Influences of renal insufficiency and ischemia on mitochondrial bioenergetics and limb dysfunction in a novel murine iliac arteriovenous fistula model

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ABSTRACT

Objective: Hand disability after hemodialysis access surgery has been common yet has remained poorly understood. Arteriovenous fistula (AVF) hemodynamic perturbations have not reliably correlated with the observed measures of hand function. Chronic kidney disease (CKD) is known to precipitate myopathy; however, the interactive influences of renal insufficiency and ischemia on limb outcomes have remained unknown. We hypothesized that CKD would contribute to access-related hand dysfunction via altered mitochondrial bioenergetics. Using a novel murine AVF model, we sought to characterize the skeletal muscle outcomes in mice with and without renal insufficiency.

Methods: Male, 8-week-old C57BL/6J mice were fed either an adenine-supplemented diet to induce renal insufficiency (CKD) or a casein-based control chow (CON). After 2 weeks of dietary intervention, the mice were randomly assigned to undergo iliac AVF surgery (n = 12/group) or a sham operation (n = 5/group). Measurements of aortoiliac hemodynamics, hindlimb perfusion, and hindlimb motor function were collected for 2 weeks. The mice were sacrificed on postoperative day 14 to assess skeletal muscle histopathologic features and mitochondrial function. To assess the late outcome trends, 20 additional mice had undergone CKD induction and sham (n = 5) or AVF (n = 15) surgery and followed up for 6 weeks postoperatively before sacrifice.

Results: The adenine-fed mice had had a significantly reduced glomerular filtration rate and elevated blood urea nitrogen, confirming the presence of CKD. The sham mice had a 100% survival rate and AVF cohorts an 82.1% survival rate with an 84.4% AVF patency rate. The aorta and inferior vena cava velocity measurements and the vessel diameter had increased after AVF creation (P < .0001 vs sham). The AVF groups had had a 78.4% deficit in paw perfusion compared with the contralateral limb after surgery (P < .0001 vs sham). Mitochondrial function was influenced by the presence of CKD. The respiratory capacity of the CKD-sham mice (8443 ± 1509 pmol/s/mg at maximal energy demand) was impaired compared with that of the CON-sham mice (12,870 ± 1203 pmol/s/mg; P = .0001). However, this difference was muted after AVF creation (CKD-AVF: 4478 ± 3685 pmol/s/mg; CON-AVF, 5407 ± 3582 pmol/s/mg; P = .198). The AVF cohorts had had impairments in grip strength (vs sham; P < .0001) and gait (vs sham; P = .012). However, the presence of CKD did not significantly alter the measurements of gross muscle function. The paw perfusion deficits had persisted 6 weeks postoperatively for the AVF mice (P < .0001 vs sham); however, the myopathy had resolved (grip strength, P = .092 vs sham; mitochondrial respiration, P = .108 vs sham).

Conclusions: CKD and AVF-induced distal limb ischemia both impaired skeletal muscle mitochondrial function. Renal insufficiency was associated with a baseline myopathy that was exacerbated by the acute ischemic injury resulting from AVF creation. However, ischemia was the primary driver of the observed phenotype of gross motor impairment. This model reliably reproduced the local and systemic influences that contribute to access-related hand dysfunction and provides a platform for further mechanistic and therapeutic investigation. (JVS—Vascular Science 2022;3:345-62.)
It has been estimated that 30% to 60% of patients with renal failure who require hemodialysis will experience access-related hand dysfunction (ARHD) after dialysis access creation. Historically, the symptoms of pain, discoordination, and weakness have been attributed to ischemia, termed the "steal syndrome." However, the noninvasive measurements of access flow and digital perfusion have varied highly and can remain normal; thus, ARHD has remained largely a clinical diagnosis. Recent evidence has also shown poor correlations between hand function and the hemodynamic changes in the upper extremity after arteriovenous fistula (AVF) placement, suggesting that pathogenic influences exist in addition to flow reversal and hypoperfusion. A unifying biologic mechanism that could account for the observed clinical heterogeneity of ARHD remains unknown, limiting therapeutic progress.

The systemic influence of chronic kidney disease (CKD) has been shown to cause a baseline myopathy. Chronic inflammation and the accumulation of oxidative stress are characteristic of the renal insufficiency milieu and have been linked to mitochondrial dysfunction within skeletal muscle tissue. We have recently shown that uremic toxin accumulation will disrupt the efficiency of energy transfer during mitochondrial respiration, thereby reducing free energy production and increasing reactive oxygen species. Impaired bioenergetics have been linked to the clinical phenotype of neuromotor dysfunction in patients with kidney failure. Metrics, such as grip strength or the 6-minute walking distance, have been used as surrogate markers of frailty but can also be used to quantify the developing muscle impairment and degree of myopathy. Additionally, muscle wasting and weakness have been independently associated with increased morbidity and mortality in those with late-stage CKD. However, it is unknown how this uremic myopathy might modulate muscle dysfunction in the upper extremity after AVF creation.

For the present study, we hypothesized that renal insufficiency could contribute to the pathogenesis of ARHD via altered mitochondrial bioenergetics, exacerbating the hemodynamic insult from access creation. Using a recently developed murine AVF model, we aimed to characterize the interactive influences of CKD and ischemia on mitochondrial respiration and hindlimb function.

**METHODS**

Animal cohorts and assessment of renal function. Male, 8-week-old C57BL/6J mice (n = 54) were housed in a light (12-hour light/12-hour dark cycle), humidity (50%), and temperature (22°C) controlled room throughout the study. The mice were fed either a casein-based control diet (CON; n = 17) or an adenine-supplemented diet (CKD; n = 37) to induce renal dysfunction, as previously described. After 2 weeks of dietary intervention, renal function was evaluated to confirm the presence of kidney disease. Blood urea nitrogen (BUN) was quantified using a commercial kit (model no. K024; Arbor Assays, Ann Arbor, MI), and the glomerular filtration rate (GFR) was calculated via fluorescein isothiocyanate-labeled inulin clearance, as previously described. The CON and CKD mice were randomly assigned to undergo arteriovenous fistula (AVF) surgery (n = 12-15/group) or sham surgery (sham; n = 5/group). A total of 34 mice were monitored for 2 weeks postoperatively and sacrificed on postoperative day (POD) 14. To determine the late outcome trends, another 20 CKD mice were monitored for 6 weeks postoperatively before sacrifice. Different groups of mice (n = 7 sham; n = 7 AVF) were used for the 6-week gait assessments. The casein and adenine diets were maintained throughout the postoperative periods. The study design and cohort assignments are summarized in Fig 1. The institutional animal care and use committee at the University of Florida and Malcom Randall Veterans Affairs Medical Center approved the present study, and our experiments adhered to the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research (National Academy Press, National Research Council, Washington, DC, 1996) and any updates.

Iliac AVF creation. Kim et al described the creation and validation of a novel murine AVF model to study ARHD and that model was used in the present study. In brief, the creation of a left common iliac AVF involved cross clamping and axially rotating the common iliac artery and vein to expose the vein anteriorly. A longitudinal venotomy (~1.0-1.2 mm) allowed for intraluminal...
exposure of the posterior–lateral vein wall, and an elliptical incision (~1 mm × 0.3 mm) was made to excise the adherent common walls of the iliac artery and vein, thereby creating the AVF. We used 0.9% sterile saline to flush the residual blood and thrombus from the exposed AVF, and the venotomy was repaired using interrupted 10-0 sutures. A single dose of intravenous heparin (0.2 IU/g) was administered via inferior vena cava (IVC) injection intraoperatively to enhance AVF patency. The sham surgeries included the dissection and cross-clamping steps but without creation of a venotomy or AVF. The clamp time (~20 minutes) was matched between the AVF and sham groups. The surgical interventions were performed with the mice under isoflurane anesthesia, and the postoperative care included buprenorphine pain control (0.1 mg/kg) and resuscitation with subcutaneous normal saline for 48 hours.

Assessment of AVF patency, hemodynamic changes, and hindlimb perfusion. Ultrasound and laser Doppler ultrasound were used to assess the hemodynamic changes after surgery. Ultrasound imaging of the aortoiliac anatomy was performed preoperatively and on PODs 3 and 13 for the 2-week cohorts and weekly after surgery for the 6-week cohorts using the Vevo 2100 Imaging System with a 40-MHz MS-550D MicroScan transducer (FUJIFILM VisualSonics, Toronto, ON, Canada). AVF patency was determined using color and pulse-wave Doppler ultrasound, as previously described.20 Additionally, the diameter and velocity measurements of the infrarenal aorta and IVC were determined using B-mode imaging and pulse-wave Doppler ultrasound at a 60° angle of insonation. The aortic flow rates were calculated from the vessel cross-sectional area (CSA) and mean velocity of multiple cardiac cycles. The hindlimb perfusion of the bilateral tibial anterior muscles and paws were measured preoperatively and on POD 0, 3, 7, and 13 for the 2-week cohorts and weekly after surgery for the 6-week cohorts via laser Doppler flowmetry (moorVMS-LDF, Moor Instruments Inc, Wilmington, DE). After a stable breathing pattern had been obtained, the laser Doppler flowmeter was placed against the tissue of interest, and data were collected for 10 seconds. The perfusion unit averages were normalized to the contralateral limb. The ultrasound and laser Doppler ultrasound assessments were performed with the mice under anesthesia and placed on a 37°C heating pad.

Assessment of hindlimb function. Hindlimb function was studied preoperatively and on POD 4, 8, and 12 for the 2-week cohorts and weekly after surgery for the 6-week cohorts. To determine the hindlimb grip strength, the mice were suspended over a T-bar connected to a test sensor (BIO-GS3; Bioseb, Vitrolles, France). Once the paw of interest had securely gripped the bar, the mouse was gently pulled away, and the strength score was measured. The highest score from five consecutive trials was recorded, and the strength was normalized to the contralateral limb. The treadmill gait assessment (DigiGait, Mouse Specifics Inc, Framingham, MA) was performed on the same postoperative days or weeks. Each mouse was placed on a treadmill, and the belt speed was gradually increased to 20 cm/s. If a mouse was unable to walk at a speed of 20 cm/s, the maximally tolerated belt speed was recorded. Once a consistent stride had been reached, a gait pattern video was recorded for 5 seconds. The gait dynamic measurements of stride length, percentage of swing stride, paw area at peak stance, and variability of the paw area and paw angle were normalized within each cohort and evaluated as a composite variable. The baseline averages were set to zero.

Analysis of mitochondrial bioenergetics. Muscle from the left hindlimb was harvested during sacrifice on POD 14 or postoperative week 6. Mitochondria were isolated from the left gastrocnemius and plantaris muscles, and an assessment of the mitochondrial bioenergetics was performed as previously described.12,20 The muscles were first minced in a Petri dish on ice after removing the excess fat and connective tissue, followed by myofilament digestion for 5 minutes with 0.025% trypsin. Each sample was centrifuged (800g for 5 minutes at 4°C) and then homogenized in chilled mitochondrial isolation medium (50 mM MOPS, 100 mM KCl, 5 mM MgSO4, and 1 mM EGTA; pH, 7.1) supplemented with 0.2% bovine serum albumin. The homogenate was centrifuged at 800g for 10 minutes at 4°C, and the supernatant was transferred to a new tube on ice, followed by centrifugation at 10,000g for another 10 minutes at 4°C. The mitochondrial pellet was gently washed and then resuspended in mitochondrial isolation medium.

After the protein assay, 20 μg of the sample was used for high-resolution respirometry (Oxygraph O2K; ARTICLE HIGHLIGHTS

- **Type of Research:** A mouse model study
- **Key Findings:** Iliac arteriovenous fistula creation in mice caused modest unilateral hindlimb ischemia and impairments in gross motor function and mitochondrial bioenergetics. Mice with renal insufficiency had the greatest derangements in oxidative respiratory capacity across the spectrum of energy requirements; however, the significance was only obvious compared with the sham mice.
- **Take Home Message:** Functional muscle outcomes after arteriovenous fistula surgery were dictated by ischemic impairment of mitochondrial respiratory capacity. Kidney disease caused a baseline myopathy that was exacerbated by the ischemic insult.
Oroboros Instruments, Innsbruck, Austria) to quantify the oxygen consumption rates in response to changes in energy demands (change in free energy adenosine triphosphate \( \Delta G_{\text{ATP}} \)), mimicking a range of energy demands from rest to intense muscle contraction. Oxidative phosphorylation (OXPHOS) conductance was quantified via the slope between the variables. Another mitochondrial sample (20 mg) was used to measure the corresponding hydrogen peroxide emission via fluorometry (Fluorolog; Horiba, Ltd, Kyoto, Japan), which was used to calculate the electron leak rate \( \frac{J_{\text{H}_2\text{O}_2}}{J_{\text{O}_2}} \).

Muscle histologic features. The tibialis anterior (TA), extensor digitorum longus (EDL), and soleus (SOL) muscles were isolated for histologic analysis. The muscles were mounted in disposable base molds (model no. 6235215; Electron Microscopy Sciences, Hatfield, PA) with embedding medium compound (Tissue-Tek O.C.T.; Sakura Finetek, Torrance, CA) and frozen in liquid nitrogen-cooled isopentane. Serial 10-µm transverse cuts were performed from the middle of the muscle using a Leica 3050S cryostat at \(-20^\circ\text{C}\), and the sections were mounted on frosted microscope slides, briefly air-dried at room temperature, and stored at \(-80^\circ\text{C}\) for subsequent analysis. Immunofluorescence microscopy was used to quantify the myofiber CSA, capillary contact, and central nuclei fibers. The frozen sections were air-dried at room temperature for 10 minutes and then fixed with 4% paraformaldehyde for 5 minutes. After multiple 1× phosphate-buffered saline (PBS) washes, the sections were permeabilized with 0.3% triton X-100 for 10 minutes. After multiple 1× PBS washes, the sections were incubated in blocking buffer (5% goat serum and 1% bovine serum albumin in PBS) for 1 hour. Thereafter, the sections were incubated with anti-laminin primary antibody (model no. L9393; 1:100; Sigma-Aldrich, St Louis, MO) overnight at \(4^\circ\text{C}\). After a series of 1× PBS washes, muscle sections were labeled with a secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG; 1:250) and biotinylated griffonia simplicifolia lectin I isolecitin B4 (1:200; GSL 1-B4; Vector DyLight 649; Thermo Fisher Scientific, Waltham, MA). After 1× PBS washes, coverslips were mounted with fluorescent mounting medium with DAPI (H-1500; Vector Laboratories, Newark, CA). Next, the slides were imaged at 20× magnification with an Evos FL2 Auto microscope (Thermo Fisher Scientific). The central nuclei fiber percentage was manually calculated, and MuscleJ was used to quantify the myofiber CSA and

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**Fig 1.** Study design. Schematic overview of the experimental timeline and mouse cohorts: 54 male, 8-week-old C57BL/6J mice were fed either a casein-base control diet (CON) or an adenine-supplemented diet to induce chronic kidney disease (CKD). After 2 weeks of dietary intervention, the mice were randomly assigned to undergo sham surgery (n = 5/group) or arteriovenous fistula (AVF) surgery (n = 12-15/group). The final cohorts were labeled by their diet and surgery assignments: CON-sham, CON-AVF, CKD-sham, and CKD-AVF. The mice were sacrificed at 2 or 6 weeks after surgery. (Created using Biorender; available at biorender.com.)
capillary contact. Muscle fibrosis was quantified using Masson’s trichrome staining (model no. HT15; Sigma Aldrich) and automated threshold selection with ImageJ (National Institutes of Health, Bethesda, MD) software (hue, 140/96; saturation, 0/255; brightness,110/255). Fibrotic areas are expressed as a percentage of the total image area.

**Statistical analysis.** The data are presented as the mean ± standard deviation, unless otherwise noted. The Student unpaired two-tailed t test was used for CKD outcomes, and analysis of the histologic data was performed using two-way analysis of variance with Tukey’s post hoc multiple comparisons. The Kaplan-Meier method and log-rank test were used to compare the survival rates. The remaining hemodynamic, mitochondrial function, and limb function outcomes were analyzed using mixed effects linear modeling. Modeling of the postoperative variables compared the group means and trends over time. An initial model compared the sham and AVF surgical groups, and a subsequent analysis evaluated the effects of CKD within each group. For the 6-week cohorts, modeling was performed separately for postoperative weeks 1 to 3 and weeks 4 to 6 owing to the clear differences in the trends for these time points. P < .05 was considered statistically significant. All statistical analysis was performed using R statistical software, version 4.02 (R Foundation for Statistical Computing, Vienna, Austria), or GraphPad Prism, version 9.0 (GraphPad Software, San Diego, CA).

**RESULTS**

**Confirmation of CKD.** The presence of renal insufficiency was confirmed in the adenine-fed mice before surgical intervention. Preoperatively, the 2-week CKD mice had had a reduced GFR (148.2 ± 100.3 μL/min; P < .0001) and elevated BUN (40.0 ± 8.1 ng/dL; P < .0001) compared with the CON group (Fig 2, A and B). Additionally, the body weight was significantly reduced in the CKD mice (24.5 ± 1.5 g; P < .0001) compared with the CON group (Fig 2, C). Similarly, the 6-week cohorts were confirmed to have preoperative renal insufficiency (sham BUN, 33.5 ± 3.6 ng/dL; AVF BUN, 35.06 ± 4.0 ng/dL).

**Surgical outcomes.** After surgery, 100% of the sham mice had survived (n = 15 of 15), and 82.1% of the AVF mice had survived (n = 32 of 39; P = .086). The Kaplan-Meier survival curve per group is shown in Fig 3, A. The mortality rate was similar between the CON-AVF (n = 2 of 12; 16.7%) and CKD-AVF (n = 5 of 27; 18.5%) cohorts. Overall, for the surviving AVF mice, the fistula patency rate was 84.4% (CON-AVF, n = 8 of 10 [80%]; CKD-AVF, n = 19 of 22 [86.4%]). All death and thrombosis events had occurred within 3 days after surgery. The mortality and fistula patency outcomes per group are shown in Fig 3, B, which were improved compared with the original report of the model. The turbulent flow of a patent AVF shown via color flow Doppler analysis is presented in Fig 3, C, and the high velocity, low resistance, aortoiliac waveforms of an AVF via pulse-wave Doppler analysis are shown in Fig 3, D. The lack of these features is suggestive of AVF thrombosis. Mice with AVF failure were excluded from analysis.

**AVF creation impairs hindlimb perfusion.** AVF creation increased the aortic and IVC diameters and the inflow and outflow velocity metrics. The aortic peak systolic velocity and end-diastolic velocity measurements had increased throughout the 2-week postoperative recovery period. The peak systolic velocity had increased from 180.3 ± 44.7 mm/s at baseline to 546.3 ± 159.2 mm/sec at POD 13 (P < .0001 vs sham), and the end-diastolic velocity had increased from 9.6 ± 14.6 mm/s to 267.5 ± 49.2 mm/s (P < .0001 vs sham). The average aortic diameter was 0.9 ± 0.4 mm on POD 13 (P < .0001 vs sham), a 70.5% increase from baseline (0.5 ± 0.7 mm). Together, the velocity and diameter increases had contributed to a 12.4-fold increase in the aortic flow rate (21.2 ± 8.2 mm3/s at baseline vs 262.9 ± 72.2 mm3/s at POD 13; P < .0001 vs sham). Similarly, the IVC diameter had increased from 0.8 ± 0.1 mm to 13.0 ± 0.1 mm for the AVF mice (P < .0001 vs sham), and the IVC peak velocity had increased from 40.5 ± 17.6 mm/s to 144.25 ± 8.2 mm/s (P < .0001 vs sham). The vascular measurements and flow dynamics for the sham mice had not changed from those at baseline. Also, renal insufficiency had not influenced any of the ultrasound measurement end points (CON-sham vs CKD-sham, P > .05; CON-AVF vs CKD-AVF, P > .05). For the 6-week AVF mice, the aortic and IVC diameters had increased during the first 3 postoperative weeks and had plateaued at weeks 4 to 6. The corresponding velocities were stable throughout weeks 1 to 3 and had decreased during weeks 4 to 6. Taken together, these adaptations caused the aortic flow rate to peak at week 3 at 205.68 ± 89.56 mm3/s. All measures of vascular diameter, velocity, and flow were elevated for the AVF mice compared with the sham mice throughout the 6-week recovery period (P < .01 for all comparisons). Supplementary Figs 1 and 2 show the vascular diameter, velocity, and flow measurement trends for the 2-week and 6-week cohorts. The 2-week AVF groups had had significantly reduced hindlimb perfusion found by postoperative laser Doppler ultrasound measurements of the paw (P < .0001 vs sham) and TA (P < .0001 vs sham). Compared with the contralateral limb, the AVF mice had had an average paw perfusion decrease of 78.39% ± 17.88% on POD 0, and this deficit had gradually recovered over time (3.0%/d; 95% confidence interval [CI], 2.03-3.90; Fig 4, A). The presence CKD did not influence the paw laser Doppler measurements between groups (AVF, P = .944; sham, P = .709). The ischemic insult was not as severe for the TA muscle (average decrease, 39.84% ± 21.67% on POD 0), and the TA perfusion deficit had recovered by 2.3%/d (95% CI,
TA perfusion was not significantly affected by CKD (AVF, \( P = .944 \); sham, \( P = .193 \); Fig 4, B). For the 6-week AVF mice, both measures of hindlimb perfusion had improved at weeks 1 to 3 and had stabilized at weeks 4 to 6 (Fig 4, C and D). TA perfusion had fully recovered at weeks 4 to 6 (Fig 4, C and D). TA perfusion had fully recovered at weeks 4 to 6 (Fig 4, C and D). TA perfusion had fully recovered at weeks 4 to 6 (Fig 4, C and D). TA perfusion had fully recovered at weeks 4 to 6 (Fig 4, C and D). TA perfusion had fully recovered at weeks 4 to 6 (Fig 4, C and D).

CKD and ischemia alter mitochondrial bioenergetics without histologic evidence of injury. At the 2-week sacrifice, the AVF mice had had significantly impaired mitochondrial respiratory function (\( JO_2 \)) compared with the sham group (\( P = .002 \); Fig 5, A). Renal dysfunction had impaired the mitochondrial function for the sham groups (CON-sham vs CKD-sham; \( P = .0001 \)). However, this difference was diminished between the AVF cohorts (CON-AVF vs CKD-AVF; \( P = .198 \)), suggesting that the AVF-induced hemodynamic changes are a major driver of limb muscle mitochondrial alterations. The average OXPHOS conductance, which describes the efficiency of the electron transport chain throughout the spectrum of energy requirements, was highest for the CON-sham group (1042.00 ± 693.18), followed by the CKD-sham (963.18 ± 126.47), CON-AVF (411.21 ± 295.76), and CKD-AVF (367.47 ± 295.23) groups (Fig 5, B). The differences between the CKD-sham and CKD-AVF groups did not reach significance owing to the baseline impairment of CKD (\( P = .09 \)). An electron leak, indicative of the propensity for reactive oxygen species generation, is shown in Fig 5, C. The incidence of an electron leak was worse for the AVF mice (\( P = .002 \)), and the pathologic influence of CKD within sham cohorts (\( P = .007 \)) was not present between the two AVF groups (\( P = .458 \)). After 6 weeks of muscle recovery, the measures of mitochondrial respiration (\( P = .108 \)) and electron leak (\( P = .411 \)) were no longer significant between the AVF and sham mice (Supplementary Fig 3).

The individual muscle weight averages and histopathologic results for the three different hindlimb muscles (TA, EDL, and SOL) for the 2- and 6-week cohorts are shown in Supplementary Figs 4 and 5. Measures of muscle atrophy (muscle mass and myofiber CSA), capillary density (number of capillary contacts), muscle regeneration (proportion of fibers with central nuclei), and muscle fibrosis were included. Most measures did not show statistically significant differences owing to significant variability for the AVF mice.

Limb function impaired by AVF creation. Normalized to the contralateral hindlimb, the grip strength was lower for the AVF mice (\( P < .0001 \)) during the first 2 postoperative weeks (Fig 6, A). However, because of the significant outcome variability, differences between the AVF groups were not statistically significant (CON-AVF, 66.09% ± 36.20%; CKD-AVF, 41.66% ± 34.86%; \( P = .171 \)). Both AVF cohorts had recovered strength over time at a similar rate (1.6%/d; 95% CI, 1.02-2.12). The recovery had plateaued after 3 weeks for the 6-week cohorts (Fig 6, B). The strength deficit for the AVF mice was 21.3% (95% CI, −4.05 to 46.7) less than that of the sham mice for weeks 4 to 6, and the difference from that of the sham mice was no longer statistically significant (\( P = .092 \)).

The treadmill analysis revealed similar trends in functional impairment for the 2-week cohorts. All the sham mice were able to walk at the standard pace of 20 cm/s postoperatively. However, 50% of the AVF mice (\( n = 3 \) of 8 CON-AVF; \( n = 6 \) of 10 CKD-AVF) had required a lower treadmill speed for analysis. The AVF mice had also had worse gait performance (\( P = .012 \)) than sham. However, the presence of CKD did not significantly influence the gait outcomes for the sham or AVF groups (\( P > .05 \); Fig 6, C). The sham group scores had increased after surgery and the AVF group scores had decreased. The 6-week cohorts showed similar differences at 3 weeks.
postoperatively \( (P = .018; \text{Fig } 6, D) \). The abnormal gait characteristics of the AVF mice (ie, negative value for the composite gait score) included a shorter stride length, larger percentage of swing stride, smaller paw area at peak stance, and greater variability in the paw area and paw angle.

**Correlation analysis.** A correlation matrix of the CKD-AVF outcomes to further evaluate the interactive influences of renal insufficiency, ischemia, muscle injury, mitochondrial health, and limb function is shown in Fig 7. The outcome measures with moderate to strong correlations included limb perfusion, limb function, mitochondrial function, and histologic parameters. The flow dynamics of the AVF and renal function had weaker associations with the other outcome measurements. The body mass did not seem to influence the outcomes in the present study.

**DISCUSSION**

The biologic mechanisms of ARHD have remained poorly understood. Although disability has classically been considered to be secondary to ischemia after AVF creation, the variability in the measured hemodynamic parameters and symptom heterogeneity suggest that other biologic factors contribute to the clinically observed phenotype. However, to date, alternative factors have not been identified or studied, limiting the development of therapeutic agents. To the best of our knowledge, the present analysis has provided the first experimental application of a novel murine AVF model to investigate the complex relationships between renal insufficiency, AVF hemodynamics, muscle physiology, and hindlimb function to better understand the pathogenesis of ARHD.

In this model, AVF creation significantly impaired hindlimb perfusion, and the ultrasound measurements were able to confirm the steal-associated pathophysiology. Specifically, mice with a patent AVF had consistently had elevated aortic and caval velocities and adaptive vessel dilation, analogous to the hemodynamic changes in humans. Clinically, 80% of patients who have undergone brachial-based AVF creation will have reduced distal extremity blood pressure after access creation, and patients who experience ARHD will often have high fistula flow rates. However, significant variability
has been reported in the AVF hemodynamics, degree of distal extremity ischemia, and neuromotor outcomes in clinical studies. In patients undergoing AVF creation, Rehfuss et al found that postoperative digital pressures were decreased in the ipsilateral upper extremity but that the biomechanical outcome trends did not match the temporal changes in hemodynamics after surgery. These findings indicate that the occurrence of postoperative ischemia does not exclusively drive hand function disability in patients with dialysis patients. Similarly, we found that the AVF flow dynamics weakly correlated with the perfusion changes and limb functional outcomes. Moreover, CKD did not cause significant differences in hindlimb perfusion or flow-mediated vascular adaptations.

The mitochondrial bioenergetics were clearly influenced by kidney dysfunction. The CKD-sham mice had had decreased mitochondrial respiratory capacity and increased electron leak compared with their control group (CON-sham), representing the baseline influence of renal insufficiency. Other preclinical studies have also found that CKD causes mitochondrial dysfunction. Using a 5/6 nephrectomy model, Yazdi et al found that renal disease impaired oxidative phosphorylation, increased reactive oxygen species generation, and reduced the mitochondrial mass in the skeletal muscle of Sprague-Dawley rats. Thome et al identified uremia as a driver of respiratory chain uncoupling and diminished efficiency of energy production. Specifically, murine muscle tissue exposed to various uremic metabolites had decreased oxidative phosphorylation conductance and respiratory capacity. Additionally, untargeted metabolomics of muscle tissue from mice with renal insufficiency revealed the accumulation of uremic toxins, which correlated with the measures of mitochondrial dysfunction and alterations in neuromuscular junction morphology.

Ischemia also causes mitochondrial impairment. Restriction of oxygen and nutrients during ischemic conditions limit oxidative phosphorylation, leading to adenosine triphosphate depletion, lactic acidosis, mitochondrial membrane imbalance, and reactive oxygen...
species generation. These conditions can severely impair mitochondrial bioenergetics via respiratory chain dysfunction.28-30 Furthermore, reperfusion injury will exacerbate local inflammatory changes and can lead to cell death.28-30 Most preclinical and translational studies investigating the mitochondriopathy of ischemia have analyzed peripheral arterial disease (PAD) pathobiology.31-34 Berru et al.22 found that mice with kidney disease had exacerbated ischemic myopathy after femoral artery ligation, a murine model of PAD, suggesting additive insults from renal disease and ischemia. However, the local and systemic influence of steal-mediated ischemia, such as increased cardiac output, arterial flow reversal, and venous hypertension, differ from the pathogenesis of occlusive disease; thus, the findings cannot be exchanged between the two pathologies. Moreover, the type, frequency, and degree of recurrent effort–related ischemia—reperfusion injury differ between PAD and ARHD.

The mitochondrial function in skeletal muscle distal to an AVF has not previously been investigated before this model. In our first description of the model, we found diminished complex I and II mitochondrial respiratory capacity after iliac AVF formation. With the comprehensive mitochondrial phenotyping platform used in the present study, we also found AVF creation impaired oxidative respiration in hindlimb muscle, confirming the ischemic injury. Both CON-AVF and CKD-AVF mice had a worse rate of oxygen flux across the spectrum of energy requirements compared with their surgical controls (CON-sham and CKD-sham). Although the CKD-AVF mice had the greatest derangement in OXPHOS conductance and electron leak, these measures of mitochondrial function were not significantly worse than those for the CON-AVF group. Therefore, the uremic myopathy observed at baseline between the CON-sham and CKD-sham cohorts was minimized after AVF creation. Likely, the overwhelming influence of regional ischemia partially masked the myopathic influence of renal insufficiency.

The fast twitch (TA and EDL) and slow twitch (SOL) muscle histologic analysis did not show significant injury from uremia or ischemia. Although our findings of minor muscle injury produced underpowered results, they actually appeared analogous to the observed patient outcomes. Severe ARHD, involving digital necrosis and extremity paralysis, has been uncommon.1,35 Patients will usually complain of variable amounts of pain, discoordination, and weakness without findings of tissue loss, which parallels the variation in our histologic outcomes. Our histopathologic results correlated with the mitochondrial respiration and gross measures of muscle function, confirming that muscle physiology will be disrupted by even a modest amount of CKD-driven myofiber atrophy and ischemic muscle injury. CKD and ischemia have both been implicated in the histologic findings of myopathy, supporting these correlations.22,25,26,29,31,36 An association between mitochondrial health and muscle capacity has been demonstrated in patients with PAD and murine

**Fig 5.** Mitochondrial bioenergetics. **A,** Oxygen consumption rate (JO₂) at progressive increases in energy demand (change in free energy adenosine triphosphate [ΔGₐₜᵢₚᵢₚ]), mimicking muscle contraction. Mitochondrial respiratory capacity was highest for control mice (casein-base control diet) with sham surgery (CON-sham). Chronic kidney disease (CKD) caused oxidative respiration differences between sham mice, and the ischemic insult of arteriovenous fistula (AVF) creation further impaired mitochondrial function. Differences were greatest at near maximal energy demand. **B,** Oxidative phosphorylation (OXPHOS) conductance between cohorts. **C,** Log electron leak rate (JH₂O₂/JO₂) at progressive increases in energy demand (ΔGₐₜᵢₚᵢₚ). Plotted mitochondrial respiration and electron leak values presented as mean ± standard error of the mean.
models of occlusive disease but has not previously been shown after AVF creation.\textsuperscript{22,37} Also, occlusive disease models have routinely produced too severe an ischemic insult to be clinically relevant for studying ARHD.\textsuperscript{22,38}

AVF creation altered hindlimb function, matching the ischemic impairment of mitochondrial function. The CKD-AVF mice had the worst measurements of grip strength and gait, which were significantly different from those of the controls (CKD-sham). The spectrum of hindlimb disability correlated well with mitochondrial impairment, the histologic assessment of muscle injury, and paw hypoperfusion, confirming that strength and stride are adequate functional assessments of muscle health and reflect the degree of ischemic injury. However, the pathologic influence of renal insufficiency on hindlimb function is less obvious. Despite a trend of worse outcomes for the CKD mice, the presence of renal disease did not significantly affect hindlimb function between the sham and AVF groups.

\textbf{Fig 6.} Limb function. Grip strength measurements of the 2-week (A) and 6-week (B) cohorts. Arteriovenous fistula (AVF) cohorts had had impaired strength compared with that of sham mice, which gradually recovered over time. Composite gait scores of the 2-week (C) and 6-week (D) cohorts. Abnormal gait characteristics of AVF mice (ie, negative value on composite gait score score) included shorter stride length, larger percentage of swing stride, smaller paw area at peak stance, and greater variability of paw area and paw angle. Gait scores had similarly recovered over time. Renal insufficiency did not influence limb function. Plotted values represent mean ± standard error of the mean. B (horizontal axis), preoperative baseline; ns, not significant.
In contrast, other studies have detected baseline grip strength deficits and decreases in the muscle contractile forces in CKD mice. The reason for these observed functional outcome differences is likely the differences in the severity of kidney disease among the CKD murine models. Zhang et al used a 5/6 nephrectomy model, and Thome et al completed 8 weeks of adenine diet induction before testing muscle function, both of which resulted in more severe CKD (average BUN, 60 mg/dL for both studies) than had the 2-week adenine diet used in our study (BUN, 40 mg/dL). Matching our sham mice results, Roshanravan et al found mitochondrial impairments in patients with mild CKD despite no difference in functional assessments. Therefore, mitochondrial impairments likely precede functional impairment in patients with early-stage renal disease. Alternatively, the estimated GFR and BUN levels correlated with the metrics of strength and exercise tolerance in patients with late-stage CKD with known functional decline. Kestenbaum et al also found that the 6-minute walking distance is associated with upper and lower extremity maximal muscle oxidative capacity, linking muscle function to mitochondrial health in patients with CKD.

For the 6-week cohorts, the flow-mediated vascular adaptations had plateaued at 3 weeks after AVF creation, and no late thrombosis or failure events had occurred, validating the durability of the model. Furthermore, the paw perfusion deficits persisted during postoperative weeks 4 to 6, confirming the steal-mediated ischemia of the distal hindlimb throughout the late time points. In humans, AVFs that have successfully matured will have similar diameter and flow increases during the first 1 to 2 months and will stabilize thereafter. Additionally, patients with >1 month of ARHD symptoms will have lower measures of digital perfusion (eg, basal digital pressure, digital brachial index, oxygen saturation), matching the persistent hemodynamic changes of this model. Measures of gross muscle function and mitochondrial function did not differ during the late recovery period (weeks 4-6). Likely, the longer recovery time after the ischemic insult diminished the myopathy seen at 2 weeks. The central nucleated fibers in the EDL and SOL muscles at 6 weeks confirmed muscular regeneration. Goldberg et al quantified skeletal muscle recovery after femoral artery ligation. Their results showed that 8 weeks of recovery demonstrated improved muscle...
contractile function, dystrophin staining, and myofiber CSA compared with the findings at 2 weeks. However, the contractile function and myofiber size had had persistent deficits at the 8-week point compared with the baseline values.

**Study limitations.** The results of the present analysis should be interpreted within the context of the study’s limitations. Measurements of renal insufficiency, fistula hemodynamics, limb perfusion, and muscle function within this model all matched the AVF outcome associations in humans, including the high variability, further supporting the validity of the model. Furthermore, most patients with ARHD will exhibit acute perfusion and hand function deficits that reach a nadir and partially recover, which has been recapitulated well in this model. However, a subset of patients will experience a late presentation of hand disability, months to years after access creation, that will frequently be related to either access remediation or vascular remodeling. Future studies are required to determine whether this model can be used to examine these late events. Also, future experiments should include sex-based comparisons because the present study was limited to male mice alone. The next iterations of the model should assess a greater severity of kidney disease based on these findings. Experiments using antioxidant and targeted mitochondrial therapeutic agents should be completed to provide preliminary data to support the development of novel treatment strategies for ARHD, especially because surgical remediation is the only current interventional strategy, which should be reserved for patients with severe disability. In addition, the highly variable hemodynamic and muscle-related outcomes in the present study were observed in both AVF mice cohorts (CON-AVF and CKD-AVF). Therefore, renal insufficiency might contribute to the symptom heterogeneity of ARHD but should not be considered the only pathogenic influence. Further investigation of other contributing influences of ARHD is warranted.

**CONCLUSIONS**

The results from the present study have shown that AVF-induced distal limb ischemia and uremia do not appear to be equal impairments of mitochondrial function. Rather, most acute changes were driven by ischemia, exacerbating the baseline impairment of CKD. The functional muscle outcomes matched the trends of the mitochondrial derangements, implicating impaired respiratory capacity as the primary driver of diminished muscle function after AVF in those with CKD. Furthermore, a variable response pattern was noted in response to the uremic and ischemic influences, analogous to the clinical heterogeneity found in human studies. Therefore, this model reliably produced the local and systemic influence that contributes to ARHD and provides a platform for further mechanistic and therapeutic investigations.

**AUTHOR CONTRIBUTIONS**

Conception and design: EA, KK, AM, KO, SB, TR, SS

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Data collection: EA, KK, BF, KH, QH, ZS, VP, TC, EK

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Supplementary Fig 1. Two-week arteriovenous fistula (AVF) flow dynamics. Ultrasound measurements of aortoiliac velocities and vessel diameters. The AVF cohorts had an increased aortic diameter (A), aortic peak systolic velocity (PSV; B), aortic end-diastolic velocity (EDV; C), aortic flow rate (D), inferior vena cava (IVC) diameter (E), and IVC peak velocity (F) compared with their surgical controls (sham). Values increased throughout the 2-week postoperative recovery period. Renal insufficiency did not influence flow dynamics. Plotted values represent mean ± standard error of the mean. B (horizontal axis), preoperative baseline; CON, casein-base control diet.
Supplementary Fig 2. Six-week arteriovenous fistula (AVF) flow dynamics. Ultrasound measurements of aortoiliac velocities and vessel diameters for 6-week cohorts. Because of trend differences, postoperative weeks 1 to 3 and 4 to 6 were analyzed separately. Aortic (A) and inferior vena cava (IVC: E) diameters had increased during first 3 postoperative weeks before plateauing. After an initial increase, the velocity measurements (B, C, and F) had attenuated during weeks 4 to 6. Together, these trends caused aortic flow (D) to peak at approximately week 3. Plotted values represent mean ± standard error of the mean. B (horizontal axis), preoperative baseline.
Supplementary Fig 3. Six-week mitochondrial bioenergetics. A,B. Mitochondrial respiratory capacity and electron leak at various levels of energy demand. Differences were not statistically significant. Plotted mitochondrial respiration and electron leak values represent mean ± standard error of the mean. ΔG<sub>ATP</sub>, Change in free energy adenosine triphosphate; ns, not significant.
Supplementary Fig 4. Two-week muscle histologic findings. Muscle mass (A–C) and muscle histologic characteristics (D–O) for three different hindlimb muscles: tibialis anterior (TA), extensor digitorum longus (EDL), and soleus (SOL). Histologic outcomes included myofiber cross-sectional area (CSA; D–F), number of myofiber capillary contacts (CCs; G–I), percentage of fibers with central nuclei fibers (CNFs; J–L), and percentage of fibrosis (M–O). No significant differences were found between arteriovenous fistula (AVF) and sham groups or chronic kidney disease (CKD) and control (CON) groups, unless otherwise indicated. *P < .05.
Supplementary Fig 5. Six-week muscle histologic findings. Muscle mass (A-C) and muscle histologic characteristics (D-O) for 6-week cohorts. No significant differences were found between arteriovenous fistula (AVF) and sham groups, unless otherwise indicated. *P≤.05; **P<.01. CC, Capillary contact; CNF, central nuclei fiber; CSA, cross-sectional area; EDL, extensor digitorum longus; SOL, soleus; TA, tibialis anterior.