Supplemental Material Table of Contents

Methods

Supplemental Table 1. Association of total quadriceps cross-sectional area with mitochondrial function.

Supplemental Table 2. Association of physical performance with mitochondrial function after adjusting by inflammation and oxidative stress markers.

Supplemental Figure 1. A. PCr recovery time constant tau (τ) was prolonged in patients on MHD compared to controls and patients with CKD with similar physical performance measured by the 6-minute walk (B).

Supplemental Figure 2. Maximal voluntary contraction (MVC) of the quadriceps muscle was diminished in the MHD group (n=18) compared to control (n=16) and CKD 3-5 (n=20) groups.

Supplemental Figure 3. Interleukin 1 beta (**A**), interleukin-8 (**B**), and interleukin-10 (**C**) levels in control individuals (n=21), patients with CKD 3-5 (n=20), and patients on MHD (n=22).

Supplemental Figure 4. Levels of total coenzyme Q_{10} (CoQ_{10}, **A**), reduced CoQ10 (**B**), in control individuals (n=21), patients with CKD 3-5 (n=20), and patients on MHD (n=21).

References

METHODS

Participants

We recruited 63 participants divided into three different groups; a) **Control**, participants with no history of CKD, b) **CKD 3-5**, patients with eGFR <60 ml/min/1.73m^2 not on maintenance hemodialysis, and c) **MHD**, patients with ESRD on maintenance hemodialysis, three times per week for at least six months, who were clinically stable and adequately dialyzed (single-pool Kt/V > 1.2). GFR was estimated from creatinine using the CKD-EPI formula. Exclusion criteria were a history of functional kidney transplant less than six months prior to the study, use of immunosuppressive drugs within one month prior to the study, active connective tissue disease, acute infectious disease, history of myocardial infarction or cerebrovascular event within three months prior to the study, advanced liver disease, gastrointestinal dysfunction requiring parenteral nutrition, active malignancy, left ventricular ejection fraction less than 40%, history of poor adherence to hemodialysis or medical regimen, use of vitamin E>60 IU/day or vitamin C >500 mg/day, and inability to undergo MRI evaluation. Patients with a history of stable cardiovascular disease were included in the study (Table 1). The study was approved by the Vanderbilt University Human Research Protection Program Committee.

31-Phosphorus magnetic resonance spectroscopy (^{31}P-MRS)

Quadriceps muscle mitochondrial bioenergetics were measured using ^{31}P-MRS protocol that has been previously published.[1] Briefly, each participant lay prone with a coil positioned over the belly of the rectus femoris muscle. The knee was flexed and suspended with elastic bands secured to a non-magnetic ergometer at one end and ankle Velcro® strap at the other end. The level of resistance of the elastic tubing was selected to an intensity that decreased PCr to approximately 30% of the baseline during the exercise protocol. After basal measurements, participants were asked to perform two knee extensions every three seconds against the

resistance of approximately 30- 40% of the MVC. The exercise protocol lasted 90 seconds (a total of 60 knee extensions) followed by four minutes of rest. The exercise/rest cycle was repeated three times. The intensity of the exercise decreases phosphocreatine (PCr) levels with minimal change in muscle pH. Spectra analysis was performed with AMARES from the jMRUI software package.[2] Spectra were used to calculate the relative concentrations of inorganic phosphate (Pi), PCr, and ATP. The recovery of PCr after the exercise was fit with a monoexponential model that calculated the time constant tau (τ), which is the time to restore approximately 63% of the recovery response.

Magnetic resonance imaging and intermuscular fat (IMAT) measurements

IMAT was calculated using nine consecutive cross-sectional images of the mid-thigh region between the patella and ischial spine. Each section was 3 mm thickness and at a 14 mm interval. The analysis was performed in all the quadriceps muscle heads using a custom-written Matlab (Mathwworks, Natick, MA) program, as previously described.[3] IMAT was defined as the fat beneath the deep fascia of the muscle. IMAT infiltration was quantified as the ratio between IMAT and muscle volumes.

Six-minute walk and maximal voluntary contraction

We measured physical performance with the six-minute walk test. Briefly, this test consists of instructing the patients to walk back and forth on an indoor 30-meter measured corridor.[4] Maximal static voluntary contraction (MVC) of the quadriceps was measured with the participant lying prone, the leg suspended by a Velcro strap, and with a knee angle of approximately 20°. The strap was attached to an ergometer by two rigid metallic rings. MVC was repeated until we obtained at least three consistent and reproducible MVCs.

Skeletal muscle biopsies

We performed muscle biopsies in a subgroup of patients (15 controls and 9 patients on MHD). Biopsies were obtained from the vastus lateralis, one of the heads of the quadriceps femoris, by a percutaneous needle biopsy with the modified Bergström technique.[5] Briefly, after proper aseptic technique and administration of local anesthesia, the needle was then inserted through a skin incision to the skeletal muscle. The inner trocar of the needle was retracted, and suction applied to pull muscle into the outer trocar. The inner trocar was then closed to cut the muscle. The procedure was repeated two more times. Muscle biopsies were immediately placed into the fixative solution, or instantly frozen in liquid nitrogen and stored at -80ºC.

Transmission electron microscopy

Samples were prepared for electron microscopy as previously described.[6] Briefly, samples were post-fixed in osmium tetraoxide (1%), dehydrated and embedded for further sectioning. Thin (80 nm) fiber transverse sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope.

Western blot analysis

One piece of the muscle biopsy was homogenized, denatured, and resolved electrophoretically in 4-20% precast acrylamide gels (Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-FL, Millipore, Billerica, MA). Membranes were then incubating overnight with primary antibodies against dynamic-related protein 1 (DRP-1, Cell Signaling, Danvers, MA, catalog number 8570), mitochondrial fission 1 protein (Fis-1, Novus Biological, Littleton, CO, catalog number NB110-56646), and optic atrophy protein 1 (OPA-1, Novus Biological, catalog number NB110-55290). After incubating the membranes with fluorescent secondary antibodies, we used the Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) to detect the antibody fluorescence intensity. Band densities were analyzed using NIH Image J software.

Measurements of inflammatory cytokines

Cytokines were measured in serum. Blood was collected in serum tubes and allowed to clot at room temperature followed by centrifugation at 2,000g for 10 minutes at 4°C. Serum was collected and stored at -80°C. Inflammatory cytokines (IL1β, IL6, IL8, IL10, and TNFα) were measured using the multiplex proinflammatory human panel 1 (Meso Scale Discovery, Rockville, MD) according to the manufacturer's instruction.

Coenzyme Q10 (CoQ10) measurements

CoQ10 was measured as previously published.[7] Briefly, 100 µl of plasma samples were mixed with 200 µl of ice-cold propanol containing coenzyme Q9 and reduced CoQ9 as internal standards. Precipitated proteins were removed by centrifugation and the supernatant was analyzed by liquid chromatography-tandem mass spectrophotometry.

Statistical Analysis

We performed standard graphing and screening to detect any outliers and verify the data accuracy. The distribution of endpoints was examined for normality. We used the Kruskal-Wallis test to compare the difference among the groups and implemented contrast for specific two-group comparisons. Differences in PCr recovery time (tau) among the groups were assessed using ANCOVA with tertile of eGFR as fixed effects, and covariates including gender, BMI, age, six-minute walk distance as covariates. We tested the association of mitochondrial function (PCr recovery time) with physical performance, IMAT, and markers of inflammation and oxidative stress using linear regression and adjusting for potential confounders. An additional comparison between Control and MHD groups for mitochondrial fragmentation and markers of mitochondrial dynamics was performed using the Wilcoxon rank-sum test. Hypotheses were tested at the level of $\alpha=0.05$. Statistical analysis was performed using SPSS version 25 (IBM, North Carolina).

Supplemental Table 1. Association of total quadriceps cross-sectional area with mitochondrial function.

	Unadjusted (n=57) β (95% CI)	**Adjusted** (n=57) β (95% CI)	**Adjusted p value**
Quadriceps CSA (cm^2)	-0.137 (-0.246, -0.028)	-0.211 (-0.372, -0.050)	0.011
Age (years)		0.164 (-0.256, 0.583)	0.436
BMI (kg/m^2)		-0.145 (-0.906, -0.615)	0.702
Gender (female)		8,559 (-4.110, 21.228)	0.180
Race (Caucasian)		-9.289 (-19.036, 0.458)	0.061

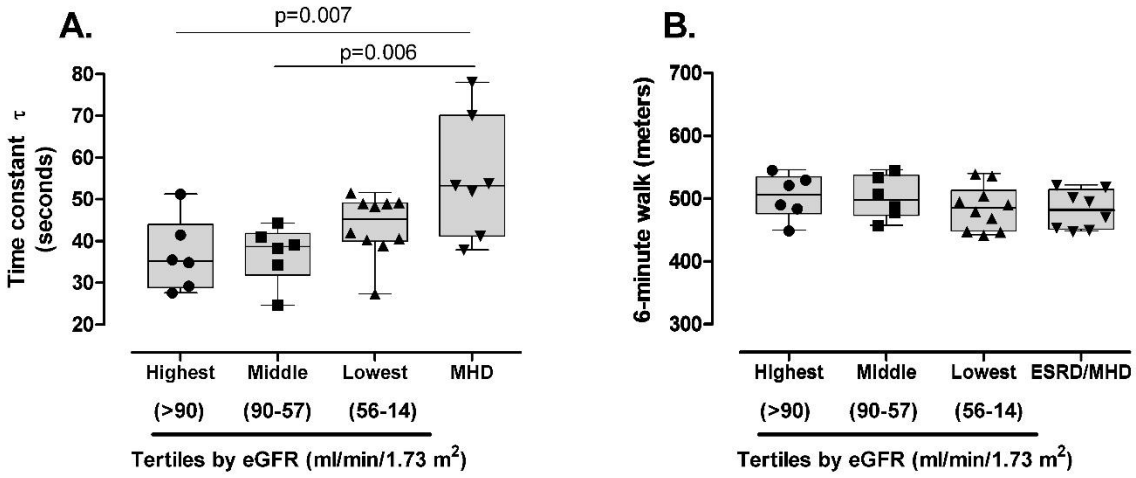
CSA, cross-sectional area; BMI, body mass index. Mitochondrial function (dependent variable) was measured using the tau time constant (in seconds). Every cm^2 increase in CSA was negatively associated with 0.137 seconds lesser in the tau time constant.

Supplemental Table 2. Association of physical performance with mitochondrial function after adjusting by inflammation and oxidative stress markers.

	Unadjusted (n=59) β (95% CI)	**Adjusted** (n=57) β (95% CI)	**Adjusted p value**
TNF (pg/ml)	3.032 (1.754, 4,310)	1.986 (0.731, 3.242)	0.003
CoQ_{10} redox ratio	-7.550 (-11.817, -3.282)	-3.034 (0.142, -7.121)	0.14
6MWT (meters)		-0.047 (-0.078, -0.016)	0.004

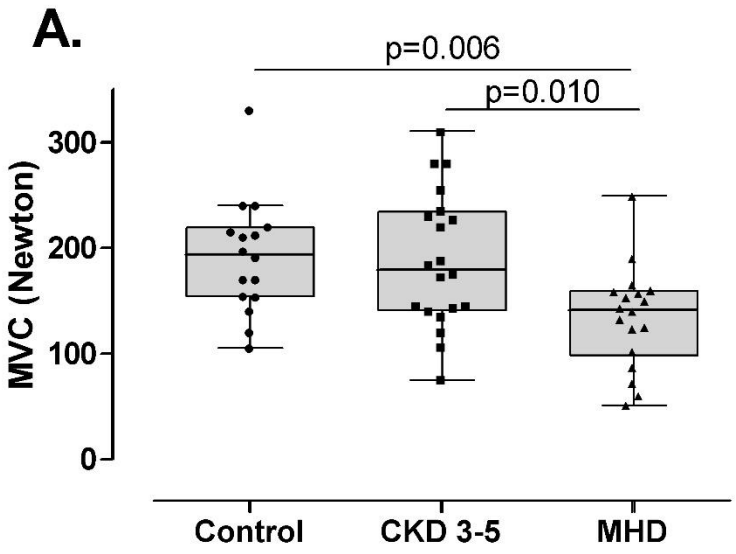
Mitochondrial function (dependent variable) was measured using the tau time constant (in seconds). Each one pg/ml of TNF increase was positively associated with 0.078 seconds greater in the tau time constant.

Supplemental Figure 1



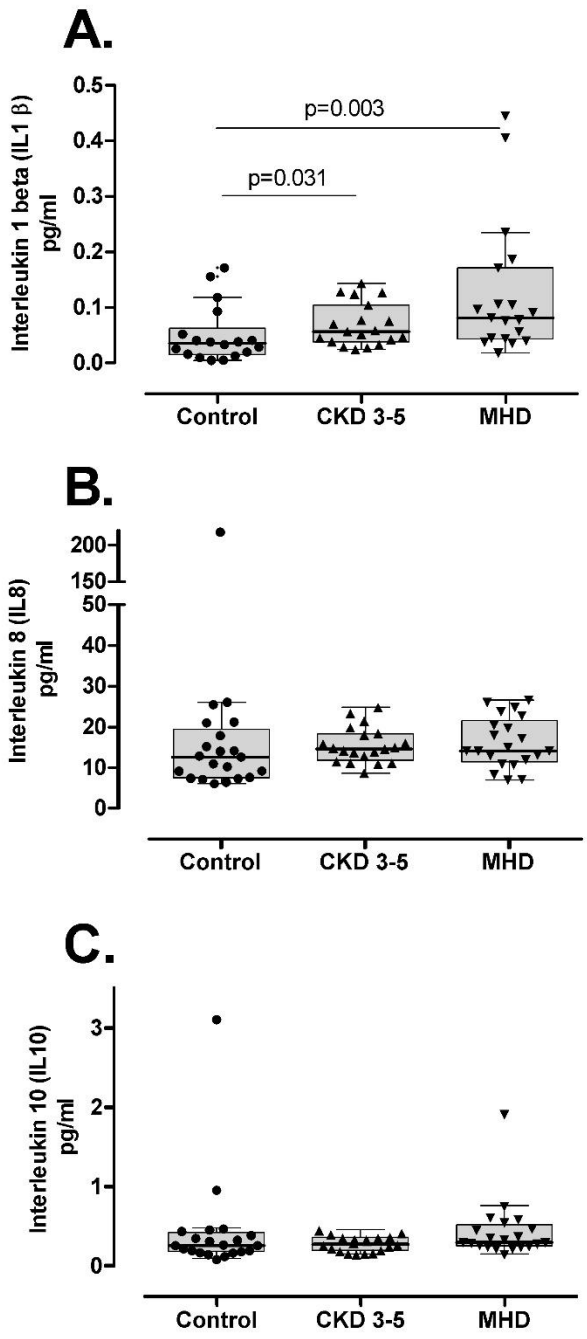
Supplemental Figure 1. A. PCr recovery time constant tau (τ) was prolonged in patients on MHD compared to controls and patients with CKD with similar physical performance measured by the 6-minute walk (B).

Supplemental Figure 2



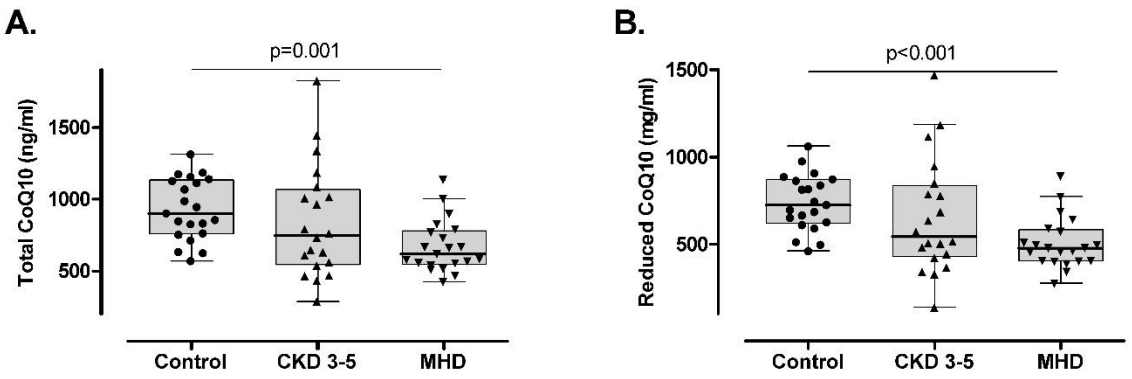
Supplemental Figure 2. A. Maximal voluntary contraction (MVC) of the quadriceps muscle was diminished in the MHD group (n=18) compared to control (n=16) and CKD 3-5 (n=20) groups.

Supplemental Figure 3



Supplemental Figure 3. Interleukin 1 beta (**A**), interleukin-8 (**B**), and interleukin-10 (**C**) levels in control individuals (n=21), patients with CKD 3-5 (n=20), and patients on MHD (n=22).

Supplemental Figure 4



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