Effects of Chronic Kidney Disease and Exercise on Musculoskeletal and Cardiovascular Health

By

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Declaration

I declare that this dissertation is my own work. It is being submitted for the degree of Doctor of Philosophy at the University of California, Davis.

It has not been submitted for any degree or examination at any another University.

Acknowledgements

At the ripe age of thirty, with zero hair remaining on my head, and in yielding defiance of imminent sarcopenia, I am quite excited to finally be finishing my scholastic career. From Julie Elias' preschool to the Keith Baar's lab at UC Davis, it has been a long but fulfilling 27 years. Without a doubt, I would not be here if I had not been the most fortunate person I have ever met, having constantly been surrounded by amazing family, friends, teachers, and mentors.

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Abstract

Chronic kidney disease (CKD) is a debilitating condition characterized by a gradual decline in kidney function, resulting in decreased glomerular filtration, increased permeability of the filtration barrier, and deficits in secondary renal functions such as vitamin D activation and erythropoietin production. This functional decline leads to secondary complications, including the accumulation of uremic metabolites, chronic acidosis, systemic inflammation, vitamin D deficiency, and low hematocrit. Through these mechanisms, CKD exerts profound systemic effects on extrarenal tissues, particularly within the musculoskeletal and cardiovascular systems. This dissertation explores the specific effects of CKD on tendon, bone, skeletal muscle, and cardiac muscle, and evaluates the efficacy of uphill treadmill exercise as a therapeutic intervention using an adenine-diet-induced CKD rat model. We report the first experimental evidence of tendon dysfunction in CKD, providing a novel platform for mechanistic research. Furthermore, we found that while chronic treadmill exercise successfully maintained skeletal muscle mass, it exhibited limited effects on bone structure and function. Notably, exercise led to weaker tendons in CKD animals, further suggesting that renal disease fundamentally impairs tendon remodeling responses. Finally, CKD completely blunted exercise-induced cardiac hypertrophy in treadmill-trained rats, a maladaptation that appears driven by altered protein turnover. Collectively, this work elucidates the tissue-specific impacts of exercise in the context of CKD, highlighting the underlying mechanisms driving these responses and identifying critical targets for future therapeutic interventions.

Table of Contents

Chapter 1: Exercise Interventions to Improve Clinical Outcomes in Non-dialysis Chronic Kidney Disease: State of the Literature

This chapter was adapted from a previously published review.
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Abstract

Exercise interventions in chronic kidney disease (CKD) have received growing interest, with over 30 meta-analyses published in the past five years. The potential benefits of exercise training in CKD range from slowing disease progression to improving comorbidities and quality of life. Still, there is a lack of large, randomized control trials in diverse populations, particularly regarding exercise in non-dialysis-dependent CKD (NDD). When exercise interventions are implemented, they often lack fundamental features of exercise training such as progressive overload, personalization, and specificity. Furthermore, the physiology of exercise and CKD-specific barriers appear poorly understood. This review explores the potential benefits of exercise training in NDD, draws lessons from previous interventions and other fields, and provides several basic tools that may help improve interventions in research and practice.

Introduction

Structured exercise is a promising form of intervention in chronic kidney disease (CKD; Figure 1) and has been added to expert clinical care recommendations[1]. Research on the effects of exercise training in CKD has mainly focused on dialysis patients, with at least 20 meta-analyses (supplementary table S1) and an umbrella review[2] published since 2018. Exercise training in non-dialysis-dependent CKD patients (NDD), who differ in both clinical care and physiological status, has received relatively less attention. Still, there have been over a dozen recent meta-analyses on the effects of exercise training on various clinical outcomes in NDD (Table 1).

While systematic reviews represent the highest quality of research on the evidence pyramid, these analyses often report conflicting or null results, and much remains to be learned from well-designed interventional studies. This narrative review will discuss the results of recent meta-analyses, draw lessons from the literature, and direct the reader to other relevant manuscripts and resources. For a similar review in dialysis patients, see the recent review by Thompson and colleagues[3] and for a quantitative review of exercise across all stages of CKD the clinical practice guidelines published in 2022 by Baker et al.[1].

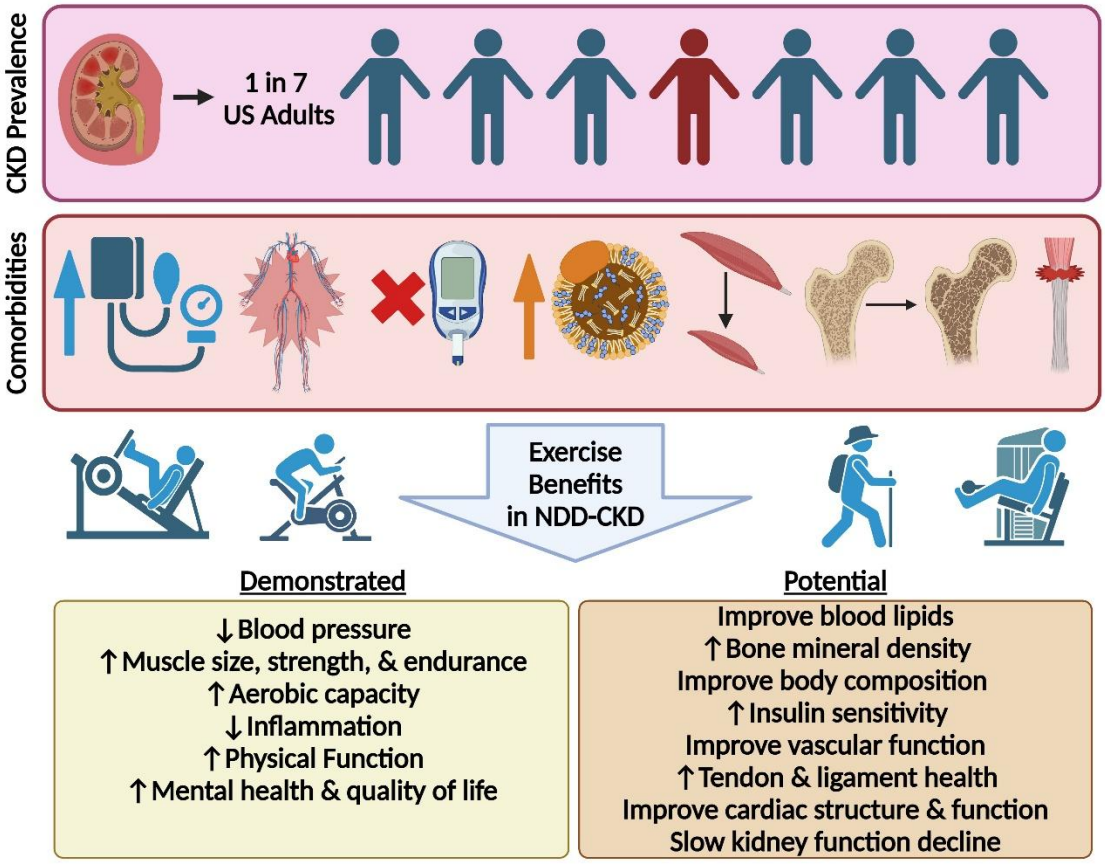

Figure 1. Exercise helps combat pathology in non-dialysis chronic kidney disease. More than 1 in 7 US adults are estimated to have chronic kidney disease (CKD) and as many as 9 in 10 are unaware of their condition[4]. Comorbidities are extremely common in CKD, specifically high blood pressure[5], cardiovascular disease[6], diabetes[7], dyslipidemia[8], muscle wasting[9], bone abnormalities[10], and potentially increased susceptibility to tendon injury[11,12]. The coincidence of such broad-ranging pathologies is likely due to the vast physiological roles of the renal system including the excretion of metabolic waste, termination of humoral signaling, regulation of blood pressure and volume, endocrine signaling, preservation of acid-base and electrolyte balance, maintenance of hematocrit levels, and involvement in the calcium-parathyroid hormone-vitamin D axis. Unsurprisingly, populations with CKD report lower health-related quality of life (HRQL)[13] and have higher rates of hospitalization and mortality compared to healthy populations[14]. Although individuals with non-dialysis dependent (NDD) CKD also often exhibit poor exercise capacity[15], engagement in regular aerobic and resistance exercise can greatly improve health and well-being in this population as covered in this review. Created with BioRender.com.

Study	CKD Stage	Exercise Types (Durations)	Evaluated Outcomes with Significant Effects[#] (effect size, 95% Confidence Intervals, # of studies)
Baião et al., 2023[16]	1-5	AT and/or RT (8-96wk)	**IL-6** (-0.64 SMD^{BP}, −1.01 to −0.27, n=5)
Ferreira et al., 2021[17]	1-3 and HD	AT+RT, guided breathing, Pilates (8-48wk)	**Depression** (-0.66 SMD^{BP}, -1.00 to -0.33, n=3), **anxiety** (-0.78 SMD^{BP}, -1.21 to -0.24, n=2)
Junqué-Jiménenz et al., 2022[18]	3-5	Home-based AT or AT+RT (12-52wk)	**6MWT** (44.8m^{BP}, 30.6 to 59.0, n=5), **sit-to-stand test time** (-0.45 SMD^{BP}, -0.64 to -0.26, n=3), **timed up and go** (-0.77s^{BP}, -1.38 to -0.16, n=2), **SF-36 role physical** (15.62^{BP}, 5.55 to 25.69, n=3) , **SF-36 general health** (8.42^{BP}, 2.36 to 14.47, n=3), **HAD scale depression** (-1.88^{BP}, -3.05 to -0.71, n=1)
Nakamura et al., 2020[19]	1-5	AT and/or RT (8-78wk)	**Aerobic capacity*** (2.75 mL/kg/min^{BP}, 1.73 to 3.76, n=4), **timed up and go** (-0.72s^{BP}, -1.21 to -0.24, n=3)
Neale et al., 2023[20]	1-5	Unspecified (12-78wk)	None
Pei et al., 2019[21]	2-5	AT (10-52wk)	**Aerobic capacity** (2.08mL/kg/min^{BC}, 1.1 to 3.05, n=17), **exercise duration** (155s^{BC}, 86 to 225, n=6), **HDL** (3.54mg/dL^{BC}, 0.43 to 6.65, n=6), **SF-36 pain** (5.94^{BC}, 1.65 to 10.23, n=6)
Thompson et al., 2019[22]	NDD 3-5	AT, AT+RT, or Tai Chi (12-156wk)	**Resting SBP after 12-16 weeks** (-4.9mmHg^{BP}, -8.8 to -1.0, n=8), **after 24-26 weeks** (-10.9mmHg^{BP}, -15.8 to -6.1, n=4), **resting DBP after 24-26 weeks** (-6.2 mmHg^{BP}, -10.9 to -1.5, n=4), **24h ambulatory SBP** (-18.0mmHg^{BP}, -29.9 to -6.1, n=1), **24h ambulatory DBP** (-9.0 mmHg^{BP}, -17.7 to -0.29, n=1)
Villanego et al., 2020[23]	NDD 1-5	AT and/or RT (12-52wk)	**Hemoglobin** (0.3 SMD^{BC}, 0.1 to 0.5, n=4), **aerobic capacity** (2.66mL/kg/min^{BC}, 1.61 to 3.71, n=9), **6MWT** (56.6m^{BC}, 28.9 to 84.3, n=6), **bicep curl repetitions** (6.8^{BC}, 4.9 to 8.6, n=2), **BMI** (-0.89kg/$m^{2\ BC}$, -1.47 to -0.31, n=10), **waist circumference** (-3.33cm^{BC}, -5.95 to -0.72, n=5)
Wu et al., 2020[24]	NDD 1-5	AT + RT (5-52wk)	**eGFR** (5.01mL/min/1.73$m^{2\ BP}$, 2.37 to 7.65, n=6; 3.01 mL/min/1.73$m^{2\ WC}$, 0.86 to 5.16, n=9), **serum creatinine** (-8.57µmol/L^{BP}, -13.71 to -3.43 n=3; -6.33µmol/L^{WC}, -10.23 to -2.44, n=5), **aerobic capacity** (0.55L/min^{BP}, 0.31 to 0.80, n=2), **SBP** (-5.2mmHg^{WC}, -7.9 to -2.5, n=7), **DBP** (-3.6mmHg^{WC}, -5.4 to -1.9, n=6)
Wu et al., 2022[25]	NDD 1-5	AT and/or RT (12-52wk)	**BMI** (-0.77kg/$m^{2\ BC}$, -1.31 to -0.23, n=14), **waist circumference** (-3.11cm^{BC}, -5.25 to -0.97, n=5), **body weight when BMI > 25** (-2.18kg^{BC}, -3.81 to -0.54, n=9), **when intervention > 48 weeks** (-2.52kg^{BC}, -4.28 to -0.77, n=5)
Wyngaert et al., 2018[26]	3-4	AT or AT+RT (12-52wk)	**eGFR** (2.16mL/min/1.73$m^{2\ BC}$, 0.18 to 4.13, n=10), **SBP** (-5.2mmHg^{WC}, -9.4 to -1.0, n=8), **aerobic capacity** (2.39mL/kg/min^{BP}, 0.99 to 3.79, n=11; 1.70mL/kg/min^{WC}, 0.65 to 2.74, n=11)
Yang et al., 2020[27]	1-5	AT or AT+RT (12-56wk)	**Urinary albumin-to-creatinine ratio** (0.21 SMD^{WC}, 0.04 to 0.38, n=7)
Zhang et al., 2019[28]	NDD 2-5	AT and/or RT (6-52wk)	**eGFR** (2.62mL/min/1.73$m^{2\ BC}$, 0.42 to 4.82, n=12), **SBP** (-5.6mmHg^{BC}, -9 to -2.2, n=9), **DBP** (-2.9mmHg^{BC}, -3.7 to -2.1, n=8), **total cholesterol after interventions^{T} < 6 months** (14.6mg/dL^{BC}, 3.8 to 25.5, n=7), **triglycerides after interventions < 3 months** (-94.8mg/dL^{BP}, -148.8 to -40.9, n=2), after **interventions > 3 months^{T}** (45.9mg/dL^{BP}, 17.8 to 73.9, n=4), **BMI** (-1.32 kg/$m^{2\ BP}$, -2.39 to -0.25, n=9),

Table 1. Significant effects of exercise interventions on clinical outcomes in chronic kidney disease from recent meta-analyses. Analyses differed in methodology such as comparison type (e.g., within-group vs. between-group), modeling (fixed vs. random effects), etc. Please see corresponding studies for caveats, estimates of clinical significance, and further information when interpreting. CKD, chronic kidney disease; AT, aerobic training; RT, resistance training; IL, interleukin; HD, Hemodialysis; SMD, standard mean difference; 6MWT, six-minute walk test; SF-36, Short Form Health Survey for evaluating health-related quality of life; HAD, Hospital Anxiety and Depression; HDL, high-density lipoprotein; NDD, non-dialysis-dependent CKD; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; eGFR, estimated glomerular filtration rate. [#] $p \leq 0.05$ and a heterogeneity $I^2 \leq 50\%$ when reported. [BP] Between group analysis of post-intervention values, [BC] Between group analysis of the change elicited by intervention. [WC] Within group analysis of the change elicited by intervention. *By subgroup analysis including only center-based exercise studies. [T]Effect in a detrimental direction.

Requirements for inducing exercise training adaptations

Exercise as medicine is a paradigm that has been around for hundreds if not thousands of years[29], but implementation in clinical practice has lagged behind[30]. Basic requirements for inducing training adaptations, such as *progressive overload, personalization, and specificity*[31,32], are briefly covered here to facilitate our discussion.

Exercise provides a stimulus to the body by stressing a system (cardiovascular, musculoskeletal, etc.) to a degree greater than it is accustomed to. Although there is an acute response to this *"overload"* from a single bout of exercise, adaptation requires consistently repeated bouts, known as exercise training. The effects of exercise training are then the sum of the responses to single exercise bouts. As the body adapts, exercise capacity improves, and the amount of stimulus needed to overload the system increases. As a result, exercise training must continually increase in frequency, intensity, volume, or duration (aka *progressive overload*) until the desired physiological outcome is attained.

Individuals with worse baseline status often show the greatest improvements in response to exercise[33]. However, the stimulus needed for large improvements in low-fit individuals would be insufficient to overload those with greater baseline fitness. Thus, a one-size-fits-all intervention is not appropriate for exercise studies. Training programs must be *personalized* to individuals by modulating frequency, intensity, time, type, volume, and progression (FITT-

VP)[34], ideally using the guidance of physiological[35] or performance markers[36]. One approach to optimizing personalization is the *"needs analysis"*. Borrowed from the field of sports performance, a *needs analysis* is a systematic process for determining the difference between the current state and a goal state to guide intervention design. In Figure 2, we present a *needs analysis* guide adapted from Scroggs and Simonson[37] for use with CKD patients.

Much like traditional medicine, the benefits of exercise are dependent on specific physiological mechanisms, however, these are often overlooked. The *principle of specificity*, states that training adaptations are specific to the elicited stimulus[38]. On a macro scale, specificity can be very simple; if improved sit-to-stand performance is desired, then the muscles involved in sit-to-stand should be exercised in a similar pattern and duration as required by the test. However, specificity becomes more complicated when trying to elicit adaptations such as decreasing chronic inflammation in NDD. It is thus imperative to know the physiological signals, adaptive mechanisms, best training stimulus, and CKD-related pathological barriers that may impede these processes. With this in mind, we have created a specificity chart for several commonly desired adaptations (Fig. 3; additional resources table S2).

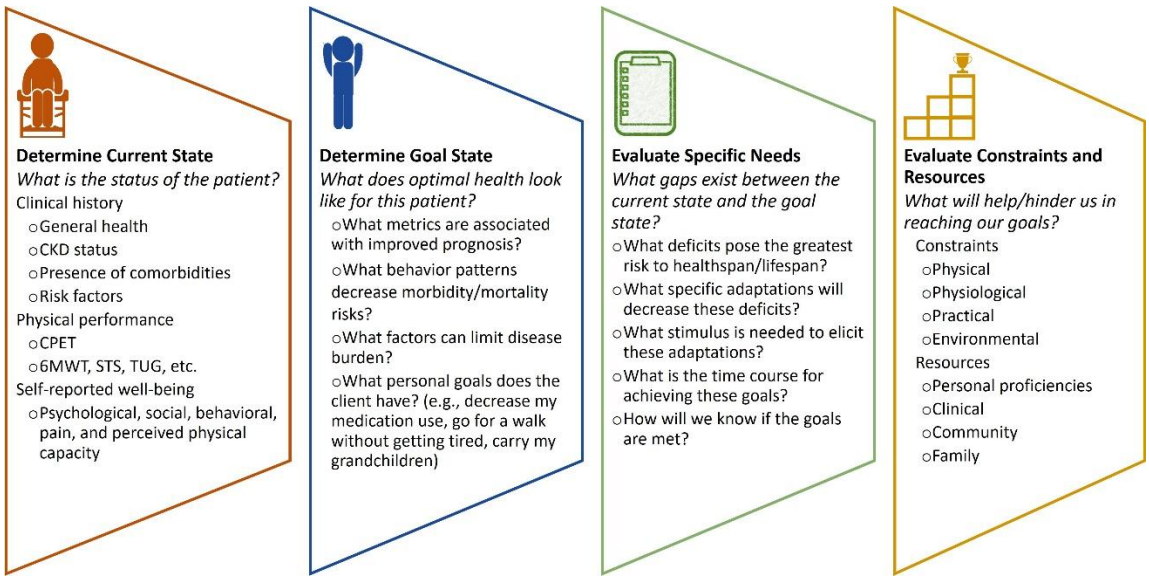

Figure 2. Needs analysis guide for developing exercise interventions for chronic kidney disease patients. Interventions should start with a comprehensive evaluation of patient status. This is followed by the determination of what optimal health looks like incorporating the desires of both the practitioner and the patient. Next, "needs" can be identified, where a need is the gap between the patient's status and the goal state. Needs should then be prioritized - as it may not be possible to give each one equal attention. Finally, the patient and practitioner should work together to evaluate potential constraints and resources that will impact progress. With this needs analysis the practitioner can then design an intervention that targets an individual's deficits while accounting for their physical limitations and constraints and leveraging their resources. CKD, chronic kidney disease; 6MWT, 6-minute walk test; STS, sit-to-stand; TUG, timed up and go; CPET, cardiopulmonary exercise test.

Challenges to Successful Exercise Interventions

Exercise adaptations are notoriously variable in healthy individuals[39]. Although these varied responses can be due to genetic differences, they are often the result of inadequate consistency in effort, intensity, and adherence across individuals or differences in baseline status[33,40,41]. The pathological state that accompanies CKD, however, clearly creates further physical, physiological, and practical hurdles to exercise adaptations.

Physical Challenges

Physical challenges are defined here as factors that make the act of regularly exercising more difficult. Individuals with CKD are afflicted with muscle wasting[9,42–44], neurocirculatory dysregulation[45], pulmonary dysfunction[46], and a lower aerobic capacity[47,48], all likely leading to decreased physical function[49] and exercise intolerance[50]. In focus groups of non-exercising patients with stage 3-4 CKD, fatigue is the most common self-reported barrier to exercise[51]. Perceived fatigue is higher in NDD patients than in healthy controls even in the absence of greater muscle fatigue (i.e., a decline in strength with prolonged contractions)[52]. Still, there is growing evidence of impaired muscle energetic function in CKD[53] that may contribute to physical limitations. This impairment is evident even during submaximal exercise whether[54,55] or not[56,57] an oxygen delivery limitation also exists and may be due to a build-up of harmful serum metabolites such as kynurenine[58,59]. Compared to healthy controls, individuals with NDD demonstrate a lower muscle oxidative

capacity, which is strongly associated with poorer six-minute walk test (6MWT) performance[60,61]. NDD also leads to greater mitochondrial uncoupling at rest[61,62], which increases the amount of oxygen needed to generate energy. Greater uncoupling during exercise would cause increased oxygen consumption, which has been associated with slower walking speeds and greater fatigability in older adults[63,64].

Across patients with CKD, decrements in muscle mass[65], physical function[66], aerobic[67] and exercise capacity[68] increase with disease severity. Other factors such as the degree of bicarbonate deficiency or metabolic acidosis further contribute to impairments in muscle oxidative capacity[61], muscle endurance, and exercise blood pressure regulation[69]. Mobility impairment[70] and musculoskeletal pain[71] are also common, which may make certain exercises more difficult. Thus many disease-related complications may contribute to decreased physical capacity in CKD. Designing interventions that stimulate exercise adaptation with minimal duration may help limit fatigue-related deterrence. For example, just two 14-minute sessions of HIIT per week for 12 weeks was sufficient to increase absolute aerobic capacity by ~10% in severely obese non-CKD individuals[72]. Due to the significant heterogeneity of CKD symptoms[73], blanket implementation of exercise programs will likely lead to inconsistent adherence and personalization may need to be valued over standardization. Providing modified or alternative exercises and pain management strategies may keep this from impeding participation. Because the physical capacity to perform exercise is an inherent prerequisite for training, interventions in CKD may need to be initiated at intensities that would normally be considered suboptimal for health benefits and progressed over time.

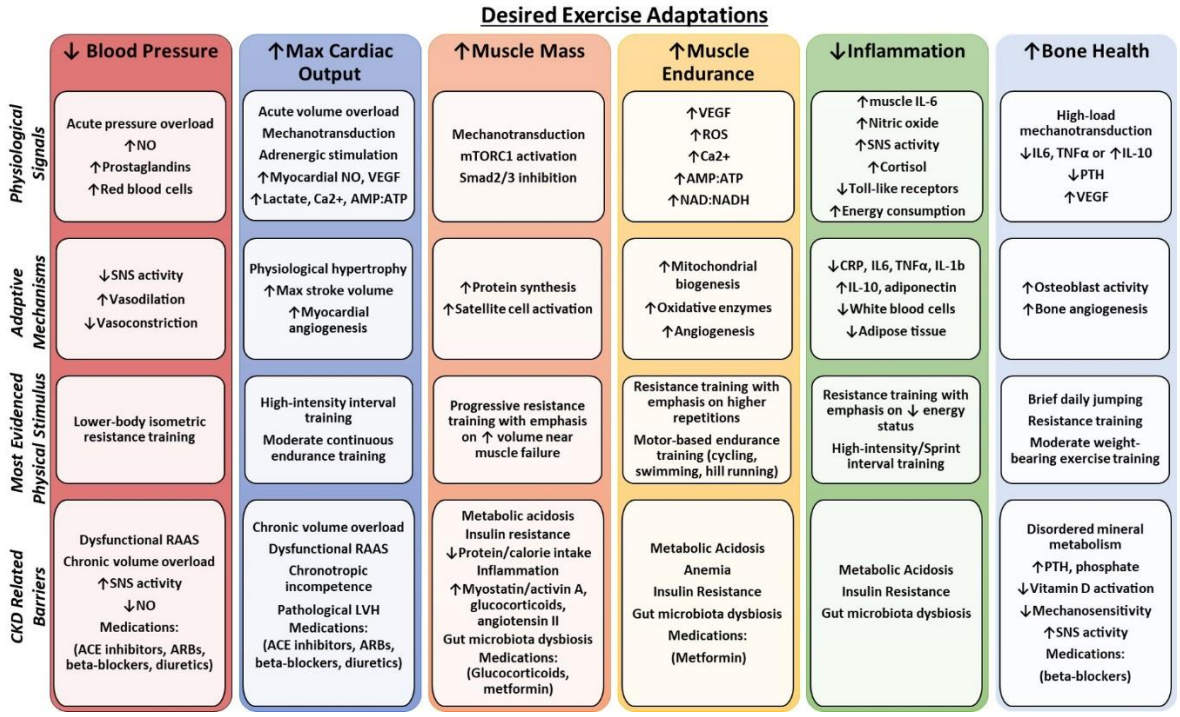

Figure 3. Specificity chart template for organizing important factors for exercise prescription in chronic kidney disease. This chart is not meant to be absolute or comprehensive but can serve as a template that can be edited and added to in conjunction with the *needs analysis.* For more information regarding exercise adaptations and their molecular signals see recent reviews by Dent[74], Egan[75], and McGee[76] and their colleagues. SNS, sympathetic nervous system; RAAS, renin angiotensin aldosterone system; NO, nitric oxide; ACE, angiotensin converting enzyme; ARBs, angiotensin receptor blockers; VEGF, vascular endothelial growth factor; IGF, insulin-like growth factor; AMP, adenosine mono-phosphate; ATP, adenosine tri-phosphate; LVH, left ventricular hypertrophy; mTORC, mammalian target of rapamycin complex; ROS, reactive oxygen species; NAD, nicotinamide adenine dinucleotide; IL, interleukin; CRP, c-reactive protein; TNF, tumor necrosis factor; PTH, parathyroid hormone. Additional resources can be found in supplementary material table S2.

Physiological Challenges

Physiological challenges include CKD features that have the potential to impair the adaptative responses to exercise training - chronic uremia, metabolic acidosis, inflammation, insulin resistance, electrolyte imbalance, volume overload, endothelial dysfunction, low hematocrit, acid-base and mineral disturbances, etc. In general, these can be separated into mechanisms that may inhibit aerobic or resistance training adaptations.

Currently, there is insufficient evidence to determine whether CKD blunts aerobic training adaptations, though cardiovascular limitations seem likely (see *Aerobic capacity* below).

Kirkman and colleagues found that NDD patients (mean±SD estimated glomerular filtration rate, eGFR=44±12 ml/min/1.73m^2) obtained only half of the increase in aerobic capacity reported in the general aging population in response to 12 weeks of aerobic training[77]. However, this trial used control data from separate studies and thus could not account for training effort, intensity, adherence, or baseline status and medication use. Evidence of blunted molecular responses to aerobic exercise that may impact adaptation is scarce. One study of muscle gene expression responses to a single bout of cycling found similar, but less pronounced, mRNA changes in end-stage CKD patients compared to healthy individuals ($r=0.79$ $p<0.01$). However, this was a small study (n=5) that also relied on external control data, making consistency hard to verify[78]. Another study found one microRNA (miR-146a; associated with inflammation) that exhibited a varying response to maximal exercise between healthy and CKD (eGFR=46±23) subjects but the relevance of this finding remains unclear[79].

Limited data suggest muscle hypertrophic signaling may be blunted by CKD, but impaired muscular adaptation has not been directly shown in humans. Aberrant molecular signaling in CKD leads to altered protein turnover and muscle wasting, purportedly due to chronic inflammation, metabolic acidosis, elevated glucocorticoids, and other mechanisms that have been extensively reviewed[42–44,80–82]. Many proposed mechanisms involve insulin/IGF-1 resistance and impaired IRS-1/PI3K/Akt signaling which are upstream of one of the main muscle hypertrophy pathways, mTORC1[83,84]. In animal models of CKD basal deficiencies in IRS-1/PI3K/Akt signaling, increased protein degradation, and decreased protein synthesis have been reported[85,86]. However, 7 days of chronic muscle overload in 5/6-nephrectomized rats has been reported to fully activate IRS-1/PI3K/Akt pathways, resulting in hypertrophy similar to controls[85]. Although a deeper evaluation of these data reveals remaining deficiencies in

mTORC1 and IRS-1 phosphorylation and questionable responses of total IRS-1 compared to other reports[87]. A similar study by Wang and colleagues found that chronic muscle overload improved but did not completely rescue muscle size and hypertrophy signaling in 5/6-nephrectomized mice[86]. While these data demonstrate the potential for impaired resistance training adaptation, one of the only human training studies that compared NDD (GFR=17±5) to healthy controls found similar increases in quadriceps strength (~2.4-fold) and endurance (~1.5-fold) between groups [88].

Practical Challenges

Practical barriers to exercise in CKD include insufficient funding for renal exercise programs, a lack of renal education opportunities for the public and practitioners, and poor accessibility of exercise equipment[89]. Patients with NDD in particular do not benefit from the greater presence of exercise professionals and programs that have fortunately been implemented in many hemodialysis clinics[90]. Well-designed home-based exercise programs may help circumvent some of these challenges. For example, Sian and colleagues elicited improvements in cardiorespiratory fitness, exercise tolerance, blood pressure, and cholesterol through just 4 weeks of unsupervised home-based high-intensity interval training in a general older adult population[91]. Additionally, a small meta-analysis (n=8 trials) of home-based exercise in CKD shows limited but consistent evidence of improvements in physical function and self-reported health metrics[18]. Given the complexities of exercise prescription in special populations, many kidney health providers feel inadequately trained to advise patients on physical exercise[92]. The Global Renal Exercise Network (GREX, https://grexercise.kch.illinois.edu/) suggests that increasing the number of exercise professionals in renal care programs is the most effective strategy for removing exercise barriers in CKD[89]. Additionally, there is also a disproportionate

burden of CKD on low-resourced and minority communities[93], requiring investigators and practitioners to address disparities in access to education, nutrition, exercise equipment, and healthcare to improve outcomes equitably. This includes a special emphasis on enrolling participants from these underrepresented groups into clinical trials to ensure the generalizability of findings.

Results and lessons from previous interventions

Despite the growing acceptance of exercise as an effective intervention in chronic kidney disease[94] and over 20 years of related research[95], there is still a lack of large, randomized control trials investigating the benefits of exercise in NDD. The 13 meta-analyses in Table 1 collectively cited only ~47 unique trials in NDD, most of which involved fewer than 50 participants. As such, there is a paucity of data to draw strong conclusions from. Furthermore, across meta-analyses, there is inconsistent use of post-intervention values versus change from baseline for calculating effect size (ES) - likely increasing variability in the findings. Given the challenges of exercise research, it may be advantageous to view even inconsistently demonstrated outcomes as potential benefits that can be obtained with adequate interventional design. With this in mind, we cover here a range of the purported benefits of exercise in NDD, though they differ in strength of evidence.

Physical function

Impaired physical function is common in CKD and is associated with worse clinical outcomes including increased risk of cardiovascular disease and mortality[49,70,96–98]. In NDD specifically, a meta-analysis by Ribeiro and colleagues found that low physical performance in NDD results in a mortality hazard ratio of 2.04[99]. Of the meta-analyses in Table 1, exercise training was found to improve 6MWT distance in two of four analyses (ES=44.8m, and 56.6m),

timed up-and-go in 2 of 2 (ES=-0.77s and -0.72s), and sit-to-stand in one of three (-0.45 standardized mean difference, SMD). When the trials used in these meta-analyses are evaluated individually, improvements in 6MWT, Sit-To-Stand, and Timed Up-and-go are more consistently shown[100–105]. At least seven studies[100–106] have demonstrated changes in 6MWT distances greater than the minimally clinically significant change of approximately 30 meters suggested for older adults with pathologies or pulmonary hypertension[107,108]. Two of these studies also found significant improvements in self-reported physical function and perception of energy/fatigue[100,103], supporting the use of these tests as metrics of overall physical well-being. Furthermore, in a retrospective longitudinal study of CKD patients (eGFR=30±27), improvement in an incremental shuttle walking test greater than 50 meters was related to a significantly lower risk of morbidity and mortality compared to "non-improvers" and unexercised controls[109]. Together the currently available data suggest that exercise can improve physical function which may lead to better long-term patient outcomes, and thus is a worthwhile target for intervention.

Aerobic capacity

Aerobic capacity is the maximal amount of oxygen (O_2) an individual can intake, deliver, and utilize during exercise. Individuals with CKD have lower aerobic capacity than healthy controls[48] which worsens with disease progression[47] and is associated with increased cardiovascular burden[110] and mortality[111]. Five of five meta-analyses in Table 1 found a significant effect of exercise training on aerobic capacity (ES=2.08 to 2.75mL/kg/min)[19,21,23,24,26]. Improvements in aerobic capacity as little as 6% have been suggested to be clinically significant[26,112,113] and a critical cut point of 17.5mL/kg/min has been shown to predict mortality[111]. One of the more impressive improvements in aerobic

capacity in NDD (eGFR=38±13), an increase of 5.8mL/kg/min, was elicited by Van Craenenbroeck et al. through three months of cycling for four daily 10-min sessions at 90% of anaerobic threshold heart rate[114]. The volume of this intervention (40min daily) is significantly greater than most other interventions (30min 2-3 days/week), likely leading to a greater effect. Whether the use of multiple shorter exercise sessions across the day in this study impacted the results is not clear, but the efficacy of similar, brief albeit vigorous, exercise has been explored in other trials and warrants consideration[115,116]. No specific aerobic exercise program has demonstrated superiority for increasing aerobic capacity and several meta-analyses suggest practically equivocal effects of moderate-intensity continuous and high-intensity interval training in general and overweight/obese populations[117,118]. Due to its complexity and interaction with kidney disease, we discuss the physiology of aerobic capacity in more detail here.

Aerobic capacity is generally considered to be limited by cardiovascular function (O_2 delivery) and improvements with training are attributed to increases in maximal cardiac output[119,120]. This is because in healthy individuals the oxidative capacity (maximal O_2 consumption) of muscle normally exceeds oxygen delivery during whole-body exercise[120,121]. In order to improve aerobic capacity in NDD, it is important to understand how the disease could impair both oxygen delivery and use[30]. In a cross-sectional study, Wallin et al. found that stroke volume, peak heart rate, and hemoglobin concentration are lower in NDD compared to controls and concluded that oxygen delivery is the main determinant of aerobic capacity decline with disease progression[68]. Wallin's study, however, did not include measures of oxygen utilization needed to evaluate the role of peripheral oxygen use, such as arterial venous O_2 difference (AVO_2D). In a similar evaluation, Chinnappa and colleagues found

that AVO_2D was a better predictor of aerobic capacity in NDD (R=0.78) compared to cardiac output (R=0.74)[122]. They further suggested that in NDD aerobic capacity reflects the ability of skeletal muscle to extract oxygen and not cardiovascular function. This claim is surprising given that in their data peak cardiac output mirrored the aerobic capacity decline with disease status. Additionally, their study showed no difference in peak AVO_2D between the NDD and healthy control groups, which does not support a muscular impairment. While AVO_2D did explain more of the variance in aerobic capacity in NDD (R^2=0.61) compared to healthy controls (R^2=0.38) or heart failure patients (R^2=0.32), this difference may instead be attributed to the varying degrees of anemia in CKD.

Several factors contribute to oxygen delivery limitations in NDD. Maximal heart rate, which is generally not modifiable through exercise training, is classically decreased in CKD[123]. This deficit resolves within two months of kidney transplant[124], suggesting the uremic milieu may blunt the cardiac adrenergic response[125]. Training-induced increases in plasma volume and hematocrit are two major adaptations that improve cardiac output and oxygen delivery and thus aerobic capacity[40,126]. Patients with CKD, however, commonly suffer from chronic plasma volume expansion without compensatory increases in hematocrit[127], which could restrict their adaptive ability. The prevalence of low hematocrit progressively increases from 8% to 53% across CKD stages 1-5[128], likely due to declines in erythropoietin production. In dialysis patients, normalizing hematocrit (increasing the oxygen-carrying capacity of the blood) improves aerobic capacity, both alone[55,129] and when combined with exercise[55,95]. However, improvement in hematocrit even to normal levels with these agents does not restore aerobic capacity to the level of healthy controls[55,95]. Furthermore, caution must be taken with the use

of exogenous erythropoiesis-stimulating agents to improve hematocrit as they significantly increase the risk of thrombovascular events and mortality[130].

In summary, impaired oxygen delivery in NDD likely limits aerobic capacity, however, the impact of impaired muscle oxygen use is unclear[68,122]. Furthermore, several physiological factors in NDD may prevent exercise training-induced improvements in aerobic capacity, and methods for overcoming these are limited. Still, there is consistent evidence that exercise training can lead to clinically meaningful improvements in aerobic capacity in NDD[19,21,23,24,26] and further research on optimizing interventions is warranted. Of note, evaluating changes in absolute (L/min) along with relative (mL/kg/min) values will help researchers isolate the effects of changes in physiological function from those of altered body composition.

Blood pressure

Hypertension is prevalent in CKD and is considered one of its main causes[4]. Of the seven meta-analyses in Table 1 evaluating blood pressure (BP), four found significant benefits of exercise training on resting systolic BP (ES= -4.9 to -10.9mmHg), and three on resting diastolic BP (ES=-2.9 to -6.2 mmHg)[22,24,26,28]. Although the effects of exercise training on BP are inconsistent and may vary with intervention duration[22], several of the analyzed studies demonstrate impressive BP reductions[100,101,131]. Aoike and colleagues found that home or center-based walking exercise three times per week for 24 weeks reduced systolic blood pressure by ~13-14mmHg, versus no change in the control group (eGFR~28±11)[100,101]. The intensity of this intervention was personalized using a heart rate monitor and cardiopulmonary exercise test results. Further, the workouts were progressed by increasing duration at weeks 4 and 8, potentially adding to their effectiveness. A study by Leehey and colleagues[131] utilized similar intervention methods resulting in a 17mmHg reduction in mean systolic BP (eGFR=44±36),

however, the non-exercise group saw a similar change - highlighting the importance of study run-ins to normalize standard of care. Other pitfalls that may lead to null findings include well-controlled BP at baseline[132,133], confounding medications, the use of an automated sphygmomanometer, and low study power[134,135]. Exercise as a means to improve BP is well-documented in other populations, with two meta-analyses of 93 and 270 trials concluding that isometric resistance training is the most effective mode[136,137]. The Edwards and colleagues analysis found mean reductions from baseline in resting SBP of 4.1, 4.5, 4.6, 6., and 8.2mmHg following high-intensity interval, aerobic, dynamic resistance, combined, and isometric resistance training respectively[136]. Both analyses also found greater effects in individuals with higher baseline BP. Thus, exercise training has the potential to improve BP in NDD, however, baseline BP and medication should be controlled for and the use of isometric exercises should be explored in exercise studies targeting BP.

Muscle strength, endurance, and size

Muscle strength is the maximal capacity to generate force and muscle endurance is the ability to sustain force. Low muscle strength and mass are prevalent in roughly 20% of NDD patients[138] and are associated with increased mortality (hazard ratio=1.46 and 1.38)[99]. Handgrip strength, knee extensor strength, and bicep curl repetitions were the only muscle-specific outcomes evaluated in the analyses in Table 1[18,19,23]. Maximal bicep curl repetitions (in 30s) alone was found to improve with training (ES=6.8 repetitions)[23] and was used as an indicator of muscle endurance in two studies[100,101]. However, unless participants were reaching failure prior to 30s this measure may more accurately reflect contraction speed and not endurance. Though this difference may seem trivial, the training methods for increasing endurance and contraction speed are different and such discrepancies in training/testing could

lead to erroneous null findings. In the meta-analyses of knee extensor[19] and handgrip strength[18], three of the five analyzed trials found significant improvements from baseline that were not seen in controls[139–141]. The two trials that failed to increase strength had a brief period (8-12 weeks) of supervised resistance training, followed by a much longer period of at-home training without a standardized plan or progression[110,142] (a fundamental component of training[31]). In contrast, one of the positive trials elicited 29-47% increases in upper and lower body strength through 12 weeks of progressive resistance training, despite patients (GFR=25) being on a low-protein diet, [141]. A major difference here was that all training was supervised by an exercise physiologist. Another study found that 8 weeks of supervised progressive resistance training resulted in a ~13% increase in isokinetic strength as well as significant increases in rectus femoris cross-sectional area and volume (eGFR=29, range[19-32])[140]. We found five resistance training studies with muscle outcomes not included in the analyses in Table 1. The three that implemented supervised in-center progressive resistance training showed robust changes in muscle size and strength in just 12 weeks[88,143,144]. The other two studies[145,146] used 12-month home-based interventions and only the one that was progressed remotely by a physiotherapist elicited significant (though modest) improvements in strength[145].

Muscle adaptations reflect the demands imposed by training. For example, to improve muscle endurance, exercise should involve repetitive or prolonged contractions to produce metabolic stress in the target muscle - a stimulus for endurance adaptations[147,148]. Cycling then, may be more effective than running at increasing leg muscle endurance because the muscles are used as a motor as opposed to a strut[149] imposing a greater energy demand. Although exercise training improves muscle strength, endurance, and size, each involves a

different combination of stimuli and adaptations that can be targeted through specific approaches. Collective analysis of resistance training interventions suggests that if muscle growth (hypertrophy) is the goal, then the volume of work near failure should be maximized; and, if increases in muscle strength are desired, training at higher loads is beneficial[150]. An important caveat regarding resistance training is that muscular adaptation only occurs in the utilized tissue (knee extension exercise will not increase handgrip strength)[151]. Factors released into serum during exercise, such as growth hormone and IGF, have not been shown to elicit hypertrophy or strength changes in unexercised muscle[152,153].

Overall, there is a shortage of resistance exercise research in the NDD population. While available meta-analyses do not identify a strong effect of exercise training on muscle function in NDD, a careful reading raises questions as to the validity of this conclusion. Results from a number of studies have shown that well-designed interventions can increase muscle strength and size in NDD in as few as 12 weeks[88,139–141,143,144]. Therefore, the design of a resistance training program (e.g., supervision and progression) appears more important than duration for eliciting adaptations. Training should be specifically chosen based on the desired adaptation and target muscles, as adaptations demonstrate mode[154] and location specificity[152,153]. In terms of muscle testing, most physical function tests involve multiple non-muscular components (balance, coordination, cardiovascular fitness, etc.) making them non-ideal for evaluating muscle function. Thus, care should be taken when selecting muscle outcome measures, suggestions for which can be found in articles from Beaudart[155] and Buckinx[156].

Inflammation and oxidative stress

Chronic inflammation is a well-accepted component of CKD[157] and is associated with mortality across all stages of the disease[158]. Exercise training has well-demonstrated anti-

inflammatory effects in other populations[159], and although there is an acute pro-inflammatory response to unaccustomed exercise in NDD, 8 weeks of training alleviates this response[160]. Only two meta-analyses[16,25] from Table 1 evaluated the effects of exercise training on inflammatory markers. One found no effect of exercise on IL-6 or C-reactive protein (CRP) levels in NDD[25] and the other found changes in IL-6 (ES=-0.64 SMD) but not CRP, IL-10 (anti-inflammatory), or tumor necrosis factor-α (TNF-α) in a subgroup analysis of NDD[16]. The latter study also found that resistance, but not aerobic or combined exercise, significantly decreased CRP and TNF-α and increased IL-10 in an analysis that pooled results from dialysis and NDD[16]. Interestingly one of the main suggested mechanisms for the anti-inflammatory effect of exercise is the transient elevation in IL-6 it causes, which purportedly triggers a post-exercise increase in IL-10 and suppression of TNF-α[159,161,162]. Muscle is responsible for the majority of this IL-6 spike during exercise, rising 1 to 100-fold depending on exercise type[163] and it is believed to be stimulated by sensors of a low-energy state[161,164]. It is thus unsurprising that exercise of greater intensity and duration is associated with greater IL-6 responses[163]. At least four exercise studies in NDD have shown convincing reductions in basal IL-6 (~2.2-4.2pg/mL) using resistance[165,166] or aerobic training[167,168]. In a similar vein, acute increases in oxidative stress with exercise are an important signal for exercise adaptions, and chronic exercise training can decrease oxidative stress[169]. Available studies in stage 3-4 CKD measuring the effects of exercise on oxidative stress in NDD show conflicting results with one finding a reduction in F2-isoprostane levels[168] and the other finding no effect[170]. Still, a recent meta-analysis containing mostly hemodialysis studies found that exercise training improves oxidative stress markers including malondialdehyde, advanced oxidation protein

products, superoxide dismutase, and F2-isoprostanes, making this an area worthy of further research.

Kidney Function

Four of nine meta-analyses in Table 1 found a significant effect of exercise training on at least one metric of kidney function[24,26–28]. The variation in methods of calculating effect sizes, study weighting, and grouping, and the large number of different metrics used do not instill confidence in these collective results. For example, the impact of exercise training on estimated GFR (eGFR) from a study by Leehey et al.[131] in stage 2-4 CKD is assigned an effect size of 1 by two analyses[23,28] and -2.41 by another[26]. The clinical relevance of these findings is also difficult to determine as it is dependent on the length of intervention and the expected rate of kidney function decline. Many individual studies have suggested that exercise can improve or slow kidney function decline in NDD patients[102,134,141,166,171–176]. Unfortunately, most studies do not evaluate the rate of renal function decline before intervention and lack the sample size to account for baseline variation. This makes it difficult to determine whether the observed improvement is real or an artifact of insufficient randomization. Furthermore, GFR is typically estimated, introducing other confounders such as the independent effects of exercise and muscle mass on creatinine levels; an issue which may be remedied through the use of cystatin C-based estimates[177]. More potentially convincing evidence comes from an ancillary analysis of the LIFE study, which included 1199 older adults, 66% of whom had an eGFR of less than 60mL/min/1.73m^2 (mean 54±17)[178]. This analysis found that a two-year exercise intervention led to roughly 0.5mL/min/1.73m^2 per year slower decline in eGFR compared to health education alone[178]. Mechanistically, it is easy to postulate that exercise may benefit kidney function indirectly by decreasing blood pressure and inflammation or improving diabetic symptoms.

Another potential mechanism is muscle-kidney crosstalk via muscle-secreted extracellular vesicles, growth factors, and myokines or exercise-induced cytokines ("exerkines")[179,180]. To date studies interrogating muscle-kidney crosstalk have only taken place in animal models where such pathways are easier to observe[181–183], but this area warrants further attention. Thus, while mechanistically plausible, the strength of the evidence for exercise-induced improvements in kidney function is modest due to limitations in the sample size and methodology of current studies. This sentiment is echoed and greatly expanded upon in a recent review by Davies et al. to which we refer the reader[184].

Adverse events

Five of the meta-analyses in Table 1 aggregated data on adverse events in analyzed trials[17–19,24,27]. Only one reported finding any adverse events related to exercise[19] and all reported events were from a singular trial (including several cases of hypotension due to weight loss, and one case each of chest pain while exercising, knee pain, Achilles pain, joint pain while exercising, and rapid atrial fibrillation with hospitalization)[168]. We have found no citations of exercise safety concerns specific to NDD and various forms of exercise including aerobic, resistance, and high-intensity interval training have been directly assessed by various literature and found to be safe[1,105,106,185,186]. Resources for performing exercise safely are provided by GREX[187,188] and the American College of Sports Medicine[34].

Gaps in Knowledge

Gaps exist in understanding exercise's effect on bone health, optimal exercise dosing, and the use of nutritional and pharmacologic therapeutics to improve exercise adaptation. We found only one study on exercise and bone health in NDD (eGFR=27±11), which showed no benefit of a 24-week walking program on serum bone metabolism markers, likely due to the low

mechanical load[189]. Still, exercise represents a promising therapy for combating bone dysfunction when impact exercise or resistance training is used[190–193]. Another interesting area that needs evaluation is the minimum effective dose of exercise, as decreasing exercise frequency or duration may help increase adherence. One of the few studies on exercise dosing showed that after 12 weeks, resistance training for one versus three sessions per week resulted in equal improvements in isometric strength, physical function, and self-reported uremic symptoms in stage 3 CKD[143]. Only muscle cross-sectional area and pennation increased more with greater frequency. Larger, longer-duration interventions are needed.

Strategies to enhance the effect of limited protein intake on exercise-induced anabolism such as maximizing essential amino acid content[194], supplementing with surplus ketoanalogues[195], and optimizing protein timing[196] lack evidence in NDD and warrant investigation. Supplementing with sodium bicarbonate (to decrease acidosis[197,198]), dietary nitrate (to improve exercise efficiency[199]), iron (to improve muscle function[200]), vitamin D (to promote musculoskeletal[201] and cardiovascular function[202]), and coenzyme-Q and nicotinamide riboside (to combat mitochondrial dysfunction[203]) has received some attention in the CKD literature, but requires further research. Creatine, which may become a conditionally essential nutrient in CKD[204], is one of the best-evidenced supplements for exercise performance[205,206] and could help combat sarcopenia, osteoporosis, and frailty[207]. Creatine supplementation does not damage healthy kidneys[208]. If creatine supplementation is shown to be safe for diseased kidneys, exploration of its use may be beneficial although this may necessitate substituting cystatin-C for serum creatinine in GFR calculations.

Incretin-mimetics may improve physical function and have gained popularity as weight loss drugs[209], but their efficacy in conjunction with exercise remains to be tested in patients with

CKD. A recent study in obese patients with heart failure with preserved ejection fraction demonstrated improvements in 6MWT performance[210]. Given the high prevalence of diastolic heart failure and obesity in patients with CKD these treatments appear promising. Semaglutide has been shown to improve renal endpoints[211] and lead to substantial weight loss, but its impact on exercise adaptation remains to be explored. However, caution is advised as potential adverse responses have been reported[212], and induction of weight loss may not benefit some patients with CKD.

Further Considerations for Research and Practice

Optimizing interventional studies is crucial for increasing the use and efficacy of exercise in CKD. The expanding use of smart devices could be leveraged to collect physical activity data, administer surveys, and encourage adherence[213]. A group from the UK recently developed a free digital platform for physical activity and emotional well-being intervention in CKD populations ("Kidney Beam", https://beamfeelgood.com/kidney-disease) which significantly improved mental health and sit-to-stand test performance in just 12 weeks[214–216]. While clinical trials using these platforms in regions with a universal healthcare system hold promise, they require validation in regions with alternative healthcare systems and racially and socioeconomically diverse patient populations to determine the generalizability and identify barriers to implementation. Furthermore, trials may benefit from the inclusion of attention control groups (with health education similar to the AWARD study[106]) to identify benefits attributable to exercise alone. The use of healthy control groups may also help delineate the effects of CKD pathophysiology from the efficacy of the intervention and potentially identify new therapeutic targets.

Concluding Remarks

Despite significant physical, physiological, and practical barriers, exercise training has been shown to consistently improve physical function and aerobic capacity, frequently improve blood pressure and muscle function, and occasionally have beneficial effects on inflammation, oxidative stress, and kidney function. In addition, meta-analyses have suggested several benefits of exercise training not discussed here (Table 1). Other potential benefits such as improved bone health remain underexplored. To date, exercise interventions have often lacked the basic requirements for inducing adaptation, namely progressive overload, personalization, and specificity. We present here several resources (Figures 2 and 3, and references throughout) to aid in avoiding these pitfalls. Future interventions may also benefit from including exercise supervision, greater sample sizes, and better control of participant baseline status. Although the number of well-designed studies of exercise training in NDD is growing, we must not wait to implement personalized progressive exercise programs into patient care. In general, the risk of exercise is low and its inclusion in practice and policy has the potential to immediately affect the health and quality of life of the patient population. There is an urgent need to ensure programs are effective in limited-resource environments and underrepresented groups, which represent a disproportionately large and underserved portion of the patient population.

Disclosure

Nothing to disclose.

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Supplementary Material

Table S1. Recent systematic reviews meta-analyses evaluating exercise interventions in hemodialysis patients.

Author/Year	Journal	Title
Alves et al., 2022	Physiotherapy Theory and Practice	Efficacy of resistance exercise during hemodialysis on improving lower limb muscle strength in patients with chronic kidney disease: a meta-analysis of randomized clinical trials
Andrade et al., 2019	Scientific Reports	Effects of intradialytic exercise on cardiopulmonary capacity in chronic kidney disease: systematic review and meta-analysis of randomized clinical trials.
Andrade et al., 2022	The Journal of Vascular Access	Effects of upper limb exercise programs on the arteriovenous fistula in patients on hemodialysis: A systematic review and meta-analysis
Burrai et al., 2021	Giornale Italiano di Nefrologia	Effectiveness of physical exercise on cardiovascular endurance and functional capacity in hemodialysis patients: a systematic review and meta-analysis
Cai et al., 2022	Annals of Palliative Medicine	A systematic review and meta-analysis of the efficacy of aerobic exercise combined with resistance training on maintenance hemodialysis patients
Clarkson et al., 2019	American Journal of Physiology: Renal Physiology	Exercise interventions for improving objective physical function in patients with end-stage kidney disease on dialysis: a systematic review and meta-analysis
Cardoso et al., 2020	BMC Nephrology	Impact of physical activity and exercise on bone health in patients with chronic kidney disease: a systematic review of observational and experimental studies
Ferrari et al., 2020	Journal of Nephrology	Intradialytic training in patients with end-stage renal disease: a systematic review and meta-analysis of randomized clinical trials assessing the effects of five different training interventions
Ferrari et al., 2023	Nephrology Dialysis Transplantation	Efficacy of six exercise-based interventions for individuals undergoing hemodialysis: a network meta-analysis of randomized clinical trials

Ferreira et al., 2019	Archives of Physical medicine and rehabilitation	Does intra dialytic exercise improve removal of solutes by hemodialysis? A systematic review and meta-analysis.
Gomes et al., 2018	Clinical Rehabilitation	Intradialytic exercise training modalities on physical functioning and health-related quality of life in patients undergoing maintenance hemodialysis: systematic review and meta-analysis
Hargrove et al., 2021	Clinical Journal of the American Society of Nephrology	Effect of Aerobic Exercise on Dialysis-Related Symptoms in Individuals Undergoing Maintenance Hemodialysis: A Systematic Review and Meta-Analysis of Clinical Trials
Hu et al., 2022	Quality of Life Research	Effects of intradialytic exercise on health-related quality of life in patients undergoing maintenance haemodialysis: a systematic review and meta-analysis
Huang et al., 2019	American Journal of Nephrology	Exercise Training and Outcomes in Hemodialysis Patients: Systematic Review and Meta-Analysis
Lu et al., 2019	AE and/or RE	Muscle mass, muscle strength, physical performance
Molsted 2019	Danish Medical Journal	Effects of strength training to patients undergoing dialysis: a systematic review
Pu et al., 2019	British Medical Journal Open	Efficacy and safety of intradialytic exercise in haemodialysis patients: a systematic review and meta-analysis
Salhab et al., 2019	Journal of Nephrology	Effects of intradialytic aerobic exercise on hemodialysis patients: a systematic review and meta-analysis.
Schardong et al., 2020	Archives of Physical Medicine and Rehabilitation	Neuromuscular electrical stimulation in chronic kidney failure: a systematic review and meta-analysis
Song et al., 2018	Journal of Pain and Symptom Management	Effects of Exercise Training on Restless Legs Syndrome, Depression, Sleep Quality, and Fatigue Among Hemodialysis Patients: A Systematic Review and Meta-analysis
Young et al., 2018	Nephrology Dialysis Transplantation	Effects of intradialytic cycling exercise on exercise capacity, quality of life, physical function and cardiovascular measures in adult haemodialysis patients: a systematic review and meta-analysis

Table S2. Specificity table: additional resources.

Exercise Adaptation	Related articles
Decreased blood pressure	[217–219]
Increased maximal cardiac output	[186,220,221]
Decreased inflammation	[16,159,167,222]
Increased muscle endurance	[223–225]
Increased muscle mass	[42,226,227]
Improved bone health	[191,228–230]

Chapter 2: Kidney disease impairs muscle, bone, and tendon function in rats

This chapter is currently in press.
Kidney disease impairs tendon function in rats
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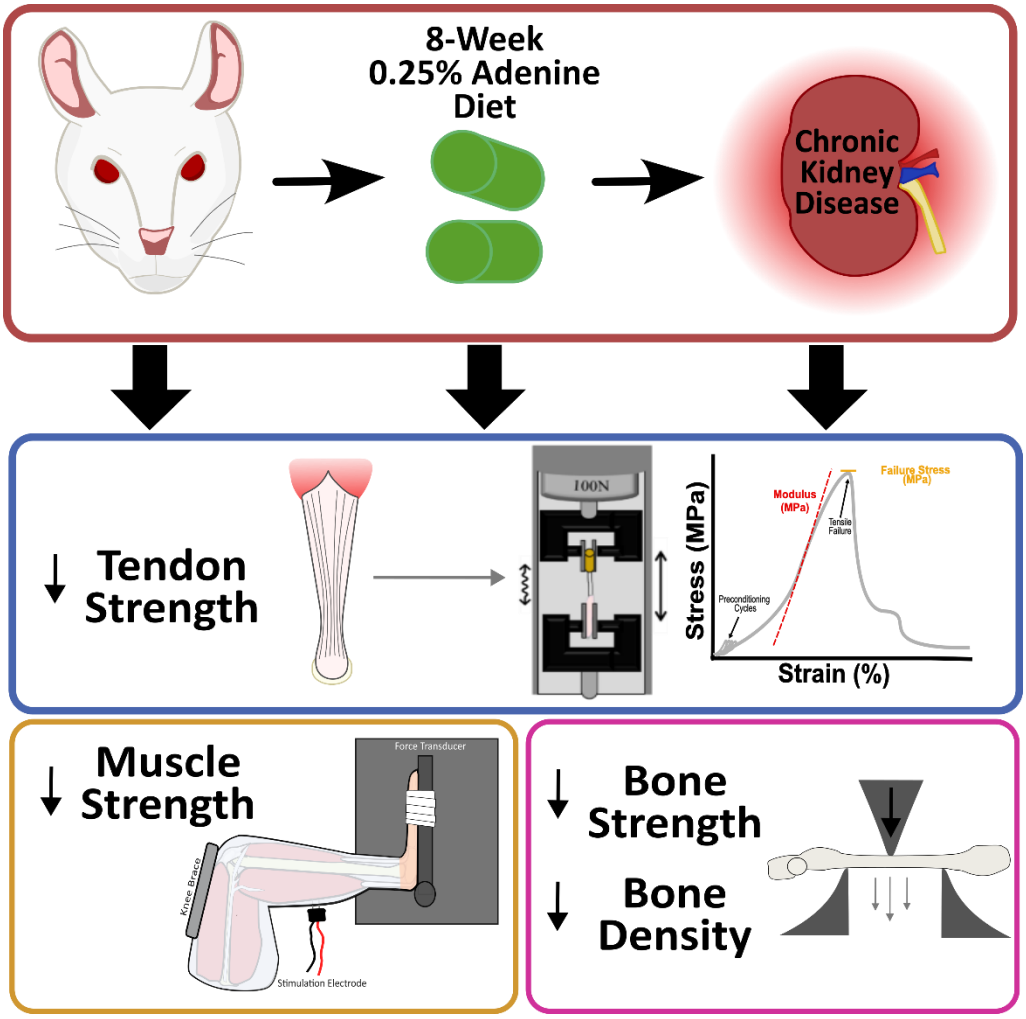

Abstract Figure Heading: This study demonstrates for the first time that tendon strength is reduced in a rodent model of chronic kidney disease (8 weeks of 0.25% adenine feeding) and confirms concurrent dysfunction in muscle and bone. These findings provide novel characterization of multiple tissues, paving the way for future investigations into the effects of CKD on tendon and integrated musculoskeletal health.

Key Points

1. Chronic kidney disease is well known to lead to musculoskeletal dysfunction, such as bone and muscle wasting.
2. Numerous case reports suggest that chronic kidney disease may also predispose patients to catastrophic tendon injuries, however, there are no mechanistic studies or animal models regarding this phenomenon - and thus, no treatments.
3. We evaluated if a rat model of chronic kidney disease previously used to study muscle and bone dysfunction (0.25% adenine in the diet), would also cause impaired tendon function.
4. Eight weeks of adenine-induced kidney disease caused tibialis anterior tendons to tear at 24% lower stress than in control animals, whilst also decreasing plantar flexor muscle strength by 13%, and femur strength by 41% (males only)
5. This work is the first experimental evidence for a direct effect of chronic kidney disease on tendon function, establishing a comprehensive multi-tissue model for future research

Abstract

Spontaneous tendon rupture occurs in a concerning number of individuals with chronic kidney disease (CKD); however, almost no data exists regarding CKD-related tendon pathology. Given that tendon ruptures have a significant impact on health and well-being, we sought to determine whether tendon mechanics are altered by kidney disease in an established rat model of CKD. Male and female Sprague-Dawley rats (age=8wk) were equally divided into a control group (CON, n=16) and a group fed a diet containing 0.25% adenine to induce kidney disease ($_{ADI}$CKD). After 9wk, Achilles and tibialis anterior (TA) tendons were excised, and maximum tensile load (MTL), failure stress, modulus, and cross-sectional area (CSA) were measured and evaluated by two-way ANOVA (main effects: CKD and sex). CKD was confirmed through elevated creatinine (1.99 vs 0.61mg/dL, CKD vs CON, P=0.001) and blood urea nitrogen (93.4 vs 21.4mg/dL, P<0.001). Plantar flexor strength was also 13% lower in ADICKD (p=0.021), and femur yield force was decreased by 41% in male ADICKD alone (p<0.001). The failure stress of TA tendons was 24% lower in CKD vs CON (P=0.038). There were no statistically significant differences in TA MTL, modulus, or CSA (p>0.097). There were no significant main effects for any parameter for the Achilles, however, post-hoc testing following a finding of group-by-sex interactions revealed that in females only, Achilles failure stress was decreased in $_{ADI}$CKD by 25% (p=0.028). To our knowledge, this is the first direct evidence that tendon weakness is caused by kidney disease, providing a model for further evaluating mechanisms and interventions.

INTRODUCTION

Chronic kidney disease (CKD) - which affects 1 in 7 US adults [4] – commonly leads to musculoskeletal dysfunction, including muscle wasting [9] and bone disorders [231]. This muscle and bone dysfunction, along with the consequent mobility impairment and decreased physical activity, are highly predictive of mortality in the CKD population [9,15] and thus has been extensively researched [232,233]. The musculoskeletal system, however, consists of multiple other tissues – such as ligaments, tendons, and cartilage – that are necessary to provide structure, maintain posture, and enable movement. These musculoskeletal tissues may also be negatively affected [4] in CKD. Tendons, for instance, have received relatively little attention in the CKD literature, which is concerning, as there are more than 60 years of case studies describing spontaneous (non-traumatic) tendon ruptures in patients with CKD [12,234–243]. These reported ruptures occur in both load bearing (Achilles and quadriceps) and non-weight bearing tendons (biceps brachii, triceps), often during normal daily activities such as walking downstairs. The site of rupture varies across case reports and may occur at the osteotendinous junction, myotendinous junction, or the tendon mid-substance [243,244].

Spontaneous tendon ruptures are debilitating injuries that commonly require surgery, costing the patient more than $4000 on average in healthcare charges [245] and a similar amount in indirect costs from missed work[246]. These injuries lead to decrements in muscle strength, endurance, and physical function that can take more than a year to fully recover from [247]. Furthermore, in hemodialysis patients, tendon rupture is associated with an increased risk of hip fracture [248] which has a generally poor prognosis. Between 1999 and 2013, 1091 cases of Achilles tendon rupture were identified amongst Medicare patients with end-stage renal disease, with an incidence rate of 3.8/10,000 person-years [249]. This is 3-4 fold greater than the rate

found in a separate study across all Medicare patients (0.67-1.08/10,000 patients per year from 2005 to 2011) [245]**.** Incidence rate further increases to 5-6/10,000 person years when including only transplant waitlist and recipient patients [249]. Furthermore, although CKD patients constitute 10% of the world population, they make up 37% of reported cases of bilateral spontaneous quadriceps tendon rupture, with nearly half of these occurring prior to the initiation of dialysis [243]. Despite this, the impact of CKD on tendon health is virtually unstudied, and purported mechanisms have not been experimentally investigated.

While no concrete link between CKD and tendon rupture has been established, evidence suggests that spontaneous rupture does not occur in healthy tendons [250] . Therefore, CKD or its treatments likely lead to degenerative changes in tendon tissue that predispose patients to catastrophic injury. Such degenerative changes likely contribute to chronic musculoskeletal pain, which limits movement and impairs quality of life in ~40% of CKD patients [251]. To date, most scientific literature on the topic of tendon dysfunction in CKD consists of case studies, which provide limited insight into the mechanistic causes.

Due to the scarcity of non-invasive techniques for interrogating tendon function and the necessity of biological samples for mechanistic research, animal models are needed to understand the impact of CKD on tendons and to test interventions targeted at improving tendon function. Therefore, the goal of this study was to evaluate the use of the adenine-diet-induced CKD model ($_{ADI}$CKD) in rats for studying CKD-related tendon dysfunction. The adenine-diet-induced model of kidney disease was first developed in rats by Yokozawa et al., in 1986 [252] and later refined in both rats [253] and mice [254]. This model was chosen because it has been well characterized and recapitulates the human CKD phenotype, including cardiovascular [253,255] and bone [256,257] abnormalities. Furthermore, as opposed to the popular 5/6

nephrectomy model, the adenine diet model does not require a surgical intervention (kidney ligation), has a better survival rate, and was identified in mice as the superior model for studying skeletal myopathy in CKD [258]. Because tendons work in conjunction with muscle and bone to enable musculoskeletal function, we studied the effect of $_{ADI}$CKD on these tissues as well.

METHODS

Animal model

All animal experiments were approved by the University of California Davis Institutional Animal Care and Use Committee under Protocol #22806 Thirty-two Sprague-Dawley rats (age 7 weeks, n=16 male and female) were obtained from Charles River Laboratories (Wilmington, MA, USA). Rats were housed two per cage with 12h light/dark cycles and ad libitum access to food and water. After acclimation, animals were split into control or $_{ADI}$CKD groups to equate maximal running speed from an exercise tolerance test (Appendix A1). Animals were kept on standard chow (AIN 93G, 0.3% phosphorus, 0.5% calcium, Bio Serv F3156, Flemington, NJ, USA) until 8 weeks of age, at which point $_{ADI}$CKD animals were switched to an adenine-containing chow (0.25% adenine, 0.9% phosphorus, 0.6% Ca, Inotiv TD.180493, West Lafayette, IN, USA). These diets were maintained for 9 weeks until sacrifice, with exercise performance testing completed prior to diet initiation and after 8 weeks on diet (Figure 1). All animals had ad libitum access to food and water. Animals and food were weighed once per week. Weekly change in food weight was divided by the number of rats in the cage to estimate consumption of each rat (Figure 1).

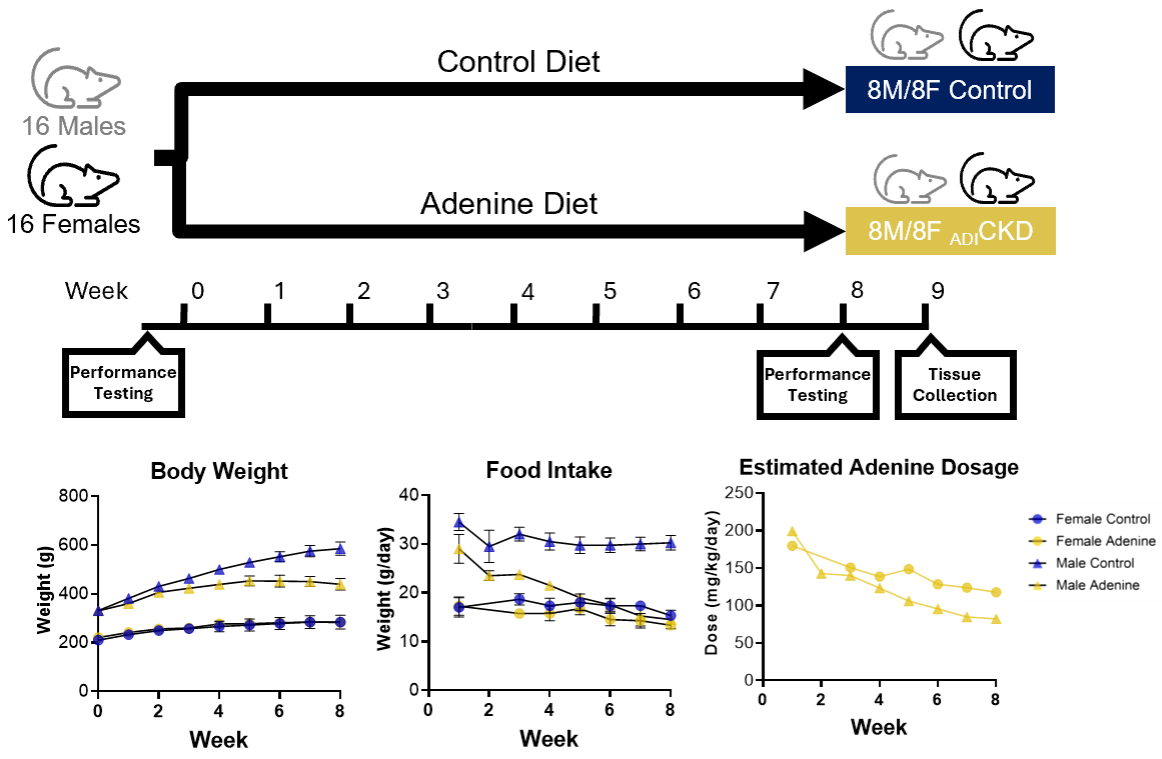

Figure 1. Study overview and time course of bodyweight, food intake, and estimated adenine dosage in rats fed control of adenine containing diets. Performance testing consisted of treadmill running and electrically stimulated muscle contraction for assessment of strength and endurance of plantar flexors (n=8 per group). Standard deviation is omitted where bars are smaller than symbol size. Standard deviation could not be accurately calculated for adenine dosage and as such should be considered an estimation. ADICKD, adenine-diet-induced chronic kidney disease.

In vivo muscle testing

Two days after the exercise tolerance test, in *vivo* muscle function was tested. Rats were anesthetized in a sealed chamber with 2% isoflurane. Once the absence of a righting response was confirmed and breathing rate had slowed, they were transferred to the force transducer platform, and anesthesia was maintained using an anesthetic nose cone. Adequate sedation was confirmed by a lack of pedal withdrawal reflex. Fur was then removed from the right hindlimb using an electric razor. Rats were placed in front of the force transducer (Aurora Scientific, Aurora, Ontario) with their knee positioned in a custom-printed brace (Experimental Setup, Fig. 3A). The hip, knee, and ankle of the tested leg were aligned at 90 degrees, and the foot was taped

to the force transducer pedal. Forceps were then used to pull the skin of the right hindlimb away from the distal third of the gastrocnemius muscle, and a needle electrode (negative) was then inserted into the skin without puncturing the muscle. The needle electrode was positioned so that when the skin was released, it pulled the electrode up against the gastrocnemius just distal to the muscle belly. A second needle electrode (positive) was then placed roughly 1-2 mm distal to the first in the same manner. Tape was wrapped around the needles approximately 2-3mm from the tips of the needles to ensure consistent separation between electrodes. Electrodes were placed distally to avoid stimulation of the hamstring muscles. The stimulation box was set to 30mA and biphasic pulse. Next, the computer was set to deliver one 150Hz stimulation every other second (InstantStim), and electrode placement was verified by the presence of a muscle twitch and force tracing. If the force tracing had signs of co-contraction, such as double peaks or negative force deflections, then electrodes were repositioned.

Amperage was set at 30mA for all experiments to keep the stimulus consistent. This value was chosen to elicit a peak twitch force without evidence of hamstring, quadricep, or dorsiflexor activation, such as changes in body position or negative deflections in the force tracing. A force frequency test was performed by stimulating at 20, 40, 60, 80, 100, 125, 150, and 200Hz (pulse width 0.2ms, duration 0.350s) with 20s of rest between contractions. Torque traces were evaluated to ensure tetanus had been achieved and a torque plateau had been reached at higher frequencies. Following the test, data were plotted as force over frequency to verify a plateau had been reached, and the peak force was selected. Torque (mN·mm) was calculated by multiplying the force reported by the system (mN) by the lever arm length used during calibration (32mm). The muscle endurance protocol was performed following a 3-minute rest period after the maximal force testing and consisted of 180 stimulations at 70Hz stimulation

(pulse width 0.2ms, duration 0.350s) with 2s between contractions, which has previously been used to evaluate muscle endurance in 5/6 nephrectomized rats [259].

Euthanasia and tissue collection

In the ninth week of diet intervention, rats were anesthetized with 2.5% isoflurane, and tissues were collected immediately before euthanasia via pneumothorax and cardiac puncture. Soleus muscles and right kidneys were excised, snap frozen in liquid nitrogen, and stored at -80° C. Left kidneys were excised and immersed in 1.5mL of 4% paraformaldehyde for 7 days at 4° C before being transferred to 70% ethanol and sent for histology. Left and right Achilles and tibialis anterior (TA) tendons were isolated along with the foot and stored in phosphate buffered saline soaked gauze at -30° C. Left and right femur and humerus bones were excised and half were stored in phosphate buffered saline soaked gauze at -80° C for biochemistry and the other half stored in 70% ethanol for micro CT. Diaphragm muscle was isolated along with several ribs, placed in Ringer's solution, and immediately mechanically tested. Blood was collected in heparin coated syringes via cardiac puncture and ~100uL was immediately tested using an i-STAT CHEM 8+ Cartridge (Abbott Laboratories, Chicago, Illinois; n=5 each group) the remaining blood was left at room temperature and allowed to clot before centrifugation at 2000 g for 15 minutes at which point serum was aspirated and stored at -80° C. Urine was collected directly from the bladder via syringe and stored at -80° C. Gastrocnemius weights for male animals alone were measured after stripping the muscle from the Achilles prior to tendon testing.

Ex vivo muscle testing

The mechanical properties of diaphragm muscle strips were examined as in previous studies [260,261] (Experimental Setup, Fig. 3B). Diaphragm muscles with the surrounding rib cage were stored in Ringer's solution on ice with bubbling oxygen until mechanical testing was

performed. Strips were cut from the diaphragm, and 7-0 sutures were tied onto the rib cage and at the myotendinous junction at the central tendon to connect it to a 300C-LR Dual-Mode motor arm and force transducer (Aurora Scientific). Optimum muscle length (L_0) was determined by performing twitches as the muscle was slowly stretched using the 701C high power follow stimulator (Aurora Scientific), with the length set at the largest twitch amplitude [262]. L_0 was measured using a caliper between the rib cage and the myotendinous junction at the central tendon. A passive mechanical testing protocol was performed where the muscles were cyclically strained 2.5% Lo at 1Hz for 5 seconds. This was repeated at 5, 7.5, 10, and 12.5% L_0. Immediately following 10% L_0 cyclic stretching, the muscle was held at 10% strain for 2 minutes to obtain a stress relaxation curve prior to undergoing 12.5% L_0 cyclic strain. At the end of the 12.5% strain, muscles were returned to L_0. A custom MATLAB script was used to fit a quadratic function to the peak stresses and to the nadir stress (following 2 minutes of relaxation). Dynamic stiffness was calculated as the tangent slope at 10% strain of a quadratic fit of the peaks, and elastic stiffness as the tangent slope at 10% strain of the quadratic fit of the nadir as previously described [261] (Fig. 4B).

Following passive mechanicals, an active fatigue protocol, consisting of an isometric twitch contraction followed by 25 maximum isometric tetanic contractions 5 seconds apart, was performed on each diaphragm strip. After the 25th contraction, there was a 5-minute waiting period before a final isometric tetanic contraction to assess muscle recovery. After completion of mechanical testing, the muscle strip was isolated, and muscle mass was measured to determine physiological cross-sectional area (PCSA). The diaphragm was then flash-frozen in liquid nitrogen and stored at -80℃ until the hydroxyproline assay was performed.

Western blotting

Western blotting analysis of pyruvate dehydrogenase and oxidative phosphorylation proteins was performed on soleus muscle samples. Tissues were powdered on dry ice, incubated on a shaker for 1 hour in 200 µL of sucrose lysis buffer, and centrifuged for 10 min at 8000RPM and 4°C. The supernatant was collected, and a detergent-compatible protein assay (DC Protein Assay, Bio-Rad Laboratories, Hercules, CA) was used to normalize protein concentrations across samples which were diluted into Laemmli sample buffer and sonicated. Samples (12uL) were loaded onto 4-20% Criterion TGX Stain-free gels (BioRad) and were run at 200V for 45 mins. The gels were activated under UV-light to fluorescently quantify total protein in each well. Proteins were transferred onto a nitrocellulose membrane at 100V for 30 mins. Membranes were ponceau stained to confirm protein transfer, washed in Tris-buffered saline w/ 0.1% Tween (TBST), and blocked in 5% skim milk in TBST for 30 mins. Membranes were then rinsed in TBST and incubated overnight at 4°C with the appropriate primary antibody diluted in TBST at 1:1000. The following day, membranes were washed in TBST (3 x 5 mins) and incubated with peroxidase-conjugated secondary antibodies in TBST at 1:10,000 for 1hr at room temperature. Following secondary antibody incubation, Immobilon Western chemiluminescent horseradish peroxidase (HRP) substrate (Millipore, Hayward, CA) was applied to the membranes for protein visualization by chemiluminescence. Images were taken using the ChemiDoc MP system and bands were quantified through Image Lab 5.0 software (BioRad). The following primary antibodies were used: PDH-E1α (Santa Cruz Biotechnology, sc-377092); Total OxPhos Rodent WB Antibody Cocktail (abcam, ab110413). Full protein gel and blot images can be found in Appendix A2.

Histology

Kidneys were sectioned at 10μm thickness, stained with Masson's trichrome, and imaged at 20x magnification by the UC Davis Center for Genomic Pathology Lab. Images were reviewed by a clinical pathologist and a subset of four slides from each $_{ADI}$CKD group were scored for interstitial fibrosis and tubular atrophy (Appendix A3).

Collagen assays

Collagen content was measured using a hydroxyproline assay[263]. Kidney, muscle, Achilles, and TA tendon samples were weighed pre- and post-drying at 120° C for 90 min (30 min for tendon) to determine water concentration. One centimeter lengths of cortical bone was cut from the middle of the femur and was demineralized in 0.5N HCl for 24 hours, placed in deionized water, sonicated for 15 minutes, then rinsed five times in deionized water [264]. Femurs were weighed once following demineralization and removal of marrow. All tissues were hydrolyzed in 6N HCl for 2 hours, following which HCl was boiled off in a fume hood. Samples and hydroxyproline standards were then suspended in 200 μL of hydroxyproline buffer (173 mm citric acid, 140 mm acetic acid, 588 mm sodium acetate, 570 mm sodium hydroxide), and samples were diluted further in hydroxyproline buffer to keep collagen content within range of the assay. Next, 150 μL of Chloramine T solution was added to each sample, before mixing and incubating for 20 min at room temperature. Following this, 150 μL of aldehyde-perchloric acid (60% 1-propanol, 5.8% perchloric acid, and 1M 4-(dimethylamino) benzaldehyde) was added to each tube, and the samples were incubated at 60°C for 15 min. Samples were cooled for 5 minutes, and 200 μL of each was added to a clear-bottomed 96-well plate and absorbance at 550 nm was read on an Epoch Microplate Spectrophotometer (BioTek Instruments Limited, Winooski, VT, USA). Collagen content was then determined by comparing sample absorbance to the standard curve, assuming hydroxyproline constitutes 13.7% amino acid composition of

collagen. Nine TA tendon samples taken from female animals, which were too small to accurately weigh, leading to collagen concentrations above 100%; these were excluded from analysis.

Bone Micro-Computed Tomography (μCT)

A subset of four femurs from each group were scanned using micro-computed tomography (SCANCO Medical, μCT 35, Brüttisellen, Switzerland) with 10μm nominal voxel size (X-ray tube potential= 55 kVp, current= 114mA, integration time= 900 ms, number of projections= 1000/180°). Trabecular bone was analyzed at the distal femoral metaphysis. Trabecular ROIs were manually drawn on transverse images, excluding the cortical surface. The regions of interest for the distal femoral metaphysis was located 1.5mm from the start of the growth plate and extended 1 mm proximaly. Analysis of cortical bone in the femoral diaphysis was performed by contouring transverse slices, with a 1mm region of interest (100 slices) centered on the midpoint of the femur. Femur midpoint was calculated from length as derived from CT scan. Microstructural parameters were determined using the manufacturer's analysis software.

Mechanical Testing of Femur Midshaft

3-point bending was used to determine structural and material properties of femora using a materials testing system (Instron, TA Instruments, New Castle, DE, USA; n=6 for controls and n=7 for $_{ADI}$CKD). The span length of the lower supports was 10 mm, and the femur was positioned to load the anterior aspect of the bone in tension. A 1–2 N preload was applied to ensure contact with the upper platen, then loading was applied at a displacement rate of 0.01 mm/sec until failure. Resulting force and displacement was recorded at 50 Hz and analyzed to determine whole bone stiffness, yield force, ultimate force, and post-yield displacement.

Tendon mechanical testing and differential scanning calorimetry

Tendons were mechanically tested using a single column tensile tester with a 100N load cell, with a BioPuls Body Temperature Bath and BioPuls Submersible Pneumatic Side Action Grips (Model 68SC-1, Instron, Norwood, MA, USA). The left and right Achilles and TA tendons were thawed in PBS-soaked gauze at room temperature before testing. Achilles and TA tendons were isolated from one another by carefully separating the small toe (and TA) from the rest of the foot. Width and depth were measured at the narrowest point of each tendon using optical coherence tomography (OQ LabScope, Lumedica Inc, Durham, NC, USA), and cross-sectional area (CSA) was calculated (width x depth). All muscle was then removed from the proximal end of each tendon using forceps, and the top 4-5mm was affixed inside a folded piece of 80-grit sandpaper (sand side in) using fast-drying cyanoacrylate. Tendons were then placed into the tensile tester with the sandpaper and either the entire foot (Achilles) or the first digit (TA) clamped into the top and bottom pneumatic grips (80psi), respectively. Gauge length was measured using digital calipers. Samples were immersed in 0.9% PBS within the BioPuls bath held at 37^{o}C, preconditioned using 10 cycles (0.10–0.25 N, 0.25 mm/s), and then elongated at 0.25 mm/s until failure (defined as an 80% decline in force). When not visually obvious, failure was confirmed by gently passing a pair of forceps through the remaining tendon tissue. Force and displacement were recorded throughout, from which the maximal tensile load (MTL; N) was determined as the highest tensile force obtained before failure – representing mechanical strength. Maximal apparent stiffness (N/mm) was calculated as the steepest slope using a least-squares fit of the load-displacement curve (excluding the preconditioning phase) – representing the greatest capacity of the tissue to resist elongation. Material properties, failure stress and maximal modulus, were calculated by normalizing MTL to CSA and maximal stiffness to CSA

and initial length, respectively. Following tensile testing, a 4mm biopsy punch was used to cut a similarly sized piece from each tendon. Each tendon was then lightly blotted to remove excess liquid, placed on the bottom of a Tzero® aluminum pan, and sealed using an aluminum hermetic lid and Tzero® press. Each sample was then weighed and placed into a differential scanning calorimeter with an autosampler (Discovery, TA Instruments, New Castle, DE, USA). Following equilibration at 40° C, samples were heated from 40 to 90° C at a rate of 10° C/min. The temperature at which material transitions began to occur (Onset Temperature) was determined as the temperature where the tangent of the melt curve intersected baseline. The temperature at which 50% of the material transitions have occurred (Peak Temperature) was determined as the temperature at which peak heat flow occurred, and the amount of energy needed to transition all of the material (Enthalpy) was calculated as the area under the melt curve.

Statistics

Muscle and tendon data were screened for outliers using the ROUT method and a Q = 1%. Outliers and any other values dependent on outlying measurements were removed from analysis. Bone and serum data were not screened for outliers due to the low n. Evaluation of the effects of adenine diet, sex, or interaction between the two was evaluated through two-way ANOVA, with the exception of in vivo performance testing for which ANCOVA was use to control for baseline performance. If an interaction was detected, a post-hoc Fisher's LSD test was used to evaluate the effect of adenine diet in males and females separately. Unpaired t-tests were used to compare protein levels (determined by Western blot) across diet interventions for each sex separately, to ensure comparisons were made only within a single blot. All statistics were performed using either GraphPad Prism (v. 10.4.2, GraphPad Software, Boston, MA, USA) or MedCalc Statistical Software (version 23.2.8, MedCalc Software Ltd, Ostend, Belgium).

RESULTS

Nine weeks of adenine feeding induced renal dysfunction in growing rats

All animals, control and adenine fed, survived the entire course of the study. Over nine weeks, the increase in body mass in adenine-fed female rats mirrored that seen in female controls. However, male controls gained significantly more weight than the male adenine group (585 vs 439g at week 8, $p<0.001$, Fig. 1). This difference in weight is likely due to a decreased consumption of food in the adenine-fed males compared to control males, leading to a similar but slightly lower estimated adenine dosage compared to the adenine-fed females (Fig. 1). Masson's trichrome staining of kidneys showed an increase in collagen deposition, 2,8-dihydroxyadenine crystals, calcium phosphate crystals, neutrophil infiltration, tubule dilation, and proximal tubule hypertrophy in the adenine-diet fed animals (Fig. 2A). Adenine feeding also led to a significant increase in kidney mass that coincided with a greater collagen concentration (by hydroxyproline assay) and water content (Fig. 2B). This increase in kidney mass was more pronounced in the left kidney (~117%) compared to the right (~57%). Serum creatinine and blood urea nitrogen (BUN) were elevated in adenine-fed animals compared to controls (2.09 vs 0.68 and 98.1 vs 28.4mg/dL, respectively), which was more pronounced in male animals, with several exceeding the upper limit of detection for BUN (140mg/dL). Hematocrit was significantly decreased in the adenine-fed group (33.7% vs 47.3%, Fig. 2C). Notably, blood glucose was elevated in male controls alone, coinciding with their higher food consumption and continuous weight gain. Collectively, these results demonstrate that nine weeks of adenine feeding led to significant renal dysfunction, which tended to be more severe in male animals. The histological and humoral phenotype of adenine-fed animals recapitulates characteristics of human CKD and is consistent with previous

descriptions of this model [252,253,255], successfully establishing the presence of renal dysfunction.

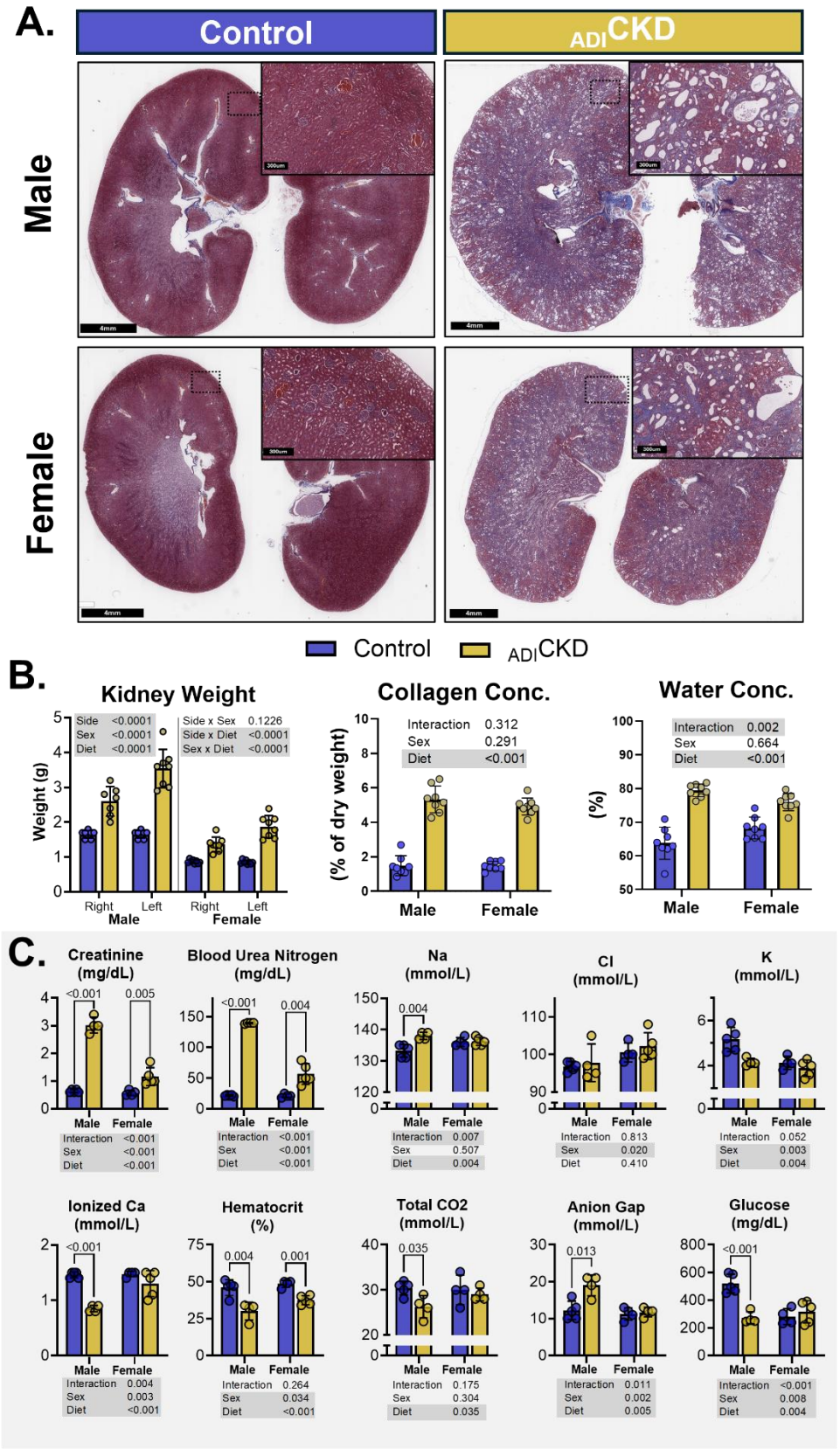
A.
Control
ADICKD
Male
Female
Control
ADICKD
B.
Kidney Weight
Side <0.0001
Sex <0.0001
Diet <0.0001
Side x Sex 0.1226
Side x Diet <0.0001
Sex x Diet <0.0001
Weight (g)
Right
Left
Right
Left
Male
Female
Collagen Conc.
Interaction 0.312
Sex 0.291
Diet <0.001
(% of dry weight)
Male
Female
Water Conc.
Interaction 0.002
Sex 0.664
Diet <0.001
(%)
Male
Female
C.
Creatinine (mg/dL)
<0.001
0.005
Male Female
Interaction <0.001
Sex <0.001
Diet <0.001
Blood Urea Nitrogen (mg/dL)
<0.001
0.004
Male Female
Interaction <0.001
Sex <0.001
Diet <0.001
Na (mmol/L)
0.004
Male Female
Interaction 0.007
Sex 0.507
Diet 0.004
Cl (mmol/L)
Male Female
Interaction 0.813
Sex 0.020
Diet 0.410
K (mmol/L)
Male Female
Interaction 0.052
Sex 0.003
Diet 0.004
Ionized Ca (mmol/L)
<0.001
Male Female
Interaction 0.004
Sex 0.003
Diet <0.001
Hematocrit (%)
0.004
0.001
Male Female
Interaction 0.264
Sex 0.034
Diet <0.001
Total CO2 (mmol/L)
0.035
Male Female
Interaction 0.175
Sex 0.304
Diet 0.035
Anion Gap (mmol/L)
0.013
Male Female
Interaction 0.011
Sex 0.002
Diet 0.005
Glucose (mg/dL)
<0.001
Male Female
Interaction <0.001
Sex 0.008
Diet 0.004

Figure 1. Adenine feeding for 9 weeks leads to CKD phenotype in rats that is more severe in males. A) Masson's trichrome staining of kidneys from control and $_{ADI}$CKD rats, collagen deposition shown in blue. B) Kidney weight, and collagen and water concentrations from hydroxyproline assays (n=8/group/sex). C) Blood markers measured at collection (week 9) using and i-STAT with CHEM 8+ cartridges (n=5/group/sex, n=4 $_{ADI}$CKD males due to cartridge error). Statistics calculated using two or three-way ANOVA with significant effects highlighted in gray, post-hoc Fisher's LSD performed when significant interactions existed. $_{ADI}$CKD, adenine-diet-induced chronic kidney disease; Na, sodium, Cl, chloride; Ca, calcium.

$_{ADI}$CKD causes bone mineral wasting and decreases mechanical strength

Femurs from all animals were imaged using Micro-CT to evaluate the effects of $_{ADI}$CKD on bone structure and mineral content. Animals with $_{ADI}$CKD had significantly lower cortical thickness and tissue mineral density compared to healthy controls. At the same time, collagen content (via hydroxyproline assay) was unchanged (Fig. 3A). Femur cross-sections from $_{ADI}$CKD animals were visibly porous and had significantly greater pore area and average pore size. However, this was more variable in female animals, with several appearing to be unaffected (Fig. 3A). There was no effect of $_{ADI}$CKD on trabecular bone volume fraction (BV/TV), and disease resulted in an increase in trabecular thickness (Fig. 3B). Trabecular bone tissue mineral density, however, was lower in $_{ADI}$CKD animals, suggesting poorer bone quality (Fig. 3B). Mechanical testing showed that $_{ADI}$CKD rats did not differ from healthy controls in terms of stiffness or post-yield displacement. However, yield force and ultimate force were significantly lower in male $_{ADI}$CKD animals (Fig. 3C).

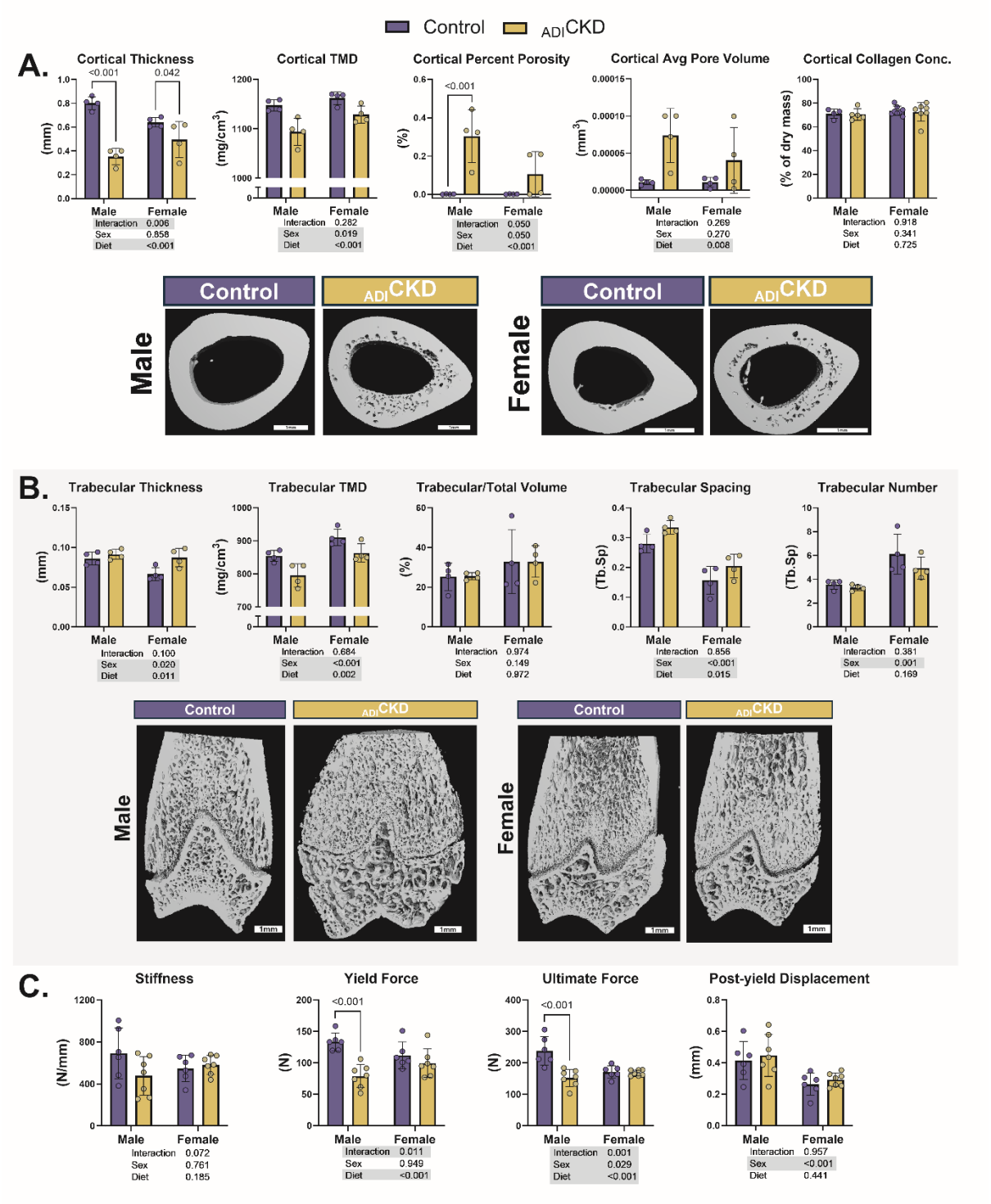

Figure 2. Bone morphology and mechanical strength is altered in ADICKD. A) Cortical bone Micro-CT measurement and images from femurs of control and ADICKD rats (n=4/group/sex). B) Trabecular bone Micro-CT measurement and images from femurs of control and ADICKD rats (n=4/group/sex). C) Three-point-bending mechanical analysis of femurs (n=6/controls, n=7/ADICKD). Statistics calculated using two -way ANOVA with significant effects highlighted in gray, post-hoc Fisher's LSD performed when significant interactions existed. ADICKD, adenine-diet-induced chronic kidney disease; TMD, tissue mineral density; Conc., concentration.

$_{ADI}$CKD led to decreased muscle strength but not endurance

Muscle strength and endurance were evaluated both *in vivo* (plantar flexors) and *ex vivo* (diaphragm). There was a significant effect of time on plantar flexor strength, with all groups increasing in maximal torque from pre-to-post diet intervention (50±35%, $p<0.001$). Strength was significantly lower in $_{ADI}$CKD after correcting for baseline differences (Fig. 4A). There was no significant effect of time on plantar flexor endurance, determined as the average torque output over 180 contractions, but there was a sex by time interaction driven by greater baseline endurance in female animals that declined over time (Fig. 4A). There were no effects of diet on plantar flexor endurance, or soleus weight, collagen concentration, or water concentration (Fig. 4A). Gastrocnemius weight was significantly lower in male $_{ADI}$CKD compared to controls (1.7±0.2 vs 2.3±0.3g, $p<0.001$) but was not measured in female animals. Muscle electron transport complex levels were not affected by $_{ADI}$CKD; however, PDH levels were lower in diseased males (Fig. 4A). A strip of diaphragm muscle was excised and mechanically tested *ex vivo* for both active (stimulated) and passive (relaxed) tension generation. There was no effect of diet on diaphragm peak force, fatigue, or recovery (Fig. 4B). Passive mechanical analyses, which are evaluated using a stress-relaxation test (Passive Mechanics, Fig. 4B), demonstrated a significant increase in both dynamic and elastic stiffness in the diaphragm muscle of $_{ADI}$CKD animals (Fig. 4B). Although there is no significant interaction, this appears to be driven by lower control values in the males.

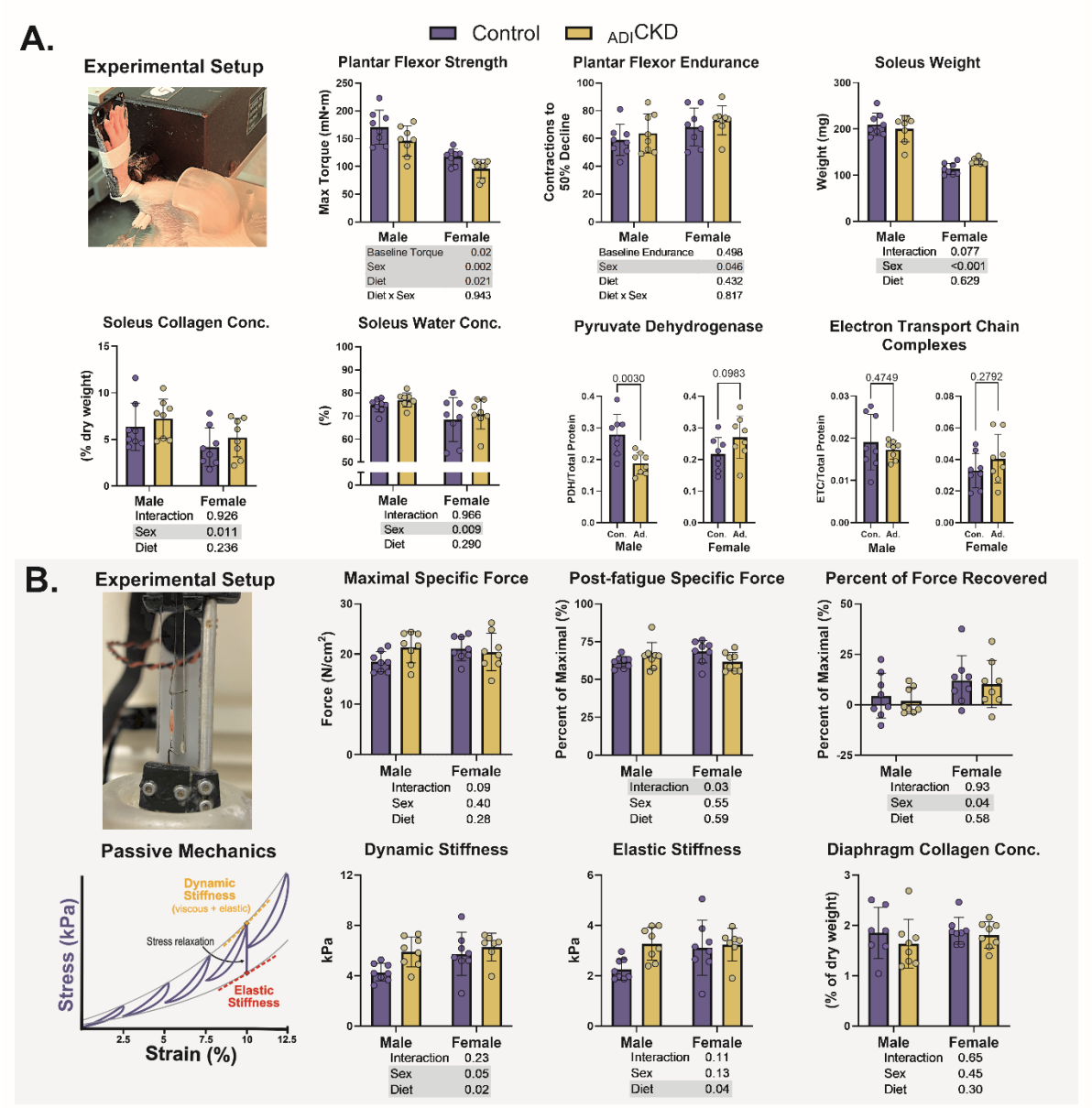

Figure 3.Plantar flexors are weaker but not more fatigable, and diaphragm is stiffer but not more fibrotic in ADICKD. A) *In vivo* assessment of plantar flexor strength and endurance measured via muscle electrical stimulation to elicit maximal torque and over 180 repeated submaximal contractions, as well as soleus mass, collagen and water content from a hydroxyproline assay, and pyruvate dehydrogenase and electron transport chain protein levels from Western Blotting (n=8/group/sex). B) *Ex vivo* assessment of a strip of diaphragm muscle during active and passive mechanical protocols, along with collagen content via hydroxyproline assay (n=8/group/sex). Statistics calculated using two -way ANCOVA (strength and endurance) or ANOVA with significant effects highlighted in gray, post-hoc Fisher's LSD performed when significant interactions existed. ADICKD, adenine-diet-induced chronic kidney disease; Conc., concentration.

ADICKD decreased tendon failure stress without changing cross-sectional area or collagen content

Both the Achilles and TA tendons were excised and tested for strength and function, collagen concentration, and extracellular matrix stability. Because these two tendons have different functions - the Achilles is an energy-storing tendon and the TA is a positional tendon - their mechanical and material properties, as well as their response to load and disease, may differ. Therefore, results were analyzed separately. There was no effect of diet on Achilles tendon mechanics, collagen concentration, or measures of extracellular matrix stability (onset temperature, peak temperature, and enthalpy; Fig. 5A). There was, however, an interaction effect on failure stress and CSA. Post-hoc testing revealed that in females only, Achilles failure stress was significantly decreased in $_{ADI}$CKD (-25%) and CSA was significantly increased (+26%, Fig. 5A). In the TA tendon, there was a significant effect of diet on failure stress, with $_{ADI}$CKD tendons being 24% weaker. There were non-significant decreases in MTL (-15%, $p=0.100$) and stiffness (-17%, $p=0.063$) in the $_{ADI}$CKD group (Fig. 5B). No significant effect of diet was found on TA tendon CSA, collagen concentration, or measures of extracellular matrix stability.

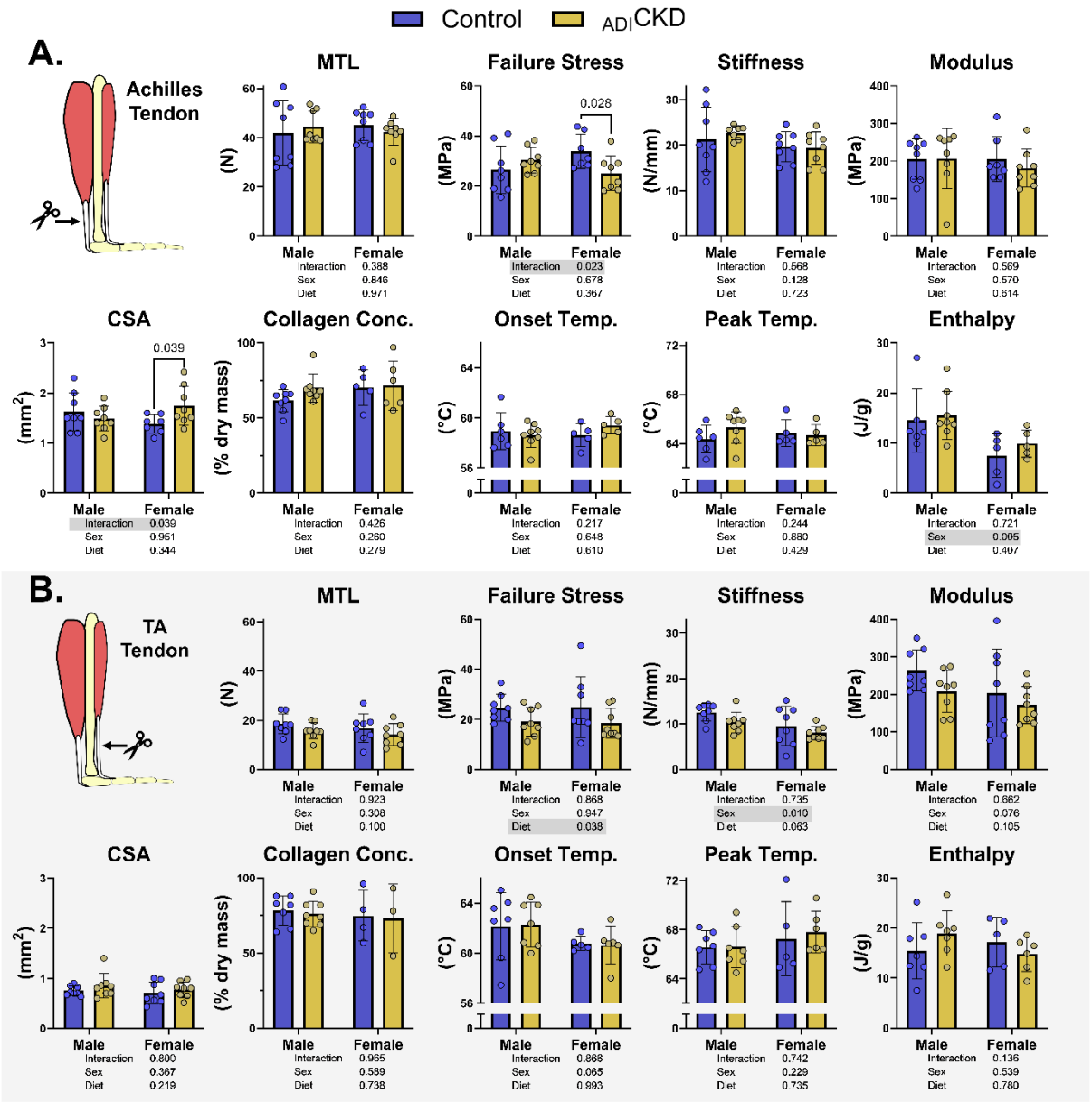

Figure 4. Tendons are significantly weakened in rats with ADICKD. *Ex vivo* assessment of tendon mechanical and material properties under tensile strain to failure, collagen content via hydroxyproline assay, and matrix thermal stability via differential scanning calorimetry in A) Achilles and B) TA tendons from control and ADICKD rats (n=8/group/sex). Statistics calculated using two -way ANOVA with significant effects highlighted in gray, post-hoc Fisher's LSD performed when significant interactions existed. ADICKD, adenine-diet-induced chronic kidney disease; MTL, maximum tensile load; CSA, cross-sectional area; Conc., concentration, Temp., temperature.

DISCUSSION

The goal of this study was to evaluate the use of $_{ADI}$CKD in rats for studying CKD-related tendon dysfunction. Nine weeks of adenine feeding successfully recapitulated the CKD phenotype previously reported in the literature [252,253]. This includes significant kidney dysfunction evidenced by kidney crystalline deposits, neutrophil infiltration, and interstitial fibrosis as well as elevated blood creatinine, blood urea nitrogen, and low hematocrit (Fig. 2). Consistent with our hypothesis, rats with $_{ADI}$CKD had significantly weaker tendons compared to controls (~25% lower failure stress, Fig. 5). This detrimental effect on tendons is evident in $_{ADI}$CKD rats in the absence of pharmaceutical treatment, suggesting that kidney impairment itself is detrimental to tendon health. To our knowledge, this is the first direct evidence of altered tendon mechanics in a model of CKD.

Muscle wasting [9], osteoporosis [265], and poor exercise capacity [15] are consistently seen in CKD, and such musculoskeletal dysfunction and consequent physical inactivity are highly predictive of mortality in CKD populations [15,70,98]. This is the first study known to the authors to combine both functional and structural analysis of muscle and bone in a CKD model into one paper. As muscle-bone crosstalk in CKD has recently become a topic of interest [191,192], the data presented here support the use of this model to mechanistically explore this area. Furthermore, the analysis of tendon tissue is completely novel and when integrated with the muscle and bone data creates a platform for comprehensive evaluation of the effects of the CKD on musculoskeletal function.

Officially termed “renal osteodystrophy”, bone dysfunction in CKD falls under the umbrella of CKD-mineral and bone disorder (CKD-MBD), which differs from bone dysfunction seen in other diseases [231,265]. General characteristics of CKD-MBD include high circulating

FGF-23 and parathyroid hormone, low concentrations of active vitamin D, and altered calcium and phosphate homeostasis [265]. This leads to decreased volume, thinning, and increased porosity in cortical bone, with less severe and sometimes opposite effects seen in trabecular bone [266–268], collectively resulting in an increased risk of fracture[269]. In line with previous studies in rats [257] and mice [270], we found that $_{ADI}$CKD led to significant bone pathology, including decreased bone mineral density and volume in cortical bone and increased porosity (Fig. 3A). Further recapitulating human disease [266–268], we saw an increase in thickness in trabecular bone despite a concomitant decrease in mineral density (Fig. 3B). In accordance with the mouse work from Metzger and colleagues [270], the bone phenotype we observed was slightly more severe in males than females. This coincided with decreased mechanical properties in the $_{ADI}$CKD males (Fig. 3C), which is evident in humans CKD in the form of higher rates of fracture [269]. We also observed a decrease in serum ionized calcium (Fig. 2C), and while we did not measure parathyroid hormone or FGF-23, others have found these to be elevated in $_{ADI}$CKD rats [257,271]. Together, our results suggest that 9 weeks of adenine feeding in rats effectively recreates relevant aspects of CKD-MBD in humans.

Cachexia or muscle protein energy wasting as a result of disease, is a well-established comorbidity in CKD with a large number of confounders that make it difficult to study in humans [43,44,272]. Several studies using animal models of CKD have reported decreases in muscle mass [200,257–259], *in vivo* strength and endurance [259], and *ex vivo* single fiber force [200,273]. These negative effects of CKD on muscle vary by sex and muscle [258], and are undoubtedly dependent on the testing protocol and model used. To our knowledge, this is the first study to evaluate muscle function *in vivo* or *ex vivo* in the rat $_{ADI}$CKD model. We found that plantar flexor strength decreased by 13% in $_{ADI}$CKD with no concomitant change in soleus mass

(Fig. 4). The gastrocnemius, which is the larger plantar flexor in rats, was 26% smaller in the $_{ADI}$CKD males suggesting that muscle atrophy may be responsible for the decline in strength; however, this measure was added mid-way through collections and therefore not confirmed in females. We designed our *in vivo* muscle function testing to be similar to that of Fusagawa and colleagues, who showed that 8 weeks after inducing CKD using a 5/6 nephrectomy, male rats had significant reductions in plantar strength (due to muscle atrophy) and also plantar flexor endurance [259]. Our study did not find any sign of reduced muscle endurance using the same protocol. We did find that $_{ADI}$CKD led to decreased levels of PDH in the soleus of male animals, but no effect was seen in female rats or in electron transport chain complexes (Fig. 4A). Animal [274–276] and human [277] studies that have found decreased PDH activity in CKD, which has been shown to contribute to mitochondrial dysfunction and impaired endurance performance; however, we measured only PDH content and did not observe endurance deficits in the current work.

We also completed an *ex vivo* assessment of diaphragm muscle active and passive tension to evaluate muscle function independent of circulation and innervation. Diaphragm function may be less influenced by any potential disease-related changes in activity levels and is particularly relevant as CKD is associated with a number of respiratory comorbidities [46]. While we did not find significant effects of $_{ADI}$CKD on diaphragm active mechanics, there were differences in dynamic and elastic stiffness (i.e., passive mechanics, Fig. 4B). Passive mechanics measure the resistance of muscle to lengthening in a relaxed state and can be indicative of changes in muscle, such as fibrosis[278] or changes in matrix orientation and cross-linking. The effect on passive mechanics was driven by the apparent deficits in control males, where signs of obesity and metabolic disorder were evident. Our study found no increase in collagen content in $_{ADI}$CKD

soleus or diaphragm, suggesting changes in passive mechanics were not due to fibrosis (Fig. 4A-B). Pathological fibrosis has been reported in the muscle of CKD patients in two studies by one group [279,280], and there is only non-quantitative evidence of muscle fibrosis in animal CKD models [281,282]. This is potentially counteracted by the fact that obesity, which is common in CKD, can decrease collagen production in muscle, bone, and tendon [283]. Collectively, our results suggest that 9 weeks of adenine feeding in 8-week-old Sprague Dawley rats induces muscle pathology in some, but not all muscles, evidenced by declines in strength and to a limited extent mass and passive mechanics. These results do not align with a recent study by Lair and colleagues, which suggested that common rodent CKD models do not induce cachexia [284]. However, the Lair et al. study used a three-week adenine diet model that is significantly shorter than most protocols and failed to induce severe uremic build-up (creatinine < 0.51 mg/dL), which is at odds with the literature [200,257–259].

Although CKD-related muscle and bone disorders have been subject to numerous investigations, almost no data exist regarding tendon pathology in CKD or its mechanisms. In this study, we found that rats with $_{ADI}$CKD disease had significantly weaker tendons (lower failure stress) than controls (Fig. 5), supporting their use as a model for CKD-induced tendon pathology. Failure stress is a material property, indicative of tissue quality. The decrease in failure stress found in this study, therefore, suggests that $_{ADI}$CKD tendons have reduced quality and are not simply smaller. As opposed to other organs that are primarily composed of parenchymal cells (liver, kidney, heart, muscle, etc.), tendons are composed of less than 20% cellular mass. The extracellular matrix, which is produced and secreted by tendon cells, makes up the remaining 80% of tendon mass and is central to tendon function (force transfer). The majority of the extracellular matrix in tendons is comprised of collagen, predominantly collagen

I, which aligns along the longitudinal axis and provides tensile strength [285]. Despite the decrease in material properties, there were no differences in tendon collagen concentration between control and ADICKD animals. Additionally, there was no effect of ADICKD on matrix thermal stability measures (Fig. 5), which can be used to roughly gauge collagen microstructure (crosslinking, fibril packing density, etc.) [286,287]. Thus, the exact structural change leading to the decrease in tendon material properties remains unknown.

Mechanical properties (MTL and stiffness) are more functionally important characteristics as they define the absolute ability of the tendon to resist elongation. An important aspect of this study is that it was completed in a strain of rats that continue to increase body weight throughout life (Fig 1.) and commonly develop insulin resistance [288]. Tendon mechanical properties increase across development [289], likely to meet the functional demands of concurrent increases in bodyweight. Thus, over the course of this study, TA and Achilles tendons should have grown in length to match any longitudinal bone growth and in CSA to maintain or increase tendon mechanical properties. In TA tendons, there were non-significant decreases in mechanical properties in ADICKD, including a 15% decrease in the amount of force necessary to rupture the tendon and a 17% decrease in tendon stiffness (Fig. 5B). Thus, ADICKD tendons did not adapt to maintain mechanical properties in response to body growth or to compensate for their decreased material properties, suggesting that tendon homeostasis was not maintained in the ADICKD animals; however, a repeated study with greater power would be necessary to validate this hypothesis. Conversely, in the Achilles, there was a significant increase in CSA in female rats with ADICKD, resulting in no apparent deficit in MTL or stiffness. Because the Achilles tendon is a load-bearing tendon, whereas the TA tendon is a positional tendon, during ambulation the Achilles tendon will experience loads close to body weight and the TA

will only be exposed to the load of the foot. Therefore, load-dependent signaling processes may have played a role in maintaining mechanical properties in the Achilles, but this requires further confirmation. Of note, there was not a significant effect of $_{ADI}$CKD on failure stress in the male Achilles tendon alone (Fig. 5A). This is likely due to the larger body size of the control males (585 vs 439g for $_{ADI}$CKD) and resulting decrease in insulin sensitivity (increased blood glucose; Fig. 2C). Since insulin-resistance is well known to impair tendon function [290,291], interpretation of the Achilles tendon data from the male rats is difficult.

The only large scale analysis to date of tendon rupture in CKD [249], suggests that rupture is less common in men than in women (adjusted incidence rate ratio, aIRR=0.75). Although our study shows less consistent effects of CKD on tendon in male rat (in agreement with Humbyrd), we believe this is due to the negative effects of obesity and inactivity in the male control group rather than a less pronounced effect of CKD. Consistent with this hypothesis, Humbyrd and colleagues also found that rupture risk increased with increasing BMI (reference BMI 0-24.99, BMI=25-29.99: aIRR=1.25, BMI=30-39.99: aIRR=1.56, BMI≥40: aIRR=1.81). Importantly, the Humbyrd study included only end-stage renal disease patients who were on dialysis or had already received a kidney transplant, which is different than our model.
In the literature, hypotheses as to why CKD leads to tendon dysfunction include chronic acidosis [292], increased parathyroid hormone[293,294], and Beta-2 microglobulin amyloidosis [295]. The most popular explanation appears to be elevated parathyroid hormone, which has been shown by two studies to be higher in dialysis patients with tendon rupture than those without [248,294]. However, the parathyroid hormone level of the rupture group in the first study [294] (1,802 pg/mL) was roughly the same as the non-ruptured group in the second study [248] (1728 pg/mL), bringing into question validity of this finding. This highlights the pitfalls of

observational studies and the need for well-controlled experimental research on the topic. Regardless of the cause, spontaneous tendon ruptures are debilitating injuries that occur as a result of pre-existing tendon pathology. In fact, in a study of biopsies from 891 spontaneously ruptured tendons, not a single healthy structure was found, while only one third of 445 tendons from healthy individuals had pathological changes [250]. Therefore, significant tendon pathology likely precedes spontaneous rupture seen in many CKD patients and may contribute to the high prevalence of reported musculoskeletal pain [71], that reduces physical activity and quality of life. Unfortunately, tendons are notoriously hard to investigate *in vivo,* which has led to equivocal results from a number of low-quality studies on the impact of CKD on tendon size and stiffness (via ultrasonography) [11,296–300]. To date, most scientific literature on the topic of tendon dysfunction in CKD consists of case studies of one or a few individuals, which limits inference of potential mechanisms. Due to the scarcity of non-invasive techniques for interrogating tendon function and the necessity of biological samples for mechanistic research, an animal model is necessary to progress toward treatment. Rats are an effective model system as they are relatively low-cost, easy to handle, have high homology with humans, and are frequently used for the study of tendinopathy [301]. Rats are also preferred over mice due to their larger tendon size, trainability, and temperament [301]. The use of rat models allows excision of whole tendons at sacrifice, enabling accurate and simple mechanical testing. The tested tendon samples can then be evaluated biochemically, allowing mechanistic and functional interrogation of the same tendon.

Additional considerations

There were limitations in this study. First, CKD typically affects adults, but here we used young rats (8wk) that had not yet fully developed. However, because tendon development involves similar mechanisms to repair [302], this approach may offer useful insight into maladaptive responses that lead to degeneration in adult CKD. Still, conducting a similar study with skeletally mature animals may be more congruent with human disease. Second, in humans, CKD must last at least 3 months to be considered chronic. Our intervention lasted just over two months, and we did not determine when kidney dysfunction onset occurred. Thus, whether we were testing acute or chronic kidney dysfunction is unclear. Despite this limitation, the intervention was sufficient in length to induce significant tendon dysfunction, which highlights the interconnection between the two pathologies. Third, the male controls in this study ate significantly more than the males on the adenine diet, leading to large differences in body weight and blood glucose. Though not quantified, these animals likely had much greater fat accumulation as well and thus may have developed other comorbidities impairing their use as "healthy" controls. Any follow-up studies may need to pair-feed animals to maintain similar body weights or rely on the use of the female animals, which maintain stable weights through adulthood and have been historically understudied.

As for the interesting finding that in ADICKD the left kidney is consistently significantly larger than the right, a potential explanation is that this occurs due to differences in renal blood flow. The left kidney has a shorter artery and longer vein, meaning there is less resistance to afferent flow and more resistance to efferent flow, while the right kidney has the opposite. This could potentially lead to differences in glomerular pressure and therefore hypertrophy, as our histology indicates obstruction induced dilation. However, this is highly speculative and at the moment the authors have no definitive evidence for this.

Amongst our three musculoskeletal tissues, it may be noted that bone is the only one where the greater burden of CKD in males appeared to result in more severe pathology. The lack of apparent dose response in muscle and tendon to CKD might suggest that the pathobiology of kidney disease occurs at lower levels of disease or that the metabolic impact of hyperphagia and the higher BMI was more detrimental to muscle and tendon than bone. Further, the mechanisms that lead to bone dysfunction in CKD likely differ from those driving the loss of muscle and tendon function. In fact, a surprising finding of Humbyrd and colleagues was that amongst individuals with ESRD, those with osteoporosis were far less likely to spontaneous rupture their Achilles than those without (aIRR=0.14)[249]. Spontaneous tendon ruptures in CKD have been reported in many different tendons of the upper and lower extremities, often occurring in load bearing tendons of the knee and ankle - although there is at least one report of a torn medial obliquus abdominus internus [12]. Our finding that the TA tendon was more consistently affected by CKD than the load bearing Achilles, should be evaluated in context with two thoughts. One, tendon loading within healthy bounds results in positive tissue adaptation that may counteract the negative effect of disease (e.g., the benefits of exercise)[303]. Two, although adult tendons are relatively stable (i.e., low protein turnover) and may need external stimulus to initiate pathology, these animals developed kidney disease while their musculoskeletal system was still growing and maturing. Thus, in this model the negative effects of CKD on tendons may be evident in more locations as CKD may impair normal tendon development. Nevertheless, the finding of worse material properties in TA tendon is good evidence for a direct systemic effect of CKD on tendon tissue, as opposed to confounding from other CKD symptoms such as sedentarism.

Conclusion

Tibialis anterior tendons from rats with $_{ADI}$CKD displayed evidence of disease-induced dysfunction, including significantly lower failure stress compared to controls. Additionally, inducing $_{ADI}$CKD in young Sprague Dawley rats led to changes in kidney structure and function, and bone pathology that mimics human CKD. Given the interdependency of tendon and bone function, the concurrence of CKD-MBD symptoms amplifies the relevance of the $_{ADI}$CKD model for use in CKD-related tendon dysfunction research. Further, while significant muscle dysfunction was not observed in this study, trends in our data and results from similar models in the literature suggest that lengthening the diet intervention may induce CKD-related muscle dysfunction. This initial investigation did not detect any changes in tendon structure, such as altered collagen content or matrix stability, necessitating more mechanistic follow-up. Importantly, while several drugs commonly used to treat CKD patients, including corticosteroids, fluoroquinolone antibiotics, and angiotensin II receptor agonists have been reported to increase tendon rupture risk [304], we believe this data to be the first evidence of a direct impact of CKD on tendon function – laying the groundwork for further research.

Disclosure

Nothing to disclose.

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Competing interests

All authors declare no competing interests

Author contributions

CMTH, BR, and KB, contributed to project design. CMTH, NKG, BO, SEB, MG, and KYJ completed data acquisition and analysis. All authors contributed to the interpretation of the work as well as drafting and revising it critically for important intellectual content. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved; and all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Supplementary Materials

Figure A1. Exercise Tolerance Test
Exercise tolerance testing was completed prior to (Pre) diet initiation and following 8 weeks on adenine or control diets (Post). Baseline test performance was used to separate rats into groups with equivalent maximal running speeds. Both pre and post-tests were completed with the same protocol as follows. Rats were accustomed to the treadmill by running for 5 minutes per day on a 10-degree grade for four consecutive days at 10, 15, 20, and 25m/min, respectively. Each time the rats were run, they were allowed to acclimate to the treadmill space for 3 min. During this time, the electrical grid was on to discourage them from residing there, but the treadmill was not moving. To encourage running, rats were prodded with a soft-bristled brush when they fell back onto the back quarter of the treadmill. On the fifth day, the first two stages of the exercise tolerance were performed with the electrical grid on (1Hz and 2mA stimulation). On the sixth day the entire exercise tolerance test was performed, consisting of running on a 10-degree grade beginning at 10m/min and increasing by 2m/min every three minutes until volitional fatigue (sitting on the electrical grid > 5s for females and > 10s for males) or until a running gait could no longer be maintained (e.g., continual hopping on and off the grid). At cessation, the electrical grid was immediately switched off, the animal was removed from the treadmill, and the maximal speed completed as well as total running time, were recorded. Animals did not show signs of exhaustion, and righting response was not slowed. There was an effect of time and sex on maximal running speed; however, there was no effect of disease status.

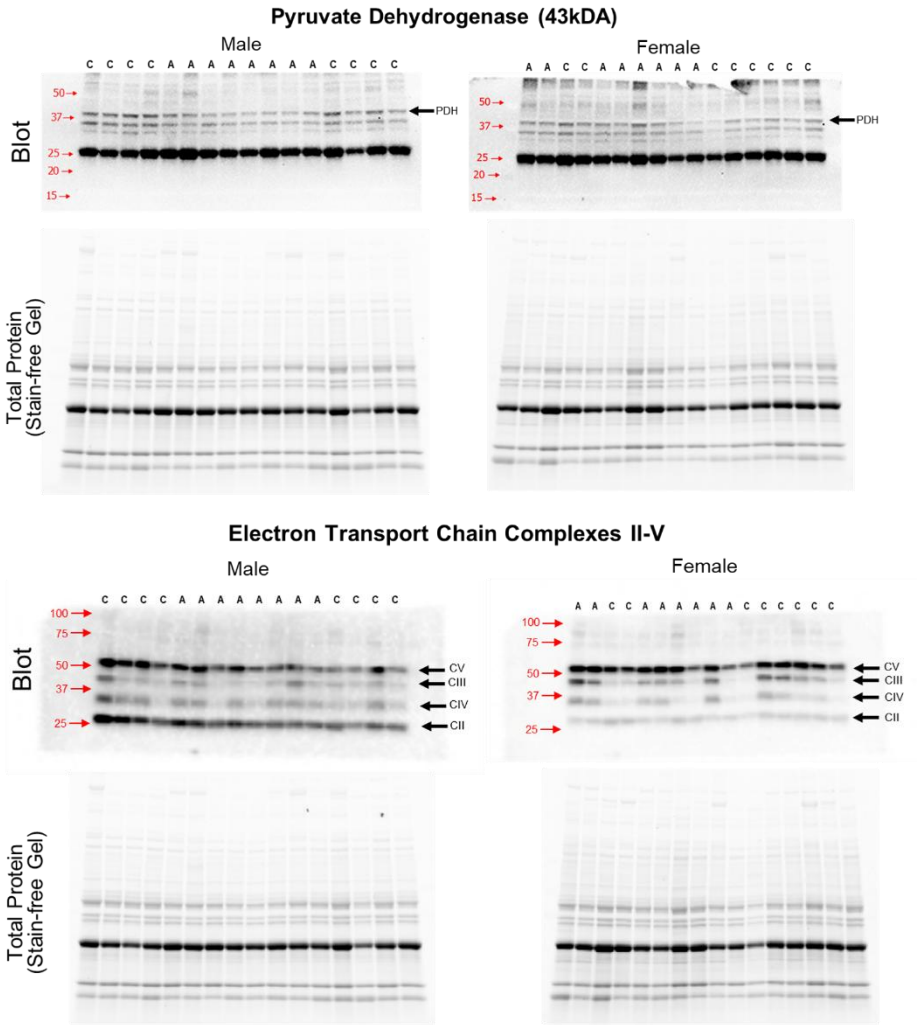

Figure A2. Western Blots
Western blotting full membranes and gels from soleus muscles of control (C) and $_{ADI}$CKD (A) rats.

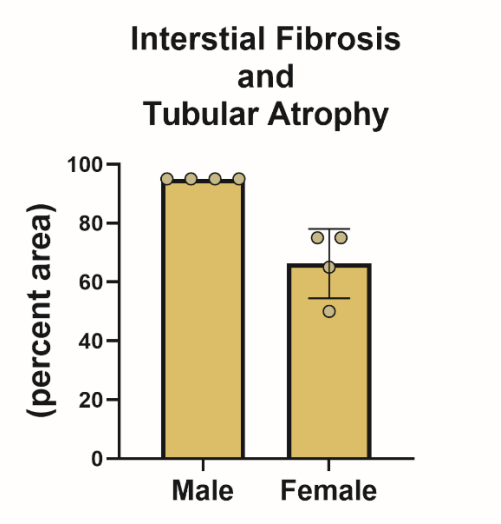

Figure A3. Interstitial Fibrosis and Tubular Atrophy Scoring
Scoring of $_{ADI}$CKD kidneys from four rats from each disease group completed by a clinical pathologist (JYJ).

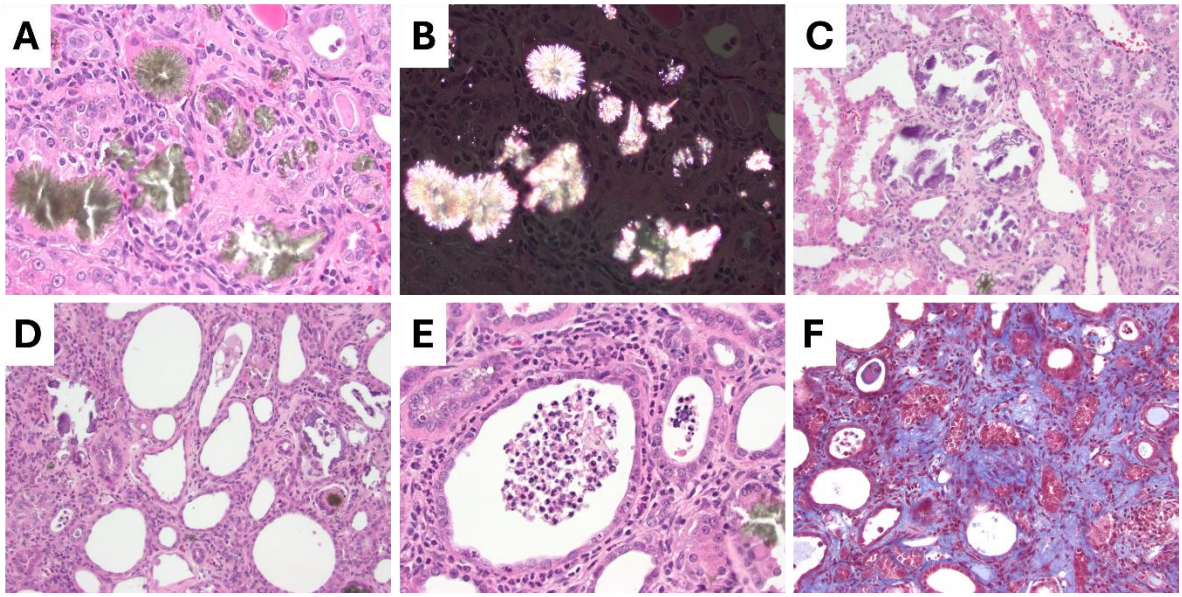

Figure A4. Histological findings in $_{ADI}$CKD animals.
A) Abundant 2,8-dihyroxyadenine crystals appear slightly brownish grey on H&E stain and B) are birefringent under polarized light (400X). C) Numerous large pale purple calcium phosphate crystals are seen on H&E stain (200X). Note the adjacent hypertrophic proximal tubules showing tubular injury. D) Frequent tubules demonstrate microcystic dilation, likely due to obstruction (H&E; 200X). E) Scattered tubules contain luminal microabscesses with neutrophils and cell debris, likely indicating acute pyelonephritis (H&E; 400X). F) Trichrome stain showing interstitial widening due to fibrosis (200X).

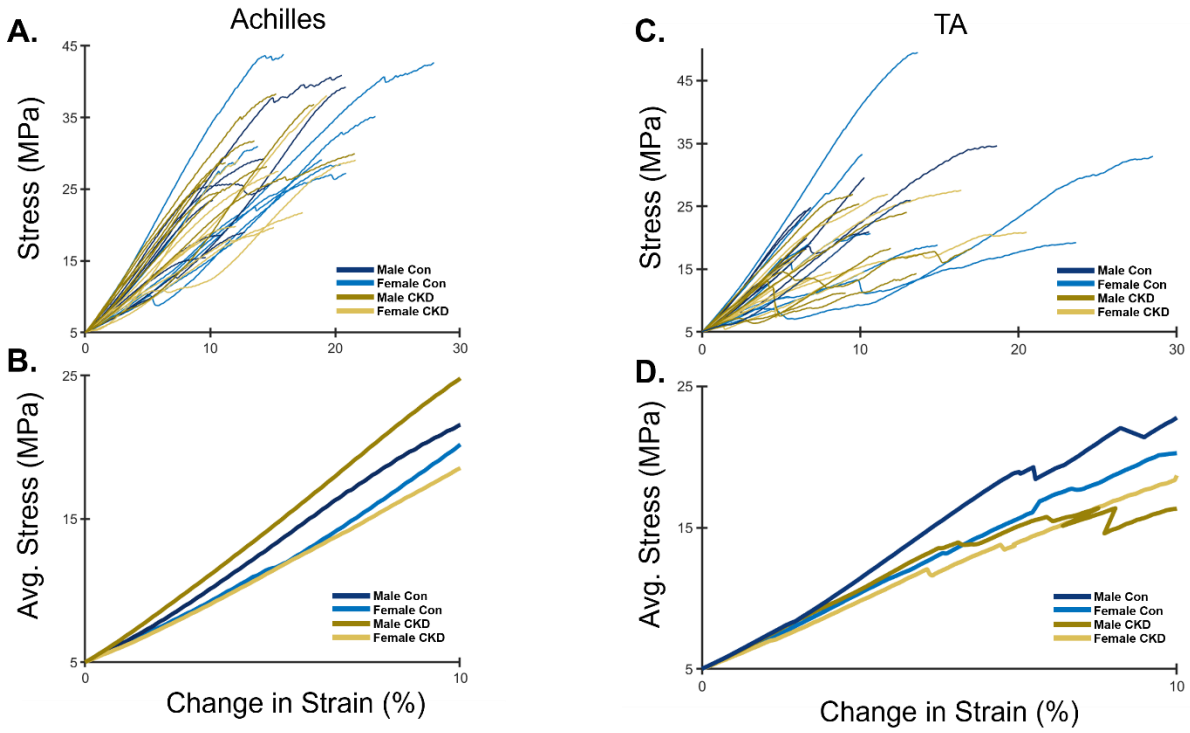

Figure A5. Stress strain curves of tendons normalized to strain at 5 MPa.
A&C) Stress strain curves of induvial Achilles and TA tendons from all four experiment groups (n=8 per group). B&D) Average stress strain curves for each group, zoomed in to highlight the typical linear region. To align traces for plotting and averaging strain was re-zeroed post-hoc from the point at which stress equaled 5 MPa. Con, control animals; CKD, animals with adenine-diet induce chronic kidney disease.

Chapter 3: Treadmill exercise benefits muscle, fails to improve bone, and negatively affects tendon in rats with kidney disease

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Key Points

1. Sixteen weeks of adenine-induced CKD led to muscle atrophy and bone wasting, but did not induce tendon weakness in skeletally mature sedentary rats
2. CKD did result in increased toe-region energy and a greater strain at 10MPa in sedentary rats Achilles tendons
3. Eight weeks of uphill treadmill exercise training prevented muscle atrophy, but not the decline in strength or endurance in rats with CKD
4. Eight weeks of uphill treadmill exercise training improved bone structure and function in healthy, but not CKD rats
5. Eight weeks of uphill treadmill exercise training led to tendon weakness in CKD rats

Abstract

We recently showed that CKD decreases tendon strength in developing rats; however, the effect of CKD on tendons in skeletally mature animals is unclear. Thus, this study set out to determine whether CKD negatively affected tendon strength in adult rats and whether treadmill running could rescue disease-induced dysfunction. Female Sprague-Dawley rats (age=7 mo, n=36) were divided into control (Con), and kidney disease-inducing 0.25% adenine diet (CKD) groups. After 8wk, groups were further split into sedentary (Sed) and treadmill running (Tread; 30min, 10% grade, 5x/week) subgroups for an additional 8wk. Achilles tendons were excised and tested via a monotonic ramp to failure. Muscle and bone structure and function were also evaluated. The effect of disease under sedentary conditions, the effect of exercise in controls, and the effect of exercise in disease were evaluated using Fisher's least square differences. CKD was confirmed through elevated creatinine (1.3 vs 0.57mg/dL, CKD vs CON, p=0.008) and blood urea nitrogen (82.4 vs 17.4mg/dL, p=0.007). Treadmill exercise improved gastrocnemius muscle mass 28% [95%CI 10, 46] and reduced diaphragm stiffness 59.9% [-115.8, -3.9], but did not significantly affect bone in CKD animals. Maximum tensile load (MTL), failure stress, CSA, and modulus were not different in CKD-Sed and Con-Sed; however, CKDsed groups did have significantly higher Toe Region Energy and strain at 10MPa (170% [45, 295] and 35% [3.7, 65.66] higher, respectively). Contrary to our hypothesis, MTL was lower in the CKD-Tread group (p=0.0254 vs CONsed), despite no evidence of CKD related deficits in tendon size or collagen content. Together, these data confirm that exercise is an effective intervention for musculoskeletal health; however, our results suggest that in the context of CKD exercise training may not improve bone and may lead to decreased tendon strength.

INTRODUCTION

Spontaneous tendon ruptures are debilitating injuries that appear to occur at disproportionately high rates in individuals with chronic kidney disease (CKD) (Shah, 2002; Humbyrd *et al.*, 2018). Tendon injury is associated with decreased physical activity (Corrigan *et al.*, 2018), increased risk of hip fracture (Alem *et al.*, 2000), and increased rates of cardiovascular complications, including deep vein thrombosis and arrythmias (Nilsson-Helander *et al.*, 2009; Barfod *et al.*, 2020; Gür *et al.*, 2023). Tendon degeneration precedes tendon rupture (Kannus, Pekka & Jozsa, Laszlo, 1991), and likely contributes to the chronic musculoskeletal pain observed in 40% of CKD patients (Caravaca *et al.*, 2016). Despite this, almost no experimental data exists regarding CKD-related tendon pathology and thus therapeutic interventions are nonexistent.

We recently found that tendon tensile strength (failure stress) was ~25% lower in Sprague Dawley rats with adenine-diet-induced CKD compared to controls rats (Hayden *et al.*, 2025). To our knowledge, this was the first experimental evidence of impaired tendon mechanics in CKD, establishing a platform for mechanistic research. As opposed to other organs that are primarily composed of parenchymal cells (liver, kidney, heart, muscle, etc.), less than 20% of tendon mass is cellular. The major function of tendon cells is to produce and secrete components of the extracellular matrix (ECM), which makes up the remaining 80% of tendon mass and is central to tendon function (force transfer). Tendon cells produce ECM as part of their normal function and this is increased in response to mechanical load (Passini *et al.*, 2021; Nakamichi *et al.*, 2022) as well as nutritional and molecular cues (Nourissat *et al.*, 2015). Thus, mechanically loading tendons is a primary strategy to prevent dysfunction and repair injury (Baar, 2017, 2019; Tam & Baar, 2025). Although the processes remain incompletely understood, physical exercise

increases collagen synthesis in human tendons (Miller *et al.*, 2005), and has been shown to improve tendon function in healthy populations (Bohm *et al.*, 2015).

Exercise is a multipotent intervention for CKD, with demonstrated benefits for musculoskeletal health (Hayden *et al.*, 2024). However, this research has mainly focused on muscle (Noor *et al.*, 2021) and to a lesser extent bone (Cardoso *et al.*, 2020), and the effects of exercise on tendon health in CKD have not been evaluated. As such, the purpose of this study was to evaluate the use of exercise training in combatting the negative effects of CKD on tendon function. Because the adenine-diet-induced CKD model also exhibits pathological changes to bone and skeletal muscle – osteodystrophy, cachexia, muscle and bone weakness – we evaluated the effect of chronic exercise on these tissues as well.

Our previous study was completed in juvenile animals; however, CKD is primarily a disease developed during adulthood. Further, in humans, kidney disease needs to be present for at least 3 months to be considered chronic, whereas our previous intervention lasted just over 2 months. While two months in a rodent model is significantly longer comparatively to three months in humans, we did not determine the onset of kidney dysfunction and thus lack an understanding of the duration of disease. For these reasons, we employed the adenine-diet model in adult animals for a period of four months. Additionally, following 8 weeks of disease induction, we used 8 weeks of uphill treadmill exercise to evaluate exercise as a therapeutic for CKD-related dysfunction.

METHODS

Animal model

Thirty-six 7-month-old female Sprague Dawley rats (retired breeders, Charles River Laboratories, Wilmington, MA, USA) were housed two/three per cage with a 12 h light and dark cycle, and ad libitum access to food and water. After four weeks of acclimation animals were equally separated into control diet (Con, AIN 93G, 0.3% phosphorus, 0.5% calcium, Bio Serv F3156, Flemington, NJ, USA) or CKD-inducing diet groups (CKD, AIN 93G + 0.25% adenine, 0.9% phosphorus, 0.6% Ca, Inotiv TD.180493, West Lafayette, IN, USA). Groups were created to equally match maximal baseline torque of the plantarflexors. Female rats were used based on two observations from our previous work (Hayden *et al.*, 2025): 1) young male rats fed a 0.25% adenine diet for 8-weeks exhibited renal impairment that was severe enough to likely be incompatible with a 16-week study, 2), the young male rats maintained on the control diet consumed chow at a rate greatly exceeding adenine diet animals, leading to an obese and hyperglycemic phenotype that made for a poor "healthy" control. Therefore, female rats alone were used in this work, as female rats from our previous study did not exhibit either of these issues and female models have historically been understudied.

After 8 weeks on Con or CKD diets, half of the animals from each group were assigned to either the sedentary (Sed) or treadmill exercise (Tread) arms for the following 8 weeks; creating four groups (Con-Sed, Con-Tread, CKD-Sed, CKD-Tread). The exercise groups completed 30 minutes of treadmill running up a 10% grade 5 days per week. The first week of treadmill running was performed at 10m/min with the subsequent weeks being 15m/min. Each time the rats were run they were allowed to acclimate to the treadmill for 3 minutes with treadmill off but the electrical grid on (1 Hz and 2 mA) to help them learn to stay on the treadmill belt area it was

shielded from light. To encourage running, rats were prodded with a soft-bristled brush when they fell back onto the back quarter of the treadmill prior to reaching the grid. Animals that refused to stay on the treadmill on a given day were allowed to sit on the grid with the electricity off. A log was kept tracking which animals had difficulty with treadmill adherence. No disease effects on adherence were noted. Food was weighed and replenished once per week. Total food consumption was divided by the number of rats in the cage to estimate intake. At 5 (4 for the exercise group) and 10 weeks, the CKD groups were fed standard chow to help prevent them from reaching humane endpoint weight loss.

Blood chemistry markers

Blood chemistry markers were measured in the afternoon in week 5, 10, and at study termination (week 16). At weeks 5 and 10, blood was sampled from the lateral saphenous vein using a hypodermic needle and a heparin coated syringe while the animals were anesthetized with 2.5% isoflurane. At study termination blood was sampled directly from the heart via cardiac puncture with a hypodermic needle and a heparin coated syringe. The left ventricle was targeted; however, this blood likely represents mixed arterial-venous blood. Immediately following collection ~100µl of blood was tested using an i-STAT CHEM 8+ Cartridge (Abbott Laboratories, Chicago, IL, USA).

In vivo muscle testing

At baseline and after 16 weeks, maximal *in vivo* muscle torque was measured. Rats were anesthetized in a sealed chamber with 2.5% isoflurane. Once the absence of a righting response was confirmed and breathing rate had slowed, they were transferred to the force transducer platform, and their muzzle was placed inside an anesthetic nose cone. Adequate sedation was confirmed by a lack of response to toe pinching. Fur was then removed from the right hindlimb

using an electric razor. Rats were placed in front of the force transducer with their knee positioned in a custom brace. The hip, knee, and ankle of the tested leg were aligned at 90 degrees and the foot was taped to the force transducer pedal (Aurora Scientific, Aurora, Ontario). Forceps were then used to pull the skin of the right hindlimb away from the distal third of the gastrocnemius muscle and a dual-needle electrode (needles spaces ~5mm apart) was then inserted into the skin without puncturing the muscle. The needle electrode was positioned so that when the skin was released it pulled the electrode up against the gastrocnemius just distal to the muscle belly, and the positive electrode was distal to the negative. This dual-needle electrode was used to maintain consistent electrode spacing and depth and was placed lower on the leg to avoid stimulation of the hamstring muscles. The stimulation box (Aurora Scientific, Aurora, Ontario) was set to ~50mA with a biphasic pulse, current was increased slightly if necessary to elicit maximal twitch force monitored using the InstantStim mode (150Hz stimulation every other second). If the force tracing had signs of co-contraction, doubles peaks, or negative force defections, the electrodes were repositioned. Plantar flexor strength was calculated as the maximal torque from the contraction with the highest peak and peak rate of force development was calculated as the greatest slope during the onset of this contraction.

For the determination of muscle strength, a force-frequency test was performed by stimulating at 20, 40, 60, 80, 100, 125, 150, 200Hz (pulse width 0.2ms, duration 0.350s) with 20s of rest between contractions. Following the tests, data were plotted in MATLAB (2020a, The MathWorks Inc., Natick, Massachusetts) and a baseline correction was applied using a custom script, before peak force was extracted. Torque was calculated by multiplying the force reported by the system by the lever arm length used during calibration (32mm). Muscle endurance was determined in a second protocol which was performed following a 3-minute rest period after the

maximal force testing. This protocol consisted of180contractions (70Hz pulse frequency, 0.2 ms pulse width, and 350 ms duration) with 2s between contractions while torque was measured as previously (Fusagawa *et al.*, 2023; Hayden *et al.*, 2025). Endurance was quantified by the average torque produced normalized to peak force during the protocol.

Euthanasia and tissue collection

Following the sixteenth week of intervention (7 days after the last bout of treadmill running and 5 days after muscle force testing), tissues were collected from animals anesthetized with 2.5% isoflurane followed by euthanasia via pneumothorax and cardiac puncture. Muscles and kidneys were snap frozen in liquid nitrogen and stored at -80° C. Achilles tendons were isolated with the gastrocnemius muscle and the foot and stored in phosphate buffered saline soaked gauze at -80° C until testing. For mechanical testing, excised femurs were stored in phosphate buffered saline soaked gauze at -80° C, whereas for micro–CT they were placed in 70% ethanol. Diaphragm muscle was isolated with several ribs, placed in cold Ringer's solution, and immediately mechanically tested. Blood was collected via cardiac puncture and was left at room temperature and allowed to clot before centrifugation at 2000 g for 15 minutes at which point serum was transferred and stored at -80° C.

Ex vivo muscle testing

Diaphragm muscle mechanical function was examined as in previous studies (Smith & Barton, 2014; Brashear *et al.*, 2021; Hayden *et al.*, 2025) (Experimental Setup, Fig. 3C). Diaphragm muscles with the surrounding rib cage were stored in Ringer's solution on ice with bubbling oxygen. Strips were cut from the diaphragm and 7-0 sutures were tied onto the rib cage and at the central tendon to connect the muscle to a 300C-LR Dual-Mode motor arm and force transducer (Aurora Scientific). Optimum muscle length (L_0) was determined by performing

twitches as the muscle was slowly stretched using the 701C high power stimulator (Aurora Scientific), with the length set at the largest twitch amplitude (Moorwood *et al.*, 2013). L_0 was measured using a caliper between the rib cage and the myotendinous junction at the central tendon. A passive mechanical testing protocol was performed where the muscles were cyclically strained 2.5% Lo at 1Hz for 5 seconds. This was repeated at 5, 7.5, 10, and 12.5% L_0. Immediately following 10% L_0 cyclic stretching, the muscle was held at 10% strain for 2 minutes to obtain a stress relaxation curve prior to undergoing 12.5% L_0 cyclic strain. At the end of the 12.5% strain, muscles were returned to L_0. A custom MATLAB script was used to fit a quadratic function to the peak stresses and to the nadir stress (following 2 minutes of relaxation). Dynamic stiffness was calculated as the tangent slope at 10% strain of a quadratic fit of the peaks, and elastic stiffness as the tangent slope at 10% strain of the quadratic fit of the nadir as previously described (Smith & Barton, 2014) (Fig. 4B).

Following passive mechanics, an active fatigue protocol, consisting of an isometric twitch contraction followed by 25 maximum isometric tetanic contractions 5 seconds apart, was performed on each diaphragm strip. After the 25th contraction, there was a 5-minute waiting period before a final isometric tetanic contraction to assess muscle recovery. After completion of mechanical testing, the muscle strip was isolated, and muscle mass was measured to determine physiological cross-sectional area (PCSA).

Bone Micro-Computed Tomography (μCT)

Femurs from rats were scanned using micro-computed tomography (SCANCO Medical, μCT 35, Brüttisellen, Switzerland) with 10μm nominal voxel size (X-ray tube potential= 55 kVp, current= 114mA, integration time= 900 ms, number of projections= 1000/180°). Trabecular bone was analyzed at the distal femoral metaphysis. Trabecular ROIs were manually drawn on

transverse images, excluding the cortical surface. The regions of interest for the distal femoral metaphysis were located 1.5mm from the start of the growth plate and extended 1 mm proximally. Analysis of cortical bone in the femoral diaphysis was performed by contouring transverse slices, with a 1mm region of interest (100 slices) centered on the midpoint of the femur. Femur midpoint was calculated from length as derived from CT scan. Microstructural parameters were determined using the manufacturer's analysis software.

Mechanical Testing of Femur Midshaft

3-point bending was used to determine structural and material properties of femora using a materials testing system (Instron, TA Instruments, New Castle, DE, USA). The span length of the lower supports was 10 mm, and the femur was positioned to load the anterior aspect of the bone in tension. A 1–2 N preload was applied to ensure contact with the upper platen, then loading was applied at a displacement rate of 0.01 mm/sec until failure. Resulting force and displacement was recorded at 50 Hz and analyzed to determine whole bone stiffness, yield force, ultimate force, and post-yield displacement.

Tendon mechanical testing, differential scanning calorimetry, and collagen assay

Achilles tendons were thawed in PBS-soaked gauze at room temperature before testing. Physiological length was measured as the distance from the calcaneus to the most distal region of the gastrocnemius muscle with the ankle at 90°, following which the gastrocnemius was removed (and weighed) and the top of the tendon was fixed inside a folded piece of 80-grit sandpaper, sand side in, using fast-drying cyanoacrylate . This left the physiological free tendon (~9mm) between the sandpaper and calcaneus exposed for testing. Cross-sectional area (CSA) was calculated by multiplying tendon width and depth measured at the narrowest point of each tendon using optical coherence tomography (OQ LabScope, Lumedica Inc, Durham, NC, USA).

Tendons were then placed into the tensile tester (Model 68SC-1, 100N load cell, Instron, Norwood, MA, USA) with the sandpaper and the entire foot clamped into the top and bottom pneumatic grips (80psi), respectively. Gauge length was measured using digital calipers and samples were immersed in a 0.9% PBS bath held at 37°C. Following 10 preconditioning cycles (0.10–0.25 N, 0.25 mm/s), tendons were mechanically tested using a pseudo-static monotonic ramp to failure (elongation at 0.25 mm/s until failure: defined as an 80% decline in force). Failure was confirmed by gently passing a pair of forceps through the remaining tendon tissue if rupture was not visually obvious. Force and displacement were recorded throughout. Mechanical strength was determined as the highest tensile force obtained before failure, or maximal tensile load (MTL; N). Maximal apparent stiffness (N/mm) was calculated as the steepest slope using a least-squares fit of the load-displacement curve (excluding the preconditioning phase) – representing the greatest capacity of the tissue to resist elongation. Failure stress and maximal modulus (material properties) were calculated by normalizing MTL to CSA and maximal stiffness to CSA and initial length, respectively. Toe region mechanics were investigated using the amount of energy needed to reach the modulus x-intercept ("toe region energy") and the amount of strain at 10MPa. Both legs for each animal were tested and the tendon with the higher MTL for each animal was used in analysis. When there was evidence of tendon slipping (e.g., strain to failure > 40%), strain dependent measures for that sample was excluded.

Following tensile testing, each tendon was separated into proximal and distal portions, lightly blotted to remove excess liquid, placed on the bottom of a Tzero® aluminum pan, and sealed using an aluminum hermetic lid and Tzero® press. Samples were then weighed and placed into a differential scanning calorimeter (DSC; Discovery, TA Instruments, New Castle, DE, USA) and heated from 25 to 90° C at a rate of 10° C/min. The melt curve (heat flow vs temperature) was

then evaluated to extract the Onset Temperature (the temperature at which material transitions begin), the Peak Temperature (temperature at which 50% of the material transitions have occurred), and the Enthalpy (the amount of energy needed to transition all of the material) was calculated as the area under the melt curve.

Collagen content was then measured using a hydroxyproline assay (Woessner, 1961). The proximal and distal Achilles samples were then removed from the DSC pans and dried at 120° C for 30 min, after which they were weighed again to determine dry weight. Tendons were then hydrolyzed in 6N HCl for 2 hours, following which HCl was boiled off in a fume hood. The samples were then resuspended in 1000 µL of hydroxyproline buffer (173 mm citric acid, 140 mm acetic acid, 588 mm sodium acetate, 570 mm sodium hydroxide), following which they were diluted 1:30 further in hydroxyproline buffer to keep collagen content within range of the assay. Next, 150 µL of Chloramine T solution was added to each sample, before mixing and incubating for 20 min at room temperature. After incubation, 150 µL of aldehyde-perchloric acid (60% 1-propanol, 5.8% perchloric acid, and 1M 4-(dimethylamino) benzaldehyde) was added to each tube, and the samples were incubated at 60°C for 15 min. Samples were cooled for 5 minutes, and 200 µL of each was added to a clear-bottomed 96-well plate and absorbance at 550 nm was read on an Epoch Microplate Spectrophotometer (BioTek Instruments Limited, Winooski, VT, USA). Collagen content was then determined by comparing sample absorbance to the standard curve, assuming hydroxyproline constitutes 13.7% amino acid composition of collagen.

Statistics

Data were screened for outliers using the ROUT method and a Q=1%. Outliers and any other values dependent on outlying measurements were removed from analysis. Outcomes were analyzed using a two-way ANOVA with factors Disease (control vs disease) and Activity

(sedentary vs exercise). Based on *a priori* hypotheses, planned comparisons were performed to assess: 1) the effect of disease under sedentary conditions (Con-Sed vs CKD-Sed), 2) the effect of exercise in controls (Con-Sed vs Con-Tread), and 3) the effect of exercise in disease (CKD-Sed vs CKD-Tread) using Fisher's least square differences. Effect sizes and 95% confidence intervals are reported. Due to unexpected negative effects of exercise on tendon mechanics an unplanned post hoc paired t-test (between Con-Sed and CKD-Tread groups) was used in several instances to estimate this effect. All statistics were performed using GraphPad Prism (v. 11.0.1, GraphPad Software, Boston, MA, USA).

RESULTS

Sixteen weeks of adenine feeding induced renal dysfunction in adult rats

Adenine feeding successfully induced kidney disease in female adult Sprague Dawley rats as determined by elevated creatinine nitrogen blood urea and levels (Fig. 1). This elevation was seen after five weeks of feeding (creatinine +0.87mg/dL 95%CI [0.24, 1.5], BUN +27mg/dL [6.7, 47] CKD-Sed vs Con-Sed), and was maintained at 10 weeks (creatinine +2.4mg/dL [0.41, 4.4], BUN +64mg/dL [29, 99] CKD-Sed vs Con-Sed), and 16 weeks (creatinine +0.72mg/dL [0.31, 1.1], BUN +65mg/dL [30, 100] CKD-Sed vs Con-Sed). Hemoglobin was also lower in CKD animals at all time points (-1.89g/dL [-2.67, -1.10] at week 5, -3.03g/dL [-4.58, -1.47] at week 10, and -6.13g/dL [-8.98, -3.27] at week 16). On average, CKD animals lost significantly more body weight than controls (-13.6% vs +7.3%, $p<0.0001$). Additionally, Con-Sed gained significantly more weight than Con-Tread (+11.7% vs +2.9%, $p=0.0160$), while there were no differences in weight change between CKD-Sed and CKD-Tread (-17.3% vs -10.0%, $p=0.2439$). Estimated food consumption was 16.4, 15.9, 13.6, and 13.2g/animal per day for Con-Sed, Con-Tread, CKD-Sed, and CKD-Tread, respectively. Four animals reached humane endpoints (e.g., body weight loss) requiring euthanasia prior to the end of the study, including three in the Con-Sed group (week 10, 13, and 14) and one in the control treadmill group (week 14). Tissues from these animals were collected in an identical manner to the rest of the study except for week 16 blood measures that were not obtained. Additionally, one animal from the Con-Sed group was euthanized due to unrelated disease one week into the study from which no tissues were collected. Data points representing animals that were euthanized early were not statistical outliers and are therefore included in analyses; however, they are colored red for transparency.

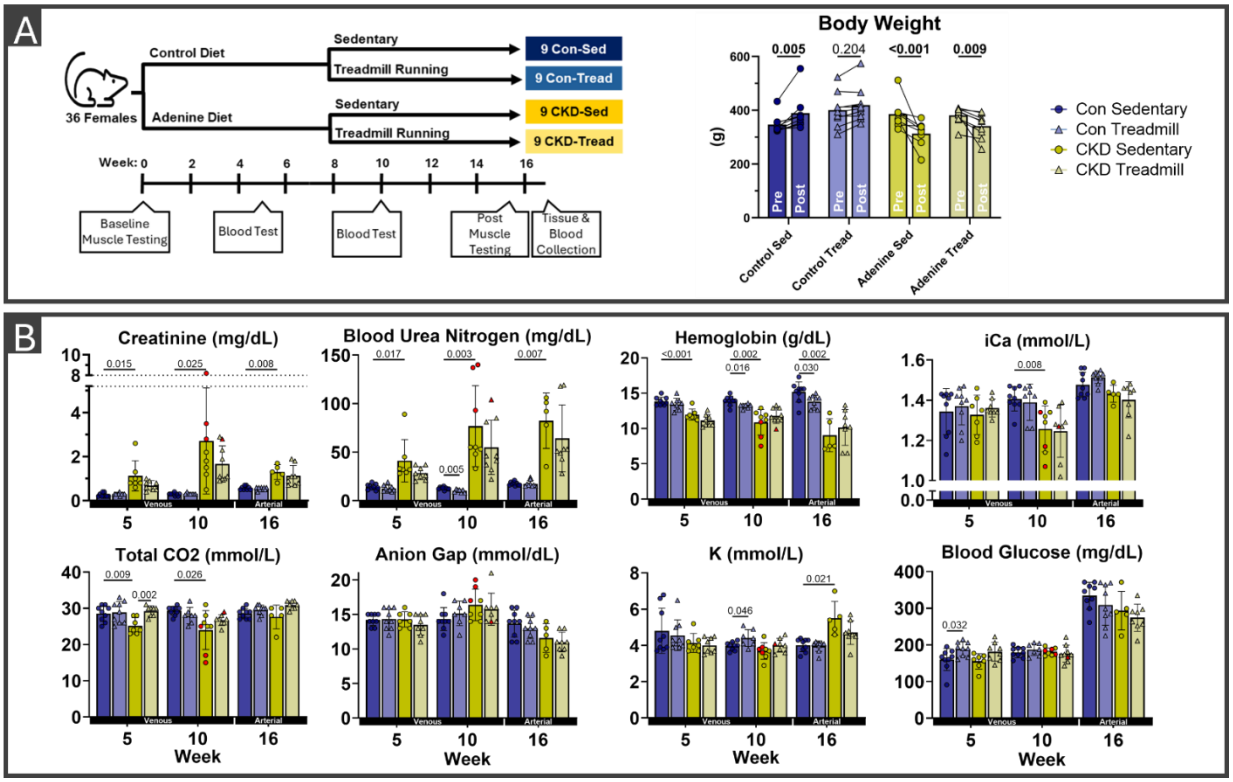

Figure 1. Eight weeks of treadmill exercise does not affect body weight or blood biochemical profile in CKD animals. A 0.25% adenine diet was used to induce and maintain chronic kidney disease over 16 weeks in 7-month-old Sprague Dawley rats. At the 8-week mark, treadmill exercise (30min/day, 5 days/week, 15m/min, 10% incline) was introduced as an intervention. A) Study design, timeline, and animal body weight by group at baseline and study completion. B) Blood chemistry profile at weeks 5, 10, and 16 across study groups. Fisher's LSD comparisons were made between sedentary groups to evaluate the effects of disease, and within control and CKD groups to evaluate the effects of exercise. Values are represented as mean ± SD. Con, control animals; CKD, chronic kidney disease. Red data points are from animals terminated prior to 16 weeks.

Treadmill training improves muscle mass but not strength or endurance in rats with CKD

Gastrocnemius muscle mass was 19% lower (95% CI, [4.4, 34]) in CKD-Sed animals compared to Con-Sed (Fig 2A). In response to eight weeks of treadmill exercise the gastrocnemius weight in the Con-Tread group was 11% [3, 26] greater than Con-Sed and the CKD-Tread group was 28% [10, 46] higher vs the CKD-Sed group (Fig 2A). Soleus mass was not significantly affected by disease (-7% [-23, +10], CKD-Sed vs Con-Sed), or treadmill exercise (+11% [-5, +27.42] Con-Tread vs Con-Sed; and +12% [-5.3, +30] CKD-Tread vs CKD-Sed). Extensor digitorum longus (EDL) mass was 14% [0.2, 27] lower in CKD-Sed vs Con-Sed, and was 16% [2.2, 29] and 18% [2.8, 34] greater in Con-Tread and CKD-Tread groups vs their

respective sedentary controls (Fig 2A). Plantar flexor strength decreased significantly in the CKD-Tread group (-10.8%, [-21.5, -0.2]), but not in the Con-Sed (-6.3%, [-17.9, +5.4]), CKD-Sed (-6.4%, [-19.6, +6.8]), or Con-Tread groups (-1.6%, [-11.6, +8.5], Fig 2B). Plantar flexor endurance decreased significantly in the Con-Sed (-13%, [-20, -6.3]), CKD-Sed (-16%, [-24, -7.9]), and CKD-Tread groups (-12%, [-19, -5.4]), but not in the Con-Tread group (-3.6, [-10, +2.9], Fig. 2B).

Treadmill training improves diaphragm compliance but not specific force or fatigue resistance

Diaphragm muscle maximal specific force and post fatigue specific force were not affected by CKD, treadmill running, or the combination of the two (Fig. 2C). There was no effect of CKD on diaphragm dynamic or elastic stiffness (Fig. 2C). Diaphragm dynamic stiffness was significantly lower in the CKD-Tread group compared to the CKD-Sed (-58.8%, [-112.4, -5.2]), but not significantly lower in Con-Tread compared to Con-Sed (-40.3%, [-81.5, +0.9], Fig. 3C). While treadmill running resulted in significantly lower elastic stiffness in both Con-Tread (-44.6%, [-86.0, -3.2]) and CKD-Tread groups (-59.9%, [-115.8, -3.9]) compared to their respective controls (Fig. 2C).

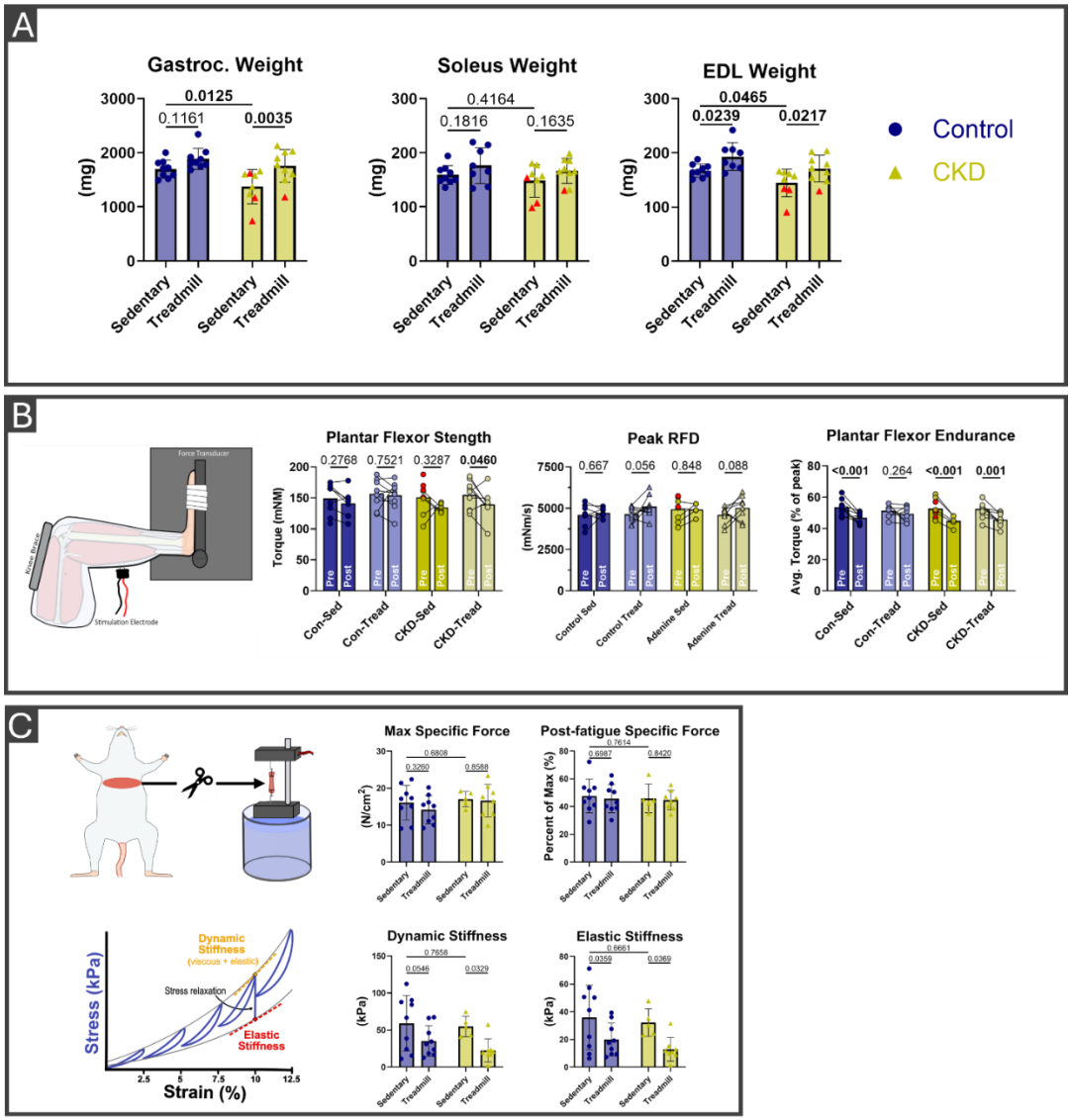

Figure 2. Eight weeks of treadmill exercise improves muscle mass and reduces diaphragm stiffness but does not affect muscle strength or endurance in CKD animals. Sprague Dawley rats (n=36 female) with and without adenine-diet-induced chronic kidney disease (CKD) completed 8 weeks of treadmill exercise (30min/day, 5 days/week, 15m/min, 10% incline). A) Muscle mass at study termination. B) Plantar flexor strength and endurance measured via direct muscle electrical stimulation *in vivo* with schematic of experimental setup. C) Active and passive mechanics of diaphragm muscle strips tested *ex vivo*, testing setup is shown in top left and schematic of passive mechanics protocol in the bottom left. Fisher's LSD comparisons were made between sedentary groups to evaluate the effects of disease, and within control and CKD groups to evaluate the effects of exercise. Values are represented as mean ± SD. Gastrcoc, gastrocnemius; pre, baseline measures performed at week 0; post, measures performed at week 16; RFD, rate of force development. Red data points are from animals terminated prior to 16 weeks.

Bone fails to significantly adapt to treadmill training in rats with CKD

Animals with CKD exhibited lower cortical thickness, tissue mineral density, and bone mineral density compared to control animals (Fig. 3A). Cortical porosity and cortical area were also higher in CKD animals compared to controls, which can be observed visually in representative images (Fig. 3A). Trabecular thickness was higher in CKD animals, while trabecular tissue mineral density and degree of anisotropy was down (Fig. 3B). Eight weeks of treadmill running resulted in lower cortical tissue and bone mineral density, but greater cortical area in control animals while no changes were seen in CKD (Fig. 3A). Similarly, treadmill running decreased trabecular tissue mineral density but increased anisotropy in control animals with no effect observed in CKD. Unlike the structural characteristics, no deficits in bone strength were observed in sedentary CKD animals (Fig. 3C). However, following treadmill running the yield force in the Con-Tread group was 36% (95% CI [6.3, 65]) higher than the Con-Sed group, with similar but nonsignificant improvements in stiffness (26.18% higher, [-1.07, +53.5]. No improvements were seen in any mechanical properties in CKD animals (Fig. 3C).

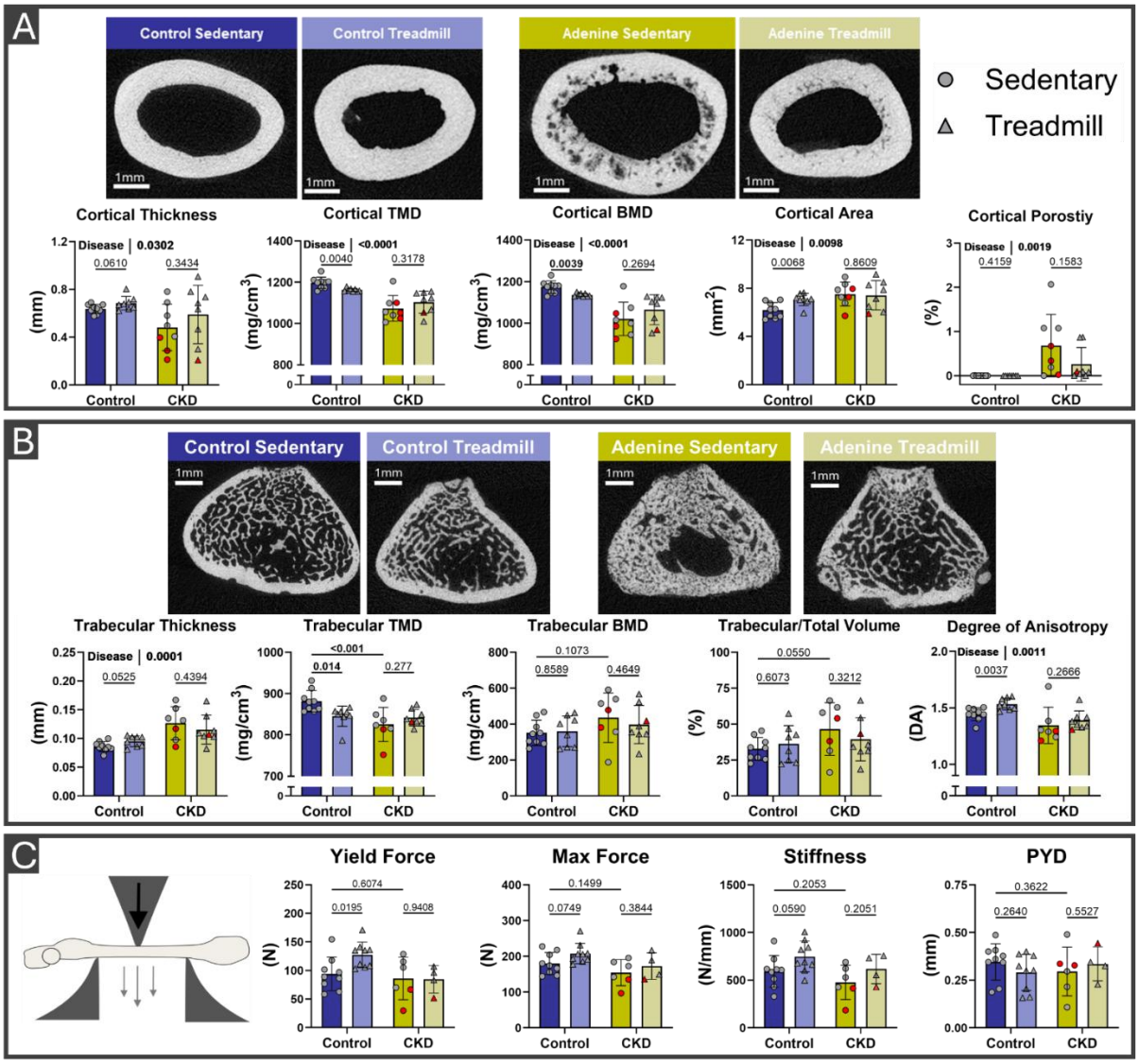

Figure 3. Eight weeks of treadmill exercise improves bone structure and mechanical strength in control but not CKD rats. Sprague Dawley rats (n=8-9 per group, female) with and without adenine-diet-induced chronic kidney disease (CKD) completed 8 weeks of treadmill exercise (30min/day, 5 days/week, 15m/min, 10% incline). A) Cortical bone structural characteristics of the femur measured via micro-CT (n≥8 per group) with representative images. B) Trabecular bone structural characteristics of the femur measured via micro-CT (n≥7 per group) with representative images. C) Mechanical strength of femurs determined via three-point bending (n=9 for controls, n=6 and 4, CKD-Sed and CKD-Tread). Fisher's LSD comparisons were made between sedentary groups to evaluate the effects of disease, and within control and CKD groups to evaluate the effects of exercise. Main effects of disease and independent t-test are used in cases of unequal variance. Values are represented as mean ± SD. TMD, tissue mineral density; BMD, bone mineral density; PYD, post yield displacement. Red data points are from animals terminated prior to 16 weeks.

Tendon toe region mechanics are altered in CKD, but strength only declines when exercise is introduced

Tendon CSA was not affected by CKD, treadmill exercise, or the combination of the two (Fig. 4A). Tendon length, however, was longer with treadmill running in the Con-Tread group alone (8.96%, 95% CI [2.2, 15] vs Con-Sed). Tendon strength (MTL), failure stress, stiffness, and modulus were not different between Con-Sed and CKD-Sed groups (Fig. 4B-C), neither was there an effect of treadmill running on these parameters in control animals. The CKD-Sed group did have altered toe-region mechanics compared to the Con-Sed group with a 170% [45, 295] higher toe region energy and a 35% [3.7, 65.66] greater strain at 10MPa of stress (Fig. 4B-C). Unexpectedly, treadmill running appeared to result in decreased tendon strength and energy at MTL in CKD animals, because these effects were the opposite of what was anticipated an unpaired t-test was used post hoc to estimate these effects. In this regard, MTL was found to be 13% [1.6, 24] lower and energy at MTL was found to be 19% [1.5, 37] lower in the CKD-Tread group compared to Con-Sed.

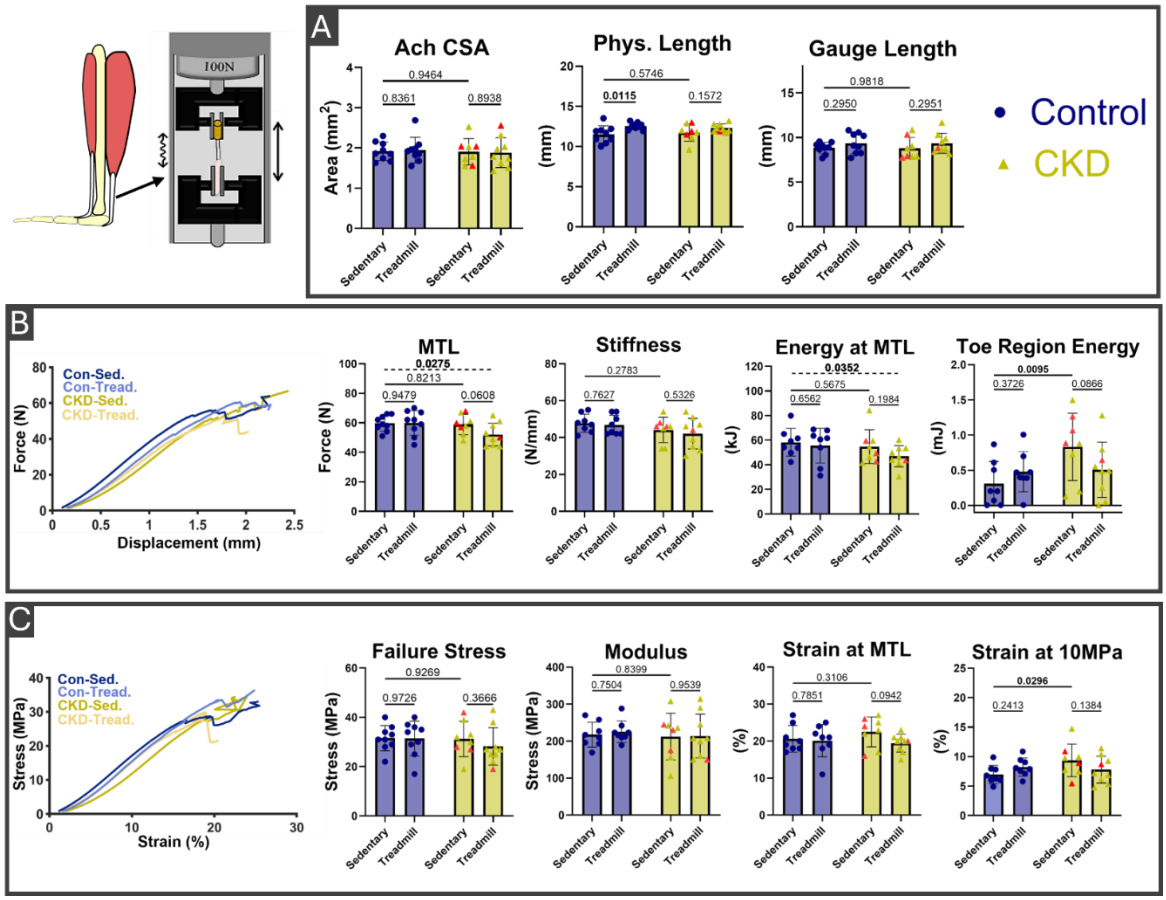

Figure 4. Eight weeks of treadmill exercise decreases tendon strength in CKD animals. Sprague Dawley rats (n=8-9 per group, female) with and without adenine-diet-induced chronic kidney disease (CKD) completed 8 weeks of treadmill exercise (30min/day, 5 days/week, 15m/min, 10% incline). A) Tendon morphology. B) Group averaged force/displacement curves and mechanical properties. C) Group averaged stress/strain curves and material properties. Fisher's LSD comparisons were made between sedentary groups to evaluate the effects of disease, and within control and CKD groups to evaluate the effects of exercise. The dotted line comparison is a post-hoc unpaired t-test. Values are represented as mean ± SD. CSA, cross sectional area; phys. length, physiological length from calcaneus to distal myotendinous junction; MTL, maximum tensile load. Red data points are from animals terminated prior to 16 weeks.

Tendon composition and thermal properties are largely unchanged by CKD and exercise

For compositional and thermal property analysis, the Achilles tendon was split into proximal and distal portions as these have been shown to exhibit distinct mechanical and transcriptional properties, and protein turnover (Arruda *et al.*, 2006; Zhang *et al.*, 2020; Disser *et al.*, 2022). Tendon collagen and water concentrations, as well as calcium content were not affected by CKD (Fig. 5A). There was no effect of exercise in the control group (Fig. 5A). In the CKD group, 8 weeks of treadmill running only affected the water concentration in the distal

tendon increasing it from 56% to 66% (a 10% increase 95% CI [1, 18]). Tendon thermal properties were unchanged by exercise in both groups (Fig. 5B), however, peak temperature in the distal tendon was 1.1°C [0.2, 2.1) higher in the CKD-Sed group compared to the Con-Sed group. There were no other significant differences noted in thermal properties.

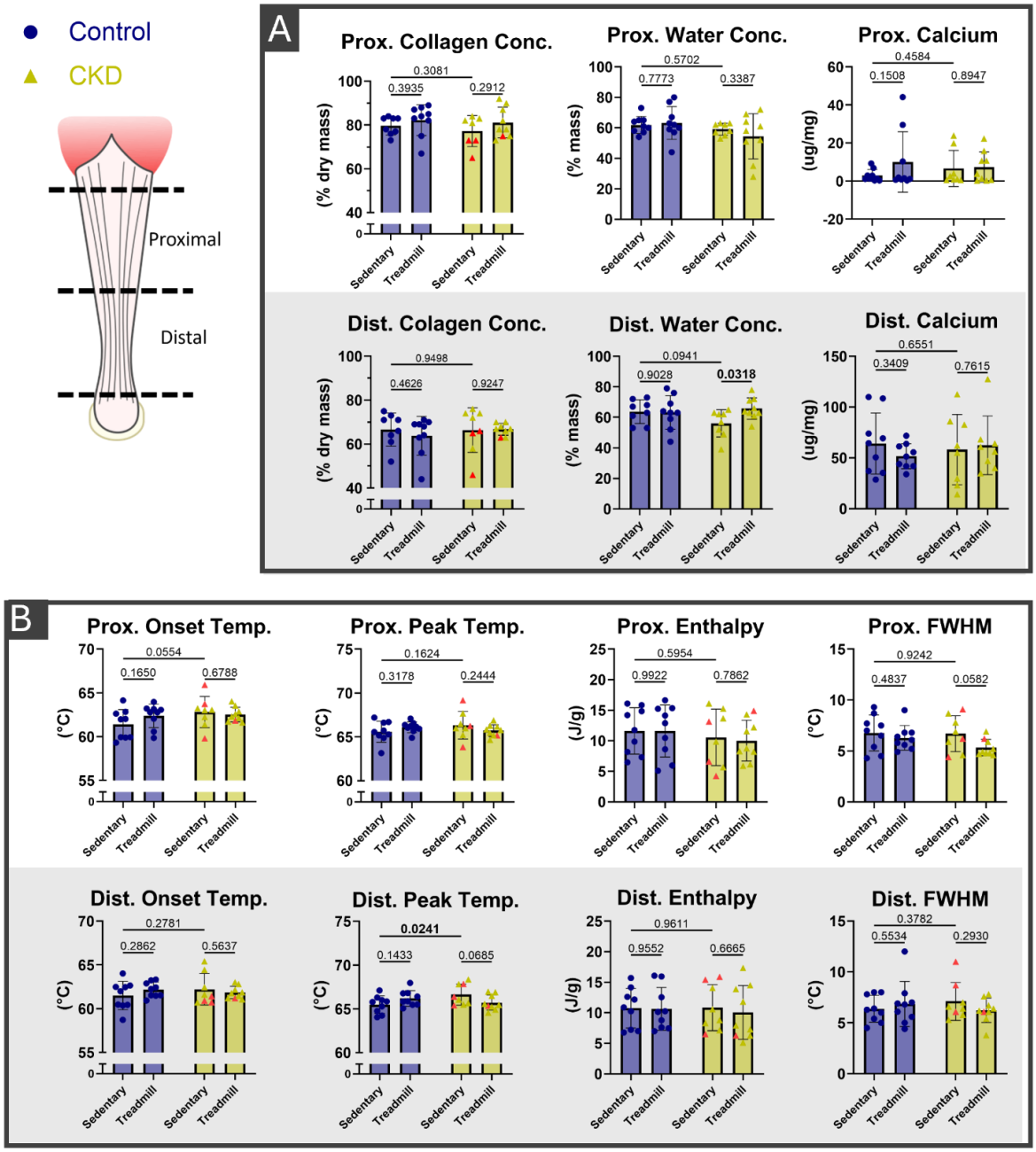

Figure 5. Minimal effects of eight weeks of treadmill exercise and CKD on tendon composition and thermal properties. Sprague Dawley rats (n=8-9 per group, female) with and without adenine-diet-induced chronic kidney

disease (CKD) completed 8 weeks of treadmill exercise (30min/day, 5 days/week, 15m/min, 10% incline). Achilles tendons were split into proximal and distal portions and analyzed separately. A) Tendon composition B) Tendon thermal properties extracted from melt curves after heating from 25-90°C on a differential scanning calorimeter. Fisher's LSD comparisons were made between sedentary groups to evaluate the effects of disease, and within control and CKD groups to evaluate the effects of exercise. Values are represented as mean ± SD. Prox., proximal tendon; Dist., distal tendon; FWHM, full width at half max. Red data points are from animals terminated prior to 16 weeks.

DISCUSSION

The goal of this study was to evaluate the efficacy of treadmill exercise training for improving tendon, as well as muscle and bone, structure and function in rats with CKD. We previously demonstrated that tendons from juvenile rats with CKD were weaker than age matched controls (Hayden *et al.*, 2025). However, because human CKD is a disease of later adulthood that progresses over long periods of time, this study used older animals (7mo vs 2mo) and a longer adenine-diet intervention (16wk vs 9wk). In this model, we found markers of renal impairment (elevated creatinine and BUN) from 5 weeks through the completion of the study, suggesting a duration in kidney disease of at least 11 weeks (Fig 1). We also found that CKD led to significant deficits in muscle and bone health in sedentary animals, and that muscle, but not bone, was positively affected by 8 weeks of uphill treadmill training (Fig. 2 and 3). Contrary to our expectations, CKD did not lead to tendon weakness in sedentary animals, although it did cause changes in viscoelasticity. Tendon strength was worse in CKD animals following chronic treadmill training. Thus, while we anticipated a more severe musculoskeletal phenotype in adult rats with a longer period of disease burden, this was only true for muscle and bone. Eight weeks of treadmill exercise had positive, negligible, and negative effects on muscle, bone, and tendon, respectively.

Muscle

We report here significant atrophy in the gastrocnemius (a plantar flexor) and EDL (a dorsiflexor) muscles in sedentary CKD animals compared to sedentary controls following 16 weeks of adenine-diet (-19% and -14% mass, Fig. 2A). Interestingly, the soleus was not significantly smaller in the CKD-Sed animals, suggesting it might be more resistant to CKD-induced atrophy (Fig. 2A). Indeed, our group and others have previously found a lack of soleus

muscle atrophy when utilizing the adenine diet model in rats (Niida *et al.*, 2020; Heitman *et al.*, 2024, 2025; Hayden *et al.*, 2025) and others have reported that the soleus is more resistant to atrophy induced by caloric restriction in healthy rats (Almurshed & Grunewald, 2000). Heitman *et al.,* also reported that the adenine diet induced an increased expression of FGF-23 in the gastrocnemius but not the soleus, quadriceps, or tibialis anterior muscle (Heitman *et al.*, 2025). Although their data also suggest that FGF-23 itself does not induce muscle atrophy, this finding demonstrates that different muscle groups can respond differently to the systemic changes induced by CKD (Heitman *et al.*, 2024, 2025). Paradoxically, both the 5/6 nephrectomy model in rats (Fusagawa *et al.*, 2023) and the adenine model in mice (Thome *et al.*, 2021, 2024) show equivalent if not greater atrophy in the soleus compared to gastrocnemius muscles. Collectively, these findings demonstrate muscle atrophy varies in rodent models of CKD and highlights the importance of reporting changes in multiple muscles.

Uphill treadmill running is a motor endurance exercise, and therefore eight weeks of led to significantly greater mass in both the gastrocnemius (+11%) and EDL (+16%) of exercised compared to sedentary control animals. Importantly, this effect was also observed in, if not surpassed by, the effects in treadmill trained CKD animals where gastrocnemius and EDL mass were 28% and 18% higher than sedentary CKD animals (Fig. 2A). It should be noted that in the CKD-Tread animals it is unclear whether exercise induces hypertrophy or prevents the muscle wasting seen with CKD. Indeed, gastrocnemius and EDL muscle mass were similar following treadmill exercise in the CKD-Tread group compared to Con-Sed (Fig. 2A). These data support the use of exercise in CKD to maintain muscle mass.

Contrary to the effects on muscle mass, treadmill exercise training did not prevent declines in muscle strength or endurance in CKD animals (Fig. 2B). Over the course of the 16-

week study the CKD-Tread group alone saw a significant decrease in muscle strength, although we suspect that similar results would have been seen in the CKD-Sed group if 4 animals were not lost to attrition. This finding, not surprisingly, suggests that treadmill exercise is not the ideal intervention for maintaining muscle strength. However, it is surprising that changes in plantar flexor muscle mass did not appear to translate to plantar flexor strength, though this the tendency for rate of force development to go up suggest this is due to the addition of sarcomeres in series rather than parallel (Fig. 2B). Muscle endurance on the other hand is a metric that might more readily improve with treadmill training, specifically with uphill training as it forces the muscle to work more as a motor opposed to as a strut (Roberts *et al.*, 1997; Dickinson *et al.*, 2000). Indeed, in control animals, treadmill training prevented the 13% decline in muscle endurance seen over 16 weeks (Fig. 2B). This effect, unfortunately, was not mirrored in CKD animals where CKD-Sed and CKD-Tread animals saw similar declines in muscle endurance over the course of the study (-16% and -13%, respectively, Fig. 2B). This suggests that some CKD related factor may be blunting exercise induced adaptations that allow muscles to resist fatigue, likely through interference with normal energy metabolism as has been previously suggested (Tamaki *et al.*, 2014; Xu *et al.*, 2020; Thome *et al.*, 2021, 2024; Fusagawa *et al.*, 2023).

Individuals with CKD suffer from diaphragm weakness and pulmonary dysfunction which is associated with dyspnea, fatigue, and adverse clinical outcomes (Faria *et al.*, 2013; Wang *et al.*, 2019; Zheng *et al.*, 2022; Moraes *et al.*, 2026). Respiratory muscle strength improves slightly with renal transplant suggesting an impact of the uremic environment on diaphragm function; although maximal inspiratory mouth pressure remained well below the lower normal limits despite relative normalization of creatinine and BUN (Tavana & Mirzaei, 2016). Specific inspiratory muscle training significantly improves respiratory muscle strength,

functional capacity, and quality of life in both hemodialysis and non-dialysis dependent individuals with CKD (Pellizzaro *et al.*, 2013; De Medeiros *et al.*, 2017; Katayıfçı *et al.*, 2024). We did not find any evidence of diaphragm muscle weakness or reduced endurance in our *ex vivo* experiments (Fig. 2C). We also did not observe any disease-induced changes in diaphragm stiffness as have been observed in other profibrotic conditions such as Duchenne's muscular dystrophy (Sahani *et al.*, 2022; Brashear *et al.*, 2022). Treadmill exercise did lead to significantly lower dynamic and elastic stiffness in the diaphragm of CKD animals, as well lower elastic stiffness in controls (Fig. 2C). While these results may be promising in terms of respiratory benefits of aerobic exercise in CKD, it is unclear at this time whether decreases in diaphragm stiffness would translate to improvements in respiratory function.

Bone

In line with our findings in young adenine-induced CKD rats, we found that cortical thickness, tissue mineral density, and bone mineral density were significantly lower in CKD while cortical porosity was significantly increased (Fig. 3A, (Hayden *et al.*, 2025)). Interestingly, cortical area was higher in CKD – perhaps as a compensatory mechanism for the increased porosity and decreased thickness. In response to exercise, cortical area was greater in the Con-Tread group versus Con-Sed, suggesting the accumulation of bone with regular loading (Fig. 3A). This greater cortical area in Con-Tread was paradoxically accompanied by a lower bone and tissue mineral density; we believe this may be due to a lower density in the newly synthesized bone which has a prolonged calcification period (Fuchs *et al.*, 2008; Donnelly *et al.*, 2010). There were no significant effects of exercise on any cortical parameter across CKD animals (Fig. 3A). Others have shown improvements in cortical porosity with treadmill running in adenine-fed rats; however, they used a 4-week long 0.75% adenine diet to induce disease prior

to 10 weeks of treadmill running on normal chow, which resulted in a much less severe CKD phenotype then seen here (creatinine 0.64mg/dL and BUN or 34mg/dL) (Harata *et al.*, 2023). Voluntary wheel running in rats with genetically induced progressive cystic kidney disease (Cy/+$_{IU}$) also reduces porosity, which is promising (Avin *et al.*, 2019). Our data do show a tendency for cortical porosity to decrease (Fig. 3A) suggesting that with a greater number of animals or longer intervention exercise may have improved porosity.

Also in line with our previous findings in young rats, we found that trabecular tissue mineral density was lower in CKD while trabecular thickness was increased (Fig. 3B, (Hayden *et al.*, 2025)). The degree of anisotropy of the trabecular bone, which represents how uniformly it is oriented, was also decreased in CKD bone – which may be the first time this has been reported (Fig. 3B). In control animals, similar to cortical bone, treadmill training also leads to slightly lower trabecular tissue mineral density, which again may be due to newly synthesized bone being less mineralized. Perhaps more interestingly, chronic exercise also led to a significantly higher degree of anisotropy in the trabecular bone of control animals, suggesting that the trabeculae remodeled to align along the axis of this new loading pattern (Fig. 3B). Also similar to our findings in cortical bone, the trabecular bone in CKD animals failed to match the exercise adaptations seen in controls with no changes seen in any trabecular bone parameter. Once again the work by Avin *et al.,* did find exercise-induced improvements in trabecular bone in voluntary run Cy/+$_{IU}$ rats, including increased trabecular bone volume, thickness, and number while decreasing spacing (Avin *et al.*, 2019). However, their model did not recapitulate the baseline elevation in trabecular thickness or volume that we and others have reported in CKD (Malluche *et al.*, 2011, 2018; Nickolas *et al.*, 2013).

To understand some of the functional relevance of CKD induced changes in bone structure, we also mechanically tested the femurs using three-point bending. We found that in the sedentary groups there were no significant differences between controls and CKD animals in yield force, max force, stiffness, or post yield displacement (Fig. 3C), similar to our previous findings in young female rats (Hayden *et al.*, 2025). Lower bone strength may be expected given the increase in porosity seen in cortical bone, but this is counteracted by the increased cortical area (Fig. 3A), maintaining bone strength by increasing its periosteal radius (Warden *et al.*, 2021). The other key finding here is that control, but not CKD bone, was stronger following treadmill training (Fig. 3C), suggesting that this sex mediated factor does not preserve exercise-induced adaptation in CKD. In support of our results, Avin *et al.* also found that bone mechanical adaptations in voluntary run CKD rats were less pronounced than the structural adaptations – with improvements in stiffness alone.

Key factors believed to contribute to CKD mineral and bone disease include elevated serum phosphate, sclerostin, parathyroid hormone (PTH), and FGF-23 as well as decreased active vitamin D and klotho (Pazianas & Miller, 2021). Exercise has also been suggested to improve bone health by reducing serum PTH and FGF-23, however, further review of this data suggest it may be an artifact of heteroscedasticity (Avin *et al.*, 2019). Due to the complex interactions among these factors, it may be that a concurrent molecular therapy alongside exercise is necessary to incur exercise-induced adaptation. Indeed, combined interventions - such as bisphosphonate administration (alendronate) and treadmill exercise (Harata *et al.*, 2023), or selective estrogen receptor modulator (raloxifene) treatment and compressive axial bone loading (Surowiec *et al.*, 2024) - have elicited beneficial bone adaptation in CKD models. Unfortunately, these pharmaceuticals still lack sufficient efficacy and safety data for use in

human CKD (Khairallah & Nickolas, 2022). Other interventions such as RANKL inhibition have been shown to improve bone health in rodent CKD models , but may act on pathways that are redundant to exercise and therefore may not make an optimal combined therapy (Metzger *et al.*, 2024).

Undoubtedly, the mechanical loading of bone during exercise is an important mediator of its benefits. To stimulate bone remodeling, bone must be loaded dynamically (opposed to statically) in a pattern that differs from its customary load (Turner, 1998). The degree of this bone remodeling is dependent on both load strain rate and magnitude (O'Connor *et al.*, 1982; Rubin & Lanyon, 1985), is maximized after relatively few loading cycles (<100, (Burr *et al.*, 2002)), and requires 4-8 hours between bouts before mechanosensitivity is completely recovered (Robling *et al.*, 2000, 2001). As suggested by Avin's group, voluntary running exercise may be a more effective intervention than forced running to achieve the load pattern necessary to improve bone biology. Indeed, benefits of voluntary running over our treadmill protocol include increased running speed (leading to a greater strain rate) and distribution of activity across the day (leading to a greater stimulus), could explain the greater adaptations seen in their model. Thus, future studies with the specific intent of improving bone health in CKD models may be more effective if they design their exercise interventions with optimal bone loading parameters in mind (Warden *et al.*, 2021).

Tendon

While muscle and bone pathology in CKD have received significant attention in the literature, the effects of CKD on tendon has largely been limited to case studies/retrospective analyses (Shah, 2002; Basic-Jukic *et al.*, 2009; Park *et al.*, 2013; Humbyrd *et al.*, 2018; Pichone *et al.*, 2024) and our previous study using an 9-week long adenine-diet model of CKD (Hayden

et al., 2025). In our previous study, we found that both the tibialis anterior and Achilles tendons were roughly 25% weaker in juvenile female rats with CKD compared to controls by means of failure stress. Because failure stress is normalized to the cross-sectional area being tested, this finding suggested that CKD impaired tendon function by effecting its internal mechanical structure and not via atrophy. In our current study, we expected to find a more severe phenotype due to the longer disease burden and older age of the animals (presumably reducing disease resilience). However, contrary to our findings in young rats, CKD was not associated with weaker tendons in adult rats after 16 weeks on the adenine diet (Fig. 4B-C). A potential reason for this finding is that CKD affects tendons by impairing their capacity to turnover matrix proteins. This would explain our results by: 1) the initial study was performed in animals that were still developing and skeletally maturing, during which time tenocytes constantly produce and assemble new ECM, 2) the tendon ECM does not turnover as readily after longitudinal growth is complete (Heinemeier *et al.*, 2013, 2018), and 3) deficits in protein turnover might not manifest in adult tendons without a stimulus to increase ECM turnover. If this is the case, then tendon dysfunction in CKD should be revealed in adult animals in response to mechanical load and damage, which increase collagen turnover (Miller *et al.*, 2005; Heinemeier *et al.*, 2018). In agreement with this idea, 8 weeks of treadmill running in adult CKD rats resulted in 13% weaker tendons which required 19% less energy to rupture compared to sedentary control animals (Fig 4B). As such, we hypothesize that high rates of tendon injury in CKD could be due to an inability of tenocytes to repair the ECM in response to loading-induced stress.

Tendons exist in a state whereby the structural component of the tendon (the ECM) is maintained by the tenocytes which balance the degradation of damaged proteins with the synthesis of new ones (Cousineau-Pelletier & Langelier, 2010). Impairment of this process in

CKD could occur via either process being underactive, overactive, or a combination of the two. Because we do not see atrophy, hypertrophy, or changes in collagen concentration in CKD tendons (Fig. 4A), the most likely option may be underactivity of both. Our DSC data also supports this hypothesis, as we found a slight elevation in distal tendon peak temperature in sedentary CKD animals (Fig. 5B). Elevated peak temperature could represent a more crosslinked tissue due to lower protein turnover. In CKD, skeletal muscle protein synthesis is believed to be decreased and protein degradation increased due to metabolic acidosis, insulin resistance, and inflammation (Wang *et al.*, 2022; Cheng *et al.*, 2022). While similar may be true for tendon, an alternative hypothesis is that CKD affects mechanotransduction, as tendon adaptation is potentially more dependent on mechanotransduction than muscle (Lambrianides *et al.*, 2024). Such an effect could be mediated through the altered toe-region mechanics we identified in sedentary CKD animals (Fig. 4B-C). The toe region in tendons is the portion of strain where tendon uncrimps and represents the region of normal physiological load (Lake *et al.*, 2023). The higher toe-region energy and greater strain at 10MPa in CKD tendon could be an indicator of greater baseline crimp. Because tendon cells are embedded in ECM and respond to longitudinal strain by increasing protein synthesis, it stands to reason that increased crimp may blunt this mechanotransduction and thereby protein turnover; however, this remains to be tested.

Additional considerations

Despite being designed to address limitations on our first study, this follow-up experiment had several drawbacks. First, due to issues maintaining healthy controls and the extreme severity of renal impairment seen in males in our first study, this work was completed only in female animals. It stands to reason that sex-based differences may exist in the response to chronic exercise training, and these should be evaluated. Second, our exercise intervention which

we hypothesized would improve CKD-induced tendon dysfunction turned out to negatively affect tendons in CKD. While this is a novel and interesting finding, our *a priori* hypotheses were not set up to test this effect directly, requiring post-hoc analysis that may be less robust. Third, we had a number of animals reach humane endpoints prior to the end of the study. While the inclusion of their data does not appear to skew the results in any direction, we do not know for sure if they would have differed by the 16-week timepoint.

Conclusion

Exercise is an important and promising intervention for many of the complications that occur with kidney disease and has been incorporated into clinical practice guidelines in many countries (Wilkinson *et al.*, 2025). Using exercise to improve musculoskeletal targets in particular is appealing as individuals with CKD suffer from considerable muscle and bone pathologies (Pazianas & Miller, 2021; Wang *et al.*, 2022) and these tissues have well-established adaptive responses to exercise (Warden *et al.*, 2021; Egan & Sharples, 2023). Still, much research is needed in regards to best practices and expected benefits of exercise in muscle, and even more so in bone (Hayden *et al.*, 2024). Further, this area is completely unexplored with regards to tendon, where more information is critical to help prevent the high rates of catastrophic injury (Shah, 2002; Humbyrd *et al.*, 2018) without worsening the issue, as has been done in other populations (Stausholm *et al.*, 2024). With these ideas in mind, we present here that 8-weeks of uphill treadmill running improves muscle mass, does not significantly affect bone health, and leads to greater tendon weakness in a rat model of CKD. The pathological response of CKD tendons to mechanical load is particularly novel and necessitates further research. Importantly, this same intervention in healthy animals induced beneficial adaptations in muscle and bone without negatively affecting tendon. Collectively, the results suggest that

different musculoskeletal tissues adapt differently to any given exercise stimulus and this should be considered when designing exercise protocols. Further understanding the benefits and mechanisms of exercise induced adaptation will help optimize and promote these interventions, thus improving the treatment of individuals with CKD.

Disclosure

Nothing to disclose.

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Data availability statement

All data points are shown in figures. The authors agree to provide any and all raw data upon reasonable request.

Competing interests

All authors declare no competing interests

Author contributions

CMTH, BR, and KB, contributed to project design. CMTH, BO, SEB, and MG completed data acquisition and analysis. All authors contributed to the interpretation of the work as well as drafting and revising it critically for important intellectual content. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved; and all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Chapter 4: Chronic kidney disease blocks exercise-induced cardiac hypertrophy in rats

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Key Points

1. Hearts of control but not CKD rats exhibited cardiac hypertrophy in response to eight weeks of uphill treadmill exercise
2. Cardiac tissue in CKD animals show chronic upregulation of both anabolic and catabolic pathways despite not exhibiting signs of pathological hypertrophy
3. One week of treadmill exercise leads to lower heart mass in control and CKD rats with a greater decrease in dry mass seen in CKD
4. Control and CKD rat hearts experience similar suppression of protein synthesis and anabolic signaling two and a half hours following exercise, while autophagy is slightly elevated in CKD

Abstract

Chronic kidney disease (CKD) is a progressive disease that ultimately culminates in renal failure. However, cardiovascular complications are the most common cause of death in this population. Exercise improves cardiovascular health in the general population and can reduce morbidity in CKD patients. However, CKD has been suggested to blunt certain exercise-induced adaptations. Here, we interrogate the finding that exercise-induced cardiac hypertrophy is blunted in the adenine diet model of CKD. In two separate experiments, we investigate the response of cardiac tissue to acute and chronic exercise in CKD animals. Following eight weeks of uphill treadmill exercise, the hearts of exercised control animals were 15% (95%CI [1.2, 28]) larger by mass compared to sedentary controls. However, the heart mass of exercise-trained CKD rats did not significantly differ from that of sedentary CKD animals (-3% mass [-20, +13] CKD Treadmill vs CKD Sedentary), which also did not differ from sedentary controls, suggesting there was also no pathological hypertrophy. This lack of cardiac hypertrophy in response to exercise coincided with an increase in a number of anabolic and catabolic markers, and was not explained by different responses to acute exercise. Thus, CKD appears to impair exercise-induced cardiac hypertrophy in the adenine-diet model in rats, but the exact cause of this blunted response is yet to be determined.

INTRODUCTION

In the US, 31 million people are estimated to have chronical kidney disease (CKD). Almost a third of these cases are believed to be caused by hypertension (Centers for Disease Control and Prevention, 2023; United States Renal Data System, 2025). Additionally, 35% of individuals with CKD are believed to concurrently suffer from cardiovascular disease (United States Renal Data System, 2025). Given the high co-occurrence of these diseases, the American Heart Association introduced the diagnosis of "cardiorenal syndrome" in 2019 to enable better treatment of these highly interdependent conditions (Rangaswami *et al.*, 2019). Crossover between conditions such as cardiovascular and kidney disease are not isolated to humans. A number of common rodent models of CKD spontaneously develop cardiovascular complications, including the adenine diet (Diwan *et al.*, 2013), 5/6 nephrectomy (Adams *et al.*, 2005), and Indiana University autosomal dominant progressive cystic kidney disease rats ($Cy/+_{IU}$) (Avin *et al.*, 2019). Among these, the adenine diet model of CKD has been suggested to best mimic both renal impairment and associated cardiovascular pathology (Diwan *et al.*, 2018; Beikoghli Kalkhoran *et al.*, 2024; Yang *et al.*, 2024). These models are invaluable for mechanistic insight into cardiorenal syndrome as they provide access to tissue samples that cannot be obtained in human studies (Khetarpal *et al.*, 2025). Because cardiovascular events are the most common cause of death among individuals with CKD, use of these models may provide priceless insight into interventions and treatments that improve the lifespan of those suffering from CKD.

Exercise is one such intervention that has long been a staple in cardiac rehabilitation (Anderson *et al.*, 2016) and has comparable benefits for participants with and without CKD (Thompson *et al.*, 2021). Research into the cardiac specific benefits of exercise in CKD has been scarce; however, exercise improves diastolic function in individuals with stage 3-4 CKD

(Howden *et al.*, 2013) and reduces left ventricular mass in dialysis patients (Graham-Brown *et al.*, 2021). The latter was a particularly interesting finding, as one of the main exercise-induced cardiac adaptations is left ventricular hypertrophy. Indeed, the interplay between so called "physiological" (exercise-induced) hypertrophy and pathological hypertrophy (as occurs in CKD and heart disease) has been a subject of great interest in the literature (McMullen & Jennings, 2007; Dorn, 2007; Shimizu & Minamino, 2016; Nakamura & Sadoshima, 2018). Still, the interactions between exercise-induced cardiac hypertrophy and cardiac pathology in CKD are largely unexplored and whether cardiac specific benefits are altered in CKD remains to be elucidated.

Because musculoskeletal disorders are also common in CKD (Pazianas & Miller, 2021; Wang *et al.*, 2022), we recently utilized the adenine-diet model in Sprague Dawley rats to investigate the effects of 8 weeks of uphill treadmill exercise on muscle, bone, and tendon in CKD (unpublished). In this study, we found that muscle, but not bone or tendon, positively adapted to chronic exercise. As cardiopulmonary adaptations to exercise have also been suggested to be blunted in CKD (Kirkman *et al.*, 2021), we further investigated the cardiac responses from this study. In the analysis presented here, we find that while the hearts of control animals exhibited exercise-induced hypertrophy, cardiac growth was not elicited in CKD animals. To begin to understand this blunted exercise response, we interrogated both chronic and acute responses to exercise in CKD animals.

METHODS

Chronic exercise adaptation experiment

Seven-month-old female Sprague Dawley rats (n=36 retired breeders, Charles River Laboratories, Wilmington, MA, USA) were equally separated into control diet (Con, AIN 93G, 0.3% phosphorus, 0.5% calcium, Bio Serv F3156, Flemington, NJ, USA) or CKD-inducing diet groups (CKD, AIN 93G + 0.25% adenine, 0.9% phosphorus, 0.6% Ca, Inotiv TD.180493, West Lafayette, IN, USA). After 8 weeks on Con or CKD diets, half of the animals from each group were assigned to either sedentary (Sed) or treadmill exercise (Tread) arms for the following 8 weeks; creating four groups (Con-Sed, Con-Tread, CKD-Sed, CKD-Tread). The exercise groups completed 30 minutes of incline treadmill running (10% grade) 5 times per week. The first week of treadmill running was performed at 10m/min with the subsequent weeks being 15m/min. After 8 weeks of treadmill training animals were rested for 7 days hours after the last exercise bout, following which all animals were anesthetized with 2.5% isoflurane and their hearts were excised (Fig. 1A).

Acute exercise response experiment

The acute exercise response experiment used the same protocol from above with the only difference being that animals were sacrificed 9 weeks into the experiment, after completing only 5 days of treadmill exercise. Three groups were utilized for this experiment Con-Sed, (n=3), Con-Tread (n=6), and CKD-Tread (n=6). On the fifth day of treadmill exercise, animals completed their treadmill exercise one at a time staggered by 30min with the study groups evenly interspersed. Exactly three hours after the start of their 30min treadmill bout, animals were euthanized and their tissues were collected.

Hematocrit measurement

Hematocrit was measured at weeks 5 and 10 from the lateral saphenous vein samples using a hypodermic needle and a heparin coated syringe while the animals were anesthetized with 2.5% isoflurane. At study termination blood was sampled directly from the heart via cardiac puncture. Immediately following collection ~100µl of blood was immediately tested using an i-STAT CHEM 8+ Cartridge (Abbott Laboratories, Chicago, IL, USA).

Protein synthesis measurement via puromycin

Puromycin dihydrochloride (500mg, AdipoGen, San Diego, CA) was mixed into 8mL of MilliQ and then brought to a final volume of 10.2 mL (90mM), this was split into 1mL aliquots and stored at -30° C. On the day of the collection the 90mM solution was diluted into PBS at a ratio of 1mL puromycin to 1.81mL PBS making a 32mM working solution. Animals were injected intraperitoneally with this solution exactly 30 min before sacrifice at a dose of 0.04umol/g body weight (a volume of 125uL/100g body weight).

Cardiac tissue collection and processing via western blot and hydroxyproline assay

At collection, hearts were rinsed in saline and compressed and blotted on gauze to remove any residual blood from the chambers. They were then weighed and frozen in liquid nitrogen. Samples were stored at -80 °C until further processing, at which time they were powdered, aliquoted into microtubes, and stored back at -80 °C without any thawing. Powdered heart tissue was added to 200 µL of sucrose lysis buffer and incubated on a shaker for 1 hour before centrifugation for 10 min at 8000RPM and 4°C. The supernatant was collected, and a detergent-compatible protein assay (DC Protein Assay, Bio-Rad Laboratories, Hercules, CA) was used to normalize protein concentrations across samples that were diluted into Laemmli sample buffer and sonicated. Samples (10uL) were loaded onto 4-20% Criterion TGX Stain-free gels (BioRad) and run at 200V for 45 mins. Proteins were transferred onto a nitrocellulose

membrane at 100V for 30 mins. Membranes were washed in Tris-buffered saline w/ 0.1% Tween (TBST), blocked in 5% skim milk or 1% fish skin gelatin in TBST for 30 mins, and rinsed in TBST before being incubated overnight at 4℃ in primary antibody. Membranes were washed in TBST (3 x 5 mins) and incubated with horseradish peroxidase-conjugated secondary antibodies in TBST for 1hr at room temperature. Total protein was quantified by using UV light to activate the trihalo compound from the stain-free gels or in the membranes after transfer (via stain-free imaging or ponceau staining). Images were taken using the ChemiDoc MP system and bands were quantified through Image Lab 5.0 software (BioRad).

Collagen concentration was measured in powdered cardiac tissue using a hydroxyproline assay (Woessner, 1961). Samples were weighed pre- and post-drying at 120°C for 30 min to determine dry mass and water concentration. Samples were hydrolyzed in 6 N HCl for 2 h, following which HCl was boiled off in a fume hood. Samples and hydroxyproline standards were then suspended in hydroxyproline buffer (173 mm citric acid, 140 mm acetic acid, 588 mm sodium acetate, 570 mm sodium hydroxide), and 150µl of chloramine T solution was added to 200µl each sample, followed by incubation at room temperature for 20 min. After this 150µl of aldehyde-perchloric acid (60% 1-propanol, 5.8% perchloric acid and 1M 4-(dimethylamino) benzaldehyde) was added to each tube, and the samples were incubated at 60°C for 15 min and cooled for 5 min. Standards and samples (200µl each) were added to a clear-bottomed 96-well plate, and absorbance at 550 nm was measured using an Epoch Microplate Spectrophotometer (BioTek Instruments Limited, Winooski, VT, USA). Collagen content was determined assuming that hydroxyproline constitutes 13.7% of the amino acid composition of collagen.

Statistics

Data were screened for outliers using the ROUT method and a Q=1%. Outcomes from the chronic training study were analyzed using a two-way ANOVA with factors Disease (control vs disease) and Exercise (sedentary vs exercise). Based on *a priori* hypotheses, planned comparisons were performed to assess: 1) the effect of disease under sedentary conditions (Con-Sed versus CKD-Sed), 2) the effect of exercise in controls (Con-Sed versus Con-Tread), and 3) the effect of exercise in disease (CKD-Sed versus CKD-Tread) using Fisher's least square differences. Outcomes from the acute training study were assessed via one way ANOVA followed by Fisher's least square difference test to assess three *a priori* comparisons: 1) the effect of exercise in controls (Con-Sed versus Con-Tread), 2) the effect of CKD on the exercise response (CKD-Tread versus Con-Tread), and 3) the effect of CKD and exercise (CKD-Tread versus Con-Sed). All statistics were performed using GraphPad Prism (v. 11.0.1, GraphPad Software, Boston, MA, USA).

RESULTS

Chronic treadmill running induces cardiac hypertrophy in control but not CKD rats

To investigate the capacity for exercise-induced cardiac hypertrophy in CKD rats, we used a 0.25% adenine diet to induce and maintain chronic kidney disease over 16 weeks in 7-month-old Sprague Dawley rats. At the 8-week mark, treadmill exercise (30min/day, 5 days/week, 15m/min, 10% incline) was introduced as an intervention (Fig. 1A). The severity and phenotype of renal impairment in this cohort have been reported previously (Chapter 3). Body weight and hematocrit levels followed different patterns between control and CKD groups, with significant reductions in hematocrit occurring both in response to exercise in controls and in response to disease (Fig. 1B). Several animals were terminated early due to reaching human endpoints, and data from these animals are not included, resulting in a reduced n in CKD-Sed and CKD-Trad groups (n=5 and n=8, respectively).

In control animals, treadmill training resulted in a statistically significant 15% (95%CI [1.2, 28]), greater cardiac mass relative to the Con-Sed group while no difference was seen in CKD animals (-3% [-20, +13]; Fig. 1). Dry mass followed a similar pattern with treadmill training in controls (+18% [2, 33] Con-Tread versus Con-Sed), while heart mass to body ratio was elevated in CKD alone (Fig. 1C) corresponding to the body weight decline seen in CKD (Fig. 1B). Water concentration and procollagen protein were significantly elevated in CKD while collagen concentration was unchanged (Fig. 1D).

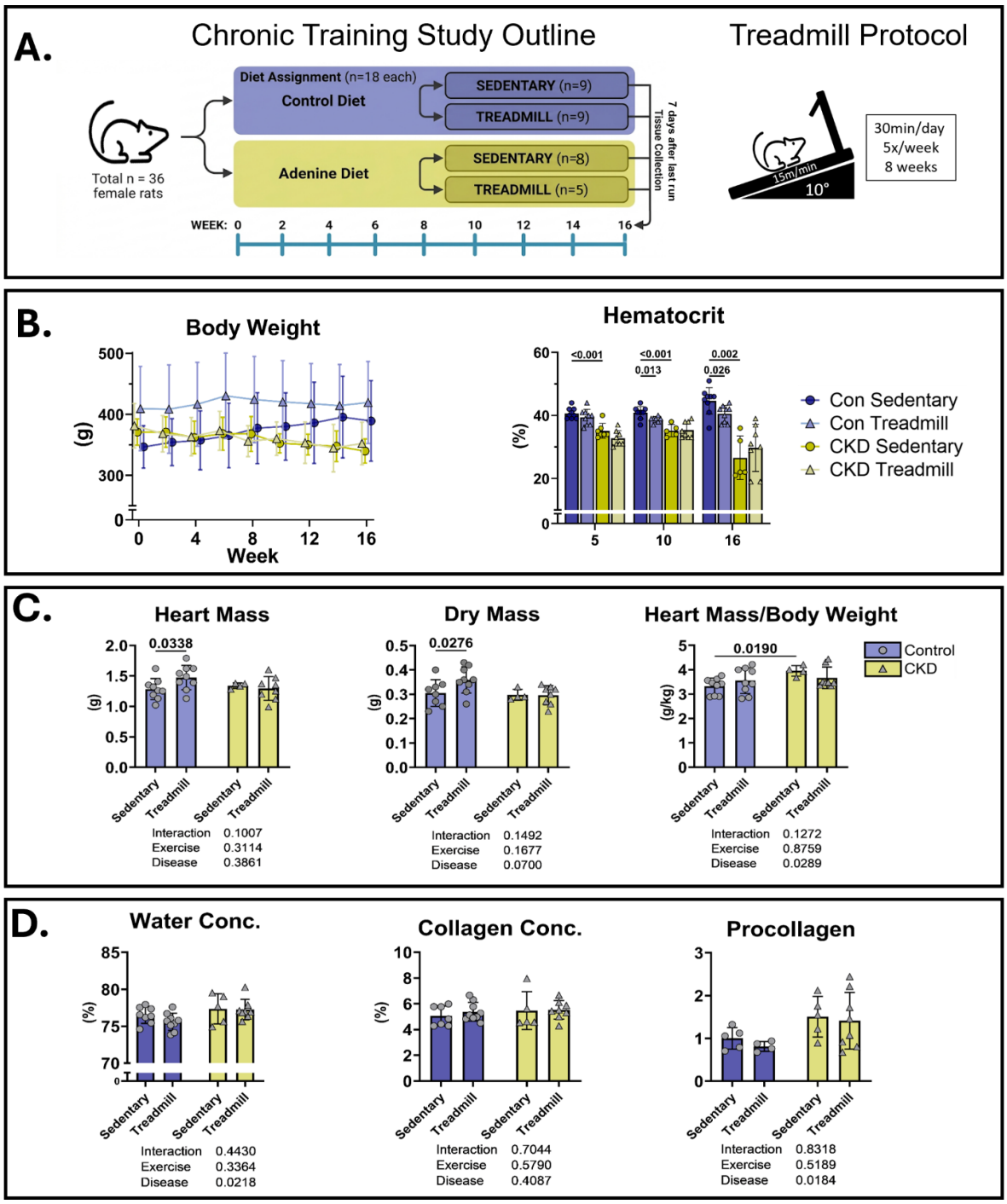

Figure 1. Exercise-induced cardiac hypertrophy is blunted in rats with CKD. A 0.25% adenine diet was used to induce and maintain chronic kidney disease over 16 weeks in 7-month-old Sprague Dawley rats. At the 8-week mark, treadmill exercise (30min/day, 5 days/week, 15m/min, 10% incline) was introduced as an intervention for 8 weeks. A) Study design, timeline, and exercise protocol. B) Body weight and hematocrit levels over the course of the study. C) Heart mass, dry mass, and mass-to-body weight ratio. D) Cardiac water concentration, collagen concentration (from hydroxyproline assay), and procollagen levels (arbitrary units, from Western blotting). Full Fisher's LSD comparisons were made between sedentary groups to evaluate the effects of disease, and within control and CKD groups to evaluate the effects of exercise. Values are represented as mean ± SD. Con, control animals; CKD, chronic kidney disease; Conc., concentration as a percent of total mass.

Cardiac markers of anabolism and catabolism are chronically increased in CKD

To determine what processes may have led to cardiac hypertrophy in control but not in CKD animals, we next investigated proteins involved in anabolic and catabolic processes in heart tissue collected 7 days after the last exercise bout. In CKD animals compared to control, phosphorylation of Akt and glycogen synthase 3β were (GSK3β) chronically high, as was total amounts of these proteins (Fig. 2A). CKD hearts also contained increased levels of autophagy pathway protein microtubule-associated protein 1 light chain 3 (LC3), in both its unactive (i.) and activated (ii.) form (Fig. 2B), as well more of the transcriptional repressor CCAAT/Enhancer-Binding Protein Beta (C/EBPβ) and E3 ubiquitin ligase F-box protein 40 (FBXO40). Exercise did not result in a chronic change in any anabolic or catabolic marker in control animals, except for an increase in LC3-I (Fig. 2B). In CKD animals, exercise resulted in less phosphorylation of GSK3β and ribosomal protein S6 (Fig. 2A)

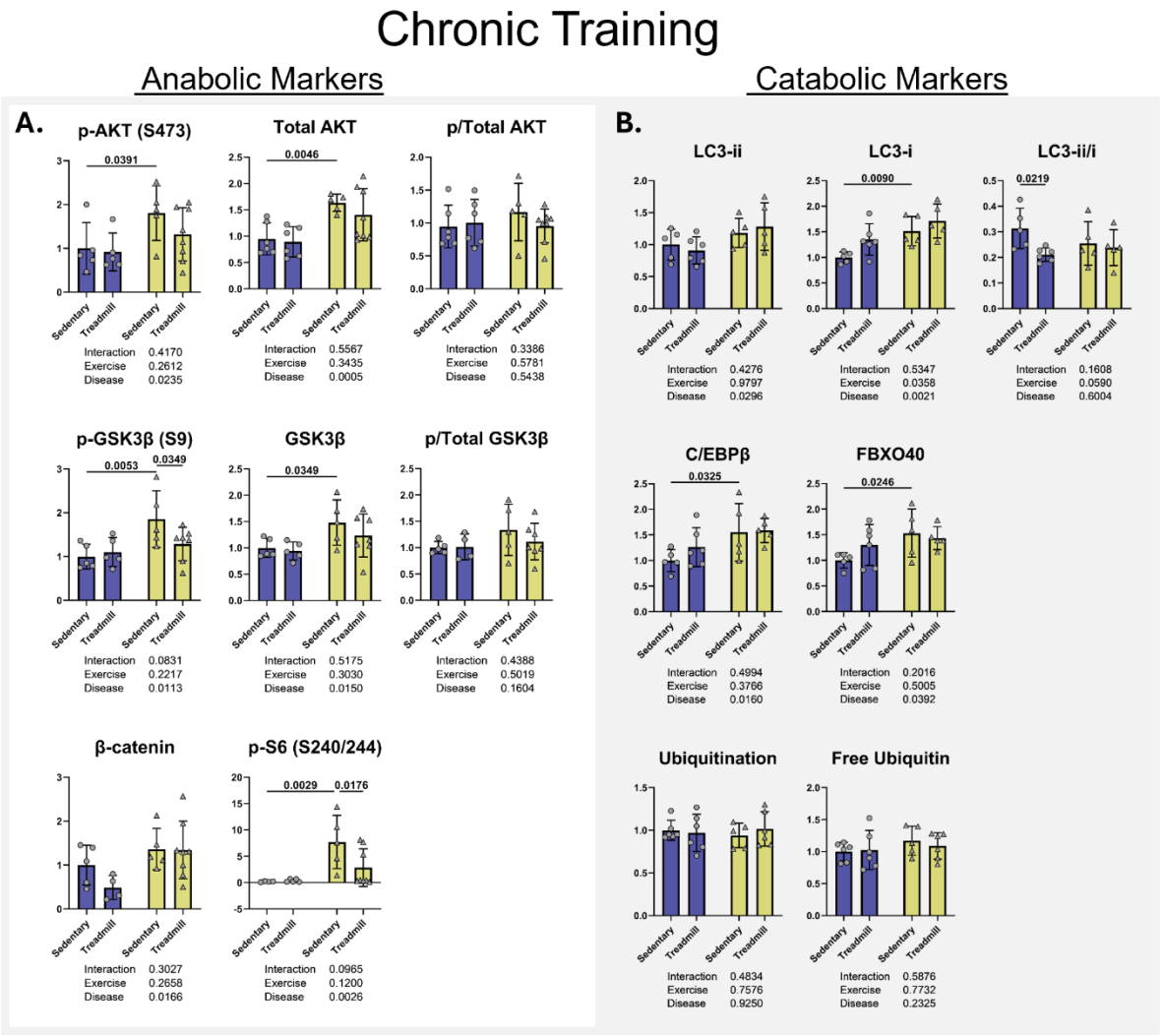

Figure 2. Both anabolic and catabolic markers are chronically elevated in cardiac tissue of CKD rats. One week following 8 weeks of treadmill exercise (30min/day, 5 days/week, 15m/min, 10% incline), both A) anabolic and B) catabolic markers are elevated in CKD. Protein levels were measured in cardiac tissue homogenate via Western blot, are normalized to total protein, and presented as fold change versus the control sedentary group. Fisher's LSD comparisons were made between sedentary groups to evaluate the effects of disease, and within control and CKD groups to evaluate the effects of exercise. Values are represented as mean ± SD. Con, control animals; CKD, chronic kidney disease; p, phosphorylated; GSK3βglycogen synthase 3β; S6, ribosomal protein S6; LC3, microtubule-associated protein 1 light chain 3; C/EBPβ, CCAAT/Enhancer-Binding Protein Beta; FBXO40, E3 ubiquitin ligase F-box protein 40.

One week of treadmill exercise acutely decreases cardiac mass in control and CKD rats

Because biochemical markers measured one week following 8 weeks of treadmill training did not explain the blunted cardiac adaptation to chronic treadmill exercise, we completed a follow-up experiment using the same model to determine whether CKD rats maintained the ability to

acutely respond to exercise stimuli (Fig. 3). In response to one week (5 sessions) of uphill treadmill running, heart mass was decreased in both Con-Tread (-15% [-25, -5]) and CKD-Tread (-21% [-31, -11]) animals compared to Con-Sed (Fig. 3C). This was largely driven by a change in water content in both groups and was accompanied by significantly lower heart dry mass in the CKD-Tread but not the Con-Tread group; resulting in a significantly altered water concentration in the Con-Tread group alone (Fig. 3C).

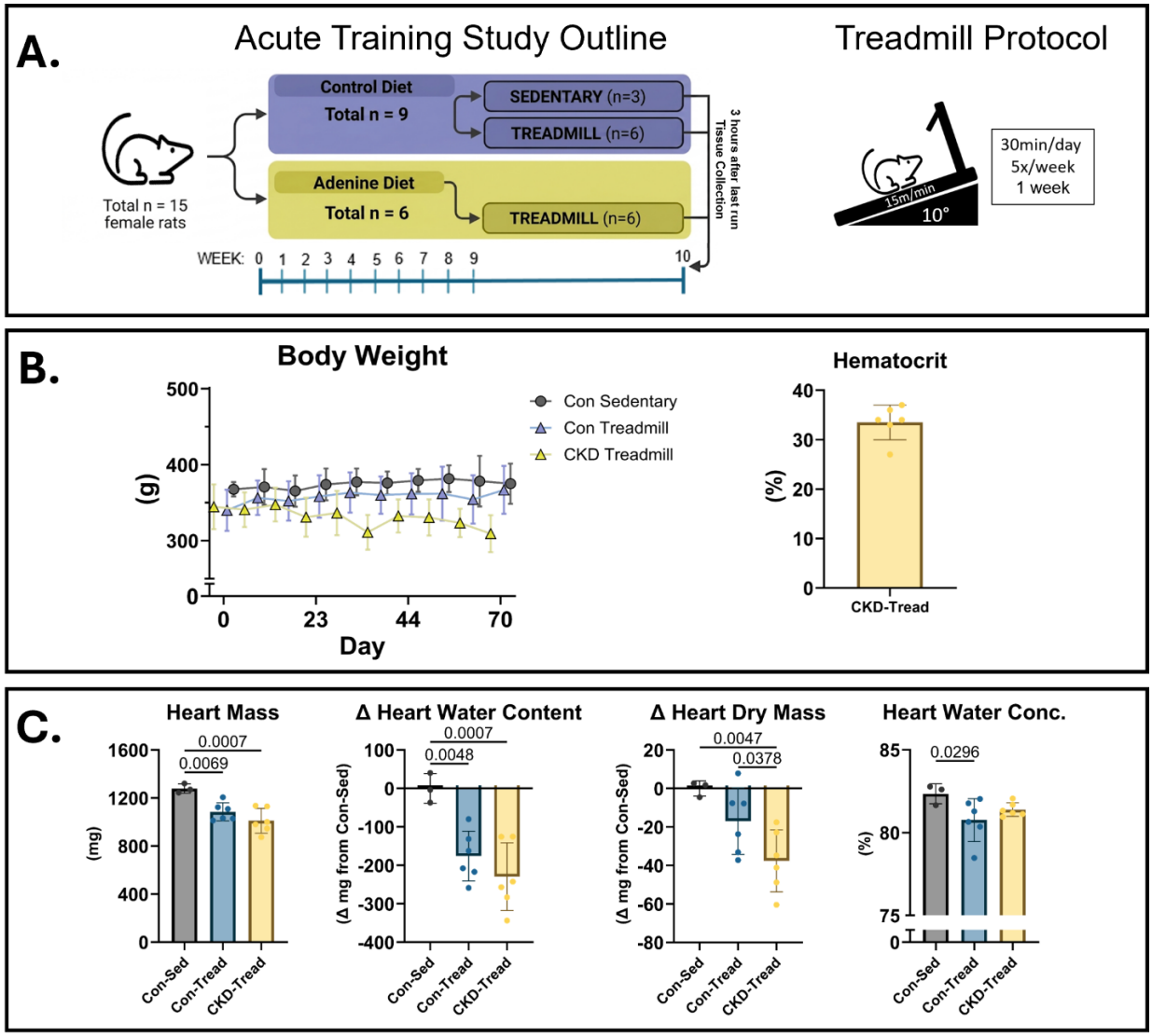

Figure 3. Heart mass is decreased in control and CKD animals following one week of treadmill running. A 0.25% adenine diet was used to induce and maintain chronic kidney disease over 10 weeks in 7-month-old Sprague Dawley rats. At the 9-week mark, treadmill exercise (30min/day for 5 days, 15m/min, 10% incline) was introduced as an intervention for one week, and animals were euthanized 3 hours following the 5th exercise bout. A) Study design, timeline, and exercise protocol. B) Body weight and hematocrit levels. C) Heart mass, water content, dry mass, and cardiac water concentration. Fisher's LSD comparisons were made between and within control and CKD groups to evaluate the effects of exercise and disease. Values are represented as mean ± SD. Con, control animals; CKD, chronic kidney disease; Sed, sedentary; Tread, treadmill run; Conc., concentration as a percent of total mass.

Molecular responses to acute treadmill exercise are similar in control and CKD rats

Next, to understand why the CKD rats experienced a greater loss of dry mass in response to one week of treadmill running compared to controls, we investigated the molecular responses involved in cardiac protein synthesis and breakdown two and a half hours following the final exercise bout. Phosphorylation of Akt, GSK3β, S6, and eukaryotic elongation factor 2 (EEF2) were not changed with exercise in either group, while phosphorylation of eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) was significantly lower following exercise in both control and CKD groups (fig. 4A). Additionally, total Akt was upregulated in both exercised groups while total GSK3β was lower in the control treadmill group alone (Fig. 4A). Free ubiquitin was reduced following exercise in both control and CKD groups, but ubiquitination of high molecular weight proteins (>50kDa) did not differ between groups. Total LC3 ii and I were not different between groups; however, the ratio of ii/i was elevated in CKD-Tread compared to Con-Tread (Fig. 4B).

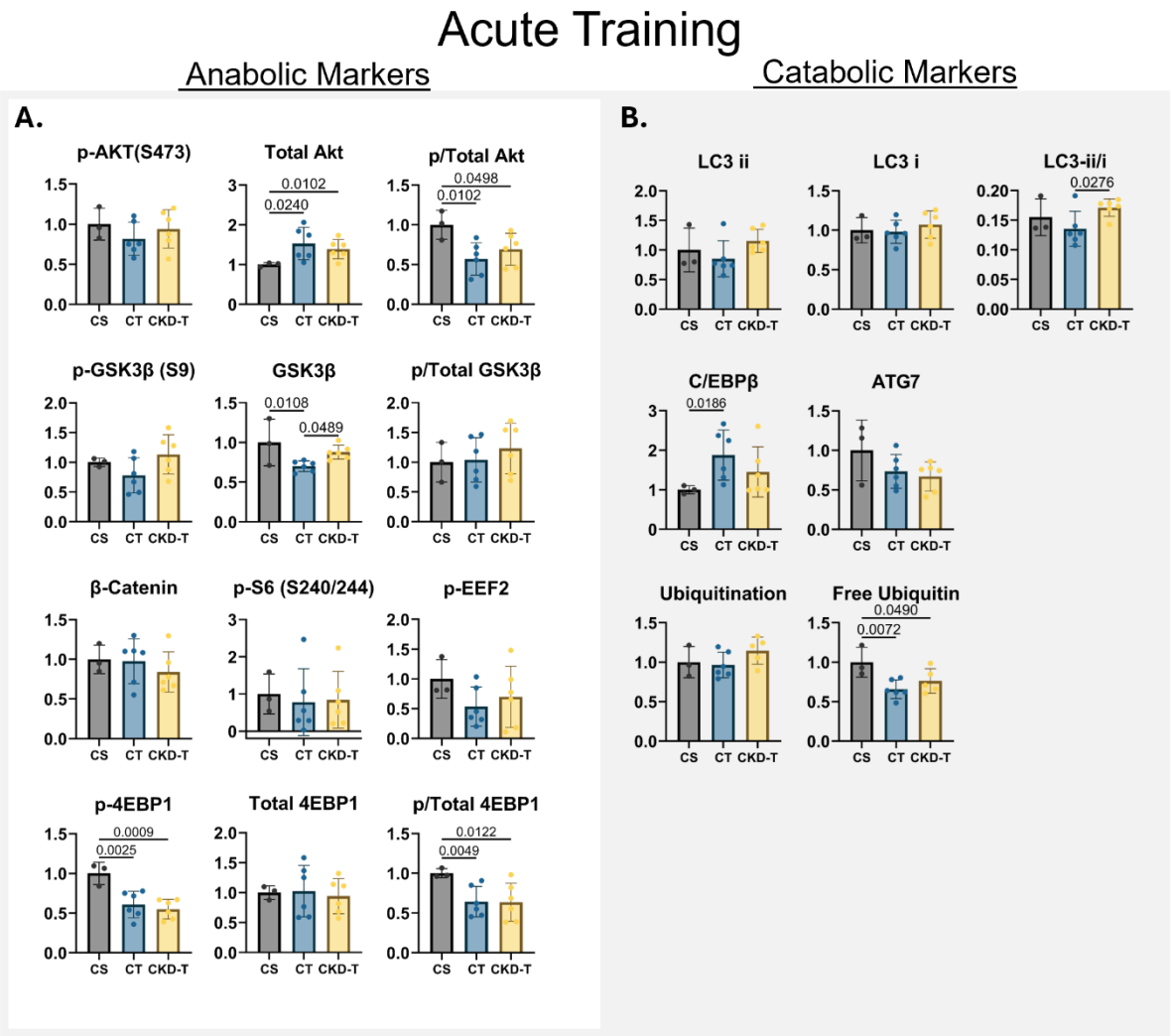

Figure 4. CKD rats have similar cardiac anabolic and catabolic responses to acute exercise. Three hours following the last exercise bout of one week of treadmill exercise (30min/day for 5 days, 15m/min, 10% incline), both A) anabolic and B) catabolic markers follow similar trends in control and CKD animals. Protein levels were measured in cardiac tissue homogenate via western blot, normalized to total protein, and are presented as fold change versus the control sedentary group. Fisher's LSD comparisons were made between and within control and CKD groups to evaluate the effects of exercise and disease. Values are represented as mean ± SD. CS, control sedentary animals; CT, control treadmill run animals; CKD-T, chronic kidney disease treadmill run animals; p, phosphorylated; GSK3βglycogen synthase 3β; S6, ribosomal protein S6; LC3, microtubule-associated protein 1 light chain 3; C/EBPβ, CCAAT/Enhancer-Binding Protein Beta; FBXO40, E3 ubiquitin ligase F-box protein 40.

Cardiac protein synthesis is suppressed acutely following treadmill exercise in control and CKD rats

To determine whether the cardiac protein synthesis response to exercise is suppressed in CKD, we used puromycin to measure protein synthesis rates from 2 to 2.5 hours post treadmill

exercise. Incorporation of puromycin into peptide chains was significantly lower in both control and CKD groups compared to sedentary controls following exercise (Fig 5).

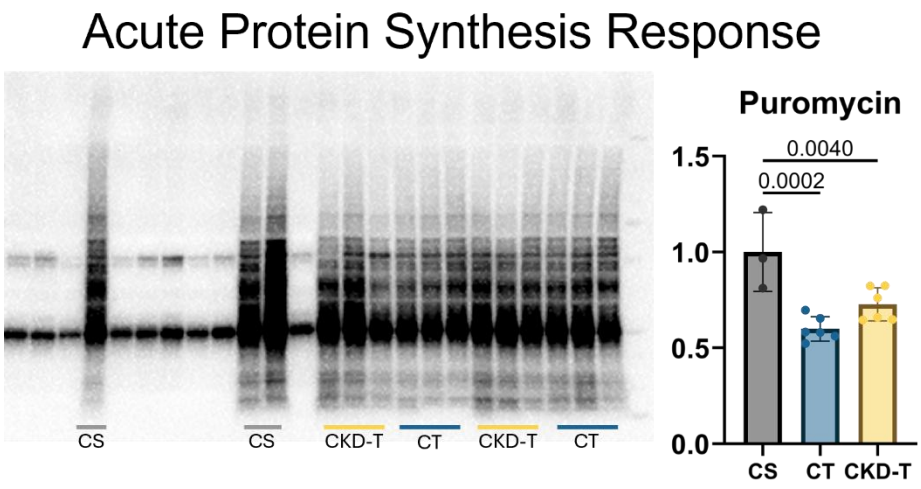

Figure 5. Cardiac protein synthesis decreases acutely following treadmill exercise in control and CKD rats. Two and a half hours after the fifth day of treadmill exercise (30min/day, 15m/min, 10% incline), puromycin incorporation into translated proteins is significantly lower in both control and CKD animals compared to sedentary controls. Protein was measured via western blot, normalized to total protein, and presented as fold change versus the control sedentary group. Fisher's LSD comparisons were made between sedentary groups to evaluate the effects of disease, and within control and CKD groups to evaluate the effects of exercise. Values are represented as mean ± SD. CS, control sedentary animals; CT, control treadmill run animals; CKD-T, chronic kidney disease treadmill run animals.

DISCUSSION

In line with the immense crosstalk between kidney and cardiac function, we find here that the hearts of rats with adenine-diet-induced CKD failed to exhibit physiological hypertrophy following 8 weeks of endurance training (Fig. 1). This is in stark contrast to treadmill-trained control rats, which exhibited 15% greater heart mass, including an 18% greater dry mass, suggesting significant protein accretion (Fig. 1).

Despite the lack of exercise-induced hypertrophy, anabolic signaling was chronically elevated in CKD one week following the last exercise bout (Fig 2A). These anabolic signals include phosphorylated Akt and S6, which are associated with protein synthesis through the canonical mechanistic target of rapamycin complex 1 (mTORC1) growth pathway. Coincident with the elevation of p-Akt, one of its targets, GSK3β, is also chronically phosphorylated. GSK3β is inhibited by phosphorylation by Akt, and this prevents it from labeling the transcriptional co-factor β-catenin for degradation, likely leading to the observed accumulation of β-catenin (Fig. 2A). The Akt/GSK3β pathway lies downstream of insulin/insulin-like growth factor -1 (IGF-1) signaling, which is known to be impaired in CKD (Semple *et al.*, 2011, 2012) and is inhibited through degradation of insulin receptor substrate by FBXO40 - shown here to be upregulated in CKD (Fig. 2B). Elevation of these pathways in CKD may result from the chronic pressure overload associated with increased blood pressure and increased cardiac output to compensate for reduced oxygen carrying capacity in the blood (decreased hematocrit Fig. 1B). Further, the increase in total Akt and GSK3β levels without concomitant increases in the phosphorylated/total ratio suggests that increased phosphorylation may not reflect increased upstream activity of this anabolic pathway.

Regardless of the cause, chronic upregulation of the mTORC1 pathway, as well as increased β-catenin, is associated with pathological cardiac hypertrophy and fibrosis (O'Neill, 2005; Chaanine & Hajjar, 2011; Balatskyi *et al.*, 2023). While heart mass to body weight ratio is higher in CKD, we believe this is due to the weight loss in the CKD group and not pathological hypertrophy, which is supported by the lack of changes in heart dry mass (Fig. 2C). Further, water made up a greater proportion of cardiac mass in CKD hearts despite having similar mass to sedentary controls, suggesting the potential presence of edema (Fig. 2C-D). We did not find signs of fibrosis, since collagen concentration was not elevated in CKD, although we did observe increased procollagen levels which suggest a profibrotic environment (Fig. 1D). We also found evidence for increased autophagy in CKD through elevated LC3-ii levels, which is associated with the formation of autophagosomes (Fig. 2B). However, without an autophagy inhibitor like colchicine, it is unclear whether the change in LC3-ii levels reflect increased autophagy or impaired clearance of autophagosomes (impaired degradation). Further, no changes were seen in protein ubiquitination in CKD, suggesting proteasomal activity was not changed (Fig. 2B). Collectively, we can theorize three explanations to the lack of hypertrophy (physiological or pathological) seen in CKD animals: 1) the chronic increase in anabolic signaling was balanced by increased catabolic signaling resulting in no net changes in protein turnover, 2) some undetermined mechanism downstream of these anabolic signals exerts greater control over protein synthesis and is blocking hypertrophy, and 3) we have captured these signals early on in the maladaptive phase before pathological effects have had an opportunity to manifest.

Since chronic signaling could not explain the lack of cardiac adaptation in CKD, we next investigated the acute response to exercise. Surprisingly, we found that cardiac mass decreased 15-20% following one week of training in both control and CKD rats (Fig. 3C). In line with this

finding, anabolic signaling was lower in Con-Tread and CKD-Tread hearts 2.5 hours after the last exercise bout (Fig. 4A) and acute cardiac protein synthesis rates were lower (Fig. 5). Further analysis of cardiac composition revealed a greater loss in dry mass in the CKD-Tread group suggesting greater protein degradation. Indeed, the LC3-ii/i ratio was increased in CKD which could suggest greater autophagy following acute exercise with disease (Fig. 4B). We did not see an acute increase in autophagy markers or protein ubiquitination following treadmill running, but free ubiquitin was reduced, suggesting exercise-induced alteration in this pathway (Fig. 4B). Thus, these results suggest that at the onset of a new exercise program cardiac mass may initially decrease via regular blunting of protein synthesis in response to acute exercise. Such a mechanism could explain how intradialytic cycling decreased left ventricular mass in end-stage kidney disease patients (Graham-Brown *et al.*, 2021), although here we show that with the acute decrease in heart mass during the transition to chronic exercise is not retained in either control or CKD animals.

Interestingly, C/EBPβ, a transcriptional repressor and possible driver of exercise-induced cardiac adaptation and hypertrophy (Boström *et al.*, 2010), was elevated both in controls acutely after exercise and chronically in CKD. Typically, C/EBPβ acts as a molecular brake preventing expression of the transcriptional coactivator CITED4 (Creb binding protein [CBP]/p300–interacting transactivator with ED-rich carboxy-terminal domain-4). CITED4 promotes physiological hypertrophy and adaptations that protect against pathological remodeling (Bezzerides *et al.*, 2016; Lerchenmüller *et al.*, 2020). When C/EBPβ is decreased through exercise or other means, CITED4 expression increases, allowing it to elicit its downstream effects. Thus, the finding that C/EBPβ is chronically upregulated in the hearts of CKD animals

suggests this may contribute to the lack of exercise adaptation in CKD, although this remains to be tested.

Conclusion

In conclusion, we demonstrate that exercise-induced cardiac hypertrophy following 8 weeks of treadmill training is blunted in adult rats with adenine-diet-induced CKD, and this occurs in the absence of indicators of pathological hypertrophy and despite chronic activation of growth pathways. Further, we find that markers of autophagy are upregulated in CKD hearts and are not affected by 8 weeks of exercise training. The potential increased protein breakdown, along with the elevated suppression of CITED4 through the C/EBPβ, may contribute to the lack of exercise adaptation in CKD. Surprisingly, we found that one week of treadmill training decreased heart mass in control and CKD animals, likely through repeated suppression of protein synthesis. This cardiac atrophy during the first week of treadmill exercise was associated with a greater loss of cardiac protein in CKD rats compared to exercised controls, which again may be due to elevated autophagy. Collectively, these results suggest that the cardiac response to exercise is altered by the CKD environment, and further research is needed to determine how the benefits of exercise may be rescued under these conditions.

Disclosure

Nothing to disclose.

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Data availability statement

All data points are shown in the figures. The authors agree to provide raw data upon reasonable request.

Competing interests

All authors declare no competing interests

Author contributions

CMTH, MG, and KB, contributed to project design. CMTH and MG completed data acquisition and analysis. All authors contributed to the interpretation of the work as well as drafting and revising it critically for important intellectual content. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved; and all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Chapter 5: Discussion

Adenine-diet-induced Chronic Kidney Disease Model

Many animal models of chronic kidney disease (CKD) exist in the literature; the majority of these utilize either mice or rats. While rodent models have long come under scrutiny, at times deserved and at times not, they enable mechanistic research that is not ethical to perform in humans. This is because rigorous research is highly dependent on both control of experimental conditions and the ability to observe experimental responses. In this light, human subjects research poses two major constraints: 1) the environment and behavior of human subjects is not easy to control, and 2) most molecular and mechanistic observations require tissue samples, which are difficult, if not impossible to obtain from living human subjects. Although someday far in the future, technology may progress to the point where all molecular observations can be made non-invasively, we will never possess the ability to completely control the environment and behavior of human subjects. Thus, model systems are required for biomedical progress. As for tissue culture and computational modelling, these tools are indispensable resources that may supplement or replace animal models in many instances. However, a key requirement for any model is that it recapitulates the system for which it is acting as a surrogate. In the case of complex multiorgan conditions such as CKD, organoids and computer models simply are not yet able to capture the complexity of the interplay between physiological systems the way that animal models can. With that in mind, much of this dissertation serves to characterize the adenine-diet-induced model of CKD in Sprague Dawley rats and determine whether it is appropriate to use to investigate the musculoskeletal and cardiac dysfunction that is known to accompany CKD.

We chose the adenine model of CKD based on the findings of Kim and colleagues, who directly compared CKD induction via adenine-diet to 5/6 nephrectomy for the investigation of CKD-related muscle myopathy (Kim *et al.*, 2021). They found similar levels of renal and skeletal muscle dysfunction in both models, but better survival rates, recapitulation of phosphate dysregulation, and more consistent uremic toxin levels. Others had also shown significant mineral bone disorder in the adenine model (Ogirima *et al.*, 2006; Metzger *et al.*, 2021; Saito *et al.*, 2021). As well as cardiac dysfunction (Shobeiri *et al.*, 2013; Verhulst *et al.*, 2017; Kashioulis *et al.*, 2018; Kieswich *et al.*, 2018). Thus, the adenine model is effective at mimicking many of the extrarenal complications seen in human CKD. In Ch.2, we add to this list by establishing that the adenine model of CKD leads to tendon weakness in growing rats. This was an important finding as it is the first model to demonstrate tendon dysfunction in CKD and the first experimental evidence that CKD (and not its treatments or overlap with diabetes) is associated with tendon weakness. Despite a large number of reports of spontaneous tendon ruptures in CKD and two reviews now finding that CKD patients make up between 37% and 61% of all known cases of bilateral knee extensor tendon ruptures (Shah, 2002; Camarda *et al.*, 2017) there is essentially no primary research on the topic. As such, our findings here provide an experimental platform for research into a yet unexplored but important comorbidity of CKD.

Responses to Exercise in CKD

Beyond our novel findings in tendon, we also report here a comprehensive analysis of muscle, bone, and cardiac abnormalities in adenine-induced CKD. More importantly, we evaluate the use of exercise as a potential intervention to treat these CKD-associated pathologies. As discussed in depth in Ch.1, exercise is a potent treatment in CKD with benefits ranging from improved blood pressure to decreased inflammation, to enhanced muscle function and physical

fitness. However, low study enrollment numbers and high variability in exercise adherence have limited our understanding of what beneficial adaptations we can anticipate from an exercise intervention. Particularly when it comes to the effects of exercise on skeletal and cardiac health (and certainly tendon health), there is a paucity of available human research. Further, as mentioned above, when adaptations are or are not obtained in such studies, very little can be done to gain mechanistic insight into the underlying causes. This limits not only our understanding of the condition, but also our ability to develop novel nutritional or pharmaceutical treatments with specific molecular targets. Once again, this is where animal models can provide valuable information that will help progress clinical care. Our results presented in Ch. 3-4 begin to do just that. We identified that 8 weeks of uphill treadmill running maintains muscle mass but not strength or endurance, does not significantly improve bone structure or strength, does not lead to cardiac hypertrophy, and actually causes tendon weakness in adult rats with CKD. Importantly, our work also included sedentary and exercised control animals, allowing us to verify that adaptation occurs in healthy animals with our intervention and to probe mechanistically what induces this response. This data allows us to now address why CKD may blunt exercise-induced adaptations and improve the design of future exercise interventions.

Remaining Gaps and Future Studies

As with any research, this dissertation leaves far more questions unanswered than answered. While we characterize many responses and non-responses to exercise in CKD, the next step is to chase each of these down and determine the etiology of the condition and how it may be rectified. Among the presented works, perhaps the most exciting and ripe for further interrogation is the tendon dysfunction we observed in CKD. Because we found tendon

weakness in growing but not skeletally mature CKD rats, and that tendon weakness manifested in the mature rats only following 8 weeks of treadmill exercise, we believe that CKD likely impairs the ability of tenocytes to grow and repair the extracellular matrix. This is different than the effects of CKD on skeletal muscle or bone, where tissue is actively lost, especially when combined with disuse. The fact that tendon gets worse with exercise is likely related to defective proteostasis in tenocytes. We had planned to address this protein turnover question in this dissertation by measuring the tendon protein synthesis response to the acute exercise in Ch. 4. However, as tendons are highly acellular, we anticipated that the puromycin technique may not yield detectable results in our tendon samples. For this reason, we attempted to implement bioorthogonal non-canonical amino acid tagging (BONCAT) in the acute exercise study design described in Ch. 4. Unfortunately, of the twelve animals dosed with our BONCAT tracer (azidohomoalanine, AHA), nine of them died within 24 hours. As such, we did not follow through with dosing the remaining twelve animals, which were in the exercise study group. This resulted in the lack of a sedentary CKD group and reduced the number of animals in the sedentary control group to three. The result, unfortunately, was reduced statistical power and ability to isolate the effects of disease from exercise.

Fortunately, we did utilize deuterated water (D_2O) as a backup method to BONCAT. D_2O is identical to water with the exception that the hydrogen contains a neutron and a proton (opposed to only a proton in standard hydrogen). This “heavy” hydrogen first gets incorporated into alanine in the liver through transamination and is subsequently used throughout the body in protein synthesis. Through the use of mass spectrometry, the level of heavy alanine incorporation can then be used to estimate protein synthesis in a given tissue over the course of the D_2O treatment (in our case, 5 days of treadmill exercise) (Belloto *et al.*, 2007). Because we have these

labeled samples, our next step is to send them out for processing. This will provide us with protein turnover data to either support or refute our hypotheses on tendon protein synthesis in CKD. Unfortunately, this data could not be obtained in time for inclusion in the dissertation.

If we do find impaired protein synthesis in CKD tendons, the next question to answer will be why this occurs. Because tendons are dependent on load to maintain and repair the extracellular matrix (Tam & Baar, 2025), one potential explanation could be impaired mechanosensing in CKD tendon. Indeed, we did find that toe region mechanics were altered in adult CKD animals in the absence of exercise. Because the toe region makes up much of the tendon strain range utilized during physical activity, this could potentially alter how much stretch is sensed by the cells within the matrix. An experimental way to test this would be to use genetically engineered mice that express a calcium sensor such as GCaMP6. Calcium release is an important secondary messenger in tendon mechanosensing (Nakamichi *et al.*, 2022) and can be used to identify stretch sensitivity. After induction of CKD with an adenine diet, tendons could be extracted from GCaMP6 mice and imaged on a fluorescence microscope under varying degrees of strain. If the slope between strain and calcium release varies between control and CKD animals, this would support our theory of altered mechanosensing. An important adjoining experiment would be to also treat a subset of control and CKD mice with angiotensin receptor blockers (ARBs). ARBs are commonly prescribed to individuals with CKD and are independently associated with a 7-fold increased risk of spontaneous tendon rupture (Nyyssönen *et al.*, 2018). Interestingly, angiotensin receptors are also mechanosensitive and can induce calcium signaling (Mederos Y Schnitzler *et al.*, 2011; Wang *et al.*, 2018). Thus, it would be very interesting and pertinent to determine whether these drugs are affecting tendon mechanosensing as well.

Optimization of exercise interventions to drive specific benefits is an often-overlooked component of exercise research and prescription. As discussed in Ch. 1, care should be taken to select the exercise mode, intensity, and timing that elicits the desired physiological response responsible for a given adaptation. While we provide both a framework and a specificity chart to guide such decisions in Ch. 1, our work here utilized a relatively nonspecific exercise intervention. Indeed, if our goal was to strengthen tendon specifically, isometric loading would likely have been beneficial (Baar, 2017). While uphill running was likely a better choice than level running for inducing bone adaptation (Tamakoshi *et al.*, 2018), an intervention that spreads out short period of load throughout the day may have had a better chance at providing benefits in adaptation-resistant CKD animals (Robling *et al.*, 2000). Further, muscle-specific adaptation could be better targeted through resistance training, whilst treadmill running, or swim training is likely a preferable intervention for cardiac hypertrophy in rats. However, because we utilized control animals in our study, we were able to determine that our intervention does elicit positive adaptations in a tissue-specific manner in the absence of CKD. Due to the clear blunting of adaptation by CKD, optimization of exercise interventions in CKD specifically presents a very interesting area of research. For instance, while our lab is currently working on determining the ideal loading protocol for tendon (research by Tam and Gilmore, soon to be published), such protocols may need modification in the context of CKD.

Translation

Translation of animal research to improve human health is central to our role as scientists. The Functional Molecular Biology Lab is promoting a ‘discovery to recovery pipeline’ to do exactly this. As mentioned earlier, model systems are only useful if their findings can scale up to the system they are modeling. Because the data presented here is only from rats, it would

be inappropriate to over-extrapolate and directly apply it to human health as is perhaps too often in the popular media. To properly translate this work, we must first use it to generate a testable hypothesis in humans. For example, from our data we believe that tendon collagen synthesis in response to exercise is impaired by CKD, we can test this in humans by measuring the serum P1NP response following exercise in individuals with CKD versus healthy controls. While P1NP is a surrogate marker, and not nearly as direct as measurements of collagen synthesis that can be performed in rodents, a finding of a lower P1NP exercise response in CKD, along with our hypothesized evidence in rats, would support further investigation. Following consistent replication of these findings - perhaps with various animal models and collagen synthesis markers in humans – we can then begin to inform decision-making for the treatment of human disease. For this, we would need to determine the answers to follow-up questions such as: how do we properly load tendons to induce positive adaptation in individuals with CKD, or how can the normal response to load be restored in CKD tendons, or simply how can tendons be safely loaded in CKD? To address these questions, it will likely be most effective and timely to return to our animal model, which allows for higher throughput and greater mechanistic insight. Thus, the cycle of translating research continues iteratively up and down the spectrum from discovery to recovery.

All of this is not to say that our findings here should have no bearing on those looking to implement exercise interventions in CKD patients. On the contrary, especially in the area of CKD-related tendon dysfunction, where available information is virtually non-existent, it is important to take into account the available information in an appropriate way. In the position of a practitioner, such as a physical therapist, I would interpret this as follows. *Research in rats has shown that uphill treadmill running may lead to tendon damage in CKD patients, because I do*

not yet know if this occurs in humans or to what degree, I will balance the potential unknown risks with the known benefits of exercise, monitor my patient for obvious symptoms of tendon pathology (such as pain), and mediate risk through good exercise practices such as slowly progressing intensity and managing load. Alternatively, a cardiac rehab specialist working with a client with CKD could use this data as a potential explanation if a lack of cardiac adaptation is identified, while holding on to the idea that this may in fact not be the case. It is my hope that we will have the opportunity to continue asking these questions, and interested parties should be on the lookout for future works that begin to address the systemic effects of CKD on musculoskeletal and cardiac tissues and how these might be treated through exercise and other interventions.

Key Takeaways

1. Exercise has many proven benefits for individuals with CKD and should be implemented immediately where possible; however, best practices and adjacent therapies for implementation remain to be optimized.
2. Tendon dysfunction is likely an underappreciated issue in CKD, beyond years of case reports of spontaneous tendon ruptures. We provide here the first experimental evidence suggesting that tendons are weakened in the presence of CKD.
3. Tendon weakness in CKD appears to be due to impairments in the growth and repair process, and may be related to altered sub-rupture tendon mechanics and mechanosensing – though this remains to be tested.
4. Eight weeks of uphill treadmill running in rats with adenine-diet-induced CKD prevents muscle atrophy but does not prevent declines in muscle strength or endurance, nor does it improve bone structure or strength.

5. Eight weeks of uphill treadmill running induces cardiac hypertrophy in control rats, but not in rats with adenine-diet-induced CKD, and this is likely due to alterations in the balance between anabolic and catabolic processes within the heart.

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