Supplemental Material

Vascular Dysfunction, Oxidative Stress, and Inflammation in Autosomal Dominant Polycystic Kidney Disease

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Summary of Supplemental Material

Supplemental (Full) Methods

Supplemental Table 1. Comparison of Autosomal Dominant Polycystic Kidney Disease Participants Included Versus Excluded from Saline and Ascorbic acid Infusions.

Supplemental (Full) Methods

Study Design and Participants

This was a cross-sectional study comparing mechanisms of vascular dysfunction in adults with early-stage ADPKD and age-matched healthy controls. ADPKD patients participated in a randomized, placebo controlled trial of spironolactone administration (NCT01853553) and data presented were collected at their baseline visit, with enrollment between July 2014 and June 2016. Healthy controls were prospectively recruited through University and community advertisement for comparison to ADPKD participants, with enrollment between October 2015 and May 2017. The study was conducted at the University of Colorado Anschutz Medical Campus Division of Renal Diseases and Hypertension Clinical Vascular Physiology Laboratory. Analysts were blinded to group (ADPKD or healthy control) in the assessment of vascular measurements and circulating and cellular markers.

All ADPKD participants randomized in the clinical trial were included in this analysis. Inclusion criteria for the trial were age between 20-55 years of age (women were required to be premenopausal) with a diagnosis of ADPKD based on the Ravine criteria (in participants $\geq$30 years)(1) and the *PKD1* genotype (or presumed *PKD1* genotype based on family history). Additional inclusion criteria were total kidney volume between 500-2,500 ml, estimated glomerular filtration (eGFR) by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation(2) ≥60 ml/min/1.73 m^2, and a history of hypertension treated with a stable maximal tolerable dose of an

angiotensin converting enzyme inhibitor (ACEi) or angiotensin receptor blocker (ARB) and currently controlled. All ADPKD participants were required to have controlled blood pressure (to <160/90 mmHg) and were on a stable antihypertensive regimen at the time of enrollment in the study. Participants were excluded if they had an average serum potassium >5.5 mEq/l or any single value >6.0 mEq/l in the past 6 months, received an aldosterone antagonist in the past 6 months, were using a potassium sparing diuretic or other medication that could contribute to hyperkalemia (e.g. non-steroidal anti-inflammatory agents), had a history of severe congestive heart failure (ejection fraction <35%), used warfarin with an INR > 2.5, or had alcohol dependence or abuse.

Healthy control participants were 23-38 years of age (recruited after partial completion of ADPKD enrollment to best match the mean age of ADPKD participants; women were required to be premenopausal). Inclusion criteria were: healthy (free from kidney disease, cardiovascular disease, or other chronic disease as assessed by self-report, physical exam including resting 12-lead electrocardiogram, and screening labs), free from hypertension based on guidelines at this time (systolic blood pressure [BP] <140/90 mmHg and no antihypertensive agents), and an eGFR by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation(2) ≥60 ml/min/1.73 m^2. Control participants were recruited to be non-hypertensive as we were interested in vascular changes in ADPKD, which complicated by hypertension in the majority of individuals,(3) as compared to healthy controls free from hypertension.

Additional exclusion criteria for both ADPKD and control participants were a history of liver disease, heart failure, recent hospitalization, use of immunosuppressive therapy within the last year, active infection or antibiotic use, antioxidant and/or omega-3 fatty acid use 4 weeks prior to participation, current smoking or history of smoking in the past 12 months, body mass index (BMI ≥40 kg/m^2; for accuracy of vascular measurements), and pregnancy or lactation.

Procedures

Demographics and Clinical Characteristics. Race and ethnicity were determined by self-report. BMI was calculated as mass in kg divided by height in m^2. Resting blood pressure was assessed under quiet resting conditions in the seated position using a semi-automated device (Vital Signs, Welch Allyn, Skaneateles Falls, NY) as the mean of 3 readings. eGFR was calculated using the CKD-EPI equation.(2) Medications were assessed during a medical history by self-report.

***Vascular Measurements*.** All measurements were made under supine, overnight fasted (water only) conditions, following standard recommendations including 24-hr abstention from physical activity and a climate controlled room.(4) Participants refrained from non-prescription medications for 48-hours prior to testing, but prescription medications were not withheld, in order to maintain blood pressure control. Brachial artery flow-mediated dilation (FMD_{BA}) was determined using duplex ultrasonography (Xario 200, Toshiba, Tustin, CA) with ECG-gated end-diastolic ultrasound images analyzed by a single blinded analyst using a commercially available software package (Vascular Analysis Tools 5.8.1, Medical Imaging Applications, Coralville, IA), as described in detail previously.(5, 6) Doppler flow of

the brachial artery was also measured and peak shear rate was calculated as a potential covariate.(5, 6) Endothelium-independent dilation (brachial artery dilation to 0.4 mg of sublingual nitroglycerin) was assessed as a standard index of smooth muscle cell sensitivity to exogenous nitric oxide.(6, 7) Nitroglycerin was administered to 12 control and 37 ADPKD participants (missing due to low HR and/or low systolic BP [n=7 control; n=16 ADPKD]; i.v. failure [n=6 ADPKD]; drug contraindication [n=1 ADPKD]; other [n=1 ADPKD not administered due to prior apparent vasovagal response]).

Carotid-femoral PWV was measured as described in detail previously.(5, 6) Briefly, a transcutaneous custom tonometer (Noninvasive Hemodynamics Workstation [NIHem], Cardiovascular Engineering Inc., Norwood, MA) was positioned at the carotid, brachial, radial and femoral arteries to non-invasively assess carotid-femoral PWV and carotid-radial PWV (an index of peripheral stiffness). Distances between sites were measured using a custom raised ruler (NIHem, Cardiovascular Engineering Inc., Norwood, MA [suprasternal notch and femoral artery]) or tape measure (all other distances). The distance from the suprasternal notch to the carotid was subtracted from the distance between the two recording sites, and carotid-femoral PWV was calculated as the distance divided by time between the foot of waveforms recorded at each site, as described previously.(8)

Additionally, as secondary indices of arterial stiffness, the tonometry assessment in conjunction with ultrasound imaging of the carotid artery also provided blinded assessment of carotid artery compliance and carotid artery β-stiffness index, as

described previously.(5, 6) Carotid intimal medial thickness (cIMT) and carotid systolic BP were also assessed.(5, 6)

The influence of oxidative stress on FMD_{BA} was assessed by infusing a supraphysiological dose of ascorbic acid known that produces plasma concentrations known to inhibit superoxide production in vitro(9) or isovolumic saline, and measuring FMD_{BA} during the "drip infusion" when peak plasma concentrations occur, as described previously.(6, 10) A priming bolus of 0.075 g of ascorbic acid/kg of fat free mass (maximal dosage set at 5.0 g) was dissolved in 150 mL of saline was infused intravenously at 5 mL/min for 20 min, followed immediately by a "drip-infusion" of 0.5 mL/min over a period where FMD_{BA} was measured. Fat free mass was estimated using a previously validated predictor equation considering age, sex, and body-mass index.(11) Infusions were performed in all 19 controls and 52 ADPKD participants (missing in n=7 due to i.v. failure; n=1 due to prior vasovagal response; n=1 due to i.v. pump malfunction). Characteristics of ADPKD participants included in the infusions did not differ from those excluded for reasons described above (Supplemental Table 1). Circulating ascorbic acid levels were measured before and after the ascorbic acid infusion to demonstrate effective elevation of plasma levels in a small sub-group of ADPKD (n=5) and control (n=5) participants by ARUP laboratories using quantitative high performance liquid chromatography.

Cellular Markers of Oxidative Stress and Inflammation. Vascular endothelial cells were obtained immediately prior to other vascular measurements from the intima of an antecubital vein (n=9-17 control participants per protein analyzed and n=32-43 ADPKD participants per protein analyzed; not available in all participants and for all

proteins due to i.v. failure or low cell yield). Cells were recovered, fixed, and slides were prepared and frozen for later staining. VE cadherin primary antibody (1:500, Abcam, Cambridge, MA) was used to identify endothelial cells. Primary antibodies used for the assessment of markers included NAD(P)H oxidase (p47phox; 1:1000, Millipore, Billerica, MA), interleukin-6 (IL-6; 1:50, Santa Cruz, Dallas, TX); nuclear factor κ B (NF κ B; 1:300, Santa Cruz, Dallas, TX), and phosphorylated endothelial nitric oxide synthase (PeNOS; 1:100, Cell Signaling, Danvers, MA) was determined by immunofluorescence staining (Nikon Eclipse Ti, Melville, NY) by a blinded analyst, as described previously.(6, 12, 13) Alexa Fluor 488 and Alexa Fluor 568 were used as secondary antibodies (Thermofisher Scientific, Waltham, MA). A minimum of 20 cells per protein were analyzed for each participant. These markers were chosen as markers of oxidative stress, inflammation, and vascular endothelial nitric oxide production.

Circulating Markers of Oxidative Stress, Inflammation, and Bioactive Lipid Mediators. Serum high-sensitivity C-reactive protein (hsCRP) and IL-6 levels were measured by ELISA (MSD, Rockville, MD). Targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of markers of oxidative stress (prostaglandins [PG], including marker 8-isoprostane, as well as PGF2α, PGD2, and PGE2), and bioactive lipid mediators (hydroxyoctadecadienoic acids [9-HODE and 13-HODE], hydroxyeicosatetraenoic acids [5-HETE, 8-HETE, 9-HETE, 11-HETE, 12-HETE], epoxyeicosatrienoic acids [8,9-EET, 11,12-EET and 14-15-EET), and hydroxyeicosapentanoic acids [5-HEPE, 12-HEPE] was also performed on serum samples using validated assays, as described in detail previously.(14-16)

Statistical Analyses

Normality was evaluated using the Shaprio-Wilk test of normality. Differences in baseline variables between groups were assessed using independent sample t-tests, Chi-square tests, or Fisher's exact tests. Differences in vascular parameters and circulating markers between groups were assessed using an independent samples t-test and changes in FMD_{BA} in response to ascorbic acid infusion were assessed using a 2x2 ANOVA. The influence of mean arterial pressure on group differences in carotid-femoral PWV, as recently recommended,(17) was analyzed by ANCOVA. Non-normally distributed variables were log-transformed prior to analysis. All data are reported as means$\pm$S.D or medians (interquartile range), with figures presented as means$\pm$S.E. Missing data for any variables is described above, and analysis was completed only on individuals with complete data for the outcome of interest. Analyses were performed using SPSS 24 and statistical significance was set at $p<0.05$. As the data were considered mechanistic and hypothesis-generating, adjustment was not made for multiple comparisons.

A sample size of 19 control subjects was calculated based on 95% power at an alpha level of 0.05 (two-sided) in order to detect a group difference of 1.9 in the change in FMD_{BA} with ascorbic acid infusion, based on previously published data assessing the effect ascorbic acid on FMD_{BA} in healthy aging compared to young healthy controls (mean$\pm$SD change in percent FMD_{BA} for each group: young healthy controls: 0.2$\pm$2.0; older adults: 2.1$\pm$0.9),(10) and assuming a similar effect size in ADPKD. While only 19 ADPKD participants were required to provide 95% power, the ADPKD group included all participants from the clinical trial. These sample sizes (n=19 controls and n=61 ADPKD participants) also provided 81% power to detect a group difference of at least 3.3±4.4 in

percent FMD_{BA} (18) and 90% power to detect a group difference of 66±77 cm/sec in carotid-femoral PWV(5) based on previous publications in ADPKD, and 98% power to detect a group difference of 0.20±0.19 in endothelial cell protein expression of NFκB based on data in healthy aging compared to young healthy controls.(12)

Study Approval

All procedures were approved by the Institutional Review Board of the University of Colorado Anschutz Medical Campus, and adhere to the *Declaration of Helsinki*. The nature, benefits and risks of the study were explained to the volunteers and their written informed consent was obtained prior to participation.

Supplemental Table 1.

Table 1: Comparison of Autosomal Dominant Polycystic Kidney Disease Participants Included Versus Excluded from Saline and Ascorbic Acid Infusions.

Variable	Included in Infusions (n=52)	Excluded from Infusions (n=9)
Age, y	34±10	35±8
Sex, % Male	48%	33%
Race/Ethnicity, % Non-Hispanic White	83%	78%
BMI, kg/m^2	27.0±4.8	25.9±5.1
Systolic BP, mmHg	120±12	120±14
Diastolic BP, mmHg	77±10	81±10
CKD-EPI eGFR, ml/min/1.73m^2	95±22	90±17
LDL Cholesterol, mg/dL	95±29	101±21
HDL Cholesterol, mg/dL	49±12	53±14
Total Cholesterol, mg/dL	164±33	175±28
Hypertension, %	100%	100%
ACEi/ARB, %	100%	100%
Diuretic, %	21%	22%
Calcium Channel Blockers, %	8%	0%
Statin, %	25%	11%
Antidepressant or Antianxiety Medication, %	13%	22%
Thyroid Medication, %	8%	0%

Data are mean±S.D.or n (%). BMI, body-mass index; BP, blood pressure; CKD-EPI eGFR; estimated glomerular filtration rate by the Chronic Kidney Disease Epidemiology Collaboration equation; LDL, low density lipoprotein, HDL, high density lipoprotein; ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker.

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