Potential Therapeutic Interventions for Cardiovascular Disease in Autosomal Dominant Polycystic Kidney Disease

Priyanka Sagar

MBBS, FRACP

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Michael Stern Laboratory for Polycystic Kidney Disease,

Centre for Transplant and Renal Research,

The Westmead Institute for Medical Research,

Faculty of Medicine and Health,

The University of Sydney

4th March 2024

Statement of Originality

This is to certify that to the best of my knowledge; the content of this thesis is my own work.

This thesis has not been submitted for any degree or other purposes.

This thesis is presented in the form of a series of manuscripts. I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged. Where appropriate, a detailed description of the contributions from each manuscript co-author has been included as part of this thesis.

Signed:

_____

Priyanka Sagar

Date:

9/1/2024

Abstract

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is an inherited condition that results from reduced or dysfunctional polycystin-1 or polycystin-2 cellular proteins. It is characterised by the formation of numerous cysts in both kidneys and affects multiple other body systems. Cardiovascular disease (CVD) is a major manifestation of ADPKD and is the main cause of mortality for affected individuals. Hypertension is the most common presentation of CVD in APDKD and increases the risk of progression of other vascular manifestations of CVD including renovascular disease, valvular heart disease, intracranial aneurysms and ischaemic heart disease.

Current treatments include lifestyle and dietary modifications and pharmacotherapy for management of hypertension and hyperlipidaemia, but these therapies are not specific to ADPKD. While these interventions are appropriate and reduce CVD risk, the pathogenesis of cardiovascular dysfunction in ADPKD is unique compared to other forms of chronic kidney disease as it is linked to the underlying inherited polycystin abnormalities and is driven by a number of mechanisms.

CVD in ADPKD is associated with the growth of kidney cysts, which activate systemic drivers of hypertension such as the renin-angiotensin-aldosterone system and the sympathetic nervous system, and lead to reduced renal clearance of vasoconstrictive molecules. Cystogenesis is driven by vasopressin (a pituitary hormone released in response to increases in plasma osmolality) which stimulates pathogenic cyclic-adenosine monophosphate growth factor pathways *via* impaired calcium-mediated signalling due to underlying lack of polycystin-1 and -2. Additionally, reduction in functional polycystin in vascular endothelial cells results reduces nitric-oxide mediated flow-induced dilatory responses predisposing to the premature development of hypertension, arterial stiffness and atherosclerosis.

Furthermore, vitamin D deficiency is common in kidney disease, and in ADPKD reduced vitamin D receptor activation may contribute to renal and vascular inflammation and fibrosis, and increased renin gene expression. Given this specific pathogenesis (which commences early in the disease progression) and the burden of disease due to cardiovascular manifestations despite current therapies, targeted interventions for CVD in ADPKD are needed.

Therefore, the aim of this thesis is to explore specific preclinical and clinical interventions in three pathogenic pathways in ADPKD involved in CVD progression. Given the clinical impact of CVD, interventions that are easily accessible, rapidly translatable, and sustainable for long-term therapy have been prioritised, leading to the selection of non-pharmacological interventions and medications that can be repurposed. The hypotheses that will be examined are: (i) increased water intake reduces the progression of cardiorenal disease in experimental polycystic kidney disease, (ii) a vitamin D receptor agonist (VDRA), paricalcitol, reduces blood pressure and proteinuria in both early and late stages of experimental polycystic kidney disease, alone and in conjunction with angiotensin converting enzyme inhibitor (ACEi) therapy, and (iii) nitrate-replete beetroot juice lowers blood pressure compared with nitrate-depleted beetroot juice in hypertensive adults with ADPKD.

The outline of the thesis is as follows:

Chapter 1 provides an overview of ADPKD and a published literature review of the cardiovascular manifestations of disease, the underlying pathophysiologic mechanisms and current clinical practice guidelines.

Chapter 2 examines the effect attenuating cyst-related CVD progression through increased water intake in the Lewis Polycystic Kidney (LPK) rat model of polycystic kidney disease.

This study demonstrated that increased water intake reduced kidney size, cyst area, kidney fibrosis, proteinuria, systolic blood pressure and cardiac hypertrophy.

Chapter 3 examines the effect of VDRAs on reducing blood pressure, cardiac hypertrophy and proteinuria in early and late experimental polycystic kidney disease (*LPK* rat model), alone and in conjunction with standard therapy of ACEi. The results demonstrated that combination treatment of VDRA and ACEi reduced blood pressure and cardiac hypertrophy in late disease stages to a greater extent than either treatment alone but resulted in severe hypercalcaemia and weight loss. There was no change in proteinuria, kidney growth, cyst disease or renal function in early or late disease stages with VDRA treatment.

Chapters 4 and 5 examines the effect of nitrate (which is a precursor for nitric oxide) supplementation with beetroot juice (BRJ) to reduce blood pressure in ADPKD in a double-blind placebo-controlled randomised clinical trial. Specifically, **Chapter 4** describes the rationale for the clinical trial and the published protocol describing the methodology, and **Chapter 5** describes the results of the trial. Sixty participants were recruited for the trial and randomised 1:1 to either nitrate-replete BRJ or nitrate-deplete BRJ. The trial demonstrated excellent adherence with BRJ supplementation with no major adverse events. The results of the trial were that nitrate-replete BRJ did not lower blood pressure significantly compared to nitrate-deplete BRJ. However, there was a significant reduction in blood pressure in both arms of the trial, suggesting there was an effect from other components of BRJ that are vasoactive and/or an effect of trial engagement. **Chapter 6** contains a summary of the studies, an examination of their strengths and limitations, and outlines future directions of this work.

In conclusion, the novel findings in this thesis are; (i) increased water intake reduced cardiorenal progression of experimental polycystic kidney disease, (ii) vitamin D receptor

activation in combination with ACEi reduced blood pressure and cardiac hypertrophy, but caused severe hypercalcaemia and weight loss cautioning its use, and (iii) nitrate-replete BRJ did not lower blood pressure compared to nitrate-deplete BRJ in hypertensive ADPKD participants, but a significant reduction in blood pressure in both trial arms suggests vasoactive components of BRJ and/or effect of trial participation. These findings suggest that non-pharmacological treatments (increased water intake and BRJ) and repurposed drugs (paricalcitol) have the potential to reduce blood pressure and progression of CVD in ADPKD, however, further studies are needed to evaluate these interventions.

Publications, Abstracts and Conference Presentations

Manuscripts Published Related to this Thesis:

1. Sagar PS, Zhang J, Luciuk M, Mannix C, Wong ATY, Rangan GK. **Increased water intake reduces long-term renal and cardiovascular disease progression in experimental polycystic kidney disease.** *PLoSOne.* 2019; 14(1): e0209186. doi: 10.1371/journal.pone.0209186

2. Sagar PS, Saravanabavan S, Munt A, Wong ATY, Rangan GK. **Effect of Early and Delayed Commencement of Paricalcitol in Combination with Enalapril on the Progression of Cardiovascular Disease in Experimental Polycystic Kidney Disease**. *Journal of Cardiovascular Development and Disease*. 2021;8:144. doi:10.3390/jcdd8110144

3. Sagar PS, Munt A, Saravanabavan S, Vahedi FA, Elhindi J, Nguyen B, Chau K, Harris DC, Lee V, Sud K, Wong N, Rangan GK. **Efficacy of beetroot juice on reducing blood pressure in hypertensive adults with autosomal dominant polycystic kidney disease (BEET-PKD): study protocol for a double-blind, randomised, placebo-controlled trial**. *Trials*. 2023;24(1):482. doi:10.1186/s13063-023-07519-2

4. Sagar PS, Rangan GK. **Cardiovascular Manifestations and Management in ADPKD.** *Kidney International Reports,* 2023; 8(10):1924-1940. doi: 10.1016/j.ekir.2023.07.017

Manuscripts submitted (under review) related to this thesis:

1. Sagar PS, Elhindi J, Chau K, Harris DC, Lee V, Sud K, Wong N, Rangan GK. **Double-Blind Randomised Controlled Trial of Nitrate-Replete Beetroot Juice on Blood Pressure Lowering in Hypertensive Adults with Autosomal Dominant Polycystic Kidney Disease.** Under Review with *Kidney International Reports (Submitted 13/12/23)*.

Conference presentations, posters and abstracts presented related to this thesis:

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2. **Sagar PS**, Zhang J, Luciuk M, Mannix C, Wong ATY, Rangan GK. Increased water intake reduces long-term renal and cardiovascular disease progression in experimental polycystic kidney disease. *Mini-Oral Presentation at the Australian and New Zealand Society of Nephrology 53th Annual Scientific Meeting; 2nd-6th September 2017.*

Manuscripts and conference posters published not related to this thesis (completed during the time of thesis enrolment):

1. Oey O, Rao P, Luciuk M, Mannix C, Rogers NM, Sagar P, Wong A, Rangan G. **Effect of dimethyl fumarate on renal disease progression in a genetic ortholog of nephronophthisis**. *Exp Biol Med (Maywood)*. 2018;243(5):428-436. doi:10.1177/1535370218759313

2. Sagar PS, Fischer E, Gangadharan Komala M, Bose B. **A case of minimal change disease during pregnancy - benefits of early diagnosis and use of corticosteroids.** Obstet Med. 2022;15(3):198-200. doi:10.1177/1753495X21990214
3. Sagar P, Vahedi F, Rangan G, on behalf of PREVENT-ADPKD Investigators. **Effect of increasing water intake for 3 years on repeated self-assessed health-related quality of life (HR-QoL) in autosomal dominant polcystic kidney disease (ADPKD).** *Poster presentation at World Congress of Nephrology, 30 March - 2 April 2023*

Contributions to Manuscripts

Part of **Chapter 1** of this thesis, **Section 1.2.3**, is published as Sagar PS, Rangan GK. Cardiovascular Manifestations and Management in ADPKD. *Kidney International Reports*, 2023; 8(10):1924-1940.

PS (Priyanka Sagar) and GR co-conceived and co-designed the idea for the manuscript. PS performed the literature review, drafted the manuscript and completed the revisions required for publication. GR reviewed and edited the manuscript on numerous occasions. All authors contributed to critical reviewing and finalisation the manuscript prior to submission.

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GR conceptualised, designed the experiment, performed the live animal studies and specimen collection. PS, ML and GR performed data curation and analysis including all experimental work on animal specimens. PS drafted the manuscript and completed the revisions required for publication. GR reviewed and edited the manuscript on numerous occasions. All authors contributed to critical reviewing and finalisation the manuscript prior to submission.

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GR conceptualised, designed the experiment, performed the live animal studies and specimen collection. PS, SS and GR performed data curation and analysis including all experimental work on animal specimens. PS drafted the manuscript and completed the revisions required for publication. GR reviewed and edited the manuscript on numerous occasions. All authors contributed to critical reviewing and finalisation the manuscript prior to submission.

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GR is the chief investigator and conceived the idea for the study. GR and PS designed the study and developed the protocol. GR, PS, SS, JE and AM were involved in protocol revisions. PS drafted the manuscript and completed the revisions required for publication. GR reviewed and edited the manuscript on numerous occasions. All authors contributed to critical reviewing and finalisation the manuscript prior to submission.

Chapter 5 of this thesis is submitted for consideration of publication. GR is the chief investigator and conceived the idea for the study. PS and GR designed the study and developed the protocol. PS, GR, FV and SS were involved with the implementation of the study, participant visits, safety and data monitoring. PS, JE, FV and GR were involved with data curation and analysis. PS and GR drafted the manuscript. All authors contributed to editing, critical reviewing and finalisation the manuscript prior to submission.

Author Attribution Statement

In addition to the statements above, in the cases where I am not the corresponding author of a published or submitted manuscript, permission to include the material has been granted by the corresponding author.

Signed:_____

Name: Priyanka Sagar

Date: 21st December 2023

As the primary supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Signed:_____

Name: Prof Gopala Rangan

Date: 21st December 2023

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Table of Contents

List of Abbreviations

25-OH vitamin D	25-hydroxy vitamin D
AAA	Abdominal Aortic Aneurysms
ACEi	Angiotensin Converting Enzyme Inhibitor
ACR	Albumin to Creatinine Ratio
ADH	Anti-Diuretic Hormone
ADMA	Asymmetric Dimethylarginine
ADPKD	Autosomal Dominant Polycystic Kidney Disease
AMBP	Ambulatory Blood Pressure Monitoring
AOBP	Automated Oscillometric Blood Pressure
ARB	Angiotensin Receptor Blocker
AVP	Arginine Vasopressin
BEET-PKD	Efficacy of Beetroot Juice on Reducing Blood Pressure in Hypertensive Adults with Autosomal Dominant Polycystic Kidney Disease
BNP	B-type natriuretic peptide
BP	Blood Pressure
BRJ	Beetroot Juice
BW	Body Weight
cAMP	Cyclic Adenosine Monophosphate
CCB	Calcium Channel Blocker
CKD	Chronic Kidney Disease
COPD	Chronic Obstructive Pulmonary Disease
CrCl	Creatinine Clearance

CRISP	Consortium for Radiologic Imaging Studies of Polycystic Kidney Disease
CTFR	Cystic Fibrosis Transmembrane Conductance Regulator
CVD	Cardiovascular Disease
DASH	Dietary Approach to Stop Hypertension
E	Enalapril
eGFR	Estimated Glomerular Filtration Rate
ERK	Extracellular Signal-Regulated Kinase
EVEREST	Effects of Oral Tolvaptan in Patients Hospitalized for Worsening Heart Failure
ET-1	Endothelin-1
FGF23	Fibroblast Growth Factor 23
GI	Gastrointestinal
HALT-PKD	Halt Progression of Polycystic Kidney Disease
HbA1c	Glycosylated Haemoglobin A1c
HREC	Human Research Ethics Committee
Ht-TKV	Height-adjusted Total Kidney Volume
HOCM	Hypertrophic Obstructive Cardiomyopathy
HW	Heart Weight
HWI	High Water Intake
ICA	Intracranial Aneurysm
ICH-GCP	International Council for Harmonisation-Good Clinical Practice
ICPMR	Institute Of Clinical Pathology And Medical Research
IDCM	Idiopathic Dilated Cardiomyopathy

IHD	Ischaemic Heart Disease
IQR	Inter-Quartile Range
KDIGO	Kidney Disease Improving Global Outcomes
KW	Kidney Weight
LPK	Lewis Polycystic Kidney
LVH	Left Ventricular Hypertrophy
MI	Myocardial Infarction
MR	Mitral Regurgitation
mTOR	Mammalian Target of Rapamycin
MVP	Mitral Valve Prolapse
NHMRC	National Health and Medical Research Council
NPHP	Nephronophthisis
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NSAIDs	Non-steroidal Anti-inflammatories
NWI	Normal Water Intake
OPTIMAAL	The Optimal Therapy in Myocardial Infarction with the Angiotensin II Antagonist Losartan
PAS	Periodic-Acid Schiff
PC	Paricalcitol
PC-1	Polycystin-1
PC-2	Polycystin-2
PCNA	Proliferating Cell Nuclear Antigen
PCR	Protein to Creatinine Ratio

PKD	Polycystic Kidney Disease
RAAS	Renin-Angiotensin-Aldosterone System
RCT	Randomised Controlled Trial
SAE	Serious Adverse Event
sBP	Systolic Blood Pressure
SGLT	Sodium Glucose Co-transporter
SNS	Sympathetic Nervous System
SPIRIT	Standard Protocol Items: Recommendations for Interventional Trials
TGF-β	Transforming Growth Factor Beta
U PCR	Urine Protein to Creatinine Ratio
Usyd	University of Sydney
UK	United Kingdom
V	Vehicle
VDRA	Vitamin D Receptor Agonist
VSMC	Vascular Smooth Muscle Cell
WIMR	Westmead Institute for Medical Research
WSLHD	Western Sydney Local Health District
α-SMA	α-Smooth Muscle Actin

List of Figures

All the figures, with the exception of Figure 1 in Chapter 1.8, are self-contained within their relevant published manuscript and thus are labelled according to journal guidelines.

Supplemental figures

The following figures have been referenced within published manuscripts and are thus labelled according to journal guidelines.

List of Tables

The following tables have been referenced within published manuscripts and are thus labelled according to journal guidelines.

Chapter 4.2

Chapter 5.2

Supplemental tables

Chapter 2.3

Chapter 3.3

Chapter 1: Introduction

1.1 Overview of Autosomal Dominant Polycystic Kidney Disease

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is the most common monogenic cause of kidney failure globally, affecting between 1:1000 to 1:2500 individuals and accounting for up to 5-10% of the end-stage kidney failure population. (1-3) ADPKD is a multi-system disorder with renal and extra-renal manifestations. The renal manifestations include the characteristic finding of numerous kidney cysts that form in childhood and can result in kidneys up to five times normal size by the 5th decade of life, which contributes to the development of chronic pain, urinary tract and cyst infections, cyst haemorrhage and kidney stones. (1, 3) Extra-renal manifestations include cysts in other organs (most commonly liver), early onset hypertension, left ventricular hypertrophy (LVH), valvular heart disease and arterial aneurysms. (1)

As an inherited condition, the diagnosis of ADPKD presents a major burden on affected patients and whole families. There is currently no cure and disease-modifying therapy (vasopressin-2 antagonists) is limited to a small subset of patients with rapidly progressive kidney disease. (4) Current treatments focus on slowing disease progression with lifestyle interventions to avoid dehydration, limit sodium intake, maintain healthy body weight and diet, and pharmacological therapy to reduce blood pressure and control lipids. (1, 4) Despite these interventions, 50% of patients develop kidney failure by the age of 60 and suffer from cardiovascular events, which is the most common cause of death in ADPKD. (2, 5)

1.1.1 Overview of pathogenesis of ADPKD

The condition is caused by autosomal dominant inheritance of pathogenic variants most commonly in the *PKD1* (~75% of tested patients) and *PKD2* (~18%) genes which encode for ciliary proteins polycystin-1 and polycystin-2, respectively. Other variants in proteins associated with polycystin function (such as *ALG5, ALG9, DNAJB11, GANAB, IFT140* or *NEK8*) have been described in patients without *PKD1* or *PKD2* pathogenic variants who have an ADPKD-like phenotype. (6, 7) Inheritance of pathogenic variants (or *de novo* mutations) in these genes are followed by a "second hit" in affected renal epithelial cells (usually in only 1-5% of nephrons). (2) Over time these affected cells grow into enlarging and innumerable kidney cysts that lead to kidney impairment by obstruction, inflammation, vascular remodelling and fibrosis of surrounding renal parenchyma. (2) Apart from causing progressive kidney disease, distortion of vascular structures by these growing kidney cysts leads to ischaemia-related activation of the renin-angiotensin-aldosterone system (RAAS) and sympathetic nervous system (SNS) driving hypertension and cardiovascular disease (CVD). (8, 9) Furthermore, as kidney impairment develops, there is further dysregulation of mediators of endothelial function leading to chronic vasoconstriction and arterial stiffness contributing to the development of hypertension and CVD, including accelerated atherosclerosis and aneurysm formation. (8, 10, 11)

1.1.2. Role of primary cilia and calcium-mediated signalling in the pathogenesis of ADPKD

ADPKD is considered a ciliopathy, as the causative pathogenic variants in the renal epithelial cell affect the cellular organelle, the primary cilium. Primary cilia are found in various cell types throughout the body and are a hair-like structure formed from microtubules that protrude from the plasma membrane and have a mechanosensory and chemosensory role. (12,

13) Polycystin-1 and -2 are transmembrane proteins on cilia that play an vital role in detecting fluid flow and in cell-to-cell communication. (12, 13) Polycystin-1 is a pivotal element of the cilia structure, and bending of the primary cilia in response to direction and pressure of fluid flow results in activation of intracellular calcium-signalling pathways, a step that appears to be primarily mediated by polycystin-2. (12) Additionally, ciliary-mediated calcium signalling is important during cellular development in determining left-to-right orientation and regulating cell growth. (12) Loss of function of these intracellular calcium pathways is a key pathogenic finding in polycystic kidney disease (PKD) and contributes to the dysregulated and uncontrolled growth seen in the cystic cells in ADPKD. (12) Additionally, primary cilia are found in vascular endothelial cells where they have a similar role to sense fluid shear stress and regulate endothelial relaxation to prevent vascular injury and stiffening. (14, 15) Impairment of these pathways contributes to the progression of renal disease and the development of CVD in ADPKD.

1.1.3. Other mechanisms of kidney cystic disease in ADPKD

There are also extracellular mediators that drive cyst growth, the most important and well-described being vasopressin, or antidiuretic hormone, which is a trigger for cytogenesis and ongoing cyst growth. (16) Vasopressin activates cyclic adenosine monophosphate (cAMP) signalling resulting in cell proliferation and chloride-driven cystic fluid secretion. (2, 16) There are multiple other pathogenic features in ADPKD including activation of growth factors (such as protein kinase A, mammalian target of rapamycin and hedgehog signalling) and increased anaerobic glycolysis, which together result in uncontrolled cystic cell proliferation leading to enlarged kidneys, obstruction of normal renal parenchyma, kidney impairment, chronic kidney pain and CVD. (2)

1.2 Pathophysiology of Cardiovascular Disease in ADPKD

CVD associated with ADPKD is a lifelong comorbidity and can lead to acute, life-threatening and chronic, debilitating consequences. As patient survival has improved over the decades with increased education and access to anti-hypertensives and renal replacement therapy, the burden of CVD and accumulation of risk factors has also increased. (5) Although the characteristic finding of ADPKD occurs in renal tubular epithelial cells, polycystin-1 and -2 proteins are present in many other cell types including vascular endothelial cells and smooth muscle cells, cardiomyocytes, and fibroblasts, where their dysfunction contributes to the development of CVD. (1, 5)

The major manifestation of CVD in ADPKD is hypertension, which affects up to 70% of patients prior to the onset of kidney impairment at a relatively early age, around the 3rd decade of life. (17) Other common manifestations that can be linked to hypertension, but also occur independently, are LVH, intracranial aneurysms (ICA) and other arterial aneurysms, ischaemic heart disease and ischaemic cardiomyopathy, mitral valve prolapse (MVP, which occurs early in disease) and mitral regurgitation (MR, which occurs later in disease). (5, 17)

1.2.1 Role of the renin-angiotensin-aldosterone system and the sympathetic nervous system on development of CVD

A major driver of CVD in ADPKD is obstructive cystic disease leading to ischaemia and activation of RAAS. Renin, angiotensin II and aldosterone activation lead to chronic vasoconstriction, sodium retention, systemic hypertension and cardiac ventricular remodelling contributing to LVH. (8, 18) RAAS activation also promotes inflammation and fibrosis with increased transforming growth factor-beta (TGF-β) and oxidant-mediated endothelial damage, and promotes increased cystic proliferation by stimulating angiogenesis,

leading to further obstruction, ischaemia and renin stimulation. (19) Cystic kidney disease also leads to sustained sympathetic nervous system activation which chronically elevates vascular tone contributing to raised blood pressure, LVH and potentially increased risk of vascular injury, malformations and rupture. (9)

1.2.2. Role of mediators of endothelial dysfunction on development of CVD

Another major driver of CVD in ADPKD is vascular endothelial cell dysfunction due to impaired primary ciliary functioning. This occurs due to loss of cellular mechanisms to sense increases in fluid shear stress or minute changes in blood pressure and results in impaired flow-mediated vasodilation, arterial stiffness and increased risk of vessel injury, which in a pathogenic cycle contributes to further local ischaemia, RAAS activation, systemic hypertension and vascular abnormalities. (14, 15) Mediators of endothelial dysfunction in ADPKD include nitric oxide (NO) which has significantly decreased activity, and asymmetric dimethylarginine (ADMA) and endothelin-1 (ET-1), which are overactive. (10, 15, 20, 21) Furthermore, normal ciliary functioning is required for regulation of vascular cell growth, vascular cellular differentiation and cell-extracellular matrix interactions in relation to mechanical stimuli, and its loss may contribute to vascular ectasia and valvular abnormalities seen in ADPKD. (14, 15, 22)

1.2.3. Detailed narrative literature review into the cardiovascular manifestations and management in ADPKD

The following literature review focuses on CVD in ADPKD and details the pathophysiology, manifestations, current guidelines for treatment, and potential future therapeutic directions.

The last comprehensive review was published in 2009 by Ecder *et al.* with more recent published reviews focusing on specific manifestations or treatment aspects of CVD in ADPKD. (23) This narrative review describes the entire range of CVD manifestations in ADPKD including hypertension, LVH and other cardiomyopathies, valvular heart disease, ICAs and other vascular abnormalities. It draws on preclinical and clinical studies, describing the pathogenesis, epidemiology, risk factors, and clinical recommendations for screening and treatment of each manifestation. The review also provides clinic practice points, highlighting the relevance of interventions with the known pathological mechanisms of disease specific to ADPKD.

This review was published in the *Kidney International Reports* journal on August 3rd 2023, and is formatted according to journal guidelines with self-contained figures, tables and references.

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REVIEW

Cardiovascular Manifestations and Management in ADPKD

Priyanka S. Sagar[1,2,3] and Gopala K. Rangan[1,2]

[1]Michael Stern Laboratory for Polycystic Kidney Disease, Westmead Institute for Medical Research, The University of Sydney, Sydney, New South Wales, Australia; [2]Department of Renal Medicine, Westmead Hospital, Western Sydney Local Health District, Sydney, New South Wales, Australia; and [3]Department of Renal Medicine, Nepean Hospital, Nepean Blue Mountains Local Health District, Sydney, New South Wales, Australia

Cardiovascular disease (CVD) is the major cause of mortality in autosomal dominant polycystic kidney disease (ADPKD) and contributes to significant burden of disease. The manifestations are varied, including left ventricular hypertrophy (LVH), intracranial aneurysms (ICAs), valvular heart disease, and cardiomyopathies; however, the most common presentation and a major modifiable risk factor is hypertension. The aim of this review is to detail the complex pathogenesis of hypertension and other extrarenal cardiac and vascular conditions in ADPKD drawing on preclinical, clinical, and epidemiological evidence. The main drivers of disease are the renin-angiotensin-aldosterone system (RAAS) and polycystin-related endothelial cell dysfunction, with the sympathetic nervous system (SNS), nitric oxide (NO), endothelin-1 (ET-1), and asymmetric dimethylarginine (ADMA) likely playing key roles in different disease stages. The reported rates of some manifestations, such as LVH, have decreased likely due to the use of antihypertensive therapies; and others, such as ischemic cardiomyopathy, have been reported with increased prevalence likely due to longer survival and higher rates of chronic disease. ADPKD-specific screening and management guidelines exist for hypertension, LVH, and ICAs; and these are described in this review.

Kidney Int Rep (2023) **8,** 1924–1940; https://doi.org/10.1016/j.ekir.2023.07.017
KEYWORDS: autosomal dominant polycystic kidney disease; cardiovascular disease; endothelial dysfunction; hypertension; intracranial aneurysms; valvular heart disease

ADPKD is the fourth most common cause of kidney failure worldwide and the most common monogenic cause of kidney impairment.[1] ADPKD is caused by autosomal dominant inheritance of pathogenic mutations (or *de novo* mutations) in either *PKD1* (∼78% of cases) or *PKD2* (∼15% of cases), which results in impaired function of proteins polycystin-1 or polycystin-2 respectively, or rarely mutations in other genes (such as *ALG5, ALG9, DNAJB11, GANAB*, or *IFT140*) that have an effect on polycystin function.[2,3] Polycystins have particular functional importance in renal tubular epithelial cells where its dysfunction leads to the hallmark finding of numerous, enlarging, fluid-filled cysts in the kidneys.[1] Cystogenesis occurs sporadically and is hypothesized to be triggered by a reduction in total functional polycystin-1 below a certain "threshold" level (∼10%–30% below normal).[4,5] The reduction may be due to a somatic mutation in the unaffected polycystin allele, localized kidney injury, environmental factors, and/or stochastic factors[4,5]

Loss of function of polycystin proteins in other cell types leads to extrarenal manifestations in ADPKD.[1,6] In vascular endothelial cells, vascular smooth muscle cells (VSMCs), cardiomyocytes, and cardiac fibroblasts, the reduction in polycystin-1 contributes to early development of CVD.[1,6] CVD is the most common cause of mortality and is a major cause of morbidity in patients with ADPKD.[6–8] The cardiovascular manifestations present in the second and third decade of life, often prior to development of kidney impairment, and the subclinical manifestations such as endothelial dysfunction and arterial stiffness present earlier.[7,9,10] The driving factors of CVD in ADPKD are enlarging cysts stimulating the RAAS and SNS combined with inherited dysregulation of vasoactive molecules (NO, ADMA, ET-1).[6,7] In addition, ciliary-related abnormalities in cell development and cell-to-cell communication in vascular endothelial cells, smooth muscle cells and cardiomyocytes predispose to the development of

Correspondence: Gopala K. Rangan, Westmead Institute for Medical Research, 176 Hawkesbury Road, Westmead, New South Wales 2145, Australia. E-mail: g.rangan@sydney.edu.au
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arterial aneurysms and cardiac valvular disease.[9,11] The development of vascular abnormalities are also hypothesized to be due to the "threshold" mechanism of disease; however, this level varies between different tissues and cell types, and is independent of kidney cystogenesis.[4,12] CVD is a significant chronic condition that has acute and detrimental consequences. Changing clinical characteristics, increased patient education, and evolving therapies have resulted in increased patient survival but with the ongoing burden of high CVD risk.[6] Clinicians and patients should be vigilant about screening and initiating management early to improve outcomes. This review discusses the epidemiological risk factors, pathogenesis, screening, and management of CVD in ADPKD. Hypertension is the most common manifestation, and its severity impacts other conditions; therefore, it will be discussed first, followed by cardiomyopathies (LVH, ischemic cardiomyopathy, and others), valvular heart disease (mitral valve prolapse [MVP] and mitral regurgitation [MR]), ICAs, and other vascular malformations.

HYPERTENSION

Epidemiology and Clinical Associations

Hypertension is the most common presenting symptom of ADPKD, has a mean onset at 27 years old and occurs in 50%–70% of patients prior to decline in kidney function.[6,7] Hypertension is also common in children with ADPKD, with a 2016 meta-analysis reporting a 20% prevalence (95% confidence interval 15%–27%).[13] Hypertension is a major modifiable risk factor for the development of CVD and a key contributor to the development of LVH and albuminuria.[14] For pregnant women with ADPKD, hypertension leads to a significantly greater risk of preeclampsia (54% vs. 8% in normotensive women with ADPKD, $P < 0.001$), maternal complications (~85% vs. ~30% in normotensive ADPKD, $P < 0.001$) and kidney function decline (0.8% of hypertensive ADPKD pregnancies progressed to kidney failure compared to 0% in the normotensive ADPKD and 0.0001% in the general population).[15,16] In addition, the presence of hypertension is linked with increased severity of cystic kidney disease in children and adults.[17] Cohort studies (including the large longitudinal CRISP cohort) of hypertensive patients with ADPKD have shown significantly greater kidney sizes, rates of kidney growth, decline in kidney function, and progression to kidney failure compared to normotensive patients with ADPKD.[10,12,18–22]. Together, these findings reflect the central role of hypertension in cardiovascular and kidney disease progression in ADPKD.

Pathogenesis of Hypertension

The pathogenesis of hypertension in ADPKD occurs through 2 main mechanisms: (i) hypertension driven by cystic growth and renal dysfunction and (ii) hypertension driven by vascular dysfunction due to abnormal polycystin function.[23,24] The key downstream mediators of hypertension are the RAAS, NO, ADMA, ET, and the SNS.

Cystic Growth and Kidney Dysfunction as Drivers of Hypertension

Kidney cyst expansion leads to nephron obstruction and intrarenal microvascular ischemia stimulating the RAAS and SNS, and driving systemic hypertension.[25] Cohort and histological studies have shown that both circulating and intrarenal renin, angiotensin II, and angiotensin-converting enzyme (ACE) levels are increased in ADPKD.[26,27] Renin, aldosterone, and ACE activity was increased compared to patients with chronic kidney disease (CKD) and essential hypertension, implying an ADPKD-specific process contributing to RAAS overactivity (than renal dysfunction alone, another driver of RAAS activation).[24,26,27] Similarly, RAAS overactivity is detected in hypertensive patients with ADPKD prior to the development of renal impairment.[27,28] Focal ischemia of the parenchyma or the juxtaglomerular apparatus directly, stimulates renin secretion and RAAS activation.[29] Graham *et al.*[30] found additional renin secreting cells lining intrarenal vessels of ADPKD kidneys, which likely contribute to unregulated renin release and increased RAAS activity, particularly in patients with early or mild cyst burden. Graham *et al.*[30] also found hyperplasia of juxtaglomerular apparatus in ADPKD kidneys, a feature also found in noncystic CKD, which likely plays a role in RAAS overactivity in late ADPKD when kidney dysfunction is established. RAAS is also a stimulator of angiogenesis and renal epithelial cell growth contributing further to cyst growth and the persistence of hypertension in vicious cycle (Figure 1).[24,29]

In addition, kidney cyst growth leads to stretching of the renal capsule and the release of neuromodulator signaling molecules from injured kidney tissue (such as bradykinin, neurokinin A, calcitonin gene-related peptide, substance P, and prostaglandins) leading to stimulation of surrounding renal sympathetic nerves and increased SNS activity.[31–33] Angiotensin II also directly increases vascular sympathetic tone, leading to raised peripheral vascular resistance and blood pressure (Figure 1).[32,33] This is reflected in the published literature, where measures of SNS activity in patients with ADPKD are significantly increased compared to noncystic CKD, essential hypertension, and healthy controls.[32,34] In patients with ADPKD who are on

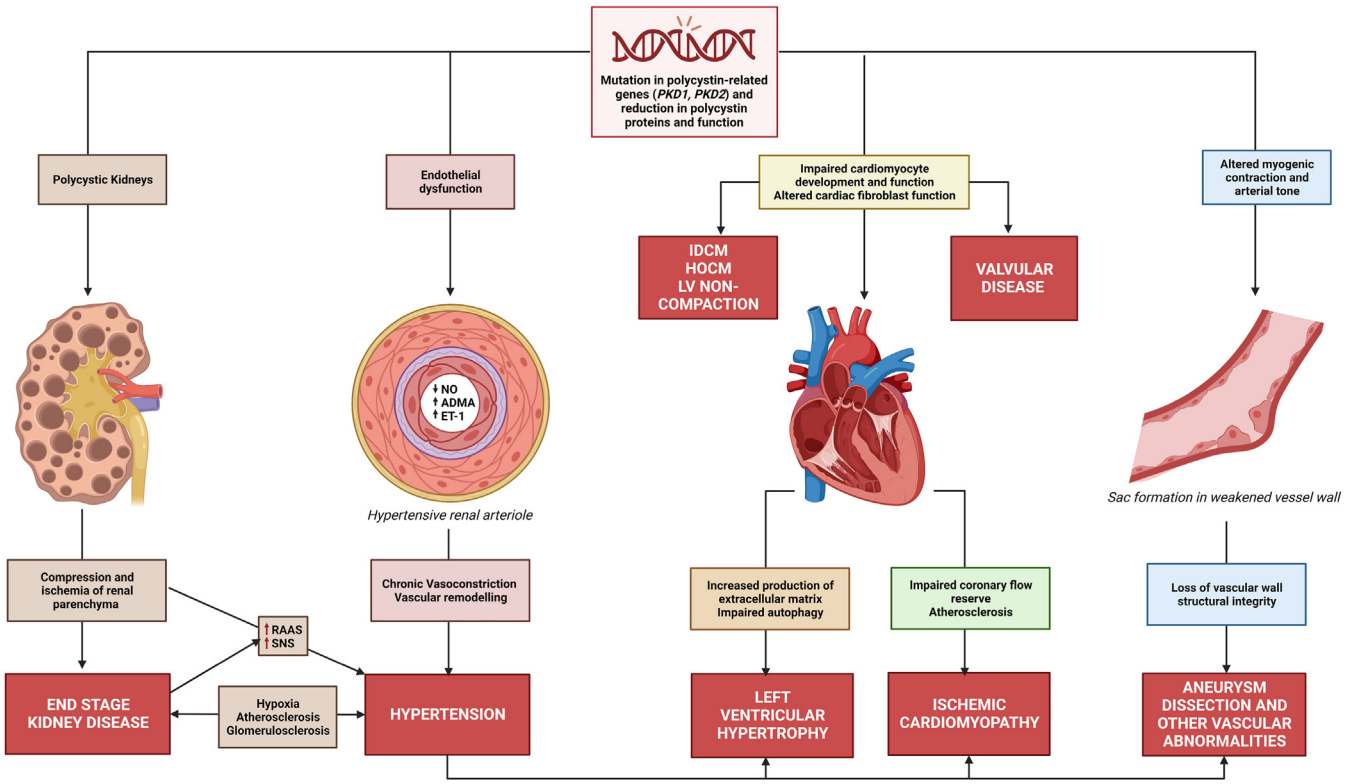

Figure 1. Pathogenesis of cardiovascular disease in ADPKD. Inherited abnormalities and reduction of functional polycystin-1 and polycystin-2 proteins impact multiple organ systems. Kidney cyst growth activates the RAAS and SNS, driving hypertension and other cardiovascular abnormalities. Endothelial dysfunction and chronic vasoconstriction lead to hypertension and vascular abnormalities, including arterial stiffness, thickening of vessel walls and atherosclerosis. These vascular changes also contribute to other arterial abnormalities such as intracranial aneurysms, dissections, and coronary artery disease. Hypertension is also a key risk factor for development of LVH and together contribute to the risk of coronary ischemia, likely predisposing patients to ischemic cardiomyopathy. Hypertensive arterial injury and development of cardiomyopathy leads to impaired end organ perfusion and atheroma development, which in turn, leads to renal injury with glomerulosclerosis and further RAAS activation in a pathogenic cycle. ADMA, asymmetric dimethylarginine; ADPKD, autosomal dominant polycystic kidney disease; ET-1, endothelin-1; HOCM, hypertrophic obstructive cardiomyopathy; IDCM, idiopathic dilated cardiomyopathy; LVH, left ventricular hypertrophy; NO, nitric oxide; RAAS, renin-angiotensin-aldosterone system; SNS, sympathetic nervous system.

dialysis, the morphological changes associated with SNS overactivity (increased renal nerve density and renal arterial sympathetic innervation) are significantly increased compared to non-ADPKD patients who are on dialysis, and these changes are thought to play a pivotal role in the persistence of hypertension in kidney failure.[33,35]

Chronic pain has an estimated prevalence of 50% to 60% in patients with ADPKD, and may contribute to SNS hyperactivity and hypertension.[36–38] Moreover, chronic pain is reported as a cause of sleep disturbance in 16.8%–20.8% of patients with ADPKD.[36,37] Pain may also contribute to poorer BP control through its potential role in loss of overnight decrease in BP and increase in early prewaking BP (a feature attributed to spikes in SNS overactivity) described in patients with ADPKD; however, the current evidence is circumstantial.[38] Further studies are required to evaluate the impact of chronic pain, SNS overactivity, and elevated BP in ADPKD. This may be of additional importance to other cardiovascular manifestations because SNS overactivity independently contributes to the development of LVH, arterial remodeling, arrhythmias, and heart failure.[39]

Vascular Dysfunction as a Driver of Hypertension and CVD

Endothelial dysfunction is pathogenic precursor to chronic CVD and has been detected in patients with ADPKD prior to the development of hypertension or LVH.[6] Endothelial dysfunction results in vascular abnormalities, including loss of flow-mediated dilation and increased arterial stiffness, which occur prior to hypertension, suggesting a role in its pathogenesis.[40,41]

Polycystins are expressed at high levels in the cilia of vascular endothelial cells where they sense fluid shear stress, detect minute changes to BP, and have a central role in flow-related endothelial response and performance.[42–45] In transgenic mice, endothelial PC-1 mediates Ca^{2+}-dependent hyperpolarization and vasodilation through the activation of nitric oxide synthase (NOS) and intermediate or small-conductance

K^+ channels.[46] PC-1 forms a complex with PC-2 which localizes to the plasma membrane of vascular endothelial cells.[46,47] The loss of either PC-1 and/or PC-2 reduces flow-mediated vasodilation and an increase in BP.[46–48] These conditional endothelial-specific knockout models provide evidence that CVD occurs prior to and independent of kidney impairment in ADPKD.[46–48]

However, as kidney impairment develops, mechanisms of hypertension common to other forms of CKD (such as increased RAAS activity and sodium retention) contribute to progression of CVD in ADPKD.[49] This was demonstrated by endothelial-*PKD1* knockout mice, where 5 of 6 nephrectomy exacerbated impaired flow-mediated dilation.[48]

Vascular endothelial cilia play a role in the regulation of cell division and endothelial-to-mesenchymal transition functions, which likely contributes to structural abnormalities such as vascular ectasias and MVP.[50] In VSMCs primary cilia, PC-1 and PC-2 play a role in cell-extracellular matrix interaction and responses to mechanical stimulation.[51] PC-2, localized to the plasma membrane on VSMCs, mediates sensing of intraluminal wall stretch, myocyte Ca^{2+} regulation, and modulation of myogenic tone.[52,53] Cell culture and animal models have demonstrated that the quantity of PC-1 and PC-2, and their relative balance, is important to modulate their action on myocyte constriction and myogenic tone.[45,54] The outcome of alterations of PC-2 in VSMCs in the current literature is conflicting. Studies of aortic arteries from $PKD2^{+/-}$ mice displayed exaggerated contraction to phenylephrine stimulus, whereas VSMC-specific *PKD2* knockout mice developed vasodilation and reduced BP in mesenteric and hindlimb arteries.[52,54] These differences may reflect variable expression between cell-specific and global constitutive knockout of *PKD2*.[45,52] Although the exact mechanisms require further elucidation, polycystin expression and function is variable between cell types and these changes are important in the pathogenesis of CVD. Due to these abnormalities in polycystin function, key mediators of endothelial function NO, ADMA, and ET-1 are dysregulated (through the mechanisms described below and contribute to hypertension and vascular complications, including aneurysm, dissection, and atherosclerosis (Figure 1).[50]

Reduced Nitric Oxide. NO is a potent driver of endothelial-mediated vasodilation and in normal conditions, PC-1 and PC-2 modulate flow-stimulated Ca^{2+}-dependent NOS activation, hyperpolarization, and relaxation in vascular endothelial cells.[46,47] The reduction or inhibition of this process leads to vasoconstriction and increased BP.[25,47] Zhang *et al*.[55] detected a >70% reduction in NO metabolites in 2 separate human ADPKD cell lines. Other clinical and preclinical studies have detected this decrease in NO levels and NOS activity in ADPKD.[55–58] This reduction has been detected prior to establishment of hypertension or kidney impairment, and has a negative linear correlation with BP in hypertensive patients.[56,59] Lorthioir *et al*.[58] demonstrated a reduction in NO release and heat-stimulated flow-mediated dilation in normotensive patients with ADPKD compared to healthy controls, which improved with infusion of dopamine (stimulation of endothelial dopamine receptors restores Ca^{2+} influx in polycystin-deficient cells). Although in contrast to other studies, baseline plasma nitrite was higher in the ADPKD group compared with controls in this study, nitrite levels did not increase in response to heating as they did in controls, demonstrating a deficiency in NO production.[58] These are supported by studies by Wang *et al*.,[59] who demonstrated a reduction in NO metabolites and maximal acetylcholine-induced vasodilation (in normal conditions acetylcholine increases NO by stimulating intracellular calcium and activating NOS) in normotensive patients with ADPKD. The effect could not be overcome or further suppressed by an NO substrate (L-arginine) or a NOS inhibitor (NG-nitro-L-arginine methyl ester, or L-NAME) implying an inherent inactivity of endothelial NOS in ADPKD.[57,59] Interestingly, ADPKD vessels responded to SIN-1, an exogenous NO-donor, to induce endothelial relaxation confirming that the endothelium in these normotensive patients were still able to respond to but unable to generate NO.[59] Although a reduction in NO is a pathological feature in diabetes and CKD-related hypertension, is it unclear if it occurs early in these diseases as it does in ADPKD.[59] Furthermore, there is persistence of NO deficiency until late stage ADPKD, with the development of characteristic sclerotic lesions in renal vessels (that are similar to those found in animals treated with NOS inhibitors), highlighting its pathogenic role in both early and late ADPKD.[60]

Increased ADMA and Oxidative Stress. ADMA is a potent NO-inhibitor and its levels are significantly increased in ADPKD compared to controls, including in patients with ADPKD with normal kidney function and BP.[25,60] ADMA suppresses endothelial NO stimulating vasoconstriction, increasing peripheral vascular resistance and promoting atherosclerosis.[25] ADMA is increased in all-cause CKD stage 3 onward, but is elevated early (CKD stage 1) in ADPKD.[60] Even modest increases in ADMA levels (as demonstrated in the study above) can inhibit vasodilation and further impair NO production.[60,61] Intravenous infusions of ADMA in healthy subjects significantly reduces renal blood flow

and vasodilatory responses; and in CKD rats, ADMA infusion resulted in glomerular capillary loss and vascular sclerosis.[62,63] Furthermore, elevated ADMA levels are a strong predictor of coronary vascular disease and mortality in patients with CKD.[64,65] Further studies are required to evaluate the long-term impacts of ADMA; however, given its role in vascular dysfunction and the documented elevated levels, it is likely an important mediator of CVD in ADPKD.

The cause of increased ADMA is not certain but has been linked to increased oxidative stress and decreased clearance early ADPKD.[25] Studies of patients with ADPKD with preserved kidney function demonstrated a significant increase in markers of oxidative stress (prostaglandin-2α, prostaglandin-D2, prostaglandin-E2, 8-isoprostane, and lipid peroxidation product 13-hydroxyoctadecadienoic acid), which are known to further decrease NO availability.[25,60,66,67] In addition, elevated oxidative stress is associated with vascular dysfunction (as measured by impaired flow-mediated dilation) further supporting the interaction of these pathways and their contribution to disease.[67]

Increased Endothelin-1. ET-1 is an endothelial-derived systemic and intraglomerular mediator of blood flow.[68] It acts as a vasoconstrictor in the renal cortex and a vasodilator in the renal medulla, and its actions are mediated by NO.[69] Focal ischemia, hypoxia, and increased angiotensin II caused by cyst growth are strong stimulators of ET-1.[69,70] It is speculated that ET-1 has a compensatory role in early ADPKD to maintain blood flow to medullary areas obstructed by cysts and to maintain sodium balance in the setting of tubular loss; however, this role becomes pathogenic as disease progresses, contributing to elevated BP and sodium retention, particularly in the setting of reduced NO mediation.[68–71]

The imbalances of these endothelial mediators, vascular dysfunction, and consequences of cyst growth lead to the development and persistence of hypertension, kidney impairment, and CVD in a pathogenic cycle (Figure 1).[63,72–74]

Management of Hypertension

Detailed screening and therapeutic recommendations, including lifestyle and pharmacological interventions are presented in Table 1. Briefly, all patients with ADPKD should be screened for hypertension at the time of diagnosis and during follow-up, and managed with standard lifestyle principles, which include low sodium diet (<100 mmol/d), regular exercise, weight loss (if overweight), and smoking cessation. Sodium intake in ADPKD can be assessed using the scored salt questionnaires and recommended dietary changes are similar to other types of CKD.[88–90] The first-line agents for antihypertensive therapy are ACE inhibitors (ACEi); or if not tolerated, angiotensin receptor blockers (ARB).[91] The effectiveness of ACEi/ARB was tested in the landmark HALT-PKD randomized controlled trials which used by a 2-by-2 factorial design to evaluate a lower BP target of 95/60 to 110/75 mm Hg (vs. standard BP targets of 120/70 to 130/80 mm Hg), and combination of ACEi-ARB (vs. ACEi) alone on annual percentage change in total kidney volume.[78] The results showed that lower BP targets were associated with slower kidney growth, lower left ventricular mass index and albuminuria.[78] These beneficial effects were greatest in participants with large kidneys (≥ 75th percentile total kidney volume) and under the age of 30 years, participants with total kidney volume greater than the median, and in male participants.[78] The combination therapy of ACEi-ARB did not alter the rate of annual total kidney volume increase or eGFR.

CARDIAC MANIFESTATIONS IN ADPKD

Left Ventricular Hypertrophy and Other Cardiomyopathies

Epidemiology and Risk Factors of LVH

LVH is a serious complication of ADPKD and its presence increases the risk of premature death and major cardiovascular events (including arrhythmias and heart failure), especially when LVH coexists with hypertension.[44,92] There is variability in the reported prevalence of LVH in ADPKD from 41% in a study in 1997 (Chapman *et al.*[93]), 21.4% in a 2019 study (Chen *et al.*[92]) and 3.9% in the HALT-PKD study (2006–2014).[44,78,92–94] Furthermore, a recent retrospective study in February 2023 (Arjune *et al.*[95]) reported a 65% incidence of LVH in patients with ADPKD compared with 55% in controls. A key difference between these studies is the imaging modality; HALT-PKD used cardiac magnetic resonance imaging (the current gold-standard to measure ventricular dimensions) and the other studies used ultrasound echocardiography (with variability from advances in echocardiogram technology in the 26 years between the 2 studies). The lower prevalence of LVH in more recent studies may also be due to altered diagnostic definitions; increased and earlier screening; and importantly, increased use of RAAS blockers (5% in the 1997 study, 63.5% in the 2019 study, and >80% before commencement of HALT-PKD).[78,92–94] Notably, when the same criteria for left ventricular mass in the HALT-PKD study was applied to the 2023 study, the incidence of LVH was found to be 5% (which is much more consistent with the HALT-PKD cohort).[94,95] The lower incidence of LVH seen in recent studies is likely due to more effective introduction of antihypertensive therapy.[92,94]

Table 1. Screening and management of cardiovascular manifestations in ADPKD

Hypertension
Screening
• Individuals with ADPKD or at-risk (family history or equivocal kidney ultrasound) should have annual BP measurement[75]
• ABPM and home BP recordings are recommended to diagnose early hypertension or masked hypertension
Targets
• BP ≤130/80.[76,77]
• A lower target 95–110 systolic and 60–75 mm Hg diastolic should be considered for selected patients in early disease stages (estimated glomerular filtration rate >60 ml/min per 1.73 m^2) and less likely to experience adverse effects. A lower target slowed rate of kidney growth and reduced left ventricular mass in the HALT-PKD studies but did not affect kidney function.[78]
• This target does not apply to patients with kidney failure. In these patients, higher BP was associated with better survival.[79]
• For children, consensus recommendation is to target blood pressure <75th percentile for their age.[80]
Non-pharmacological interventions
• Sodium restriction to <100 mmol/d (2.3 g sodium/d or 6 g salt/d).
• Smoking cessation, particularly because it is an independent risk factor for CVD and CKD.
• Maintenance of healthy weight (body mass index 20–25).
• Regular exercise for at least 30 minutes 5 days a week (as compatible with cardiovascular health).
Pharmacological interventions
• ACEi, or if not tolerated, ARB should be used as first-line agents for hypertension.
◦ Should be implemented with low-sodium diet to optimize effectiveness.[81]
◦ Dual RAAS blockade is not recommended.[78]
◦ Caution should be exercised with their use in child-bearing aged women due to their teratogenic risks.
• Choice of second-line agent is based on patient factors and comorbidities.
• Patients whose BP does not drop overnight (as evaluated by ABPM) may benefit from dosing antihypertensive at night.
• Other modifiable cardiovascular risk factors (such as hypercholesterolemia, smoking, and coronary artery disease) should be assessed and treated as per CKD guidelines.[82]
Left ventricular hypertrophy and other cardiomyopathies
Screening
• Diagnosis of cardiomyopathies are made by echocardiography.
• Screening is not recommended for LVH because management is currently focused on BP reduction.
• Patients with a family history of ventricular noncompaction or idiopathic dilated cardiomyopathy should have a screening echocardiogram.
Management
• Lifestyle interventions to reduce modifiable CVD risk factors as described for hypertension.
• Patients with LVH and hypertension should be treated with ACEi or ARB as first-line therapy.[76]
• Specific management of ischemic cardiomyopathy includes ACEi or ARB, beta-blockers, aspirin, statins, and revascularization where possible[83]
• Management of other cardiomyopathies should be individualized and include fluid management and optimization of cardiac function with ACEi/ARB, beta-blockers, diuretics, aldosterone antagonists, and digitalis, where indicated.
Valvular heart disease
Screening
• Adults and children with a heart murmur should be screened with an echocardiogram.
• Asymptomatic screening is not recommended.
Management
• Management is based on severity and patient comorbidities, and follows principles of heart failure management, which includes diuretic therapy, beta-blockers, and ACEi as needed.
• Patients with valvular disease have a higher risk of arrhythmias, and may require screening and treatment with anti-arrhythmics and anticoagulation.[84]
Intracranial aneurysms
Imaging should be done urgently for any patient with ADPKD and neurological symptoms (severe or atypical headache, history of transient ischemic attack, neurological deficits, cranial nerve palsy) because it may be due to an ICA or other intracranial vascular abnormality
Screening
• Screening is performed by computer tomographic angiogram or magnetic resonance angiogram (gold standard, "time of flight" MRI can be done without gadolinium)
• Screening is suggested for patients:
◦ With a family or personal of ICA or
◦ If in high-risk professions (e.g., commercial pilots) or
◦ Prior to major elective surgery (e.g., kidney transplant)
• Repeat screening scans every 5–10 years[85]
• Asymptomatic screening is controversial and consensus recommendations do not suggest screening due to low yield of significant ICAs and risk of undue patient anxiety[77,81]
Management
• Management is dependent on patient and aneurysm characteristics and should be made in consultation with neurosurgery.
• Small ICAs (<5 mm) may be monitored 6-monthly or 12-monthly and then possibly at longer intervals if they remain stable.
• Larger ICAs may require endovascular or surgical intervention.
• Ongoing risk factor modification with smoking cessation, rigorous blood pressure management to target BP <130/80 mm Hg and control of classic CVD risk factors.[85,86]
Abdominal Aortic Aneurysms
There are no ADPKD-specific guidelines for management of AAA and patients should be managed as per the general recommendations below.[87]
Screening
• Opportunistic screening during other investigations (e.g., ultrasound, CT, or MRI of the abdomen) is recommended by expert opinion.
• Single-episode ultrasonography screening is recommended for:
◦ Male patients aged >65 years old with a smoking history
◦ Patients aged >65 years old with an immediate family history of AAA
◦ Adult patients with personal or immediate family history of Marfan, Loeys-Dietz, or similar syndrome
◦ Patients with known thoracic or popliteal aortic aneurysms
Management
• is dependent on patient and aneurysm characteristics and should be made in consultation with vascular surgery.
• may involve active surveillance or intervention with either open repair or endovascular aneurysm repair.

AAA, abdominal aortic aneurysms; ABPM, ambulatory BP monitoring; ACEi, angiotensin-converting enzyme inhibitors; ADPKD, autosomal dominant polycystic kidney disease; ARB, angiotensin receptor blockers; BP, blood pressure; CT, computed tomography; CVD, cardiovascular disease; CKD, chronic kidney disease; ICA, intracranial aneurysm; IDCM, idiopathic dilated cardiomyopathy; LVH, left ventricular hypertrophy; MRI, magnetic resonance imaging; PKD, polycystic kidney disease; RAAS, renin-angiotensin-aldosterone system.

Hypertension is a major risk factor in the development of LVH, and studies in ADPKD demonstrate a positive linear correlation with higher BPs and the presence of LVH.[96] In children with ADPKD, elevated or borderline (above the 75th percentile) BP led to significant higher rates of LVH compared with children with lower BP.[97,98] Particularly in "borderline" hypertensive patients, masked hypertension and loss of overnight decrease in systolic BP is a contributor to the development of LVH.[98,99] Although LVH is a well-known consequence of hypertension, multiple studies of normotensive children and young adults with ADPKD have also shown increased rates of LVH compared with non-ADPKD controls, including those with similar 24-hour BP readings.[24,92,93,100] Chen *et al*.[92] reported a similar prevalence of LVH in hypertensive (22.4%) and normotensive (17.9%) groups. Chapman *et al*.[93] reported a higher prevalence of LVH in normotensive ADPKD (23%) compared to healthy controls (16%); however, hypertensive ADPKD was significantly higher than both (48%). Therefore, hypertension is clearly a major driving factor for LVH in ADPKD; however, there are other predisposing mechanisms that lead to the increased prevalence of LVH in normotensive patients with ADPKD.

Kidney dysfunction and increased age are independent risk factors for the presence of LVH in ADPKD, similar to the non-ADPKD population.[38,93,94] Total kidney volume is also associated with LVH, independent of BP and kidney function.[92] Furthermore, high early morning or prewaking BP has been demonstrated in patients with ADPKD with LVH who did not have any other risk factors, suggesting that SNS overactivity may contribute to the development of LVH in ADPKD.[38]

Pathogenesis of LVH

The most commonly described trigger for LVH is an increase in volumetric burden on the left ventricle (usually due to hypertension or cardiac valvular disease) resulting in compensatory increase in myocardial hypertrophy leading to LVH. Over time, increased deposition of extracellular matrix and myocardial fibrosis results in ventricular stiffness and diastolic dysfunction.[101] Angiotensin II, a mediator of this process, is overactive in ADPKD.[24]

The dysfunction of polycystins in experimental knockout models of *PKD1* and *PKD2* show significant cardiac abnormalities, and this may explain the incidence of LVH in normotensive patients with ADPKD. Primary cilia, expressing PC-1 and PC-2, are present in cardiomyocytes (particularly endocardial-facing) during early fetal development and contribute to cardiac left–right axis development, valvulogenesis, and myocardial regeneration; however, these cilia are no longer expressed in adult animal models.[102,103] Instead, in adult rat cardiomyocytes, PC-1 localizes to the plasma membrane where it functions as a mechanosensor independently or in complex with PC-2.[104] Studies of cardiomyocytes in PC-1 knockout mice demonstrate reduced L-type calcium channels and intracellular signaling, increased cell proliferation leading to cardiac wall thickening, and reduced cardiac contractility compared to controls.[104,105] Primary cilia, which highly express PC-1 are also found in cardiac fibroblasts and these cells normally accumulate at the site of myocardial injury and regulate matrix deposition and fibrosis.[106] Impairment of fibroblastic cilia results in increased cardiac hypertrophy and fibrosis.[106] In the $PKD1^{RC/RC}$ mouse phenotype (which has a reduction but not complete loss of PC-1 expression), there is a dosage effect of PC-1 on the severity of the cardiac hypertrophy.[107]

PC-2 localizes to the sarcoplasmic reticulum and loss of PC-2 in murine cardiomyocytes leads to reduced cardiac shortening and cardiac dyssynchrony.[108,109] Furthermore, decreased PC-2 alters cardiomyocyte beta-adrenergic pathways in murine $PKD2^{+/-}$ without hypertension or cystic disease, and these changes may contribute to high incidence of atrial fibrillation reported in patients with ADPKD.[108,109] In addition, reduced PC-1 and PC-2 in ADPKD leads to dysregulation of mammalian target of rapamycin, an important regulator of autophagy, a process which maintains cardiomyocyte size, function, and structure.[110] PC-2 also has a role in inducing and regulating autophagy via its Ca^{2+-} conductance effects, independent of the mammalian target of rapamycin pathway.[111] Preclinical PKD studies show that reduced autophagy leads to left ventricular hypertrophy, dilation and contractile dysfunction.[44,110] These mechanisms together likely contribute to the development of LVH in ADPKD.

Management of LVH

Recommendations for management of LVH include rigorous BP control with use of a RAAS blocker as first-line therapy because ACEi and ARBs reverse the hypertrophic changes in the left ventricle (Table 1).[76] The current focus of treatment is based on BP reduction; however, it is not clear if this alters cardiac outcomes, particularly given that there is a subset of patients with LVH without hypertension.[92,93] In addition, in the large cohort HALT-PKD studies, the rate of serious cardiovascular events were unchanged despite a reduction in left ventricular mass index in the group with lower BP targets.[78] Further longitudinal studies with serial imaging are required to characterize the effects of treatment in patients with LVH and ADPKD.

It should be noted that LVH is a structural finding and does not accurately reflect cardiac function, particularly in those patients without other manifestations of CVD.[112] Therefore, the assessment of cardiac function should accompany investigations for LVH, including echocardiographic determination of ejection fraction and possible inclusion of newer methods to detect cardiac strain, such as 2-dimensional strain or global longitudinal strain.[112,113] Furthermore, there are studies reporting increased cardiac strain in patients with ADPKD (detected by increased left ventricular global longitudinal strain on echocardiography) with normal ejection fractions and preserved kidney function.[112,114] This subclinical sign of cardiac dysfunction is associated with decreased functional capacity (measured by 6-minute walk and timed up and go tests) and quality of life in CKD; however, further evaluation in ADPKD populations are required to determine its predictive long-term risk.[113,115]

Ischemic Cardiomyopathy

Ischemic heart disease is a major complication of CKD, but specific data in ADPKD is limited. Two large cohort studies, from China and Taiwan, reported a >2-fold higher incidence of myocardial infarction in patients with ADPKD, which was associated with increased severity of coronary disease and poorer cardiovascular outcomes compared with non-ADPKD controls.[116,117] In particular, Yang *et al.*[117] showed that those with ADPKD had a higher incidence of ST-elevation myocardial infarction (75% compared with 59%), sudden cardiac death (11.5% vs. 4.6%), need for coronary artery bypass grafting (7.7% vs. 5.4%) and higher overall mortality (13.5% vs. 6.2%). Most prior large ADPKD studies excluded patients with a history of ischemic heart disease and this may contribute to the relative paucity of data on ischemic cardiomyopathy in this group. However, clinical and preclinical studies show early vascular changes (as described above) lead to impairment of flow-mediated dilation and impaired coronary flow reserve in ADPKD.[118] The presence of hypertension and LVH contributes to these vascular changes leading to the premature development of arterial stiffness and cardiac dysfunction.[6,119] Increased levels of angiotensin II and ADMA also contribute to early atherosclerosis of coronary arteries.[50,119] Furthermore, in normotensive patients with ADPKD with normal kidney function, coronary artery velocity reserve was significantly decreased and carotid intima-media thickness was increased compared to non-ADPKD controls, suggesting changes to coronary vasculature that predispose to ischemia occur at an early stage.[119] Studies of human, mouse, and rat cardiac fibroblasts with loss of functional PC-1 demonstrate impaired myocardial healing from ischemic injury and alterations in scar architecture, increasing the risk of developing cardiomyopathy following myocardial infarction.[106] As with ischemic cardiomyopathy from other causes, the management principles in ADPKD include classic CVD risk factor modification, treatment with RAAS blockers, beta-blockers, aspirin, statin, and revascularization where possible.[83]

Other Cardiomyopathies

Other rare cardiomyopathies are reported in ADPKD, including idiopathic dilated cardiomyopathy, which is characterized by left ventricular dilatation and systolic dysfunction; and hypertrophic obstructive cardiomyopathy, which is characterized by asymmetrical LVH and diastolic dysfunction.[92] In a retrospective study of 667 patients with ADPKD who had echocardiographs, 58 had primary cardiomyopathy identified; idiopathic dilated cardiomyopathy was found in 5.8%, hypertrophic obstructive cardiomyopathy in 2.5%, and left ventricular noncompaction in 0.3%.[120] *PKD1* mutations were found in 100% ($n = 2$) of patients with noncompaction and those with *PKD2* mutations had twice the expected incidence of idiopathic dilated cardiomyopathy (36.8%, $n = 7$), suggesting that polycystin expression impacts the pathogenesis of these conditions.[120] Mutations in *PKD2* are linked with impaired intracellular calcium flux leading to decreased cardiac contractility, thin ventricular walls, and dilated cardiomyopathy; and (as described earlier in this review) mutations in *PKD1* can lead to cardiac hypertrophy and reduced myocyte fractional shortening, predisposing to the development of cardiomyopathies.[107,109,121]

Diastolic dysfunction is commonly reported in patients with ADPKD, usually in late disease stage with established kidney failure; however, it has also been reported in early disease prior to development of hypertension, LVH, and kidney impairment.[122–124] As with the LVH, polycystin-related dysfunction of cardiomyocytes and vascular cells (as described earlier in this review) are likely involved in the pathogenesis of diastolic dysfunction.[6] There is no specific ADPKD management guidelines for these cardiomyopathies and recommended therapy is described in Table 1.

Valvular Heart Disease

The patterns of cardiac valvular and structural heart disease vary as ADPKD progresses. In early disease, the most common valvular abnormality is MVP; and as disease progresses to kidney failure, mitral and aortic valve calcification and regurgitation are more common.[122] Identification of these conditions are important because their presence can lead to systolic and diastolic

dysfunction, heart failure, and (for MR in particular) arrhythmias.[125]

MVP is considered a classic extrarenal manifestation of ADPKD; however, recent reports of its prevalence vary between 0% and 26%.[125–131] In a study in 2001 (Lumiaho *et al.* [126]) of 109 patients with *PKD1* mutations, MVP was found in 26%, which was significantly higher than unaffected relatives (14%) and unrelated healthy controls (10%). In this study, MVP was present in greater portion in younger and normotensive groups with no association with gender or kidney function.[126] Hossack *et al.*[127] reported a similar prevalence of MVP (26%) in a study in 1988 in a cohort that predominantly had *PKD1* mutations detected by gene linkage. In a study in 1995 by Ivy *et al.*,[130] MVP was prevalent in 12% of children with ADPKD (compared to 3% non-ADPKD affected children from the same families) and its presence was associated with more severe renal phenotype (>10 cysts). However, a recent study in 2022 by Savis *et al.*[131] of 102 children and young adults with ADPKD, found 1 patient (0.98% prevalence) with MVP and 9 patients (8.8%) with changes that may represent early MVP changes or may be normal variation. Arjune *et al.*[95] found 6 out of 141 patients (4%) with MVP and another 2 studies in adults did not find any patients with MVP (Saggar-Malik *et al.*[128] and Miyamoto *et al.*[125]). Therefore, there is significant variability in the reported prevalence of MVP, and applicability of previous studies are limited by differences in genotype (greater portion of *PKD1* mutations), disease stage, presence of other comorbidities, changes in echocardiography technology, and diagnosis guidelines.[126,131,132] Further studies are needed to determine the true prevalence.

The presence of MVP in children, young adults, and normotensive patients suggests a connective tissue basis for its pathogenesis. Although the exact mechanism is still to be elucidated, likely mechanisms are: (i) polycystin-related ciliary dysfunction causing valvular cell growth abnormalities, altered valve geometry, myxomatous degeneration, and prolapse, or (ii) dysfunction of papillary muscle cell (which contain polycystin proteins) leading to abnormal valve biomechanics.[6,129,131]

MVP predisposes to MR because changes in the valve geometry can lead to separation of valve leaflets, particularly in the presence of increased left-sided preload as caused by hypertension and volume overload from kidney impairment and sodium retention.[129] In keeping with this, unlike MVP, the presence of other valvular abnormalities are associated with later disease stages when there is significant kidney dysfunction and progression of CVD.[133] Aortic valve calcification, mitral valve calcification, and MR were significantly more common findings in an ADPKD dialysis population (38%, 28%, and 8.6%, respectively) than MVP (4.3%).[133] Lumiaho *et al.*[126] also found a greater prevalence of grade 2 or 3 MR in *PKD1* patients (12.8% vs. 2.7%) and this was associated with age, systolic BP, and impaired kidney function, with no cases of MR in patients with normal kidney function. In a recent study of 65 patients with ADPKD of which approximately 30% had MR, Miyamoto *et al.*[125] found an increased prevalence of MR in those with *PKD1* mutations over *PKD2* or other ADPKD mutations (46.9% vs. 8.3% vs. 19.0%, respectively; $P = 0.02$); however, there were no other significant differences in prevalence of aortic regurgitation, mitral stenosis, or aortic stenosis between genotypes.[125] Arjune *et al.*[95] found a higher prevalence of 63% of MR in a study of 141 patients with ADPKD with CKD stages 1-4, although the authors have stipulated that most of these cases were of mild MR. Differences in diagnostic criteria may contribute to variability in the reported prevalence and uniform reporting guidelines in ADPKD would be beneficial in future research to evaluate the extent of these cardiac valvular manifestations.[134]

Current guidelines suggest echocardiographic evaluation of any cardiac murmur in ADPKD. Management of valvular abnormalities is based on the severity of patients' overall condition and follows the principals of heart failure management (Table 1).[84] There are no specific recommendations for ADPKD valvular disease.

VASCULAR MANIFESTATIONS

Intracranial Aneurysms

Epidemiology and Risk Factors for ICAs

Compared to the general population, ICAs are found in higher prevalence in the ADPKD population (9%–12% vs. 2%–3%) and present at a younger age (~40 years old vs. 51 years old).[85,86,135,136] Rupture of an ICA and resulting subarachnoid hemorrhage is catastrophic, and leads to death (in 30%–60%) or significant neurological deficits.[136–138] ICAs in patients with ADPKD are usually small (<5 mm in diameter) and found in the anterior circulation, with the exception of one large Finnish registry study where most ICAs were found in the middle cerebral artery, similar to the general population in Finland.[136,137,139,140]

The major known risk factor for the presence of ICAs in ADPKD is a positive personal or family history; and in this group, the prevalence of ICAs is reported as high as 22%.[135] The classic risk factors for ICAs such as the presence of hypertension, smoking and older age, also apply to the ADPKD population.[86] Furthermore, in studies from French and Japanese ADPKD cohorts,

females were significantly more likely to develop aneurysms than males.[136,139] In 2 Japanese studies, there was an association with more severe disease (reduced kidney function and larger kidney size) and the presence of ICAs.[141,142] Patients with *PKD1* mutations had a >2-fold increase in the risk of both ruptured and unruptured ICAs compared to those with *PKD2* mutations, with no significant influence from the type or location of the mutation.[136] These results from November 2022 are in contrast to with a previous case series from early 2003 that suggested that mutations in the 5′ region of *PKD1* correlated with increased risk of ICAs and rupture.[143]

Pathogenesis of ICAs

The increased incidence of ICAs in patients with ADPKD is due to inherited dysfunction of vascular endothelial and smooth muscle cells and is compounded by hypertension and CVD. Impaired ciliary function results in the vessels' inability to sense and react to fluid shear stress and loss of myogenic tone, leading to increased arterial wall stress, loss of structural integrity, sac formation, and expansion into an aneurysm (Figure 1).[85,123,136,144]

Management of ICAs

Given the high prevalence of ICAs in the ADPKD population, the decision to screen presymptomatic patients should be made in conjunction with the patient, taking into consideration their medical history and the potential for anxiety.[85,86,145] Screening is recommended for patients with family or personal history of ICAs.[85] Management should be decided in consultation with neurosurgery (Table 1).[85]

Other Vascular Malformations

The vessel wall abnormalities that lead to ICAs also predispose to the development of other vascular abnormalities. There have been case reports of dissections and aneurysms in most major vessels including aortic, coronary, carotid, cervicocephalic, and vertebral arteries.[132,144] Abdominal aortic aneurysms are reported as high as 5% to 10% in patients with ADPKD (vs. 2%–4% in the general population) with case reports of rupture resulting in devastating consequences.[138,146] Risk factors of hypertension, kidney dysfunction, age, and smoking predispose to abdominal aortic aneurysms formation and there are no specific recommendations for patients with ADPKD above the general guidelines (Table 1).[138] Dolichoectasias, which is the elongation and dilation of an arterial segment that can predispose to dissection or rupture, has been reported with increased incidence in a study of 178 patients with ADPKD (2%–2.5% in ADPKD vs. 0.06% in the general population).[147] Although these vascular malformations are a known feature of ADPKD, there have been no large registry, longitudinal, or systematic studies to determine the true burden of disease or provide specific screening guidelines. Expert opinion suggests that it is not unreasonable to extensively investigate patients with a strong personal or family history of vascular complications.[144] Management is specific to the vascular characteristics and organ involved, and treatment decisions should be made in conjunction with surgical or interventional teams.

CONCLUSIONS AND FUTURE DIRECTIONS

Longitudinal population studies in patients with ADPKD over the last decade have documented reduced rates of hypertension, LVH, and progression to kidney failure due to earlier diagnosis, engagement with healthcare professionals, and more rigorous CVD risk management.[17,148,149] Despite this, CVD remains the main cause of mortality, and patients with ADPKD experience more severe cardiovascular events than the general population and have risk factors that are not optimally controlled.[117,138,148] The pathogenesis of CVD is complex and evolves with progression of ADPKD during life. The clinical landscape of ADPKD therapies has changed with the introduction and increasing use of the vasopressin-2 receptor antagonist, tolvaptan, which is the first disease-modifying therapy that attenuates kidney cyst growth.[150] In addition, there are promising phase 3 trials with repurposed drugs (e.g., cystic fibrosis transmembrane conductance regulator modulator GLPG2737 [NCT04578548], and metformin [NCT04939935]) and evolving novel therapies (such as micro RNA inhibitors RGLS8429, which is currently undergoing a phase 1b trial) targeted at reducing cyst burden and maintaining kidney function.[151,152] It will be important to determine the impact of therapies that reduce cyst burden on CVD risk and outcomes, especially at different disease stages. The effect of statins to reduce oxidative stress and improve endothelial function have also been investigated in ADPKD.[153] Statin trials so far have had contradictory results, with a 3-year randomized controlled trial showing reduction in total kidney volume in children and young adults; however, other studies such as secondary analysis of the HALT-PKD study showed no effect. Nevertheless, a current trial (NCT03273413) is underway and further test its effects.[153–155]

Other research strategies in CVD and ADPKD should include further elucidation of the factors leading to endothelial dysfunction, particularly because much of

the research into some pathways, such as the role of ADMA, was published over a decade ago. In addition, because ADPKD-specific CVD can precede clinical kidney disease, development of therapies targeted at polycystin-related cardiovascular pathways are also required. Recently, dopamine receptor agonism was demonstrated to correct the reduction of Ca^{2+} influx, NO release, and flow-mediated vasodilation that occurs in ADPKD vascular endothelial cells and further research is required to determine its potential as a therapeutic target.[58,156] Other novel therapies could include approaches to attenuating early endothelial dysfunction by acting on the ADMA pathway, reducing SNS overactivity, and improving cardiomyocyte function.

Ongoing long-term longitudinal studies are required to determine the impact of any intervention on attenuating CVD in patients with ADPKD. To facilitate this, standardized outcome measures in ADPKD trials would be beneficial. Recent comprehensive reviews of the clinical ADPKD literature found significant variability study reporting, particularly in composite outcomes.[157,158] The 2020 international Delphi survey identified potential core outcome domains taking into account patient or care-giver and healthcare professionals' priorities, which may be considered in developing standardized outcomes.[159] Current therapeutic guidelines are appropriate and are based on general CVD and CKD recommendations; however, we await the release of the upcoming new version of the Kidney Disease: improving Global Outcomes ADPKD guidelines and determine the impact of recent trials on management recommendations.[157]

DISCLOSURE

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1.3 Considerations for therapeutic interventions for CVD in ADPKD

As described in the preceding section, the current goal of management of CVD in ADPKD focuses on the treatment of hypertension (with RAAS blockers and other agents when required) and lifestyle modifications to reduce cardiovascular risk factors. Large studies in general and chronic kidney disease (CKD) cardiovascular risk factor modification have shown that lifestyle interventions such as dietary changes to incorporate restriction of sodium, reduction of highly-saturated fat intake, and maintaining a healthy weight range through exercise, are effective and should be used in conjunction with other therapies (anti-hypertensives, lipid-lowering therapy and chronic pain management if required), and thus similar lifestyle modifications are recommended in ADPKD. (24-26)

When considering the development of new therapeutic interventions, patient and caregiver considerations are vital. There have been two major ADPKD patient/carer/physician surveys and feedback forums recently. (27, 28) These identified that the key research priority in ADPKD were treatments that can slow or halt progression of disease, including the associated cardiovascular complications, and within that, a key focus of future research should include the impact of lifestyle modifications on disease progression. (27, 28) Patients, healthcare professionals and stakeholders highlighted the importance of avoiding interventions that are onerous or difficult to comply with long-term, given the potential to intervene in childhood and the chronic nature of the disease. (28) Patients and caregivers have also identified that psychological stress related to the diagnosis and symptoms from multi-system disease are prominent features of ADPKD and thus the condition requires interventions that are holistic and focus on optimising quality of life. (28)

Another consideration in developing therapeutic interventions in ADPKD is prioritising testing of drug therapies already found to be efficacious and safe in other clinical conditions

that share similarities with ADPKD. An article by Panchapakesan *et al.* highlighted the importance of drug repurposing in kidney disease, to enable rapid access of potential disease-modifying therapy. (29) As described in the preceding sections, there are elements to the pathogenesis of ADPKD that are shared with other forms of CKD such as RAAS activation, accumulation of pathogenic and vasoactive metabolites such as ADMA due to kidney impairment, increased oxidative stress from increased kidney inflammation and fibrosis. If found to be effective, use of therapies already approved for other indications can significantly reduce the time and resources required to make the intervention accessible for ADPKD patients.

Therefore, ideal interventions from a patient perspective are those that can attenuate CVD and its consequences urgently but do not diminish quality of life or have intrusive side effects. A potential avenue for discovering an effective therapy is repurposing interventions already available in other diseases with shared pathogenesis. Furthermore, there is an interest in lifestyle modification in ADPKD, as patients and their families can be aware of the diagnosis at a young age and potentially can to intervene early with a lifelong therapy. These factors were considered when developing the hypotheses in this thesis, along with consideration of the specific pathological pathways that could be targeted in ADPKD.

1.4 The role of vasopressin in mediating CVD in ADPKD

As described, vasopressin is a key mediator of cyst growth and disease progression in ADPKD. Its role and actions in progression of CVD are considered below.

1.4.1. Physiological actions of vasopressin

Vasopressin, also known as antidiuretic hormone (ADH) or arginine vasopressin (AVP), is a neuro-hypophyseal hormone released to maintain plasma osmolality within the physiological range. (30) When there are increases in plasma osmolality (primarily driven by relative increases in sodium chloride compared to free water) or decreases in effective blood volume, osmoreceptors neurons in the brain (specifically within the circumventricular organum vasculosum of the lamina terminalis and in the anterior wall of the third ventricle) sense changes in the osmolality, and stimulate the sensation of thirst (with the goal to increase free water consumption) and release of vasopressin from magnocellular neurons in the hypophysis along the pituitary axis and into the bloodstream. (30-32) In the kidney, vasopressin binds to vasopressin-2 receptors on the basolateral membrane of the distal convoluted tubule and collecting duct stimulating the expression of aquaporin channels on the apical membrane to increase water permeability and increase plasma volume. (32, 33) It also has some action on stimulation of sodium reabsorption *via* increased renal epithelial sodium channel expression which increases net water permeability but at the cost of reduced sodium excretion. (34) Vasopressin also stimulates increased facilitated urea transporter-A1 activity to increase concentration of urea in the medulla to improve counter-current concentration capacity of the tubules and increase water resorption. (34)

While the strongest stimulus for vasopressin release is change in plasma osmolality, changes in arterial stretch also impact vasopressin release. Baroreceptors in the aortic arch and carotid sinus tonically inhibit secretion of vasopressin *via* cranial nerves V and IX to the pituitary. (30) When there are changes in arterial stretch caused by arterial underfilling, which occurs in states such as volume depletion, cardiac failure, or systemic vasodilation, this tonic inhibition is stopped and vasopressin is released to increase plasma volume by preventing renal water loss. (30) Vasopressin also has a strong vasoconstrictor effect in muscle,

mesenteric/splanchnic and skin beds which results in shunting of blood volume to other vital circulatory systems, an important response in dehydration states. (31)

Angiotensin II and noradrenaline have also been suggested as direct stimulants for vasopressin release. (35)

1.4.2. Copeptin as a surrogate marker for vasopressin

Physiological levels of plasma vasopressin range from being undetectable to 3 pg/ml and small changes in vasopressin release impact urinary water/solute excretion and plasma osmolality. (30) Vasopressin is unstable in plasma and has a relatively short half-life of 16-20 minutes which makes it difficult to measure in research settings. (36) Vasopressin is produced as a pro-hormone and cleaved in the pituitary stalk into three peptides when released: functional vasopressin, neurophysin and copeptin. (30, 35) Copeptin is produced in a 1:1 ratio with vasopressin and is a more stable and easier to measure. (36) Copeptin has a good correlation with vasopressin and hence is used in preclinical and clinical studies as a measure of vasopressin levels. (35, 36)

1.4.3. Role of vasopressin in ADPKD disease progression

A hallmark discovery in ADPKD was the finding that vasopressin was a key mediator of cystogenesis and cyst growth. (16) In ADPKD-affected renal tubular epithelium, vasopressin-2 receptor stimulation leads to dysregulated calcium-mediated intra-cellular signalling pathways and upregulation of cAMP signalling resulting in uncontrolled cell proliferation, cyst formation and chloride-driven fluid secretion into cystic structures. (16, 37) In rats with PKD, concurrent congenital vasopressin deficiency resulted in almost complete lack of

cystogenesis by interrupting this pathogenic pathway, highlighting vasopressin as a key driver of cyst growth. (16)

Vasopressin levels are increased in patients with ADPKD and this has been associated with increased severity of renal and cardiovascular disease. (34, 38) In the Consortium for Radiologic Imaging Studies of Polycystic Kidney Disease (CRISP) study, which had a cohort of 102 ADPKD patients, baseline copeptin levels (a surrogate marker for vasopressin) were independently associated with ADPKD disease progression in patients with early disease. (38) Specifically, higher copeptin levels were associated with poorer kidney function, lower effective renal blood flow, larger kidney volume, and increased albuminuria. (38) Furthermore, as cystic disease progresses and kidney architecture becomes more disrupted, renal concentrating ability is impaired, leading to increased risk of perturbations to plasma osmolality, potentially driving volume loss and increasing stimulus for vasopressin release, contributing to ongoing disease progression. (30, 39) Vasopressin also likely plays a role in increasing blood pressure and disease progression through latent vasopressin-1a receptor stimulation in vascular cells leading to vasoconstriction. (40)

Suppression of vasopressin action is a potential therapeutic intervention to reduce the cAMP growth signalling and slow the progression of ADPKD. This has been explored through two mechanisms – vasopressin receptor antagonism and increasing water intake to suppress vasopressin release.

1.4.4. Role of vasopressin in cardiovascular disease

As described earlier, vasopressin release is partially mediated by changes in arterial stretch, and stimulation of vasopressin-1a receptors leads to arterial vasoconstriction. (30, 35) Therefore, there has been interest in vasopressin (and its surrogate measure, copeptin) as a

novel functional marker of cardiovascular states and hemodynamic changes. (35) Vasopressin release has also been linked to acute stress responses. (35)

An increase in vasopressin levels has been demonstrated in heart failure, particular in those who have recently suffered an acute hemodynamic event such as an acute myocardial infarction (MI). (35, 41) The Optimal Therapy in Myocardial Infarction with the Angiotensin II Antagonist Losartan (OPTIMAAL) study (consisted of 224 patients) showed copeptin to be a more sensitive marker for morbidity and mortality with heart failure than B-type natriuretic peptide (BNP) and N-terminal pro-BNP (markers commonly used in clinical settings) in patients who acutely suffered a MI. (41) Similarly, another study of 274 patients found that increased levels of copeptin correlated with left ventricular dysfunction in both early and late (median follow up 155 days) follow up after a MI. (42) Furthermore, in patients with established heart failure, copeptin levels correlated with severity of disease (as measured by New York Heart Association classification of heart failure). (43) Treatment of these patients and improvement in their cardiac index was correlated with lower levels of copeptin. (43)

In rodent models, vasopressin has been shown to stimulate cardiac fibroblasts to induce myocardial fibrosis and increase cardiac hypertrophy, and sustained increased levels may contribute to cardiac remodelling and increased risk of arrhythmias. (44-46)

Copeptin levels were also increased in a study of 362 patients with acute ischaemic stroke and correlated with severity of National Institutes of Health Stroke Scale scores. (35, 47) Copeptin independently predicted mortality and functional status post-stroke suggesting its importance as a hemodynamic marker. (47)

Given the increased levels of vasopressin in heart failure, there have been multiple clinical trials trialling the use of vasopressin-2 receptor antagonist, Tolvaptan, to treat congestive heart failure by blocking the action of vasopressin in the kidney and aiding free water

removal. (48) The first large-scale randomised controlled trial (RCT) was the Effects of Oral Tolvaptan in Patients Hospitalized for Worsening Heart Failure (EVEREST) trial of 4133 patients hospitalised with heart failure, which did not show a benefit to mortality or heart failure hospitalisations. (48) There was improvement in dyspnoea and weight loss/oedema, which has been replicated in some subsequent trials, however it did not lead to any change in long or short term cardiovascular outcomes. (49-53) Recent studies in heart failure have also shown (inconsistently) that Tolvaptan use may worse kidney impairment, although this appears to occur at higher doses. (50, 54) Therefore, though there is a detectable benefit in free water removal in some studies, vasopressin antagonism is not routinely used in the treatment of congestive heart failure due to the lack of effect on clinical outcomes and potential risk of kidney injury, except in the circumstances of concurrent severe hyponatraemia. (49, 53, 55)

1.4.5. Vasopressin reception antagonism in ADPKD

There have been a number of preclinical studies and large RCTs performed to test the efficacy of vasopressin-2 antagonists in ADPKD that have concluded that its use is successful in slowing disease in individuals with severe and progressive kidney disease. (56) Since then, the vasopressin-2 antagonist Tolvaptan has been approved for use in the specific clinical setting of rapidly progressive ADPKD, where it is effective in slowing trajectory of kidney failure and reducing cyst growth, kidney pain and urinary tract infections. (57) Furthermore, post-hoc analyses of Tolvaptan in the large RCT TEMPO 3:4, showed a modest reduction in blood pressure over three years (-3/-1.4 mmHg *vs* +1/+0.2 mmHg). (58) There is no other long-term clinical trial data on the impact of vasopressin-2 antagonists on attenuating other manifestations of CVD associated with ADPKD.

It is important to note that only a subset of patients are eligible for this treatment. In Australia, eligibility is determined by rate of decline of estimated glomerular filtration rate (eGFR) of greater than or equal to 5 mL/min/1.73 m^2 within one year, or an average decline in eGFR of greater than or equal to 2.5 mL/min/1.73 m^2 per year over a five-year period, and treatment must be initiated before eGFR falls below 30 ml/min/1.73m^2. These relatively stringent criteria render most patients ineligible. (57) Additionally, treatment can have adverse effects of intolerable polyuria (which occurred in 10% of patients commenced on vasopressin-2 antagonist Tolvaptan in clinical trials) and liver derangement (occurred in ~5% of patients in clinical trials) leading to its cessation. (57, 59) Therefore, despite its efficacy, there is an ongoing need for a therapeutic option that can be used more widely and with fewer adverse effects.

1.4.6. Water intake and suppression in ADPKD

As increases in water intake can decrease plasma osmolality and thus remove the stimulus for neuro-hypophyseal vasopressin release, increasing water intake has been proposed as a safer and more accessible alternative to lower vasopressin levels and potentially progression of ADPKD. (30) In healthy volunteers, increasing water intake also enhances urinary sodium excretion and this natriuresis may have beneficial effects towards blood pressure reduction, particularly as previous studies have shown that high sodium intake may contribute to more severe disease in ADPKD. (60, 61)

Two preclinical studies have been performed to test the effect of increased water intake on reducing vasopressin-stimulated cyst growth in experimental polycystic kidney disease using the *pck* rat model and the *Pkd1*$^{RC/RC}$ mouse model. (62, 63) Studies by Nagao *et al.* in the *pck* rat demonstrated significant reduction in renal expression of vasopressin-2 receptors, reduced

activation of pathogenic growth factor pathways (B-Raf/MEK/ERK pathways), 28% reduction in kidney/body weight ratio (a reliable surrogate marker of cyst growth) and improved kidney function. (62) Hopp *et al*. demonstrated similar beneficial effects in the *pck* rat with reduction in urinary vasopressin, renal cAMP levels, lower kidney weights, cystic and fibrotic indexes on kidney tissue and plasma urea levels. (63) These effects were not replicated in the $Pkd1^{Rc/RC}$ mouse potentially due to differences in vasopressin response to changes in plasma osmolality between animal models and variable manifestation of PKD in the mouse model with a slower progression of renal disease contributing to lack of detectable effect. (63)

In both studies, water intake was increased using 5% glucose added to drinking water which enhances thirst without significant metabolic effects and promotes increased net consumption of water. (62, 63) Hopp *et al*. also used 1% hydrated agar to increase water intake to test other forms of increases water intake, and this had a similar effect in the *pck* rat to 5% glucose. (63)

The first RCT (PREVENT-ADPKD) testing the effect of prescribed increased water intake in patients was published in 2021. (64) Prior to this, clinical evidence for increased water intake was inconclusive and not incorporated into clinical practice guidelines as the only trials were small non-randomised observation trial (n=30) and two short uncontrolled trials, which did not reach significant endpoints. (65-67) The results of PREVENT-ADPKD showed that prescribed high water intake did not significantly slow the rate of kidney growth over three years compared to ad-libitum water intake, however the control participant group had ~1.5L higher water intake than average population, potentially impacting results. (64) Therefore, the recommendations from these results were that water intake in ADPKD should be directed by thirst and dehydration should be avoided. Hypertension was included in the composite

secondary outcome measure and was unchanged by increased water intake. (64) Other manifestations of CVD in ADPKD were not explored in this RCT.

1.5 Role of Vitamin D in CVD in ADPKD

As discussed earlier, there is a reduction in functional polycystin proteins in ADPKD that leads to disruption of calcium-mediated signalling pathways in renal epithelial cells that allows for increase in cAMP and activation of the MEK/ERK cell proliferation pathway, promoting uncontrolled cyst growth and RAAS activation. (68) Vitamin D is a key calcium mediator and has been considered as a therapeutic intervention for the reduction of CVD in ADPKD by targeting these dysregulated calcium signalling pathways.

1.5.1 Actions of Vitamin D

Vitamin D is secosteroid prohormone that has pleiotropic intra- and extra-cellular effects. (69) It is best known for its calcium-related actions on bone health, however it has widespread action in many tissues, including renal epithelial cells. Important other actions of vitamin D include (i) its role as an intranuclear regulator of cellular differentiation and control of proliferation, (ii) immunomodulatory actions to reduce inflammation and fibrosis, (iii) suppression of renin gene expression and thus RAAS activation, (iv) reduction of blood pressure and cardiovascular risk, and (v) protective effect on podocytes in the glomerulus leading to reduction in proteinuria. (29, 69-73) These features make it an important potential pathological contributor to CKD and CVD.

Vitamin D is an intranuclear modulator of renin gene expression, which it achieves by binding to the renin gene promoter region and supressing renin expression. (74) In vitamin-D

receptor null mice, renin gene expression and plasma angiotensin II were increased, and subsequently the mice developed hypertension and cardiac hypertrophy. (75) Similar responses were found in wild-type mice when vitamin D activation was inhibited, and conversely, RAAS activation decreased when treated with vitamin D. (75) In healthy humans, low vitamin D levels are correlated with high RAAS activity and blunted renal plasma flow to angiotensin II (an inverse measure of RAAS activity). (76)

Vitamin D also has an role in immune system function and the vitamin D receptor is expressed in a wide range of immune cells including macrophages, neutrophils, dendritic cells, and B and T lymphocytes. (77) Its role in each of these cell types is variable. The phagocytic action of innate immune cells are modulated by vitamin D, which has downstream effects on reducing autoimmunity and inflammation. (77, 78) Similarly, macrophages and dendritic cells have the ability to convert vitamin D to its active form and suppress their own maturation and survival, an important balance to prevent overexpansion. (77, 79) Furthermore, in active inflammation, vitamin D receptor activation supresses dendritic cell migration and antigen presentation. (77, 80, 81) However, the impact of vitamin D activation is stimulus-specific, as vitamin D enhances the activity of macrophages and production of antimicrobial peptides in response to bacterial and viral pathogens. (82) Within the adaptive immune system, vitamin D has actions to reduce the production of pro-inflammatory cytokines such as IL-12, IFN-γ, IL-6, IL-8 TNF-α, and IL-9, and upregulates the production of anti-inflammatory cytokines such as IL-4, IL-5, and IL-10. (82) Overall, actions of vitamin D appear to induce an anti-inflammatory and tolerant state. (77, 82)

1.5.2 Vitamin D and its role in chronic kidney disease

"Active" Vitamin D is formed by the hydroxylation of 25, hydroxyvitamin D to 1,25 dihydroxyvitamin D (also known as calcitriol) primarily in the proximal tubule in the kidney, and its conversion is reduced in CKD leading to vitamin D deficiency. (69)

In large population studies (NHANES III), vitamin D deficiency was associated with hypertension. (69) Vitamin D deficiency contributes to progression of CKD and CVD by aggravating vascular damage through the loss of vitamin D suppression of nuclear factor-κβ, a profibrotic growth factor, and through loss of suppression of renin gene stimulation in ischaemic kidney injury states. (29, 83) In ADPKD, vitamin D deficiency may contribute to increased renin gene expression, leading to increased RAAS activity driving hypertension and LVH. Furthermore, increased angiotensin II may contribute to increased cyst proliferation as it stimulates renal epithelial cell growth, leading to progression of kidney disease in a vicious cycle. (70, 84) In CKD and vitamin D deficiency, there is also a loss of protective effect on renal glomerular podocytes in inflammatory states from vitamin D induced changes to macrophage activity (as described earlier), leading to increased proteinuria. (73, 78)

In preclinical and clinical studies in CKD, vitamin D deficiency can be treated with vitamin D receptor agonists (VDRAs) where their use is associated with reduced risk of cardiovascular death, improved LVH, reduced inflammation measured by IL-6 and IL-18 and reduced fibrosis measured by reduced TGF- β and apoptosis. (85, 86) However, its use (especially in high doses and in kidney impairment) is cautioned by the risk of hypercalcemia and potential to worsen vascular calcification by increasing serum calcium levels. (85, 86)

1.5.3. Use of vitamin D receptor agonists in ADPKD

The effect of VDRAs in ADPKD, including its long and short-term effects on attenuating kidney and cardiovascular disease, has not been explored. If shown to be effective, a potential benefit of utilising VDRAs in ADPKD is that it would be repurposing a drug that is already available and tested in the CKD, allowing for rapid accessibility to patients to address this vital clinical need. (29) An important consideration in testing VDRAs is that vitamin D activation may have different effects in PKD compared with other forms of CKD. In a preclinical study by Rangan *et al.*, induced vitamin D deficiency worsened proteinuria and hypertension in LPK rats but also had a mild inhibitory effect on cyst growth, suggesting that vitamin D activation may improve CVD and inflammation/fibrosis, but also may potentially contribute to cystic enlargement. (70) Conversely, a small observational study of 72 ADPKD patients found an association with vitamin D deficiency and more severe kidney disease, but this was not independently predictive of kidney volume. (87) Therefore, the impact of VDRA therapy on cardiovascular and kidney disease in ADPKD is not known.

1.6 Role of nitric oxide in endothelial dysfunction and CVD in ADPKD

1.6.1 Reduced nitric oxide activity in ADPKD

Apart from RAAS activation, another important pathophysiological mechanism in the development of hypertension and CVD in ADPKD is the early development of endothelial dysfunction. (88) As with cystic disease, the function of primary cilia and polycystin proteins play an important role in the development of endothelial dysfunction. As described previously, the endothelium is necessary for healthy vascular functioning and has a major role in maintaining homeostasis of vasoactive molecules including NO, ET-1, ADMA and vascular endothelial growth factors. (89) Functional balance of these mediator molecules by

the endothelial cell are vital to prevent vessel injury, arterial stiffness and the development of atherosclerosis. (89)

Primary cilia are an essential component of the mechanosensory function in ciliated vascular endothelial cells, where they act as fluid shear stress sensors that detect minute changes in blood pressure. (14, 15) Changes in flow result in activation of intracellular calcium cascade signalling within the endothelial cell to autoregulate vascular dilation. (14, 15) This vasodilatory action occurs primarily through the tonic release of NO. (14, 15, 89) This flow-mediated vasodilation is impaired in ADPKD due to loss of functional polycystin-1/-2 resulting in depleted calcium signalling, decreased activation of NO-mediated relaxation and sustained vasoconstriction. (14, 15)

In keeping with this, Zhang *et al.* detected >70% decrease in NO metabolites in human ADPKD cell lines, and other clinical studies have detected similar significant reductions in NO levels or nitric oxide synthase (NOS) activity. (20, 90-92) Calcium-mediated NO release and vasodilation are also influenced by ADMA, a potent NO inhibitor which is increased in ADPKD. (8, 21) Increased ADMA levels are also an independent marker of increased risk of atherosclerotic disease and worse cardiovascular disease outcomes. (8) High ADMA levels are detected in patients with ADPKD and this is thought to be due to reduced renal clearance of ADMA molecules as well as increased generation in the setting of inflammation and oxidative stress induced by kidney cysts and surrounding fibrosed parenchyma. (8, 21)

The classical pathway for NO production in vascular endothelial cells is by conversion of *L-arginine* to *L-citrulline* by NOS. (20) Clinical studies have shown a reduction in functional NO production in response to heat stimulus and when treated with NO donors, which demonstrates an intrinsic deficiency of NO production *via* the classical endothelial pathway. (92, 93) This was confirmed in a series of studies by Wang *et al.* which demonstrated a

reduction in NO metabolites and NO-mediated dilation in response to acetylcholine. (20, 91, 93) Importantly, this effect could not be restored by NO substrate L-arginine or inhibited by NOS inhibitor (NG-nitro-L-arginine methyl ester) confirming a loss of activity in the classical NO pathway. (20, 91, 93) Despite this, ADPKD vessels responded to SIN-1, an exogenous NO donor that does not require conversion with NOS, confirming that the endothelium was able to respond to NO normally but unable to generate NO *via* the classical pathway. (20, 93) Similarly, in another clinical study, Lorthioir *et al.* showed that dopamine infusion was successful at restoring Ca^{2+} influx into polycystin-deficient cells and NO release by stimulating endothelial dopamine receptors (an alternative pathway to NO production); but due to the short-acting nature of dopamine infusions this is not a viable chronic therapeutic option. (92)

1.6.2. Dietary nitrate supplementation and effect on blood pressure

A potential pathway to increase NO delivery to vessels is *via* dietary/inorganic nitrate supplementation through the entero-salivary *nitrate-nitrite-NO* pathway, which uses alternative substrates and enzymes to the classic *L-arginine* pathway. (94) In this pathway, nitrous-converting bacteria produce nitrate reductases which is secreted in saliva and acts on consumed nitrate in food to directly convert nitrate to nitrite and then NO in the acidic environment of the stomach and duodenum. (89, 94, 95) It is proposed that then NO directly acts on the endothelium to stimulate vasodilation, improve endothelial function and lower blood pressure. (89, 94)

Dietary nitrates are found in high concentration in beetroots and leafy vegetables such as rocket, spinach, celery and lettuce with up to 250mg/100g of nitrate (compared with low nitrate foods like eggplants, mushrooms and garlic which contain less than 20mg/100g). (96)

Consumption of these foods are thought to contribute to the anti-hypertensive effect of the Dietary Approach to Stop Hypertension Diet (DASH diet) which is part of first-line therapy in hypertension management guidelines and has been shown to lower systolic blood pressure by ~5mmHg. (26, 96)

Therefore, multiple clinical studies have been conducted to test the impact of high nitrate-containing foods or supplements on blood pressure, endothelial function and extensions of these functions such as improved exercise endurance, muscle contractility and cognitive function. (97-99) These studies have shown that dietary nitrate supplementation lowers blood pressure and improves endothelial function in healthy volunteers, CKD and hypertensive participants from other causes. (89, 94, 100, 101) Other studies show improvements in blood pressure, vascular function and endurance, but inconsistent results on possible "downstream" effects of cognitive function and exercise oxygen utilisation. (97-99) With regards to hypertensive populations, there have been multiple clinical trials on the effect of dietary nitrate supplementation which have shown significant reduction of blood pressure. (95, 100, 102, 103) The largest RCT by Kapil *et al.* showed a 7mmHg reduction in systolic BP after 4 weeks of daily nitrate supplementation. (95) With other disease states such as CKD, hypercholesterolemia, COPD, obesity, current active smoking, and heart failure, the effect of NO supplementation has been variable. (95, 104-111) Blood pressure was lowered significantly by 4-12mmHg in CKD, obesity and heart failure, but no effect was observed in current smokers, patients with COPD or hypercholesterolemia suggesting that nitrate supplementation may not be able to counteract certain vascular abnormalities. (95, 104-112)

There was one preclinical study of sodium nitrate supplementation in experimental polycystic kidney disease (the $Pkd1^{Rc/RC}$ mouse model) which examined its effect on cyst growth and did not find a benefit. (90) This mouse model does not develop hypertension therefore the impacts of NO on endothelial function were not explored. (90) There have been no clinical

studies of nitrate supplementation in ADPKD. Despite the benefit seen in hypertensive and CKD participants, given the intrinsic dysfunction of NO production in ADPKD vasculature and inhibitors of vascular health that arise from worsening kidney disease (such as increased ADMA, oxidative stress and RAAS activation), it is unclear if nitrate supplementation will result in a blood pressure reduction in ADPKD.

1.6.4. Beetroot juice as a form of nitrate supplementation

The most common form of nitrate supplementation in the previous studies was with daily dosing of concentrated beetroot juice, which is commercially available. (94, 100, 101, 112) Previous clinical trials reported excellent compliance with BRJ (>90%) and the only side effects reported were non-harmful beet-coloured urine and faeces. (94, 95, 112) Being a food product, beetroot juice (BRJ) is an attractive therapeutic option as it is accessible, relatively easy to incorporate as a lifestyle modification and likely to have limited side effects which (as previously described) is a therapeutic preference highlighted by the ADPKD community. (27, 94) Some concerns have been raised in the past about excessive inorganic nitrates in polluted drinking water and preserved red meats increasing the risk of gastric cancer and methemoglobinemia, however these concerns have been alleviated for dietary nitrate derived from fruit and vegetable sources following a comprehensive WHO report and US National Toxicology Report. (95, 113, 114) The WHO report stated that increased vegetable intake associated with dietary nitrate supplementation is probably beneficial against cancer. (113)

1.7 Other potential therapeutic targets for CVD in ADPKD

There are several other therapeutics targeted towards reducing cardiorenal disease progression in ADPKD in various stages of development. These include interventions

towards reducing sympathetic nervous system activation, cystic growth factor modulation, directly increasing polycystin-1 expression, and repurposing drugs such as sodium-glucose cotransporter-2 (SGLT-2) inhibitors, non-steroidal MRAs and combination endothelin receptor antagonist/angiotensin receptor blockers which improve CVD outcomes in other forms of CKD.

1.7.1 Reducing sympathetic nervous system activity

Excessive SNS activity is a contributor to systemic elevated blood pressure (particularly through renal arterial sympathetic nerve activation), arterial remodeling, LVH (both in the presence of and independently of hypertension), arrhythmias, and heart failure. (9, 115) Disease progression in ADPKD triggers SNS activity in multiple ways. Firstly, as cysts growth and kidneys enlarge, there is stretching of the renal capsule and activation of sympathetic nerves that line the capsule. (116) Secondly, cyst growth leads to obstruction and ischaemia of renal parenchyma, and this injured tissue releases a number of neuromodulatory molecules, such as adenosine, bradykinin, neurokinin A, calcitonin gene-related peptide, substance P, and prostaglandins, that activate the SNS. (9) Thirdly, angiotensin II, which as described above is overactive in ADPKD, is a stimulator of the SNS and has been shown to facilitate noradrenaline secretion from adrenergic nerves and amplify adrenergic response to stimuli. (115, 117) Increased SNS activity (measured by quantitative histology of nerve density from ADPKD nephrectomy kidneys and increased muscle sympathetic nerve activity) has been documented in ADPKD patients regardless of impairment of kidney function, and is higher in ADPKD than in patients with matched kidney impairment with non-cystic causes of disease, those with essential hypertension, and in healthy controls. (9, 118, 119) It has also been implicated as a cause of difficult to control hypertension in ADPKD dialysis patients, as

the stimulus from large kidneys is maintained despite loss of kidney function. (9) Given the close link between RAAS activity and the SNS, the most common intervention for hypertension caused by SNS-overactivity is RAAS blockade with ACEi or angiotensin receptor blockers (ARBs). There are limited data on other interventions to reduce SNS activation. Renal nerve denervation has been examined as a possible therapy, but preclinical studies demonstrated a mixed response (with a reduction of blood pressure in the Han:SPRD-Cy/+ rat model but no change in the LPK model) and there are only a few case reports of successful reduction of blood pressure in ADPKD patients with difficult to control hypertension. (119-122) Large prospective studies are required to establish the benefits of renal denervation as a therapeutic option to manage hypertension in ADPKD.

1.7.2 Endothelin-1 Receptor Antagonism

ET-1 is an important mediator of intraglomerular and systemic blood flow which works primarily by regulating NO. (10, 123) It is produced by endothelial cells and its action is variable and site-specific. In the renal cortex it acts as a vasoconstrictor but in the medulla is a vasodilator. (10) ET-1 is thought to have a beneficial effect in early tubular loss where vasoconstriction of the vasculature in the renal cortex leads to better medullary blood flow and sodium balance, however as disease progresses and the vasoconstrictory stimulus becomes chronic, this effect becomes pathogenic leading to sodium retention and increased blood pressure. (10) Focal renal ischaemia and angiotensin II are strong stimulators of ET-1 production. (10) ET-1 can also bind to two endothelin receptor subtypes in the kidney and vasculature – ETA and ETB which have opposing effects. ETA receptor activation causes vasoconstriction, increases cell proliferation, and has proinflammatory and profibrotic actions in the glomerulus and tubules where it plays a role in the progression of renal fibrosis and

CKD, whereas ETB receptor activation stimulates NO release and vasodilation, and has anti-fibrotic effects. (124, 125) ETB also blocks vasopressin action and can promote salt and water removal. (126) In preclinical animal models, ETA receptor activation has been shown to advance progression of kidney disease (including cyst growth, fibrosis and vascular disease with increased glomerulosclerosis). (123, 127) Similarly, in ADPKD patients, higher ET-1 levels correlate with poorer kidney function and larger kidney size, and ET-1 has been suggested as a non-invasive biomarker of disease severity. (123, 128)

Endothelin antagonism has been explored in other forms of chronic kidney disease, in particular diabetic nephropathy and IgA nephropathy, and has been shown to have renoprotective and anti-hypertensive effects, but ADPKD patients have been excluded from these studies. (124, 129-132) One small short-term study by Dhaun *et al*. of 7 patients with non-diabetic kidney disease included 1 patient with ADPKD and showed an anti-hypertensive and renoprotective benefit with ET receptor antagonism. (133)

Preclinical studies in animal models of polycystic kidney disease have not shown a clear benefit with endothelin receptor antagonism. (126, 127, 134) Two studies have been performed; one with non-selective ET-1 antagonism in the Han:SPRD rat which resulted in progression of kidney disease and tubular cell proliferation, and the other in the $Pkd2^{WS25/-}$ mouse which showed that either selective ETB blockade progressed cystic renal disease. (127, 134) These changes were mitigated by combined ETA/ETB blockade demonstrating the importance of balance between receptor activation in polycystic kidney disease. (126, 127) Additionally, ETA blockade in the $Pkd2^{WS25/-}$ mouse promoted tubular cell proliferation but did not change cystic disease. (127) There have been no clinical studies of endothelin antagonism in ADPKD.

Therefore, given the pathophysiology of disease in ADPKD, it is possible that endothelin antagonism, particularly when paired with ARBs, may be beneficial as it is in other forms of CKD. However, the preclinical studies suggest a cautious approach is required to ensure this therapy does not promote cyst growth. (126, 132)

1.7.3 Sodium-glucose co-transporter 2 inhibitors

SGLT2 inhibitors reduce urinary glucose resorption, increase sodium excretion and have potent hemodynamic effects to lower blood pressure. (135) Numerous preclinical and several large RCTs have shown that SGLT2 inhibitors lower blood pressure, reduce proteinuria and improve renal and cardiovascular outcomes. (135)

Preclinical studies of SGLT2 inhibitors in PKD have had conflicting results. One study showed that treatment with SGLT2 inhibitor (Dapagliflozin) induced glucosuria and osmotic diuresis which resulted in hyperfiltration, progression of proteinuric kidney disease and increased cyst growth in the *pck* rat. (136) Other studies in the *Han:SPRD* PKD rat model showed that nonselective SGLT inhibition with phlorizin induced osmotic diuresis and retarded cyst growth, and selective SGLT2 inhibition improved kidney function and albuminuria but did not affect cyst growth. (137, 138) Differences between these studies may be due to variability in the cystic phenotypes between these two models. (136)

A retrospective observation study of 23 ADPKD patients given dapagliflozin for ~8.5 months (range 2-13 months) showed possible worsening of renal function and increase in cyst volume, but this was an uncontrolled study with methodological limitations. (139) ADPKD patients have been excluded from large clinical trials testing the effect of SGLT2 inhibitors in non-diabetic kidney disease, however given its known positive hemodynamic and cardiorenal protective effects, further studies are required to elucidate its potential in ADPKD. (140)

1.7.4 Non-steroidal mineralocorticoid antagonists

As previously described, aldosterone activity is increased in ADPKD leading to sodium retention, hypertension, and increased cardiovascular risk. (141) Mineralocorticoid receptor activation leads to proinflammatory and profibrotic pathways in kidney, cardiac and vascular tissues. (141) Use of traditional steroidal mineralocorticoid antagonists, such as spironolactone, have been shown to improve perivascular fibrosis in the *LPK* rat model of experimental PKD. (142) An RCT of 60 patients with early ADPKD demonstrated a reduction in blood pressure with 24 weeks of treatment with spironolactone but no change in vascular or endothelial function. (143)

Non-steroidal mineralocorticoid antagonists (MRAs), such as Finerenone, have been shown to improve cardiovascular outcomes including heart failure, all-cause mortality, and the risk of renal and cardiovascular events in patients with CKD and type 2 diabetes. (141, 144) There have been no studies in ADPKD, but given the response to spironolactone and the lack of classical steroidal side effects associated with non-steroidal MRAs, they are a potential therapeutic option for CVD in ADPKD.

1.7.5 Tyrosine kinase inhibitors

As discussed earlier, increased cAMP activation is a key pathogenic feature of ADPKD. Increased cAMP promotes the upregulation of protein kinase A (PKA), which stimulates downstream tyrosine receptor kinase growth pathways implicated in cystogenesis. (145) However, PKA can also be upregulated independently of cAMP in PKD. (145) Tyrosine kinase receptor inhibitor and modulator of PKA expression, Tesevatinib, has been shown to ameliorate cystic disease in animal models of PKD, however results from phase II clinical trial reports no significant change in height-adjusted total kidney volume (Ht-TKV) or renal

function. (145, 146) Further studies are required to evaluate the efficacy of interventions in this pathway.

1.7.6 Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Modulators

Chloride-driven fluid section into kidney cysts is an important pathogenic pathway in the progression of cystic disease (and thus, cardiovascular disease). Preclinical studies have shown that cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel, is found on renal epithelial cells that line kidney cysts in ADPKD. (140) Its activity is stimulated by PKA and cAMP and increased levels of activation of these pathways in PKD contributes to increased fluid secretion into cystic structures and cystic growth. (140) CFTR modulator GLPG2737 promotes net absorption of cystic fluid, reduces cyst growth and improves kidney function in preclinical PKD1-knockout mouse models. (140, 145) A phase II trial for patients with rapidly progressive ADPKD was conducted and terminated early due to lack of efficacy in April 2023 with results pending. (147)

1.7.7 Therapies directed at polycystin proteins or their expression

Another potential therapeutic avenue in ADPKD is preventing or reducing cyst growth by targeting the primary ciliary dysfunction and the pathological growth mechanisms that occur at a subcellular level and cause the disease. Though the inherited mutation in ADPKD is typically in polycystin-1 or -2 proteins, there are a number of preclinical studies that have identified modulators of ciliary function apart from polycystins that are potential targets to possibly offset the loss of polycystin function.

RGLS432 is a short oligonucleotide inhibitor of microRNA-17 (miR-17), a micro-RNA family expressed primarily in kidney tissue and implicated in cyst growth. (148) MiR-17 appears to bind to PKD1 and PKD2 and inhibits its expression. (148) Use of RGLS432 in human cell lines *in vitro* and preclinical polycystic mouse models has been shown to attenuate cyst growth, likely through its action on increasing polycystin protein expression above the threshold to cause PKD. (148) A phase Ib multiple ascending dose study of RGLS432 in patients with ADKPD is underway and recently advanced to the third cohort, with a favourable safety and side effect profile demonstrated in first and second cohorts treated with lower doses. (149)

Another target is CDK5, a growth factor kinase involved in cellular differentiation and cyst growth, and in a study by Husson *et al.* inhibition of CDK5 with R-roscovitine or its analog S-CR8 resulted in structurally shorter cilia which reduced progression of cystic disease in a juvenile rodent model of polycystic kidney disease. (150) Similarly, INPP5E, a dephosphorylating enzyme which results in murine cystic kidney disease when inactivated, may potentially be an inhibitor of PI3K/Akt/mTORC1 growth factor signalling pathways in renal epithelial cells, potentially reducing growth of cystic epithelia. (151) These targets are in very early preclinical stages, however they are potential interventions to consider in attenuating this multi-system disease in the future.

The interventions discussed in this section were not examined further in this thesis but their role in slowing CVD progression in ADPKD could form the basis for further research.

1.8 Hypothesis and Aims

The aim of this thesis is to explore interventions to attenuate CVD by specifically targeting known pathological drivers of disease in ADPKD using non-pharmacological or repurposed therapies as they are accessible, pragmatic and rapidly translatable.

Potential targets include those that attenuate kidney cystic disease (as cyst growth is a key stimulator of major systemic drivers of CVD such as RAAS overactivity), or those that directly reduce vascular and endothelial dysfunction. Based on this, the following domains were examined in this thesis; (i) the vasopressin pathway, responsible for cyst growth and has physiological hemodynamic actions, (ii) the vitamin D receptor pathway, a modulator of intracellular calcium, cellular growth and RAAS and, (iii) the nitric oxide pathway, a key mediator of vasodilation. The specific interventions chosen in each of these areas were increased water intake, paricalcitol (a vitamin D receptor agonist) and dietary nitrate supplementation with beetroot juice.

Our hypotheses are summarised in Figure 1 below.

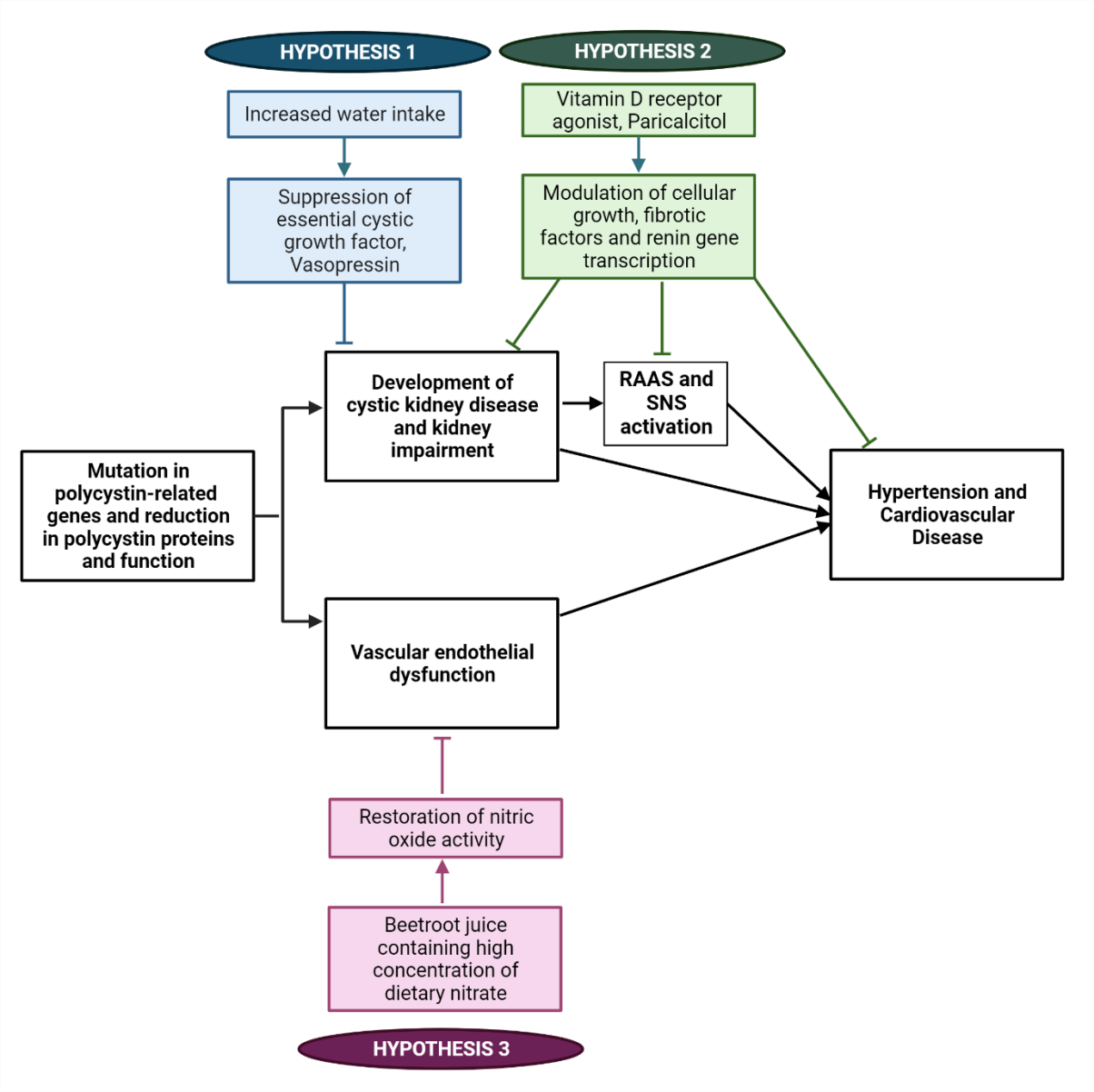

Figure 1: Summary of the drivers of cardiovascular disease in ADPKD and the hypotheses investigated in this thesis.

The hypotheses examined in this thesis are:

(i) Increased water intake reduces cardiorenal disease progression in experimental polycystic kidney disease (Chapter 2)

(ii) Treatment with vitamin D receptor agonists reduces the progression of cardiovascular disease in early and late stages of experimental polycystic kidney disease, independently and in conjunction with ACEi therapy (Chapter 3)

(iii) Nitrate-containing beetroot juice lowers blood pressure compared to nitrate-depleted beetroot juice in hypertensive ADPKD patients (Chapter 4 and 5).

The specific aims investigated in this thesis are to test the:

(i) Effect of increased water intake in the Lewis Polycystic Kidney experimental rodent model of polycystic kidney disease on attenuating cardiorenal disease in early and late stages of disease progression

(ii) Effect of vitamin D receptor agonist, Paricalcitol, on reducing cardiorenal disease in both early and late stages of disease in the Lewis Polycystic Kidney experimental model of polycystic kidney disease, and in conjunction with standard ACEi therapy

(iii) Efficacy of dietary nitrate supplementation with beetroot juice (containing 400mg nitrate/day) on reducing blood pressure compared with nitrate-depleted beetroot juice in a randomised controlled trial of hypertensive patients with ADPKD.

Chapter 2: The effect of increased water intake on cardiorenal disease progression in experimental polycystic kidney disease

2.1 Preface

Vasopressin is a key mediator of cystogenesis and cyst expansion, and drives progression of kidney and cardiovascular disease in ADPKD through multiple mechanisms including RAAS activation and vasoconstriction. Decreases in plasma osmolality can suppress vasopressin release, and this is achievable by increasing water intake. The study described in this chapter aims to test the hypothesis that increased water intake can attenuate kidney and cardiovascular disease in an experimental model of PKD.

Two preclinical studies have been performed to date to test the effect of increased water intake on reducing vasopressin-stimulated cyst growth in PKD using the *pck* rat model and the *Pkd1*$^{RC/RC}$ mouse model. (62, 63) Both models used in these previous studies do not develop the significant cardiovascular phenotype of human ADPKD, therefore the impact of increased water intake on CVD was not determined. The *Lewis Polycystic Kidney (LPK)* rat is a chronic model of kidney cystic disease that develops early significant hypertension and left ventricular disease similar to human PKD. (152) To explore the effects of increased water intake on CVD, the *LPK* rat model was used for this study.

Notably, during the time of analysis and writing the manuscript for publication, the first RCT testing the effect of increased water intake in patients with ADPKD ("PREVENT-ADPKD") was in development. (3)

2.2 Increased water intake reduces long-term renal and cardiovascular disease progression in experimental polycystic kidney disease

The following manuscript detailing the study was published in peer-reviewed journal *PLOS One* on January 2nd 2019. The formatting and writing style are in keeping with the journal requirements. References are self-contained within the manuscript. Section 2.3 contains supplementary material referenced in this manuscript.

PLOS ONE

RESEARCH ARTICLE

Increased water intake reduces long-term renal and cardiovascular disease progression in experimental polycystic kidney disease

Priyanka S. Sagar[1,2]*, **Jennifer Zhang**[1,2], **Magda Luciuk**[1,2], **Carly Mannix**[1,2], **Annette T. Y. Wong**[1,2], **Gopala K. Rangan**[1,2]

1 Centre for Transplant and Renal Research, Westmead Institute for Medical Research, The University of Sydney, Sydney, NSW, Australia, **2** Department of Renal Medicine, Westmead Hospital, Western Sydney Local Health District, Sydney, NSW, Australia

* priyanka.sagar@sydney.edu.au

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Abstract

Polycystic kidney disease (PKD) is the most common inherited cause of kidney failure and currently has limited treatment options. Increasing water intake reduces renal cyst growth in the *pck* rat (a genetic ortholog of autosomal recessive PKD) but it is not clear if this beneficial effect is present in other models of PKD. In this study, we tested the hypothesis that high water intake (HWI) reduces the progression of cystic renal disease in Lewis polycystic kidney (LPK) rats (a genetic ortholog of human nephronophthisis-9). Groups of female and male LPK (n = 8–10 per group) and Lewis (n = 4 per group) rats received water *ad libitum* supplemented with or without 5% glucose [to simulate HWI or normal water intake (NWI) respectively] from postnatal weeks 3 to 16. Water intake increased ~1.3-fold in the LPK +HWI group compared to LPK+NWI rats between weeks 3 to 10 but the differences were not significant at later timepoints. In LPK rats, HWI reduced the increases in the kidney to body weight ratio by 54% at week 10 and by 42% at week 16 compared to NWI (both $p<0.01$). The reduction in kidney enlargement was accompanied by decreases in the percentage renal cyst area, percentage renal interstitial collagen and proteinuria (all $p<0.05$). At week 16, HWI reduced systolic blood pressure and the heart to body to weight ratio by 16% and 21% respectively in males LPK rats (both $p<0.01$). In conclusion, a modest increase in water intake during the early phase of disease was sufficient to attenuate renal cystic disease in LPK rats, with secondary benefits on hypertension and cardiovascular disease. These data provide further preclinical evidence that increased water intake is a potential intervention in cystic renal diseases.

Introduction

Polycystic kidney disease (PKD) is the most common inherited cause of end-stage renal disease (ESRD) [1–3]. It is characterised by the formation and growth of numerous fluid-filled kidney cysts and cystic tubular dilatations, which cause progressive nephron obstruction,

2018. The other authors have declared that no competing interests exist. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

tubulointerstitial injury and renal impairment with secondary hypertension and cardiovascular disease [2]. Arginine vasopressin (AVP) is a neuro-hypophyseal hormone that regulates water homeostasis and is released in response to elevated serum osmolality [4]. In the *pck* rat (a genetic ortholog of autosomal recessive PKD), circulating AVP was critical for triggering the formation of renal cysts as well as contributing to their continued growth *via* activation of cyclic adenosine monophosphate (cAMP)-mediated transepithelial fluid secretion and cell proliferation [5–7]. Furthermore, pharmacological receptor antagonists of AVP, such as tolvaptan, reduce renal cyst growth and the decline in renal function in both experimental and human PKD, confirming the importance of AVP in renal cyst growth [3, 8].

Increasing the intake of water attenuates AVP release, and has been hypothesised to be an easily-accessible and safe therapeutic intervention to reduce renal cyst growth in PKD [2, 9, 10]. To date, there have been only two preclinical studies that have evaluated the efficacy of increased water intake in PKD [11, 12]. In this regard, Nagao *et al.* demonstrated that increased water intake (induced by adding 5% glucose to the drinking water) over 10 weeks reduced the progression of kidney enlargement and the percentage cyst area in *pck* rats [11]. Furthermore, Hopp *et al.* replicated these findings in the *pck* rat (using hydrated agarose gel and 5% glucose) but unexpectedly did not observe a protective effect in a hypomorphic model of autosomal dominant PKD ($Pkd1^{RC/RC}$ mice) [12].

It is not known if the beneficial effects of high water intake attenuates the long-term progression of kidney enlargement in other models of PKD. The Lewis polycystic kidney (LPK) rat model is a hypertensive rat model of cystic renal disease due to a point mutation in *Nek8* and is genetically orthologous to human nephronophthisis (NPHP)-9 [13]. Interestingly, the renal phenotype of LPK rats varies from NPHP and is characterised by progressive nephromegaly (due to diffuse collecting duct ectasia from postnatal weeks 3 to 20), late-onset end-stage renal failure and hypertension, with similarities to autosomal recessive PKD [14, 15]. The LPK rat model provides a valuable preclinical tool to evaluate and understand the chronic efficacy of therapeutic interventions in PKD. In the present study, the hypothesis that an high water intake (HWI) reduces the progression of cystic renal disease in the LPK rat model, was investigated.

Methods

Animals

All animals were obtained from the breeding colony at Westmead Hospital and allowed food and water *ad libitum*. The study was approved by the Western Sydney Local Health District Animal Ethics Committee (Protocol number 4100) and conducted according to the Australian Code for the Care and Use of Animals for Scientific Purposes [16].

Experimental design

Following weaning, LPK and control Lewis littermates were equally divided into two groups that had free access to tap water either supplemented with or without 5% glucose [to simulate HWI or normal water intake (NWI) respectively] from postnatal week 3 until week 16 (Fig 1). Groups of rats were sacrificed at either week 10 or week 16 (LPK n = 66; Lewis n = 32). Both male and female animals were examined to determine the gender-specific effects of increased water intake. The sample size and time-points were based on the natural history of the LPK model, with the period between weeks 3 and 10 being characterised by rapid kidney growth (that is, early PKD), whereas the interval between weeks 10 to 16 marking the onset of progressive tubulointerstitial inflammation and fibrosis, hypertension and worsening renal function (that is, late PKD) [15].

The rats were placed in metabolic cages for 14 hours, at weeks 10 and 16, and sacrificed on the following day. At the time of sacrifice, rats were anaesthetised by an intraperitoneal injection of ketamine/xylazine, blood was collected by cardiac puncture, and both kidneys and heart were rapidly removed by surgical dissection as described in previous studies [15, 17].

The addition of 5% glucose to drinking water is a well-established model to increase water intake and is not known to have any detrimental effects [11, 12]. It been used in several previous studies to investigate the effects of HWI on the progression of PKD and CKD in small animal models [11, 12]. Moreover, Hopp *et al*. validated that tap water supplemented with 5% glucose was equivalent to providing food enriched with hydrated agarose gel on the efficacy on renal cyst growth in the *pck* rat [12].

Measurement of water intake

Water intake was assessed by two methods during the study. In the first method, water intake was measured in each rat at two time-points (weeks 10 and 16) while they were *individually-*

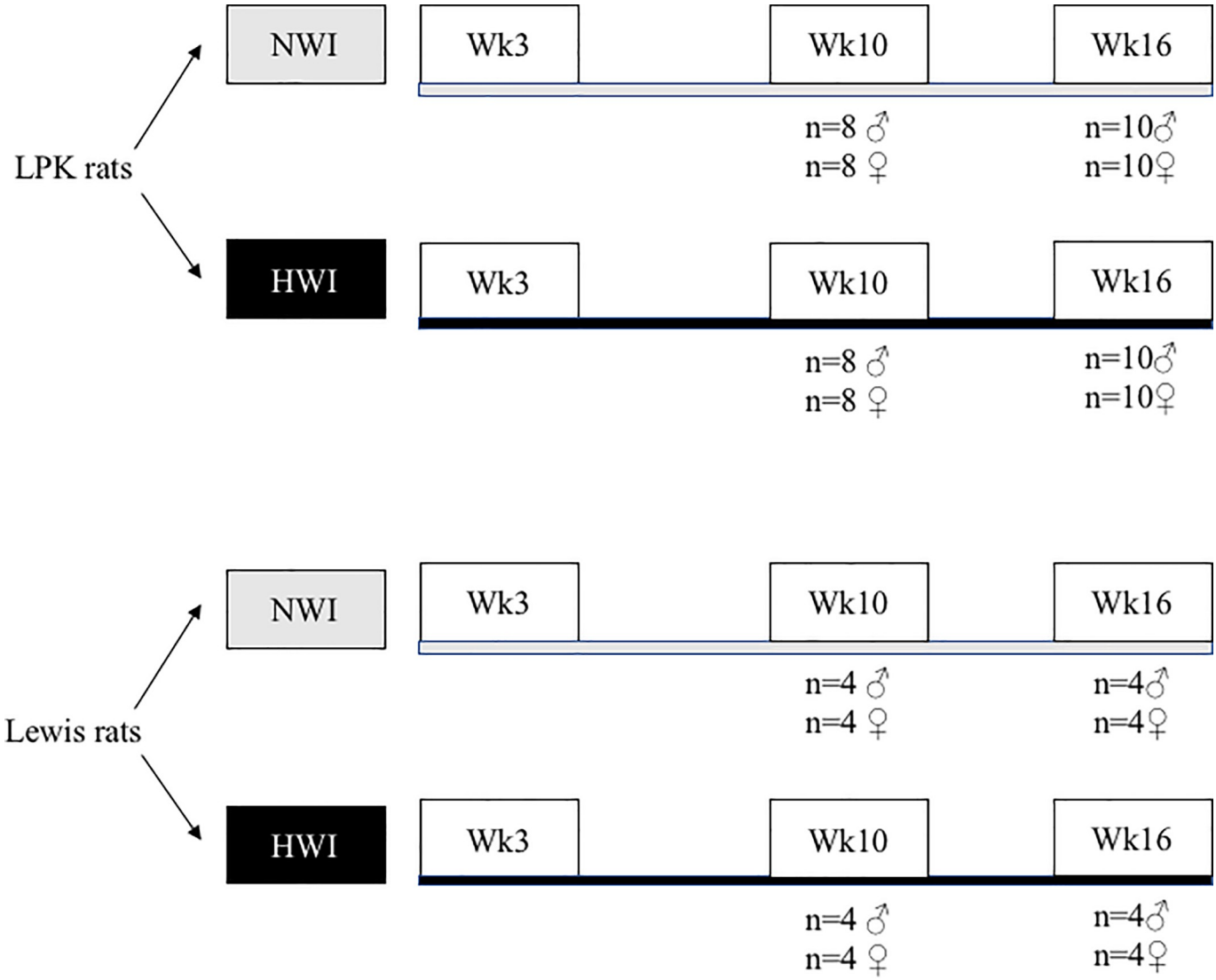

Fig 1. Experimental design. Female and male Lewis and LPK litter-mates were equally divided into two groups—HWI (tap water supplemented with 5% glucose) or NWI (normal water intake). The rats were sacrificed at either week 10 (LPK n = 33, Lewis n = 15) or week 16 (LPK n = 33, Lewis n = 17).

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housed in metabolic cages (for 14 hours). In the second method, the consumption of water was measured continuously for entire the duration study while rats were *group-housed* in standard cages (n = 3–4 littermates) by measuring the weight of water bottles. In this method, water intake was recorded every morning (between 0700–0900 hrs) second daily (arbitrarily designated Day 0 and Day 2) and calculated by the following formula:

$$\text{Water intake per rat/day (mls)} = \frac{(\text{Bottle Weight on Day 0} - \text{Bottle Weight on Day 2})}{(\text{Number of Rats in Cage X No.of Days Since Last Measurement})}$$

Renal function and other biochemistry

The serum urea, creatinine, random glucose, and osmolality were measured from blood collected from all rats at the time of sacrifice, as previously described [15][17]. The urine volume, creatinine and osmolality were measured from samples collected while rats were placed in metabolic cages on the day prior to sacrifice. The creatinine clearance was corrected for body weight, and calculated as described previously [15, 17]. The serum sodium and albumin were measured in a subset of rats from each group. In addition, the osmolyte intake was estimated by multiplying the urine volume with urinary osmolality.

Histology and quantitative image analysis

Coronal sections of kidney and heart were fixed in either methyl carnoy solution or 10% neutral buffered formalin, and embedded in paraffin. Tissue sections for histology or immunohistochemistry were cut at 4μm in thickness. Whole-slide imaging of stained slides was performed (magnification x 20) using a digital slide-scanner (Hamamatsu Nanozoomer, Hamamatsu Photonics, Shizuoka, Japan). To determine the renal cyst area, methyl carnoy-fixed samples were stained with Periodic-Acid Schiff (PAS) and the cystic ('white') areas was quantified by the area morphometric analysis using imaging analysis software (Aperio Imagescope, v12.3.2.8013 Leica Biosystems, Wetzlar, Germany). The proportion occupied interstitial fibrosis in renal and cardiac tissue was quantified by Sirius Red collagen staining. Renal inflammation was determined by interstitial monocyte and myofibroblast accumulation, using antibodies reactive against either CD68 (1:400, MCA341R; Serotec, Kidlington, U.K.) or α-smooth muscle actin (α-SMA) (1:1000, A2547; Sigma–Aldrich, St. Louis, MO, USA) respectively, as previously described [18]. All immunohistochemical stains were analysed in whole-slide images using a standardized algorithm to measure positive pixels in the Aperio Imagescope software. The percentage monocyte and myofibroblast infiltration, and interstitial collagen deposition indexes were calculated using the formula: $\left(\frac{Positive\ Pixels}{Total\ Pixels}\right) \times 100$. The surface area of myofibroblasts, monocyte or interstitial collagen deposition was calculated per mm^2 by the formula: $\left(\frac{Positive\ Pixels\ x\ Total\ Section\ Area}{Total\ Pixels}\right)$.

Evaluation of blood pressure and cardiovascular disease

To assess the progression of cardiovascular disease in LPK rats, serial tail-arterial blood pressure measurements were performed in male Lewis (n = 8) and male LPK rats (n = 14) between weeks 14 to 16 of age as previously described [15, 17]. In addition, cardiac weight was measured in all rats at the time of sacrifice and corrected for body weight.

Statistical analysis

The data was analysed using JMP statistical software and results presented as mean ± SD. A *P* value less than 0.05 was defined as statistically significant. The differences between LPK and Lewis groups were analysed by the Tukey-Kramer (HSD) or Wilcoxon each pair test.

Results

General effects of increased water intake

Both Lewis and LPK rats tolerated increased water intake (induced by 5% glucose in drinking water) with no apparent adverse effects, and no deaths occurred during the study. In general, as expected, male rats weighed significantly more than female rats in all groups ($p<0.01$, S1 Table). Moreover, the body weight of male Lewis rats was higher when compared to male LPK male rats, whereas no difference was observed between female Lewis and female LPK rats.

When comparing the NWI and HWI groups, there was no differences in body weight between the LPK and Lewis groups at the commencement of the intervention (at week 3). In addition, the growth of Lewis rats between week 3 and 10 or 16 were the same in both the HWI and NWI groups. In LPK male rats, there was no difference in body weight at week 10 between the HWI and NWI groups. However, at week 16, males in the LPK+HWI group had a higher body weight compared to the LPK+NWI group ($p = 0.01$, S1 Table), whereas there was no difference for female LPK rats. Also, as shown in Table 1, the serum glucose and albumin were the same between HWI and NWI group at both timepoints for both Lewis and LPK rats.

Changes in water consumption in the experimental groups

Water consumption while rats were housed individually in metabolic cages. As discussed in the Methods, water consumption was assessed in two ways: (i) while rats were housed individually in metabolic cases at two specific timepoints prior to sacrifice (i.e. weeks 10 and 16); and (ii) while rats were group-housed in standard cages (discussed below). The data for water intake is shown in Table 2. In general, LPK+NWI rats had a higher water intake than Lewis+NWI rats (~1.4-fold higher at week 10, $p = 0.0043$; and 1.7-fold more at week 16, $p = 0.0368$), most likely due to loss of urinary concentrating ability and increased thirst response in PKD-affected LPK rats compared to normal Lewis rats [19, 20]. When comparing the HWI and NWI groups, Lewis+HWI rats had a 1.8- and 3.7-fold increase in water intake compared to the Lewis+NWI at week 10 and 16 respectively (both $p<0.0001$; Table 2). Similarly, in the LPK+HWI group, water intake increased 1.7-fold compared to the LPK+NWI group at week 10 ($p<0.0001$), and at week 16 the magnitude of increase in water intake was ~1.2-fold ($p = 0.0963$). The estimated osmolyte intake was similar in all groups at week 10, but at week 16 it was reduced in LPK+NWI group compared to the Lewis+NWI group (Table 2).

Water consumption while rats were housed in groups in standard cages. To provide serial measurements of fluid intake during the study, water consumption was also assessed continuously while rats were group-housed in standard cages, as shown in Fig 2. Overall, for both Lewis and LPK rats, water intake in the HWI groups ($n = 48$) was at least 1.3-fold higher than the NWI groups. For Lewis rats, the increase in water intake in the HWI group was statistically significant at all timepoints throughout the study period (Fig 2A). However, for the LPK+HWI group water consumption was higher compared to the LPK+NWI until week 10 ($p<0.0001$, Fig 2B). After week 10, the water intake of the LPK+HWI group was not consistently higher than LPK+NWI rats ($p = 0.9888$).

Effect of increased water intake on urine volume and biochemistry

Urine volume and urine osmolality. The results for urine volume and osmolality while rats were housed individually in the metabolic cages are shown in Table 2. In general, the urine volume tended to be lower than the volume of water consumed possibly in part due to

Table 1. Effects of increased water intake on body weight, kidney weight, kidney cyst area, and serum glucose and albumin.

	Lewis		LPK	
	NWI	HWI	NWI	HWI
Week 10	**n = 8**	**n = 8**	**n = 17**	**n = 16**
Body weight at week 3 (g)	32±3	31±3	31±6	32±5
Body weight at week 10 (g)	202±56	185±44	174±29	188±32
Kidney: body weight (%)	0.8±0.0	0.7±0.0	7.3±0.8*	3.4±0.7†
Kidney cyst area (mm^2)	7.7±3.3	5.7±112.8	112.8±15.4*	59.8±15.0†
Kidney section area (mm^2)	45.4±11.1	38.0±4.0	174.7±22.5*	111.5±20.0†
Kidney cyst area: kidney section area (%)	17.1±5.7	14.9±5.2	64.6±4.6*	53.4±5.3†
Serum glucose (mmol/L)	13±2.4	12.7±2.4	10.7±2.6	12.6±4.2
Serum albumin (g/L)	29±3	29±1	29±4	29±2
Week 16	**n = 9**	**n = 8**	**n = 17**	**n = 16**
Body weight at week 3 (g)	35±4	34±2	31±7	31±6
Body weight at week 16 (g)	241.1±66.8	250.4±70.8	211.5±49	257±63.5
Kidney: body weight (%)	0.7±0.1	0.7±0.0	7.8±1.0*	4.4±0.9†
Kidney cyst area (mm^2)	9.0±4.9	8.7±3.5	143.4±29.4*	103.1±31.6†
Kidney section area (mm^2)	46.4±11.9	46.7±10.4	210.2±40.6*	163.5±38.6†
Kidney cyst area: kidney section area (%)	18.6±5.5	18.0±4.0	68.2±4.6*	61.9±6.6§
Serum glucose (mmol/L)	12.1±3.2	13±2.2	10±2.3	10.6±1.9
Serum albumin (g/L)	30±1	33±1	31±3	31±3

NWI, normal water intake; HWI, high water intake; KW:BW% (mg), kidney weight to body weight ratio expressed as a percentage. Values represented as mean ± SD.

*p<0.001 versus age-matched NWI Lewis rat

†p<0.001 versus age-matched LPK NWI rat

‡p<0.05 versus age-matched NWI Lewis rat

§p<0.05 versus age-matched NWI LPK rat. Unless otherwise indicated differences between the groups are not statistically significant

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behavioural factors and/or urine evaporation in the metabolic cage [21, 22]. In Lewis rats, there was a trend for an increase in urine volume in the HWI group at week 10 (p = 0.0728) and this statistically significant at week 16 (5.8-fold; p<0.0001) when compared to the NWI group. In LPK rats, the urine volume increased 1.5-fold in HWI group (p = 0.0484) at week 10 but was not statistically different at week 16 (p = 0.5829) compared to NWI group.

The changes in urine osmolality in the experimental groups are shown in Table 2, and were consistent with the effects of increased water intake but also highlighted the effect of renal disease progression in PKD. Comparing the NWI groups, the urine osmolality was higher in Lewis rats compared to LPK rats at both week 10 and 16 (p<0.0001) most likely due to the loss of urinary concentrating ability in PKD [19, 20]. In the Lewis rat groups, there was a significant decrease in urine osmolality with HWI compared to NWI at both timepoints (p<0.0001),. In contrast, in LPK rats, there was no difference between the LPK+HWI and LPK+NWI groups at either week 10 (p = 0.5503) or week 16 (p = 0.9256), indicating that it is a less sensitive biomarker to gauge the serial effects of changes in fluid intake in PKD, as suggested in previous studies [23].

Serum osmolality, sodium and urea. As shown in Table 2, the serum osmolality and urea were higher in LPK+NWI rats compared to Lewis+NWI rats, possibly secondary to increased urinary loss of solutes in PKD [20]. In Lewis rats, serum osmolality, urea and sodium were not

Table 2. Changes in water intake (as determined while housed in metabolic cages) and urine osmolality, estimated osmolyte intake and serum sodium/urea.

	Lewis		LPK	
Variables	**NWI**	**HWI**	**NWI**	**HWI**
Week 10	**n = 8**	**n = 8**	**n = 16**	**n = 11**
24 hr water intake (mls)	29.5±7.7	54.7±13.8‡	41.1±7.6*	68.5±17.4†
24 hr urine volume (mls)	13.2±7.4	27.6±12.4	18.9±8.0	29.2±14.1§
Urine osmolality (mmol/kg)	987.8±606.5	451.1±184.2‡	588.4±158‡	434.2±82.5
Estimated osmolyte intake (mmol/day)	11.3+2.8	12.8+2.6	10.3+3.2	11.7+3.6
Serum osmolality (mmol/kg)	313.1±13.0	308.4±12.9	354.1±27.9‡	310.1±28.4†
Serum sodium (mmol/L)	144±2	142±3	147±4	136±11†
Serum urea (mmol/L)	6.5±1.5	5.6±0.8	20.2±5.8*	9.3±3.0†
Week 16	**n = 9**	**n = 6**	**n = 13**	**n = 12**
24 hr water intake (mls)	27.0±7.4	92.4±28.4	46.7±10.1*	64.4±18.1
24 hr urine volume (mls)	10.1±4.2	58.1±43.6*	20.2±8.2	29.2±9.7
Urine osmolality (mmol/kg)	1624.2±649.5	370.3±341.1*	481.4±179.7*	411.8±78
Estimated osmolyte intake (mmol/day)	14.2±2.1	12.4±3.6	9.3±3.6*	11.4±3.1
Serum osmolality (mmol/kg)	324.6±44	311±14.9	356.2±32.4	324.9±24.4†
Serum sodium (mmol/L)	145±7	146±4	146±5.0	141±8
Serum urea (mmol/L)	7.2±1.5	5.8±0.9	29.0±8.9*	16.7±7.4†

Values represented as mean ± SD.

*p<0.001 versus age-matched NWI Lewis rat

†p<0.001 versus age-matched LPK NWI

‡p<0.05 versus age-matched NWI Lewis rat

§p<0.05 versus age-matched NWI LPK rat.

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altered in the HWI groups compared to the NWI rats (Table 2). In contrast, in LPK+HWI rats the serum osmolality and urea were significantly lower compared to LPK+NWI rats at both time points (p<0.0001). Interestingly at week 10, this decrease was also associated with a reduction in the serum sodium in the LPK+HWI group compared to LPK+NWI (p = 0.0071), but statistical significance was not present at week 16 (p = 0.4692).

Effect of increased water intake on kidney enlargement and renal cystic disease

Kidney enlargement. The results for kidney enlargement (as determined by the kidney to body weight ratio) are shown in Table 1. The kidney size was increased by 10- and 11-fold in LPK+NWI rats compared to the Lewis+NWI group at week 10 and 16 respectively (p<0.0001 for both). In Lewis rats, increased water intake did not alter kidney size. In contrast, in the LPK+HWI groups there was a 54% and 43% reduction in the progression of kidney enlargement at week 10 and 16 respectively compared to the LPK+NWI group (p<0.0001).

Percentage kidney cyst area. HWI attenuated the progression of renal cyst area in LPK rats, as determined by histological analysis. The percentage cyst area was reduced by 47% and 28% at week 10 and 16 respectively in LPK+HWI rats compared to the LPK+NWI group (both p<0.0001), with an overall decrease in cystic index percentage (cyst area: renal section area) by 17% and 9%, at week 10 and 16 (both p<0.0001) in the LPK+HWI compared to LPK+NWI (Table 1 and Fig 3).

Renal interstitial myofibroblast and monocyte accumulation. Renal interstitial myofibroblast and monocyte accumulation were examined by immunohistochemistry for α-SMA

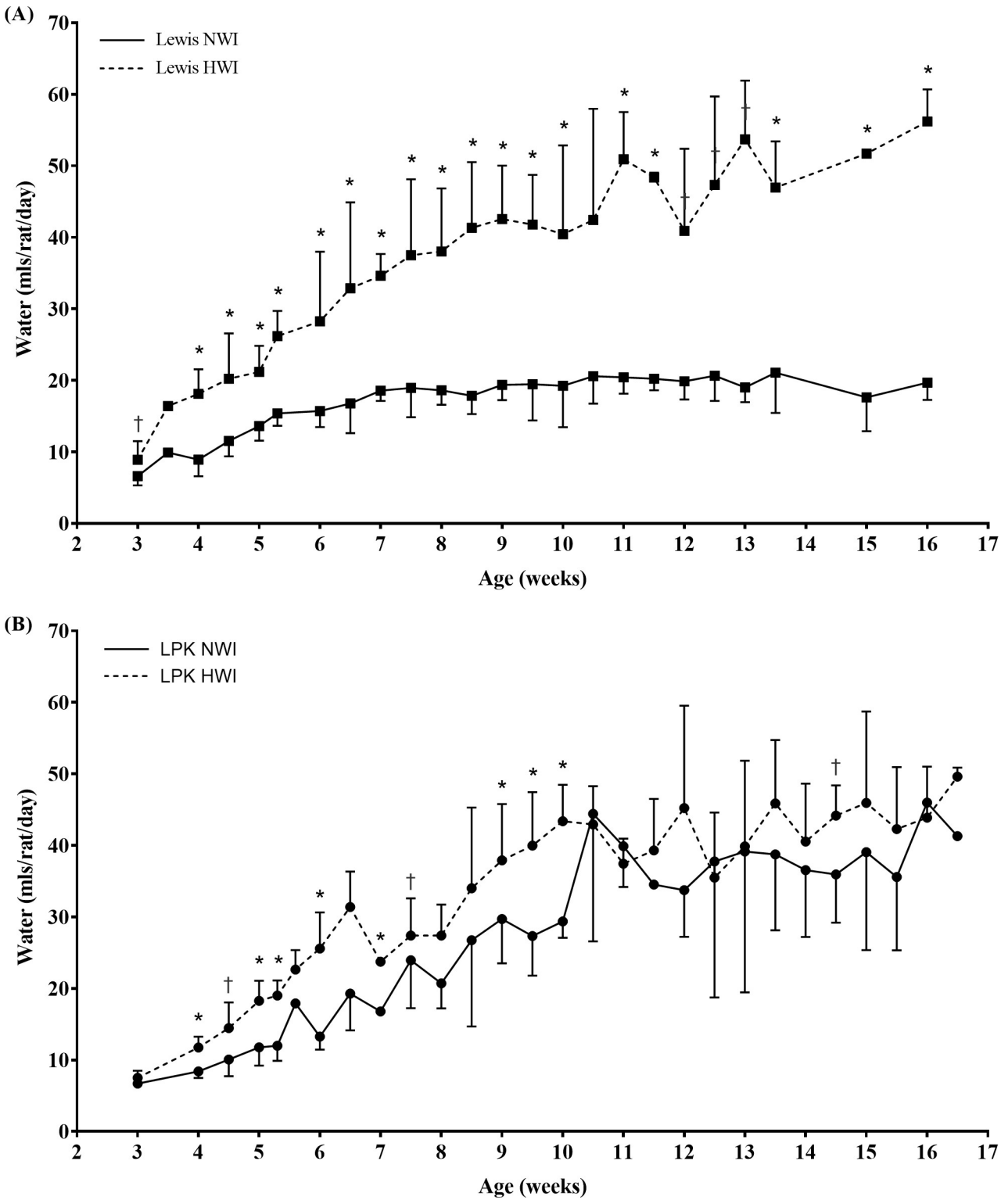

Fig 2. Water intake while rats were group housed in standard cages. (A) Water intake in the Lewis rat groups. Water intake in the Lewis+HWI rats was significantly increased water intake at all timepoints during the study period compared to the NWI group. *$p<0.001$ and †$p<0.05$ versus age-matched NWI Lewis rat. (B) Water intake in the LPK rat groups. Water intake in the LPK+HWI group was increased only up to week 10, after which it was similar to LPK+NWI rats. *$p<0.001$ and †$p<0.05$ versus the age-matched NWI group. Please see Methods for details on calculation.

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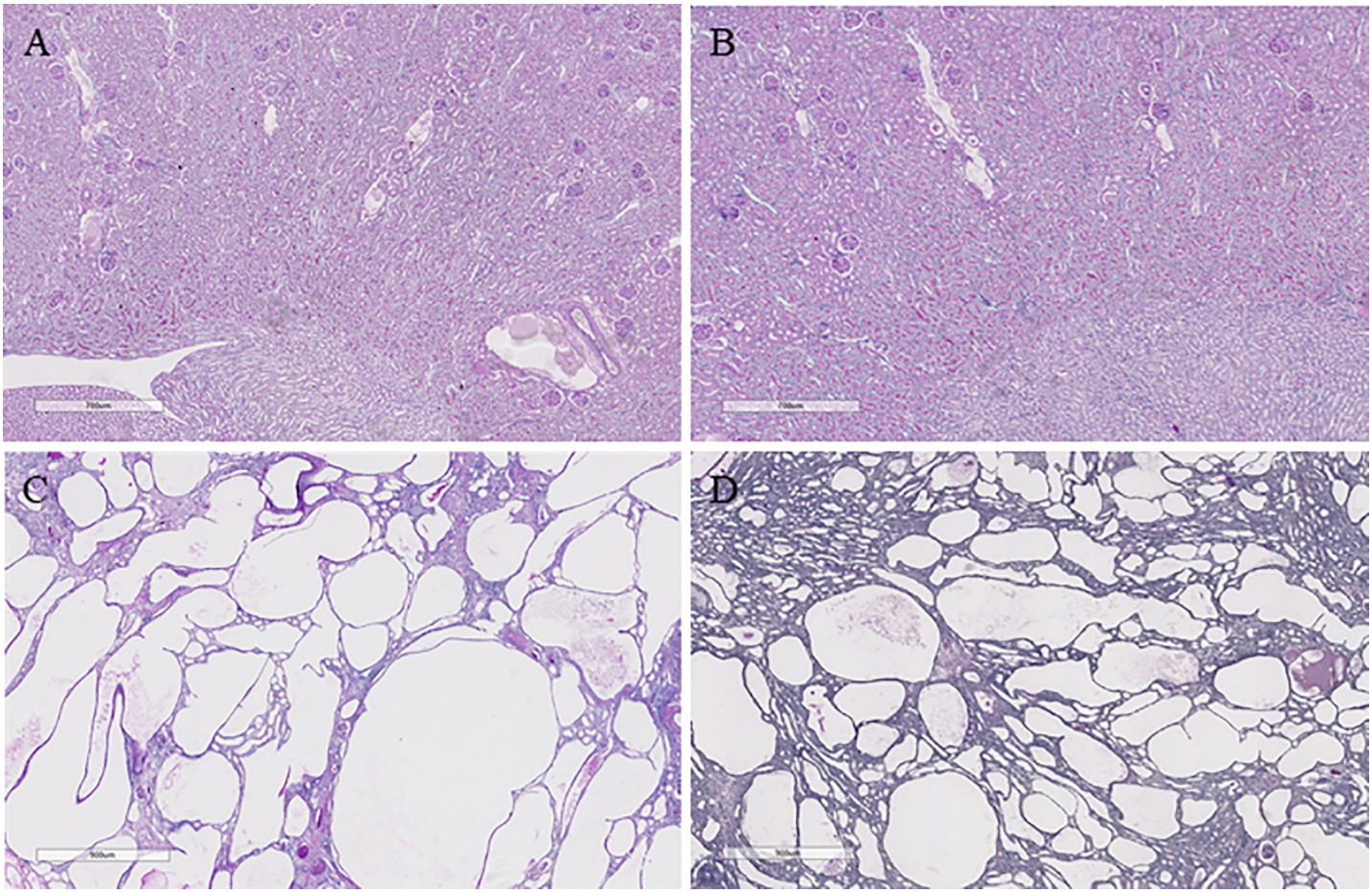

Fig 3. Representative photomicrographs of periodic acid Schiff-stained sections of kidney showing effects on cyst growth at week 10 in Lewis+NWI (A), Lewis+HWI (B), LPK+NWI (C) and LPK+HWI (D). There was no change in renal structure in the Lewis rats with HWI, whereas in LPK rats there was a marked attenuation in the renal cystic tubular dilation (D vs. C).

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and CD68 respectively. As shown in Table 3, myofibroblast infiltration was increased in the LPK+NWI group (with 12% of total section area positively stained for myofibroblasts) compared with Lewis+NWI (where 3% of the total section area positively stained for myofibroblasts), at both timepoints ($p = 0.0152$). However, HWI did not alter the progression of myofibroblast accumulation in either LPK or Lewis rats, at either timepoint ($p = 1.0000$). Representative photomicrographs of α-SMA in LPK and Lewis groups are shown in S1 Fig. Similarly, interstitial monocyte (CD68+) infiltration was increased in the LPK+NWI groups compared to the Lewis+NWI rats (2.1-fold increase in positively stained monocyte area in LPK+NWI at week 10 ($p = 0.0208$) and 2.2-fold at week 16 ($p = 0.0036$)). However, HWI did not affect monocyte infiltration in either Lewis or LPK HWI groups in comparison to NWI groups. These results were unchanged when analysed according to gender (S2 Table).

Renal fibrosis. Renal fibrosis as determined by Sirius red staining for collagen deposition, was increased in LPK+NWI rats at week 16 ($p<0.0001$; Table 3 and Fig 4) but not at week 10 ($p = 0.1023$) when compared to Lewis+NWI. In LPK rats, interstitial collagen deposition was not altered by HWI at week 10, but at week 16, it was significantly reduced (by 50%;

Table 3. Effect of increased water intake on renal myofibroblast and monocyte infiltration and interstitial collagen deposition.

	Lewis		LPK	
Variables	**NWI**	**HWI**	**NWI**	**HWI**
Week 10	**n = 7**	**n = 8**	**n = 17**	**n = 15**
Renal section area (mm^2)	45.8±11.3	39.8±4.5	173.1±24.5*	110.5±18.5†
Myofibroblast infiltration (mm^2)	1.24±1.1	1.17±0.5	9.76±5.4‡	6.60±3.5
Myofibroblast index (%)	3.2±3.0	3.3±1.5	12.3±7.1‡	10.4±4.5
Monocyte infiltration (mm^2)	0.33±0.2	0.33±0.3	0.69±0.5‡	0.62±0.3
Monocyte index (%)	1.0±0.4	1.1±0.9	1.2±0.7	1.3±0.6
Interstitial collagen deposition (mm^2)	3.6±2	1.6±1.3	27.5±17.8	17.1±7.4
Interstitial collagen deposition index (%)	7.6±3.1	4.3±3.7	16.2±11.4	15.4±6.0
Week 16	**n = 7**	**n = 8**	**n = 17**	**n = 15**
Renal section area (mm^2)	45.9±11.6	46.6±10.1	214.5±41.5*	158.6±39.2†
Myofibroblast infiltration (mm^2)	1.29±0.6	1.08±0.6	11.2±6.6‡	10.2±5.1
Myofibroblast index (%)	3.0±1.2	2.4±1.3	12.4±7.5‡	12.6±6.2
Monocyte infiltration (mm^2)	0.26±0.2	0.33±0.4	0.56±0.3‡	0.45±0.1
Monocyte index (%)	0.7±0.5	0.9±1.1	0.8±0.5	0.8±0.3
Interstitial collagen deposition (mm^2)	3.9±2.5	2.8±2.0	51.5±33.9*	25.1±10.7§
Interstitial collagen deposition index (%)	8.7±5.0	5.9±4.1	25.0±17.6‡	16.6±7.9

*$p<0.001$ versus age-matched NWI Lewis rat

†$p<0.001$ versus age-matched LPK NWI

‡$p<0.05$ versus age-matched NWI Lewis rat

§$p<0.05$ versus age-matched NWI LPK rat

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p = 0.0043) when compared to NWI (Table 4 and Fig 4). There were no changes between Lewis NWI and HWI groups (Table 4 and Fig 4).

Effects of increased water intake on renal function and proteinuria

Serum creatinine and creatinine clearance. LPK rats developed significant and progressive renal dysfunction over time (as determined by elevation in the serum creatinine and reduction in the endogenous creatinine clearance) compared to Lewis rats (Table 4). HWI did not affect renal function in Lewis rats. Similarly, in LPK rats the decline in renal function was not affected by HWI (Table 4 and S3 Table). Both LPK groups developed significantly elevated serum creatinine compared to Lewis rats at week 16 ($p<0.0001$). There was no difference in serum creatinine or calculated creatinine clearance corrected for body weight between HWI and NWI in either Lewis or LPK groups at both time points (Table 4) or when sub-analysed by gender (S3 Table) ($p>0.2$ in all cases).

Proteinuria. As shown in Table 4, there was a significant increase in proteinuria in the LPK+NWI rats compared to the Lewis+NWI group (p = 0.0263). In LPK rats, HWI reduced proteinuria by 70% (p = 0.0117) at the week 16 but not at week 10 (Table 4). There was no difference in proteinuria between the Lewis+HWI and Lewis+NWI groups at either timepoint (Table 4).

Effects of high water intake on cardiovascular disease

Systolic blood pressure. The effects of HWI on systolic blood pressure in male animals are shown in Fig 5. The systolic blood pressure was higher in LPK+NWI rats compared to

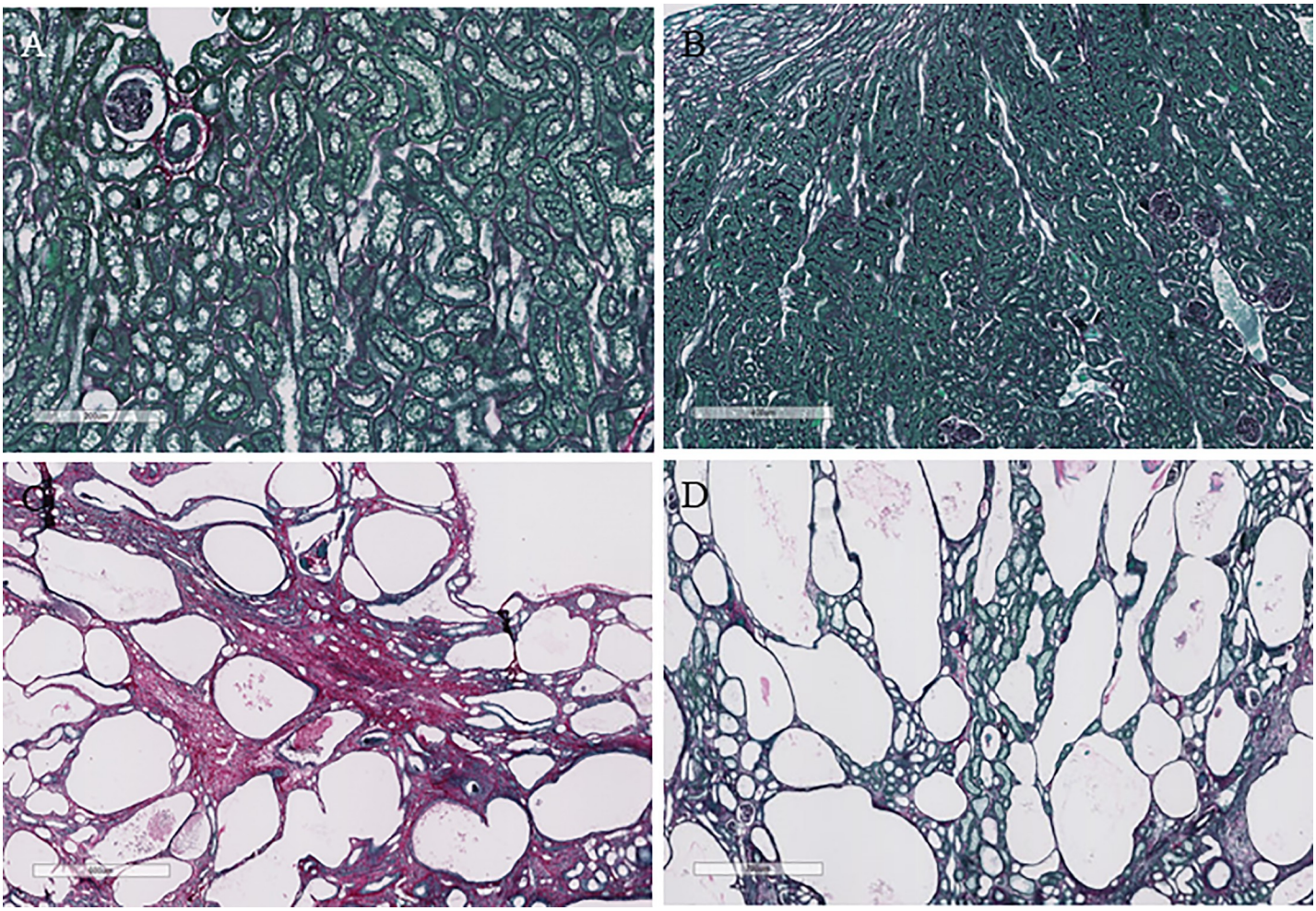

Fig 4. Representative photomicrographs of Sirius red stained sections of kidney tissue showing interstitial collagen deposition at week 20 in Lewis+NWI (A), Lewis +HWI (B), LPK+NWI (C) and LPK+HWI (D). There was no change in renal fibrosis in the Lewis rats with increased water intake, whereas in LPK rats (D) there was a marked attenuation in interstitial collagen deposition in comparison to LPK+NWI (C).

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Lewis+NWI group at week 16 (Lewis+NWI: 86.8±5.9; LPK+NWI: 159±14 mmHg; $p<0.0001$). In LPK rats at week 16, HWI reduced the systolic blood pressure by 15% (LPK+HWI: 134±9 mmHg) when compared to LPK+NWI ($p<0.0022$). There was no difference between Lewis +NWI and Lewis+HWI groups (Fig 5).

Cardiac enlargement and fibrosis. In LPK rats, cardiac enlargement is most likely due to the secondary effects of hypertension [14]. As shown in Table 5, there was a significant increase in cardiac enlargement (as determined by the heart weight to body weight ratio, HW: BW) in LPK+NWI group compared to Lewis+NWI rats at both week 10 and 16. Consistent with effects on systolic blood pressure, the LPK+HWI group had significantly reduced HW: BW ratio (by 21%, $p<0.0082$) compared with LPK+NWI at week 16. In contrast, increased water intake did not affect the HW:BW ratio in Lewis rats (Table 5).

A subset of cardiac tissue from each rat group was also tested for collagen deposition with Sirius red staining, and there was no difference in fibrosis between NWI and HWI in both Lewis and LPK rats (S2 Fig).

Table 4. Effect of increased water intake on renal function.

	Lewis		LPK	
Variables	**NWI**	**HWI**	**NWI**	**HWI**
Week 10	**n = 8**	**n = 8**	**n = 17**	**n = 16**
Serum creatinine (μmol/L)	26±6	26±4	47±23	49±13
CrCl/BW (ml/min/g)	6.4±1.9	7.4±1.7	2.8±0.9*	3.0±1.1
Urine PCR (mg/mmol Cr)	39.8±29.9	116.8±88.9	297.7±499.0	334.2±261.4
Week 16	**n = 8**	**n = 8**	**n = 17**	**n = 16**
Serum creatinine (μmol/L)	33±10	33±9	93±42*	71±31
CrCl/BW (ml/min/g)	5.7±1.4	5.4±1.6	1.7±1.4*	2.0±1.4
Urine PCR (mg/mmol Cr)	23.1±15.3	149.4±101.1	1171.2±1387.8‡	346.6±370.4§

Urine PCR; urine protein creatinine ratio; CrCl/BW, creatinine clearance corrected for body weight. Values represented as mean ± SD.

*p<0.001 versus age-matched NWI Lewis rat

†p<0.001 versus age-matched LPK NWI

‡p<0.05 versus age-matched NWI Lewis rat

§p<0.05 versus age-matched NWI LPK rat

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Discussion

PKD is an insidious and life-long disease with limited treatment options. The current study provides several new insights regarding the effects of increasing water intake in PKD: (i) an increase in water intake markedly attenuated long-term the progression of kidney enlargement, renal cyst growth and renal fibrosis in LPK rats, when initiated during the early stages of renal disease. It is noteworthy that while cystic renal disease was slowed it was not completely abrogated and the decline in renal dysfunction was not altered; (ii) remarkably, only a modest

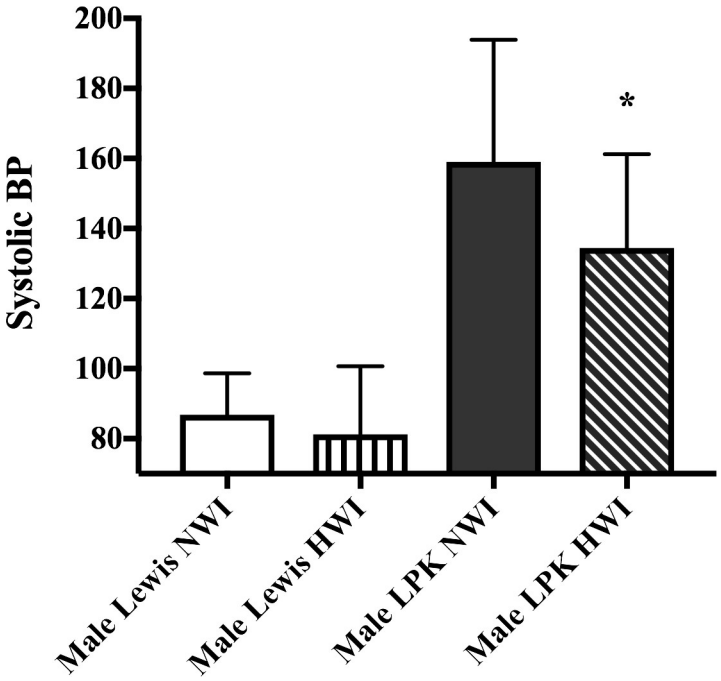

Fig 5. Effect of increased water intake on systolic blood pressure at week 16 in male Lewis and LPK rats. The data is expressed as mean ± SD (n = 4 for Lewis+NWI; n = 4 for Lewis+HWI; n = 8 for LPK+NWI, n = 10 for LPK+HWI); *p<0.01 when compared to Male LPK NWI group.

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Table 5. Effect of increased water intake on cardiac enlargement.

	Males				Females			
	Lewis		LPK		Lewis		LPK	
Variables	NWI	HWI	NWI	HWI	NWI	HWI	NWI	HWI
Week 10	**n = 4**	**n = 4**	**n = 7**	**n = 8**	**n = 3**	**n = 4**	**n = 9**	**n = 8**
Cardiac weight (g)	0.77±0.06	0.68±0.04	1.09±0.07*	1.14±0.08	0.48±0.08	0.51±0.03	0.86±0.07*	0.84±0.08
HW:BW (%)	0.31±0.03	0.3±0.001	0.55±0.07*	0.53±0.06	0.34±.03	0.35±0.02	0.57±0.06*	0.52±0.05
Week 16	**n = 4**	**n = 4**	**n = 8**	**n = 10**	**n = 5**	**n = 4**	**n = 9**	**n = 6**
Cardiac weight (g)	0.85±0.13	0.88±0.05	1.34±0.16*	1.34±0.12	0.64±0.07	0.57±0.13	0.97±0.13*	1.04±0.09
HW:BW (%)	0.28±0.02	0.28±0.02	0.57±0.10*	0.45±0.06§	0.34±0.02	0.30± 0.07	0.53±0.06*	0.57±0.06

HW:BW%: heart weight to body weight ratio expressed as a percentage. mean ± SD.

*p<0.001 versus age-matched NWI Lewis rat

†p<0.001 versus age-matched LPK NWI

‡p<0.05 versus age-matched NWI Lewis rat

§p<0.05 versus age-matched NWI LPK rat.

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increase in water intake (between 1.4 to 1.7-fold increase above the control group) was sufficient to demonstrate a sustained benefit on kidney enlargement LPK rats; and (iii) finally, increased water intake also reduced systolic blood pressure and cardiac enlargement during the later stages of disease. These data extend previous results obtained in the *pck* rat and support the hypothesis that increased water intake reduces the progression of renal cyst growth in experimental PKD [11, 12].

AVP is a critical driver of postnatal kidney cyst growth, and the suppression of its endogenous production is considered a potential therapeutic intervention in PKD [5, 24]. The release of AVP from the pituitary gland is stimulated by an increase in the serum osmolality sensed by specific osmoreceptors [25]. As expected AVP levels have a close proportional relationship to serum osmolality in both humans and Lewis rats, that is reliable even if renal function is impaired. [4, 10]. This is not the case for other surrogate markers of AVP such as copeptin, which accumulates in renal failure [26]. Pharmacological receptor antagonists of AVP consistently reduce renal cyst growth and renal function decline in preclinical models and humans, but off-target effects (massive, rapid-onset and persistent aquaresis and increased risk for liver function derangement) are barriers for universal application in all patients with PKD [3, 27]. Thus, decreasing serum osmolality and subsequent release of endogenous AVP through increased water intake has been considered as an alternative therapy that is simple, easily accessible, and likely to have fewer adverse effects [24].

The efficacy of increased water intake on reducing kidney enlargement in LPK rats in this study is especially interesting because the efficacy is similar to that previously obtained with sirolimus (a very potent anti-proliferative agent), and thus highlights the importance of AVP in promoting cyst growth in PKD [28]. Two previous experimental studies have shown that increasing water intake in the *pck* rat was associated with a reduction in the progression of renal cystic disease [11, 12]. Nagao *et al.* measured the downstream effects of AVP and found the increased water intake reduced the expression of renal AVP V_2 receptors, B-raf and ERK cellular proliferation pathways, and PCNA-positive renal cells likely driving the beneficial effects observed [11]. Similarly, Hopp *et al.* found that increased water intake downregulated ERK phosphorylation and renal cAMP production [12]. In the current study, there was a sustained reduction in serum osmolality (a surrogate marker for AVP) in the LPK+HWI group compared to the LPK+NWI rats at both the early and late stages of disease.

A surprising and unexpected finding of the present study was the relatively modest increase in daily water intake (1.4- to 1.9-fold increase) required to gain the beneficial effect, and this was lower than that observed in previous studies using the *pck* rat [11, 12]. The reasons for this discrepancy are not entirely clear but could be due to differences between physiological changes that occur in the animal models (*pck* rat vs. LPK rat) and methods of water intake measurements. For instance, Nagao *et al*. measured water intake only once during the study period in a metabolic cage at week 13.5 of age [11]. In contrast, Hopp *et al*. estimated water intake extrapolated from urine volume measured in metabolic cages at weeks 6, 8, and 10 of age [12], which may be influenced by urine concentrating capacity [19, 20].

In the present study, water intake was measured using two separate methods: (i) while housed individually in metabolic cages which we considered to be the gold-standard because it allows data measurement from each specific rat; and (ii) while rats were in standard housing cages which was the usual environment of the animals, and allowed continuous monitoring of fluid intake. While the pattern of consumption between the groups was the same between the housing and metabolic cages, the absolute volume of water consumed was increased in the metabolic cage compared to the housing cages (Table 2 and Fig 2). This was possibly due to a change in usual behaviour in the rats while in the individual metabolic cages, compared to the housing cages [22]. The difference in the volume of water consumed between the two environments may also contribute to the much higher targets of 3 to 4-fold increases in water intake reported in previous studies in comparison to this study. Additionally, there may be differences between the sensitivity of endogenous AVP to ingested water between the LPK and *pck* model that contributed to a lower water intake requirement to reduce serum osmolality and thirst drive. Further studies should be undertaken to determine if there is an optimal dose and duration of increased fluid intake to attenuate renal cyst growth in other animal models and also test this hypothesis in humans.

Although a detailed analysis of fluid intake was undertaken, a limitation of this study is that food intake was not measured. The assessment food intake is relevant given recent evidence showing that calorie restriction reduces renal cyst growth and fibrosis, and also that glucose metabolism is altered in renal cysts in PKD [29, 30]. That being said, the results of this study (including data for body weight, osmolality, serum glucose and albumin) do not suggest that differences in caloric intake or a metabolic effect of 5% glucose are likely to confound the observed effects on kidney enlargement. This is because there were no changes in body weight between the HWI and NWI groups. In relation to this, sub-analysis by gender showed that in male rats, body weight was reduced in the LPK+NWI group compared to LPK+HWI at week 16 (S1 Table). Two possible explanation are as follows: (i) food intake was reduced secondary to renal failure-induced anorexia, as serum urea was elevated in the LPK+NWI group compared to LPK+HWI rats (Table 2 and S3 Table). The absence of changes in serum albumin between these two groups does not necessarily argue against this hypothesis, given that is an insensitive marker of nutrition; (ii) net caloric intake was higher in the LPK+HWI groups given the addition of 5% glucose to drinking water, leading to body weight gain. Moreover, regardless of the mechanism, the attenuating effects of 5% glucose on kidney enlargement were observed in all LPK groups, regardless of gender and in the absence of an increase in body weight. These data, in combination with the results showing that there was a sustained decrease in serum osmolality in the LPK+HWI rat groups (Table 2), suggest that the findings are consistent with differences in hydration status between the groups [19].

Despite an improvement in renal cystic disease in the LPK+HWI rats the decline in renal function was not affected. Similarly, Hopp *et al*. did not demonstrate a change in serum creatinine with 5% glucose, and Nagao *et al*. reported a change in serum urea which may reflect hydration, but creatinine levels were not measured [11, 12]. The absence of a benefit of

increased water intake on renal function in LPK rats is consistent with previous studies from our laboratory (using other therapeutic agents such as sirolimus), and likely reflects the persistent changes in abnormal cystic microarchitecture that continued in increased water intake groups despite reduction in cyst size [15, 31]. In addition, the sustained activation of non-AVP related growth factors may also contribute to renal impairment and sustained renal inflammation as demonstrated by the persistent α-SMA and ED-1+ cell accumulation seen in LPK +HWI rats, which was not suppressed by increased water intake [32, 33].

An important observation in the current study is that increased water intake reduced hypertension in male LPK rats. The mechanism of this effect could be related to the two main sites of action of AVP; at vascular smooth muscle where it causes vasoconstriction, and at the renal distal tubule where it increases blood volume, cardiac output and arterial pressure through activation of the protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway [28, 34]. In cystic renal diseases, AVP-mediated stimulation at these sites result in hypertension and associated cardiovascular disease (such as concentric cardiac hypertrophy and diastolic impairment) [28, 34]. The beneficial effects observed in this study could be due to inhibition of these pathways. This finding is in keeping with clinical trials which showed that the use of AVP antagonists improved of hypertension and heart failure [35]. Given the cardiovascular disease burden in PKD, these results provide an important off-target protective benefit of increased water intake. The effect of increased water intake on blood pressure was also mirrored by a reduction in the heart to body weight ratio. While these data are supportive of the reduction in hypertension, a caveat that must be considered is that the absolute heart weight was similar in both groups, and therefore the differences in the ratio in male rats could simply be due to the body weight (S1 Table).

The strengths of the current study include the long duration of the study (which allowed sampling of the effects of hydration at an intermediate and late stage of PKD), the study of cardiovascular disease biomarkers (hypertension and the HW:BW ratio), the high frequency of water intake measures to ensure accurate recording of increased water consumption, the detailed renal histological analysis and finally the inclusion of both male and female animals which allowed us to confirm that there was no gender-bias on the protective effects of increased water intake. There were also several limitations; (i) while plasma osmolality reliably correlates with a reduction in AVP, no downstream markers of AVP measured; (ii) as discussed earlier, caloric intake and food consumption were not determined, but as mentioned above, the effects of this on the current study is unlikely to be significant; (iii) other methods to induce increase water intake (such as agarose gel) were not examined, and (iv) finally, the effects of increased water intake during the later phase of PKD (that is, between week 10 and 16) were not investigated.

In conclusion, the results of this study show that increased water intake reduces the progression of renal disease in the LPK rat model of PKD. Uniquely, our study shows that, an early and relatively modest increase in water intake is sufficient for long-term beneficial effects in LPK rat, and that the intervention also reduces systolic blood pressure and cardiovascular enlargement. Future preclinical and clinical studies should determine: (i) whether there is optimal amount and duration of increased water intake for slowing renal cystic renal disease; (ii) the comparative efficacy of increased water intake compared to pharmacological receptor blockade of AVP; (iii) the additive effects (if any) of increased water intake with other proven therapeutic interventions, and (iv) finally the mechanisms underlying the lowering of systolic blood pressure with increased water intake in PKD. Hitherto, few preclinical studies have investigated the effects of increased water intake, but the results of the current paper suggest that this line of investigation could be of immense value to the PKD community and other stakeholders.

Supporting information

S1 Table. Gender and effect of increased water intake on body weight, kidney weight, and cyst size at week 10.
(DOCX)

S2 Table. Gender and effect of increased water intake on renal inflammation and myofibroblast infiltration at week 10.
(DOCX)

S3 Table. Gender and effect of increased water intake on renal function, serum sodium and albumin.
(DOCX)

S1 Fig. Representative photomicrographs of alpha-smooth muscle actin stained sections of renal tissue at week 10. (A) Lewis NWI, (B) Lewis HWI, (C) LPK NWI, (D) Lewis HWI. Please see methods section for further details on staining protocol.
(TIF)

S2 Fig. Representative photomicrographs of sirius red stained sections of cardiac tissue at week 16. (A) Lewis NWI, (B) Lewis HWI, (C) LPK NWI, (D) Lewis HWI. Red-stained areas are consistent with the deposition of collagen. Please see methods section for further details on staining protocol.
(TIF)

Author Contributions

Conceptualization: Priyanka S. Sagar, Annette T. Y. Wong, Gopala K. Rangan.

Data curation: Priyanka S. Sagar, Magda Luciuk, Carly Mannix, Annette T. Y. Wong, Gopala K. Rangan.

Formal analysis: Priyanka S. Sagar, Jennifer Zhang, Magda Luciuk, Gopala K. Rangan.

Funding acquisition: Annette T. Y. Wong, Gopala K. Rangan.

Investigation: Priyanka S. Sagar, Annette T. Y. Wong, Gopala K. Rangan.

Methodology: Priyanka S. Sagar, Jennifer Zhang, Magda Luciuk, Carly Mannix, Annette T. Y. Wong, Gopala K. Rangan.

Project administration: Priyanka S. Sagar.

Resources: Carly Mannix, Annette T. Y. Wong, Gopala K. Rangan.

Supervision: Annette T. Y. Wong, Gopala K. Rangan.

Validation: Priyanka S. Sagar.

Writing – original draft: Priyanka S. Sagar.

Writing – review & editing: Priyanka S. Sagar, Magda Luciuk, Carly Mannix, Annette T. Y. Wong, Gopala K. Rangan.

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2.3 Supplementary Material

S1 Table: Gender and effect of increased water intake on body weight, kidney weight, and cyst size at week 10

	Males				Females			
	Lewis		LPK		Lewis		LPK	
Variables	NWI	HWI	NWI	HWI	NWI	HWI	NWI	HWI
Week 10	**n = 4**	**n = 4**	**n = 8**	**n = 8**	**n = 3**	**n = 4**	**n = 9**	**n = 8**
Body weight (g)	246±16	225±17	200±18*	216±19	143±11	145±6	150±10	161±7
Kidney weight (g)	1.9±0.1	1.6±0.2	14.3±1.5*	7.4±1.3†	1.2±0.1	1.1±0.0	11.1±1.1*	5.2±0.8†
Kidney: body weight (%)	0.79±0.01	0.73±0.03	7.18±0.86*	3.49±0.85†	0.83±0.01	0.74±0.03	7.40±0.77*	3.22±0.40†
Renal section area (mm2)	53.9±3.7	34.1±4.3*	186.5±22.9*	124.1±18.0§	34.1±4.3	35.9±1.2	164.2±17.0*	97.1±9.7†
Cyst area (mm2)	9.3±3.6	6.3±2.9	117.7±18.2*	66.8±17.0†	5.7±1.3	5.1±1.9	108.4±11.3*	51.8±6.8†
Cyst area: renal section area (%)	17.8±7.8	15.5±5.5	63.1±5.8*	53.4±6.5	16.3±2.1	14.3±5.7	66.0±3.0*	53.4±4.0§
Serum glucose (mmol/L)	13.7±2.4	13.3±0.7	10.2±1.9	13.1±4.5	12.8±3	12.2±3.5	11.2±3.1	12.1±4
Serum albumin (g/L)	28±3	28±1	27±1	28±1	30±1	30±1	31±4	31±2
Week 16	**n = 4**	**n = 4**	**n = 8**	**n = 10**	**n = 5**	**n = 4**	**n = 9**	**n = 6**
Body weight (g)	309±25	316±19	242±48*	301±29†	187±18	185±6	184±31	184±16
Kidney weight (g)	2.2±0.1	2.0±0.2	17.2±3.0*	13.5±2.6§	1.4±0.2	1.2±0.1	15.2±3.3*	7.8±2.0†
Kidney: body weight (%)	0.70±0.02	0.63±0.03	7.18±0.73*	4.49±0.64†	0.74±0.07	0.67±0.04	8.25±0.92*	4.31±1.31†

Renal section area (mm2)	53.9±3.7	34.1±4.3*	186.5±22.9*	124.1±18.0§	34.1±4.3	35.9±1.2	164.2±17.0*	97.1±9.7†
Cyst area (mm2)	12.8±5.2	11.0±3.7	153.3±24.7*	119.5±22.3§	6.0±1.0	6.4±0.7	134.6±31.8*	73.4±23.1§
Cyst area: renal section area (%)	21.5±7.4	19.0±5.6	67.8±4.3*	63.9±4.1	16.2±2.2	17.0±1.6	68.6±4.8*	58.4±9.0§
Serum glucose (mmol/L)	13.4±3.4	11.6±1.5	10.8±3.1	10.2±0.8	10.7±2.5	14.4±1.9	9.2±1.1	11.2±3.1
Serum albumin (g/L)	30±1	33±1	30±1	30±2			31±4	31±4

*p<0.001 versus age-matched NWI Lewis rat, †p<0.001 versus age-matched LPK NWI, ‡p<0.05 versus age-matched NWI Lewis rat, §p<0.05 versus age-matched NWI LPK rat

<u>S2 Table:</u> Gender and effect of increased water intake on renal inflammation and myofibroblast infiltration at week 10

	Males				**Females**			
	Lewis		**LPK**		**Lewis**		**LPK**	
Variables	**NWI**	**HWI**	**NWI**	**HWI**	**NWI**	**HWI**	**NWI**	**HWI**
Week 10	**n = 4**	**n = 4**	**n = 8**	**n = 8**	**n = 3**	**n = 4**	**n = 9**	**n = 8**
Renal section area (mm2)	54.6±3.4	43.8±2.1‡	186.6±24.9*	124.0±14.9§	34.2±3.0	35.9±1.2	131.2±17.7*	97.1±10.2§
Myofibroblast infiltration (mm^2)	1.0±0.7	0.9±0.4	10.3±6.1	7.7±3.9	1.5±1.6	1.4±0.5	9.3±5.2	5.5±2.7

Myofibroblast index (%)	2.0±1.3	2.3±1.2	10.5±5.3	10.7±4.5	4.5±4.0	4.3±1.3	13.9±8.2	10.1±4.7
Monocyte infiltration (mm^2)	0.4±0.3	0.3±0.1	0.6±0.2	0.7±0.3	0.3±0.1	0.4±0.4	0.8±0.7	0.5±0.3
Monocyte index (%)	0.9±0.6	0.9±0.6	1.0±0.2	1.5±0.6	1.0±0.3	1.3±1.1	1.3±1.0	1.2±0.6
Interstitial collagen deposition (mm^2)	4.9±1.7	1.1±0.6	33.1±23.6	19.5±8.4	1.9±0.7	2.1±1.7	22.4±9.3	14.6±5.8
Interstitial collagen deposition index (%)	9.0±0.3	2.5±1.3	19.0±15.9	15.9±7.2	5.7±2.1	6.0±4.7	13.8±4.7	15.0±5.1
Week 16	**n = 4**	**n = 4**	**n = 8**	**n = 8**	**n = 3**	**n = 4**	**n = 9**	**n = 8**
Renal section area (mm2)	56.9±6.3	55.6±3.4‡	230.7±31.9*	179.6±26.3§	37.2±4.7	37.5±3.2	200.2±45.5*	120.6±28.4§
Myofibroblast infiltration (mm^2)	1.5±0.9	1.4±0.5	11.6±4.5‡	13.5±2.7	1.1±0.3	0.7±0.5	10.8±8.3‡	4.8±2.8
Myofibroblast index (%)	2.9±1.8	2.6±0.9	11.4±5.8	15.6±5.4	3.1±0.8	2.3±1.8	13.2±9.0	7.7±3.9
Monocyte infiltration (mm^2)	0.4±0.2	0.3±0.2	0.6±0.2	0.4±0.1	0.2±0.1	0.4±0.6	0.5±0.3	0.5±0.1
Monocyte index (%)	0.9±0.6	0.7±0.6	0.8±0.3	0.7±0.2	0.5±0.4	1.1±1.5	0.9±0.7	1.1±0.3
Interstitial collagen deposition (mm^2)	4.5±3.5	3.9±1.6	43.7±21.4	27.0±11.8	3.4±1.7	1.7±1.9	58.5±42.2	21.8±8.5§
Interstitial collagen deposition index (%)	8.3±6.7	7.3±2.9	20.1±12.8	15.2±7.4	9.2±4.0	4.5±5.0	29.3±20.7	19.2±9.1

*p<0.001 versus age-matched NWI Lewis rat, †p<0.001 versus age-matched LPK NWI, ‡p<0.05 versus age-matched NWI Lewis rat, §p<0.05 versus age-matched NWI LPK rat

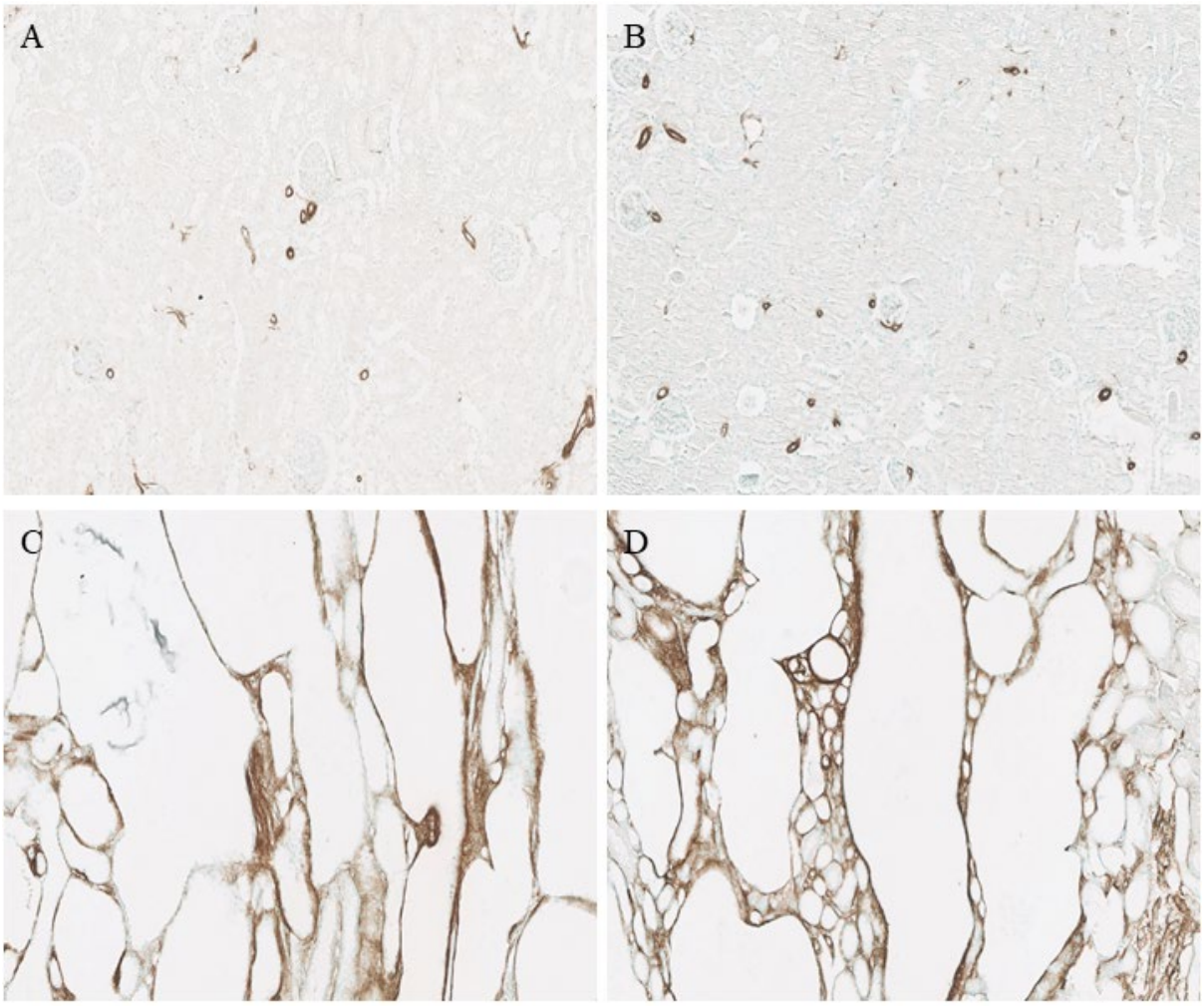
S3 Table: Gender and effect of increased water intake on renal function, serum sodium and albumin.

	Males				Females			
	Lewis		LPK		Lewis		LPK	
Variables	**NWI**	**HWI**	**NWI**	**HWI**	**NWI**	**HWI**	**NWI**	**HWI**
Week 10	**n = 4**	**n = 4**	**n = 8**	**n = 8**	**n = 3**	**n = 4**	**n = 9**	**n = 8**
Serum creatinine (umol/L)	24±7	23±3	53±32	50±17	28±6	28±3	40±4‡	48±8
Serum urea (mmol/L)	6.8±2	6.1±0.6	21.6±7.1*	9.2±3.4†	6±0.2	5.2±0.8	19.1±4.5*	9.6±2.6†
Urine PCR (mg/mmol Cr)	51.6±42.4	82.9±62.0	694.9±851.4	362.0±147.2	31.4	82.8±32.8	139.9±69.7	46.3±16.6§
CrCl/BW(ml/min/g)	7.00±2	8.50±1	3.00±1.07*	3.50±1.31	5.67±2.08	6.25±1.50	2.67±0.71‡	2.50±0.53
Serum sodium (mmol/L)	143±1	142±5	146±5	129±11	146±1	142±2	148±2	145±3
Week 16	**n = 4**	**n = 4**	**n = 8**	**n = 8**	**n = 3**	**n = 4**	**n = 9**	**n = 8**
Serum creatinine (umol/L)	35±13	27±2	114±49‡	78±36	31±9	39±8	73±22‡	58±17
Serum urea (mmol/L)	8.1±1.8	6.4±0.5	31.6±10.4*	19±8.1†	6.5±0.8	5.1±0.6	26.7±7.2*	12.4±3.5†
Urine PCR (mg/mmol Cr)	25.5±17.8	179.6±138.3	1716.1±1854.6	272.6±178.0§	15.6	119.2±47.7	686.8±530.6	470±570.4
CrCl/BW(ml/min/g)	5.25±1.71	6.5±1.29	1.38±1.41*	2.33±1.32	6±1.22	4.25±0.95	1.89±1.36*	1.4±1.51
Serum sodium (mmol/L)	143±5	144±3	144±3	142±2	147±9	148±4	147±6	138±14

Urine PCR; urine protein creatinine ratio; CrCl/BW, creatinine clearance corrected for body weight. Values represented as mean ± SD.

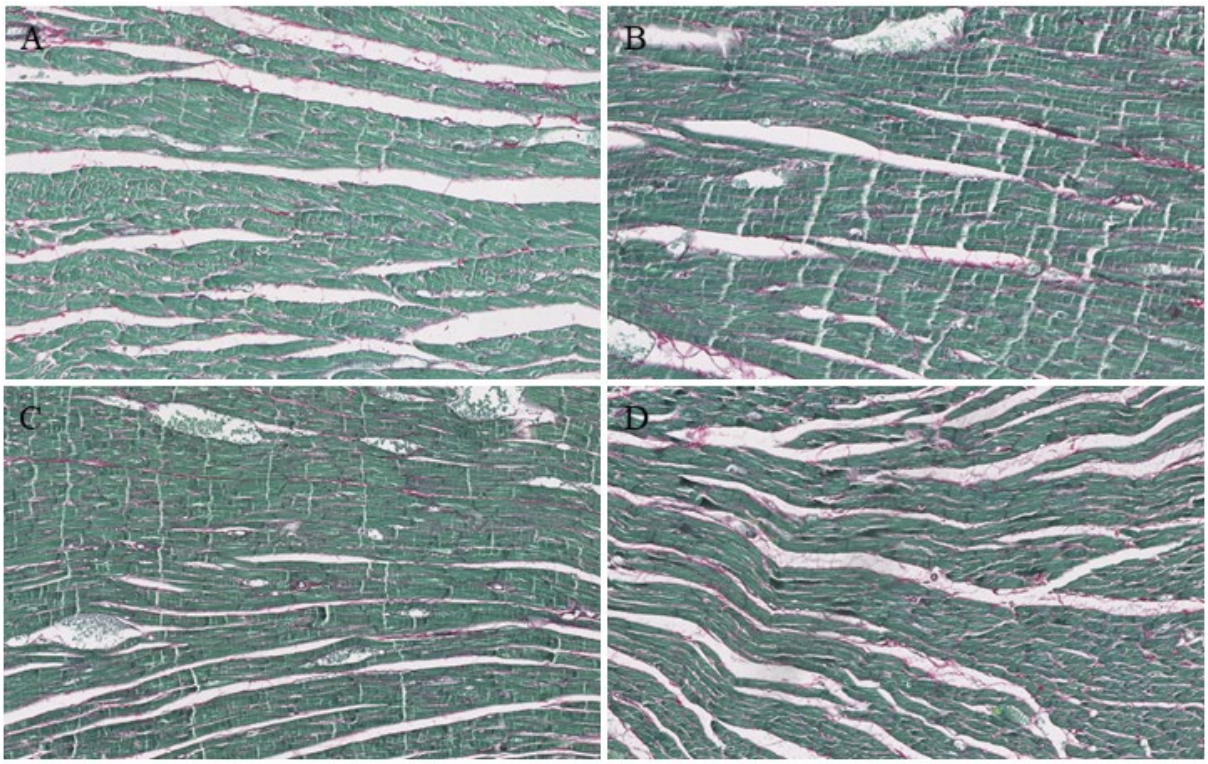
*p<0.001 versus age-matched NWI Lewis rat, †p<0.001 versus age-matched LPK NWI, ‡p<0.05 versus age-matched NWI Lewis rat, §p<0.05 versus age-matched NWI LPK rat

<u>S1 Figure</u>: Representative photomicrographs of alpha-smooth muscle actin stained sections of renal tissue at week 10.



(A) Lewis NWI, (B) Lewis HWI, (C) LPK NWI, (D) Lewis HWI. Please see methods section for further details on staining protocol.

S2 Figure: Representative photomicrographs of sirius red stained sections of cardiac tissue at week 16.



(A) Lewis NWI, (B) Lewis HWI, (C) LPK NWI, (D) Lewis HWI. Red-stained areas are consistent with the deposition of collagen. Please see methods section for further details on staining protocol.

Chapter 3: Effect of vitamin D receptor agonists on cardiorenal disease progression in experimental polycystic kidney disease

3.1 Preface

Vitamin D is a mediator of many cellular and paracellular functions, including suppression of renin gene expression, inflammation and fibrosis, and angiogenic growth factor modulation. (70) Vitamin D deficiency is common in all kidney diseases including ADPKD, and activation of the vitamin D receptor may modulate the development of kidney and vascular inflammation/fibrosis, RAAS-driven hypertension and LVH. (87) VDRAs are commonly used in treatment of vitamin D deficiency in CKD and, if found to be effective in ADPKD, could be rapidly repurposed and accessible to patients.

Paricalcitol (19-nor-1,25-dihydoxy vitamin-D2) is an "active" form of vitamin D and is a potent activator of the vitamin D receptor in the multitude of tissues which express the receptor. (153) Paricalcitol has a similar side effect profile compared to other VDRAs and can be administered intraperitoneally, and so was chosen as the intervention for this study. (153)

The study described in this chapter aims to explore the effect of vitamin D receptor activation on reducing cardiovascular and cystic disease in early and late experimental PKD. The hypothesis is that treatment with VDRA will attenuate cardiorenal disease progress in the *LPK* rat model of PKD.

Given the potential for vitamin D receptor activation to have an impact in early growth factor modulation and in the later inflammatory and hypertensive states, this study had two separate experimental designs. The first examines the effect of paricalcitol from postnatal week 3 to 10 or "early PKD", which is during a period of rapid microscopic cyst growth but prior to the onset of detectable kidney impairment or elevated blood pressures. (152) The second

examines the effect of paricalcitol from postnatal week 10 to 20, when there is detectable kidney and cardiovascular disease. Given that standard treatment of RAAS blockade is indicated at this disease stage and the expected effects of VDRAs are synergistic with RAAS blockade, the effect of enalapril, an ACEi, in conjunction with VDRA was tested, along with VDRA alone, enalapril alone and vehicle alone.

3.2 Effect of early and delayed commencement of paricalcitol in combination with enalapril on the progression of experimental polycystic kidney disease

The following manuscript describing the study was published in the peer-reviewed *Journal of Cardiovascular Development and Disease*, first online on 29th October 2021 and then in print in November 2021. The formatting and writing style are in keeping with the journal requirements. References are self-contained within the manuscript. Section 3.3 contains supplementary material referenced in the manuscript.

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Effect of Early and Delayed Commencement of Paricalcitol in Combination with Enalapril on the Progression of Experimental Polycystic Kidney Disease

Priyanka S. Sagar [1,2], Sayanthooran Saravanabavan [1,2], Alexandra Munt [1,2], Annette T. Y. Wong [1,2] and Gopala K. Rangan [1,2,*]

[1] Michael Stern Laboratory for Polycystic Kidney Disease, Westmead Institute for Medical Research, The University of Sydney, Sydney, NSW 2145, Australia; priyanka.sagar@sydney.edu.au (P.S.S.); sayan.saravanabavan@sydney.edu.au (S.S.); alexandra.munt@sydney.edu.au (A.M.); annette.wong@sydney.edu.au (A.T.Y.W.)
[2] Department of Renal Medicine, Westmead Hospital, Western Sydney Local Health District, Sydney, NSW 2145, Australia
* Correspondence: g.rangan@sydney.edu.au

Abstract: Vitamin D secosteroids are intranuclear regulators of cellular growth and suppress the renin-angiotensin system. The aim of this study was to test the hypothesis that the vitamin D receptor agonist, paricalcitol (PC), either alone or with enalapril (E) (an angiotensin-converting enzyme inhibitor), reduces the progression of polycystic kidney disease. Preventative treatment of Lewis polycystic kidney (LPK) and Lewis control rats with PC (0.2 µg/kg i.p. 5 days/week) or vehicle from postnatal weeks 3 to 10 did not alter kidney enlargement. To evaluate the efficacy in established disease, LPK rats received either PC (0.8 µg/kg i.p; 3 days/week), vehicle, E (50 mg/L in water) or the combination of PC + E from weeks 10 to 20. In established disease, PC also did not alter the progression of kidney enlargement, kidney cyst growth or decline in renal function in LPK rats. Moreover, the higher dose of PC was associated with increased serum calcium and weight loss. However, in established disease, the combination of PC + E reduced systolic blood pressure and heart-body weight ratio compared to vehicle and E alone ($p < 0.05$). In conclusion, the combination of PC + E attenuated cardiovascular disease but caused hypercalcaemia and did not alter kidney cyst growth in LPK rats.

Keywords: polycystic kidney disease; paricalcitol; vitamin D receptor agonists

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1. Introduction

Polycystic kidney diseases (PKD) are a group of inherited conditions associated with the development of numerous enlarging renal cysts that lead to life-threatening end-stage kidney disease (ESKD) [1]. Autosomal dominant polycystic kidney disease (ADPKD) is the most prevalent form of PKD in adults and affects 6.5 million people worldwide [1]. Early-onset hypertension occurs prior to the onset of renal impairment and, in addition to left ventricular hypertrophy, contributes to increased mortality, with cardiovascular disease as the most common cause of death for ADPKD patients [2–5]. In ADPKD patients, hypertension presents on average in their mid-thirties, and they have a greater prevalence of cardiovascular risk factors compared to the general CKD population [5]. Current medical management of ADPKD is focused on the treatment of hypertension with renin-angiotensin inhibitors, such as angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, along with dietary and lifestyle modification to reduce sodium intake and maintain a healthy body mass index, however these interventions only partially slow disease progression [4,6]. Recently, the vasopressin receptor-2 antagonist, tolvaptan, was introduced as the only disease modifying drug for ADPKD, but its universal implementation is limited by off-target adverse effects that include polyuria and idiosyncratic hepatotoxicity. Hence

additional pharmacological approaches for PKD that have minimal adverse effects and utilise re-purposed medicines are needed [1,6,7].

The vitamin D group of fat-soluble secosteroids (D_1–D_5), are widely utilised in chronic kidney disease (CKD) for the management of secondary hyperparathyroidism. Aside from their classical function in maintaining calcium homeostasis, the vitamin D secosteroids are also intranuclear regulators of cellular growth and differentiation [1,8–10] and in vivo have been found to have additional renal- and vascular-protective properties, including the suppression of inflammation, fibrosis [11–16], and blood pressure [1,8,13]. In this regard, vitamin D receptor knockout mice develop severe hypertension [17] and, conversely, vitamin D receptor agonists (VDRAs) such as paricalcitol lower blood pressure in conjunction with inhibitors of the renin-angiotensin-aldosterone system (RAAS) in experimental CKD [18]. Data on the role of the vitamin D system on the progression of PKD are limited. In a hypertensive chronic model of PKD, the Lewis Polycystic Kidney Disease (LPK) rat model, chronic vitamin D deficiency exacerbated hypertension (as expected) but mildly reduced kidney cyst growth, suggesting that the vitamin D system may have divergent effects in PKD [19].

Paricalcitol (19-nor-1,25-dihydoxy vitamin-D_2) is 1000-fold more potent than calcitriol but carries a similar risk of adverse effects, such as hypercalcemia [20]. Based on previous studies in the LPK rats, we hypothesised that paricalcitol may reduce blood pressure and proteinuria. However, as chronic vitamin D deficiency caused a mild reduction in kidney cyst growth in LPK rats, it was important to evaluate whether paricalcitol exacerbated kidney cyst growth. To our knowledge the role of VDRAs in PKD has not been previously investigated [21]. Therefore, the aim of this study was to investigate whether VDRAs have therapeutic effects in PKD and to test the hypothesis that VDRAs reduce the progression of proteinuria and hypertension in the Lewis Polycystic Kidney Disease rat model of PKD (LPK rat) [19]. To test this hypothesis, two sequential chronic experiments were performed to evaluate the preventative (early treatment) and therapeutic (delayed treatment) effects of paricalcitol on the progression of PKD.

2. Materials and Methods

2.1. Animals

Animals were housed at the animal research facility in the Institute for Clinical Pathology at Westmead Hospital under standard conditions (artificial light, light-dark cycle 1800-0600) with access to water and standard rat chow ad libitum [19]. LPK and Lewis/SSN rats were obtained from the breeding colony at Westmead Hospital [19]. The LPK rat model is a genetic ortholog of human nephronophthisis (NPHP)-9 but phenotypically resembles human autosomal recessive PKD with rapid early and marked cystogenesis between postnatal weeks 3 to 10, the onset of hypertension at week 6, followed by progressive cystic related tubulointerstitial disease leading to renal failure and death soon after week 20 [22–24]. All experiments were conducted according to the Australian Code for the Care and Use of Animals for Scientific Purposes [25], and the study ethics protocol was approved by the Western Sydney Local Health District Animal Ethics Committee (Protocol number 4100).

2.2. Experimental Design

To determine the effects of early and delayed treatment paricalcitol on the progression of early and established PKD in the LPK rat, two experiments were performed. In Experiment 1, LPK (n = 16) and Lewis (n = 6) rats were treated from postnatal week 3 until week 10 with either vehicle (V) or paricalcitol (PC) at 0.2 µg/kg administered intraperitoneally (i.p.) 5 days/week with the dose determined by previous studies in rat models of CKD, to minimise the risk of any adverse effects on serum calcium elevation [26]. Rats were weighed daily and the average weight per group was used to determine the dose of paricalcitol. At week 10, rats were placed in metabolic cages for 16 hours for urine collection and sacrificed the following day.

The design of Experiment 2 was based on the results of Experiment 1 and aimed to determine whether a higher dose of PC together with a combination of angiotensin blockade had efficacy in established disease, based on previous studies in models of hypertension and CKD [11,27]. Enalapril, an angiotensin-converting enzyme inhibitor, was used for renin-angiotensin blockade. In Experiment 2, LPK and Lewis rats were treated with either vehicle (V), paricalcitol (PC; 0.8 μg/kg i.p.; 3 days/week), enalapril (E; 50 mg/L in tap water), or a combination of both paricalcitol and enalapril (PC + E) from postnatal week 10 until week 20, with dosing based on previous studies [11,15,28,29]. As mentioned, the dose and frequency of PC was modified in Experiment 2 (compared to Experiment 1) based on previous studies to determine if a higher weekly dose had an effect on kidney enlargement and also to evaluate the effects on blood pressure and proteinuria [11,15]. Rats were weighed weekly and water intake was measured twice a week (as previously described [24]) to ensure that the amount of E consumed was similar between combined and single treatment groups. Rats were placed in metabolic cages for 16 hours at weeks 13, 16 and 19 to collect urine for analysis of renal function. At sacrifice, rats were anaesthetized by an intraperitoneal injection of ketamine/xylazine, blood was collected by cardiac puncture, and both kidneys and heart were removed by surgical dissection as described in previous studies [23].

2.3. Assessment of Serum Calcium, Phosphate, Albumin and Renal Function

Serum 25-hydroxy (OH) vitamin (Experiment 1 only), corrected calcium, phosphate, creatinine and albumin were analyzed at the Institute for Clinical Pathology and Medical research (ICPMR) at Westmead Hospital, as previously described [19]. In Experiment 2, urine volume, proteinuria, urine calcium and creatinine were additionally analyzed and creatinine clearance was calculated and corrected for body surface area [19].

2.4. Tail-Cuff Systolic Blood Pressure

Serial measurements of tail-cuff systolic blood pressure were undertaken non-invasively in conscious rats using a tail-sensor (MacLab, AD Instruments) and tail-cuff inflation at week 13, 16 and 19 as previously described [23]. The systolic blood pressure was defined as the appearance of the tail arterial pulse wave with cuff deflation. Five measurements were undertaken at each session for each rat and the mean calculated.

2.5. Histology

For experiment 2, coronal slices of kidney and heart were immersion-fixed in methyl carnoy solution and embedded in paraffin. Tissue sections, cut at 4 microns in thickness, were stained with Periodic-acid Schiff. Because cystic kidney disease in the LPK rat at week 20 is diffuse and very severe, only qualitative analyses of the sections were performed.

2.6. Statistical Analysis

The data was entered into JMP Pro statistical software (Version 15.2, SAS institute) and Prism (version 9.1.0, GraphPad) and presented as mean and standard deviation. Comparison between groups was performed by ANOVA followed by post-hoc analysis with Tukey–Kramer honestly significant difference test. A p value less than 0.05 was defined as statistically significant.

3. Results

3.1. Effect of Early Treatment with Paricalcitol on Disease Progression in LPK Rats

3.1.1. Renal function, Proteinuria and Serum Calcium

There were no deaths or adverse events. At Week 10, LPK + V rats developed increased proteinuria and urine volume, and impairment of endogenous creatinine clearance without significantly increased serum creatinine compared to the Lewis + V rats, and this was not affected by treatment with PC (Table 1). There was also no change in serum calcium in PC treated rats at week 10 compared with V (Table 1). There was a significant reduction in 25,

hydroxy-vitamin D in both LPK rat groups compared with Lewis rats, as expected with early kidney disease ($p < 0.01$, Table 1).

Table 1. Effect of early treatment with paricalcitol on renal function and disease progression at week 10.

	Lewis + V (n = 3)	Lewis + PC (n = 3)	LPK + V (n = 8)	LPK + PC (n = 8)
Serum Ca (mmol/L)	2.5 ± 0.1	2.4 ± 0.1	2.3 ± 0.4	2.3 ± 0.2
Serum Albumin (mg/dL)	27 ± 2	27 ± 2	27 ± 4	28 ± 4
Serum Creatinine (µmol/L)	28 ± 2	35 ± 16	38 ± 7	42 ± 5
Serum 25-OH vitamin D	133 ± 9	120 ± 13	82 ± 14 *	95 ± 14 *
Urine volume (mls/16hrs)	5 ± 1	5 ± 1	11 ± 4 *	10 ± 3 *
CrCl (µmol/L/g BW)	7.8 ± 0.9	7.0 ± 2.8	4.5 ± 1.5 *	3.4 ± 1.2 *
Urine PCR (mg/mmol Cr)	5.4 ± 0.7	5.3 ± 0.8	162.7 ± 49.1 *	147.2 ± 62.9 *[1]

[1] Abbreviations: LPK, Lewis polycystic kidney rat; V, vehicle; PC, paricalcitol; Ca, corrected calcium; 25-OH vitamin D, 25-hydroxy vitamin D; CrCl, endogenous creatinine clearance corrected for body weight (BW); PCR, protein to creatinine ratio. Data expressed as mean ± standard deviation. * $p < 0.01$ compared to Lewis + V.

3.1.2. Renal and Cardiac Enlargement

The two-kidney weight to body weight ratio is a surrogate marker of cyst growth and increased 6-fold in LPK + V rats compared to the Lewis + V rats at week 10. Treatment with PC did not alter the progression of kidney enlargement (Table 2). Cardiac enlargement, as determined by the heart-to-body weight ratio which is a surrogate marker for left ventricular hypertrophy in LPK rats, was 1.3-fold higher in LPK + V rats compared to the Lewis + V rats and was not affected by PC treatment (Table 2).

Table 2. Effect of early treatment with paricalcitol on renal and cardiac enlargement at week 10.

	Lewis + V (n = 3)	Lewis + PC (n = 3)	LPK + V (n = 8)	LPK + PC (n = 8)
Week 10 BW (g)	282 ± 5	283 ± 11	232 ± 14 *	232 ± 14 *
Right KW (g)	1.1 ± 0.1	1.1 ± 0.1	6.7 ± 0.6 *	6.9 ± 0.9 *
Left KW (g)	1.1 ± 0.1	1.1 ± 0.1	6.9 ± 0.8 *	6.8 ± 0.8 *
2KW:BW ratio (%)	0.8 ± 0.0	0.8 ± 0.0	5.9 ± 0.5 *	5.7 ± 0.5 *
HW (g)	0.83 ± 0.05	0.83 ± 0.04	0.94 ± 0.07 *	1.00 ± 0.05 *
HW:BW ratio (%)	0.29 ± 0.01	0.29 ± 0.03	0.40 ± 0.02 *	0.41 ± 0.03 *[1]

[1] Abbreviations: LPK, Lewis polycystic kidney rat; V, vehicle; PC, paricalcitol; BW, body weight; KW, kidney weight; HW, height weight. Data expressed as mean ± standard deviation. * $p < 0.01$ compared to Lewis + V.

3.2. Effect of Delayed Treatment with Paricalcitol on Disease Progression in LPK Rats

3.2.1. General Health of LPK and Lewis Rats

Two LPK rats died in the study. At week 18, one rat in the LPK + V group developed hemiplegia and was suspected to have suffered a cerebrovascular event and was euthanized early. At week 19, another rat in the LPK + PC was found dead, with autopsy showing the likely cause of death was ESKD. Both rats were excluded in the final timepoint analyses. There was no difference in body weight between Lewis and LPK rats treated with either V, PC, E or PC + E up to week 16. From week 16 onwards, LPK rats from all groups were smaller than the PKD-unaffected Lewis rats. LPK rats treated with PC alone or in combination with E consistently had lower body weights from week 18 onwards (Figure 1, Table S1).

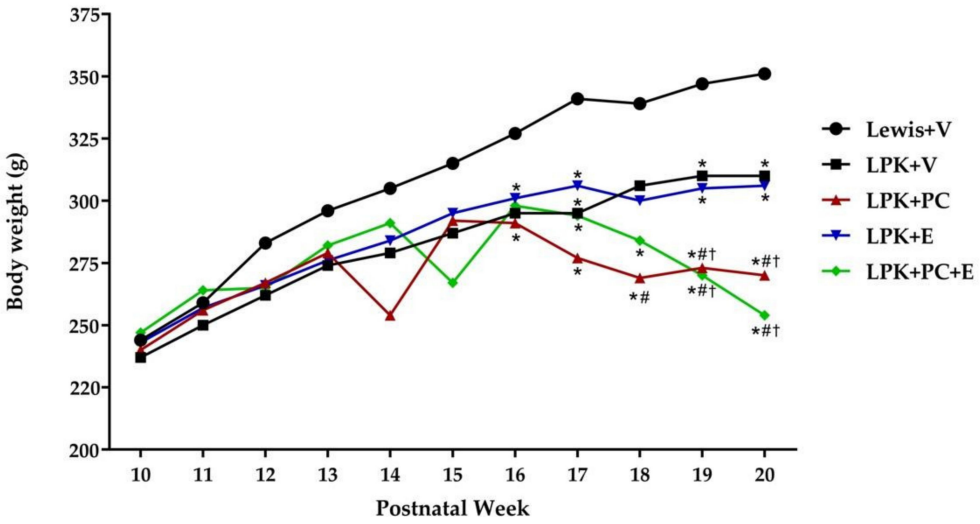

Figure 1. Body weight over time in late PKD. Abbreviations: LPK, Lewis polycystic kidney rat; V, vehicle; PC, paricalcitol; E, enalapril; g, grams. Data represented as mean. * p = <0.05 compared to Lewis + V group, # $p < 0.05$ compared to the LPK + V group. † $p < 0.05$ compared to the LPK + E group.

3.2.2. Water Intake and Urine Output in Lewis and LPK Rats

Water intake was increased in all LPK rat groups compared to Lewis + V (Table S2). At week 13, 16 and 19, LPK rats urine output was significantly increased compared to Lewis rats, as expected, probably due to loss of renal concentrating ability (Table S3). There was no change in urine output between LPK groups (Table S3).

3.2.3. Renal Function, Serum Calcium, Serum Phosphate and Urinary Calcium Excretion

At week 20 renal function, as determined by both serum creatinine and endogenous creatinine clearance, was significantly impaired in LPK rats compared to the Lewis + V and this was not affected by treatment with PC, E or a combination of PC + E (Table 3). At Week 20, LPK rats receiving PC developed a 1.3-fold increase in serum calcium compared to the LPK + V group ($p < 0.01$, Table 3). Serum phosphate was elevated in LPK + PC + E rats compared to LPK + V and LPK + E groups, but there was no significant difference between any other group (Table 3). The urinary calcium to creatinine ratio was increased in all LPK groups compared to Lewis controls at week 13 and 19 (Table S4). At week 19, the urinary calcium to creatinine ratio was increased in LPK + PC compared with LPK + V and LPK + E (Table S4).

Table 3. Effect of delayed treatment of paricalcitol, enalapril and combination therapy on renal function and serum calcium in LPK rats at week 20.

	Lewis + V (n = 6)	**LPK + V (n = 7)**	**LPK + PC (n = 6)**	**LPK + E (n = 7)**	**LPK + PC + E (n = 7)**
Serum Ca (mmol/L)	2.7 ± 0.0	2.7 ± 0.1	3.6 ± 0.1 *	2.8 ± 0.0	3.6 ± 0.1 *
Serum PO_4 (mmol/L)	2.2 ± 0.4	2.0 ± 0.2 #	2.1 ± 0.2	2.1 ± 0.2 #	2.6 ± 0.5
Serum Cr (µmol/L)	32 ± 2	144 ± 33 *	136 ± 15 *	124 ± 16 *	164 ± 41 *
CrCl (µmol/L/g BW)	8.1 ± 1.5	1.4 ± 0.7 *	1.4 ± 0.3 *	1.7 ± 0.2 *	1.4 ± 0.3 *[1]

[1] Abbreviations: LPK, Lewis polycystic kidney rat; V, vehicle; PC, paricalcitol; E, enalapril; Ca, corrected calcium; PO4, phosphate; CrCl, endogenous creatinine clearance corrected for body weight (BW). Data expressed as mean ± standard deviation. * $p < 0.01$ compared to Lewis + V. # $p < 0.05$ compared to LPK + PC + E.

3.2.4. Progression of Proteinuria

The LPK + V group developed significant proteinuria compared to the Lewis + V group and this increased between weeks 13 and 19 (Figure 2, Table S5). Treatment with PC alone caused a delayed reduction in proteinuria by 41% which was detected only at week 19 compared to LPK + V. In contrast, continuous treatment of LPK rats with E consistently reduced the progression of proteinuria by 46.9%, 53.7% and 69.0% on weeks 13, 16, and 19, respectively. This reduction was also detectable with the combination of PC + E at weeks 16 and 19, however, the combination of PC + E had no additional anti-proteinuric effect when compared to E alone ($p = 0.09$, Figure 2, Table S5).

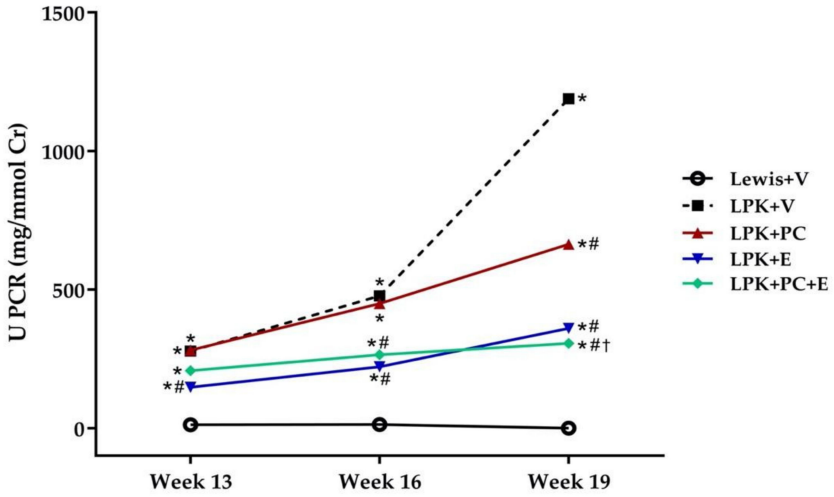

Figure 2. Effect of delayed paricalcitol treatment on the progression of proteinuria in LPK rats. Abbreviations: U PCR, Urine protein creatinine ratio measured in mg/mmol creatinine; LPK, Lewis polycystic kidney rat; V, vehicle; PC, paricalcitol; E, enalapril. Data represented as mean. * $p < 0.05$ compared to Lewis rat, # $p < 0.05$ vs. LPK + V group, † $p < 0.05$ compared to LPK + PC group.

3.2.5. Kidney Enlargement and Cyst Growth

The 2KW:BW ratio, which is a surrogate measure of kidney cyst growth in LPK rats, was increased 11.8-fold in the LPK + V group compared to the Lewis + V rats, and delayed treatment with PC did not affect this increase (Table 4). By light microscopy, LPK rats developed diffuse and advanced cystic kidney disease with little normal renal parenchyma, and the progression of these changes was not affected by treatment with either PC, E or PC + E (Figure S1). In addition, there were focal areas of intra-tubular crystals present in all LPK groups (Figure S2).

Table 4. Effect of delayed treatment of paricalcitol, enalapril and combination therapy on renal enlargement LPK rats at week 20.

	Lewis + V ($n = 6$)	**LPK + V ($n = 7$)**	**LPK + PC ($n = 6$)**	**LPK + E ($n = 7$)**	**LPK + PC + E ($n = 7$)**
Final BW (g)	355 ± 19	302 ± 19 *	266 ± 12 *#	314 ± 19 *	258 ± 238 *#
Right KW (g)	1.13 ± 0.08	10.22 ± 1.79 *	8.57 ± 1.67 *	12.00 ± 1.82 *	9.31 ± 1.60 *
Left KW (g)	1.12 ± 0.05	12.30 ± 1.90 *	10.01 ± 0.46 *	13.0 ± 1.87 *	10.74 ± 2.28 *
2KW:BW (%)	0.63 ± 0.03	7.44 ± 0.70 *	6.99 ± 0.60 *	7.97 ± 0.90 *	7.74 ± 0.86 *

Abbreviations: LPK, Lewis polycystic kidney rat; V, vehicle; PC, paricalcitol; BW, body weight; KW, kidney weight. Data expressed as mean ± standard deviation. * $p < 0.01$ compared to Lewis + V. # $p < 0.05$ compared to LPK + V.

3.2.6. Progression of Hypertension and Cardiac Enlargement

Tail-cuff systolic blood pressure increased in LPK + V rats between weeks 13 and 19 compared to the Lewis + V groups (Figure 3, Table S6). Treatment with PC alone did not

alter the progression of hypertension in LPK rats (Figure 3, Table S6). In contrast, treatment with enalapril reduced systolic blood pressure at week 13 ($p = 0.0073$ compared with LPK + V) but this was not sustained for the remainder of the study ($p = 0.3651$ and $p = 0.9995$ at weeks 16 and 19 compared with LPK + V, respectively, Figure 3, Table S6). Moreover, the combination of PC + E led to a sustained attenuation of systolic blood pressure at both week 13 and week 19 compared to LPK + V ($p = 0.0009$ and $p = 0.0196$, respectively) and was not different to Lewis + V group ($p = 0.1008$ and $p = 0.2987$ at week 13 and 19, respectively, Figure 3, Table S6).

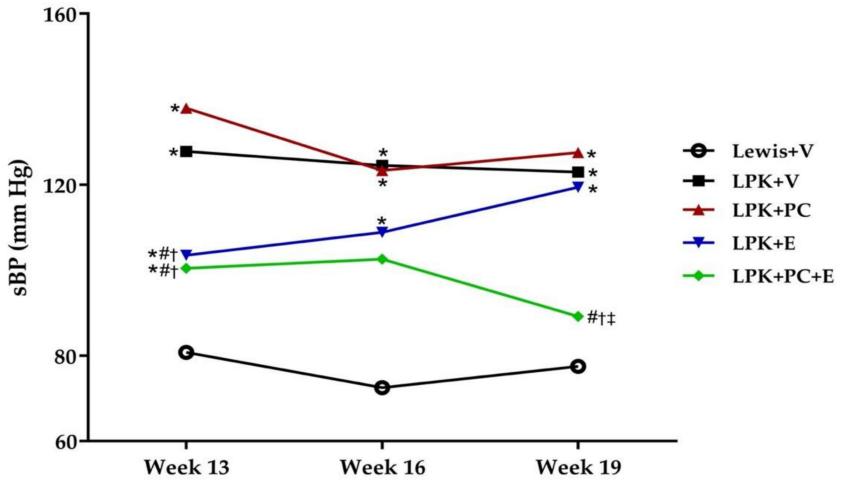

Figure 3. Effect of delayed paricalcitol treatment on the progression of hypertension in LPK rats. sBP, systolic blood pressure; LPK, Lewis polycystic kidney rat; V, vehicle; PC, paricalcitol; E, enalapril. Data represented as mean. * $p < 0.05$ compared to Lewis + V; # $p < 0.05$ compared to LPK + V. † $p < 0.01$ compared to LPK + PC. ‡ $p < 0.01$ compared to LPK + E.

The heart-to-body weight (HW:BW) ratio was increased 1.8-fold in the LPK + V group compared to the Lewis + V group. Treatment with either PC or E did not alter the HW:BW ratio compared to LPK + V, whereas the combination of PC + E reduced the HW:BW by 17.8% ($p = 0.0002$, compared to LPK + V rat group; Table 5). By light microscopy, there were no differences between the LPK rat treatment groups in cardiac histology (Figure S3).

Table 5. Effect of delayed treatment of paricalcitol, enalapril and combination therapy on cardiac enlargement LPK rats at week 20.

	Lewis + V ($n = 6$)	LPK + V ($n = 7$)	LPK + PC ($n = 6$)	LPK + E ($n = 7$)	LPK + PC + E ($n = 7$)
HW (g)	0.90 ± 0.05	1.36 ± 0.16 *	1.11 ± 0.12 *#	1.27 ± 0.07 *	0.95 ± 0.12 #
HW:BW (%)	0.25 ± 0.01	0.45 ± 0.03 *	0.42 ± 0.03 *	0.40 ± 0.02 *	0.37 ± 0.03 *#[1]

[1] Abbreviations: LPK, Lewis polycystic kidney rat; V, vehicle; PC, paricalcitol; BW, body weight; HW, height weight. Data expressed as mean ± standard deviation. * $p < 0.01$ compared to Lewis + V. # $p < 0.05$ compared to LPK + V.

4. Discussion

In this study we evaluated the effect of early and delayed treatment with paricalcitol on the long-term progression of a hypertensive model of PKD. The results showed that: (i) continuous treatment with paricalcitol either in early or established PKD did not have any disease-modifying effects on renal function or kidney cyst growth; (ii) combination treatment in the late phase of disease led to a sustained reduction in systolic blood pressure and cardiac enlargement to a greater extent than enalapril alone; (iii) paricalcitol reduced proteinuria; however, not to the extent of enalapril therapy; (iv) the above effects of paricalcitol in late disease occurred at the cost of increased serum calcium, weight loss and

increase urinary calcium excretion. Taken together, the beneficial effect of PC on blood pressure and proteinuria in LPK rats are similar to that demonstrated in other models of hypertension and CKD but without disease-modifying effects and potential for risk of adverse effects due to elevation in serum calcium [11,30].

Recognition of the non-classical actions of vitamin D metabolites and VDRAs has led to their evaluation as therapeutic agents for a broad range of chronic conditions including cancer [10], cardiovascular disease [31], autoimmune disorders [32] and CKD [33,34]. Several clinical and experimental studies have suggested that VDRAs provide cardiovascular protection and reduce blood pressure and proteinuria [35]. However, knowledge regarding the role of vitamin D signalling in PKD remains incomplete. In LPK rats, chronic dietary deficiency of vitamin D exacerbated proteinuria but unexpectedly caused a small but significant reduction in kidney cyst growth [19]. In contrast, in a small longitudinal cohort study of humans with ADPKD, low levels of 25-OH vitamin D did not predict changes in total kidney volume or blood pressure [36]. An important disease-specific finding of the current study was that neither early nor delayed treatment with paricalcitol altered kidney cyst growth [19].

Hypertension occurs early in PKD due to local hyperactivation of the RAAS with cyst formation [2]. The results of the current study support these findings by showing that treatment with paricalcitol in combination with enalapril reduced blood pressure. The findings demonstrate the known relationship between vitamin D and VDR signalling on reducing the activity of circulating and local tissue activation of RAAS in PKD [37]. The reduction in cardiac enlargement is likely secondary to this sustained blood pressure reduction and its protective effects on left ventricular hypertrophy. Given the significant role of cardiovascular disease in the morbidity and mortality of PKD [5,38], the potential to further enhance the effects of RAAS blockade with VDRAs holds potential, with the caveat of provoking hypercalcemia.

Previous observational data suggested that VDRAs were associated with a benefit on mortality in end-stage kidney failure [39], but caution has been raised in the CKD Stage 3-4 subgroup due to adverse outcomes, particularly hypercalcaemia and augmentation of fibroblast growth factor 23 (FGF23) levels, as reviewed recently [35]. Relevant to the former concern, in the current study, delayed treatment with paricalcitol elevated serum calcium during the period of rapid decline in renal function and markedly reduced body weight gain. The latter was not due to dehydration (as water intake was similar in all LPK groups). Unfortunately, food intake was not measured but we hypothesize that the weight loss was due to anorexia secondary to elevated calcium. Previous studies in non-CKD models have shown that vitamin D analogs cause hypercalcemia and lead to anorexia and weight loss [40,41]. In contrast, the dose regimen has been well tolerated in 5/6 nephrectomised rats [11,12,15,26] suggesting that other factors specific to the LPK disease model may be responsible for the sensitivity to hypercalcemia. In any case, our findings suggest that while paricalcitol may have some beneficial effects in PKD, the use of VDRAs with lower calcaemic-inducing activity may be required in future studies. In addition, given that serum levels of FGF23 are elevated innately in PKD models [42,43] and increased by paricalcitol [30], further understanding of the effects of VDRAs on this endpoint is also needed.

Paricalcitol partially reduced proteinuria compared to control LPK rats. This effect was independent of blood pressure and kidney size as there was no change to either with paricalcitol therapy alone. These findings are consistent with human observational studies and uraemic rat models which show that VDRAs reduce proteinuria, and this may be through inhibition of podocyte injury and preservation of slit diaphragm proteins [44–46], in addition to its anti-inflammatory effects [13,14]. Similarly, the combination of paricalcitol and enalapril therapy in 5/6 nephrectomy rats resulted in a marked reduction in inflammation and fibrosis through suppression of RAAS and *TGF-β* gene expression [13]. This anti-inflammatory and anti-fibrotic action is unique to paricalcitol compared with calcitriol, thought to be due to differential gene expression [13,47,48]. In the present study, histological disease in the LPK rats

at week 20 showed advanced cystic kidney disease, and there were no differences between the treatment groups. However, these data do not exclude the possibility that glomerular and/or tubulointerstitial disease, either by ultrastructure changes or gene expression, could have been altered at an earlier time point.

The main strength of the current study is that it has examined both early and delayed efficacy of paricalcitol in a robust hypertensive model of PKD. There were some limitations in the current study. First, although the molecular mechanisms of kidney cyst formation share common features, disease-specific phenotypic differences between the various subtypes of PKD may be present. In that regard, in this study, the hypothesis was investigated using a genetic ortholog of NPHP-9 rather than that of ADPKD. It is noteworthy that in a post-hoc analysis of the HALT-PKD consisting of 864 individuals with ADPKD, levels of vitamin D metabolites did not predict either change in Ht-TKV and eGFR decline consistent with the results of this study [42]. In any case, further interventional studies using genetic orthologs of ADPKD are needed to fully evaluate the role of VDRAs. Second, the dose of paricalcitol used in the two experiments was different and may not necessarily be applicable to human disease. In Experiment 2, the dose was increased to 0.8 µg/kg/day three times per week based on previous studies [11,30]. This elevated serum calcium in LPK rats and caused weight loss, and our results cannot completely exclude the possibility that this may have masked a protective effect (if any) on kidney disease. Therefore, further studies investigating the role of other VDRAs with anti-calcaemic activity in in vitro and in vivo genetic ortholog models of ADPKD would be helpful [49]. In addition, it would be interesting to evaluate the effects of combining VDRAs with calcium-sensing receptor agonists, given that they suppress parathyroid hormone-induced augmentation of renal cyclic AMP and have been shown to attenuate kidney enlargement as well as serum calcium [50]. Thirdly, we did not evaluate 1,25-hydroxy vitamin D levels in this study to verify any additional bioactivity of paricalcitol. Finally, it is also possible that higher doses of enalapril may have led to a sustained reduction in blood pressure in the delayed treatment experiment.

5. Conclusions

In conclusion, this study shows that paricalcitol in combination with enalapril leads to a sustained reduction in blood pressure and attenuates cardiac enlargement in PKD. Paricalcitol also reduced proteinuria, but this effect was not superior to ACE inhibition. While these results verify that the protective effects of VDRAs observed in generic models of CKD also apply to PKD, neither paricalcitol nor the combination with enalapril altered the progression of kidney cyst growth. In addition, paricalcitol elevated serum calcium and reduced body weight despite being used at doses similar to other CKD models [11,30], adding a significant caveat to its potential clinical application for attenuating cardiovascular disease in PKD.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jcdd8110144/s1, Table S1: Effect of delayed paricalcitol treatment on body weight (g) over time. Table S2: Mean water intake (mls/day) in LPK and Lewis rats over the treatment period in the delayed paricalcitol treatment. Table S3: Effect of delayed paricalcitol treatment on urine volume (mls) in LPK rats. Table S4: Effect of delayed paricalcitol treatment on urinary calcium excretion (urine calcium:creatinine ratio) in LPK rats. Table S5: Effect of delayed paricalcitol treatment on the progression of proteinuria in LPK rats. Table S6: Effect of delayed paricalcitol treatment on the progression of systolic blood pressure in LPK rats. Figure S1: Effect of delayed treatment (weeks 10 to 20) with paricalcitol, enalapril and combination of paricalcitol with enalapril on progression of cystic kidney disease in LPK rats. Figure S2: Intra-tubular crystal formation in LPK rats at week 20. Figure S3: Effect of delayed treatment (weeks 10 to 20) with paricalcitol, enalapril and combination of paricalcitol with enalapril on cardiac histology in LPK rats.

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writing—review and editing, P.S.S., G.K.R., S.S, A.M. and A.T.Y.W.; visualization, P.S.S.; supervision, G.K.R., S.S. and A.T.Y.W.; project administration, G.K.R.; funding acquisition, G.K.R. All authors have read and agreed to the published version of the manuscript.

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3.3 Supplementary Material

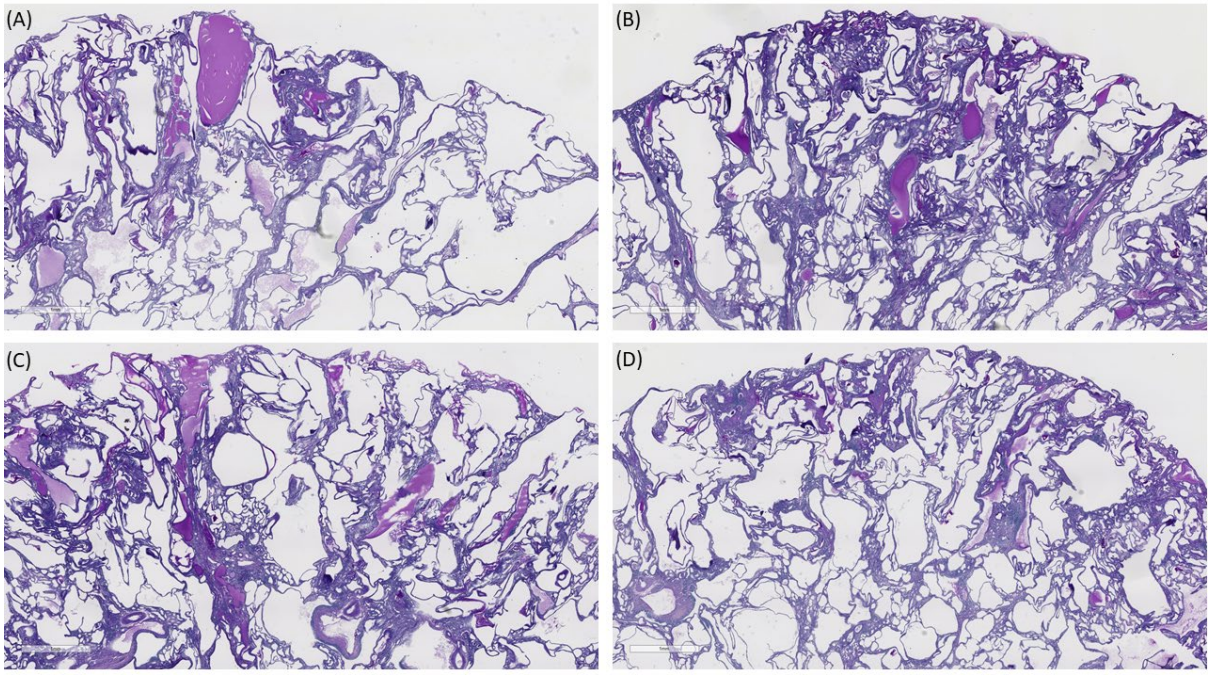

Figure S1. Effect of delayed treatment (weeks 10 to 20) with paricalcitol, enalapril and combination of paricalcitol with enalapril on progression of cystic kidney disease in LPK rats. Shown are representative images of Periodic acid-Schiff staining of kidneys of LPK rats at week 20 following delayed treatment with either: (A) vehicle, (B) paricalcitol, (C) enalapril or (D) combination of paricalcitol and enalapril. There were no differences in renal histology between the treatment groups. Scale bars = 5 m **Table S1:** Effect of delayed paricalcitol treatment on body weight (g) over time

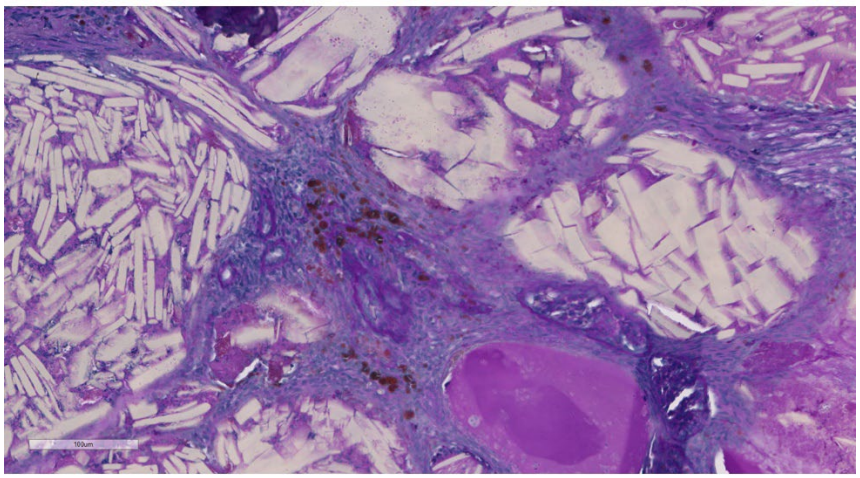

Figure S2. Intra-tubular crystal formation in LPK rats at week 20. Focal areas of intra-tubular crystals were present in all LPK rat groups. A representative example is shown in a LPK rat treated with paricalcitol. Scale bar = 100 μm.

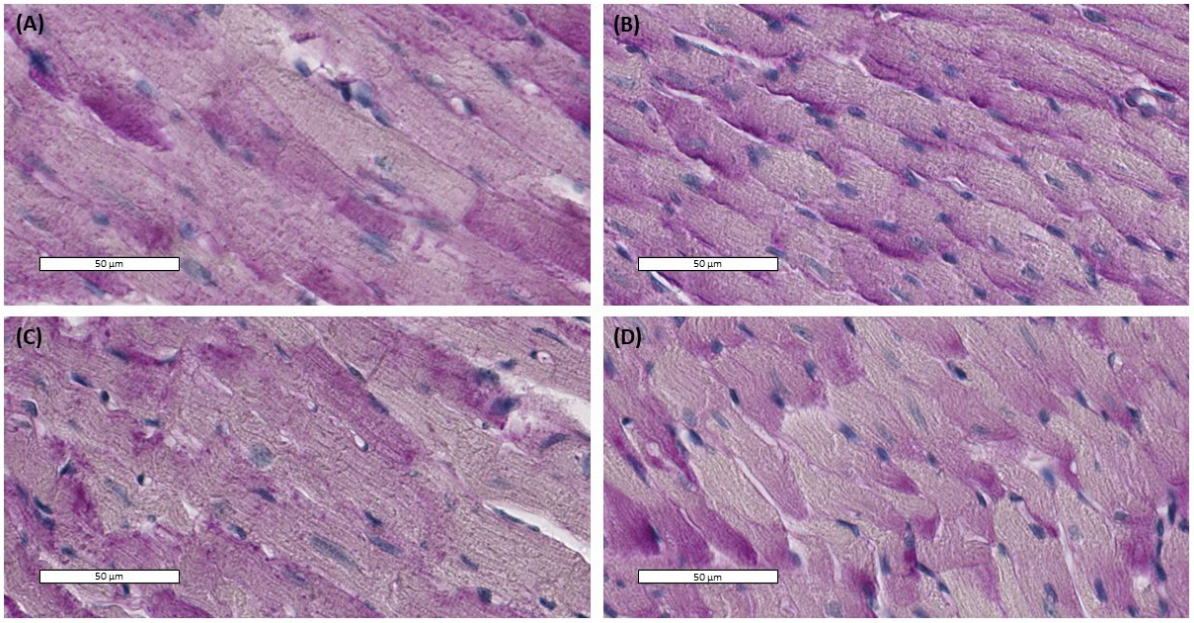

Figure S3. Effect of delayed treatment (weeks 10 to 20) with paricalcitol, enalapril and combination of paricalcitol with enalapril on cardiac histology in LPK rats. Shown are representative images of Periodic acid-Schiff staining of cardiac muscle of LPK rat at week 20 following delayed treatment with (A) vehicle, (B) paricalcitol, (C) enalapril or (D) paricalcitol and enalapril combination starting at week 10. Scale bars = 50 µm.

Table S1: Effect of delayed paricalcitol treatment on body weight (g) over time

Week	Lewis+V (n=6)	LPK+V (n=7)	LPK+PC (n=5)	LPK+E (n=7)	LPK+PC+E (n=7)
10	244.5±16.5	238.42±13.3	241.2±9.7	242.1±19.1	246.6±13.9
11	259±12.6	252.9±17.3	256.3±10.3	256.1±21.4	263.6±17.6
12	282.5±15.8	266.1±19.2	267.7±11.4	265±21.1	264.6±16.8
13	295.8±19.2	276.7±21.5	279.8±10.7	275.9±24.4	282.3±11.6
14	305±15.1	284±21.6	284.7±9.3	283±28.3	291.1±14.7
15	315.2±19.2	295.1±19.8	291.7±6.4	294.9±22.2	296.7±14.1
16	326.8±18.8	300.3±19.4*	292.7±10.1*	302.1±20.1	297.6±16.9
17	333.3±13.9	300.3±20.7*	279.8±17.5*	302±24*	294.1±25.3*
18	339±10.6	310.2±14.8	278.7±12.3*#	300.7±17.4	276.8±17.4*
19	347.3±11.4	310±18.7*	272.7±17.4*#†	308.2±16.9*	269.8±19.3*#†
20	350.7±9.3	312±12.5*	272.4±9.8*#†	323.3±3.8*	256.8±16.1*#†

[1]Abbreviations: LPK, Lewis polycystic kidney rat; V, vehicle; PC, paricalcitol; E, enalapril. Data expressed as mean±standard deviation. *p=<0.05 compared to Lewis+V group, #p<0.05 compared to the LPK+V group. †p<0.05 compared to the LPK+E group.

Table S2: Mean water intake (mls/day) in LPK and Lewis rats over the treatment period in the delayed paricalcitol treatment

Lewis+V (n=6)	LPK+V (n=7)	LPK+PC (n=5)	LPK+E (n=7)	LPK+PC+E (n=7)
22.1±2.8	43.1±5.0*	45.6±4.8*	41.9±4.5*	46.1±6.4*

[1]Abbreviations: LPK, Lewis polycystic kidney rat; V, vehicle; PC, paricalcitol; E, enalapril. Data expressed as mean+standard deviation. *p<0.01 compared to Lewis+V;

Table S3: Effect of delayed paricalcitol treatment on urine volume (mls) in LPK rats.

Week	Lewis+V (n=6)	LPK+V (n=7)	LPK+PC (n=5)	LPK+E (n=7)	LPK+PC+E (n=7)
13	5±2	14±7*	14±5*	15±2*	*13±3**
16	5±2	22±3*	*21±7**	*19±3**	*22±6**
19	4±1	*17±4**	*18±4**	*19±3**	*19±5*[1]

[1]Abbreviations: LPK, Lewis polycystic kidney rat; V, vehicle; PC, paricalcitol; E, enalapril. Data expressed as mean±standard deviation. *p<0.01 compared to Lewis+V

Table S4: Effect of delayed paricalcitol treatment on urinary calcium excretion (urine calcium:creatinine ratio) in LPK rats.

Week	Lewis+V (n=6)	LPK+V (n=7)	LPK+PC (n=5)	LPK+E (n=7)	LPK+PC+E (n=7)
13	0.09±0.03	0.27±0.06*	0.30±0.08*	0.25±0.05*	0.25±0.08*
16	0.10±0.08	0.22±0.05	0.33±0.10*	0.21±0.03	0.27±0.11*
19	0.13±0.07	0.32±0.06*#	0.44±0.05*	0.29±0.08*#	0.34±0.09*[1]

[1]Abbreviations: LPK, Lewis polycystic kidney rat; V, vehicle; PC, paricalcitol; E, enalapril. Data expressed as mean±standard deviation. *p<0.01 compared to Lewis+V; #p<0.05 compared to LPK+PC.

Table S5: Effect of delayed paricalcitol treatment on the progression of proteinuria in LPK rats

Week	Lewis+V (n=6)	LPK+V (n=7)	LPK+PC (n=5)	LPK+E (n=7)	LPK+PC+E (n=7)
13	12.1±19.1	277.7±42.8*	281.2±64.7*	147.4±17.5*#	277.5±145.8*
16	12.5±13.1	477.2±179.0*	448.9±82.6*	221.2±58.8*#	265.1±102.7*#
19	3.5±4.4	1129.4±316.3*	663.7±287.9*#	350.0±165.2*#	296.4±137.4*#[1]

[1]Abbreviations: LPK, Lewis polycystic kidney rat; V, vehicle; PC, paricalcitol; E, enalapril. Data expressed as mean±standard deviation. *p<0.01 compared to Lewis+V; #p<0.05 compared to LPK+V.

Table S6: Effect of delayed paricalcitol treatment on the progression of systolic blood pressure in LPK rats

Week	Lewis+V (n=6)	LPK+V (n=7)	LPK+PC (n=5)	LPK+E (n=7)	LPK+PC+E (n=7)
13	81±5	128±15*	138±11*	104±17*#†	100±6*#†
16	73±5	124±26*	123±17*	109±12*	103±12
19	77±4	119±18*	127±10*	117±12*	94±16#†‡

[1]Abbreviations: LPK, Lewis polycystic kidney rat; V, vehicle; PC, paricalcitol; E, enalapril. Data expressed as mean±standard deviation. *p<0.05 compared to Lewis+V; #p<0.05 compared to LPK+V. †p<0.01 compared to LPK+PC. ‡p<0.01 compared to LPK+E.

Chapter 4: Development of the study protocol for a randomised controlled trial testing the efficacy of beetroot juice to reduce blood pressure in autosomal dominant polycystic kidney disease

4.1 Preface

This chapter describes the study protocol of the first RCT testing the efficacy of BRJ on reducing blood pressure in hypertensive adults with ADPKD. The rationale for the study was that there is evidence that endothelial-derived NO is reduced and may mediate hypertension and endothelial dysfunction in ADPKD. As described earlier, BRJ is a rich natural source of dietary nitrate and there are several clinical trials that have established that increases in nitrate and NO levels have an anti-hypertensive effect in CKD and other chronic disease populations. Furthermore, as BRJ is a dietary or lifestyle modification, if found to be effective it would be rapidly accessible and easily implementable for the PKD community.

This trial aimed to determine if BRJ is efficacious in reducing blood pressure in ADPKD as an adjunct therapy. The hypothesis was that daily supplementation with nitrate-replete BRJ (containing 40mg nitrate/day) for 4 weeks would reduce blood pressure compared to placebo juice, which is nitrate-deplete.

The study protocol was approved by the Western Sydney Local Health District Human Ethics Research Committee (approval number 2020_ETH01718). The protocol underwent several revisions and the final approved protocol was version 7, approved on 27th June 2022.

The following manuscript is the published trial protocol detailing the outcomes, study design, and statistical analysis plan.

4.2 Efficacy of *beet*root juice on reducing blood pressure in hypertensive adults with autosomal dominant *p*olycystic *k*idney *d*isease (BEET-PKD): study protocol for a double-blind, randomised, placebo-controlled trial

The following trial protocol was published in the peer-reviewed journal *Trials*, first online on 29th July 2023. The formatting and writing style are in keeping with the journal requirements. References are self-contained within the manuscript. Section 4.3 contains supplementary material referenced in the manuscript.

Trials

STUDY PROTOCOL

Open Access

Efficacy of *beet*root juice on reducing blood pressure in hypertensive adults with autosomal dominant *p*olycystic *k*idney *d*isease (BEET-PKD): study protocol for a double-blind, randomised, placebo-controlled trial

Priyanka S. Sagar[1,2], Alexandra Munt[1,2], Sayanthooran Saravanabavan[1,2], Farnoosh Asghar Vahedi[1,2], James Elhindi[3], Beatrice Nguyen[1], Katrina Chau[4,5], David C. Harris[1,2], Vincent Lee[2,6], Kamal Sud[6,7], Nikki Wong[6,7] and Gopala K. Rangan[1,2*]

Abstract

Background In autosomal dominant polycystic kidney disease (ADPKD) impaired nitric oxide (NO) synthesis, in part, contributes to early-onset hypertension. Beetroot juice (BRJ) reduces blood pressure (BP) by increasing NO-mediated vasodilation. The aim of this double-blind, randomised, placebo-controlled study is to test the hypothesis that BRJ reduces systolic and diastolic clinic BP in hypertensive adults with ADPKD.

Methods Participants with ADPKD and treated hypertension ($n=60$) will be randomly allocated (1:1) to receive a daily dose of either nitrate-replete (400 mg nitrate/day) or nitrate-deplete BRJ for 4 weeks. The co-primary outcomes are change in mean systolic and diastolic clinic BP before and after 4 weeks of treatment with daily BRJ. Secondary outcomes are changes in daily home BP, urinary albumin to creatinine ratio, serum and salivary nitrate/nitrite levels and serum asymmetric dimethylarginine levels before and after 4 weeks of BRJ.

Discussion The effect of BRJ in ADPKD has not been previously tested. BRJ is an accessible, natural dietary supplement that, if effective, will provide a novel adjunctive approach for treating hypertension in ADPKD.

Trial registration ClinicalTrials.gov NCT05401409. Retrospectively registered on 27th May 2022.

Keywords Polycystic kidney disease, Beetroot juice, Dietary interventions in hypertension

*Correspondence:
Gopala K. Rangan
g.rangan@sydney.edu.au
Full list of author information is available at the end of the article

Administrative information

This table and the following study protocol contain all administrative information as required by the SPIRIT reporting guidelines and World Health Organization Trial Registration Data Set for clinical trial registration [1].

Data category	Information
Public and Scientific Title	Double-blind, randomised, placebo-controlled study to determine the effect of beetroot juice on reducing blood pressure in hypertensive adults with autosomal dominant polycystic kidney disease
Primary registry and trial identifying number	NCT05401409 [ClinicalTrials.gov]. Registered on 27th May 2022 There are no secondary identifying numbers
Protocol Version	Version 7 (approved on 27th June 2022)
Funding	This study was funded by a grant from PKD Australia to GR. PS was supported by an ICPMR Jerry Koutts Scholarship. The funding bodies have no role in the study design, execution, analyses, interpretation of the data, or decision to submit results
Author details	1. Priyanka S Sagar, Michael Stern Laboratory for Polycystic Kidney Disease, Westmead Institute for Medical Research (WIMR), The University of Sydney (USyd), Australia & Department of Renal Medicine, Westmead Hospital, Western Sydney Local Health District (WSLHD), Australia 2. Alexandra Munt, Michael Stern Laboratory for Polycystic Kidney Disease, WIMR, USyd, Australia & Department of Renal Medicine, Westmead Hospital, WSLHD, Australia 3. Sayanthooran Saravanabavan, Michael Stern Laboratory for Polycystic Kidney Disease, WIMR, USyd, Australia & Department of Renal Medicine, Westmead Hospital, WSLHD, Australia 4. Farnoosh Asghar Vahedi, Michael Stern Laboratory for Polycystic Kidney Disease, WIMR, USyd, Australia & Department of Renal Medicine, Westmead Hospital, WSLHD, Australia 5. James Elhindi, Research and Education Network, Westmead Hospital, WSLHD, Australia 6. Beatrice Nguyen, Michael Stern Laboratory for Polycystic Kidney Disease, WIMR, USyd, Australia 7. Katrina Chau, Department of Renal Medicine, Blacktown Hospital, WSLHD, Australia & Blacktown Clinical School, Western Sydney University, Australia 8. David C Harris, Michael Stern Laboratory for Polycystic Kidney Disease, WIMR, USyd, Australia & Department of Renal Medicine, Westmead Hospital, WSLHD, Australia 9. Vincent Lee, Department of Renal Medicine, Westmead Hospital, WSLHD, Australia & Faculty of Medicine and Health, USyd, Australia 10. Kamal Sud, Department of Renal Medicine, Nepean Hospital, Nepean Blue Mountains Local Health District (NBMLHD), Australia & Faculty of Medicine and Health, USyd, Australia 11. Nikki Wong, Department of Renal Medicine, Nepean Hospital, NBMLHD, Australia & Faculty of Medicine and Health, USyd, Australia 12. Gopala K Rangan, Michael Stern Laboratory for Polycystic Kidney Disease, WIMR, USyd, Australia & Department of Renal Medicine, Westmead Hospital, WSLHD, Australia

Data category	Information
Trial sponsor and contact information	Western Sydney Local Health District Human Ethics Research Committee WSLHD Research Governance Office Phone: + 61 2 8890 9007 WSLHD-researchoffice@health.nsw.gov.au
Role of the trial sponsor	The study sponsor has oversight of human ethics research committee and trial governance but no role in the design, execution, analyses, interpretation of the data or the decision to submit results
Contact for public and scientific enquires	GR [g.rangan@sydney.edu.au]
Health conditions studies	Autosomal Dominant Polycystic Kidney Disease and Hypertension

Background

Autosomal dominant polycystic kidney disease (ADPKD) is the most common monogenic cause of kidney failure in adults and is due to pathogenic variants in either *PKD1* or *PKD2* [2]. Hypertension occurs in the third decade of life and is a crucial treatable risk factor to prevent cardiovascular morbidity and mortality in ADPKD [3–5]. Multiple pathological mechanisms drive hypertension in ADPKD; primarily renin–angiotensin–aldosterone system (RAAS) activation, endothelial dysfunction and sympathetic nervous system overactivity [3, 6].

Nitric oxide (NO) is a key mediator of vasodilation and can be produced by vascular endothelial cells via the conversion to L-arginine to L-citrulline by *nitric oxide synthase* (NOS) [6, 7]. Previous studies demonstrate that the functional loss of polycystin-1 (protein encoded by *PKD1*) leads to impaired intracellular calcium-mediated signalling within the endothelial-NOS pathway [7–11]. In a study of nine hypertensive ADPKD patients, defective endothelium-dependant relaxation was associated with an ~ eightfold reduction in NOS activity and twofold reduction in NO metabolites compared to healthy controls ($P < 0.01$) [6]. Additionally, patients with ADPKD ($n = 27$) have increased levels of asymmetric dimethylarginine (ADMA), an endogenous competitive inhibitor of NOS and independent biomarker for endothelial damage, which strongly predicts risk for future cardiovascular events [8, 12–14]. In our previous work, long-term treatment with oral sodium nitrate did not reduce kidney cyst growth in a murine genetic ortholog of ADPKD ($Pkd1^{RC/RC}$ mice) but the effects on blood pressure (BP) could not be determined as this model does not have a hypertensive phenotype [8].

Beetroots, along with spinach, rocket and lettuce, contain the highest concentration of naturally occurring nitrate with an average of 250 mg/100 g compared with < 20 mg/100 g found in very low-concentration nitrate vegetables such as eggplants [15]. BRJ increases serum NO metabolites, promotes vasodilation and reduces BP [16–19]. The anti-hypertensive effect of BRJ is attributed to the direct conversion of dietary nitrate to NO by nitrous-converting bacteria in the oral mucosa (the entero-salivary *nitrate-nitrite-NO* pathway) [17]. Several randomised controlled trials testing the effect of beetroot juice showed a 7–12-mmHg decrease in systolic BP in individuals with hypertension, stage 3 chronic kidney disease (CKD), obesity and heart failure with preserved ejection fraction [16, 19–23]. In context, the dietary approach to stop hypertension (DASH) study showed an average reduction in systolic BP of 5.2 mmHg which, based on the 10-year Framingham risk score is associated with a 13% reduction in coronary heart disease, myocardial infarction and stroke [24, 25]. Moreover, BRJ is well accepted by study participants as it is a natural substance with no reported harmful effects and has up to 90% self-reported compliance [16, 26]. There are no expected or reported drug interactions with BRJ [16, 27–29].

Given the high burden of CVD in ADPKD patients, there is a need for safe, accessible therapies that can reduce their long-term risks [30]. BRJ may be well-suited to fulfil this need, particularly given the underlying reduction of endogenous endothelial NOS activity and NO metabolites [9, 31, 32]. To our knowledge, there have been no current or previous studies investigating the anti-hypertensive effects of BRJ in ADPKD. Thus, the aim of this study is to test the hypothesis that daily supplementation with BRJ (containing 400 mg nitrate/day) for 4 weeks will lower BP in hypertensive participants with ADPKD, using a randomised, double-blind, placebo-controlled trial design. The secondary aims are to test the effect of BRJ on home BP readings, serum and salivary NO metabolites, serum ADMA, and urinary albumin to creatinine ratio over 4 weeks.

Methods/design

Study objectives

BEET-PKD is a randomised, double-blind, placebo-controlled, superiority trial with 1:1 allocation of 60 participants with ADPKD and hypertension. The primary objective of this study is to evaluate the effect of 4 weeks of daily BRJ (containing 400 mg dietary nitrate/day) consumption on clinic BP in hypertensive ADPKD participants. The secondary objectives are to evaluate the effect of BRJ on daily home BP readings, nitrate metabolite levels, serum ADMA levels, and urinary albumin to creatinine ratio and monitor the safety of BRJ.

Study sites

The study sites will be Westmead Hospital and Westmead Institute for Medical Research, Sydney, NSW, Australia. The study will be performed in accordance with the Declaration of Helsinki and strictly follow the most recent version of the study protocol that has been approved by the Human Research Ethics Committee (HREC) of the study's sponsor, Western Sydney Local Health District (WSLHD) and meets the International Council for Harmonisation-Good Clinical Practice (ICH-GCP) guidelines. The study ethics approval number is 2020_ETH01718.

Inclusion and exclusion criteria

A total of 60 participants who fulfil the inclusion criteria of age over 18, diagnosis of ADPKD, diagnosis of hypertension (defined as being prescribed at least one regular anti-hypertensive medication), with an estimated glomerular filtration rate (eGFR) > 30 ml/min/1.73 m^2 (Table 1), and who do not fulfil the exclusion criteria, at the time of randomisation are eligible. The participant's diagnosis of ADPKD will be made by the treating nephrologist based on standard criteria (family history of ADPKD and renal ultrasound). The exclusion criteria (Table 1) are based on comorbidities that could potentially confound study outcomes [33–35]. In particular, participants with severe uncontrolled diabetes (HbA1c > 10%) will be excluded due to previous studies showing that severe hyperglycemia resulted in diminished vascular responsiveness and endothelial damage [36]. Participants with well-controlled diabetes will be permitted as the incidence of type 2 diabetes in ADPKD is < 1% in the population offered in this study and it is not likely to influence the primary outcome. Participants with severe uncontrolled hypercholesterolemia and current cigarette smoking will be excluded as these states result in direct endothelial injury and suppression of nitric oxide production which will likely interfere with the action of BRJ [12, 37].

Study design

At Visit 1 participants will be screened for eligibility and instructed on correct technique for BP measurement. Pre-intervention clinic BP will be measured using a standardised protocol (Additional file 1), and blood, urine and saliva samples collected. A 1-week run-in period will be used to determine baseline home BP and adherence to daily measurements. At visit 2, BP compliance will be checked and participants will be asked to commence their randomly allocated (1:1 randomisation) BRJ daily, which is either nitrate-replete (400 mg nitrate/70 ml dose) or nitrate-depleted (0 mg nitrate/70 ml dose), for 4 weeks. Visit 2 will occur via telehealth to reduce participant burden. Over the 4-week period, participants will continue to check their BP daily and report results and any side effects by responding to the daily reminder text messages. After 4 weeks, at visit 3, participants will return for post-intervention standardised clinic BP measurement and blood, urine and saliva sample collection. Further details of the parallel assignment study design and participant's timeline have been provided in Fig. 1 (a schema of the study design), Fig. 2 (an overview of the study schedule, interventions and procedures in a "Standard Protocol Items: Recommendations for Interventional Trials" ("SPIRIT") figure) and Additional file 2 (a SPIRIT checklist) [1].

Study intervention

The study intervention is a single oral daily dose of either nitrate-replete BRJ (70 mL Beet-IT Sport™; James White Drinks, UK; 400 mg nitrate/70 mL) or 70mls BRJ placebo (Nitrate-depleted Beet It shot; James White Drinks, UK) which are both identically packaged (Additional file 3). In a recent meta-analysis, 20/22 studies examining blood pressure-lowering effects of BRJ used an intervention

Table 1 Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
• Diagnosis of Autosomal Dominant Polycystic Kidney Disease • Age > 18 years old • eGFR > 30 mL/min/1.73 m^2 • Treatment with at least 1 anti-hypertensive	• Inability to provide informed consent • Labile, unstable, uncontrolled hypertension and/or changes in BP treatment 28 days prior to the screening visit • Non-compliance with study procedures and/or daily BP measurements during the screening period • Medical conditions or treatments that might interfere with the generation of NO metabolites or the primary endpoint (e.g. nitrate drugs, cigarette smoking; unwilling to stop using antiseptic mouthwash; severe, uncontrolled hypercholesterolaemia; pregnancy or post-partum and lactating) • Any serious or other medical conditions that might interfere with follow-up or stability of blood pressure (e.g. current active malignancies; uncontrolled diabetes mellitus with elevated HbA1c > 10%) • Dislike of taste of BRJ • Allergy to beetroot • Enrolled in other clinical trials

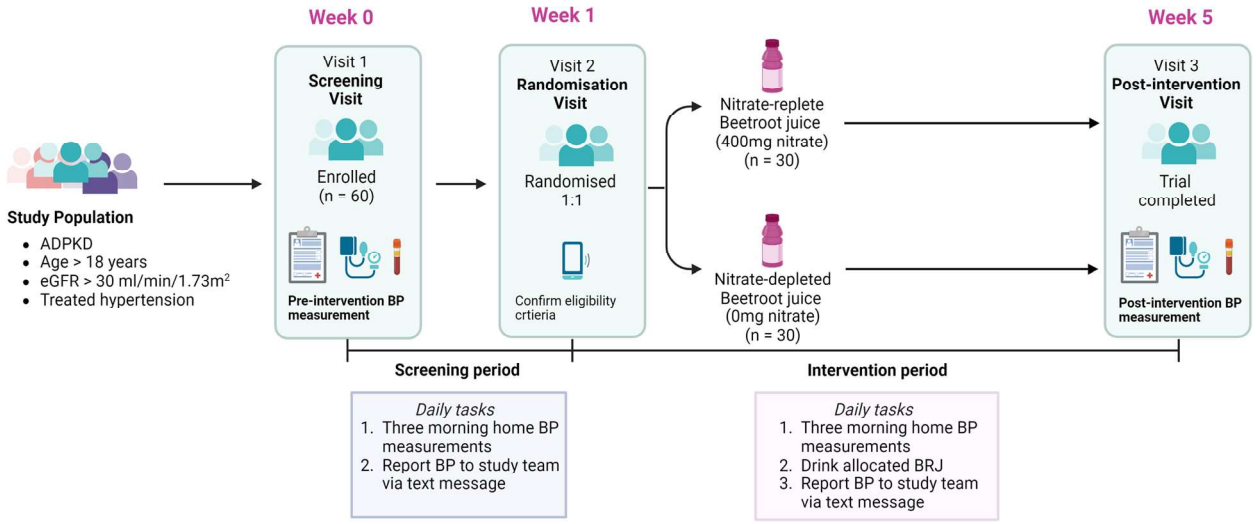

Fig. 1 Schema of the BEET-PKD trial design.

Abbreviation: *BP*, blood pressure

produced commercially by James White Drinks, UK [25]. Eleven of 18 studies also used nitrate-deplete BRJ from this provider [25]. To maintain reproducibility, the current study has used the same provider. Participants will self-administer the intervention at the same time each morning within an hour of waking and prior to BP measurement. The placebo (BRJ with only nitrate content removed) was selected to exclude confounding from other vasoactive components found in BRJ such as vitamin C, magnesium, polyphenols, betaine, and flavonoids that may contribute to an antihypertensive effect [17, 38].

Determining dose and duration of the intervention

Previous randomised controlled trials investigating the efficacy of BRJ have used a daily dose equivalent to at least 400 mg nitrate/day [16, 17, 21, 39, 40]. In a study of treated and untreated hypertensive patients ($n=68$), BRJ reduced BP after 1 week of daily dosing (~400 mg nitrate content; $p<0.01$) and this effect was maintained for the 4-week trial duration with a mean decrease in BP of 7.7/2.4 mmHg (3.6–11.8/0.0–4.9, $p<0.001$ and $p=0.050$) at the end of week 4 [16]. In older (mean ages 61–62 ± 1), overweight (mean BMI 29.4 ± 1.2 and 30.5 ± 1.3) individuals with high-normal blood pressure ($n=24$), a daily dose of BRJ (400 mg nitrate) for 3 weeks reduced daily home systolic BP (−7.3 ± 5.9 mmHg, $p=0.02$) during the intervention but this was not maintained 1 week after the last dose [21]. In contrast, normotensive and treated hypertensive patients ($n=27$) treated with 1 week of daily dose of BRJ (600 mg nitrate) observed no effect [40]. Similarly, hypertensive participants with type 2 diabetes mellitus (mean HbA1c% 7.6 + 1.1%) treated with daily BRJ (450 mg nitrate) for 2 weeks showed no change in BP or endothelial function [36]. The authors reported that the lack of effect in the latter studies may be due to the relative short duration of intervention, interference of multiple pharmacological antihypertensives, low nitrate diet or chronic endothelial damage resulting in lack of vascular responsiveness to NO. Therefore for reproducibility and comparability, the current study will administer "Beet-IT Sport Shot" (70 mL; James White Drinks, UK) containing ~400 mg nitrate per dose for a duration of 4 weeks [39].

Adherence to the intervention

To improve adherence and ensure the timing of BRJ consumption and BP readings are consistent, participants will receive a scheduled daily text message through a secure web-based messaging platform (MessageMedia, Victoria, Australia) as previously described [41]. During the screening period, the message will ask participants to measure their BP and reply with their readings in the order they took the measurements. In the intervention period, the message will remind participants to take their BRJ, measure their BP and reply with the readings in the order they were measured. Adherence to the study

	STUDY PERIOD		
	Screening Visit (Visit 1)	**Randomisation Visit (Visit 2)**	**Post-Intervention Visit (Visit 3)**
TIMEPOINT (Weeks)	**0**	**1**	**5**
ENROLMENT:			
Eligibility screen	X		
Informed consent	X		
Allocation	X		
Medical history & Anthropometrics	X		
Confirm eligibility		X	
Randomisation		X	
INTERVENTIONS:			
Nitrate-replete beetroot juice (400mg nitrate)		♦—	—♦
Nitrate-deplete beetroot juice (0mg nitrate)		♦—	—♦
ASSESSMENTS:			
***Primary Outcome:* Office blood pressure measurement**	X		X
Secondary Outcome:* Daily Home blood pressure measurement	—♦	♦— —♦	—♦
Blood, urine and saliva sample collection	X		X
Adverse events recording			X

**Home blood pressures measured daily between screening visit and randomisation visit and between randomisation visit and post-intervention visit*

Fig. 2 SPIRIT figure for the BEET-PKD trial

procedures will be verified in real-time by replies to text messages.

Concomitant care

Participants will be advised to continue their usual medications, diet, physical activity, and other lifestyle factors as directed by their treating nephrologist. Participants must be on stable anti-hypertensive medications for 28 days prior to commencing the trial.

Protocol deviations

Any instances where participants discontinue or deviate from the trial protocols will be recorded as a "protocol deviation" and immediately discussed with the trial team and PI to assess for any potential SAEs. If the intervention is discontinued due to adverse events or participants' request, participants will be encouraged to continue to record their home BP daily and attend remaining study visits and this data will be analysed as the randomised population, i.e. "intention to treat".

Study endpoints

Primary endpoints

The co-primary endpoints will be mean change in clinic systolic and diastolic BP from baseline to after 4 weeks of daily BRJ. This endpoint was selected taking into consideration the potential variability between participants' individual BP ranges which may differ between groups

and lead to biased results. This endpoint is similar to other trials of BRJ in hypertensive patients [16, 40].

Secondary endpoints

The secondary endpoints are mean changes in daily home blood pressures during the screening period and the 4-week intervention, and change in mean serum and salivary NO metabolites, mean serum ADMA levels, and mean urinary albumin to creatinine ratio from baseline to end of 4 weeks of daily BRJ. All adverse events will be assessed to determine the safety of the intervention (see the "Safety monitoring and reporting" section below).

Measurement methods

Measurement of BP

The BP will be measured three times at clinic visits ("clinic BP") using a validated automated oscillometric blood pressure (AOBP) device (Model: A&D UA-611, Tokyo, Japan) Standardised office blood pressure conditions will be used for all clinic readings as described in Additional file 1, adapted from the 2021 Kidney Disease Improving Global Outcomes (KDIGO) Clinical Practice Guidelines for the Management of Blood Pressure in Chronic Kidney Disease and the 2020 International Society of Hypertension Global Hypertension Practice Guidelines [42, 43]. An average of the second and third readings will be analysed for the endpoint, in accordance with the International Society of Hypertension 2020 guidelines and the 2018 European Society of Cardiology/European Society of Hypertension Guidelines [43, 44]. Home BP readings will be measured using the same AOBP device (Model: A&D UA-611, Tokyo, Japan) which will be provided to the participants for the duration of the trial. At the first visit, participants will be trained on how to correctly measure their blood pressure following the Heart Foundation of Australia's self-measurement instructions and a detailed instruction card on the correct technique which will be attached to the device [45]. As with the clinic BP, home BP will be measured three times and an average of the second and third reading used for data analysis.

Measurement of urinary albumin:creatinine ratio, NO metabolites and ADMA

Serum samples to measure NO metabolites and ADMA levels, and saliva samples to measure NO metabolites will be collected at visit 1 and the final visit (visit 3) on the last day of the intervention. Urine samples for urinary albumin to creatinine ratio will be collected at visit 1 (or if participants have recently completed a measurement at an accredited lab in the 6 months prior to the visit, this measurement may be used) and at visit 3 on the last day of the intervention. See Additional file 4 for details on the collection, storage and methods of measurement of biological samples.

Determination of sample size

In a previous study of daily BRJ (400 mg nitrate) supplementation in hypertensive patients ($n=68$) for 4 weeks, systolic BP was significantly reduced by 7.7 mmHg (3.6–11–8 mmHg, $p<0.001$) at the end of the intervention [16]. The average baseline BP of the two groups were 148 mmHg and 149 mmHg, with standard deviations of the two populations being 10 and 11. We expected to have a similar hypertensive population and a test for differences in two independent means was implemented in Stata SE Version 14.2 (StataCorp, TX, U.S.A) to calculate sample size. To detect this difference in systolic BP with $\alpha=0.05$ and power of 0.80, 28 participants are required in each treatment group. To account for drop-out, the sample size was increased to 30 participants per treatment group (total $n=60$). Participants will be referred from the Western Renal Service which provides a catchment of 1.2 million people and 400 potential participants with ADPKD, and therefore a single-centre study with multiple referral centres was considered adequate for this study.

Recruitment

Participants will be recruited from the Western Renal Service (Westmead, Blacktown and Nepean hospitals) which services a catchment area of 1.2 million people and 400 potential participants with ADPKD. If required, other local centres such as Concord, Royal North Shore and Liverpool Hospitals. Multiple strategies will be used to facilitate recruitment. Potential participants will be identified from the Principal Investigator's patient database and databases of previous clinical trial participants who have opted-in to be contacted about future trials. Potential participants will also be identified from treating nephrologists, either through direct referral to the study team or review of clinic letters and local databases. Participants will also be recruited passively by advertising through the PKD Australia's website and newsletter.

Study staff (study doctors or senior researchers) will obtain written informed consent from all participants prior to commencing the trial. All participants will receive the consent form and information about the trial prior to visit 1 and are able to contact the study team with questions. During visit 1, study staff will go through the information and consent forms and have an informed discussion with each participant. Specifically, participants will be informed of the expected adverse effects of beeturia and beet-coloured faeces [17]. Participants

will also be informed of the potential for gastrointestinal side effects and asked to report any symptoms to the trial team immediately.

Randomisation and blinding

Participants will be randomised using a simple randomisation program created by the study biostatistician, which uses a random number generator (version 3.6.2, R Core Team). This program creates a list that allocates treatment or placebo (1:1) to 60 unique randomisation IDs and will be generated by the study biostatistician. No stratification factors or blocking will be used. When a participant is randomised, they will be assigned a randomisation ID. Prior to commencement of the trial, a research staff member who is not involved with any other study procedures will receive the randomisation IDs and label the relevant BRJ with the study ID as allocated by the list. The study team will provide the participant with their labelled BRJ at Visit 1. The remaining members of the study team and the participants will remain blinded to the treatment allocation until data lock and statistical analysis have been completed. Emergency unblinding procedures will only occur if the safety of trial participants is at risk or should any evidence of harms arise, as per the process described below (in the "Safety monitoring and reporting" section and Additional file 6).

Statistical analysis plan

Primary endpoint analysis

The co-primary endpoints are the change in the mean of second and third systolic and diastolic BP measurements taken at Visit 1 (pre-intervention) and at Visit 3 (at the end of week 4 of the intervention). Initially, the sample mean and standard deviation will be reported at baseline and follow-up. Moreover, their mean difference and standard deviation will be reported. Secondly, the main analysis will be conducted using a Gaussian linear mixed effects (LME) model with a first-order autoregressive correlation structure (AR(1)). The repeated measures are the BP results at Visit 1 and Visit 3. An interaction parameter between the visit and arm of the study will be the determinant of a significant difference in BP change between the two arms. The model will be adjusted for the following prognostic factors: age, eGFR and baseline serum nitrate/nitrite levels. The sensitivity analysis will only be adjusted for age and eGFR. Should any variables differ significantly between the two arms, they too will be adjusted for to eliminate the possibility of a confounding effect. No subgroup analyses will be undertaken. All data analysis will be completed in R version 3.6.2 (R Core Team). All (potential) confounding variables will be summarised using *t*-tests or rank sum tests for continuous variables and chi-squared tests or Fisher's exact tests for categorical variables. Means (standard deviations) and counts (column percentages) as well as relevant p-values will be reported. Hypotheses will be conducted with a two-sided alternative and p-values less than 0.05 will be considered statistically significant.

Secondary endpoint analysis

The secondary endpoint of home BP readings is a set of (at most) 28 daily systolic and diastolic BP measurements averaged from daily home readings. Again, a Gaussian LME model with an AR(1) correlation structure will be utilised. The repeated measures are the BP results at each available home reading. An interaction parameter between the day and arm of the study will be the determinant of a significant difference in the BP change between the two arms. The model will be adjusted for the prognostic variables of age, eGFR and baseline nitrate/nitrite levels as described above. The secondary endpoints of change in serum and salivary NO metabolites, serum ADMA and albumin to creatinine ratio will have their differences summarised with sample means, standard deviations, sample mean differences, and sample standard deviations of differences. They too will undergo assessment with a Gaussian LME model as above.

Missing values

Missing values will not be imputed. An advantage of LME models is that they can automatically tolerate missing values by adjusting the respective covariance estimates. Moreover, missing data for the primary outcome is expected to be minimal as this BP data will be collected at the study visits by the investigators.

Data collection and storage

All participants will receive a unique study ID for data collection and analysis. Visit data will be collected on paper-based forms which will be stored in a secure location and will also be scanned to the sponsor's secure server. Visit forms, screening pathology, home BP readings, protocol deviations and adverse outcome reports will be de-identified with the participant study ID and stored on a database on the same secure server. Any identifying information will be stored separately and securely with controlled access.

Data monitoring

BRJ is a food supplement that is readily available for consumption from commercial food outlets. Reported side effects are non-harmful pigmentation of faeces and urine (beeturia) [17]. Previous clinical trials have not reported any serious adverse effects (SAEs) and thus they are

anticipated to be rare in this trial and no interim analyses will be undertaken [17, 25]. Due to the nature of the intervention and the size of the trial, the data monitoring will be undertaken by the trial investigators (who have no competing interests to declare) and there are no interim analyses or audits planned. All data will be stored securely in the Western Sydney Local Health District and will be available for audit as requested by the Human Research Ethics Committee. The funding body has no role in the data monitoring.

Safety monitoring and reporting

The trial staff will monitor for adverse effects or other trial issues at each study visit systematically by asking participants if they have experienced any symptoms, and these will be reviewed in at least fortnightly meetings between the principal investigator (PI) and trial staff. All SAEs will be reported immediately to the PI and the WSLHD HREC, investigated in detail and documented in accordance with the ICH-GCP guidelines. All adverse events will be graded by consensus amongst investigators and reported using the "BEET-PKD Adverse Event Reporting Guide" on an adverse event form (guide and example form provided in Additional file 5). The decision to stop or change the intervention is made by the PI and trial team and is based on the severity of the individual participant's symptoms and, in the setting of mild non-harmful adverse events, the participant's wishes. In the setting where the intervention is changed, it is reported as a protocol deviation. If there is a SAE, unblinding will occur according to the flowchart in Additional file 6 (adapted from the University of Leicester, UK) and the unblinded research staff will reveal the relevant participant's allocation to the PI. The decision to terminate the trial will be taken by the PI and WSLHD HREC and will be based on the review of SAEs. Any reported adverse events related or not related to the intervention will be reported in the publication of trial results.

Post-trial care

As all participants currently receive specialist care within public hospitals and given the nature of BRJ, with its limited and mild side effect profile reported in previous clinical trials (beet-coloured pigmentation of urine and faeces), the investigators do not anticipate a need for additional special provisions for post-trial care [17].

Trial registration

Ethics approval for this study was obtained in May 2021. A submission to the clinical trials registry (ClinicalTrials.gov) was uploaded on 5th April 2022, pre-dating the recruitment of the first participant on 5th May 2022, but was not finalised pending HREC approval of a minor amendment. The trial registration was finalised on 27th May 2022 (registration no: NCT05401409).

Protocol amendments

The study was approved by the Western Sydney Local Health District Human Research Ethics Committee (Approval no. 2020_ETH01718). The investigators will seek approval of the WSLHD HREC prior to the implementation of any changes to the study protocol and notify the health authorities in accordance with local regulations if required. Trial participants, trial registries, journals, and study investigators will be notified as appropriate. Minor changes and clarifications of the protocol that have no impact on the conduct of the study will be documented in a memorandum.

Dissemination of results

The results of the study will be disseminated at national and international scientific meetings and submitted for publication in peer-reviewed journals. Participants will be notified when results of the study are available to the public.

Discussion

There is currently no cure for ADPKD and the search for therapies that will slow disease progression is a major priority for PKD patients, community groups and healthcare providers [31, 32]. In particular, there is interest in developing dietary and lifestyle interventions for ADPKD, and this was highlighted in a recent patient priorities survey, where it was ranked 7 out 17 of major research priorities [32].

BRJ is an attractive novel therapy in the treatment of hypertension as it is all-natural, accessible and has no adverse side effects in human studies to date. There are no expected or reported drug interactions with beetroot juice [28, 29, 46]. There has only been one RCT describing the effects of BRJ in CKD and no studies on an ADPKD cohort [19]. As described earlier, there is an inherent reduction in NO and endothelial NOS activity in ADPKD, which could potentially be corrected by dietary nitrate supplementation via the entero-salivary *NO-nitrite-nitrate* pathway [6, 8]. Previous studies have shown that supplementation with BRJ increases NO metabolites in healthy volunteers and chronic diseases including essential hypertension, diabetes, heart failure, and COPD [16, 20, 36, 47]. Of particular interest, BRJ increases NO metabolites in hypercholesterolemia, which is associated with decreased NO endothelial production, via this alternate entero-salivary pathway [22]. This pathway appears to be attenuated in other conditions such as active smoking, likely due to direct inhibitory effects [37]. This trial aims to investigate if NO

metabolites can be increased using BRJ in ADPKD, given its intrinsic decreased NO state, and furthermore, if that will result in a reduction in BP.

In conclusion, this study will be the first RCT investigating the efficacy of BRJ in reducing BP in hypertensive participants with ADPKD. If the results show a significant decrease in BP, a follow-up study would be warranted to test the efficacy of BRJ over a longer time course and evaluate the potential impacts on chronic cardiovascular outcomes in ADPKD. Moreover, if the results show that NO can be increased with BRJ supplementation, other sources of dietary nitrate (e.g. spinach, rocket, lettuce) could also be explored for BP-lowering effects. Additionally, this study will contribute to the existing evidence on the impacts of dietary interventions in managing chronic kidney diseases, an area of keen interest from the patient community.

Trial status

The current study protocol (version 7) was accepted on 27th June 2022 by the Western Sydney Local Health District Human Research Ethics Committee. Trial recruitment commenced on 5th May 2022 and was completed on 24th March 2023. The trial was registered on 27th May 2022 with ClinicalTrials.gov (NCT05401409), URL: https://clinicaltrials.gov/ct2/show/NCT05401409.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13063-023-07519-2.

Additional file 1. Standardised clinic measurement of blood pressure in the BEET-PKD clinical trial. Description: Table describing the standardisation of blood pressure measurement in the BEET-PKD trial.

Additional file 2. SPIRIT checklist. Description: Checklist for key study protocol elements in this manuscript

Additional file 3. Photo of nitrate-replete and nitrate-deplete beetroot juice bottles. Description: Photo of nitrate-replete and nitrate-deplete beetroot juice bottles.

Additional file 4. Storage and analysis of biological samples in the BEET-PKD clinical trial. Description: Document describing the storage and analysis of biological samples in the BEET-PKD clinical trial.

Additional file 5. BEET-PKD Adverse Event Reporting Guide and Example form. Description: Adverse event reporting guide used by Investigators and Blank adverse event reporting form

Additional file 6. Flowchart to guide unblinding in the BEET-PKD study. Description: Flowchart to guide unblinding in the BEET-PKD study (adapted from the University of Leicester, United Kingdom).

Acknowledgements

Figure 1 was created using Biorender.com.

Authors' contributions

GR is the chief investigator and conceived the idea for the study. GR and PS designed the study and developed the protocol. GR, PS, AM and FV are involved in the implementation of the trial, participant study visits and safety and data monitoring. SS and JE are involved in the randomisation and blinding. PS, FV, BN and JE are involved in the data curation or data analysis. PS and GR drafted the initial version of the manuscript and all authors contributed to editing, critically revising and finalisation of the manuscript. All authors read and approved the final manuscript. Authors for any future publications (including the results manuscript) will include all those who have contributed to the trial implementation and production of the manuscript. Those who have been involved in the development of the study protocol but not the implementation of the trial will be listed in the acknowledgements of future publications. The investigators do not intend to use professional writers.

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Availability of data and materials

The results of the datasets have not been reported in this manuscript. Details of data accessibility (including the public access to the full protocol, model consent form, participant datasets and statistical code) will be included in the publication containing the final results of the study.

Declarations

Ethics approval and consent to participate

The study was approved by the Western Sydney Local Health District Human Research Ethics Committee (Approval no. 2020_ETH01718). Adequate information will be provided to all participants in both oral and written form by the study doctors or senior study investigators present at the study visits, and participants must provide consent in writing prior to commencing any study procedures. At the time of initial consent, participants will be informed of the potential for sharing or re-use of deidentified participant data for the means of future research relating to ADPKD or other related medical conditions and given the option to provide extended consent. The data will be stored in a secure web-based platform and room at WSLHD and will be retained for a minimum of 15 years, as specified in the consent form.

Consent for publication

Not applicable as this manuscript does not contain any details, images or videos related to an individual person.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Michael Stern Laboratory for Polycystic Kidney Disease, Westmead Institute for Medical Research, The University of Sydney, Sydney, NSW 2145, Australia. [2]Department of Renal Medicine, Westmead Hospital, Western Sydney Local Health District, Sydney, NSW 2145, Australia. [3]Research and Education Network, Westmead Hospital, Western Sydney Local Health District, Sydney, NSW 2145, Australia. [4]Department of Renal Medicine, Blacktown Hospital, Western Sydney Local Health District, Sydney, NSW 2148, Australia. [5]Blacktown Clinical School, Western Sydney University, Blacktown, NSW 2148, Australia. [6]Faculty of Medicine and Health, The University of Sydney, Sydney, NSW 2145, Australia. [7]Department of Renal Medicine, Nepean Hospital, Nepean Blue Mountains Local Health District, Sydney, NSW 2750, Australia.

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Publisher's Note

4.3 Supplementary material

Additional File 1: Standardised clinic measurement of blood pressure in the BEET-PKD clinical trial

Environment	• Quiet clinical space • The investigator will leave the room once the participant is appropriately prepared and comfortable with using the BP machine
Participant preparation and equipment	• Participant is sitting in a chair with feet on the floor and back supported • Relaxed, in the sitting position for at least 5 minutes prior to measurement • Not speaking during the rest period or during the measurement • Arm exposed so that cuff is fitted over bare skin • Arm is resting on a table roughly at heart level • Validated AOBP device (Model: A&D UA-611, Tokyo, Japan) used • Correct cuff size used so that 80% of the arm is covered by the cuff
Measurement	• The same arm is used for all clinic and home measurements (arm choice is based on which arm the participant is most dextrous and comfortable with to ensure correct technique with unobserved home BP measurements) • Measure BP three times with 1 minute interval between readings
Data analysis	• An average of the second and third readings will be taken for outcome analysis

BP, blood pressure; AOBP, automated oscillometric blood pressure. Adapted from Kidney Disease: Improving Global Outcomes (KDIGO) Blood Pressure Work Group. KDIGO 2021 Clinical Practice Guideline for the Management of Blood Pressure in Chronic Kidney Disease. Kidney Int. 2021;99(3S):S1–S87.

Additional File 2: SPIRIT checklist

		Reporting Item	Page and Line Number	Reason if not applicable
Administrative information				
Title	#1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	Page 1, lines 1-3	
Trial registration	#2a	Trial identifier and registry name. If not yet registered, name of intended registry	Page 2, line 42 Page 3, line 48	
Trial registration: data set	#2b	All items from the World Health Organization Trial Registration Data Set	Page 2, line 42-43 Page 3, line 48 Page 6, line 110 Page 7, line 135 (Table 1) Page 9 line 156 Page 11, line 170-173 Page 22 line 451-452 Page 15 line 276-277 Page 2 line 35-38, page 6 101-206 Page 23 line 458-459, page 3 line 48 Page 22 line 452 Page 23, line 473-475	
Protocol version	#3	Date and version identifier	Page 3, line 48 Page 22, line 450	
Funding	#4	Sources and types of financial, material, and other support	Page 3, line 48 Page 24, line 481-486	
Roles and responsibilities: contributorship	#5a	Names, affiliations, and roles of protocol contributors	Page 1-2, line 4-24 Page 3, line 48 Page 24, line 489-495	
Roles and responsibilities: sponsor contact information	#5b	Name and contact information for the trial sponsor	Page 3, line 48	
Roles and responsibilities: sponsor and funder	#5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to	Page 3, line 48 Page 19, line 371-372	

		submit the report for publication, including whether they will have ultimate authority over any of these activities	Page 24, line 482-486	
Roles and responsibilities: committees	#5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	Page 19, line 366-369	
Introduction				
Background and rationale	#6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	Page 4-6, line 54-97, Page 11 line 170-200	
Background and rationale: choice of comparators	#6b	Explanation for choice of comparators	Page 11 line 170-200	
Objectives	#7	Specific objectives or hypotheses	Page 6, line 90-97	
Trial design	#8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, non-inferiority, exploratory)	Page 6, line 101-102 , Page 9 line 150, 156, and Figure 1	
Methods: Participants, interventions, and outcomes				
Study setting	#9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	Page 6, line 109-110	
Eligibility criteria	#10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	Page 7, line 118-133, Table 1	
Interventions: description	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	Page 9 line 150-151 Page 10-11 line 170-180 (Figure 1 and 2),	
Interventions: modifications	#11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving / worsening disease)	Page 19 line 382-384, and Additional file 5	
Interventions: adherance	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	Page 9, line 147-148 Page 12 line 203-210	
Interventions: concomitant care	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	Page 12, line 213-215	
Outcomes	#12	Primary, secondary, and other outcomes, including the specific measurement	Page 2, line 35-38 Page 6 line 92-97,	

		variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	line 101-106 Page 13-15, line 227-266 Figure 2 Page 4, line 60-61 and 67-72,	
Participant timeline	#13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	Page 8-10, line 144-159, Figure 1 and Figure 2	
Sample size	#14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	Page 15, line 269-280	
Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	Page 15-16, line 283-291	
Methods: Assignment of interventions (for controlled trials)				
Allocation: sequence generation	#16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	Page 16, line 302-307	
Allocation concealment mechanism	#16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	Page 16, line 302-310	
Allocation: implementation	#16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	Page 16-17, line 302-310	
Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	Page 16-17, line 307-314	
Blinding (masking): emergency unblinding	#17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	Page 16-17, line 312-314, Page 20, line 385-389 and Additional File 6	
Methods: Data collection, management, and analysis				
Data collection plan	#18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if	Page 14-15, line 242-266 Additional file 4	

		known. Reference to where data collection forms can be found, if not in the protocol		
Data collection plan: retention	#18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	Page 12 line 209-210 Page 13 line 220-223	
Data management	#19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	Page 18 line 355-359 Page 19 line 369-371	
Statistics: outcomes	#20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	Page 17-18, line 317-346	
Statistics: additional analyses	#20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	Page 17, line 325-329, Page 18 line 341-343	
Statistics: analysis population and missing data	#20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	Page 13, line 220-223 Page 18, line 349-352	
Methods: Monitoring				
Data monitoring: formal committee	#21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	Page 19, line 362-372	
Data monitoring: interim analysis	#21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	Page 19, line 365-366	
Harms	#22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	Page 19-20, line 375-390, Additional file 5	
Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	Page 19, line 365-366	
Ethics and dissemination				
Research ethics approval	#24	Plans for seeking research ethics committee / institutional review board (REC / IRB) approval	Page 7, line 114-115 Page 20, line 399-400	

			Page 23, line 458-459	
Protocol amendments	#25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC / IRBs, trial participants, trial registries, journals, regulators)	Page 20-21, line 405-412	
Consent or assent	#26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	Page 16 line 292-299 Page 23 line 459-462	
Consent or assent: ancillary studies	#26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	Page 23, line 462-466	
Confidentiality	#27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	Page 18, line 355-360 Page 22, line 462-465	
Declaration of interests	#28	Financial and other competing interests for principal investigators for the overall trial and each study site	Page 24, line 478	
Data access	#29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	Page 23, line 473-475 Page 19, line 369-372	The results of datasets have not been reported in this manuscript. Details of data accessibility will be included when the results of the study are published. This has been explained on Page 23, line 473-475
Ancillary and post trial care	#30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	Page 20, line 393-396	
Dissemination policy: trial results	#31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	Page 21, line 415-417	
Dissemination policy: authorship	#31b	Authorship eligibility guidelines and any intended use of professional writers	Page 24-25, line 496-500	
Dissemination policy: reproducible research	#31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	Page 23, line 473-475	
Appendices				
Informed consent materials	#32	Model consent form and other related documentation given to participants and authorised surrogates	Page 23, line 473-475	The consent form will be published with the final results and complete study protocol at the completion of the study. This has been explained on Page 23, line 473-475
Biological specimens	#33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular	Page 9, line 147 and 155. Page 14-15 line	

		analysis in the current trial and for future use in ancillary studies, if applicable	260-266, Additional file 4	

It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license. This checklist can be completed online using https://www.goodreports.org/, a tool made by the EQUATOR Network in collaboration with Penelope.ai

Additional File 3: Photo of nitrate-replete and nitrate-deplete beetroot juice bottles

Additional File 4: Storage and analysis of biological samples in the BEET-PKD clinical trial

Preparation and storage of samples

Blood, urine and saliva samples will be collected at Visit 1 and 3 for urinary albumin to creatinine ratio (ACR), NO metabolites and ADMA levels. Blood will be collected in a gel separator tube and allowed to clot. The tube will then be centrifuged at 1000xg for 15 minutes after which the serum will be aliquoted and stored at -30ºC. Urine will be transported to the lab on ice. 25mls of the urine sample will be sent for formal laboratory measurement of urinary ACR (Institute of Clinical Pathology and Medical Research (ICPMR), Westmead Hospital). The remaining urine sample will be centrifuged at 1000xg for 5 minutes. 12.5mls of urine will be mixed with 50mg sodium hydroxide (as a preservative), the remaining sample will be aliquoted and all samples will be stored at -30ºC. Saliva samples will also be collected for future post-hoc analysis of oral microbiome.

Analysis of serum and salivary nitrate/nitrite by enzyme-linked immunosorbent assay (ELISA)

Nitrate/nitrite analysis of serum and saliva will be performed using commercially available colorimetric Nitric Oxide Assay kits, based on the Griess assay, according to the manufacturer's instructions. Briefly, nitrate is reduced to nitrite by the addition of nitrate reductase to the plasma and saliva samples, followed by the addition of Griess reagent to form a deep purple azochromophore for measurement of nitrate/nitrite using a microplate reader (OD 540 nm).(1) Saliva samples will be deproteinised prior to the initial reduction step, as recommended by the manufacturer.

Analysis of serum asymmetric dimethylarginine (ADMA) by ELISA

Serum ADMA analysis will be performed using a commercially available quantitative sandwich ELISA kit, according to the manufacturer's instructions. Briefly, the sample is added to a microplate coated with immobilised anti-ADMA antibodies to bind to ADMA present in the sample. Unbound substances will then be removed, followed by the addition of biotin conjugated ADMA specific antibodies. A substrate solution is then added and absorbance at 450 nm will be measured using a microplate reader to determine the amount of ADMA in the samples.

References

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Additional File 5: BEET-PKD Adverse Event Reporting Guide and Example form

(Contained on pages 118-120)

1. **An AE is any untoward medical occurrence, unfavourable and unintended sign, symptoms or disease in the participant temporarily associated with the clinical study whether or not considered related to the procedures. AEs will be documented from the time of randomization until the final visit. Changes to health between the time of consent and the time of randomization will be recorded as Medical History.**
    a. Procedures are defined as intervention and or study requirements e.g. blood tests, MRI.

2. **Any AE that result in the following outcomes is considered as serious:**
    a. Death
    b. Life-threatening
    c. Requires in-patient hospitalization OR significant testing or treatment upon presentation to ED OR if ambulance is called by participant
        i. Significant testing or treatment= beyond triage, beyond pain relief or prescription given (e.g. given IV antibiotics or pain relief or fluids OR CT scan/other imaging)
        ii. Please note, the AE or AEs that prompted presentation to the hospital should only be labelled serious. E.g. if participant has cough and then develops macrohaematuria and fever, presents to ED because of macrohaematuria and fever, then only these should be marked as serious despite concomitant cough.
    d. Significant disability or incapacity
    e. Congenital abnormality or birth defect
    f. Medically significant e.g. ESRF, doubling of serum creatinine, significant drop in kidney function

3. **Intensity:**
    a. Mild – easily tolerated, causing minimal discomfort and not interfering with everyday activities.
        i. Asymptomatic/incidental finding/symptoms not affecting usual lifestyle or able to take (For example) paracetamol and carry on with usual day/activities
    b. Moderate – sufficiently discomforting to interfere with normal activities.
        i. Symptoms prompt time off work(still able to complete ADLs)/GP/Allied health visit and/or requires a prescription treatment or requires day surgery
    c. Severe – prevents normal everyday activities.
        i. Symptoms prompt time off work (bedridden) (with/without GP visit) or presentation to hospital or requires surgery/operation as an outpatient

    NB: Where classifying AEs where all necessary information was not collected at time of reporting and is unable to be reported or not remembered by participant, the AE should be classified to the best of ability.

4. **Causality:**
    a. Not related – no causal relationship between the study procedures/intervention and the AE.
    b. Unlikely- temporally associated with study procedures/interventions but are not likely to have any reasonable association with the AE.
    c. Possible – temporally associated with the study procedures/intervention but could have been caused by patient's clinical state or other modes of therapy administered.
    d. Definite – temporally associated with study procedures/intervention, abates upon discontinuation of procedure/ intervention and reappears when reintroduced (e.g. Beeturia)

Additional Notes

1. **What constitutes an AE?**
    a. Any PKD related checklist finding – new condition and worsening are separate AEs
    b. Any change in normal or expected health of participant (From participant perspective)
    c. Any pain
    d. Any event in which procedures are completed, investigations done or treatment provided
    e. Any signs or symptoms (in some cases even if self-reported, e.g. refer to Oedema)
    f. Any official diagnoses by participant's doctor or written in a discharge summary
    g. Any 'flare-up' or worsening of symptoms (increase in frequency or intensity) of a chronic condition noted in medical history
        i. Marked as ongoing if no specific/noticeable ceasing of symptoms
    h. Any new conditions detected or diagnosed after randomization even though it may have been present prior to the start of the study (e.g. liver cysts identified at Month 18 MRI where participant had not noted them in screening, and were not reported in screening MRI)
    i. Any conditions or events noted in a doctors letter that the participant has failed to/not remembered to disclose.
    j. Where a participant has one AE that is then diagnosed (e.g. flank pain > pyelonephritis, bursitis > arthritis), then the AE is labelled the former symptom or diagnosis up until the diagnosis or confirmed diagnosis. The end date of the former (e.g. flank pain) is the date of diagnosis of the latter (pyelonephritis). There are many reasons for this including: diseases/illnesses have varied signs and symptoms and merely labelling as the disease state does not capture this; even if a symptom is associated with a diagnosis, it might not be related to that and the role of the study/intervention should still be considered – having only a diagnostic AE or disease term does not allow for this scrutiny; promotes integrity by only recording diagnoses or associations between symptoms/signs and condition which are official and complete by the participants

medical team/the investigator and study team does not assume. Please also note that where symptom led to medically significant event (e.g. hospitalization) and then later clinical impression /diagnosis made, both are considered AEs, both are considered serious.

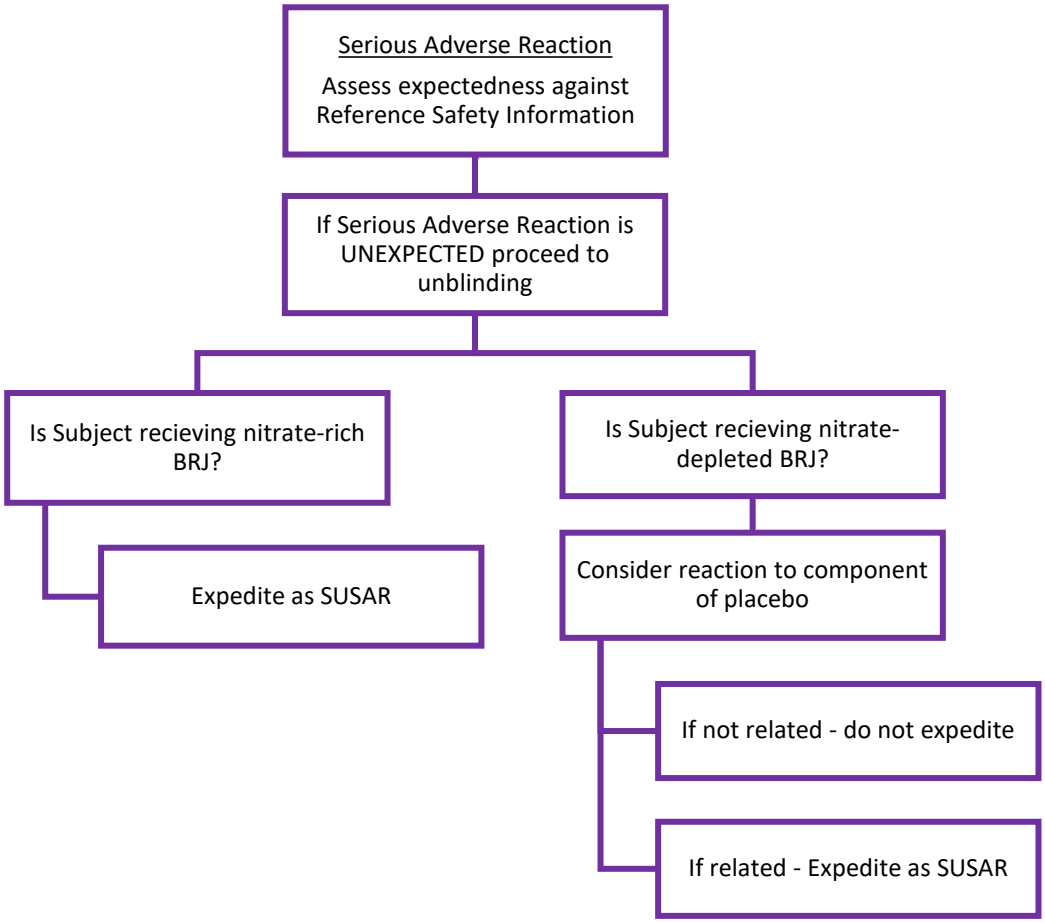
2. **Start and End Date**
    a. Where a participant cannot remember an AE date, a proxy date will be used that as closely resembles as possible when the event occurred. For example (1) "It happened Mid May 2019" = 15/05/2019 or (2) "It happened start of May 2019" = 01/05/2019.
    b. This extends to end dates where a participant cannot remember exactly how long symptoms occurred for or when they resolved. For example (1) "It happened Mid May and lasted a few days" = 15/05/2019-18/05/2019 or (2) "I had a cold at the end of May and it lasted a couple of days" = 31/05/2019-2/05-2019.
    c. Where participants cannot give a rough indication of how long symptoms occurred for or when they resolved OR when participants are still experiencing the AE at the time of current visit where it is reported and if the AE is not reported at the consequent study visit, the date of the consequent study visit can be entered as end date. For example (1) Pt. reported "I have a sinus infection at the moment" at Visit 3 (01/05/19). Pt. did not report sinus infection at Visit 4 (01/06/19), but cannot remember when symptoms ceased, end date of sinus infection is (01/06/19).
        i. This is to ensure we are capturing the longest period for which symptoms could have been present
3. **Is it a PKD related event? (Please refer to table below for specific AEs and examples)**
    a. Any findings from the PKD checklist are PKD related events.
    b. Pain (abdominal, flank and back) that have no other reasonable explanation can be considered PKD related.
    c. Often TBC based on Investigator interpretation / refer to AE classification table.
    d. If an AE occurs as a result of Tolvaptan e.g. polyuria, then it is *not* a PKD related event.
4. **Treatment or diagnostic procedure**
    a. All investigations, procedures or treatments should be entered on concomitant medication form.
    b. If treatments are unknown but the AE could not be resolved otherwise according to medical judgement, 'unspecified treatment' or 'unspecified medical treatment' or 'unspecified hospital treatment' can be entered as a proxy.
    c. If participant sees a specialist/doctor and cannot remember investigations or procedures done, please enter 'Specialist review' for example.
    d. If participant sees an allied health practitioner, this should be recorded. For example as 'physio treatment'.
    e. All OTC supplements should be captured. If they are not taken for medical need or taken without symptoms, indication is "wellbeing"

BEET-PKD Study Adverse Event Form

Screen ID S_____ Randomization ID R_____

Event Term	
Narrative	
Start Date	
Is this a PKD disease related event?	
Is this a serious event (SAE)?	
Date when the event became serious:	
Seriousness criteria	☐ Result in death ☐ Life-threatening ☐ Requires or prolongs hospitalisation ☐ Results in disability or incapacity ☐ Result in congenital anomaly or birth defect ☐ Medically significant:
Intensity	☐ Mild ☐ Moderate ☐ Severe
Action taken with study treatment	☐ Unchanged/no action ☐ Withdrawal of treatment ☐ Dose increased ☐ Dose decreased
Causality	☐ Not related ☐ Unlikely ☐ Possible ☐ Definite
Any treatment or diagnostic procedure given?	
Outcome	☐ Fatal ☐ Ongoing ☐ Permanent residual effect ☐ Resolved
Resolved date	

Additional File 6: Flowchart to guide unblinding in the BEET-PKD study (adapted from the University of Leicester, United Kingdom)



Abbreviations: SUSAR; suspected unexpected serious adverse event

Chapter 5: Results of a double-blind randomised controlled trial of nitrate-replete beetroot juice on lowering blood pressure in hypertensive adults with autosomal dominant polycystic kidney disease

5.1 Preface

Chapter 5 describes the results of the BEET-PKD trial, a novel RCT examining the efficacy of BRJ in reducing blood pressure in hypertensive adults with ADPKD. This is the only trial to date reporting the effects of nitrate supplementation on blood pressure in ADPKD. The aim of this study was to test the hypothesis that the daily dosing of BRJ (400mg nitrate) reduces blood pressure in hypertensive adults with ADPKD. A 4-week double-blind, two-armed, randomised (1:1), placebo-controlled trial was performed. The co-primary endpoints were change in office systolic and diastolic blood pressure after 4 weeks of BRJ. The secondary endpoints were change in home blood pressure and urine albumin:creatinine ratio after 4 weeks of BRJ.

The trial recruited 60 participants, commenced on 5th May 2022 and was completed on 24th March 2023.

5.2 Double-Blind Randomised Controlled Trial of Nitrate-Replete Beetroot Juice on Blood Pressure Lowering in Hypertensive Adults with Autosomal Dominant Polycystic Kidney Disease

This manuscript is under review for publication by the peer-reviewed journal *Kidney International Reports*. The formatting and writing style are in keeping with the journal requirements. References are self-contained within the manuscript. Section 5.3 contains supplementary material referenced in the manuscript.

Double-Blind Randomised Controlled Trial of Nitrate-Replete Beetroot Juice on Blood Pressure Lowering in Hypertensive Adults with Autosomal Dominant Polycystic Kidney Disease

Authors: Priyanka S Sagar[1,2,3], James Elhindi[4], Katrina Chau[5,6], David C Harris[1,2], Vincent Lee[2,7], Kamal Sud[3,7], Nikki Wong[3,7], Gopala K Rangan[1,2]

Affiliations:

[1]Michael Stern Laboratory for Polycystic Kidney Disease, Westmead Institute for Medical Research, The University of Sydney, Sydney, NSW 2145, Australia

[2]Department of Renal Medicine, Westmead Hospital, Western Sydney Local Health District, Sydney, NSW 2145, Australia

[3]Nepean Kidney Research Centre, Department of Renal Medicine, Nepean Hospital, Nepean Blue Mountains Local Health District, Sydney, NSW 2750, Australia

[4]Research and Education Network, Westmead Hospital, Western Sydney Local Health District, Sydney, NSW 2145, Australia

[5]Department of Renal Medicine, Blacktown Hospital, Western Sydney Local Health District, Sydney, NSW 2148, Australia

[6]Blacktown Clinical School, Western Sydney University, Blacktown, NSW 2148, Australia

[7]Faculty of Medicine and Health

INTRODUCTION

Reduced circulating levels of nitric oxide metabolites are hypothesised, in part, to drive hypertension in autosomal dominant polycystic kidney disease (ADPKD). [1] Beetroot juice (BRJ), a rich dietary source of nitrate, and increases nitric oxide metabolites *via* the entero-salivary nitrate-nitrite-NO pathway, and lowers blood pressure. [2] In the largest randomised controlled trial of 68 hypertensive patients, nitrate-replete BRJ reduced systolic blood pressure by a mean of 9.0±7.8 mmHg after four weeks compared to 0.1±8.0 mmHg in the nitrate-deplete BRJ group. [3] In this study, we tested the hypothesis that oral nitrate supplementation with BRJ lowers blood pressure in ADPKD. We undertook a 4-week double-blind two-parallel group randomised placebo-controlled trial and compared a daily shot of either nitrate-replete BRJ (400 mg/day) or nitrate-deplete BRJ on clinic blood pressure (primary endpoint), home blood pressure, urine albumin-to-creatinine ratio and adverse effects (secondary endpoints) (Supplementary Methods).

RESULTS

Recruitment, follow-up and baseline characteristics. Three hundred and seventy six participants were screened for eligibility, 313 were excluded and 3 were screen failures leaving 60 participants that were randomised (Supplementary Fig S1). One participant in the nitrate-replete group ceased BRJ due to pregnancy but continued follow-up; 2 in the nitrate-deplete group ceased BRJ (1 lost to follow-up; 1 due to side effects but continued follow-up). The baseline characteristics did not differ between the BRJ groups (Table 1 and Supplementary Tables S2 and S3) and the majority of visits occurred in the morning (Supplementary Table S2 S4).

Effect of BRJ on nitric oxide metabolites. Serum nitrate/nitrite increased 3.6-fold in the nitrate-replete group at 4 weeks compared to the nitrate-deplete group ($P<0.05$, Supplementary Fig. S5). The mean change from baseline in the nitrate-replete BRJ group was 20.08 μM ($p<0.01$) compared with 0.97 μM ($p=0.30$) in the nitrate-deplete BRJ group.

Primary Endpoint. At 4 weeks, in the nitrate-replete BRJ, the mean systolic and diastolic clinic blood pressure decreased from baseline (systolic -5.36±12.69; diastolic -3.55±7.08 mmHg; $p<0.05$) (Fig. 1). In the nitrate-deplete BRJ groups only systolic blood pressure decline (-7.65±14.07 mgHg; $p=0.01$; diastolic -2.73±7.81 mgHg, $p=0.07$). There was no inter-group differences between the nitrate-replete and nitrate-deplete BRJ groups (systolic, $p=0.51$; diastolic, $p=0.67$) or when adjusted for age, eGFR and baseline serum nitrate.

Secondary Endpoints. Home blood pressure decreased in both nitrate-replete [systolic -0.51 (-1.40, 0.38); diastolic -0.24 (-0.85, 0.38) mmHg/week] and nitrate-deplete BRJ groups [systolic -0.55 (-1.42, 0.31); diastolic -0.47 (-1.07, 0.13) mmHg/week] (Supplementary Fig. S6). However, this was not different between the groups (systolic $p=0.95$; diastolic $p=0.59$) or when adjusted for age, eGFR and baseline serum nitrate. Albuminuria was unchanged after 4

weeks in both groups (0.20 mg/mmol; and -0.40 mg/mmol, respectively), and there no inter-group difference (p=0.91) or when adjusted for age, eGFR and baseline nitrate levels.

Treatment adherence. The number of returned empty BRJ bottles, did not differ between the two groups (100%, IQR 96.67-100 in both groups). The majority of participants consumed BRJ in the morning (87% in nitrate-replete group; 97% in the nitrate-deplete group) and similar in both groups (p=0.32; Supplementary Table S7). Adherence with daily home blood pressure measurements, which was calculated by response to text messages, was the same in both groups (100%, IQR 100-100 in nitrate-replete group; 100%, IQR 92.83-100 in the nitrate-deplete group).

Adverse effects. There were no inter-group differences in the frequency of adverse effects (Supplementary Table S8). Gastrointestinal symptoms (change in bowel habit, bloating, gastritis) were the most frequent side effect occurring in 38% (23/60) of participants. Beeturia and beet-coloured faeces occurred in 37% (22/60) and 48% (29/60), respectively. Of note, 20% (12/60) perceived that side effects (improved bowel habits 13%; increased energy in 5%) were beneficial. Three percent (2/60) also reported positive symptoms of intentional weight loss due to increased satiety.

DISCUSSION

In this 4-week double blind randomised placebo controlled trial, a single daily shot of BRJ (either nitrate replete or nitrate deplete) lowered clinic blood pressure in hypertensive participants with ADPKD. Moreover, home blood pressure, albuminuria, treatment adherence, discontinuations and adverse effects were similar in both groups.

The increase in nitrate and effect size of blood pressure reduction with BRJ in this study was consistent with previous studies[2, 3]. These data suggest that the anti-hypertensive effect of BRJ in ADPKD does not require nitrate and is secondary to multiple other non-nitrate components of BRJ (such as betalains, flavonoids, polyphenols, saponins, and vitamin C which cause vasodilation) [2, 3]. In this regard, higher serum betaine was associated with lower blood pressure in the Guangzhou Nutrition and Health prospective observational cohort study consisting of 1339 patients[4]. A second explanation is that the blood pressure reduction with BRJ was due to a placebo-associated effect[5]. In a meta-analysis of 52 hypertension trials in non-ADPKD patients (n=7451), Patel *et al.* found that the systolic blood pressure decreased by mean of 5.92 mmHg in non-resistant hypertensive placebo groups. [5] Multiple factors could explain blood pressure improvements with placebo, including better in-trial compliance, regression to the mean and/or unintentional bias[5]. In our study, adherence was similar in both groups; multiple home blood pressure measurements were performed; participants were only included if their hypertension was stable; and the trial design was double-blind reducing bias. Nevertheless, the possibility of a placebo-associated effect, having some role, cannot be entirely excluded.

In previous observational cohort studies serum levels of nitric oxide metabolites are reduced by ~50% in ADPKD, and *in vitro* intracellular nitric oxide is decreased by ~90% in human ADPKD-mutated kidney cell lines[1, 6]. The mechanisms for the reduction in nitric oxide

have been hypothesised to be due to impaired nitric oxide synthase activity mediated by polycystin dysfunction together with increased asymmetric dimethyl arginine[1, 6, 7]. The results of this clinical trial suggest that oral nitrate does not have a major anti-hypertensive effect in ADPKD, and is consistent with previous animal studies of PKD[6].

Nearly 40% of participants reported gastrointestinal side effects which is higher than previous BRJ trials[3]. This could be due to mass effects of polycystic kidneys which leads to increased sensitivity to gastrointestinal symptoms[8]. Of note, some participants perceived improvement in frequency and consistency of stools as being positive. BRJ also contains oxalic acid (300-525 mg/L in juice) [2], and in animal models of polycystic kidney disease oxalate consumption predisposes to microtubular crystal formation[9]. In the current study, no episodes of renal colic occurred but the duration was insufficient to evaluate this completely.

The main strengths of study are the double-blind and pragmatic trial design which reduced bias and participant burden (as reflected by excellent engagement and adherence). The limitations were that a non-BRJ group was not included; the duration was 4 weeks and included only two timepoints; changes in diet during the trial were not evaluated; and long-term safety of BRJ in ADPKD has not been assessed.

In conclusion, a daily shot of BRJ lowered blood pressure in ADPKD independent of nitrate levels. The anti-hypertensive effects of BRJ could be due to biologically active non-nitrate components. These data provide further evidence of the benefits of healthy eating (comprising vegetables) together with regular blood pressure monitoring in ADPKD.

DECLARATIONS

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Conflict of Interest: GR is a member of the scientific advisory board of PKD Australia. No other conflicts of interest are declared.

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DATA SHARING

De-identified individual participant data that underlie the results reported in this article will be available to researchers in a public repository 12 months following publication of this article. Requests for the repository location and data should be directed to the corresponding author.

LIST OF SUPPLEMENTARY MATERIAL

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Table 1: Baseline characteristics

Characteristic	Nitrate-replete BRJ group (n = 30)	Nitrate-deplete BRJ group (n = 30)
Age	50 (14)	49 (12)
Female Sex	43.3% (13)	46.7% (14)
Self-reported ethnicity *Caucasian*	67% (20)	73% (22)
Asian	27% (8)	23% (7)
Other	7% (2)	3% (1)
Height (cm)	174.4 (10.5)	171.7 (9.1)
Weight (kg)	84.6 (16)	85.9 (18.7)
BMI	28 (5)	29 (6)
Age at diagnosis of ADPKD	31 (13)	33 (14)
Family history of ADPKD *Yes*	73% (22)	77% (23)
No	27% (8)	13% (4)
Unknown	0.00% (0)	10% (3)
Age at diagnosis of hypertension	36 (12)	35 (11)
Years since hypertension diagnosis *	10 (5 – 20)	11 (5 – 20)
Number of anti-hypertensives*	1 (1 – 2)	1 (1 – 2)
Anti-hypertensive drug class *ACEi/ARB*	93% (28)	93% (28)
Diuretic	10% (3)	17% (5)
CCB	30% (9)	43% (13)
Beta-blocker	23% (7)	30% (9)
Alpha-blocker	10% (3)	3% (1)
Combination therapy	10% (3)	17% (5)
Comorbidities *Type 2 Diabetes Mellitus*	0.00% (0)	3% (1)
IHD	0.00% (0)	0.00% (0)
Stroke	3% (1)	3% (1)
Hypercholesterolemia	17% (5)	30% (9)
No. prescribed medications*	2 (1 – 3)	3 (2 – 5)
No. total supplements*	4 (3 – 7)	4 (2 – 6)
Creatinine (μmol/L)*	97 (85 – 148)	105 (85 – 135)
eGFR (mL/min/1.73m^2)*	63 (44 – 87)	61.5 (44 – 82)
Total cholesterol (mmol/L)	4.93 (0.91)	4.90 (0.88)
Triglycerides (mmol/L)	1.10 (0.95 – 1.70)	1.50 (0.90 – 2.20)
Fasting glucose (mmol/L)	4.8 (0.5)	5.0 (0.6)

Note: Continuous variables sufficiently approximated by normal distribution are summarised by sample mean and standard deviation, and those that are not sufficiently approximated by a normal distribution were summarised by sample median and IQR (marked by *). Categorical variables are summarised by sample proportions and count.

Abbreviations: ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; CCB, calcium channel blocker; IHD, ischemic heart disease; eGFR, estimated glomerular filtration rate.

5.3 Supplementary material

METHODS

Study Population

The study protocol has been previously published. [1] Participants aged over 18 years old with ADPKD and hypertension on at least one anti-hypertensive therapy with estimated glomerular filtration rate (eGFR) >30ml/min/1.73m^2 were eligible. Participants who had labile or uncontrolled blood pressure, medical conditions that might interfere with the generation of NO metabolites or the primary endpoint (such as the use of nitrate drugs, smoking, pregnancy), any serious medical conditions that might interfere with stability of blood pressure (uncontrolled diabetes mellitus, active malignancies), dislike or allergy to beetroot, or were unable to comply with trial procedures in the screening period, unable to provide informed consent, or enrolled in other trials concurrently were excluded.

Study Design

This was an investigator-initiated, single-centre, double-blind, two-group (1:1) parallel randomised controlled trial. Recruitment commenced on 5th May 2022 and completed on 24th March 2023. The study was undertaken on the Westmead Health Precinct that includes both Westmead Hospital and the Westmead Institute for Medical Research. The trial comprised of three visits. At Visit 1, participants underwent baseline history, blood pressure measurement, and blood and urine sample collection. Participants measured their blood pressure daily at home for one week until Visit 2 to ensure eligibility. Compliance with blood pressure recording and other inclusion criteria were checked at Visit 2 after which participants commenced their allocated intervention and daily home blood pressure measurements for four weeks.

Participants received a daily oral dose (70 ml) of either treatment (nitrate-replete) (Beet-IT Sport Nitrate 400 Shot; James White Drinks, UK; 400mg nitrate/70mL) or placebo (nitrate-deplete) BRJ (nitrate-depleted Beet-IT Sport Nitrate 400 Shot, James White Drinks, UK) which were identical in taste, smell, appearance, and packaging. Participants were sent a daily text message as a reminder to drink their BRJ and report their home blood pressure. Replies to these messages were used to verify adherence in real-time. Participants were asked to consume the BRJ with food at the same time every day. After 28 days, participants attended Visit 3 for clinic blood pressure measurements and post-intervention sample collection.

The study was approved by the Western Sydney Local Health District Human Research and Ethics Committee and performed in accordance with the ICH Good Clinical Practice guidelines. The trial was registered on clinicaltrials.gov (NCT05401409). Trial funding was provided by PKD Australia. The trial steering committee consisted of the authors, and given the size and nature of the trial the safety and data monitoring was undertaken by the trial investigators, in accordance with ICH-GCP guidelines and the Human Research Ethics Committee approved protocol. [1]

Primary and Secondary Endpoints

The primary outcomes are change in mean systolic and diastolic blood pressure at Visits 1 and 3. The secondary outcomes are change in mean systolic and diastolic home blood pressure; change in serum total nitrate/nitrate levels; and change in urine albumin to creatinine levels between baseline and after 4 weeks of treatment with daily BRJ consumption.

Study procedures and laboratory methods

Measurement of clinic and home blood pressure. In the clinic, blood pressure was measured three times, in a seated position after at least 5 minutes of rest, in a quiet room and without the investigator present, in accordance with the 2020 International Society of Hypertension Global Hypertension Practice Guidelines. [2, 3] For home blood pressure measurement, participants were provided with an automated sphygmomanometer (A&D oscillometric device Model: UA-611, Tokyo, Japan), instructed on correct measurement technique[2, 3], and were asked to measure their blood pressure in similar conditions for their daily home measurements.

Measurement of total serum nitrate/nitrite and other methods. Total serum nitrate/nitrate was measured using a colorimetric assay (#780001, Cayman Chemical, Ann Abhor, MI, USA), as an indirect measure of NO production induced by nitrate replete BRJ. Other study procedures and methods have been described in the published study protocol. [1]

Randomisation and Allocation Concealment

Sixty participants were randomised 1:1 using a software program (version 3.6.2, R Core Team) designed by the study biostatistician. A staff member not involved with study procedures packaged the BRJ with a unique ID, which was sequentially allocated to each randomised participant. All study staff and participants were therefore blinded to their allocation until trial completion.

Statistical methods

The sample size of 60 participants was determined by expecting a reduction of 7.7mmHg (3.6-11-8 mmHg) with daily supplementation of 400mg nitrate in BRJ, based on

previous studies in a hypertensive population.[4] Using Stata SE Version 14.2 (StataCorp, TX, U.S.A), a test for differences in two independent means was implemented, and it was found that 28 participants in each group were required for 80% power and a 0.05 level of significance. To account for dropout, the sample size was increased to 30 participants in each group (n=60 total participants). The statistical analysis plan has been described in detail previously.[1] Briefly, the primary and secondary outcomes were summarised by their sample mean (and standard deviation) at baseline, 4-week follow-up, and paired change. A linear mixed effects model with a first-order autoregressive correlation structure for time within participant, modelled the primary and secondary outcomes. Significance of an interaction parameter between treatment arm and time was used to assess the difference in effect between arms. The models were adjusted for age, eGFR, and baseline total serum nitrate/nitrite as prognostic factors. When serum nitrate was the response variable, it was log-transformed to ensure conformity with normality assumptions.

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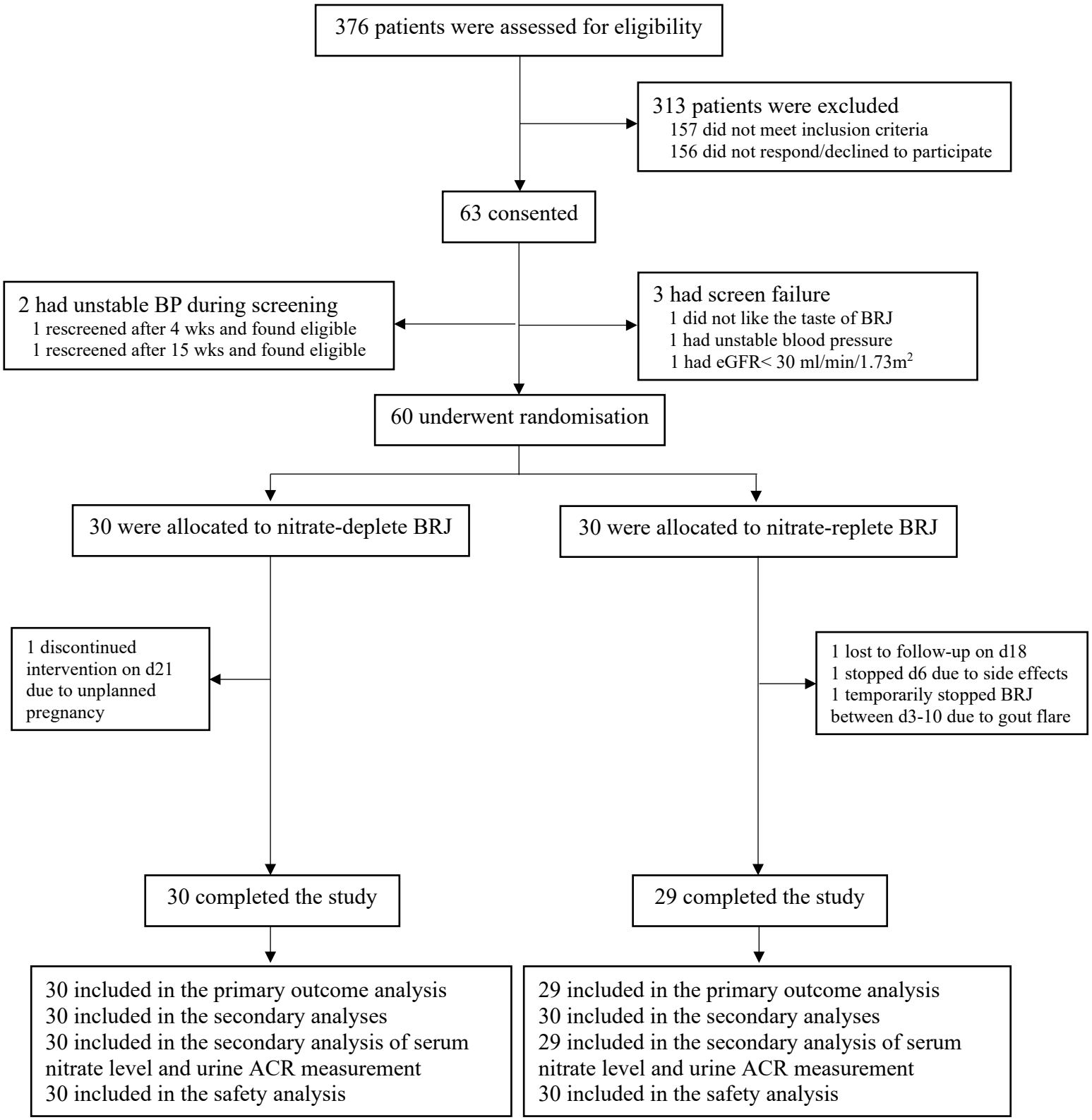
Supplementary Fig. S1: Flowchart of participant recruitment, randomisation and completion.

As shown in Supplementary Fig. S1, 376 participants were screened for eligibility, 313 were excluded leaving 63 participants who completed the informed consent. Three participants were then excluded due to screen failure leaving 60 participants who were randomised and underwent group allocation. Of the 60 randomised participants, 3 were re-screened and had delayed entry into the study due to an initially unstable blood pressure which was then managed by their treating nephrologist. Overall, 1 participant in the nitrate-replete BRJ group discontinued study intervention but continued with follow-up due to pregnancy, and 2 participants in the nitrate-deplete BRJ group ceased study intervention (1 was lost to follow-up and the other discontinued due to side effects but had follow-up measurements performed).

Abbreviations: ACR, albumin to creatinine ratio; BRJ, beetroot juice; d, day; eGFR, estimated glomerular filtration rate.


376 patients were assessed for eligibility
313 patients were excluded
157 did not meet inclusion criteria
156 did not respond/declined to participate
63 consented
2 had unstable BP during screening
1 rescreened after 4 wks and found eligible
1 rescreened after 15 wks and found eligible
3 had screen failure
1 did not like the taste of BRJ
1 had unstable blood pressure
1 had eGFR< 30 ml/min/1.73m2
60 underwent randomisation
30 were allocated to nitrate-deplete BRJ
30 were allocated to nitrate-replete BRJ
1 discontinued intervention on d21 due to unplanned pregnancy
1 lost to follow-up on d18
1 stopped d6 due to side effects
1 temporarily stopped BRJ between d3-10 due to gout flare
30 completed the study
29 completed the study
30 included in the primary outcome analysis
30 included in the secondary analyses
30 included in the secondary analysis of serum nitrate level and urine ACR measurement
30 included in the safety analysis
29 included in the primary outcome analysis
30 included in the secondary analyses
29 included in the secondary analysis of serum nitrate level and urine ACR measurement
30 included in the safety analysis

Supplementary Table S2: Additional baseline characteristics

Additional baseline history characteristic were collected including history of precipitating factors leading to diagnosis, and confirmatory test to diagnose ADPKD (Suppl. file 1). 40-43% of participants were diagnosed due to screening from family history and 100% had their diagnosis confirmed by renal tract imaging. Participants with a family history had a median of 3 affected relatives in both groups. History on other manifestations and complications of ADPKD were also collected and did not differ between nitrate replete or placebo groups (supplemental file 2). The most common extra-renal manifestation was liver cysts which was present in 43-50% of participants. The most common complication was urinary tract infection in 33-37% followed by kidney stones in 20-30%.

Characteristic	Nitrate-replete group (n = 30)	Nitrate-deplete group (n = 30)
Australian born	43.3% (13)	63.3% (19)
Employment status ***Employed***		
Full time	75.9% (22)	86.7% (26)
Part time	24.1% (7)	13.3% (4)
Diagnosis precipitating factor		
Screening (family history)	40.0% (12)	43.3% (13)
Pain	10.0% (3)	20.0% (6)
Hypertension	26.7% (8)	6.7% (2)
Incidental	6.7% (2)	16.7% (5)
Other	16.7% (5)	13.3% (4)
Diagnosis confirmatory test		
Radiographic	100% (30)	100% (30)
Age at diagnosis of ADPKD	30.9 (13.31)	33.1 (13.51)
Family history of ADPKD		
Yes	73.3% (22)	76.7% (23)
No	26.7% (8)	13.3% (4)
Unknown	0% (0)	10.0% (3)
Number of Affected Relatives*	3(2 – 4)	3 (2 – 4)

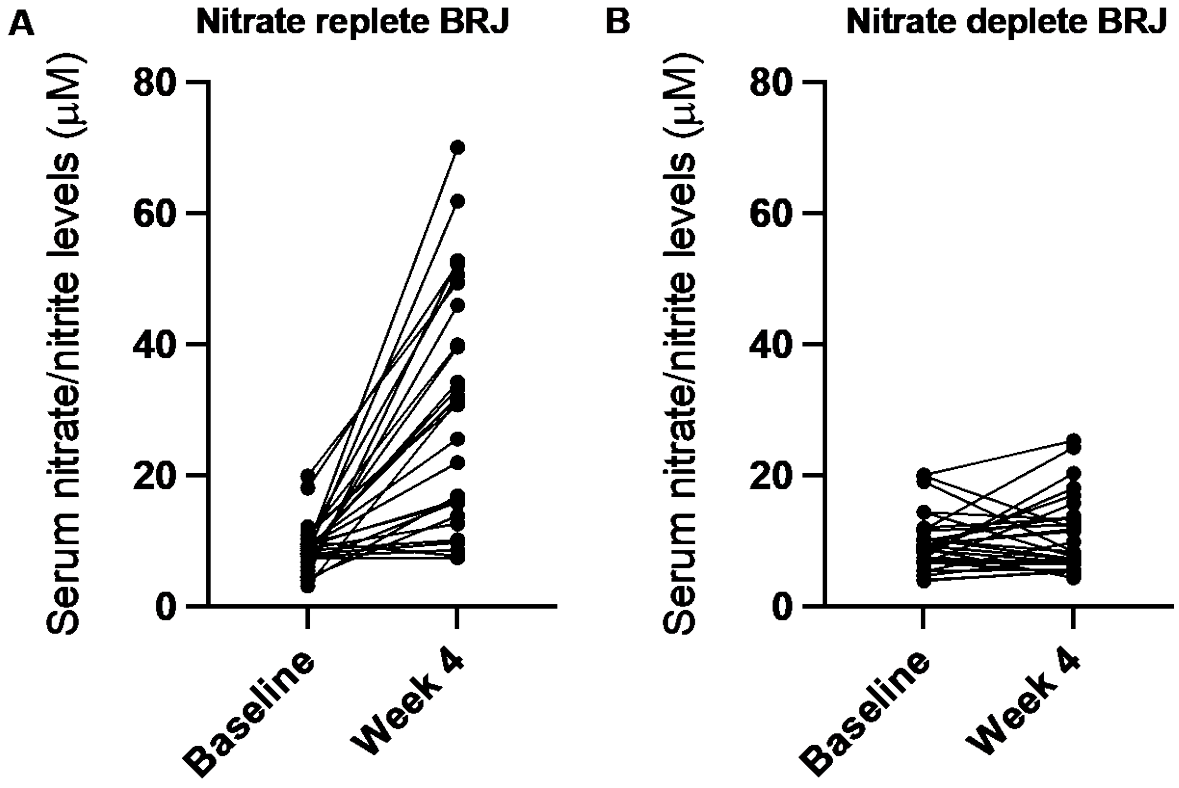
Supplementary Table S3: Additional baseline characteristics (extra-renal manifestations)

Characteristic	Nitrate-replete group (n = 30)	Nitrate-deplete group (n = 30)
Liver cysts	43.3% (13)	50.0% (15)
Other cysts	13.3% (4)	16.7% (5)
Kidney cyst infection	20.0% (6)	16.7% (5)
Kidney cyst rupture	30.0% (9)	13.3% (4)
Proteinuria symptoms	20.00% (6)	20.0% (6)
Macrohematuria symptoms	20.0% (6)	20.0% (6)
Intracranial aneurysm	3.3% (1)	6.7% (2)
Previous cerebral imaging	70.0% (21)	60.0% (18)
Kidney stones	30.0% (9)	20.0% (6)
Urinary tract infection	33.3% (10)	36.7% (11)
Colonic diverticulum	3.3% (1)	13.3% (4)
Valvular/cardiac disease	23.3% (7)	10.0% (3)
Hernias	16.7% (5)	26.7% (8)
Fertility Issues	10.0% (3)	3.3% (1)

Supplementary Table S4: Time of clinic visits and blood pressure measurements

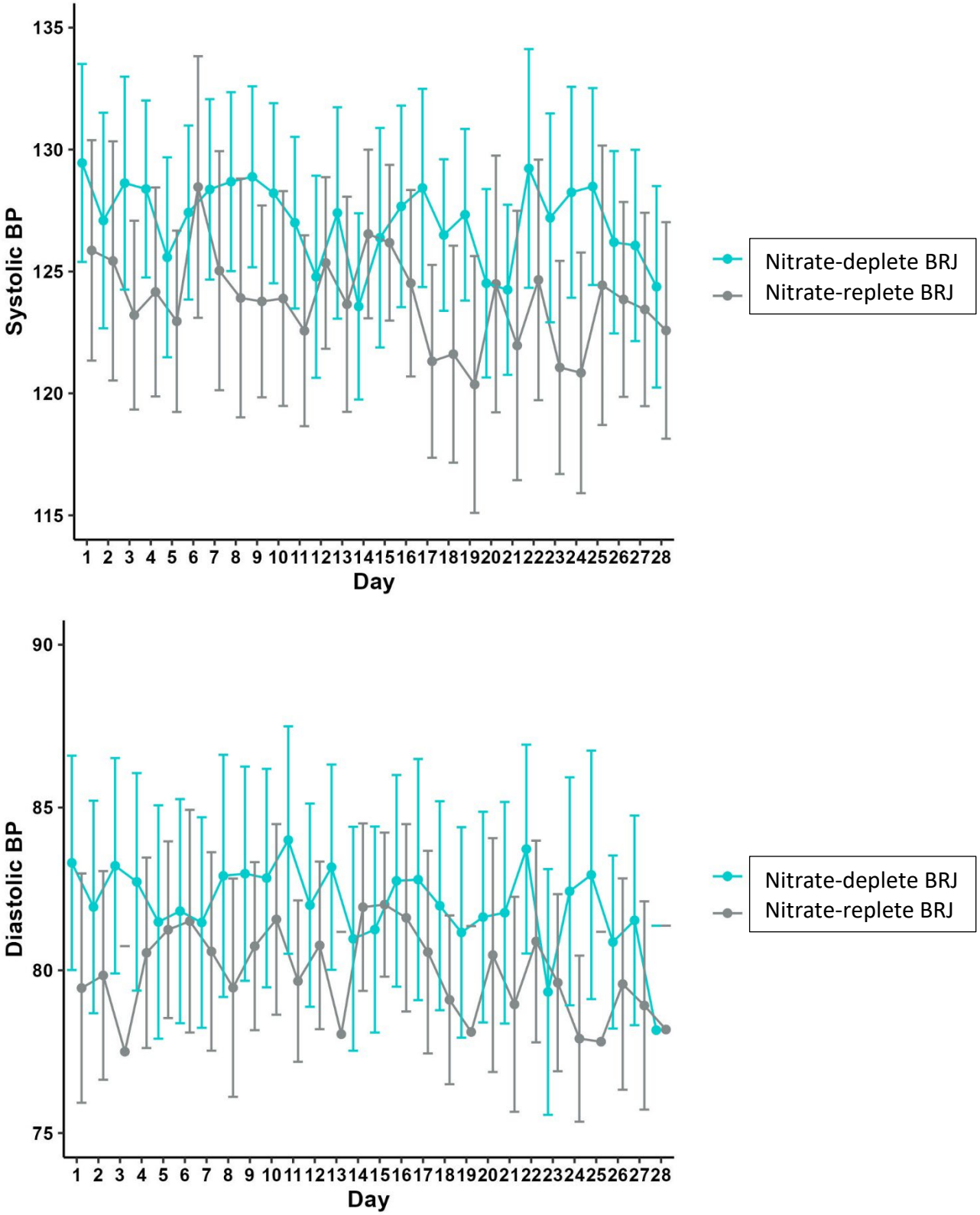
Time	Nitrate-replete group			Nitrate-deplete group		
	Baseline	Follow-Up	Change in timing	Baseline	Follow-Up	Change in timing
Morning	55.2% (16)	51.8% (15)	17.2% (5)	50.0% (15)	60.0% (18)	16.7% (5)
Afternoon	44.8% (13)	48.3% (14)		50.0% (15)	40.0% (12)	

Supplementary Fig. S5: Individual total serum nitrate/nitrite levels in the study groups (A, nitrate replete BRJ and B, nitrate deplete BRJ) at baseline and after 4 weeks of treatment. The median serum nitrate/nitrite levels increased 3.6-fold at week 4 in the nitrate replete BRJ group compared to baseline, where there were minimal changes in the nitrate deplete BRJ group.

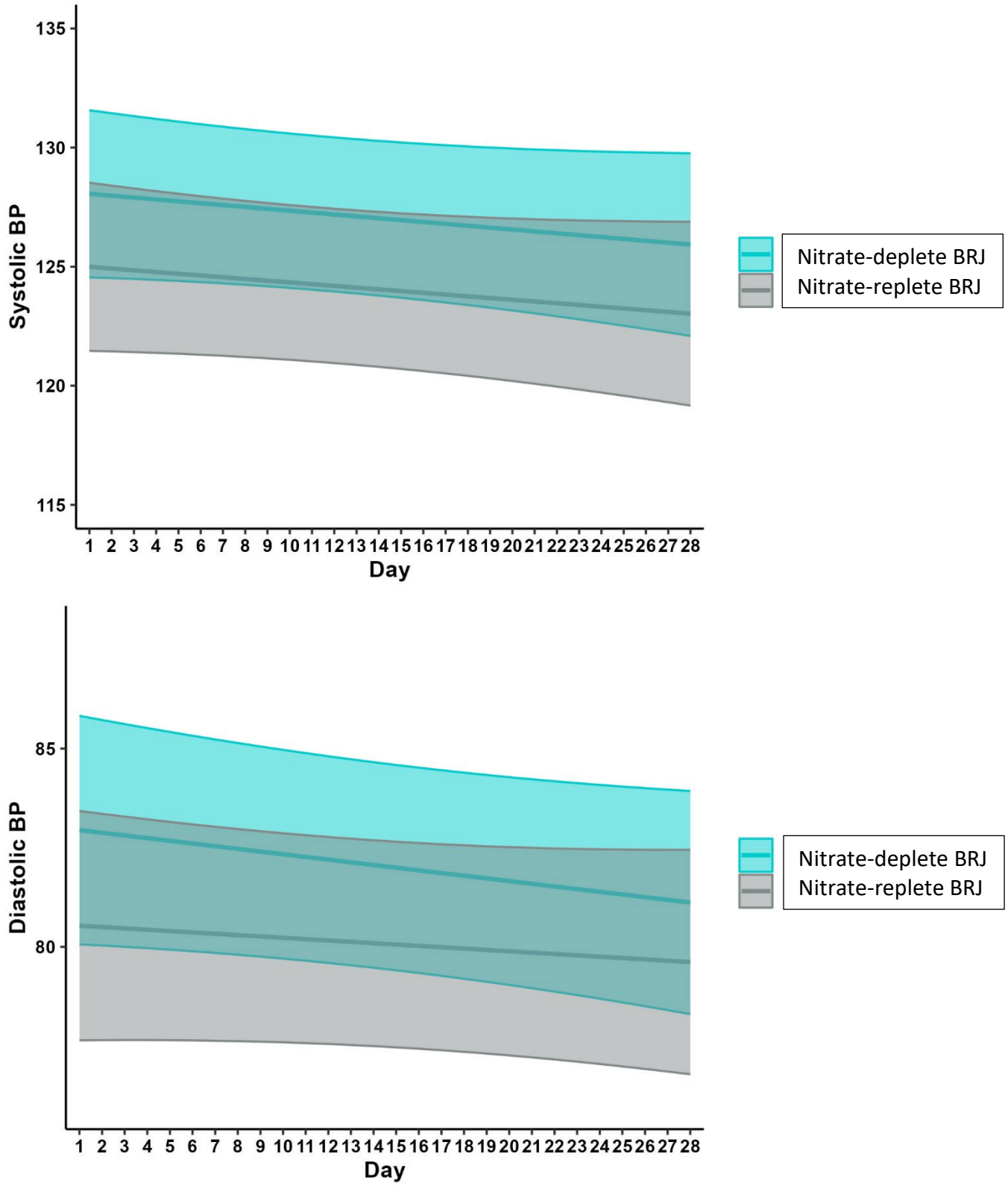


Supplementary Fig. S6: Mean daily home blood pressure measurements (panel A, raw mean values; panel B, smoothed model) during the intervention period.

A. Home blood pressure plots (raw values)



B. Home blood pressure measurements (smoothed model)



Supplementary Table S7: Self-reported time of BRJ intake in participants

BRJ Time	Nitrate-replete group (n = 29)	Nitrate-deplete group (n = 30)	p-value
Morning	86.2% (25)	96.7% (29)	0.32
Afternoon	6.9% (2)	0% (0)	
Evening	6.9% (2)	3.3% (1)	

Supplementary Table S8: Adverse events

Event	Nitrate-replete BRJ group (n = 29)	Nitrate-deplete BRJ group (n = 30)	Absolute difference (95% CI)	p-value
Gastro-intestinal (excl. beet faeces)	45% (13)	33% (10)	11% (-17%, 40%)	0.52
Change in bowel habit	41% (12)	27% (8)	15% (-13%, 42%)	0.36
Bloating	17% (5)	7% (2)	11% (-9%, 30%)	0.39
Gastritis	3% (1)	10% (3)	-6.55% (-22.57%, 9.46%)	0.63
Beeturia	52% (15)	23% (7)	28% (1%, 55%)	0.05
Beet-coloured faeces	62% (18)	37% (11)	25% (-3%, 53%)	0.09
Perceived positive symptoms	24% (7)	17% (5)	7% (-16%, 31%)	0.70
Improved stool frequency/ softness	14% (4)	10% (3)	4% (-16%, 24%)	0.96
Increased energy	3% (1)	7% (2)	-3% (-18%, 11%)	1.00

Chapter 6: Discussion and conclusions

6.1 Key findings of this thesis

This thesis explored potential novel and easily translatable therapeutic targets to reduce the burden of cardiovascular disease in ADPKD. The specific aims were to examine: (i) increased water intake as a mechanism to reduce cyst growth and CVD progression in experimental PKD, (ii) vitamin D receptor activation as a pathway to reduce the progression of cardiorenal disease in experimental PKD, and (iii) dietary nitrate supplementation with beetroot juice as an adjunct to anti-hypertensive therapy to lower blood pressure in hypertensive ADPKD patients. The novel findings of this thesis were: (i) increased water intake reduced blood pressure, cardiac hypertrophy, cyst growth/area and renal fibrosis in the LPK rat but did not affect kidney function decline, (ii) vitamin D receptor activation reduced blood pressure and cardiac hypertrophy in late disease when used in conjunction with ACE inhibitors in the LPK rat, however it did not attenuate kidney disease and resulted in hypercalcaemia and weight loss, (iii) nitrate-replete beetroot juice did not lower blood pressure compared to nitrate-deplete beetroot juice in hypertensive patients with ADPKD, however both groups had a significant reduction in blood pressure over the course of the trial. These projects provide further insight into the pathological pathways responsible for the development and progression of CVD in polycystic kidney disease, either through success of the intervention, as with increased water intake and VDRA treatment, or through unexpected findings, such possible non-nitrate-mediated effects on blood pressure associated with BRJ consumption, which can guide future research directions and therapeutic development.

6.2 Efficacy of increased water intake on CVD progression in experimental PKD

Chapter 2 explored the efficacy of increased water intake on cardiorenal progression of experimental polycystic kidney disease. The study showed that in the LPK rat model, an 1.4-1.9-fold increase in water intake from week 3 to week 10 of life led to decreased cyst total area, percentage kidney weight to body weight, kidney fibrosis, blood pressure and percentage heart weight to body weight.

The strengths of this study include the use of the LPK rat model (which has a mutation in *NEK8/NPHP9*), which allowed examination of water intake on cardiovascular PKD, as it has strong hypertensive phenotype similar to human disease. (7, 152) Use of the LPK rat model also provided an opportunity to observe the effects of chronic treatment as it has a relatively slow progression of renal and cardiovascular disease over 16 weeks, similar to human disease progression. (152) Another strength is that this study performed detailed histological analysis of kidney and cardiac tissue which allowed characterisation of inflammation and fibrosis, which is an important pathological feature leading to progression of disease in ADPKD. Other strengths include the high frequency of recordings (second daily) of water consumption despite the chronic nature of the study which improve the accuracy of the findings, and the use of both male and female rats which allowed the confirmation that gender-bias did not play a role in the response to water intake. Additionally, increasing water intake is a lifestyle intervention which, as described earlier, is an interest area for patients and stakeholders in the PKD community. Water intake has been discussed amongst PKD researchers as a possible therapy for the last two decades and this study provided further evidence for exploration of this intervention. (30)

This study had limitations. Firstly, the method of using 5% glucose in drinking water was successful at significantly increasing water intake in the high water intake group during the

first 10 weeks of life, but after this point both groups were drinking similar volumes of water, likely due to increased thirst induced by loss of concentrating ability as kidney impairment progressed. Therefore, despite the significant changes detected at week 16 (likely from early effect of increased water intake), the effects of sustained increased water intake throughout the entire disease could not be determined by this study. Secondly, although plasma osmolality is a reliable surrogate marker for vasopressin, vasopressin (or copeptin) was not directly measured. Thirdly, caloric intake was not measured, and this may have contributed to differences seen in body weight between high water intake and normal water intake male LPK rats. Finally, measured water intake varied between measurement in metabolic cages (which is considered the gold-standard) and second daily averaged housing cage readings likely due to changes in the rats' behaviour from to the altered metabolic cage environment. This made it difficult to determine the exact increase in water intake required for the changes observed. Despite these limitations, this study provided further preclinical evidence for water intake as possible therapeutic intervention to reduce cystic growth, hypertension and cardiac disease.

Since this study was undertaken, the randomised controlled trial (the "PREVENT-ADPKD" trial) of increased water intake in adult ADPKD participants was reported. This study showed that prescribed high water intake did not slow the growth of total kidney volume over 3 years in participants with ADPKD compared to *ad libitum* water intake, although the results may have been impacted by ~1.5L above average intake of water in the control group compared with the general population. (64)

6.3 Efficacy of VDRA therapy on CVD progression in experimental PKD

Chapter 3 examined the effect of the vitamin D receptor agonist paricalcitol on early and late stage experimental PKD. Paricalcitol was administered to LPK rats from postnatal week 3 to 10 (during the phase of rapid cystogenesis but without detectable kidney impairment or CVD) as the first part of this study (Experiment 1). Paricalcitol did not have an effect on kidney function, proteinuria, serum calcium, kidney or cardiac enlargement compared with vehicle (control) in this early stage. Experiment 2 examined the effect of paricalcitol on late stage PKD where there is progression of kidney impairment, hypertension and CVD. The impact of paricalcitol was tested alone and compared to vehicle, ACEi enalapril, and combination with paricalcitol and enalapril. The results showed significant and sustained decrease in blood pressure and cardiac enlargement (measured by %heart weight:body weight) in the group treated with combined paricalcitol and enalapril. This effect was not seen in the group treated with paricalcitol alone, and a reduction in blood pressure was only detectable at week 13 in the group treated with enalapril alone, suggesting synergistic action between paricalcitol and enalapril was required for this effect to be sustained. Treatment with paricalcitol alone decreased proteinuria by 41% at the week 19 timepoint but this effect was not sustained, as it was in the enalapril-treated group (which had a sustained reduction by 46.9%, 53.7% and 69.0% on weeks 13, 16, and 19, respectively) and there was no added benefit of combination paricalcitol and enalapril above ACEi therapy alone. There was no change in kidney size/cyst growth or kidney function with any intervention compared to vehicle, which was a notable finding given preclinical studies have suggested that the vitamin D deficiency may reduce cyst growth. (70) Importantly, our study found that serum calcium was elevated 1.3-fold in the both groups treated with paricalcitol which, as expected, was associated with increase in urinary calcium excretion. Furthermore, rats treated with paricalcitol had lower body weights from week 18 to 20, which we hypothesise is likely due

to anorexic effects from hypercalcaemia (although food intake was not measured) based on other animal studies. It is unknown if the hypercalcaemia masked any protective effects on kidney impairment from paricalcitol, but this adverse effect cautions its use at the current dose in further trials.

The strengths of this study were that it examined both early and late disease stages, and within those stages, measurements were taken at multiple timepoints which allowed for more detailed assessment of the effect of paricalcitol and enalapril. The study also utilised a drug class readily available to patients and therefore, had it been found to be effective, further clinical testing and potential application would be possible in a timelier manner than a completely new agent. A limitation of the study is that food intake was not measured, therefore while anorexia from hypercalcaemia is the most likely cause of decreased body weight recorded, it is not known with certainty. Furthermore, the dosing of enalapril was based on other non-PKD rodent models of PKD and it is possible that a higher dose may have led to a sustained blood pressure reduction in the LPK rat, but this was not tested in the study.

6.4 Efficacy of nitrate-replete beetroot juice supplementation on blood pressure reduction in ADPKD

Chapters 4 and 5 describe the first double-blind randomised controlled trial testing the effect of nitrate supplementation with beetroot juice on lowering blood pressure in hypertensive patients with ADPKD. The study achieved its planned recruitment of 60 participants. There was excellent compliance with the study procedures and BRJ consumption as measured by submitted home blood pressure readings, returned BRJ bottles and measured 3.6-fold increase in serum nitrate/nitrite metabolites in the nitrate-replete group. The results of the trial showed a significant decrease in blood pressure -5.36/-3.55 mmHg in the group treated with

nitrate-replete BRJ and a significant decrease in systolic blood pressure in the nitrate-deplete group of -7.65 mmHg but not in the diastolic blood pressure (-2.73 mmHg). This disproved the hypothesis that nitrate-replete BRJ would lower blood pressure compared to nitrate-deplete BRJ in hypertensive ADPKD, and suggests that dietary nitrate supplementation does not correct the underlying intrinsic NO deficit in ADPKD.

There was no change in the secondary outcomes (home blood pressure readings and albuminuria) between nitrate-replete and nitrate-deplete group. The most common adverse event was beeturia and beet-coloured faeces, followed by gastrointestinal side effects. There was no difference in the frequency of these events between nitrate-replete or nitrate-deplete groups.

The strengths of this study include the trial design, which was practical and pragmatic, leading to excellent compliance with daily home blood pressure readings and BRJ consumption. Limitations are that the primary outcome was based on only two blood pressure readings (at first and last visit), which increases the risk of environmental factors, such as whitecoat hypertension, missed doses of anti-hypertensives on the day, or changes to daily routine to accommodate the visit itself, influencing the outcome. Furthermore, other changes in diet or changes in lifestyle as a result of trial participation were not measured and adjusted for. Participants may have had consumption of high-nitrate foods in the placebo arm or commenced non-pharmacological interventions known to reduce blood pressure such as low salt diets, weight reduction or increased exercise during the trial period.

The significant reduction in blood pressure in both arms of this study may be due non-nitrate vasoactive components of BRJ (such as betaine, flavonoids, vitamin C) or the effect of trial participation (increased compliance with medications and other lifestyle changes to reduce blood pressure). The magnitude of change detected is similar to that expected of an effective

dietary intervention (as it is similar to the reported change from other BRJ studies or trials of the "Dietary Approach to Stop Hypertension" diet). This trial suggests that healthy eating with consumption of vegetables along with regular blood pressure monitoring should be the focus of future clinical trials in this area. (26, 95)

6.5 Conclusions and future directions

Cardiovascular disease is a major burden for those with ADPKD, both as the main cause of mortality and a key cause of long-term complications and comorbidity. It is an urgent clinical problem for individuals, carers and healthcare providers in the PKD community and treatments to reduce the impacts of CVD are one of the key priorities of research initiatives in PKD.

Increased water intake was successful at reducing cardiorenal disease progression in experimental PKD. Prescribed high water intake went on to be tested (unrelated to this thesis) in a randomised controlled trial setting and found that it did not attenuate cyst growth compared to *ad libitum* intake. Given that the vasopressin pathway has impacts on CVD progression in ADPKD, further studies into its role should be directed at evaluating vascular outcomes as well. VDRA therapy in experimental PKD was successful at reducing blood pressure and cardiac enlargement, however due to the adverse effects of hypercalcaemia and weight loss, additional safety and dose titration studies are required prior to considering it as a potential therapy in ADPKD. BRJ, both with and without nitrate, significantly reduced blood pressure providing multiple potential pathways to consider for the effect observed, including the effect of non-nitrate vasoactive components and the effect of trial participation. Future directions could include studies focused on holistic healthy diets and patient engagement in blood pressure monitoring in ADPKD. Taken together, the findings of this

thesis contribute towards the understanding of the pathways that influence disease progression in PKD. Further evaluation into methods of modulation of vasopressin and vitamin D, and the effect of BRJ is required. The inclusion of cardiovascular outcomes should be considered in all studies in ADPKD, even those focussed on purely cyst reduction, as shared pathways influence cardiovascular disease and long-term outcomes for individuals with this condition.

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