Influences of Renal Insufficiency and Ischemia on Mitochondrial Bioenergetics and Limb Dysfunction in a Novel Murine Iliac Arteriovenous Fistula Model

Problem: Up to 60% of CKD patients with hemodialysis access have hand disability. Hypothesis: Renal insufficiency contributes to access-related myopathy via mitochondrial dysfunction, exacerbating the hemodynamic insult of access creation.

CONCLUSIONS
- CKD causes baseline mitochondrial dysfunction. The myopathy is further impaired by ischemia after AV fistula creation.
- This model reliably produces influences that contribute to limb disability, which can be used for future mechanistic and therapeutic investigation.
Influence of Renal Insufficiency and Ischemia on Mitochondrial Bioenergetics and Limb Dysfunction in a Novel Murine Iliac Arteriovenous Fistula Model

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ARTICLE HIGHLIGHTS

Type of Research: Mouse model study

Key Findings: Iliac arteriovenous fistula creation in mice caused modest unilateral hindlimb ischemia, as well as impairments of gross motor function and mitochondrial bioenergetics. Mice with renal insufficiency had the greatest derangements in oxidative respiratory capacity across the spectrum of energy requirements, but significance was only obvious compared to sham mice.

Take Home Message: Functional muscle outcomes after arteriovenous fistula surgery are dictated by ischemic impairment of mitochondrial respiratory capacity. Kidney disease causes a baseline myopathy that is exacerbated by the ischemic insult.
ABSTRACT

Objective: Hand disability after hemodialysis access surgery is common, yet poorly understood. Arteriovenous fistula (AVF) hemodynamic perturbations do not reliably correlate with observed measures of hand function. Chronic kidney disease (CKD) is known to precipitate myopathy, but the interactive influences of renal insufficiency and ischemia on limb outcomes are currently unknown. We hypothesized that CKD contributes to access-related hand dysfunction (ARHD) via altered mitochondrial bioenergetics. Using a novel murine AVF model, our objective was to characterize 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 two weeks of dietary intervention, mice were randomly assigned to undergo iliac AVF surgery (N=12/group) or a sham operation (N=5/group). Measurements of aorto-iliac hemodynamics, hindlimb perfusion, and hindlimb motor function were collected for two weeks. Mice were sacrificed on post-operative day 14 to assess skeletal muscle histopathology and mitochondrial function. To assess late outcome trends, 20 additional mice underwent CKD induction and sham (N=5) or AVF (N=15) surgery and were followed for 6 weeks post-operatively before sacrifice.

Results: Adenine-fed mice had significantly reduced glomerular filtration rate and elevated blood urea nitrogen, confirming the presence of CKD. Sham mice had a 100% survival rate, and AVF cohorts had an 82.1% survival rate with an 84.4% fistula patency rate. Aorta and IVC velocity measurements, as well as vessel diameter increased after AVF creation (p<0.0001 vs. sham). AVF groups had a 78.4% deficit in paw perfusion compared to the contralateral limb after surgery (p<0.0001 vs. sham). Mitochondrial function was influenced by CKD. Respiratory capacity of CKD_Sham mice (8443pmol/sec/mg±1509 at maximal energy demand) was impaired
compared to CON_Sham mice (12870 pmol/sec/mg±1203, p=0.0001), but this difference was muted after AVF creation (CKD_AVF: 4478pmol/sec/mg±3685, CON_AVF: 5407pmol/sec/mg±3582, p=0.198). AVF cohorts had impairments in grip strength (vs. Sham, p<0.0001) and gait (vs. Sham, p=0.012), but CKD did not significantly alter measurements of gross muscle function. Paw perfusion deficits persisted 6 weeks post-operatively for AVF mice (p<0.0001 vs sham), but the myopathy recovered (grip strength: p=0.092 vs sham, mitochondrial respiration: p=0.108 vs sham).

**Conclusion:** CKD and AVF-induced distal limb ischemia both impair skeletal muscle mitochondrial function. Renal insufficiency is associated with a baseline myopathy that is exacerbated by the acute ischemic injury from AVF creation. However, ischemia is the primary driver of the observed phenotype of gross motor impairment. This model reliably produces local and systemic influences that contribute to ARHD and provides a platform for further mechanistic and therapeutic investigation.

**Clinical Relevance:** ARHD remains a common hemodialysis access surgery complication. Due to poor mechanistic insight, current therapies are limited to surgical remediation of hemodynamic changes in the limb for only the most severe clinical manifestations. Further, expectant management or access revision are not ideal options especially among patients with mild to moderate ARHD due to perioperative risk and implications on long-term access durability. Therefore, skeletal muscle mitochondrial-based therapy represents an intriguing treatment strategy, but characteristics of mitochondrial function after AVF placement are unknown. In this study, we characterized ischemic and uremic influences on skeletal muscle physiology to identify pathogenic drivers of ARHD.
Keywords:
Arteriovenous fistula; Hand dysfunction; Hemodialysis; Mitochondria; Steal Syndrome

Conflict of interest statement:
No conflicts of interest are declared by the authors.

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INTRODUCTION

It is estimated that 30%-60% of renal failure patients requiring hemodialysis experience access-related hand dysfunction (ARHD) after dialysis-access creation.\(^1\) Historically, symptoms of pain, discoordination and weakness have been attributed to ischemia, called ‘steal syndrome.’ However, non-invasive measurements of access flow and digital perfusion are highly variable and can be normal, so ARHD remains largely a clinical diagnosis.\(^2-6\) Recent evidence has also shown poor correlation between hand function and hemodynamic changes in the upper extremity after arteriovenous fistula (AVF) placement, which suggest pathogenic influences beyond flow reversal and hypoperfusion.\(^1\) Indeed, a unifying biologic mechanism that accounts 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 accumulation of oxidative stress are characteristic of the renal insufficiency milieu and are linked to mitochondrial dysfunction within skeletal muscle tissue.\(^7-11\) In fact, we have recently shown that uremic toxin accumulation disrupts the efficiency of energy transfer during mitochondrial respiration, thereby reducing free energy production and increasing reactive oxygen species.\(^12\) Impaired bioenergetics are linked to the clinical phenotype of neuromotor dysfunction in kidney failure patients.\(^8, 11, 13, 14\) Metrics, such as grip strength or 6-minute walking distance, are surrogate markers of frailty but also quantify developing muscle impairment and degree of myopathy.\(^15, 16\) Additionally, muscle wasting and weakness are independently associated with increased morbidity and mortality in late stage CKD.\(^13, 17-19\)

However, it is unknown how this uremic myopathy modulates muscle dysfunction of the upper extremity after AVF creation.
Herein, we hypothesized that renal insufficiency contributes to ARHD pathogenesis 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 two weeks of dietary intervention, renal function was evaluated to confirm presence of kidney disease. Blood urea nitrogen (BUN) was quantified using a commercial kit (Arbor Assays K024), and glomerular filtration rate (GFR) was calculated via fluorescein isothiocyanate-labeled inulin clearance as previously described. CON and CKD animals were then randomly assigned to undergo arteriovenous fistula surgery (AVF, N=12-15/group) or sham operation (Sham, N=5/group). Mice were monitored for two weeks post-operatively and sacrificed on post-operative day (POD) 14. To determine late outcome trends, 20 CKD mice were monitored for 6 week post-operatively before sacrifice. Of note, different groups of mice (n=7 sham, n=7 AVF) were used for the 6 week gait assessments. Casein and adenine diets were maintained throughout the post-operative periods. Study design and cohort assignments are summarized in Figure 1. The Institutional Animal Care and Use Committee at University of Florida and Malcom Randall Veterans Affairs Medical Center
approved this study and experimentation 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 Arteriovenous Fistula Creation.**

Kim et al. describes the creation and validation of a novel murine AVF model to study ARHD, which was utilized for this study. Briefly, 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 x 0.3 mm) was made to excise the adherent common walls of the iliac artery and vein, thereby creating the fistula. 0.9% sterile saline was used to flush residual blood and thrombus from the exposed AVF and the venotomy was repaired using interrupted 10-0 suture. A single dose of intravenous heparin (0.2 IU/g) was administered via IVC injection intraoperatively to enhance AVF patency. Sham surgeries included dissection and cross-clamping steps, but without creation of a venotomy or fistula. Clamp time (~20 minutes) was matched between AVF and sham mice. Surgical interventions were performed under isoflurane anesthesia and post-operative care included buprenorphine pain control (0.1 mg/kg) and resuscitation with subcutaneous normal saline for 48 hours.

**Assessment of Fistula Patency, Hemodynamic Changes, and Hindlimb Perfusion**

Ultrasound and laser Doppler were used to assess hemodynamic changes after surgery. Ultrasound imaging of the aortoiliac anatomy was performed preoperatively and on POD 3 and 13 for the 2-week cohorts and weekly after surgery for the 6-week cohorts using Vevo 2100 Imaging.
System (VisualSonics) with an MS-550D (40 MHz) MicroScan transducer. AVF patency was determined using color and pulse-wave Doppler as previously described. Additionally, diameter and velocity measurements of the infrarenal aorta and IVC were determined using B-mode imaging and pulse-wave Doppler at a 60° angle of insonation. Aortic flow rates were calculated from vessel cross sectional area and mean velocity of multiple cardiac cycles. Hindlimb perfusion of the bilateral tibialis 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). After a stable breathing pattern was obtained, the laser Doppler flowmeter was placed against the tissue of interest and data was collected for 10 seconds. Perfusion unit averages were normalized to the contralateral limb. Ultrasound and laser doppler assessments were performed on a 37°C heating pad under anesthesia.

**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 hindlimb grip strength, animals were suspended over a T-bar connected to a test sensor (BIO-GS3, BIOSEB). Once the paw of interest securely gripped the bar, the mouse was gently pulled away and strength score (g) was measured. The highest score from 5 consecutive trials was recorded and strength was normalized to the contralateral limb. Treadmill gait assessment (DigiGait, Mouse Specifics Inc.) was performed on the same post-operative days/weeks. Each mouse was placed on a treadmill and belt speed was gradually increased to 20cm/s. If a mouse was unable to walk at a speed of 20cm/s, the maximally tolerated belt speed was recorded. Once a consistent stride was reached, a gait pattern video was recorded for 5 seconds. Gait dynamic measurements of stride length, percent
swing stride, paw area at peak stance, as well as variability of paw area and paw angle were
normalized within each cohort and evaluated as a composite variable. Baseline averages were set
to zero.

**Analysis of Mitochondrial Bioenergetics**

Muscle from the left hindlimb was harvested during sacrifice on POD14 or post-operative
week 6. Mitochondria were isolated from left gastrocnemius and plantaris muscles and assessment
of mitochondrial bioenergetics was performed as previously described. The muscles were first
minced in a petri dish on ice after removing excess fat and connective tissue, followed by
myofilament digestion for 5 minutes with 0.025% trypsin. Sample was centrifuged(800xG for 5
min at 4°C) and then homogenized in chilled mitochondrial isolation medium(MIM)(50mM
MOPS, 100mM KCl, 5mM MgSO4, and 1mM EGTA, pH=7.1) supplemented with 0.2% bovine
serum albumin. The homogenate was centrifuged at 800xG for 10 minutes at 4°C, and the
supernatant was then transferred to a new tube on ice followed by centrifugation at 10,000xG for
10 more minutes at 4°C. The mitochondrial pellet was gently washed and then re-suspended in
MIM.

Following protein assay, 20 ug of the sample was used for high-resolution
respirometry(Oroboros OxygraphO2K), which quantified oxygen consumption rates in response
to changes in energy demand(ΔG_{ATP}), mimicking a range of energy demands from rest to intense
muscle contraction. OXPHOS conductance was quantified via the slope between variables.
Another mitochondrial sample(20 ug) was used to measure corresponding hydrogen peroxide
emission via fluorometry(Horiba Fluorolog), which was used to calculate electron leak
rate(JH₂O₂/JO₂).
**Muscle Histology**

Tibialis anterior (TA), extensor digitorum longus (EDL) and soleus (SOL) muscles were isolated for histological analysis. The muscles were mounted in disposable base molds (#6235215, Electron Microscopy Science) with embedding medium compound (Tissue-Tek O.C.T, Sakura Finetek) 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°C, and the sections were mounted on frosted microscope slides, briefly air-dried at room temperature and stored at -80°C for subsequent analysis. Immunofluorescence microscopy was used to quantify myofiber cross-sectional area (CSA), capillary contact (CC), and central nuclei fibers (CNF). Frozen sections were air-dried at room temperature for 10 minutes and then fixed with 4% paraformaldehyde for 5 minutes. After multiple 1x PBS washes, the sections were permeabilized with 0.3% triton X-100 for 10 minutes. Following multiple 1x PBS washes, sections were incubated in blocking buffer (5% goat serum and 1% BSA in PBS) for 1 hr. Thereafter, sections were incubated with anti-laminin primary antibody (Sigma L9393, 1:100) overnight at 4°C. Following a series of 1x 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 (GSL I) isolectin B4 (Vector Dylight 649, GSL I-B4, 1:200). Following 1x PBS washes, coverslips were mounted with fluorescent mounting medium with dapi (Vector Laboratories, H-1500). Consequently, slides were imaged at 20x magnification with an Evos FL2 Auto microscope (ThermoScientific). CNF percentage was manually calculated, and MuscleJ was used to quantify myofiber CSA and CC. Muscle fibrosis was quantified using Masson’s trichrome staining (Sigma HT15) and automated threshold.
selection with ImageJ software (Hue:140/196, Saturation:0/255, Brightness:110/255). Fibrotic areas were expressed as a percentage of total image area.

Statistical Analysis.

Data is presented as mean ± SD unless otherwise noted. Student’s unpaired two-tailed t-test was used for CKD outcomes, and analysis of histology data was performed using two-way ANOVA with Tukey’s post-hoc multiple comparisons. Kaplan-Meier methods and log-rank testing were used to compare survival rates. Remaining hemodynamic, mitochondrial function, and limb function outcomes were analyzed using mixed-effect linear modeling. Modeling of post-operative variables compared group means and trends over time. An initial model compared sham and AVF surgical groups, and subsequent analysis evaluated the impact of CKD within each group. For the 6-week cohorts, modeling was performed separately for post-operative weeks 1-3 and weeks 4-6 due to clear differences in trends for these time points. P<0.05 was considered significant. All statistical analysis was performed using R statistical software (V.4.02, the R foundation for Statistical Computing) or GraphPad Prism (V.9.0).

RESULTS

Confirmation of CKD

The presence of renal insufficiency was confirmed in adenine-fed mice before surgical intervention. Preoperatively, the 2-week CKD mice had reduced GFR (148.2 μl/min±100.3, p<0.0001 vs. CON) and elevated BUN (40.0 ng/dL±8.1, p<0.0001 vs. CON) (Figure 2A&2B). Additionally, body weight, was significantly reduced in CKD mice (24.5 g±1.5, p<0.0001 vs. CON).
CON)(Figure 2C). Similarly, the 6-week cohorts were confirmed to have pre-operative renal insufficiency (Sham BUN: 33.5ng/dL±3.6, AVF BUN: 35.06ng/dL±4.0).

Surgical Outcomes

After surgery, 100% of the sham mice survived (N=15/15), and 82.1% of the AVF mice survived (N=32/39) (p=0.086). Figure 3A shows the Kaplan-Meier survival curve per group. Mortality rate was similar between CON_AVF (N=2/12, 16.7%) and CKD_AVF cohorts (N=5/27, 18.5%). Of the surviving AVF mice, there was an 84.4% (CON_AVF N=8/10, 80%; CKD_AVF N=19/22, 86.4%) fistula patency rate. All death and thrombosis events occurred within 3 days of surgery. Figure 3B depicts mortality and fistula patency outcomes per group, which are improved compared to the original publication of the model.20 Figure 3C demonstrates the turbulent flow of a patent fistula on color flow Doppler analysis and Figure 3D shows the high velocity, low resistance aorto-iliac waveforms of an AVF on pulse-wave Doppler analysis. Lack of these features was suggestive of fistula thrombosis. Mice with AVF failure were excluded from analysis.

Arteriovenous Fistula Creation Impairs Hindlimb Perfusion

AVF creation increased aortic and IVC diameters, as well as inflow and outflow velocity metrics. Aortic peak systolic velocity (PSV) and end diastolic velocity (EDV) measurements increased throughout the two-week post-operative recovery period. PSV increased from 180.3mm/sec±44.7 at baseline to 546.3mm/sec±159.2 on POD13 (p<0.0001 vs. sham) and EDV increased from 9.6mm/sec±14.6 to 267.5mm/sec±49.2 (p<0.0001 vs. sham). Average aortic diameter was 0.9mm±0.4 on POD13 (p<0.0001 vs. sham), which was a 70.5% increase from
baseline (0.5mm±0.7). Together, velocity and diameter increases contributed to a 12.4-fold increase in aortic flow rate (21.2mm³/sec±8.2 baseline, 262.9mm³/sec±72.2 POD13) (p<0.0001 vs. sham). Similarly, IVC diameter increased from 0.8mm±0.1 to 1.3mm±0.1 for AVF mice (p<0.0001 vs. sham), and the IVC peak velocity increased from 40.5mm/sec±17.6 to 144.2mm/sec5±8.2 (p<0.0001 vs. sham). Sham mice vascular measurements and flow dynamics did not change from baseline. Also, renal insufficiency did not influence any of the ultrasound measurement end-points (CON_Sham vs. CKD_Sham: p>0.05, CON_AVF vs. CKD_AVF: p>0.05). For 6-week AVF mice, aortic and IVC diameters increased during the first three postoperative weeks and plateaued weeks 4-6. Corresponding velocities were stable throughout weeks 1-3 and decreased weeks 4-6. Taken together, these adaptions caused aortic flow rate to peak at week 3: 205.68mm³/sec±89.56. All measures of vascular diameter, velocity, and flow were elevated for the AVF mice compared to sham throughout the 6-week recovery period (p<0.01 for all comparisons). **Supplemental figures 1&2** show vascular diameter, velocity, and flow measurement trends for 2-week cohorts and 6-week cohorts.

2-week AVF groups had significantly reduced hindlimb perfusion on post-operative Laser Doppler measurements of the paw (p<0.0001 vs. Sham) and TA (p<0.0001 vs. Sham). Compared to the contralateral limb, AVF mice had an average paw perfusion decrease of 78.39%±17.88 on POD0, and this deficit gradually recovered over time (3.0% per day [95%CI:2.03, 3.90]) (Figure 4A). CKD did not influence paw laser Doppler measurements between groups (AVF: p=0.944, Sham: p=0.709). The ischemic insult was not as severe for the TA muscle (average decrease of 39.84%±21.67 on POD0), and the TA perfusion deficit recovered 2.3% per day [95%CI:1.32, 3.26]. TA perfusion was not significantly impacted by CKD (AVF: p=0.944, Sham: p=0.193) (Figure 4B). For the 6-week AVF mice, both measures of hindlimb perfusion improved
weeks 1-3 and stabilized weeks 4-6 (Figure 4C&D). TA perfusion fully recovered (p=0.883 vs sham weeks 4-6), while paw perfusion plateaued at a 36.2% [95%CI:27.0,45.4] deficit (p<0.0001 vs sham weeks 4-6).

**CKD and Ischemia Alter Mitochondrial Bioenergetics without Histological Evidence of Injury**

At time of 2-week sacrifice, AVF mice had significantly impaired mitochondrial respiratory function (JO₂) compared to Sham (p=0.002) (Figure 5A). Interestingly, renal dysfunction impaired mitochondrial function for Sham groups (CON_Sham vs. CKD_Sham, p=0.0001), but this difference diminished between AVF cohorts (CON_AVF vs. CKD_AVF, p=0.198), suggesting the AVF-induced hemodynamic changes are a major driver of limb muscle mitochondrial alterations. Average OXPHOS conductance, which describes the efficiency of the electron transport chain throughout the spectrum of energy requirements, was highest for CON_Sham (1042.00±96.93), followed by CKD_Sham (693.18±126.47), CON_AVF (411.21±295.76), and CKD_AVF (367.47±295.23) (Figure 5B). Differences between CKD_Sham and CKD_AVF did not reach significance due to the baseline impairment of CKD (p=0.09).

Figure 5C shows electron leak, which is indicative of the propensity for reactive oxygen species generation. Electron leak was similarly worse for AVF mice (p=0.002), and the pathologic influence of CKD within Sham cohorts (p=0.007) was not present between the two AVF groups (p=0.458). Interestingly, after 6-weeks of muscle recovery, measures of mitochondrial respiration (p=0.108) and electron leak (0.411) no longer reached significance between AVF and sham mice (Supplemental Figure 3).

**Supplemental Figures 4&5** depicts individual muscle weight averages and histopathological results for 3 different hindlimb muscles (TA, EDL, and SOL) for 2- and 6-week
cohorts. Measures of muscle atrophy (muscle mass and myofiber cross sectional area), capillary density (number of capillary contacts), muscle regeneration (proportion of fibers with central nuclei), and muscle fibrosis were included. Most measures did not have statistically significant differences due to significant variability for the AVF mice.

**Limb Function is Impaired by AVF Creation**

Normalized to the contralateral hindlimb, grip strength was lower for AVF mice (vs. Sham, p<0.0001) during the first two post-operative weeks (Figure 6A). However, due to significant outcome variability, differences between the AVF groups were not significant (CON_AVF 66.09%±36.20, CKD_AVF 41.66%±34.86, p=0.171). Both AVF cohorts recovered strength over time at a similar rate: 1.6% per day [95%CI:1.02,2.12]. Recovery plateaued after three weeks for the 6-week cohorts (Figure 6B). Strength deficit for the AVF mice was 21.3% [95%CI:-4.05,46.7] less than the sham mice for weeks 4-6, which was no longer significantly different from sham mice (p=0.092).

Treadmill analysis revealed similar trends in functional impairment for the 2-week cohorts. All sham mice were able to walk at the standard pace of 20cm/sec post-operatively, but 50% of AVF mice (N=3/8 CON_AVF, N=6/10 CKD_AVF) required lower treadmill speeds for analysis. AVF mice had worse gait performance (vs. Sham, p=0.012), but CKD did not significantly influence gait outcomes for sham or AVF groups (p>0.05) (Figure 6C). Sham group scores increased after surgery and AVF group scores decreased. 6-week cohorts showed similar differences for 3 weeks post-operatively (p=0.018) (Figure 6D). Abnormal gait characteristics in AVF mice (i.e. negative value on the composite gait score) included a shorter stride length, larger
percent swing stride, smaller paw area at peak stance, and greater variability of paw area and paw angle.

**Correlation Analysis**

Figure 7 depicts a correlation matrix of CKD_AVF outcomes to further evaluate interactive influences of renal insufficiency, ischemia, muscle injury, mitochondrial health, and limb function. Outcome measures with moderate to strong correlations included limb perfusion, limb function, mitochondrial function, and histological parameters. Flow dynamics of the fistula and renal functional had weaker associations with other outcome measurements. Body mass did not seem to influence outcomes in this study.

**DISCUSSION**

The biological mechanisms of ARHD remain poorly understood. While disability is classically considered to be secondary to ischemia after AVF creation, variability in 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 therapeutic development. This analysis provides the first experimental application of a novel murine AVF model, investigating the complex relationships between renal insufficiency, fistula hemodynamics, muscle physiology, and hindlimb function to better understand ARHD pathogenesis.

In this model, AVF creation significantly impaired hindlimb perfusion, and ultrasound measurements were able to confirm steal-associated pathophysiology. Specifically, mice with a patent AVF consistently had elevated aortic and caval velocities and adaptive vessel dilation,
which is analogous to hemodynamic changes in humans.\textsuperscript{23} Clinically, 80\% of patients undergoing a brachial-based AVF have reduced distal extremity blood pressure after access creation, and patients that experience ARHD often have high fistula flow rates.\textsuperscript{3, 24} However, there is a significant variability surrounding the AVF hemodynamics, degree of distal extremity ischemia, and neuromotor outcomes in clinical studies.\textsuperscript{1-6} In patients undergoing AVF creation, Rehfuss et al. found that post-operative digital pressures decreased in the ipsilateral upper extremity, but biomechanical outcome trends did not match the temporal changes in hemodynamics after surgery.\textsuperscript{1} These findings indicate that post-operative ischemia does not exclusively drive hand function disability in dialysis access patients. Similarly, we found AVF flow dynamics weakly correlated with perfusion changes and limb functional outcomes. Moreover, CKD did not cause significant differences in hindlimb perfusion or flow-mediated vascular adaptations. Mitochondrial bioenergetics were clearly influenced by kidney dysfunction. CKD\_Sham mice had decreased mitochondrial respiratory capacity and increased electron leak compared to their control group(CON\_Sham), representing the baseline influence of renal insufficiency. Other preclinical studies have similarly found that CKD causes mitochondrial dysfunction.\textsuperscript{12, 25-27} Using a 5/6 nephrectomy model, Yazdi et. al. found that renal disease impaired oxidative phosphorylation, increased reactive oxygen species generation, and reduced mitochondrial mass in skeletal muscle of Sprague-Dawley rats.\textsuperscript{27} Recently, 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.\textsuperscript{12} Additionally, untargeted metabolomics of muscle tissue from mice with renal insufficiency revealed accumulation of uremic toxins, which
correlated with measures of mitochondrial dysfunction and alterations in neuromuscular junction
morphology.26

Ischemia causes mitochondrial impairment as well. Restriction of oxygen and nutrients
during ischemic conditions limit oxidative phosphorylation, leading to ATP depletion, lactic
acidosis, mitochondrial membrane imbalance, and reactive oxygen species generation. These
conditions severely impair mitochondrial bioenergetics via respiratory chain dysfunction.28-30
Further, reperfusion injury exacerbates local inflammatory changes and can lead to cell death.28-30
A majority of preclinical and translational studies investigating the mitochondriopathy of ischemia
analyze peripheral arterial disease(PAD) pathobiology.31-34 Berru et al. found that mice with
kidney disease had exacerbated ischemic myopathy after femoral artery ligation, a murine model
of PAD, suggesting additive insults of renal disease and ischemia.22 However, the local and
systemic influences of steal-mediated ischemia, such as increased cardiac output, arterial flow
reversal, and venous hypertension, differ from the pathogenesis of occlusive disease, and findings
cannot be exchanged between the two pathologies.20 Moreover, the type, frequency and degree of
recurrent effort-related ischemia-reperfusion injury differs between PAD and ARHD.

Importantly, mitochondrial function in skeletal muscle distal to an AVF has not previously
been investigated prior to this model. In our first description of the model, we found diminished
complex I and II mitochondrial respiratory capacity following iliac AVF formation. With the
comprehensive mitochondrial phenotyping platform used in this study, we also found AVF
creation to impair oxidative respiration in hindlimb muscle, confirming ischemic injury. Both
CON_AVF and CKD_AVF mice had worse rate of oxygen flux across the spectrum of energy
requirements compared to their surgical controls(CON_Sham & CKD_Sham). Although
CKD_AVF mice had the greatest derangement in OXPHOS conductance and electron leak, these
measures of mitochondrial function were not found to be significantly worse than the CON_AVF group. Therefore, the uremic myopathy observed at baseline between CON_Sham and CKD_Sham cohorts is minimized after AVF creation. Likely, the overwhelming influence of regional ischemia partially masks the myopathic influence of renal insufficiency.

Fast twitch(TA & EDL) and slow twitch(SOL) muscle histological analysis did not show significant injury from uremia or ischemia. Although our findings of minor muscle injury produced underpowered results, they actually appear analogous to observed patient outcomes. Severe ARHD, involving digital necrosis and extremity paralysis, is uncommon. Rather, patients usually complain of variable amounts of pain, discoordination and weakness without findings of tissue loss, which parallels variation in our histological outcomes. In fact, our histopathology results correlated with mitochondrial respiration and gross measures of muscle function, confirming that muscle physiology is disrupted by even a modest amount of CKD-driven myofiber atrophy and ischemic muscle injury. CKD and ischemia have both been implicated in histological evidence of myopathy, supporting these correlations. An association between mitochondrial health and muscle capacity has been demonstrated in PAD patients and murine models of occlusive disease, but has not previously been shown after AVF creation. Also, occlusive disease models routinely produce too severe of an ischemic insult to be clinically relevant for studying ARHD.

AVF creation altered hindlimb function, matching the ischemic impairment of mitochondrial function. CKD_AVF mice had the worst measurements of grip strength and gait, which were significantly different from controls(CKD_Sham). The spectrum of hindlimb disability correlated well with mitochondrial impairment, histological 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 in CKD mice, renal disease did not significantly impact hindlimb function between sham or AVF groups.

In contrast, other studies have detected baseline grip strength deficits and decreases in muscle contractile forces in CKD mice. The reason for these observed functional outcome differences is likely due to the differences in severity of kidney disease among 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, which both caused more severe CKD (average BUN ~60mg/dL for both studies) than the 2-week adenine diet used in this study (BUN 40mg/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 a functional impairment in patients with early stage renal disease. Alternatively, eGFR and BUN levels correlate with metrics of strength and exercise tolerance in late stage CKD patients with known functional decline. Kestenbaum et al. also found that six 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, flow-mediated vascular adaptations plateaued three weeks after AVF creation and no late thrombosis/failure events occurred, which validate durability of the model. Further, paw perfusion deficits persisted during post-operative weeks 4-6, confirming steal-mediated ischemia of the distal hindlimb throughout late time points. In humans, AVFs that successfully mature have similar diameter and flow increases during the first 1-2 months and
stabilize thereafter.\textsuperscript{44-46} Additionally, patients with >1 month of ARHD symptoms have lower measures of digital perfusion (e.g. basal digital pressure, digital brachial index, O2 saturation), matching the persistent hemodynamic changes of this model.\textsuperscript{47} Measures of gross muscle function and mitochondrial function did not differ during the late recovery period (weeks 4-6). Likely, longer recovery time after the ischemic insult diminished the myopathy seen at 2 weeks. Central nucleated fibers in the EDL and SOL muscles at 6 weeks confirm muscular regeneration. Goldberg and colleagues quantified skeletal muscle recovery after femoral artery ligation.\textsuperscript{48} Their results showed that 8 weeks of recovery demonstrated improved muscle contractile function, dystrophin staining, and myofiber CSA compared to 2 weeks, however contractile function and myofiber size had persistent deficits at the 8 week timepoint compared to baseline.

The results of the current analysis should be interpreted within the context of its limitations. Measurements of renal insufficiency, fistula hemodynamics, limb perfusion, and muscle function within this model all match AVF outcome associations in humans, including high variability, further supporting the validity of the model.\textsuperscript{1, 2, 8, 14, 16, 41, 43} Further, a majority of ARHD patients exhibit acute perfusion and hand function deficits that nadir and partially recover, which is recapitulated well in this model.\textsuperscript{1} However, a subset of patients experience a late presentation of hand disability, months to years after access creation and frequently related to either access remediation or vascular remodeling. Future studies would need to be performed to see if this model can be used to examine these late events. Also, future experiments need to include sex-based comparisons since the current study was limited to only male mice. Importantly, next iterations of the model should assess greater severity of kidney disease based on these findings. Experiments using antioxidant and targeted mitochondrial therapeutics need to be completed to provide preliminary data to support development of novel treatment strategies for ARHD, especially since
surgical remediation is the only current interventional strategy and is reserved for patients with severe disability.\textsuperscript{24} It is important to note that highly variable hemodynamic and muscle-related outcomes in this study were observed in both AVF mice cohorts(CON_AVF and CKD_AVF). Therefore, renal insufficiency may contribute to symptom heterogeneity of ARHD but it is not the only pathogenic influence. Further investigation into other contributing influences of ARHD is warranted.

\section*{CONCLUSION}

In conclusion, AVF-induced distal limb ischemia and uremia do not appear to be equal impairments of mitochondrial function. Rather, a majority of acute changes are driven by ischemia, exacerbating the baseline impairment of CKD. Functional muscle outcomes match the trends of the mitochondrial derangements, implicating impaired respiratory capacity as the primary driver of diminished muscle function after AVF in CKD patients. Further, variable response pattern are noted in response to uremic and ischemic influences, analogous to the clinical heterogeneity of human studies. Therefore, this model reliably produces local and systemic influences that contribute to ARHD, and provides a platform for further mechanistic and therapeutic investigation.

\section*{AUTHOR CONTRIBUTIONS}

Conception and Design: EMA, KK, AJM, KAO, SAB, TER, STS
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Obtained Funding: STS

Overall Responsibility: STS

REFERENCES


**Figure 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 CKD. After 2 weeks of dietary intervention, the mice were randomly assigned to undergo sham surgery (N=5/group) or 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’. Mice were sacrificed 2 or 6 weeks after surgery. This figure was created with Biorender.com.

**Figure 2: Characteristics of Renal Insufficiency.** (A-C) Pre-operative measurements of renal function and muscle wasting. CKD mice had depressed GFR (A), elevated BUN (B), and decreased body weight (C) compared to the dietary control group (CON). ****P<0.001 vs. Control. N=17/group.

**Figure 3: Mortality and AVF Patency Outcomes.** (A) Kaplan-Meier survival curve of the four mouse cohorts. 100% of the sham mice survived and each AVF group had an 82.1% survival rate. All deaths occurred ≤3 days after surgery. (B) Number of patent fistulas, thrombosed fistulas, and post-operative mortalities for all AVF mice. (C & D) Ultrasound visualization of a patent AVF. AVF characteristics included turbulent flow on color Doppler assessment of the left iliac vasculature (C) as well as high velocity pulsatile waveforms within the left iliac vein on pulse-wave Doppler assessment (D).

**Figure 4: Hindlimb Perfusion.** (A&B) Laser doppler measurements of the left hindlimb for the 2-week cohorts, normalized to the contralateral limb. AVF cohorts had unilateral Paw (A) and
tibialis anterior (TA) (B) perfusion deficits after fistula creation. Paw ischemia was worse than TA. Hindlimb perfusion gradually recovered during the 2-week recovery period. Renal insufficiency did not influence hindlimb perfusion. (C&D) Hindlimb laser doppler measurements of the 6-week cohorts. Both Paw (A) and TA (B) perfusion plateaued after a few weeks of recovery. Only the distal hindlimb maintained a persistent perfusion deficit throughout the recovery period. Plotted values represent mean ± SEM. B = pre-operative baseline.

Figure 5: Mitochondrial Bioenergetics. (A) Oxygen consumption rate (JO2) at progressive increases in energy demand (ΔGATP), mimicking muscle contraction. Mitochondrial respiratory capacity was highest for CON_Sham mice. CKD caused oxidative respiration differences between sham mice, and the ischemic insult of AVF creation further impaired mitochondrial function. Differences were greatest at near maximal energy demand. (B) OXPHOS conductance between cohorts. (C) Log electron leak rate (JH2O2/O2) at progressive increases in energy demand (ΔGATP). Plotted mitochondrial respiration and electron leak values represent mean ± SEM.

Figure 6: Limb Function. Grip strength measurements of the 2-week (A) and 6-week (B) cohorts. AVF cohorts had impaired strength compared to sham mice, which gradually recovered over time. Composite gait scores of the 2-week (C) and 6-week (D) cohorts. Abnormal gait characteristics of the AVF mice (i.e. negative value on the composite gait score score) included a shorter stride length, larger percent swing stride, smaller paw area at peak stance, and greater variability of paw area and paw angle. Gait scores similarly recovered over time. Renal insufficiency did not influence limb function. Plotted values represent mean ± SEM. B = pre-operative baseline.
Figure 7: Correlation Analysis. Correlation matrix of outcomes for the 2-week CKD_AVF mice. Impairments in hindlimb perfusion, limb function, and mitochondrial function, as well as histological assessment of muscle injury, correlated well with each other. Fistula hemodynamics and renal function only weakly matched these trends.

Supplemental Figure 1: 2-week AVF Flow Dynamics. (A-F) Ultrasound measurements of aortoiliac velocities and vessel diameters. AVF cohorts had increased aortic diameter (A), aortic PSV (B), aortic EDV (C), aortic flow rate (D), IVC diameter (E), and IVC peak velocity (F) compared to their surgical controls (Sham). Values increased throughout the 2-week post-operative recovery period. Renal insufficiency did not influence flow-dynamics. Plotted values represent mean ± standard error (SEM). B = pre-operative baseline.

Supplemental Figure 2: 6-week AVF Flow Dynamics. (A-F) Ultrasound measurements of aortoiliac velocities and vessel diameters for 6-week cohorts. Due to trend differences, post-operative weeks 1-3 and weeks 4-6 were analyzed separately. Aortic (A) and IVC (E) diameters increased during the first three post-operative weeks before plateauing. After an initial increase, velocity measurements (B, C, F) attenuated during weeks 4-6. Together, these trends caused aortic flow (D) to peak around week 3. Plotted values represent mean ± SEM. B = pre-operative baseline.
Supplemental Figure 3: 6-week Mitochondrial Bioenergetics. (A&B) Mitochondrial respiratory capacity and electron leak at variable levels of energy demand. Differences were not significant. Plotted mitochondrial respiration and electron leak values represent mean ± SEM. ns = not significant.

Supplemental Figure 4: 2-week Muscle Histology. Muscle mass (A-C) and muscle histological characteristics (D-O) for three different hindlimb muscles: tibialis anterior (TA), extensor digitorum longus (EDL), and soleus (SOL). Histology outcomes included myofiber cross sectional area (CSA) (D-F), number of myofiber capillary contacts (CC) (G-I), percentage of fibers with centrally located nuclei (CNF) (J-L), and percentage of fibrosis (M-O). No significant differences were found comparing AVF to Sham or CKD to Control unless otherwise indicated. *p ≤ 0.05.

Supplemental Figure 5: 6-week Muscle Histology. Muscle mass (A-C) and muscle histological characteristics (D-O) for 6-week cohorts. No significant differences were found comparing AVF to Sham unless otherwise indicated. *p ≤ 0.05, ** p < 0.01.