Double-Blind, Randomised Placebo-Controlled Study to Determine the Effect of *BEET*root Juice on Reducing Blood Pressure in Hypertensive Adults with Autosomal Dominant *P*olycystic *K*idney *D*isease (BEET-PKD)

Version No.8: 3rd Feb 2023

Trial registration: NCT05401409

Investigators

Principal Investigator: Professor Gopi Rangan, PhD, MB BS, FRACP, MBA, MDiagGenomics

Associate Investigators:

Title	First Name	Last Name	Qualifications
Dr.	Priyanka	Sagar	MBBS, FRACP
Mr	James	Elhindi	BSc
Professor	David	Harris	MD, FRACP
A/Professor	Kamal	Sud	MB BS, FRACP
A/Professor	Vincent	Lee	PhD, MB BS, FRACP
Dr	Katrina	Chau	PhD, MB BS, FRACP
Dr	Nikki	Wong	BMedSci MBBS FRACP

Institution:

Western Renal Service: Department of Renal Medicine, Westmead Hospital, Corner of Hawkesbury and Darcy Roads, Westmead, Sydney, NSW, Australia 2145; Department of Renal Medicine, Nepean Hospital, Derby Street, Kingswood, Sydney, Australia, NSW 2747

Michael Stern Laboratory for PKD, Centre for Transplant and Renal Research, Westmead Institute for Medical Research, The University of Sydney, 176 Hawkesbury Road, Westmead, NSW 2145

ABSTRACT

Introduction: The endogenous production of nitric oxide (NO) is reduced in autosomal dominant polycystic kidney disease (ADPKD) and may contribute to early-onset hypertension, which is present in 60% of patients. Beetroot juice (BRJ) is a natural dietary source of nitrate and reduces blood pressure in other study populations. The aim of this study is to evaluate the efficacy of BRJ on reducing blood pressure in hypertensive adults with ADPKD.

Methods and analysis: ADPKD patients with hypertension (n=60) will be randomised (1:1) to receive either nitrate-rich or nitrate-depleted (placebo) BRJ using a randomised, double-blind, placebo-controlled study design. The effect of BRJ on blood pressure will be assessed over a 4-week study period. The co-primary endpoints are a change from baseline in mean automated clinic systolic and diastolic blood pressure. Secondary endpoints are changes in daily home blood pressure, changes in albuminuria, NO metabolites (plasma nitrate and nitrite), plasma asymmetric dimethylarginine (ADMA) levels (a naturally occurring endogenous inhibitor of NO increased in ADPKD), and adverse events.

Ethics and dissemination: Approval was obtained from the Western Sydney Local Health District (WSLHD) Human Research Ethics Committee (HREC). The results of this study will be disseminated at national and international Scientific Meetings and submitted for publication in peer-reviewed international journals.

Trial registration: NCT05401409 (clinicaltrials.gov; National Library of Medicine)

Summary

Study Title	Double-Blind, Randomised Placebo-Controlled Study to Determine the Effect of *Beet*root Juice on Reducing Blood Pressure in Hypertensive Adults with Autosomal Dominant *P*olycystic *K*idney *D*isease (BEET-PKD Study)
Rationale	Autosomal dominant polycystic kidney disease is the most common genetic cause of kidney failure. Previous studies have shown that the endogenous production of nitric oxide is reduced in ADPKD and this may lead to early-onset hypertension, which is present in 60% of patients. Beetroot juice (BRJ) is natural dietary source of oral nitrate and reduces blood pressure and improves exercise performance in other populations but its role in ADPKD is unknown. The aim of this study is to evaluate the efficacy and safety of BRJ in reducing blood pressure in adults with ADPKD.
Primary Objective	Determine the efficacy of daily consumption of BRJ (400 mg nitrate) on decreasing clinic systolic and diastolic blood pressure in ADPKD participants with hypertension over 4 weeks.
Secondary Objectives	• Determine the effect of BRJ on decreasing home systolic and diastolic blood pressure readings • Determine the effect of BRJ on reducing albuminuria • Determine the effect of BRJ on increasing NO metabolites (plasma/urine), and reducing asymmetric dimethylarginine (plasma) levels
Exploratory Objective	• Characterisation of the baseline oral microbiome in ADPKD using salivary bacterial genomic data • Determine the effect of 4 weeks of BRJ consumption on the oral microbiome in ADPKD patients • Measure the effect of BRJ on urinary the development of urinary *N-nitroso* compounds
Study Design	This is a randomised, double blind placebo-controlled trial. Participants with treated hypertension (defined as being on at least one anti-hypertensive medication; n=60) will be included in the study. The total duration of the study is 5 weeks. There will be a 7 day run-in period where participants will be asked to record BP daily to determine adherence and baseline blood pressures. Participants will then be randomised (1:1) to receive an oral daily dose of either BRJ (400mg nitrate) or nitrate-depleted BRJ for 28 days. Blood pressure will be measured in clinical and at home using a standard and validated automated oscillometric blood pressure (AOBP) device provided to all participants. Blood, urine and saliva samples will be collected before and after the intervention.
Primary Endpoint	Reduction in mean automated clinic systolic and diastolic blood pressure relative to baseline
Secondary Endpoint	• Change from baseline in the mean home systolic and diastolic blood pressure • Change in urinary albumin to creatinine ratio • Change from baseline in plasma and urine nitrate, NO metabolites, and ADMA levels
Planned Sample Size	A total of 60 treated hypertensive participants (1:1; male and female; aged 18-70 years old) with ADPKD (as per the inclusion/exclusion criteria) will be randomised.
Inclusion Criteria	• Diagnosis of ADPKD • Age over 18 years old • eGFR > 30 mL/min/1.73m2 • Treatment with at least one anti-hypertensive
Exclusion Criteria	• Inability to provide Informed Consent • Labile, unstable uncontrolled hypertension and/or changes in blood pressure treatment in the last 28 days prior to the screening visit that in the opinion of the investigators would confound interpretation of the primary endpoint • Non-compliance with daily BP measurements during the screening period

	• Medical conditions or treatments that may interfere with the generation of NO metabolites (such as regular use nitrate drugs, cigarette smoking; unwilling to stop using antiseptic mouthwash; uncontrolled severe hypercholesterolemia • Any serious or other medical conditions that may interfere with follow-up or stability of blood pressure (such as current active malignancies requiring treatment; uncontrolled Diabetes Mellitus (elevated HbA1c > 10%) • Dislike of taste of beetroot juice • Allergy to beetroot • Patients enrolled in other clinical trials • Pregnant or Lactating
Study Procedures	• Clinic and home blood pressure measurement • Collection of blood for serum creatinine, eGFR, NO biomarkers and ADMA levels • Collection of urine for albuminuria and NO metabolites • Collection of saliva samples for genomic analysis of oral bacteria
Samples size and statistical analysis	In a previous study of daily BRJ (400mg nitrate) supplementation in hypertensive patients (n=68) for 4 weeks, systolic BP was significantly reduced by 7.7mmHg (3.6-11-8 mmHg, $p<0.001$) at the end of the intervention. The average BP of the two groups were 148 mmHg and 149 mmHg, with standard deviations of the two populations being 10 and 11.[1] We expected to have a similar hypertensive population and to detect this difference in systolic BP with an $\alpha=0.05$, and power of 0.80 requires a sample size of n=28 in each treatment group, and to account for drop-out, the group size was therefore increased to 30 (total n=60). The pre-specified research aim was to determine whether once-daily administration of dietary nitrate lowered blood pressure in participants with ADPKD., The pre-specified co-primary endpoints are the change in 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, 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 Visit and Arm of 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. 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 Day and Arm of 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 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.
Total duration of the study	35 days (5 weeks) including 7 day run-in period and 28 day intervention period
Timeline	Study activity commenced in May 2022 and will conclude in April 2023.

Keywords

Autosomal Dominant Polycystic Kidney Disease, Cardiovascular Diseases, Humans, Dietary Inorganic Nitrate, Beetroot Juice, Vasodilation, Nitrate-depleted beetroot juice, Endothelial Dysfunction, Blood pressure, Inflammatory Mediators

Administrative information

Title	Double-Blind, Randomised Placebo-Controlled Study to Investigate the Effect of Beetroot Juice on Blood Pressure in Hypertensive Participants with Autosomal Dominant Polycystic Kidney Disease (BEET-PKD)
Trial registration	The trial has been registered with clinicaltrials.gov (National Library of Medicine). Trial ID NCT05401409
Protocol version	Protocol Version 8, 3rd Feb 2023
Funding	Research grant from PKD Australia awarded in June 2020 ($A30,000). PS doctorate studies are funded by the ICPMR Jerry Koutts Scholarship
Author details	GR, PS, DH, KS, VL, KC, NW
Name and contact information for the trial sponsor	Western Sydney Local Health District (WSLHD)
Role of sponsor	HREC approval and scientific review
Human Research Ethics Committee Approval (HREC)	Approval was received on 28th May 2021 from the Western Sydney Local Health District Human Research Ethics Committee, and approval of the amendments in this protocol will be obtained through the HREC

1. INTRODUCTION

1.1 Background and Rationale

1.1.1 ADPKD and the clinical problem

Autosomal dominant polycystic kidney disease (ADPKD) is the most common genetic kidney disease, with a population prevalence of ~1:1000.[2] World-wide it is the most common genetic cause of chronic kidney disease (CKD) and kidney failure in adults.[3] It is caused by loss-of-function pathogenic variants predominantly in *PKD1* (~85% of genetically resolved cases) or *PKD2* (~15% of genetically resolved cases), which encode the polycystin-1 (PC-1) and -2 (PC-2) proteins respectively. *In vivo,* PC-1/2 exists as a membrane complex which has a critical role in modulating extracellular signals to internal molecular pathways that govern cellular differentiation, the cell cycle [4] and the endogenous production of nitric oxide (NO) *via* activation of NO synthase (NOS).[5-7] Loss of PC-1/2 function leads to the progressive formation and growth of multiple fluid-filled kidney cysts, kidney failure and hypertension, which reduces quality of life and causes premature death.[4] ADPKD is a significant burden to the healthcare system due to the cost of kidney replacement therapy (A$160M per year in Australia alone) [8] and chronic disease complications.

Hypertension develops in 60% of patients with APDKD by 35 years of age and occurs prior to any reduction in kidney function [8]. It is a major independent risk factor for kidney function decline and cardiovascular complications. [9] Since the availability of dialysis and kidney transplantation, cardiovascular disease is the leading cause of death in ADPKD [10]. Uncontrolled hypertension is associated with increased proteinuria, haematuria, and rate of growth of total kidney volume, as well as decreased renal blood flow [8]. Morbidity and mortality in ADPKD is also associated with increased vascular aneurysm formation and left ventricular hypertrophy, which are driven by hypertension.[11]

In the absence of curative therapies, current medical management is aimed at slowing the rate of kidney cyst growth through lifestyle modification (limiting dietary solute intake and avoiding volume depletion which stimulates AVP release; limiting caloric intake), reducing blood pressure with angiotensin inhibitors and the selective use of tolvaptan in individuals at high-risk of kidney failure. [10]

1.1.2 Potential Role of Reduced NO in the Pathogenesis of ADPKD

The pathogenesis of hypertension in ADPKD is, in part due to, increased intra-renal angiotensin II production in cystic epithelial cells, as well as local renal ischemia due to compression of intra-renal micro-vessels by expanding renal cysts. Hence, angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers are preferred first-line agents in hypertensive ADPKD patients.[8] In addition, the deficiency in endogenous NO synthesis is a central molecular signature of ADPKD and may also be key driver of hypertension and vascular aneurysm formation [12]. Preclinical and observational cohort studies suggest that the reduction in endothelial NO biosynthesis in ADPKD is due to the loss of PC-1/2-mediated activity of NOS [13]. In addition, serum asymmetric dimethylarginine (ADMA), a competitive inhibitor of NOS and an independent predictor of cardiovascular events, is elevated in ADPKD (likely due to reactive oxidative stress)[14] and may also be involved in relative deficiency of NO in ADPKD.[15] The endothelial NO deficiency in ADPKD leads

to endothelial dysfunction, vascular resistance, oxidative stress and inflammation resulting in hypertension and in the long-term, contributing to kidney injury, cardiac dysfunction and aneurysm formation (Figure 1).

1.1.3 Entero-salivary Pathway for increasing Endogenous NO generation in ADPKD

The entero-salivary pathway is a recently described mechanism by which dietary nitrate is converted to nitrite by commensal anaerobic bacteria on the dorsal surface of the tongue.[16] The ingested nitrite is absorbed into the circulation through the stomach where enzymatic and non-enzymatic systems reduce nitrite to NO.[17] Thus, through the entero-salivary nitrate-nitrite-nitric oxide pathway, the dietary intake of inorganic dietary nitrate may restore the endogenous NO deficit in ADPKD, reduce blood pressure and improve endothelial dysfunction.[16] Recently, we found that intracellular levels of NO are reduced by over 75% in human ADPKD cells.[18] However, long-term supplementation of drinking water with oral sodium nitrate intake did not alter the progression of cystic kidney disease in mice with ADPKD, but efficacy on blood pressure reduction was unable to be established due to lack of cardiovascular phenotype in this model. [18]

1.1.4 Beetroot Juice (BRJ) as a Dietary Source of Nitrate and potential effects in ADPKD

Nitrates are commonly found in high concentrations in vegetables such as beetroot (*beta vulgaris*), rocket, lettuce, and spinach (~250mg/100g). BRJ has been used as a source of oral nitrate in several studies.[19] In addition to its high nitrate content, BRJ contains other vasoactive components such as vitamin C, magnesium, polyphenols, betaine, and flavonoids that can contribute to improved antihypertensive effects. Vitamin C and polyphenols also participate in non-enzymatic systems of reduction of nitrate to NO.[19] Findings from numerous studies support the efficacy of BRJ in lowering blood pressure; Kapil *et al* showed a reduction of 7.7mmHg in systolic blood pressure (sBP) and 2.4mmHg in diastolic blood pressure (dBP) in drug-naïve and treated hypertensive patients given nitrate-rich BRJ,[20] Kemmner *et al.* showed a 8.2mmHg reduction in sBP in patients with stage 3 CKD.[21] In addition, in a meta-analysis of multiple studies have shown a reduction in sBP including in healthy volunteers with an average reduction in sBP of 4.41-4.4mmHg.[22-27] Previous studies in other populations suggest that a reduction in ~5mmHg is associated with significant reduction in stroke mortality, and with usual targets of achieving a 10-15mmHg reduction in sBP with drug monotherapy or strict adherence to the DASH diet (dietary approach to stop the hypertension diet).[26, 28] In other studies, BRJ increased plasma nitrate levels and improved blood pressure within 4 hours after the consumption of 300mg of nitrate in patients with chronic kidney disease (average eGFR 41 ml/min/m^2). Additionally, dietary nitrate load reduced renal resistive index, a marker for cardiovascular mortality and progression of renal disease, in patients with CKD.[21] Moreover, consumption of nitrate-rich BRJ protected against ischaemia-induced endothelial dysfunction and attenuated platelet aggregation in short-term studies.[29, 30] Preliminary studies in *Pkd1*$^{RC/RC}$ mice showed that betaine (a component of beetroot) reduced renal cyst growth [31, 32]).

1.1.5 Safety of BRJ as medium-term intervention in chronic diseases

BRJ is made up of naturally occurring substances, and has been well-tolerated in previous clinical studies with only minimal side effects such as betacyanin pigmented urine (beeturia) and faeces.[19, 20, 26, 33] While there is a theoretical risk of increased production of *N-nitroso* compounds (one of the potential downstream metabolites of nitrate) which has been linked with the development of gastric cancer, these compounds are linked with the consumption of dietary nitrate from red meat and cured meats, rather than nitrate in vegetables, and as a result are hypothesized to be metabolised differently from nitrate-rich vegetables (likely due to the presence of heme-protein).[34, 35] Large case-control population studies have shown that a nitrate-rich vegetable diet is protective against gastric cancer, however a nitrate-rich red meat and cured meat based diet was correlated with a higher risk of gastric cancer.[20, 34-36] Furthermore, a recent large US National Toxicology Program reported a two year long study in which rats who were fed high nitrate in drinking water did not have an increased risk of cancer.[37] Subsequently, large international health surveillance bodies (such as European Commission Scientific Committee for Food and World Health Organisation) have assuaged concerns regarding the cancer risk from nitrate consumption from vegetables, such as beetroot.[34, 35] As a result, the Australian Food Standards Report into nitrate consumption in Australia does not recommend restriction of dietary nitrates from vegetables, especially given the clear health benefit of increased consumption of vegetables in daily diet.[38] Therefore, while the significance of these *N-nitroso* compounds in the a high vegetable nitrate diet are unclear, this study will measure them as part of our exploratory objectives to determine if consumption of BRJ increases the excretion of these compounds.

1.1.6 Study Hypothesis

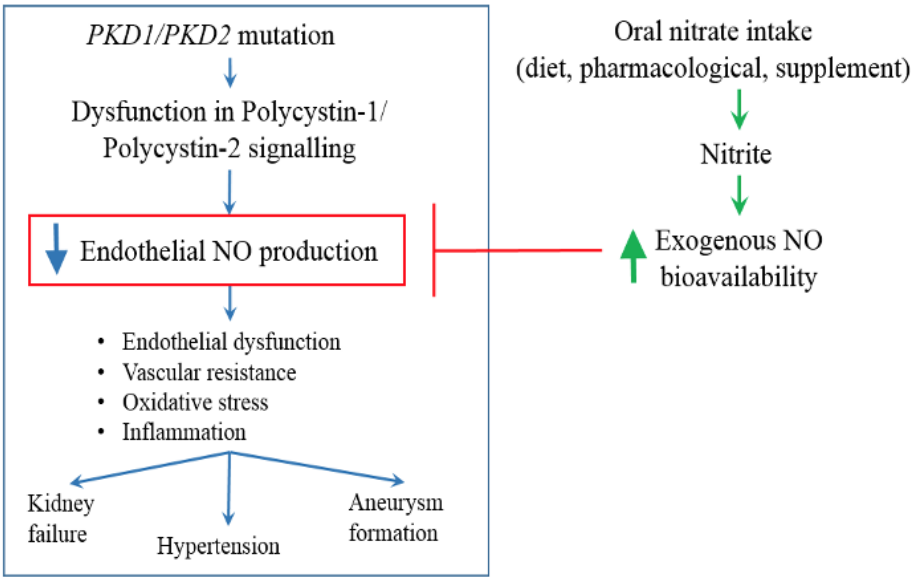

Figure 1: Potential role of exogenous oral nitrates in restoring NO deficit in ADPKD

2 RESEARCH PLAN AND TRIAL DESIGN

This is a double-blind (participant/investigator) randomised controlled clinical trial to determine the efficacy, safety, and feasibility of BRJ on lowering blood pressure in participants with treated hypertension in ADPKD.

2.1 Objectives

2.1.1 Primary Objective:

Determine the efficacy of daily consumption of BRJ (400 mg nitrate) on decreasing clinic systolic and diastolic blood pressure in hypertensive participants with ADPKD over 28 days.

2.1.2. Secondary Objectives:

a) Determine the effect of BRJ on decreasing home systolic and diastolic blood pressure readings
b) Determine the effect of BRJ on reducing urinary albumin to creatinine ratio
c) Determine the effect of BRJ on NO metabolites (plasma/urine), and ADMA (plasma) levels;

2.1.3. Exploratory Objectives

(a) Characterisation of the oral microbiome in ADPKD using salivary bacterial genomic data
(b) Determine the effect of 4 weeks of BRJ consumption on the oral microbiome in ADPKD patients
(c) Determine if there are any changes in the excretion of *N-nitroso* compounds in urine with BRJ consumption

2.2 Endpoints

2.2.1 Primary Endpoint

Change in mean automated clinic systolic and diastolic blood pressure before and after treatment with BRJ.

2.2.2 Secondary Endpoints

The secondary outcomes assessing treatment efficacy in this study will be the change from baseline in the following:

(a) mean daily home systolic and diastolic systolic and diastolic blood pressures
(b) change in urinary albumin to creatinine ratio
(c) levels of NO metabolites (nitrate and nitrite) in plasma and urine; and
(d) plasma ADMA levels after dietary nitrate supplementation using BRJ.

To assess safety of chronic nitrate supplementation, the frequency and type of adverse events will be evaluated

To assess adherence to the study procedures, the following will be assessed:

(a) Number/percent of completed/missed measurement of blood pressure as represented by response to automated text messages or completion of patient diary

(b) Of measurements captured, number/percent of those who consumed BRJ and took measurements in morning period (6am-9am).

2.2.3 Exploratory outcome

To assess the effect of BRJ on the oral microbiome of ADPKD patients, the change from baseline percentage of known nitrous-converting bacteria compared with other oral flora before and after BRJ compared with nitrate-depleted BRJ.

2.3 Inclusion and Exclusion Criteria

A total of 60 treated hypertensive participants with ADPKD (as per the inclusion criteria, **Table 1**) will be recruited.

Table 1. Participant inclusion and exclusion criteria

Inclusion Criteria	Exclusion Criteria
♦ Diagnosis of ADPKD ♦ Age over 18 years old ♦ eGFR > 30 mL/min/1.73m^2 ♦ Receiving at least one anti-hypertensive	♦ Inability to provide Informed Consent ♦ Labile, unstable uncontrolled hypertension and/or changes in blood pressure treatment in the last 28 days prior to the screening visit that in the opinion of the investigators would confound interpretation of the primary endpoint ♦ Non-compliance with daily BP measurements during the screening period ♦ Medical conditions or treatments that may interfere with the generation of NO metabolites (such as regular use nitrate drugs, cigarette smoking; unwilling to stop using antiseptic mouthwash; uncontrolled severe hypercholesterolemia ♦ Any serious or other medical conditions that may interfere with follow-up or stability of blood pressure (such as current active malignancies requiring treatment; uncontrolled Diabetes Mellitus (elevated HbA1c > 10%) ♦ Dislike of taste of beetroot juice ♦ Allergy to beetroot ♦ Patients enrolled in other clinical trials ♦ Pregnant or Lactating

If participants have uncontrolled hypertension they will be advised to consult with their general practitioner and nephrologist for appropriate management. If drug management is required, participants must have stable

blood pressure control treated for at least 28 days before screening can be repeated. The exclusion criteria are based on comorbidities that could potentially confound study endpoints. [39-41] In particular, participants with severe uncontrolled diabetes (HbA1c>10%) were excluded based on previous studies that showed lack of effect of BRJ in these participants due to major biochemical perturbations and resulting diminished vascular responsiveness and endothelial damage.[33] However, studies with participants with well-controlled diabetes or early and pre-diabetes were responsive to beetroot juice supplementation and had decreased blood pressure and improved endothelial function after its use, which informed the current study's inclusion of participants with diabetes and controlled HbA1c%.[1, 42] Participants with severe uncontrolled hypercholesterolemia and current cigarette smoking were excluded as they sustain direct endothelial injury and suppression of nitrate metabolism pathways which directly interferes with beetroot juice supplementation.[43, 44]

2.4 Study Design

The total duration of the study is 35 days. Patients will be screened at Visit 1 and eligible patients will be provided with a blood pressure monitor (oscillometric BP device: UA-651, A&D Medical, Tokyo, Japan) to commence baseline home blood pressure monitoring for a 7 day run-in. Their compliance to daily blood pressure readings will be assessed via automated text message each morning (to which participants will reply with their readings), or with a phone call by study staff on Day 2 and 5 if they opt-out of text messages, and at Visit 2. Replying via text message will be encouraged as the preferred option as it will allow for real-time monitoring and data recording and allow patients to easily contact the study team if they have any issues. Participants will be randomly allocated nitrate-rich BRJ (400mg nitrate) or nitrate depleted BRJ (placebo) in a sealed bag and asked not to open it until instructed to do so after Visit 2.

At Visit 2 (conducted via phone), participants' home blood pressure records will be reviewed for compliance. Participants who have recorded <80% of the daily blood pressure readings will be deemed non-compliant and excluded from the trial. Additional exclusion criteria assessed at Visit 2 includes eGFR, fasting lipid and glucose levels, use of antiseptic mouthwash (from V1 to V2 and willingness to comply for remainder of study). Any excluded participants will be asked to return all study equipment and the sealed bag of BRJ. Participants who meet all criteria will be asked to commence the intervention that they were allocated the following morning.

The intervention is BRJ commercially packaged BRJ in 70ml bottles. Participants will be instructed to drink one bottle per day (70ml) and measure their blood pressure at the same time every day. Participants will be asked to send their blood pressures via text or record it in their diary. A daily reminder text message will be sent, unless participants opt-out of this option.

After 28 days, participants will return for Visit 3 for post-treatment assessment and sample collection.

Blood and urine samples will be collected at Visit 1 and 3 for measurement of NO metabolites, ADMA levels and urinary albumin to creatinine ratio. A saliva sample will be collected at Visit 1 and 3 for future genomic analysis of oral bacteria.

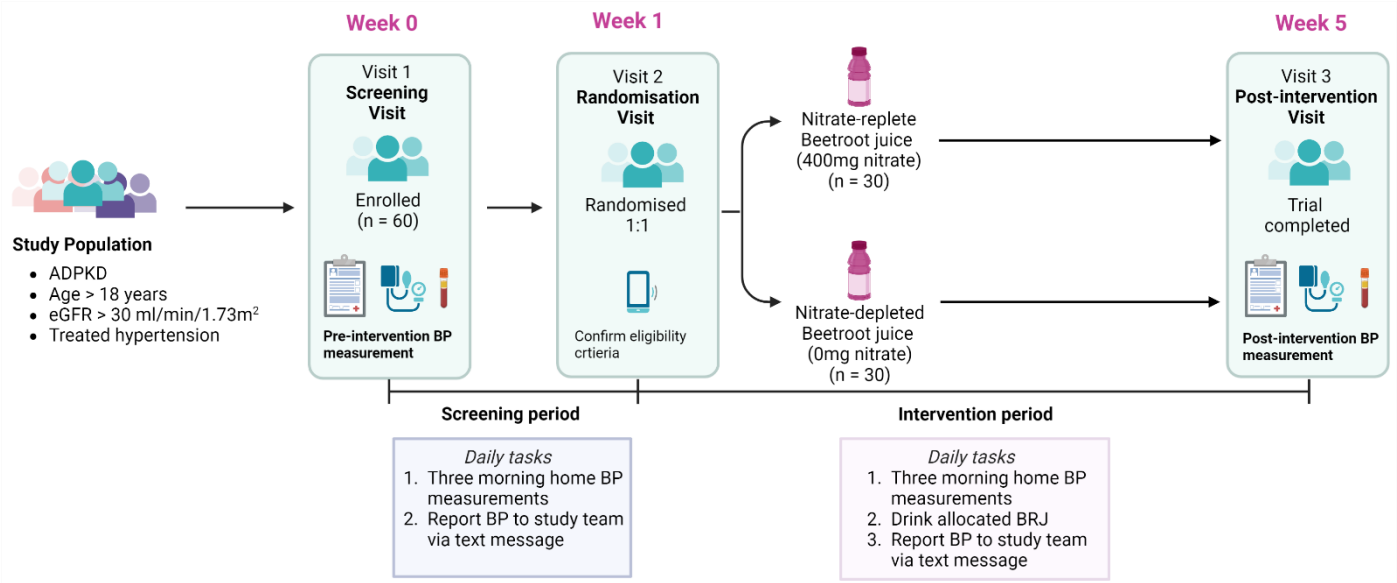

Figure 2. Study design and participant timeline

The study will be carried out in accordance with the Declaration of Helsinki and is approved by the Western Sydney Local Health District (WSLHD) Human Research Ethics Committee (HREC). Due to the impact of COVID-19, study visits will be performed in the Westmead Institute of Medical Research (WIMR) and at the Department of Renal Medicine, Westmead Hospital to allow flexibility on where participants can be seen based on local public health policy. Participants will be recruited from nephrologists in the Western Renal Service (Westmead, Auburn, Blacktown, Nepean Hospitals) providing a catchment area of 1.2 million people and 400 potential participants with ADPKD.

2.5 Recruitment

2.5.1 Recruitment targets

A total of 60 participants with ADPKD (as per the inclusion criteria, **Table 1**) will be randomised.

6.5.2 Recruitment methods

Participants will be recruited from the Western Renal Service. 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 in future. Potential

participants will further be identified from Western Renal Service nephrologists, either through direct referral to the study team or review of clinic letters and, if available, local databases. Participants will also be recruited passively through flyers (Appendix D) in renal clinics in WSLHD or by internet advertising from the PKD Foundation of Australia via their website and newsletter. Where possible, identified and interested participants will have discussed with their treating nephrologist their suitability for the study and then pre-screened by the study team, by telephone call to determine their eligibility. This process will improve the efficacy of the screening period and reduce participant burden.

To ensure equitable access to the trial from regional areas, a standard travel compensation of $30 will be provided for each study visit for participants from regional NSW. “Regional NSW” is defined by the NSW government as all areas of the state except the metropolitan areas of Greater Sydney, Newcastle and Wollongong. A map delineating these areas can be found on https://www.nsw.gov.au/regional-nsw-today. This compensation is not offered to participants from Greater Sydney as they do not incur the same travel costs.

6.5.3 Informed Consent

Each investigator has both ethical and legal responsibility to ensure that participants being considered for inclusion in this trial are given a full explanation of the protocol. This shall be documented on a written Master PICF, approved by the WSLHD HREC. Written informed consent will be obtained from all participants. Investigators may discuss the availability of the trial and the possibility for entry with a potential participant without first obtaining consent. However, informed consent must be obtained and documented prior to initiation and completion of any trial procedures.

Specifically, participants will be informed of the expected side effects of beeturia and beet-coloured faeces.(17) As this is a dietary intervention, participants will also be informed of the potential for gastrointestinal side effects and asked to report any symptoms to the trial team immediately. At the time of initial consent, participants will be informed of expectations regarding sharing or re-use of participant data for the means of future research relating to ADPKD. They will be giving the potential to provide extended consent for future use of their data in similar projects relating to ADPKD.

Once the appropriate essential information has been provided to the participant and fully explained in layman’s language by the investigator and it is felt that the participant understands the implications of participating, the HREC-approved written PICF shall be personally signed and dated by both the participant and the person obtaining the consent (investigator), and by any other parties required by the HREC. The participant shall be given a copy of the signed PICF and the original shall be kept on file by the investigator.

6.5.4. Trial registration

The trial has been registered on clinictrials.gov (National Library of Medicine) with registration no. NCT05401409.

2.6 Statistical Methods

2.6.1 Determination of Sample size

In a previous study of daily BRJ (400mg nitrate) supplementation in hypertensive patients (n=68) for 4 weeks, systolic BP was significantly reduced by 7.7mmHg (3.6-11-8 mmHg, $p<0.001$) at the end of the intervention. The average BP of the two groups were 148 mmHg and 149 mmHg, with standard deviations of the two populations being 10 and 11.[1] We expected to have a similar hypertensive population and to detect this difference in systolic BP with an $\alpha=0.05$ and power of 0.80, 28 participants are required in the each treatment group. To account for drop-out, the sample size was increased to 30 participants per treatment group (total n=60).

2.6.2 Statistical and Analytical Plans

Primary Endpoint Analysis

The co-primary endpoints are the change in 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, 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 Visit and Arm of 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 Day and Arm of 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 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.

2.7 Study Intervention

2.7.1. Method of Administration

Participants will self-administer the intervention at the same time. The intervention will be an oral dose of nitrate-rich BRJ (70mL Beet-IT Sport^{TM}; James White Drinks, UK; 400mg nitrate/70mL) taken once daily while the placebo is an identically packaged nitrate-depleted beetroot juice (identical drink and volume with nitrate content removed). The trial team will document the expected time of BRJ consumption at Visit 1 and confirm this at Visit 3.

2.7.2. Dose of BRJ

A minimum dose of $\geq$ 5mmol of NO_3^- per serving has been shown to be required to enhance exercise performance in most healthy individuals.[45] Beet-IT Sport^{TM} (James White Drinks) contains 6.41 ± 0.60 mmol of NO_3^-, or 400-500mg nitrate per serving.[46] Beetroot juice also contains other bioactive compounds that may provide minimal additive BP lowering effects such as vitamin C, magnesium, betaine and flavonoids.[47] Potentially confounding effects of other vasoactive components of beetroot juice will be limited by using the identical drink with depleted nitrate content (manufactured by James White Drinks). The nitrate-depleted placebo beetroot juice is identical in taste, appearance and packaging.

In previous studies, BRJ lowered blood pressure after at least 21 days of continuous supplementation. A significant reduction in home systolic blood pressure during the 3rd and 4th week of intake occurred after ingesting BRJ (6.41mmol NO_3^-, 400mg nitrate, 70 ml, James White Beet-IT Sport Shot) in older, overweight individuals with high-normal blood pressure.[48] No effect was observed in blood pressure measured by clinic or ambulatory blood pressure (AMBP), but a reduction in 7.3 mmHg was seen in home systolic blood pressure readings. This was potentially attributed to participants not being asked to record their physical activity during the 24-hour ABPM recording period, and lower nitrate concentration consumed than reported by manufacturer and differences in nitrate metabolism in older patients.[48] Drug naïve hypertensive patients demonstrated a reduction in clinic, home and ambulatory systolic blood pressure that persisted throughout the entire 4 weeks of daily a single dose of BRJ (250mL, 6 mmol NO_3^-, James White Beet-IT Beet Juice) in treated participants

on an unaltered diet.[20] The reduction in clinic BP was 7.7/2.4mmHg (3.6-11.8/0.0-4.9,p<0.001 and p=0.050).[20] The home blood pressure measurements over the 4 weeks demonstrated an increasing magnitude of the effects with time; comparable with a standard dose of antihypertensive medication, with a reversal occurring only on washout. However, in contrast, in 2015, Bondonno *et al*. showed no effect on home and clinic blood pressure after 1 week of treatment with BRJ (2 x 70mL,600mg nitrate; James White Beet-IT Shot) in individuals with high-normal blood pressure and treated hypertensives, despite parallel increased nitrate metabolism. This could be due to the relatively short duration of supplementation, and potential interference of multiple anti-hypertensive pharmacologic agents (2-3 per individual), low-nitrate diet, and/or extended time period between each dose.[49] Finally, in 2013, Gilchrist et al. observed no effect on blood pressure, macro or microvascular endothelial function after 2 weeks of BRJ (250mL, 450mg nitrate; James White Beet-IT) in hypertensive individuals with Type 2 diabetes mellitus. This was also potentially attributed to the relatively short duration of intervention, and the numerous biochemical perturbations associated with diabetes which leads to diminished vascular reactivity and impaired responsiveness NO.[33] Therefore for reproducibility and comparability, the current study will administer "Beet-IT Sport Shot" (70mL; James White Drinks, UK) containing ~400mg nitrate per dose for a duration of 4 weeks.[46]

2.7.3. Source of BRJ

BRJ will be purchased from James Whites Drinks (United Kingdom) as it is the brand most commonly used in previous trials. The manufacturer also provides a standardised placebo where the taste and appearance are identical to the nitrate-replete BRJ. The content of the placebo is identical to the active compound but has nitrate extracted is shown in Table 2.

Table 2: Product specifications of the BRJ (nitrate replete and nitrate depleted) according to manufacturer

Parameter	BEET-IT SPORT SHOT	BEET-IT PLACEBO
Volume	Per 70 ml	per 70 ml
Nitrate	0.4 g	0
Energy	305 kJ	305 kJ
Carbohydrates	15.4 g (of which 14.g sugars)	15.4 g
Protein	2.5 g	2.5 g
Salt	0.2 g	0.2 g

2.7.4. Management of BRJ

The product will be stored in batches at room temperature (according to recruitment rate), according to the manufacturer's specifications, in a storage room. BEET-IT is a natural product containing beetroot juice (BRJ) that is available over the counter and freely available for purchase by the community. Based on the TGA's Food-Medicine Interface Guidance Tool https://www.tga.gov.au/community-qa/food-and-medicine-regulation) BEET-IT is classified as a 'food' and not a 'medicine' (drug) (Table 3). Therefore, the oversight, dispensing and recording of BEET-IT consumption will be undertaken by study staff without involvement of pharmacy.

Accountability, shipping record (shipment dates, batch numbers and method of shipment), storage conditions (location, temperature, amount of ambient light) of the investigational product (nitrate-replete and -depleted BRJ) will be maintained in data logs according to ICH-GCP guidelines and will be the responsibility of an Associate Investigator (AI). This AI will be unblinded to the product (nitrate-rich vs nitrate-depleted) for appropriate storage, labelling and management of the product to ensure participant-facing investigators remain blinded. Nitrate-rich and nitrate-depleted BRJ will have identical packaging however the AI will be in independent contact with the manufacturer and be aware of which batch numbers correlate with nitrate-rich and nitrate-depleted BRJ, without the other investigators aware of which BRJ batch is being allocated, and so that investigators with participant contact can disseminate the product without unblinding themselves.

Table 3: Food-Medicine Interface Guidance Tool applied to BEET-IT

	Food-Medicine Interface Guidance Tool Question	BEET-IT
1	Is the product for oral use for humans?	Yes
2	Is there a (post 2010) s.7 declaration in relation to the product that it is a therapeutic good?	No
3	Is the product covered by a s.7AA declaration?	No
4	Is the product 'goods' for which there is a standard in the Food Standards Code?	No
5	Is the product goods which, in Australia or NZ, have a tradition of use as foods for humans in the form in which the thing is presented?	Yes
Conclusion	The product is not a 'therapeutic good'. It is likely to be 'food' within state/territory food regulation legislation and/or regulated under other state/territory legislation	

2.8 Randomisation and Blinding

2.8.1 Blinding

Blinding and storage will be undertaken as per Management of Investigational Product by an AI who is not participant facing and does not conduct study procedures. All other investigators and research staff will be blinded to the treatment allocation. Nitrate-rich and placebo BRJ will be identical in appearance and taste with a batch number allocated to each participant. Treatment assignment in the groups will be blinded to participants and all other investigators until data lock and statistical analysis.

Both participants and outcome assessors will be blinded to participants' treatment allocations, to reduce the risk of bias in the study. Awareness of the intervention assigned to participants can introduce ascertainment bias in the measure of outcomes; particularly subject ones, performance bias in the decision to discontinue or modify study interventions (e.g. with dosing changes of beetroot juice), concomitant interventions, and exclusion bias in the decision to withdraw from the trial or exclude a participant from the analysis.

Emergency unblinding procedures, namely, revealing the assigned intervention to participants and investigators, will only occur in certain circumstances with the intention to increase the safety of trial participants, should any evidence of harms or other relevant conditions arise. However, in some cases (i.e. minor, reversible harms), stopping and then cautiously re-introducing the assigned intervention in the affected participant can avoid both unblinding or causing further harm.

2.8.2 Randomisation

Eligible participants will be randomised 1:1 at Visit 1 to receive either dietary nitrate or placebo 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 study IDs. No stratification factors will be used. Participants will not consume the intervention until instructed to by trial staff at Visit 2.

If a participant is deemed ineligible between Visit 1 and 2, they will be asked to return the sealed beetroot juice given to them to the trial staff. The AI who is responsible for blinding will reallocate the BRJ as per the randomised number list created by the statistician.

2.8.4 Contamination

Bias and contamination could be introduced by a participant's expectations and prior knowledge of the hypothesised benefits of inorganic nitrates in ADPKD. To mitigate this, both groups will be educated on that fact that these benefits have not been proven in human studies in ADPKD; the consent form will be written in an objective, neutral manner to accurately reflect current evidence.

2.9 Concomitant care

Participants will be requested to avoid the use of mouthwash from Visit 1 until study completion as the blood pressure lowering effects of dietary nitrate have been reported to be abolished by the use of antiseptic mouthwash due to the removal of normal oral commensal bacteria.[50]. Participants will be advised to continue their usual medications, diet, physical activity, and other lifestyle factors as directed by their treating nephrologist. As described above (section 2.1), participants must be on a stable blood pressure regime for 28 days at the time of screening. All participant medications, supplements and procedures will be recorded at screening and prompted for at each study visit. If a participant requires a change in blood pressure medication as indicated by their own doctor during the study, they will be withdrawn from the study. Where a participant requires antibiotics in the month prior to commencing the study, or during the study period, it will be recorded as its impact on the oral microbiome ability to covert BRJ to NO is not known.

2.10 Study adherence

2.10.1 Adherence tools

In the screening phase (visit 1 to visit 2) Participants will be required to record home BP in the morning in triplicate in a seated position after 5 minutes rest and either reply to the reminder text message or transcribe into provided diary. In the intervention phase (visit 2 to visit 3), participants will be advised to take their dose of juice at the same time each day with food and continue to measure their BP in the morning. Participants will receive text messages with reminders to take BRJ and measure their BP. After 28 days of intervention, participants will be required to return to the clinic for a post-treatment assessment and biomarker collection. Adherence to the study protocol will be verified in real-time by reply to text messages or at Visit 3 by reviewing the participant diary. Patients will be encouraged to reply to the reminder message with their blood pressure readings as this will allow for monitoring of adherence in real-time. If participants chose not to reply to text messages, they will be contacted once per week via text message to ensure they do not have any unforeseen issues with the intervention.

2.10.2 Text messaging management

The management of the text message delivery will be by the MessageMedia web-based software program (Message Media, Victoria, Australia). The software program will allow a standard text message to be tailored and scheduled for delivery. The program will keep a log of all messages sent to each study participant and those which fail to be delivered. Data exports will be compliant with privacy legislation. There will be no access to these data by any third party, including the software developers.

During the screening period (unless participants opt out), participants will receive the following text message with their first name:
"Hi <First name>, this is your reminder to measure your blood pressure. Please respond to this message with your measurements in the order they were measured. Please also reply if any issues."

During the intervention period (unless participants opt out), they will receive the following message:
"Hi <First name>, this is your reminder to consume your beetroot juice with food and measure your blood pressure. Please respond to this message with your measurements. Please also reply if any issues."

2.10.3 Adherence according to measurement recording

Adherence will be assessed by the following;

- The time at which blood pressure measurements are received via MessageMedia will be considered as the time of BRJ ingestion (unless otherwise stated) and blood pressure measurements unless otherwise indicated by the participant; OR
- Evaluation of the participant diary (Appendix B) where the time of dose and blood pressure are recorded.

- Where participants miss a dose and associated blood pressure readings and alert study staff on the same day, they will be advised to consume BRJ with food and take blood pressure in triplicate as soon as possible on the same day.
- Where participants miss a dose and associated blood pressure readings and study staff are not alerted on the same day, participants will be advised to miss dose (i.e. not 'catch up the dose') and resume the usual protocol the following morning.
- Where participants do not respond to automated text messages for 2 consecutive days, study staff will contact them. Participants will be contacted by phone up to 3 times before they are considered lost to follow up. In

2.11 Study Schedule and Visits

2.11.1 Visit Schedule Overview

Refer to Appendix A: Schedule of Assessments

2.11.2 Visit Outlines

Study visits will occur at WIMR or at the renal department in WSLHD. If participants are unable to attend their visit due to unforeseen circumstances, the visit will be rescheduled as soon as possible. If Visit 3 is rescheduled, a protocol deviation will be filled in. Participants will be encouraged to attend follow up within 72 hours of their scheduled visit.

Details of each visit are described below.

Visit 1

The screening visit (Visit 1) will include:

- Study discussion, consent obtained, PICF signature and date
- Eligibility assessment: inclusion and exclusion criteria check
    - Taste test of nitrate depleted BRJ to ensure they can maintain the intervention (taste is identical to nitrate-rich BRJ)
- Collection of the following information/data:
    - Demographics
    - Family history of ADPKD
    - Medical history and ADPKD history
    - Prior and concomitant medication, over-the-counter medications, supplements
- Height, body weight and BMI
- Clinic blood pressure
- Blood sample collection
- Urine sample collection

- Saliva sample collection
- Provision of blood pressure monitor, associated instructions, blood pressure participant diary
- Randomisation
- Dispensing beetroot juice in sealed bag with clear instructions on bag not to open until after Visit 2

Visit 2

Visit 2 will be held via phone and include:

- Eligibility assessment: inclusion and exclusion criteria check (including home blood pressure compliance/adherence based on text message response)
- Patient interview to collect the following data:
    - Confirmation of Visit 1 Medical/ADPKD history
    - Adverse Events
    - Medical history and ADPKD history since last visit
    - Changes to concomitant medications and supplements since last visit
- The investigator will also instruct the participant to open the sealed package to commence the BRJ the following day and continue to do daily home BP readings at the same time each morning

Visit 3

Visit 3 will include:

- Collection of the following information/data:
    - Adverse Events
    - Medical history and ADPKD history since last visit
    - Changes to concomitant medications and supplements since last visit
- BP recording reconciliation and confirmation based on text message response (compliance/adherence)
- Clinic blood pressure
- Blood sample collection
- Urine sample collection
- Saliva sample collection
- Return of blood pressure monitor

2.12 Trial Procedures

2.12.1 Blood pressure measurements

Clinic Blood Pressure: Clinic blood pressure will be taken at all visits. Blood pressure measurements will be taken in triplicate (1 minute between readings) in a seated position using a validated blood pressure monitor (oscillometric BP device: UA-651 A&D Medical, Tokyo, Japan) with an appropriately sized upper-arm cuff

after at least 5 minutes rest. In accordance with International Society of Hypertension 2020 guidelines and the 2018 European Society of Cardiology/European Society of Hypertension Guidelines,[51] an average of the second and third readings at each time-point will be taken for analysis.

Home Blood Pressure: Participants will be provided a validated blood pressure monitor (oscillometric BP device: UA-651 A&D Medical, Tokyo, Japan) with an appropriately sized upper-arm cuff and given training and instruction on taking measurements (Appendix C). At the screening visit, research staff will provide demonstration and training to the participants to ensure that they can obtain blood pressure readings at home. Participants will also be provided an information card with instructions. Participants will be required to consume oral dose of beetroot juice prior to record home blood pressure every morning upon waking. Blood pressures will be taken in triplicate (with 1 minute rest between readings) in a seated position after 5 minutes rest and recorded via text message reply or in the participant diary. As with the clinic blood pressures, an average of the second and third reading will be used for data analysis.

2.12.2 Sampling procedures

Blood: At visit 1 and 3, after five minutes of rest (as plasma nitrate levels are affected by rapid changes in posture [52]), venous blood will be collected by venepuncture into one 8mL SST tube, which will immediately be centrifuged at 1000 x g (15 min at 4 ºC) to separate the serum. The serum will be stored in aliquots and frozen at -30 ºC until measurement. Serum samples will be used for measurement of NO metabolites (nitrate and nitrite) using a nitric oxide assay and asymmetric dimethylarginine ADMA (a NOS inhibitor and recognized marker of endothelial dysfunction) using ELISA.

To determine if patients fit the inclusion criteria at visit 1 (ie. Serum creatinine, absence of diabetes and uncontrolled hyperlipidemia), patients can provide results of a recent blood test or will have routine bloods performed.

Urine: Two urine samples will be collected at Visit 1 and 3. After participants return their urine collection bottles, one sample will be weighed and stored at -30 ºC for measurement of NO metabolites (nitrate/nitrite and *N*-nitroso compounds), and the other sample will be sent for formal urine albumin to creatinine ratio measurement. The sample to be stored for N-nitroso measurement will be aliquoted and preserved in sodium hydroxide based on previous research that this improves the preservation of volatile N-nitroso compounds if any are present.[53]

Saliva: Whole saliva samples will be collected from participants at Visit 1 and 3 for genomic analysis of oral bacteria. Participants will be asked to rinse their mouths with bottled water at least 10 minutes prior. A spit saliva sample will be collected in sterile tube. Samples will then be aliquoted into 1.5mL Eppendorf tubes and stored at -80 °C.

2.13 Analysis of samples

2.13.1 Analysis of plasma and urine nitrate/nitrite by ELISA

Nitrate/nitrite analysis of plasma and urine will be performed using the commercially available colorimetric Nitric Oxide Assay Kit (ab65328, Abcam, Cambridge, U.K), based on the Griess assay, according to manufacturer's instructions. Briefly, nitrate is reduced to nitrite by the addition of nitrate reductase to the plasma and urine 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).

2.13.2 Analysis of urine *N*-nitroso compounds by chemiluminescence

Urine analysis of *N*-nitrosamine and *N*-nitrosamide will be performed by chemiluminescence as described by Berends *et al*.[54]

2.13.3 Analysis of plasma asymmetric dimethylarginine (ADMA) by ELISA

Serum ADMA analysis will be performed using a commercially available quantitative sandwich enzyme-linked immunosorbent assay (ELISA) Kit (K7828, Immundiagnostik AG, Bensheim, Germany) according to manufacturer's instructions. Briefly, sample is added to a microplate coated with immobilised anti-ADMA antibodies to bind to ADMA present in the sample. Unbound substances are then removed followed by the addition of biotin conjugated ADMA specific antibodies. Between washes avidin conjugated Horseradish Peroxidase (HRP) is added. A substrate solution is then added developing a colour at an intensity proportional to the amount of ADMA in the samples.

2.13.4 Genomic analysis of oral bacteria from saliva samples

Saliva samples will be centrifuged at 15,000 x g for 10 minutes at 4 °C to separate the bacterial pellet. DNA will be extracted using the GenElute Bacterial DNA Extraction Kit (Sigma-Aldrich) according to manufacturer's instructions and modified for lysis. Oral microbiome profiling will then be performed by PCR gene amplification of 16S rRNA and pyrosequencing and analysed as described by Kapil *et al*. (2018).[55]

3 DATA MANAGEMENT

3.1 Data handling

Data will be collected on paper-based CRFs designed specifically for the study. Data will be kept confidential and each CRF have a unique participant identification number (ID) on each form. No participant names will appear in the forms. The CRF will be placed into a folder labelled with the participants' unique participant ID and will be stored in a secure location. All CRFs and relevant documentation will also be scanned to a secure server on the University of Sydney cloud system. Only the Principal Investigator and researchers will have access to documents linking participant personal information to Study ID code numbers, and this will be stored in a locked cabinet at WIMR and via password in the trial database.

The data management plan and electronic case report forms have been developed and harmonised using National Institutes of Health-Clinical Data Interchange Standards Consortium (NIH-CDISC) terminology for ADPKD to enable future data sharing if participants have provided 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 initial consent form.

3.2 Data Dissemination

The results of the study will be disseminated at national and international Scientific Meetings and submitted for publication in leading peer-reviewed international journals, after the last participant has completed the final study visit and/or in the event of early termination of the trial for any reason. If the participants opted to receive the study results upon its completion, they will be contacted by email or message.

3.3 Publications

The results of the clinical trial will be submitted to medical journals for publication by the Investigators. The results of the trial are the property of the Investigators, the Western Renal Service and Westmead Hospital.

4 SAFETY MONITORING AND REPORTING

BRJ is a food supplement that is available for over-the counter consumption from commercial food outlets. Previous clinical trials have not reported any serious adverse effects.[19, 26] The trial staff will monitor for adverse effects at each study visit and review in at least fortnightly meeting between the Principal Investigator and Study Staff.

All serious adverse events will be reported immediately Lead Investigator and in turn to the WSLHD HREC but the Investigator will also note any trends in adverse events and report these also the WSLHD HREC. Initial reports of adverse effects will be followed by detailed written reports based on Case Report Forms (CRF). These CRF will identify the participant by their unique participant identifying number; the participant's name and medical record number will be removed. In the absence of a Data Safety Monitoring Board for this study, a flowchart (adapted from the University of Leicester, United Kingdom) will be used to guide unblinding (Figure 3). In the event of any premature unblinding (accidental unblinding or unblinding due to a serious adverse event) the Investigators will promptly document and report these events to the WSLHD HREC in accordance with ICH-GCP guidelines.

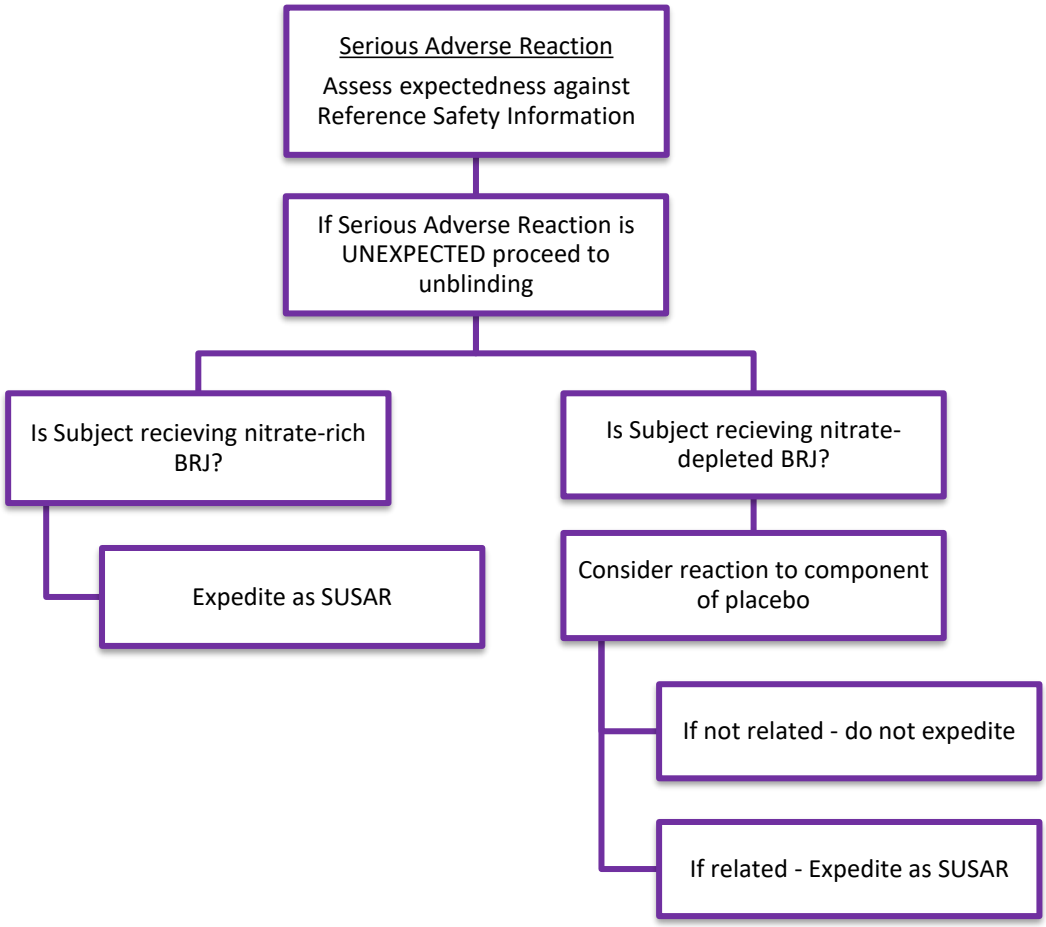

Figure 3: Flowchart to guide unblinding in the BEET-IT study (adapted from the University of Leicester, United Kingdom). SUSAR; suspected unexpected serious adverse event.

5 ETHICS AND DISSEMINATION

5.1 Research ethics approval

Before commencing the study, approval will be obtained from the Western Sydney Local Health District Human Research Ethics Committee. This study protocol, patient information and consent form, and participant diary will be reviewed and approved by ethics review bodies.

5.2 Ethical conduct of the study

The investigator will ensure that this study is conducted in accordance with the ethical principles founded in the most recent revision of the Declaration of Helsinki. The study will be conducted in accordance with the approved study protocol and Standard Operating Procedures that meet the guidelines provided by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) E6 for Good Clinical Practice (GCP) in clinical studies.

5.3 Protocol amendments

Formal changes to the study protocol including changes of study objectives, study design, patient population, sample sizes, study procedures, or significant administrative aspects which may impact on the conduct of the study, potential benefit of the participant or may affect participant safety, will require approval by WSLHD HREC prior to implementation and notified to the health authorities in accordance with local regulations. 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. The HREC may be notified of such changes at the discretion of the Polycystic Kidney Disease research team.

5.4 Participant Information and Consent

Adequate information will be provided to the participant in both oral and written form and consent will be obtained in writing prior to the performance of any study specific procedure. The content and process of obtaining informed consent will be in accordance with all applicable regulatory and HREC requirements. The Investigator, or a person designated by the Investigator, will fully inform the participant about all pertinent aspects of the study including the fact that the protocol has been granted the approval of the HREC.

5.3 Confidentiality

As outlined in the consent form, data collected from the study will be de-identified. A secure web-based database will record all protocol-required information and collection of data on each participant, including quantitative results from the study procedure and reporting of adverse effects. Before being analysed, the participant data is de-identified (removal of name, medical record number). All participants will receive a unique study number and identifying information will be stored separately and securely will controlled access. Participants' study information will not be released without the participants permission.

REFERENCES

1. Kapil, V., et al., *Dietary nitrate provides sustained blood pressure lowering in hypertensive patients: a randomized, phase 2, double-blind, placebo-controlled study.* Hypertension, 2015. **65**(2): p. 320-7.
2. Lanktree, M.B., et al., *Prevalence estimates of polycystic kidney and liver disease by population sequencing.* 2018. **29**(10): p. 2593-2600.
3. Mallett, A., et al., *The prevalence and epidemiology of genetic renal disease amongst adults with chronic kidney disease in Australia.* 2014. **9**(1): p. 1-9.
4. Cornec-Le Gall, E., A. Alam, and R.D.J.T.L. Perrone, *Autosomal dominant polycystic kidney disease.* 2019. **393**(10174): p. 919-935.
5. Nauli, S.M., et al., *Endothelial cilia are fluid shear sensors that regulate calcium signaling and nitric oxide production through polycystin-1.* Circulation, 2008. **117**(9): p. 1161-71.
6. AbouAlaiwi, W.A., et al., *Ciliary polycystin-2 is a mechanosensitive calcium channel involved in nitric oxide signaling cascades.* 2009. **104**(7): p. 860-869.
7. Nauli, S.M., et al., *Loss of polycystin-1 in human cyst-lining epithelia leads to ciliary dysfunction.* 2006. **17**(4): p. 1015-1025.
8. Rangan, G., et al., *Recent advances in autosomal-dominant polycystic kidney disease.* Intern Med J, 2016. **46**(8): p. 883-892.
9. Chapman, A.B., K. Stepniakowski, and F. Rahbari-Oskoui, *Hypertension in Autosomal Dominant Polycystic Kidney Disease.* Advances in Chronic Kidney Disease, 2010. **17**(2): p. 153-163.
10. Rangan, G., et al., *Current and emerging treatment options to prevent renal failure due to autosomal dominant polycystic kidney disease.* 2020. **8**(8): p. 285-302.
11. Ecder, T. and R.W. Schrier, *Cardiovascular abnormalities in autosomal-dominant polycystic kidney disease.* Nature Reviews Nephrology, 2009. **5**: p. 221.
12. Wang, D., et al., *The expression and activity of renal nitric oxide synthase and circulating nitric oxide in polycystic kidney disease rats.* Apmis, 2004. **112**(6): p. 358-68.
13. Wang, D., et al., *Endothelial dysfunction and reduced nitric oxide in resistance arteries in autosomal-dominant polycystic kidney disease.* Kidney Int, 2003. **64**(4): p. 1381-8.
14. Wang, D., et al., *Asymmetric Dimethylarginine and Lipid Peroxidation Products in Early Autosomal Dominant Polycystic Kidney Disease.* American Journal of Kidney Diseases, 2008. **51**(2): p. 184-91.
15. Thomas, S.R., et al., *Oxidative stress and endothelial nitric oxide bioactivity.* 2003. **5**(2): p. 181-194.
16. Lundberg, J.O., E. Weitzberg, and M.T.J.N.r.D.d. Gladwin, *The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics.* 2008. **7**(2): p. 156-167.
17. Lidder, S. and A.J. Webb, *Vascular effects of dietary nitrate (as found in green leafy vegetables and beetroot) via the nitrate-nitrite-nitric oxide pathway.* Br J Clin Pharmacol, 2013. **75**(3): p. 677-96.
18. Zhang, J.Q.J., et al., *Long-term dietary nitrate supplementation does not reduce renal cyst growth in experimental autosomal dominant polycystic kidney disease.* PLoS One, 2021. **in press (accepted 25th Feb 2021)**.
19. Bonilla Ocampo, D., et al., *Dietary Nitrate from Beetroot Juice for Hypertension: A Systematic Review.* Biomolecules, 2018. **8**(4): p. 134.
20. Kapil, V., et al., *Dietary nitrate provides sustained blood pressure lowering in hypertensive patients: a randomized, phase 2, double-blind, placebo-controlled study.* 2015. **65**(2): p. 320-327.
21. Kemmner, S., et al., *Dietary nitrate load lowers blood pressure and renal resistive index in patients with chronic kidney disease: A pilot study.* Nitric Oxide, 2017. **64**: p. 7-15.
22. Stanaway, L., et al., *Acute Supplementation with Nitrate-Rich Beetroot Juice Causes a Greater Increase in Plasma Nitrite and Reduction in Blood Pressure of Older Compared to Younger Adults.* Nutrients, 2019. **11**(7).
23. Jones, T., et al., *The effects of beetroot juice on blood pressure, microvascular function and large-vessel endothelial function: a randomized, double-blind, placebo-controlled pilot study in healthy older adults.* 2019. **11**(8): p. 1792.
24. Larsen, F., et al., *Effects of dietary nitrate on blood pressure in healthy volunteers.* 2006. **28**(355 (26)): p. 2792-3.
25. Bailey, S.J., et al., *Dietary nitrate supplementation reduces the O2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans.* J Appl Physiol (1985), 2009. **107**(4): p. 1144-55.
26. Bahadoran, Z., et al., *The Nitrate-Independent Blood Pressure-Lowering Effect of Beetroot Juice: A Systematic Review and Meta-Analysis.* Adv Nutr, 2017. **8**(6): p. 830-838.

27. Ashor, A.W., J. Lara, and M. Siervo, *Medium-term effects of dietary nitrate supplementation on systolic and diastolic blood pressure in adults: a systematic review and meta-analysis.* J Hypertens, 2017. **35**(7): p. 1353-1359.
28. Chobanian, A.V., et al., *The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: the JNC 7 report.* Jama, 2003. **289**(19): p. 2560-72.
29. Webb, A.J., et al., *Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite.* Hypertension, 2008. **51**(3): p. 784-90.
30. Raubenheimer, K., et al., *Acute Effects of Nitrate-Rich Beetroot Juice on Blood Pressure, Hemostasis and Vascular Inflammation Markers in Healthy Older Adults: A Randomized, Placebo-Controlled Crossover Study.* Nutrients, 2017. **9**(11).
31. Siervo, M., et al., *Inorganic Nitrate and Beetroot Juice Supplementation Reduces Blood Pressure in Adults: A Systematic Review and Meta-Analysis.* Journal of Nutrition, 2013. **143**(6): p. 818-826.
32. Tug, A.Y., et al., *Betaine Supplementation Ameliorates Renal Disease Severity in Experimental ADPKD [Abstract].* Journal of the American Society of Nephrology, 2019. **30**: p. 626.
33. Gilchrist, M., et al., *Effect of dietary nitrate on blood pressure, endothelial function, and insulin sensitivity in type 2 diabetes.* 2013. **60**: p. 89-97.
34. Organization, W.H., *Nitrate and nitrite in drinking-water*. 2011: [Online].
35. Commission, E., *OPINIONS OF THE SCIENTIFIC COMMITTEE FOR FOOD ON: Nitrates and Nitrite*. 1995, Office for Official Publications of the European Communities: Luxembourg.
36. Zamani, H., et al., *The benefits and risks of beetroot juice consumption: a systematic review.* Crit Rev Food Sci Nutr, 2021. **61**(5): p. 788-804.
37. Program, N.T., *NTP technical report on toxicology and carcinogenesis studies of sodium nitrite in f344/n rats and b6c3f1 mice (drinking water studies).* 2001, National Institutes of Health; Durham: National Institutes of Health; Durham.
38. Zealand, F.S.A.N., *SURVEY OF NITRATES AND NITRITES IN FOOD AND BEVERAGES IN AUSTRALIA*. 2011, Food Standards Australia New Zealand [Online].
39. Küng, C.F. and T.F.J.H. Lüscher, *Different mechanisms of endothelial dysfunction with aging and hypertension in rat aorta.* 1995. **25**(2): p. 194-200.
40. Jeserich, M., et al., *Vitamin C improves endothelial function of epicardial coronary arteries in patients with hypercholesterolaemia or essential hypertension—assessed by cold pressor testing.* 1999. **20**(22): p. 1676-1680.
41. Tsuchiya, M., et al., *Smoking a single cigarette rapidly reduces combined concentrations of nitrate and nitrite and concentrations of antioxidants in plasma.* 2002. **105**(10): p. 1155-1157.
42. Mills, C.E., et al., *A randomised, factorial trial to reduce arterial stiffness independently of blood pressure: Proof of concept? The VaSera trial testing dietary nitrate and spironolactone.* Br J Clin Pharmacol, 2020. **86**(5): p. 891-902.
43. Siervo, M., et al., *Post-challenge hyperglycaemia, nitric oxide production and endothelial dysfunction: the putative role of asymmetric dimethylarginine (ADMA).* Nutr Metab Cardiovasc Dis, 2011. **21**(1): p. 1-10.
44. Bailey, S.J., et al., *Improvement in blood pressure after short-term inorganic nitrate supplementation is attenuated in cigarette smokers compared to non-smoking controls.* Nitric Oxide, 2016. **61**: p. 29-37.
45. Jones, A.M., et al., *Dietary nitrate and physical performance.* 2018. **38**: p. 303-328.
46. Gallardo, E.J. and A.R. Coggan, *What's in Your Beet Juice? Nitrate and Nitrite Content of Beet Juice Products Marketed to Athletes.* Int J Sport Nutr Exerc Metab, 2019. **29**(4): p. 345–349.
47. Siervo, M., et al., *Inorganic Nitrate and Beetroot Juice Supplementation Reduces Blood Pressure in Adults: A Systematic Review and Meta-Analysis.* The Journal of Nutrition, 2013. **143**(6): p. 818-826.
48. Jajja, A., et al., *Beetroot supplementation lowers daily systolic blood pressure in older, overweight subjects.* Nutrition Research, 2014. **34**(10): p. 868-875.
49. Bondonno, C.P., et al., *Absence of an effect of high nitrate intake from beetroot juice on blood pressure in treated hypertensive individuals: a randomized controlled trial.* 2015. **102**(2): p. 368-375.
50. Petersson, J., et al., *Gastroprotective and blood pressure lowering effects of dietary nitrate are abolished by an antiseptic mouthwash.* 2009. **46**(8): p. 1068-1075.
51. Asayama, K., T. Ohkubo, and Y. Imai, *In-office and out-of-office blood pressure measurement.* Journal of Human Hypertension, 2021.
52. Liddle, L., et al., *Changes in body posture alter plasma nitrite but not nitrate concentration in humans.* Nitric Oxide, 2018. **72**: p. 59-65.

53. Xu, L., et al., *Urinary levels of N-nitroso compounds in relation to risk of gastric cancer: findings from the shanghai cohort study.* PLoS One, 2015. **10**(2): p. e0117326.
54. Berends, J.E., et al., *Consumption of nitrate-rich beetroot juice with or without vitamin C supplementation increases the excretion of urinary nitrate, nitrite, and N-nitroso compounds in humans.* 2019. **20**(9): p. 2277.
55. Kapil, V., et al., *Sex differences in the nitrate-nitrite-NO• pathway: Role of oral nitrate-reducing bacteria.* 2018. **126**: p. 113-121.

APPENDIX A

Schedule of Assessments

Week	-1 (Screening and Run-in)							1								2						
Day	**-7**	**-6**	**-5**	**-4**	**-3**	**-2**	**-1**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Study visit	X(1)							X(2)														
Eligibility assessment	X							X														
Informed consent	X																					
Taste test BRJ	X																					
Randomization	X																					
Demographics	X																					
Medical history	X																					
Concomitant medications	X							X														
Adverse events								X														
Vital Signs	X							X														
Weight	X																					
BRJ dispensing	X																					
BRJ Intervention									X	X	X	X	X	X	X	X	X	X	X	X	X	X
Clinic BP and HR measure	X																					
Home BP and HR measure		X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X
Compliance text messages		X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X
Blood collection	X																					
Urine collection	X																					
Saliva collection	X																					

Week	3							4						
Days	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**
Study visit														X(3)
Eligibility assessment														
Informed consent														
Randomization														
Demographics														
Medical history														X
Concomitant medications														X
Adverse events														X
Vital Signs														X
Weight														
BRJ dispensing														
BRJ Intervention	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Clinic BP and HR measure														X
Home BP and HR measure	X	X	X	X	X	X	X	X	X	X	X	X	X	
Compliance text messages	X	X	X	X	X	X	X	X	X	X	X	X	X	
Blood collection														X
Urine collection														X
Saliva collection														X

APPENDIX B

Participant Diary (BEET-PKD)

Day	Time of beetroot juice dose	Time of blood pressure	BP Measurement *after 5 mins of rest* Top number (systolic BP)/Bottom Number (diastolic BP)		
			Reading 1	***Reading 2***	***Reading 3***
SCREENING PERIOD					
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
INTERVENTION PERIOD					
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					

14			
15			
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28			

BP – blood pressure

APPENDIX C

BEET-PKD: How to measure your blood pressure

1. Take your blood pressure at the same time every day*
2. Please use the A&D Blood pressure machine provided to you by the study coordinators
3. Sit quietly for 5 minutes before you start to take the measurements
4. During the measurement, sit in a chair with your feet on the floor and your arm supported on a table so your elbow is at about heart level.
5. The cuff should be placed on bare skin, not over a shirt. Fit the cuff on your arm so that it is above your elbow and covering most of your upper arm
6. Refrain from talking during the measurement
7. Press the blue "start" button on your device and the machine will take the reading
8. Please record your reading by writing down both the top and bottom numbers for blood pressure in your diary or in a text message to the study team on your phone
9. Press start again, to take a total of three readings
10. Please write down or send the readings in the order that you took them (ie. First reading sent first, third reading sent third). This is important for the study.

**If you are in the phase of the trial where you have to take beetroot juice, please measure your blood pressure immediately after your dose.*

APPENDIX D

Do you have Polycystic Kidney Disease and High Blood Pressure?

Beetroot juice can lower blood pressure but we don't know if it will help people with polycystic kidneys.

We are looking for people with ADPKD to participate in a 5 week clinical study to help us understand this better

BEET-PKD Clinical Trial

IF YOU ARE INTERESTED PLEASE CONTACT THE CLINICAL TRIALS TEAM ON (02) 8627 3529 OR EMAIL BEETPKDTRIAL@WIMR.ORG.AU

Your participation will be invaluable in helping to learn more about treating polycystic kidney disease

This research study has been approved by the Western Sydney Local Health District HREC

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