at 37°C for 60 minutes. Primary antibodies were mixed with blocking buffer and incubated on sections overnight at 4°C. Slides were then washed in TBST and incubated in 0.1% Sudan Black B in 70% ethanol for 20 minutes. Then incubated in a secondary antibody in a humid chamber protected at 37°C. Slides were then washed, fixed with 10% NBF, washed in TBST, stained with DAPI in TBS, rinsed in TBST, and mounted using Prolong Gold (Cat# P36930; ThermoFisher). No primary and secondary only controls were used as controls to control for specificity of antibodies.

Histology:

Kidney tissue samples were excised from rats and immediately rinsed in PBS, weighed, and fixed in 10% neutral buffered formalin for 24 hours at ambient temperature, followed by paraffinization. 5µm sections on Superfrost Plus slides (Cat# 12-550-15; Fisher Scientific) were used for all histology and immunofluorescence applications.

Hematoxylin and Eosin- Rehydrated samples were placed in hematoxylin solution for 1 minute, rinsed in running tap water, placed into eosin for 45 seconds, and dipped ten times in 2x 95% and 2x 100% ethanol, 2x5 minutes in xylenes for and mounted using Permount (Cat# SP15-100; Fisher Scientific).

Collagen- Deparaffinized kidney samples were stained using a Sirius Red/Fast Green collagen staining kit (Cat# 9046; Chondrex).

Quantification:
Quantification occurred in a semi-blinded manner in which researchers quantified animals without knowledge of specific treatments for each animal being counted. Researchers only had access to the animal ID without knowledge of the treatment when quantifying but were, however, aware of the overall experimental design and potential outcomes.

_Fibrosis_- 10 images from cortical regions of Sirius red-stained sections were imaged at 100x magnification for quantification. Using Photoshop (Adobe), a grid was placed over each image, and intersections with Sirius red stain were counted as positive, with other intersections counted as negative with intersections overlaid on negative space excluded from the total number of potential intersections. The total number of positive intersections was divided by the total possible number of intersections to obtain the percent of fibrosis.

_Cystic index_- 10 cortical images of hematoxylin and eosin sections were imaged at 100x magnification for quantification. Using Photoshop (Adobe), a grid was placed over each image, and intersections overlaid on cysts were counted as positive, with other intersections as negative. Empty space intersecting outside of the tissue was excluded from the total number of potential intersections. The total positive intersections were divided by the total possible number of intersections to obtain the cystic index.

_Smooth Muscle Actin_- 10 images from cortical regions were taken from each kidney of Smooth muscle actin-stained sections at 200x magnification. Using Photoshop (Adobe), a grid was placed over each image, and intersections with smooth muscle actin stain
were counted as positive and other intersections counted as negative, with intersections overlaid on negative space excluded from the total number of potential intersections. The total number of positive intersections was divided by the total possible number of intersections to obtain the percent of smooth muscle actin positive area.

Ki67- 5 images of Ki67 stained sections were imaged at 200x from cortical regions. At least 1000 cells were counted for each animal. Cell number was determined with DAPI using FIJI (ImageJ) (23) to automate cell counting. Ki67-positive cells were manually counted and classified by location as interstitial (existing outside of tubules and cysts) or cystic/tubular (existing in cysts or tubules). The number of positive cells from all five images for each location was divided by the number of cells counted to obtain the percentage of Ki67 positive cells.

Cyst Number and Cyst Size- Whole kidney sections were imaged using a dissecting scope, and individual cysts were manually counted using the wand tool in FIJI (ImageJ).

Depictions of Synergism- Figures that investigate possible synergism between BHB and citrate when used in combination were derived using descriptions from Tallarida (24). To depict synergism, the effect size of PKD parameters were plotted relative to the amount of BHB and Citrate administered. The greatest effect measured using the least amount of BHB and Citrate was then plotted on an isobologram to depict if the combination of BHB/Citrate produces an effect with a reduced dose.
Ultra-High Pressure Liquid Chromatography Mass Spectrometry:

Chemicals and Materials:

D-beta-hydroxybutyric acid (D-BHB) (CAS:625-72-9, Cat# 54920, Sigma Aldrich)
L-beta-hydroxybutyric acid (L-BHB) (CAS:6168-83-8, Cat# 54925, Sigma Aldrich)
Sodium d₄-DL-beta-hydroxybutyrate (Na d₄-DL-BHB) (CAS:1219804-68-8, Cat# 14158, Cayman Chemical)
S-1-(2-pyrrolidinyl methyl)-pyrrolidine (S-PMP) (CAS:51207-66-0, Cat# P12411G, Sigma Aldrich)
triphenylphosphine (TPP) (CAS 603-35-0, Cat# 140420250, Sigma Aldrich)
2,2’-dipyridyl disulfide (DPDS) (CAS:2127-03-9, Cat# D11145G, Sigma Aldrich)

Solvents:

LCMS Grade Water with 0.1% Formic Acid (v/v) (CAS:7732-18-5, 64-18-6, Cat# LS118-4, Fisher Scientific)
LCMS Grade Acetonitrile with 0.1% Formic Acid (v/v) (CAS:75-05-8, 64-18-6, Cat# LS120-4, Fisher Scientific)
LCMS Grade Acetonitrile (CAS:75-05-8, Cat# A995-4, Fisher Scientific)
LCMS Grade Methanol (CAS:67-56-1, Cat# A456-4, Fisher Scientific)
MilliQ Water (CAS:7732-18-5, Millipore Sigma)

Materials:

96-well 0.2um polypropylene vacuum filtration plate (Cat# PI90036, ThermoScientific)
Software- MassLynx (V4.1, Waters Inc.) was used to acquire and process data.
The following methods were modified from a previously published method (Tsutsui, H. et al., 2012).

**UPLC-ESI-QToF** - A Waters Acquity H-class Ultra-High-Pressure Liquid Chromatography (UPLC) system coupled to a Waters Xevo G2-XS QToF Quadrupole Time-of-Flight Mass Spectrometer was used for the analysis, and the column employed was a Waters BEH C18 UPLC column (1.7 µm, 100 x 2.1 mm). The following parameters were used for the analysis:

**UPLC Parameters:**
- Injection Volume: 1.00 µL
- Column Temperature: 40°C
- Flow Rate: 0.350 mL/min
- Gradient Profile:
  - 0.00 min – 100% H₂O-FA; 0% ACN-FA
  - 5.00 min – 90% H₂O-FA; 10% ACN-FA
  - 8.00 min – 0% H₂O-FA; 100% ACN-FA
  - 9.00 min – 95% H₂O-FA; 5% ACN-FA
  - 11.01 min – 100% H₂O-FA; 0% ACN-FA
- Autosampler Temperature: 10°C

**QToF Parameters:**
- Polarity: positive
- Analyzer: sensitivity mode
Capillary Voltage: 0.50 kV
Sampling Cone Voltage: 30 V
Source Temperature: 120°C
Source Offset: 80
Desolvation Temperature: 350°C
Cone Gas Flow: 50 L/hr
Desolvation Gas Flow: 1000 L/hr
LM Resolution: 10
HM Resolution: 15
Sample Infusion Flow Rate: 10 µL/min

Standard Solutions- D-BHB and L-BHB were made at 1,000.00 mg/L in ACN. The internal standard Na-d4-DL-BHB was made at 10.00 mg/L in a solution of 90:10 ACN:MilliQ water (v/v), and Na-DL-AHB was made at 1,000.00 mg/L in the same 90:10 solution. The S-PMP, TPP, and DPDS reagents were each made to 20.00 mM in ACN.

Working Solutions- D-BHB and L-BHB were made through 10-fold dilutions of the standards with ACN to make solutions with 100.00 mg/L, 10.00 mg/L, 1.00 mg/L, and 0.10 mg/L concentrations.

Calibration Solutions- The calibration range for D-BHB and L-BHB ranged from 1 µg/L to 1000 µg/L. To a microcentrifuge tube was added an appropriate aliquot of the analyte, 100.0 µL of internal standard, 100.0 µL of S-PMP, 100.0 µL of TPP, 100.0 µL of DPDS,
and then diluted with the appropriate amount of ACN to reach a final volume of 1.00 mL. The solution was then vortexed and allowed to react at room temperature overnight to ensure a complete reaction. Once reacted, 100.0 µL of calibration solution was transferred to an LCMS vial and diluted with 900. µL of a solution of 98:1.6:0.4 H₂O-FA:MeOH:ACN (v/v/v). The LCMS vial was then vortexed and loaded into the instrument for analysis. Calibration solutions were made in triplicate.

Sample Preparation- To a microcentrifuge tube was added 10.00 µL of the sample (serum or urine), 100.0 µL of internal standard, and 890. µL of ACN. The tube was then vortexed and centrifuged for 5 minutes at 15,000 rpm, and the supernatant was filtered through a 96-well 0.2 um polypropylene vacuum filtration plate. The filtered sample was then transferred to a microcentrifuge tube, and the solvent was evaporated in a vacuum centrifuge at 40°C. To the remaining residue was added 700. µL of ACN, 100.0 µL of S-PMP, 100.0 µL of TPP, and 100.0 µL of DPDS. The solution was then vortexed and allowed to react at room temperature overnight to ensure a complete reaction. Once reacted, 100.0 µL of sample solution was transferred to an LCMS vial and diluted with 900. µL of 98:1.6:0.4 H₂O-FA:MeOH:ACN (v/v/v). The LCMS vial was then vortexed and loaded into the instrument for analysis.

Calibration Curve- Calibration curves were constructed for both D-BHB and L-BHB by plotting the average peak area ratios of D-BHB and L-BHB to the internal standard against the corresponding concentration of analyte and then using the method of least
squares linear regression to compute the equations of the lines. The calibration was acceptable with an $R^2$ value $\geq 0.995$.

Quantification of D-BHB and L-BHB From Samples- The concentrations of D-BHB and L-BHB from the samples were calculated by determining the peak area ratios of D-BHB and L-BHB to the internal standard and then using the equations of the lines to determine the corresponding concentration.

Inductively Coupled Plasma-Optical Emission Spectrometry:

Chemicals and Materials

The ICP grade 70% nitric acid (CAS: 7697-37-2, Cat# A509P212) and ICP grade 30% hydrogen peroxide (CAS: 7722-84-1, Cat# 02003185) were purchased from Fisher Scientific. The ICP grade 100 ug/mL multi-analyte (Ca, K, Mg, Na) custom solution in 5% nitric acid, ICP grade 100 ug/mL yttrium in 2% nitric acid (CAS: 7440-65-5, Cat# MSY-100PPM-125ML), and ICP grade 5% nitric (CAS: 7697-37-2, Cat# IV-ACID-BLANK-1L) were purchased from Inorganic Ventures. MilliQ water was used throughout the analysis (CAS: 7732-18-5, Millipore Sigma).

ICP-OES Instrument and Parameters

An Agilent 5800 Inductively Coupled Plasma-Optical Emission Spectrometer was used for the analysis with the following parameters:

Viewing Mode: Radial; RF Power: 1.20 kW; Viewing Height: 8 mm; Read Time: 5 s;
Stabilization Time: 15 s; Replicates: 3; Pump Speed: 12 rpm; Nebulizer Flow: 0.70 L/min; Plasma Flow: 12.0 L/min; Aux Flow: 1.00 L/min.

The following wavelengths (nm) were used to monitor the analytes:

- **Sodium**: 589.595, 588.995, 818.3;
- **Magnesium**: 279.553, 280.270, 285.213;
- **Potassium**: 766.490, 771.531, 404.721;
- **Calcium**: 393.366, 396.847, 422.673;
- **Yttrium**: 377.433, 371.030, 362.073.

**Software**

ICP Expert Pro Software (v.7.5.4.11997) was used to acquire and process data.

**Calibration Solutions**

Calibration solutions were made from 100 µg/mL to 0.1 µg/mL by serial diluting the multi-analyte standard with 5% nitric acid in 50 mL polypropylene tubes. A 100.0 µL aliquot of 100 µg/mL yttrium was added as an internal standard.

**Calibration Curve**

Calibration curves were constructed for each analyte by plotting the average wavelength intensity ratio of each analyte to yttrium against the corresponding concentration of the analyte. The least squares linear regression method was then used to compute the equations of the lines of best fit. The calibration curve for each analyte was acceptable, with an $R^2$ value ≥ 0.995.
Sample Preparation

A 200.0 µL aliquot of urine was added to a tared digestion vessel, and its mass was recorded. The vessel was tared again, a 100.0 µL aliquot of internal standard was added, and its mass was recorded. An aliquot of 200.0 µL of 70% nitric acid was then added to the vessel, and the sample sat at room temperature uncapped for 1 hour to predigest. The vessel was then placed on a heating block at 110°C, and the sample was allowed to digest for 2 hours. After heating, the vessel was removed from the block and cooled to room temperature. An aliquot of 50.0 µL of 30% hydrogen peroxide was added to the sample, and the vessel was returned to the heating block at 110°C. The sample was digested uncapped for 14 hours, after which the vessel was removed, brought to room temperature, and its mass was recorded. The sample was then diluted to 10 mL with 5% nitric acid, and its final mass was recorded and capped until analysis.

Quantification of Analytes from Samples

The concentrations of the analytes from the urine samples were calculated by determining the wavelength intensity ratios of each analyte to yttrium and then using the equations of the lines to determine the corresponding concentration.
Results

*BHB ameliorates PKD in a dose-dependent fashion in juvenile rats*

We reported that 160mM D/L-BHB supplemented in drinking water for five weeks almost completely halts PKD progression (20) and that administering citrate in water can effectively remove microcrystals that exacerbate PKD in Cy/+ rats (5). Here, our study aims to titrate the effective dose of BHB and citrate and test whether their combination can again alter PKD progression in Cy/+ rats (Figure 1A). For our experimental analyses, we focus primarily on male Cy/+ rats. Male Cy/+ rats exhibit more aggressive kidney disease, have the propensity to form microcrystals, and succumb to advanced kidney disease at approximately six months of age. In contrast, female rats survive 12 months or more (14) and are inherently resistant to microcrystal formation (5), precluding them from studying the effects of microcrystal-induced kidney injury. We have included female rats in our study (Supplemental) for completeness, allowing for appreciation of the mechanism of BHB and citrate outside of their effect on microcrystal inhibition.

Like our previous experiments (20), we administered BHB to juvenile Cy/+ rats from P21 to P56 using a 160mM starting concentration and halved it twice to 80mM and 40mM, respectively. BHB significantly reduced cystic disease in a dose-dependent manner (Figure 1B), including a reduction in 2-kidney to bodyweight (Figures 1C and S2A), cystic area (Figures 1D and S2B), cyst number (Figure S1C) and cyst size (Figure S1D), suggesting that BHB affects both cystogenesis and cyst expansion. BHB also reduced serum creatinine, suggesting improved creatinine clearance (Figure 1E).
We examined disease hallmarks of PKD and found 80mM and 160mM BHB strongly inhibited collagen deposition (Figures 1F and S2C) and myofibroblasts (Figures 1G and S2D). Ki67 staining revealed decreased proliferation with 160mM BHB in tubule/cystic cells and a non-significant inhibitory trend in interstitial cells in both males and females (Figures 1H and S2E). Interrogation of PKD-associated signaling pathways found that BHB decreased pSTAT3\textsuperscript{Y705} and pERK1/2\textsuperscript{T202/Y204} expression, alongside a reduction in Kidney Injury Molecule 1 (KIM-1) (Figure S1H), suggesting that BHB may suppress aberrant activation of these pathways and prevents kidney injury.

We observed a slight increase in animal mass (Figure S1A) for all BHB-supplemented Cy/+ groups despite a slight decrease in total calorie consumption (Figure S1B). Both 80mM and 160mM BHB groups consumed more water than controls (Figure S1B).

The blood glucose of Cy/+ rats was slightly decreased relative to wild-type rats. It was restored to wild-type levels with BHB (Figures S1E and S2F), suggesting that the metabolic demand from glycolytic cystic kidneys may lead to systemic blood glucose depletion, similar to previous observations (15, 16, 25). We supplemented BHB as a racemic mixture, so both BHB isomers were measured to report serum BHB levels accurately. Serum BHB levels increased dose-dependently following BHB supplementation, with greater concentrations in wild-type rats (Figures S1F, S1G and S2G, S2H).

Citrate administration ameliorates PKD in a dose-dependent fashion
To test the dose-dependent response of citrate, we supplemented rats with 120mM citrate in drinking water and halved the concentration twice to 60mM and 30mM, respectively. Citrate strongly decreased disease progression in a dose-dependent fashion (Figure 2A). All citrate-supplemented rats exhibited decreased 2-kidney to bodyweight ratios (Figures 2B and S4A), cystic indices (Figures 2C and S4B), cyst number (Figure S3C), and cyst size (Figure S3D), implying citrate inhibits both cystogenesis and cyst expansion. Additionally, citrate improved serum creatinine compared to water alone (Figure 2D).

We measured food and water intake and found that 30mM and 60mM citrate reduced water consumption and that all citrate-supplemented rats consumed fewer calories (Figure S3B) without decreasing animal mass (Figure S3A).

Blood glucose levels were restored and comparable to wild-type with 120mM citrate (Figure S4C) and trended upwards with 30mM and 60mM in males (Figure S3E). Citrate did not lead to detectable changes in serum D-BHB (Figures S3F and S4D).

We investigated collagen deposition (Figures 2E and S4E) and myofibroblast expression (Figures 2F and S4F) and found that only 120mM citrate significantly decreased both markers. Ki67 staining showed citrate did not significantly affect proliferation in males (Figure 2G) but decreased tubule Ki67-positivity in females (Figure S4G). A whole-kidney analysis found the most potent effects from 120mM citrate, decreasing pSTAT3\(^{Y705}\), and similar to BHB, reduced KIM-1 expression dose-dependently. No impact on pERK1/2\(^{T202/Y204}\) expression was observed (Figure S3G).

A combination of BHB and citrate ameliorates PKD in juvenile rats
Our previous data using BHB and citrate supplementation suggested their beneficial effect on disease progression may be caused via distinct mechanisms: 1) Mimicking the state of ketosis and 2) the removal and prevention of injurious tubule microcrystals. We therefore hypothesized that a combination of BHB and citrate (BHB/Citrate) may be more efficacious than either administered alone and would allow for reduced dosing.

To test this, we titrated BHB and citrate in combinations determined from the experiments described above and administered them in drinking water to rats from P21 to P56 (Figure 1A). These combinations are 40mM BHB/30mM Citrate, 40mM BHB/60mM citrate, and 80mM BHB/60mM Citrate, labeled as 40/30, 40/60, and 80/60 respectively. BHB/Citrate dramatically reduced kidney size (Figure 3A) and the appearance of cysts (Figure 3B). 2-kidney to bodyweight (Figure 3C) and cystic area (Figure 3D) were also significantly reduced. Interestingly, both 40/60 and 80/60 combinations dramatically reduced cystic area, similar to BHB or citrate alone (Figures S6A, S6E), suggesting a potential synergistic effect with 40/60 supplementation (Figure S6F). We also observed a reduction in cyst number (Figures S5C and S6E) and cyst size (Figure S5D) in a dose-dependent manner. Like BHB and citrate alone, BHB/Citrate reduced serum creatinine levels in Cy/+ rats (Figure 3E) and was more pronounced than with citrate alone (Figure 2D).

Like with BHB alone, blood glucose trended upwards with BHB/Citrate in Cy/+ rats but without a significant difference in wild-type rats (Figure S5E). Again, similar to BHB alone, there was a slight increase in liver mass with BHB/Citrate supplementation (Figure S5A). 80/60 supplementation increased water consumption but did not increase overall
caloric intake (Figure S5B). BHB/Citrate supplementation did not significantly alter steady-state serum total-BHB levels (Figures S5F, S5G), suggesting that exogenous BHB is rapidly metabolized.

BHB/Citrate supplementation significantly reduced collagen deposition in all BHB/Citrate-supplemented concentrations (Figure 3F), was as effective as BHB alone and was more effective than citrate alone (Figures S6B, S6E). Similarly, myofibroblasts were dramatically reduced with 40/60 and 80/60 (Figures 3G and S6C). These effects required less BHB and citrate than when using BHB or citrate alone, suggesting a potential synergistic activity when combined (Figures S6E, S6F).

We assayed Ki67 and found that 40/60 and 80/60 diminished signals in both interstitial and tubule cells (Figure 3H). BHB or citrate alone showed no significant effect on interstitial cells, with only 160mM BHB affecting tubule/cystic cells (Figure S6D). A comparable impact on total-Ki67 (tubule and interstitial) cell positivity was achieved with 40/60 (Figure S6E), again implying a synergistic effect (Figure S6F). Whole-kidney analysis revealed BHB/Citrate decreased p-STAT3 Y705 in a dose-dependent manner and, unlike BHB or citrate alone, diminished p-ERK T202/Y204 in all BHB/Citrate-supplemented groups (Fig S5H).

*A combination of lower doses of BHB and citrate prevents kidney injury and partially reverses PKD in adult Cy/+ rats*

To test whether reduced doses of BHB and citrate may act together to affect existing cystic disease in adult rats, we supplemented animals from P56-P84 with either 40mM
BHB and 60mM citrate (40/60) or 80mM BHB and 30mM citrate (80/30), respectively (Figure 4A), and compared this to controls receiving water or molar equivalent Na\(^+\)/K\(^+\) solutions derived from sodium and potassium chloride (40/60 Salt and 80/30 Salt).

Supplementation with BHB/Citrate ameliorated PKD in adult male rats with reduced cystic disease visible in H and E kidney sections (Figure 4B). BHB/Citrate produced a decrease in kidney mass (Figure S8A), a nonsignificant reduction in 2-kidney to bodyweight (Figure 4C), a partial reversal in cystic area (Figures 4D and S8B) and cyst number (Figure 4E), decreased cyst size (Figure S8C), and improved serum creatinine (Figure 4F). Only cyst number was significantly reduced in females with BHB/Citrate (Figure S7D). We observed no effect on blood glucose (Figure S8D), but steady-state blood BHB levels slightly increased (Figures 4G and S7F, S8F).

Surprisingly, supplementation with salt caused a reduction in cystic area in female Cy/+ rats (Figure S8B). However, the decline in the cystic area did not accompany improvements in cyst number (Figure S7D) or any other measured outcome. This effect is likely explained by the significantly increased water intake induced by salt supplementation (Figure S8E). Increased water intake improves PKD in rodents (26, 27) and has been explored in clinical studies (28, 29).

Mitochondrial damage and mitochondrial loss are known features of PKD progression in the Cy+ rat (19). To explore if BHB/Citrate could alter metabolism features, we measured mitochondrial DNA number to determine if BHB/Citrate might promote mitochondrial biogenesis. We found that BHB/Citrate did not affect mitochondrial DNA number in Cy/+ kidneys but did lead to an increase in wild-type rat kidneys and was accompanied by an increase in the mitochondrial biogenic marker PGC1\(\alpha\) (Figure 4H).
This effect was specific to BHB/Citrate since salt alone did not affect mitochondrial number.

Collagen deposition did not differ significantly between BHB/Citrate-supplemented cohorts, with only a non-significant downward trend in 40/60 males (Figure 4I). Similarly, SMA-1 positive cells decreased with 40/60 supplementation but did not reach significance (Figure 4J). This effect was similar to Ki67 signaling, trending downwards with BHB/Citrate in tubule/cystic cells without reaching significance (Figure 4K).

Precipitation of microcrystals is a substantive source of kidney injury, leading to activation of the innate immune response, including STAT3 phosphorylation in tubule epithelial cells. Whole-kidney lysates revealed both 40/60 and 80/30 diminished p-ERK$^{T202/Y204}$ and that only 80/30 decreased p-STAT3$^{Y705}$. Both 40/60 and 80/30 reduced the expression of KIM-1, whereas salt alone did not (Figure 4L).

A previous report found that BHB can preserve and support glomeruli during hyperglycemia (30). To this end, we assessed whether BHB/Citrate supplementation might similarly improve glomerular health. We labeled glomeruli in cystic kidneys using the glomerular marker podocin and scored glomeruli for injury (Figure S8G). We found more injured glomeruli within cystic kidneys than within wild-type controls and that BHB/Citrate decreased glomerular injury compared to water alone (Figure 4M) without affecting the total glomeruli number (Figure S8H), implying that part of BHB/Citrate’s benefit involves preventing glomerular injury.

Overall, the measured reduction in cyst number, cystic area, glomerular sparing, and the absence of KIM-1 and p-STAT3$^{Y705}$ suggests that BHB/Citrate can effectively prevent kidney injury, thereby slowing PKD disease progression.
**BHB and Citrate alter mineral and citrate excretion**

Improper mineral handling and reduced citrate excretion are common features of advanced chronic kidney disease (31, 32). The Cy/+ rat exhibits progressive kidney function decline, dysfunctional mineral handling, and concomitant muscle and bone wasting (33). The orthologous Ksp-Cre:Kdfl/fl mouse also exhibits reduced serum Mg$^{2+}$, Ca$^{2+}$, Na$^{+}$, and PO$_{4}^{3-}$ with associated kidney wasting (34). We previously reported that decreased urinary citrate was associated with more rapid kidney function decline and larger total kidney volume in individuals with ADPKD (5). A recent report using a more extensive patient set also found that hypocitraturia correlates with worse PKD progression (35). The clinical experience suggests that a standard or elevated urinary citrate level may be protective in ADPKD, presumably due to citrate's beneficial effect on calcium crystal precipitation and kidney stone risk. To this end, we investigated whether BHB and citrate may alter urine composition to exert their beneficial effects.

To determine if BHB and citrate alter urine composition, we placed adult Cy/+ rats in metabolic cages for 24-hour urine collections following three days with 160mM BHB, 120mM citrate, or 80mM BHB/30mM citrate (80/30) with a 3-day washout period in between each treatment (Figure 5A). Urine was collected and analyzed using UPLC and Inductively Coupled Plasma-Optical Emission Spectrometry (ICP), with the results summarized in Table 1 detailing mineral and metabolite excretion.

The most striking difference between wild-type and Cy/+ rats was that Cy/+ rats exhibited significant hypermagnesuria and reduced calcium excretion. Elevated urine
magnesium excretion normalized to wild-type levels following administration of BHB, citrate, or 80/30 (Figure 5B). BHB supplementation increases calcium excretion in Cy/+ rats and coincides with a significant increase in citrate excretion and urinary citrate concentration in most individuals (Figures 5C, 5D). The addition of BHB generally increased water intake in cystic rats, while citrate did not (Figure 5E). BHB, citrate, and 80/30 generally increased creatinine excretion in most animals (Figure 5F).
Discussion

In this study, we built upon our previous research. We more closely investigated BHB, the principal ketone produced during ketosis, and citrate, the eponymous citric acid cycle intermediate, alone and in combination for their ability to alter PKD progression. We hypothesized that combining BHB and citrate may ameliorate disease progression by simultaneously acting on distinct mechanisms that cause PKD progression, namely mimicking the effects of ketosis and preventing kidney injury by tubule microcrystals. A limitation of this study was the use of the non-orthologous *Anks6* rat model of kidney disease (Cy/+). This model exhibits proximal tubule-derived cysts and many human chronic kidney disease features. While translationally limiting to ADPKD, these disease features (e.g., the propensity to form microcrystals) make its inclusion in this study relevant.

Our study found that both BHB and citrate very effectively halt the progression of PKD in juvenile rats, and a combination of BHB and citrate recapitulated these findings with decreasing amounts of BHB and citrate. We found that early supplementation in juvenile rats dramatically slows the progression of cystic disease during the time window of most rapid progression in this model. In contrast, adult Cy/+ rats exhibit little additional cystic progression and serve as a model of fully established PKD. Remarkably, treatment with BHB and citrate in adult rats resulted in partial disease reversal, including significant reductions in signs of kidney injury, total cystic area, and total cyst number. This aligns with other observations, including our own, that reversing existing renal cystic disease is possible (20, 36, 37). However, since we did not test BHB or citrate alone in adult rats, we cannot speak to the direct comparative effect of combining BHB and citrate.
Our experiments showed increased creatinine excretion following supplementation with BHB and citrate, suggesting improved renal function. Increased creatinine clearance is consistent with human trials in healthy individuals, wherein intravenous BHB administration increases glomerular filtration independent of an alkali load, putatively via a macula densa feedback mechanism (38). We also found that BHB/citrate prevented glomerular injury in adult rats, similar to a previous report showing improvement of glomerular health with BHB in streptozotocin-induced diabetic nephropathy (30). Our human studies in ADPKD suggest ketogenic metabolic therapy increases kidney function (21, 39, 40). Ketogenic metabolic therapy also effectively reduces hypertension (41), arguing against hyperfiltration due to vasoconstriction in our experiments.

Sodium chloride supplementation is known to accelerate the progression of PKD in Cy/+ rats (42). Yet, sodium/potassium BHB salts did not exacerbate disease progression in our study. Instead, they may confer protection from sodium-induced injury, similar to prior reports utilizing butanediol to increase BHB (43). Additionally, exogenous BHB was shown to be consumed primarily by the heart and kidney (44), decreasing heart glucose uptake and improving cardiac output (45) and may inhibit any deleterious effects of high-salt intake in the context of chronic kidney disease.

We should note that sodium and potassium were delivered as chloride salts to our control animals, and this increase in Cl⁻ may promote fluid secretion into cyst lumens via the activity of the cystic fibrosis transmembrane conductance regulator (CFTR) protein, a known contributor to PKD progression in the Cy/+ rat (46, 47). Both the 40/60 salt and 80/30 salt controls showed an increase in overall kidney size in this way, implicating that Cl⁻ may have exacerbated the disease.
We observed improved serum creatinine in 40/60 salt rats, likely secondary to those animals' significant increase in water intake. However, the improvement with BHB/Citrate supplementation was accompanied by a slight decrease in water intake, arguing against any beneficial effect on creatinine by BHB/Citrate being solely due to increased water intake. There were, however, significant differences in feeding behavior between juvenile BHB, citrate, and BHB/Citrate-supplemented groups (Figures S1B, S3B, S5B). Increasing BHB supplementation correlated with increased water intake, likely caused by the coincident increase in sodium. In support of this hypothesis, the lowest BHB (40mM) supplemented animals did not increase water intake and consumed similar amounts of sodium to water-only rats. Interestingly, all BHB-supplemented juveniles consumed comparably reduced calories, implying that BHB may have an anorectic effect even at the lowest dose tested. Citrate alone also produced a similar reduction in calories while reducing water intake similarly between all citrate dosages. This effect of citrate was also observed in our metabolic cage experiments when rats were given citrate alone over four days. Citrate-supplemented water is slightly sour to the taste and may cause an aversion until animals are fully habituated. The taste would explain the reduced water intake but not the calorie reduction. This is important since our lab and others have demonstrated that caloric reduction beneficially alters PKD progression (48, 49). While overall calorie intake is decreased with BHB and citrate alone when combined, this effect vanishes with only the highest concentration of BHB/Citrate tested (80/60), causing increased water intake. We interpret these data to mean that the effect of BHB/Citrate combined is not simply an artifact of caloric restriction or increased water intake but is caused by an intrinsic property of the combination.
We propose that the beneficial effects of BHB/Citrate predominantly come from preventing kidney injury, putatively caused by tubule microcrystals and their subsequent activation of the injury response. This is supported by the measurable reduction in cystogenesis and injury-associated markers (e.g., p-STAT3, KIM-1, SMA-1, collagen, and Ki67) following BHB/Citrate supplementation. BHB is known to exert pleiotropic effects apart from its role as an energy molecule (50), with many of these effects overlapping with those of citrate. Both participate in the tricarboxylic acid (TCA) cycle, fatty acid synthesis, act as signaling molecules to affect gene regulation, alter energy production, and inhibit inflammation (51, 52). We found that BHB alone, the highest dose of citrate alone, and BHB/Citrate strongly inhibited collagen deposition while decreasing SMA-1 and Ki67 expression. It is known that the inflammasome-related protein NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) is activated in tubule epithelial cells in response to microcrystal-induced kidney injury (53) and is a requirement for collagen deposition (54). NLRP3 is directly inhibited by BHB (52), meaning that BHB/Citrate could act upstream and downstream of microcrystal-induced injury, inhibiting microcrystal formation and any subsequent injury response. This makes NLRP3 signaling modification a strong candidate for the mechanism of action of BHB/Citrate.

BHB and citrate were delivered as salts, meaning alkali is likely to contribute beneficial effects as PKD kidneys are known to possess an acidic microenvironment. In the Cy/+ rat, this can be exacerbated by ammonium chloride and improved by sodium bicarbonate (55). Bicarbonate supplementation improves outcomes in chronic kidney disease (56), and decreased serum bicarbonate levels are associated with faster
progression of PKD (57). Tanner et al. used citrate supplementation and found a
dramatic improvement in the Cy/+ rat, concomitantly increasing urine pH. Notably, citrate
converts into bicarbonate in the kidney, acts as a glycolysis inhibitor (58), binds excess
calcium to prevent kidney stone formation, and putatively cystogenesis (5). Each of
these mechanisms may contribute to citrate’s effect but are beyond the scope of the
study.

Disrupted mitochondrial energetics and decreased mitochondrial number (19) are
a feature of PKD (25, 59). To our knowledge, we report here that BHB/Citrate increases
mitochondrial DNA number in kidneys for the first time. Whether BHB/Citrate also
increases mitochondrial health in these kidneys (e.g., via increased aerobic capacity or
energy production) remains to be determined. This effect alone could impact metabolic
health and warrants further study.

Finally, we examined urine parameters following BHB and citrate supplementation
to determine if the mineral composition or kidney output was affected. We found that
BHB/Citrate supplementation reduced markers associated with increased microcrystal
formation by normalizing mineral excretion and increasing urinary citrate. Decreased
citrate excretion has been associated with increased total kidney volume and reduced
eGFR (5), whereas increased citrate excretion is associated with slower disease
progression (35). It may be that BHB/Citrate confers benefits by increasing urinary citrate
and lowering the potential of injury from microcrystals, a putative trigger of cystogenesis.
However, since we did not fully assess all parameters associated with lithogenic risk, we
cannot fully evaluate the effect of BHB/Citrate in that respect. Recently, it was reported
that tolvaptan decreased markers of lithogenesis in ADPKD patients after one year of
treatment with the drug (60). It is tempting to speculate that much of the benefit of tolvaptan, a strong aquaretic causing dilute urine, may come from preventing kidney injury due to microcrystal formation rather than solely its action on the vasopressin receptor.

Altogether, we have shown that a combination of BHB and citrate reduces PKD progression in both an adult and juvenile model of kidney disease by altering cellular signaling to prevent kidney injury, reducing proliferation and fibrosis, improving kidney health by increasing creatinine excretion, preserving glomerular health, and by normalizing mineral excretion and increasing urinary citrate. A combination of BHB and citrate is preferred over using one or the other alone. First, the combination shows a synergistic effect in juveniles by reducing markers of cystic disease using less BHB and citrate. Second, combining the two compounds reduces the overall salt load, as citrate can partially substitute the effects of BHB and be administered as citric acid.

BHB and citrate are widely available, affordable, and categorized as Generally Recognized as Safe (GRAS) in the USA. Both have a long history of use in food and as supplements, neither producing substantial negative safety signals. This makes BHB and citrate attractive for the management of individuals with ADPKD. A novel medical food product, KetoCitra®, based on BHB and citrate, has already been used in conjunction with a diet and lifestyle intervention program (40), and several clinical trials are forthcoming to investigate the short- and long-term effects in ADPKD.
Supplemental Material

Supplemental Figures:
URL: https://figshare.com/s/3b42455829efd370a8bc
DOI: https://doi.org/10.6084/m9.figshare.c.6927814

Supplemental Animal Use Table:
URL: https://figshare.com/s/83c53452670b5624eee7
DOI: http://doi.org/10.6084/m9.figshare.24556129
Perspectives and Significance

In clinical practice, ADPKD is known to be relentlessly progressive. The sole approved drug for ADPKD only slows this progression. We show that supplementation with a combination of two simple, endogenous compounds, BHB, and citrate, not only dramatically slows PKD progression in juvenile rats but even partially reverses existing cystic disease in adult rats. This result is highly significant because the observed effect size with BHB/Citrate supplementation far exceeds the effect size of pharmacological intervention with vasopressin receptor antagonists in PKD rodent models that underpinned the development of tolvaptan as an approved therapy for ADPKD. Given that BHB and citrate are safe and widely used supplements and food additives, these results suggest that BHB/Citrate supplementation may benefit human ADPKD without the side effects and toxicities associated with current pharmacological therapy.
Acknowledgments

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References


42. Keith DS, Torres VE, Johnson CM, Holley KE. Effect of Sodium Chloride, Enalapril, and Losartan on the Development of Polycystic Kidney Disease in Han:SPRD Rats. *American journal of kidney diseases : the official journal of the


Figure Legends

Figure 1: BHB ameliorates PKD in a dose dependent manner

A) Treatment scheme for all juvenile BHB and/or citrate experiments (Figures 1-3). B) Hematoxylin and eosin stained kidneys from 8-week male wild-type and Cy/+ rats supplemented with water or increasing concentrations of BHB salts in drinking water. Scale bar=1mm and 100µm, respectively. C) 2-kidney to the bodyweight of water and BHB-supplemented male wild-type and Cy/+ rats. D) Cystic area of water and BHB-supplemented male Cy/+ rats. E) Serum creatinine of male wild-type and Cy/+ rats. F) Sirius Red and Fast Green stain and quantification of water and BHB-supplemented male Cy/+ rats. Scale bar=50µm. G) Smooth muscle actin (SMA-1) immunofluorescence and quantification of water and BHB-supplemented male Cy/+ rats. Scale bar=50µm. H) Ki67 immunofluorescence stain and quantification of water or BHB-supplemented male Cy/+ rats. Scale bar=50µm.

Wild-type: N=12 (Water), N=6 (40mM BHB), N=9 (80mM), N=12 (160mM BHB)
Cy/+: N=12 (Water), N=10 (40mM BHB), N=10 (80mM), N=7 (160mM BHB)

(Standard deviation and means represented. One-way ANOVA followed by ad hoc Tukey’s test was used for multiple comparisons. *=P<0.05, **=P<0.01, ***=P<0.001, ****=P<0.0001)

Figure 2: Citrate ameliorates PKD in a dose-dependent manner

A) Hematoxylin and eosin stained kidneys from 8-week male wild-type and Cy/+ rats supplemented with water or increasing concentrations of citrate in drinking water. Scale bar=1mm and 100µm, respectively. B) 2-kidney to the bodyweight of water and citrate-
supplemented male wild-type and Cy/+ rats. C) Cystic area of water and citrate-supplemented male Cy/+ rats. D) Serum creatinine of male wild-type and Cy/+ rats. E) Sirius Red and Fast Green stain and quantification of water and citrate-supplemented male Cy/+ rats. Scale bar=50µm. F) Smooth muscle actin (SMA-1) immunofluorescence and quantification of water and citrate-supplemented male Cy/+ rats. Scale bar=50µm. G) Ki67 immunofluorescence stain and quantification of water or citrate-supplemented male Cy/+ rats. Scale bar=50µm. Wild-type: N=12 (Water), N=5 (30mM Citrate), N=4 (60mM Citrate), N=4 (120mM Citrate) Cy/: N=12 (Water), N=5 (30mM Citrate), N=7 (60mM Citrate), N=12 (120mM Citrate) (Standard deviation and means represented. One-way ANOVA followed by ad hoc Tukey’s test was used for multiple comparisons. *=P<0.05, **=P<0.01, ***=P<0.001, ****=P<0.0001) 

Figure 3: A combination of BHB and Citrate ameliorates PKD progression

A) Gross kidney images of 8-week water and BHB/Citrate-supplemented male wild-type and Cy/+ rats. Scale=1cm. B) Hematoxylin and eosin stained kidneys from 8-week male wild-type and Cy/+ rats supplemented with water or increasing concentrations of BHB/Citrate in drinking water. Scale bar =1mm and 100µm, respectively. C) 2-kidney to the bodyweight of water and BHB/Citrate-supplemented male Cy/+ rats. D) Cystic area of water and BHB/Citrate-supplemented male Cy/+ rats. E) Serum creatinine of water and BHB/Citrate-supplemented male wild-type and Cy/+ rats. F) Sirius Red and Fast Green stain and quantification of water and BHB/Citrate-supplemented male Cy/+ rats. Scale
bar=50µm. G) Smooth muscle actin (SMA-1) immunofluorescence and quantification of water and BHB/Citrate-supplemented male Cy/+ rats. Scale bar=50µm. H) Ki67 immunofluorescence stain and quantification of water or BHB/Citrate-supplemented male Cy/+ rats. Scale bar=50µm.

Wild-type: N=12 (Water), N=9 (40/30 B+C), N=3 (60/40 B+C), N=4 (80/60 B+C)
Cy/+: N=12 (Water), N=5 (40/30 B+C), N=6 (60/40 B+C), N=6 (80/60 B+C)

(Standard deviation and means represented. One-way ANOVA followed by ad hoc Tukey’s test was used for multiple comparisons. *=P<0.05, **=P<0.01, ***=P<0.001, ****=P<0.0001)

Figure 4: BHB/Citrate partially reverses PKD in adult Cy/+ rats, preventing kidney injury and preserving glomerular health

A) Timeline of BHB/Citrate supplementation in adult rats. B) Hematoxylin and eosin stained kidneys from 12-week male Cy/+ rats supplemented with water, BHB/Citrate, or salt in drinking water. Scale bar=1mm and 100µm, respectively. C) 2-kidney to the bodyweight of water, BHB/Citrate, and salt-supplemented male wild-type and Cy/+ rats.

D) Cystic area changes between 8-week and 12-week water, BHB/Citrate, and salt-supplemented male Cy/+ rats. E) Quantification of the number of cysts per mm² from whole kidney sections from water, BHB/Citrate, and salt-supplemented male Cy/+ rats. F) Serum creatinine of water, BHB/Citrate, and salt-supplemented male wild-type and Cy/+ rats. G) Serum total-BHB (L-BHB and D-BHB) values of water, BHB/Citrate, and salt-supplemented male wild-type and Cy/+ rats. H) Mitochondrial number of water, BHB/Citrate, and salt-supplemented 12-week male wild-type and Cy/+ rats, and Western
blot of the mitochondrial marker PGC1α in water and 80/30-supplemented male wild-type and Cy/+ rats. All of the samples are on the same blot; the image break is to remove lanes not included in this study. I) Sirius Red and Fast Green stain and quantification of water, BHB/Citrate, and salt-supplemented male Cy/+ rats. Scale bar=50µm. J) Smooth muscle actin (SMA-1) immunofluorescence and quantification of water, BHB/Citrate, and salt-supplemented male Cy/+ rats. Scale bar=50µm. K) Ki67 immunofluorescence stain and quantification of water, BHB/Citrate, and salt-supplemented male Cy/+ rats. Scale bar=50µm. L) Western blot of whole-kidney lysates from water, BHB/Citrate, and salt-supplemented male wild-type and Cy/+ rats. (Salt 1=40/60 Salt, Salt 2=80/30 Salt) M) Glomerular injury scoring per mm² of kidney area from 12-week-old water male wild-type and Cy/+ rats and male Cy/+ rats supplemented with 40/60 BHB/Citrate or 80/30 BHB/Citrate. Glomeruli were scored as follows:

0: No obvious morphological changes; normal

1: Morphological change, e.g., changes in shape and structure

2: Morphological changes as well as decreased filling of glomeruli space, increase in distance between Bowman’s capsule and podocin staining

Wild-type: N=12 (Water), N=10 (40/60), N=9 (80/30), N=10 (Salt 1), N=7 (Salt 2)

Cy/+: N=12 (Water), N=9 (40/60), N=7 (80/30), N=8 (Salt 1), N=9 (Salt 2)

(Standard deviation and means represented. One-way ANOVA followed by ad hoc Tukey’s test was used for multiple comparisons. *=P<0.05, **=P<0.01, ***=P<0.001, ****=P<0.0001)
Figure 5: **BHB and Citrate normalize mineral excretion and increase citrate excretion**

A) Treatment scheme for metabolic cage experiments. B) Change in 24-hour urinary magnesium and calcium excretion from BHB, citrate, and BHB/Citrate-supplemented male wild-type and Cy/+ rats. C) Change in 24-hour urinary citrate excretion in BHB, citrate, and BHB/Citrate-supplemented male wild-type and Cy/+ rats. D) Change in 24-hour urinary citrate concentration in BHB, citrate, and BHB/Citrate-supplemented male wild-type and Cy/+ rats. E) Change in 24-hour water intake of BHB, citrate, and BHB/Citrate-supplemented male wild-type and Cy/+ rats. F) Change in 24-hour urine creatinine excretion from water, BHB, citrate, and BHB/Citrate-supplemented male wild-type and Cy/+ rats.

Wild-type: N=5; Cy/+: N=7

(Standard deviation and means represented. Paired and unpaired t-test was used for comparisons. *=P<0.05, **=P<0.01, ***=P<0.001, ****=P<0.0001)

Table 1: **Metabolic cage experiment animal and urine parameters**

Analysis of 24-hour metabolic cage rat experiments. Rats were supplemented with water, 160mM BHB, 120mM citrate, or 80mM/30mM BHB/Citrate combined.

(Values represent mean and standard deviation. Paired and unpaired t-tests are used for analyses when appropriate. *=P<0.05, **=P<0.01, ***=P<0.001, ψ=P<0.0001. Parentheticals indicate the statistical difference between wild-type and Cy+ rats.)
**BHB and Citrate Ameliorate Polycystic Kidney Disease**

### Experimental Design

<table>
<thead>
<tr>
<th>Juvenile Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>P21</td>
</tr>
<tr>
<td>+BHB</td>
</tr>
<tr>
<td>+Citrate</td>
</tr>
<tr>
<td>+BHB/Citrate</td>
</tr>
<tr>
<td>5 Weeks</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Adult Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>P21</td>
</tr>
<tr>
<td>Water</td>
</tr>
<tr>
<td>+BHB/Citrate</td>
</tr>
<tr>
<td>+K/NaCl</td>
</tr>
<tr>
<td>5 Weeks</td>
</tr>
</tbody>
</table>

### Outcomes

- **BHB and citrate prevent cystic disease progression in juvenile and adult Cy/+ rat kidneys**

Increased urinary citrate and improved mineral excretion

**CONCLUSION**

BHB and citrate effectively prevent disease progression in the Cy/+ rat, increasing urinary citrate and preserving kidney health.
### Table 1: Metabolic cage experiment animal and urine parameters

Analysis of 24-hour metabolic cage rat experiments. Rats were supplemented with water, 160mM BHB, 120mM citrate, or a combination of 80mM/30mM BHB/Citrate. (Values represent mean and standard deviation. Paired and unpaired t-test used for analyses when appropriate. *=P<0.05, **=P<0.01, ***=P<0.001, ψ=P<0.0001, Parentheticals indicate the statistical difference between wild-type and Cy+ rats.)

<table>
<thead>
<tr>
<th></th>
<th>Wild-Type</th>
<th></th>
<th></th>
<th>Cy+</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>BHB</td>
<td>Citrate</td>
<td>BHB/Citrate</td>
<td>Water</td>
<td>BHB</td>
</tr>
<tr>
<td><strong>N</strong></td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Animal Mass g</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>278.5 ±6.0</td>
<td>298.0 ±6.9</td>
<td>317.0 ±8.7</td>
<td>345.0 ±9.9</td>
<td>281.7 ±10.2</td>
<td>286.7 ±17.8</td>
</tr>
<tr>
<td>Water Intake mL/kg/d</td>
<td>194.5 ±49.7</td>
<td>267.8 ±56.7**</td>
<td>117.3 ±32.8*</td>
<td>116.0 ±13.8*</td>
<td>155.5 ±33.1</td>
<td>201.0 ±62.1*</td>
</tr>
<tr>
<td>Food Intake g/kg</td>
<td>80.4 ±4.2</td>
<td>70.3 ±6.1</td>
<td>59.2 ±6.8</td>
<td>56.3 ±11.2</td>
<td>58.7 ±10.1</td>
<td>67.6 ±6.3</td>
</tr>
<tr>
<td>BHB Intake µmol/g/day</td>
<td>N/A</td>
<td>42.2 ±8.9</td>
<td>N/A</td>
<td>9.1 ±1.1</td>
<td>N/A</td>
<td>33.5 ±11.3</td>
</tr>
<tr>
<td>Citrate Intake µmol/g/day</td>
<td>N/A</td>
<td>14.0 ±3.9</td>
<td>3.5 ±0.4</td>
<td>N/A</td>
<td>18.0 ±2.9</td>
<td>6.0 ±1.8</td>
</tr>
<tr>
<td>Urine Volume mL/kg/d</td>
<td>128.7 ±45.6</td>
<td>191.1 ±44.8*</td>
<td>61.6 ±16.9*</td>
<td>75.9 ±9.3*</td>
<td>87.6 ±16.5</td>
<td>124.6 ±44.6*</td>
</tr>
<tr>
<td>Urine Creatinine mg/kg/d</td>
<td>41.8 ±3.9</td>
<td>51.1 ±6.5*</td>
<td>39.2 ±6.0</td>
<td>47.3 ±6.2*</td>
<td>39.8 ±4.5</td>
<td>44.5 ±3.8**</td>
</tr>
<tr>
<td>Urine Creatinine mg/dL</td>
<td>35.1 ±10.0</td>
<td>28.1 ±7.8</td>
<td>71.7 ±10.8**</td>
<td>58.3 ±12.6*</td>
<td>46.3 ±6.7</td>
<td>38.6 ±10.5</td>
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<tr>
<td>Sodium Consumption mg/kg/d</td>
<td>241.4 ±12.6</td>
<td>811.9 ±132.4</td>
<td>177.6 ±20.3</td>
<td>299.2 ±46.3</td>
<td>176.0 ±30.4</td>
<td>653.9 ±151.9</td>
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<tr>
<td>Sodium Excretion mg/kg/d</td>
<td>320.9 ±43.1</td>
<td>521.9 ±104.4**</td>
<td>366.0 ±264.0</td>
<td>366.0 ±191.2</td>
<td>281.7 ±65.8</td>
<td>362.5 ±74.1</td>
</tr>
<tr>
<td>Magnesium Excretion mg/kg/d</td>
<td>8.3 ±4.4</td>
<td>5.7 ±1.5</td>
<td>3.6 ±2.0</td>
<td>4.6 ±5.1</td>
<td>16.3 ±3.2[*]</td>
<td>4.6 ±2.3 Ψ</td>
</tr>
<tr>
<td>Calcium Excretion mg/kg/d</td>
<td>3.6 ±1.5</td>
<td>4.9 ±0.8</td>
<td>3.2 ±1.6</td>
<td>3.3 ±3.1</td>
<td>1.8 ±0.8[**]</td>
<td>2.9 ±0.6</td>
</tr>
<tr>
<td>Potassium Excretion mM</td>
<td>70.4 ±26.4</td>
<td>74.6 ±26.7</td>
<td>291.5 ±103.9</td>
<td>124.5 ±61.9</td>
<td>86.0 ±29.3</td>
<td>78.7 ±29.5</td>
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<tr>
<td>Potassium Excretion mg/kg/d</td>
<td>35.7 ±13.6</td>
<td>88.4±24.6</td>
<td>51.0 ±20.9</td>
<td>74.2 ±36.8</td>
<td>36.4 ±16.1</td>
<td>72.6±32.1</td>
</tr>
<tr>
<td>Calcium Excretion mM</td>
<td>3.1 ±1.9</td>
<td>1.3 ±0.5</td>
<td>2.4 ±1.1</td>
<td>2.4 ±2.6</td>
<td>8.0 ±2.5[*]</td>
<td>1.7 ±1.0</td>
</tr>
<tr>
<td>Magnesium Excretion mM</td>
<td>0.8 ±0.5</td>
<td>0.7 ±0.2</td>
<td>1.3±0.5</td>
<td>1.1±1.0</td>
<td>0.5±0.3</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>Calcium Excretion mM</td>
<td>7.3 ±3.9</td>
<td>11.4 ±2.8*</td>
<td>14.0 ±3.8*</td>
<td>10.5 ±3.3</td>
<td>13.6 ±3.6*</td>
<td>16.6 ±16.6**</td>
</tr>
<tr>
<td>Urine Citrate mg/kg/d</td>
<td>158.8 ±46.7</td>
<td>401.0 ±34.4**</td>
<td>169.8 ±76.2</td>
<td>447.5 ±80.0**</td>
<td>169.2 ±30.3</td>
<td>304.8 ±48.6***</td>
</tr>
</tbody>
</table>

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