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RESEARCH ARTICLE | *Translational Physiology*

Skeletal muscle fibrosis is associated with decreased muscle inflammation and weakness in patients with chronic kidney disease

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Abramowitz MK, Paredes W, Zhang K, Brightwell CR, Newsom JN, Kwon H, Custodio M, Buttar RS, Farooq H, Zaidi B, Pai R, Pessin JE, Hawkins M, Fry CS. Skeletal muscle fibrosis is associated with decreased muscle inflammation and weakness in patients with chronic kidney disease. *Am J Physiol Renal Physiol* 315: F1658–F1669, 2018. First published October 3, 2018; doi:10.1152/ajprenal.00314.2018.—Muscle dysfunction is an important cause of morbidity among patients with chronic kidney disease (CKD). Although muscle fibrosis is present in a CKD rodent model, its existence in humans and its impact on physical function are currently unknown. We examined isometric leg extension strength and measures of skeletal muscle fibrosis and inflammation in vastus lateralis muscle from CKD patients ($n = 10$) and healthy, sedentary controls ($n = 10$). Histochemistry and immunohistochemistry were used to assess muscle collagen and macrophage and fibro/adipogenic progenitor (FAP) cell populations, and RT-qPCR was used to assess muscle-specific inflammatory marker expression. Muscle collagen content was significantly greater in CKD compared with control (18.8 ± 2.1 vs. 11.7 ± 0.7% collagen area, $P = 0.008$), as was staining for collagen I, pro-collagen I, and a novel collagen-hybridizing peptide that binds remodeling collagen. Muscle collagen was inversely associated with leg extension strength in CKD ($r = -0.74$, $P = 0.01$). FAP abundance was increased in CKD, was highly correlated with muscle collagen ($r = 0.84$, $P < 0.001$), and was inversely associated with TNF-α expression ($r = -0.65$, $P = 0.003$). TNF-α, CD68, CCL2, and CCL5 mRNA were significantly lower in CKD than control, despite higher serum TNF-α and IL-6. Immunohistochemistry confirmed fewer CD68+ and CD11b+ macrophages in CKD muscle. In conclusion, skeletal muscle collagen content is increased in humans with CKD and is associated with functional parameters. Muscle fibrosis correlated with increased FAP abundance, which may be due to insufficient macrophage-mediated TNF-α secretion. These data provide a foundation for future research elucidating the mechanisms responsible for this newly identified human muscle pathology.

chronic kidney disease; fibrosis; inflammation; skeletal muscle

INTRODUCTION

Impaired physical function is a major determinant of poor overall health and quality-of-life in patients with chronic kidney disease (CKD) (4, 52, 55, 65). A number of deficits in physical function have been described, including loss of muscle strength, reduced exercise capacity, and the development of mobility impairment and disability (3, 17, 28, 34, 40, 50, 53, 54, 60, 64). Altered muscle physiology contributes to these functional deficits (39, 63).

Prior research has focused on intrinsic muscle fiber deficits and has scarcely examined how kidney disease affects the interstitial muscle extracellular matrix (ECM). The paucity of such investigations is important because the ECM performs several important functions: It provides structural integrity; transfers force both longitudinally and transversely within muscle; protects muscle fibers from injury; and regulates the function of myogenic progenitor cells that reside within the ECM (2, 7, 22, 23, 41, 44). It has been reported that excess ECM accumulation and fibrosis negatively impact muscle force production (19), suggesting that alterations of the ECM can have significant functional implications.

Muscle fibrosis was recently identified in an animal model of advanced CKD, but this has not been investigated in humans; thus its relevance to clinically meaningful functional outcomes is unknown (14, 79). We sought to determine whether skeletal muscle fibrosis was present in humans with CKD, fibrotic burden was associated with a clinically relevant functional outcome, and muscle fibrosis was associated with alterations in cellular constituents that are crucial for appropriate muscle repair pathways.

MATERIALS AND METHODS

Study Population

We performed cross-sectional comparisons between ambulatory participants with non-dialysis-dependent stage 4 or 5 CKD and healthy sedentary volunteers. CKD patients were recruited for this study from a prospective cohort study of patients with CKD stages 4 and 5 [estimated glomerular filtration rate (eGFR) <30 ml·min^{-1}·1.73 m^{-2}] who were not receiving renal replacement therapy with dialysis. Patients were eligible for participation in the parent study if they were ≥21 yr of age, able to provide informed consent, and were ambulatory. Exclusion criteria included bilateral lower extremity amputa-

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tions, use of immunosuppressive medications in the prior 3 mo, and an active cancer diagnosis or receiving treatment for cancer. Patients were ineligible for a muscle biopsy if they were taking anticoagulant medications. Controls were generally healthy with no physical limitations to activity. They were required to be sedentary, which was defined as not being engaged in strenuous work, regular brisk leisure physical activity, or a formal exercise session more than once per week for at least the previous 3 mo (30, 46). Medical history data were collected via standardized questionnaire and medical record review. The study protocol was approved by the Institutional Review Board of the Albert Einstein College of Medicine. Written informed consent was obtained from all participants before inclusion in the study.

Study Design

Following screening, an enrollment visit was conducted during which questionnaires and physical function testing were administered. Health-related quality-of-life was assessed with the 36-Item Short Form Health Survey (SF-36), and functional independence was assessed using the Katz Index of Activities of Daily Living (31).

Physical function. Unilateral knee extensor strength was measured using isometric dynamometry with a handheld dynamometer (Manual Muscle Test System, Lafayette Instrument, Lafayette, IN). To ensure assessment of maximum strength, subjects were instructed to perform a maximal exertion contraction, and two trials were recorded. The highest result achieved in the biopsied leg was used for analysis. Results were normalized for body weight. To measure gait speed, participants walked a 4-m course at their usual pace, with the fastest time used for analysis. Endurance capacity was measured by the 2-min walk test (62): participants were asked to walk back and forth over a 50-foot course as far as possible over 2 min. The distance covered is highly correlated with 6-min walk distance (5).

Physical activity. After the enrollment visit, all participants wore a triaxial accelerometer (Actigraph GT3X-BT, Actigraph, Pensacola, FL) around the waist for 7 consecutive days to measure physical activity level. Participants were instructed to wear the accelerometer at all times, except when showering, bathing, or swimming. Data processing was performed using 60-s epochs in ActiLife 6.13.3. Wear-time validation was performed as per Troiano et al. (70). Intensity levels of physical activity were defined as sedentary time [$<$100 counts/min (cpm)] and light (100–1,951 cpm), moderate (1,952–5,724 cpm), vigorous (5,725–9,498 cpm), and very vigorous ($\geq$9,499 cpm) intensity (16, 47). Sedentary time was classified according to daily time spent in sedentary bouts of 10 or more consecutive minutes, excluding sleep time (70).

Laboratory testing. Serum creatinine was measured by a modified kinetic Jaffé reaction in the clinical laboratory at Montefiore Medical Center. eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (36). Timed 24-h urine collections were performed after the provision of detailed instructions. Urine urea nitrogen (UUN) was measured enzymatically.

Body composition and nutrition. Body composition was assessed using whole-body dual-energy X-ray absorptiometry scans (Lunar Prodigy Advance DXA System, GE Medical Systems Lunar; Madison, WI; software v13.31). The appendicular skeletal muscle mass index (ASMI) was calculated as the total lean mass of the four extremities divided by the square of the height (10). Daily dietary protein intake was calculated as $6.25 \times$ [UUN (g/day) + weight (kg) $\times$ 0.031] (45). Protein-energy wasting (PEW) was defined, with slight modification based on the data available, according to the International Society of Renal Nutrition and Metabolism, as satisfying at least one criterion in at least three of four categories (15): Serum chemistry: serum albumin $<$3.8 g/dl; body mass: BMI $<$23 kg/m^2, $\geq$10 lb. unintentional weight loss over 12 mo, or total body fat percentage $<$10%; Muscle mass: low muscle mass as recommended by the European Working Group on Sarcopenia in Older People (ASMI $<$5.45 kg/m^2 in women and $<$7.26 kg/m^2 in men) (10); Dietary intake: dietary protein intake $<$0.6 g·kg^{-1}·day^{-1}.

Study data were collected and managed using REDCap (Research Electronic Data Capture) electronic data capture tools hosted at the Albert Einstein College of Medicine (25).

Muscle Biopsies

Patients were scheduled for muscle biopsies after completing the accelerometer wear period. On the morning of the study, participants were admitted to the Clinical Research Center study room at 8:00 AM after a 12-h overnight fast. Serum samples were collected and stored at −80°C. Tissue (100–150 mg) was obtained from the vastus lateralis via an incision site 15 cm proximal to the superior border of the patella using a 12-gauge biopsy needle (Bard Monopty, Bard Biopsy Systems, Tempe, AZ). Muscle tissue was blotted to remove extraneous blood and was immediately frozen in liquid nitrogen and then stored at −80°C. Serum TNF-α (ALPCO Diagnostics, Salem, NH) and IL-6 Chemiluminescence (R&D Systems, Minneapolis, MN) were measured in duplicate by ELISA.

Histochemistry and Immunohistochemistry

Tissue from one control participant was unavailable for histochemistry and immunohistochemistry. Frozen tissue was sectioned (7 μm) on a cryostat (HM525 NX, ThermoFisher), and slides were air-dried for 1 h. For collagen staining, slides were fixed for 1 h at 56°C in Bouin's fixative then incubated in picro-sirius red, washed in 0.5% acetic acid, dehydrated, equilibrated with xylenes, and then mounted with cytoseal XYL (ThermoFisher, Waltham, MA).

For collagen 1 and pro-collagen 1 staining, slides were fixed in ice-cold acetone (−20°C) for 10 min, rinsed with PBS, and blocked in 1% BSA in PBS for 1 h at room temperature (RT). Slides were then incubated with primary antibody: collagen 1 [ab34710, 1:200, Abcam, Cambridge, MA) and pro-collagen (SP1.D8, mouse monoclonal, supernatant, Developmental Studies Hybridoma Bank (DSHB)] overnight at 4°C. The SP1.D8 pro-collagen 1 antibody was obtained from the DSHB and deposited by H. Furthmayr, created by the NICHD of the National Institutes of Health (NIH), and maintained at The University of Iowa, Department of Biology (Iowa City, IA). The next day, slides were incubated in goat anti-mouse IgG1 AF555 (ThermoFisher), goat anti-rabbit AF488 (ThermoFisher) for 1 h at RT. Slides were costained with 4′,6-diamidino-2-phenylindole (DAPI).

For collagen 4 and collagen-hybridizing peptide (CHP), immunohistochemical methods were modified according to the manufacturer's instruction (3Helix, Salt Lake City, UT). In brief, sections were fixed in ice-cold acetone (−20°C) for 10 min and blocked in 2.5% normal horse serum for 1 h at RT. 3Helix-5-FAM conjugate (3Helix) was diluted to a working solution (20 μM) and placed on a heating block at 80°C for 5 min to denature 3Helix trimers, then quickly cooled on wet ice for 2 min. Immediately after cooling, anti-collagen 4 (ab6586, rabbit, 1:200, Abcam) was added to the 3Helix-PBS solution, and slides were incubated overnight at 4°C. The next day, slides were washed in PBS and incubated with goat anti-rabbit AF555 (ThermoFisher), and then costained with DAPI.

Platelet-derived growth factor receptor-α (PDGFRα) immunohistochemical methods have been published previously (18). Briefly, sections were fixed in 4% paraformaldehyde, followed by a blocking step in 2.5% normal horse serum (Vector Laboratories, Burlingame, CA), and then incubated overnight with goat anti-PDGFRα (AF-307-NA, 1:100, R&D Systems) and AF488-conjugated wheat germ agglutinin (WGA; W11261; ThermoFisher) at 4°C. The following day, slides were incubated in rabbit anti-goat AF555 (A-21431, ThermoFisher), costained with DAPI (D35471, ThermoFisher), and then mounted with fluorescent mounting media.

For CD68, CD11b, and CD206 macrophages, immunohistochemical protocols were adapted from Reidy et al. (61). Sections were fixed in −20°C acetone for 10 min followed by blocking for 1 h in 2.5%

normal horse serum at RT and subsequently incubated overnight at 4°C with AF488-conjugated WGA and one of three primary antibodies against either CD68 (1:100, M0814, Dako, Santa Clara, CA), CD11b (1:100, MON1019-1, Cell Sciences, Newburyport, MA), or CD206 (1:100, AF2534, R&D Systems) diluted in 2.5% normal horse serum. On *day 2*, sections were incubated for 1 h with one of two secondary antibodies: rabbit anti-goat IgG AF555 (1:500, A-21431, ThermoFisher) or goat anti mouse IgG1 AF555 (1:500, A-21121, ThermoFisher). Sections were then costained with DAPI and then mounted with fluorescent mounting media.

Image Acquisition and Analysis

Images were captured at ×200 total magnification using a Zeiss AxioImager M1 microscope (Carl Zeiss, Thornwood, NY), and analysis was carried out using AxioVision Rel software (v4.9). Picrosirius red staining was quantified to measure expansion of collagen between muscle fibers using the thresholding feature of FIJI software (https://fiji.sc/), and the area occupied by collagen was expressed relative to the total muscle area (mm^2). Fibro/adipogenic progenitors (FAPs; PDGFRα+/DAPI+) were identified as previously described (18). Briefly, FAPs were identified with PDGFRα+ staining surrounding a DAPI+ nucleus, to denote the presence of PDGFRα+ cell surface expression that has been used by others to identify FAPs in human skeletal muscle (71). We only identified cells as FAPs when the PDGFRα+ staining pattern exhibited clear cell surface/membrane staining, i.e., no overlap with a DAPI+ nucleus. PDGFRα is a cell surface protein, not a transcription factor, and this distinction was our justification for the inclusion of cells that only display PDGFRα surrounding a DAPI+ nucleus. Our assessment is in line with others in the field who have quantified FAPs in human muscle biopsies (71). Cell counts were normalized to the total area of the muscle cross section (mm^2). Collagen 1 and collagen 4 were quantified to measure expansion of key components of the ECM using the thresholding feature of Zeiss AxioVision software (v4.9), and the area occupied by collagen 1 and 4 were independently expressed relative to the total muscle area (mm^2). CHP analysis was conducted in a similar manner to determine areas of active collagen remodeling. The binding area of the CHP was determined using the thresholding feature of AxioVision and expressed relative to the total muscle area (mm^2). Muscle macrophage content was quantified as either CD68+/DAPI+, CD11b+/DAPI+, or CD206+/DAPI+, and cellular density was normalized per unit area (mm^2). All immunohistochemical images were analyzed by a single assessor in a blinded manner to control or CKD subject status.

RT-qPCR

Muscle samples were homogenized with QIAzol Lysis Reagent (Qiagen), and then the aqueous phase was used for RNA isolation. Total RNA (1 mg) isolated with an RNeasy Mini Kit (Qiagen Sciences) was reverse transcribed to cDNA using the SuperScript VILO cDNA synthesis kit (Invitrogen). TaqMan (Applied Biosystems) RT-qPCR was performed for measurement of mRNA using standard curves. Gene expression was adjusted by comparison with human RPL7 expression. Primer-probe mixtures for human RPL7 were customized, and other primer-probe mixtures were from Applied Biosystems. Primers and probes used to detect human RPL7 mRNA were forward primer 5′-AAGAAGCGAATTGCTTTGACAGA-3′, reverse primer 5′-CAAATCCTCCATGCAGATGATG-3′, and probe 5′-[6-FAM] ACGCTTTGATTGCTCGATCTCTTGGTAAATACG-[TAMRA-6-FAM]-3′. Tissue from one CKD patient was unavailable for RT-qPCR.

Statistics

Baseline characteristics, histochemistry, immunohistochemistry, and gene expression data were compared between CKD patients and controls using χ^2 tests or Fisher's exact test for categorical variables and two-tailed *t*-tests or Wilcoxon rank-sum tests for continuous variables. Correlations were tested by graphically assessing the existence of a linear fit between variables and calculating Pearson correlation coefficients. All analyses were performed with Stata 13.1 (StataCorp, College Station, TX). A P value <0.05 was considered statistically significant.

RESULTS

Participant Characteristics

Ten patients with CKD and 10 control individuals participated in this study (Table 1). Age did not differ between the groups ($P = 0.89$). Compared with the controls, participants in the CKD group were more likely to have hypertension. Control participants did not have other comorbidities. Among the CKD patients, 60% had diabetes, 20% had coronary artery disease, 20% had congestive heart failure, and none had a diagnosis of peripheral vascular disease. Their median eGFR was 12.9 $ml \cdot min^{-1} \cdot 1.73\ m^{-2}$ (interquartile range, 7.7–18.1), reflective of their advanced kidney disease. Based on the Physical Component Score from the SF-36, compared with controls, CKD patients had poorer self-reported physical functioning. Physical activity levels did not differ between the groups. All CKD patients and controls performed all activities of daily living independently. Lean body mass, ASMI, serum albumin, and dietary protein intake did not differ between CKD patients and controls. No participants met criteria for PEW.

Skeletal Muscle Collagen and Muscle Strength

Muscle collagen content was quantified using histochemistry with picro-sirius red staining. Total muscle collagen was increased in patients with CKD compared with controls (Fig. 1, *A–C*, 18.8 ± 2.1 vs. 11.7 ± 0.7% collagen area, $P = 0.008$). Staining for type I collagen, one of the main fibrillar collagens in skeletal muscle and structural components of the ECM (57), was greater in the CKD patients (Fig. 1, *E–G*). The number of pro-collagen I-expressing cells was also higher in CKD (Fig. 1, *E–H*). There was no difference in staining for type IV collagen, which is the major basement membrane collagen (Fig. 1, *I–K*) (57).

We performed several analyses to address possible confounding. Adjustment for race in a linear regression model did not affect the difference in muscle collagen between CKD and controls: CKD patients had 7.2% [95% confidence interval (CI) 1.4–13.0; $P = 0.02$] greater muscle collagen content after adjustment, compared with a 7.1% (95% CI 2.1–12.0; $P = 0.008$) difference in an unadjusted model. Muscle collagen content did not differ between diabetic and nondiabetic CKD patients (19.7 ± 3.6 vs. 17.4 ± 0.9%, respectively; $P = 0.63$), and was significantly greater than controls irrespective of diabetes status (Fig. 1*D*). Greater muscle collagen was also present in CKD after excluding patients with either coronary artery disease or congestive heart failure (20.2 ± 2.7% ($n = 7$), $P = 0.004$ vs. control). To address the possibility that hypertension could explain the increase in muscle collagen, we compared muscle collagen content in controls with and without hypertension and found no difference (12.7 ± 1.3 vs. 11.2 ± 0.8, respectively; $P = 0.33$). In addition, muscle collagen in patients with CKD was not associated with the number of antihypertensive medications used ($r = -0.11$, $P = 0.77$). To

Table 1. *Participant characteristics*

	Control	Chronic Kidney Disease	*P*
Age, yr	61.4 ± 15.2	62.2 ± 7.8	0.89
Women – *n* (%)	4 (40)	3(30)	1.0
Race/ethnicity – *n* (%)			0.09
Non-Hispanic white	4 (40)	0	
Black	4 (40)	8 (80)	
Hispanic/multiracial	2 (20)	2 (20)	
Hypertension – *n* (%)	3 (30)	9 (90)	0.02
Diabetes – *n* (%)	0	6 (60)	0.01
Coronary artery disease – *n* (%)	0	2 (20)	0.47
Congestive heart failure – *n* (%)	0	2 (20)	0.47
Peripheral vascular disease – *n* (%)	0	0	....
Medication – *n* (%)			
ACE inhibitor or ARB	1 (10)	5 (50)	0.14
Statin	1 (10)	7 (70)	0.02
Vitamin D_2 or D_3	2 (20)	6 (60)	0.19
Activated vitamin D analogs	0	2 (20)	0.47
Body mass index, kg/m^2	28.7 (27.2–30.0)	33.4 (24.4–39.8)	0.17
eGFR, ml·min^{-1}·1.73 m^{-2}	80.0 (69.5–89.0)	12.9 (7.7–18.1)	<0.001
SF-36 Physical Component Score	51 ± 3	36 ± 9	<0.001
SF-36 Mental Component Score	49 ± 4	47 ± 7	0.50
Daily physical activity, h/day*			
Sedentary time†	8.1 ± 1.7	8.5 ± 1.7	0.66
Light intensity	4.2 ± 1.8	4.7 ± 1.3	0.54
Moderate intensity	0.5 (0.3–0.6)	0.1 (0.1–0.3)	0.10
Vigorous intensity	0 (0–0.04)	0 (0–0)	0.40
Quadriceps strength, kg/kg body wt	0.21 ± 0.10	0.22 ± 0.04	0.72
Gait speed, m/s	1.3 ± 0.2	1.1 ± 0.2	0.25
2-Min walk distance, ft	546 ± 100	455 ± 96	0.05
Lean body mass, kg	49.6 ± 9.1	56.3 ± 14.6	0.24
Appendicular skeletal muscle mass index, kg/m^2	7.27 ± 1.18	8.16 ± 2.15	0.26
Serum albumin, g/dl	4.38 ± 0.24	4.32 ± 0.46	0.72
Dietary protein intake, g/day§	60.7 ± 21.2	56.2 ± 20.2	0.87

Data are presented as means ± SD or median (interquartile range) for continuous variables. *Accelerometer data were unavailable for one control participant who did not wear the Actigraph device. No very-vigorous intensity activity was recorded for any of the participants. †Sedentary time was classified according to daily time spent in sedentary bouts of 10 or more consecutive min, excluding sleep time. §Calculated from 24-h urine urea nitrogen excretion. Urine collections were unavailable in 2 control participants. ACE, angiotensin converting enzyme; ARB, angiotensin II receptor blocker; eGFR, estimated glomerular filtration rate; SF-36, 36-Item Short Form Health Survey.

address possible confounding by medication use, we also compared muscle collagen between users and non-users of medications that could impact skeletal muscle and found similar results regardless of medication use (Table 2). Furthermore, muscle collagen was not associated with the total number of medications used by CKD patients ($r = -0.11$, $P = 0.76$). Muscle collagen content was not associated with muscle mass ($r = 0.39$, $P = 0.10$ for LBM; $r = 0.43$, $P = 0.07$ for ASMI) and remained significantly greater in CKD after adjustment for age, sex, and either lean body mass (5.9%, 95% CI 0.6–11.3; $P = 0.03$) or ASMI (6.1%, 95% CI 1.0–11.1; $P = 0.02$).

We next examined dynamic collagen remodeling using a novel collagen hybridizing peptide (CHP). CHP binds only remodeling, unfolded collagen chains as the static collagen triple helix is inaccessible to peptide binding (26). CHP staining is also indicative of microstructural damage to collagen fibrils (80). CHP staining was significantly greater in CKD patients compared with controls (Fig. 1, *I*, *J*, and *L*).

To examine whether muscle fibrosis might impact physical function, we next examined isometric knee extensor strength from the biopsied leg. Higher collagen content was associated with lower knee extensor strength in CKD patients ($r = -0.74$, $P = 0.01$; Fig. 2*A*). In the overall cohort including controls, muscle collagen content was not associated with knee extensor strength ($r = -0.12$, $P = 0.63$; Fig. 2*B*), but was inversely associated with 2-min walk distance ($r = -0.51$, $P = 0.03$; Fig. 2*C*), with a similar trend for gait speed ($r = -0.43$, $P = 0.07$; Fig. 2*E*). In the CKD group alone, although not reaching statistical significance, these associations were of similar magnitude (2-min walk distance: $r = -0.45$, $P = 0.19$; Fig. 2*D*; gait speed: $r = -0.44$, $P = 0.20$; Fig. 2*F*).

FAP Cells and Skeletal Muscle Inflammation

FAP cells expressing the PDGFRα surface marker have recently been implicated in the development of muscle fibrosis, including in an animal model of CKD (14, 72). Using immunohistochemical quantification, PDGFRα+ cells, located in the interstitial space between muscle fibers, were more abundant in the muscle of CKD patients (Fig. 3, *A–C*), and their abundance strongly correlated with muscle collagen content ($r = 0.84$, $P < 0.001$ overall; $r = 0.90$, $P < 0.001$ in CKD) (Fig. 3, *D* and *E*). FAP pools expand in response to muscle damage; subsequent reduction in FAP numbers is dependent upon signaling by proinflammatory macrophages (35). Specifically, in the absence of TNF-α secreted by proinflammatory macrophages, the expansion of FAP pools persists and causes fibrosis (35). To determine whether differences in inflammatory gene expression could explain our findings, mRNA was

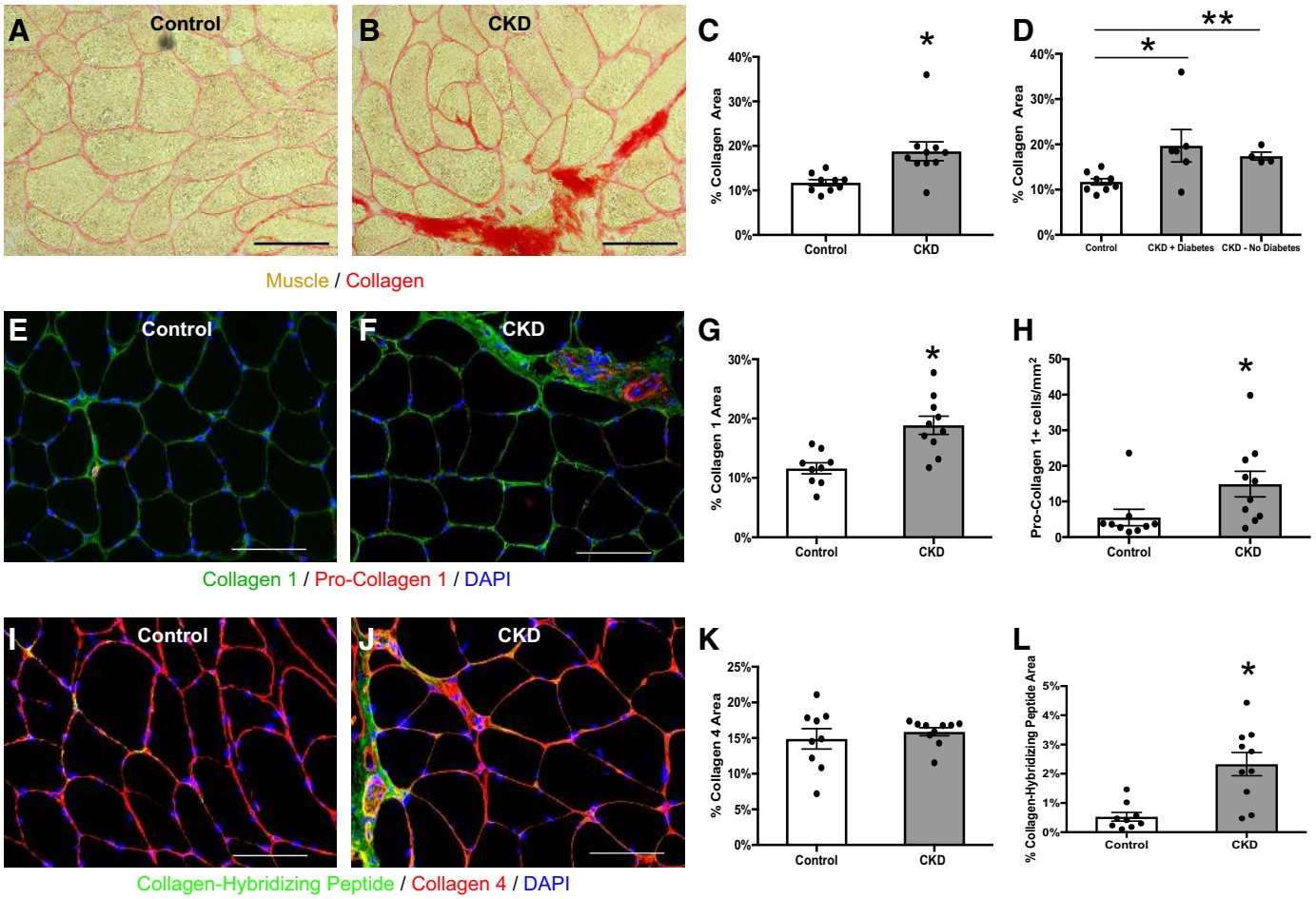

Fig. 1. Increased collagen content within the m. vastus lateralis muscle of chronic kidney disease (CKD) patients. *A* and *B*: representative histochemical image of picro-sirius red collagen stain in control (*A*; $n = 9$) and CKD (*B*; $n = 10$) muscle biopsies. Scale bar = 100 μm. *C*: quantification of collagen content within the muscle represented as mean percentage of total muscle area ± SE. *D*: quantification of collagen content stratified by diabetes status ($n = 6$, CKD with diabetes; $n = 4$, CKD without diabetes). *P* values were calculated for comparisons between each CKD subgroup and control. *E* and *F*: representative immunohistochemical images demonstrating staining for collagen 1 (green), pro-collagen 1 (red), and DAPI (blue) in control (*E*) and CKD (*F*) muscle biopsies. Scale bar = 100 μm. *G* and *H*: quantification of collagen 1 content (*G*) and pro-collagen 1+ cells (*H*) within the muscle represented as mean percentage of total muscle area ± SE. *I* and *J*: representative immunohistochemical images demonstrating staining for collagen hybridizing peptide (CHP; green), collagen 4 (red), and DAPI (blue) in control (*I*) and CKD (*J*) muscle biopsies. Scale bar = 100 μm. *K* and *L*: quantification of collagen 4 content (*K*), and CHP binding (*L*) represented as mean percentage of total muscle area ± SE. *$P < 0.05$. **$P < 0.001$.

quantified using qRT-PCR in whole muscle lysates. TNF-α expression was inversely correlated with both FAP abundance ($r = -0.65$, $P = 0.003$) (Fig. 4*A*) and with muscle collagen content ($r = -0.56$, $P = 0.01$) (Fig. 4*B*), and TNF-α expression was significantly reduced in the muscle of CKD patients compared with controls (Fig. 4*C*), despite higher systemic inflammatory markers (CKD vs. control: IL-6, 3.4 (2.6–5) vs. 2.4 (1.6–3.4) pg/ml, $P = 0.04$; TNF-α, 10.2 (8.1–16.4) vs. 7.0 (6.2–9.6) pg/ml, $P = 0.09$). We hypothesized this could be due to differences in muscle macrophage content or phenotype. Muscle expression of the macrophage marker CD68 and chemokines CCL2 and CCL5 was also significantly reduced in CKD patients (Fig. 4, *D–F*). Immunohistochemical quantification demonstrated fewer CD68+ cells (Fig. 5, *A–C*; monocyte and panmacrophage marker) and fewer CD11b+ cells (Fig. 5, *D–F*; proinflammatory macrophage marker) in CKD patients

Table 2. *Muscle collagen content based on medication use**

	Control	CKD		*P* Value	
	Non-user	Non-user	User	CKD Non-user vs. Control	CKD Non-user vs. CKD User
ACE inhibitor or ARB	11.9 ± 0.8	19.9 ± 4.4	17.6 ± 0.7	0.04	0.62
Statin	11.7 ± 0.8	17.7 ± 1.0	19.2 ± 3.1	0.002	0.75
Vitamin D†	11.8 ± 0.9	16.0 ± 2.3	20.7 ± 3.1	0.07	0.31

Quantification of collagen content within the muscle is presented as mean percentage of total muscle area ± SE. *User and non-user refer to use of each medication class separately. †Vitamin D_2 or D_3 or activated vitamin D analogs. CKD, chronic kidney disease; ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker.

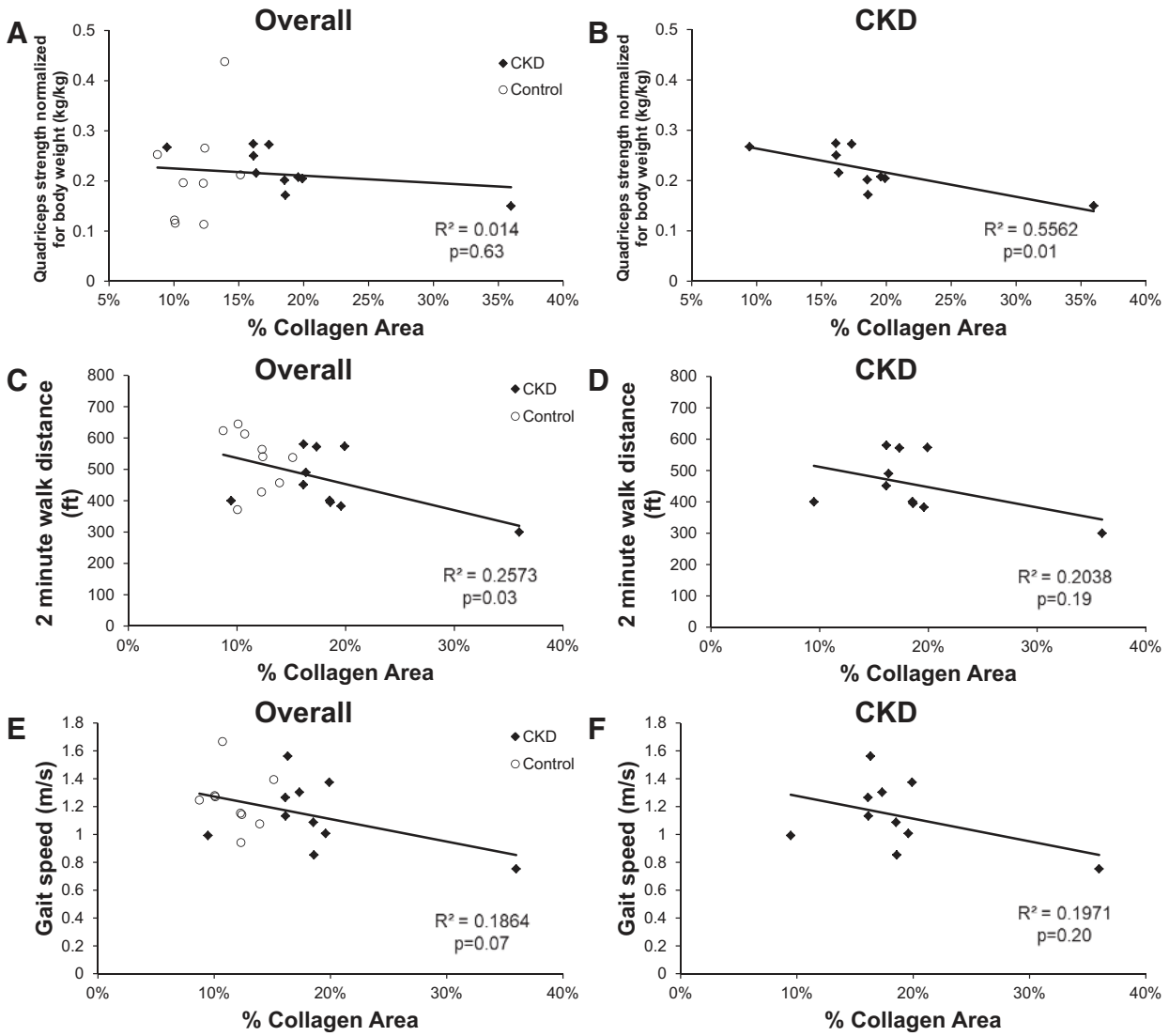

Fig. 2. Correlation of collagen content within the m. vastus lateralis muscle with physical function in all participants (*A*, *C*, and *E*) and CKD patients (*B*, *D*, and *F*). *A* and *B*: unilateral knee extensor strength was measured in the leg to be biopsied using isometric dynamometry with a handheld dynamometer. *C* and *D*: gait speed was measured by having participants walk a 4-m course at their usual pace. *E* and *F*: endurance capacity was measured by asking participants to walk back and forth over a 50-ft course as far as possible over 2 min.

compared with controls but no difference in CD206+ cells (Fig. 5, *G–I*; anti-inflammatory macrophage marker) between the two groups.

DISCUSSION

Preventing functional decline and loss of mobility among patients with CKD is a major unmet need: mobility impairment leads to falls, hospitalizations, and significant morbidity (6, 51, 56). Based on our results, this functional impairment may partly be due to skeletal muscle fibrosis. Muscle collagen content was increased in CKD patients; based on collagen I staining, this was explained by increased fibrillar collagen deposition. As pro-collagen I staining was also greater in CKD, the increased muscle collagen is likely, at least in part, due to increased collagen synthesis; increased CHP staining suggests there is also increased collagen proteolytic remodeling in CKD. As muscle collagen content was inversely associated with measures of physical function, it is possible that collagen deposition in CKD could be pathological and have functional implications.

An effect of excess muscle collagen on physical function could be due to impaired transfer of force generated by myofibril contractile units. Maximal transfer to tendons of the force generated by myofibril contraction requires intact ECM (20, 33, 42, 49, 57, 68, 69). Whereas single-fiber force was preserved in an animal model of muscle fibrosis, whole-muscle force per cross-sectional area was reduced (19). Compared with isolated muscle fiber bundles without ECM, the load-bearing potential of fiber bundles with intact ECM is about five times greater (38, 48, 58). Therefore, while producing minimal changes in muscle mass, pathological alteration of the ECM can cause meaningful changes in muscle function.

Impaired force generation could also explain the association of muscle collagen with endurance capacity; alternatively, since fibrosis may be accompanied by capillary rarefaction (12), muscle collagen could be a marker for impaired muscle

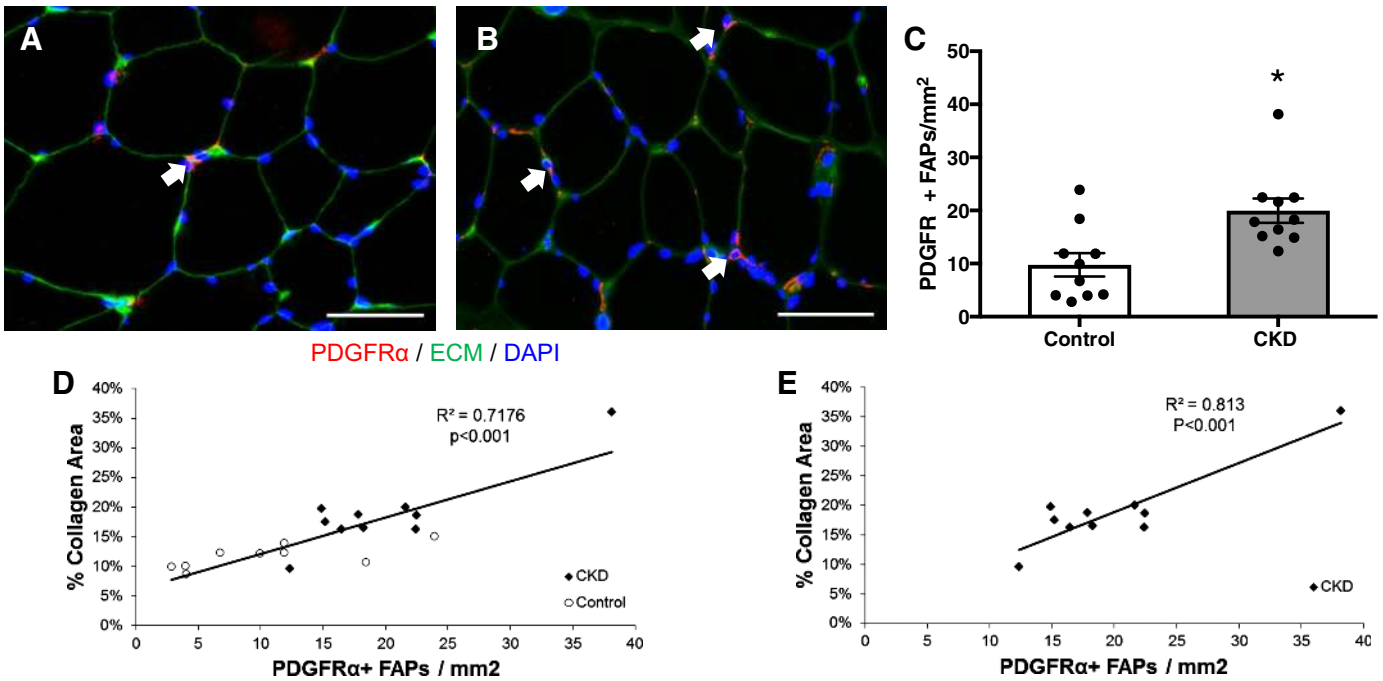

Fig. 3. Increased fibrogenic/adipogenic progenitor (FAP) cell abundance within the m. vastus lateralis muscle of CKD patients. *A* and *B*: representative immunohistochemical image demonstrating PDGFRα+ muscle FAPs (PDGFRα+ cell surface expression surrounding DAPI+ nucleus; white arrows, red), extracellular matrix (ECM; green) and DAPI (blue) in control (*A*) and CKD (*B*) muscle biopsies. Scale bar = 50 μm. *C*: quantification of FAP content within the muscle represented as mean number of PDGFRα+ FAPs per total muscle area ± SE. *D* and *E*: correlation of collagen content within the m. vastus lateralis muscle with FAP content in all participants (*D*) and CKD patients (*E*). *$P < 0.05$.

perfusion, which would promote fatigability and limit endurance. Another possibility is that fibrosis accompanies muscle atrophy, and that loss of muscle mass explains the associations of muscle collagen with physical function. Our data, however, did not support this hypothesis: muscle mass was not lower in the CKD patients than controls; greater muscle collagen content was not associated with lower muscle mass; and after adjusting for muscle mass, muscle collagen remained higher in CKD.

No prior studies have examined muscle ECM homeostasis in humans with CKD. However, muscle fibrosis has been observed in the partial nephrectomy model of uremia (14, 79), and our results confirm the relevance of this finding for human pathology. As in that animal model, FAP content was increased in human CKD muscle, and here FAP abundance was highly correlated with muscle collagen content. Prior work has shown that FAPs are elevated in severe fibrotic muscle diseases such as Duchenne muscular dystrophy (72). More recently, elevated FAPs were seen in association with muscle fibrosis following anterior cruciate ligament injury (18). Here, we report increased FAPs in association with lesser expansion of muscle collagen than is seen in classic fibrotic muscle diseases, and not following known injury. To the best of our knowledge, this is the first report of such an occurrence.

Although the CKD patients had not experienced clinically apparent muscle injury, increased CHP staining, compared with controls, may indicate subclinical muscle damage. Subfailure damage, in which macroscale damage is not detected, causes unfolding of collagen fibrils (80), which could provide a signal for the initiation of repair mechanisms. While such damage has not been described previously in patients with advanced CKD, studies have documented disrupted sarcomeres and altered muscle architecture (1, 13, 37, 67). Previously, there were no tools to detect molecular level damage to collagen in the absence of macroscale damage (80); therefore, such damage may have been undetected. If subfailure damage to collagen is present, its etiology merits further study: one possibility is increased susceptibility to muscle contraction-induced injury in CKD patients. This could be an initiating factor of the fibrotic process; conversely, because the ECM provides structural support to muscle fibers, fibrosis itself could increase susceptibility to damage (43). Such hypotheses require additional study; regardless, the CHP results, in conjunction with pro-collagen I staining, support increased collagen remodeling in patients with CKD.

An underlying cause of muscle fibrosis in CKD may be ineffective macrophage-mediated muscle repair. The initial response to injury is characterized by influx of pro-inflammatory macrophages and concomitant expansion of the FAP pool, which is needed for repair of the ECM (29). Appropriate repair, and prevention of fibrosis, is dependent upon the macrophage-FAP interaction. TNF-α, secreted by proinflammatory macrophages, causes contraction of the FAP pool via apoptosis; if TNF-α is absent, persistently expanded FAP pools result, causing fibrosis (9, 14, 35, 72). Our findings link muscle TNF-α with muscle fibrosis in CKD. Lower TNF-α expression correlated with both greater FAP abundance and greater collagen content. Furthermore, we found lower muscle expression of TNF-α and other inflammatory and macrophage genes in CKD compared with control; this was remarkable given the greater systemic inflammation in the CKD patients. CKD is recognized as a state of increased inflammation (24); thus the discrepancy between muscle inflammation and systemic inflammation is rather striking. As proinflammatory macro-

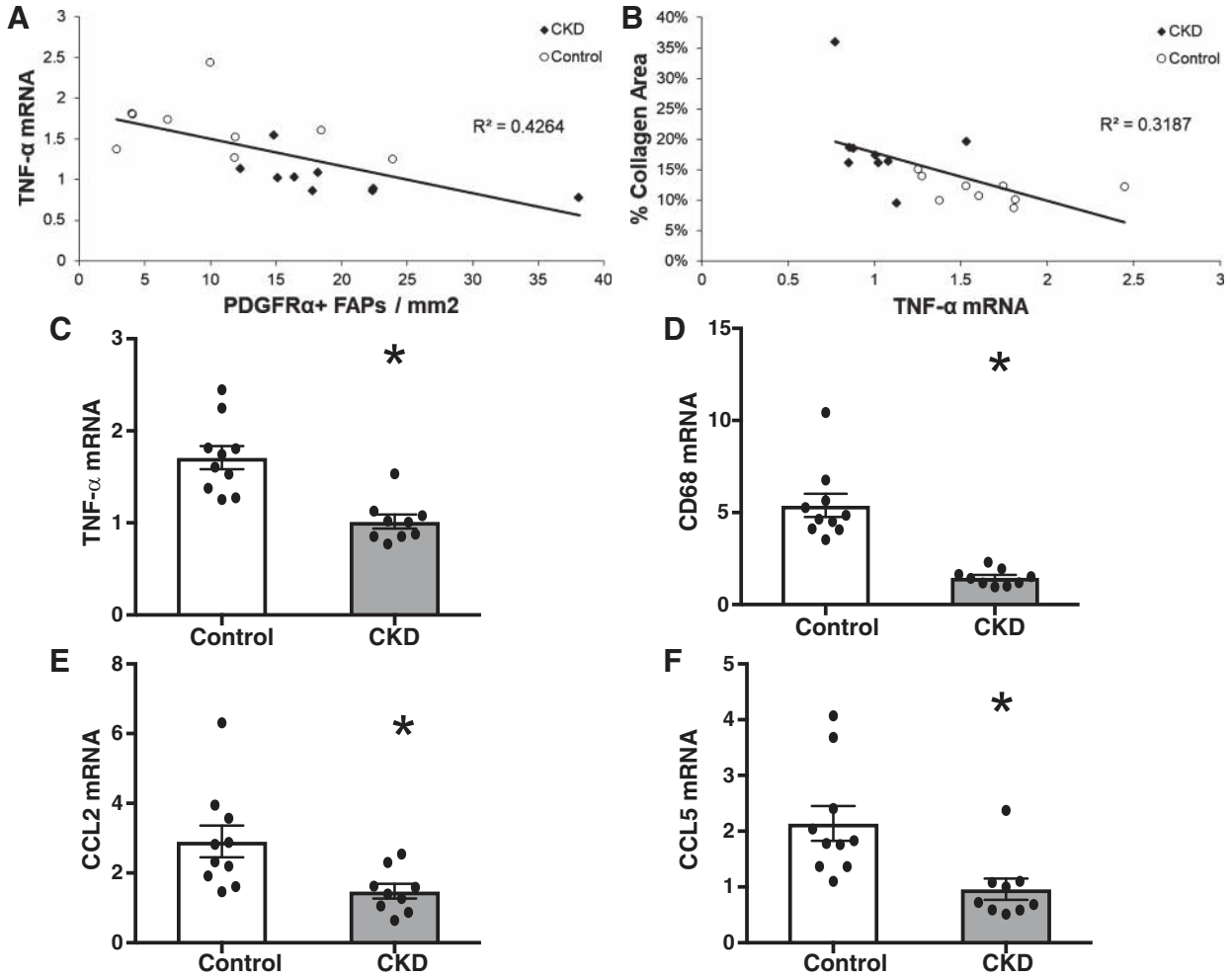

Fig. 4. Reduced mRNA expression of inflammatory and macrophage genes within the m. vastus lateralis muscle of CKD patients. *A* and *B*: correlation of TNF-α expression with PDGFRα+ muscle fibrogenic/adipogenic progenitor (FAP) cell abundance (*A*) and muscle collagen content (*B*). *C–F*: mRNA expression of TNF-α, CD68, CCL2, and CCL5 in control ($n = 10$) and CKD ($n = 9$) muscle biopsies. Gene expression was normalized to RPL7 expression. Data are means ± SE. *$P < 0.05$.

phages are a likely source of TNF-α, the reduction of TNF-α in CKD muscle is consistent with the reduced macrophage content detected. It is noteworthy that the associations between muscle collagen, FAPs, and TNF-α appeared similar in CKD patients and controls. This suggests both an important role for macrophage-mediated regulation of FAPs in human skeletal muscle homeostasis and that loss of this regulation contributes to muscle fibrosis.

Given the importance of inflammation in CKD-associated morbidity, several factors may explain our finding of lower skeletal muscle inflammation in CKD patients. First, the possibility of a divergence between systemic inflammation and that within muscle has been hinted at by prior literature. Both aerobic and resistance exercise in non-dialysis-dependent CKD patients reduce systemic inflammation (8, 27, 75), yet in skeletal muscle inflammatory gene expression did not decline (76). Second, prior studies have either examined end-stage renal disease patients receiving dialysis (as opposed to non-dialysis-dependent CKD, as in our study); studied patients during the hemodialysis treatment itself, which may be proinflammatory (59); sampled non-locomotor muscles, e.g., rectus abdominus, as opposed to vastus lateralis in our study (21, 73, 74, 78); or relied on systemic markers of inflammation only (11). Each of these differences may explain the divergence with our findings. Hemodialysis is thought to be a proinflammatory stimulus; for this and other reasons, end-stage renal disease patients appear to have a greater inflammatory burden than non-dialysis-dependent CKD patients, which might translate to differences within the muscle. Our data set does not enable us to explore whether such differences exist. Inflammatory cytokine expression might differ between locomotor and non-locomotor muscles; this could be especially important for a muscle such as the rectus abdominus that lies in close proximity to abdominal fat depots, which are recognized sources of inflammation (32). Furthermore, few studies in pre-dialysis CKD patients have included comparison with a non-CKD control group, and all sampled rectus abdominus during placement of a peritoneal dialysis catheter (21, 73, 74, 78). Two other differences between those four studies and ours deserve mention: the mean eGFR of those patients was below 10 $ml \cdot min^{-1} \cdot 1.73\ m^{-2}$, and many experienced wasting and malnutrition. Although it is possible that our findings would have differed had we studied patients with lower eGFR who were initiating dialysis, given the relatively advanced CKD in

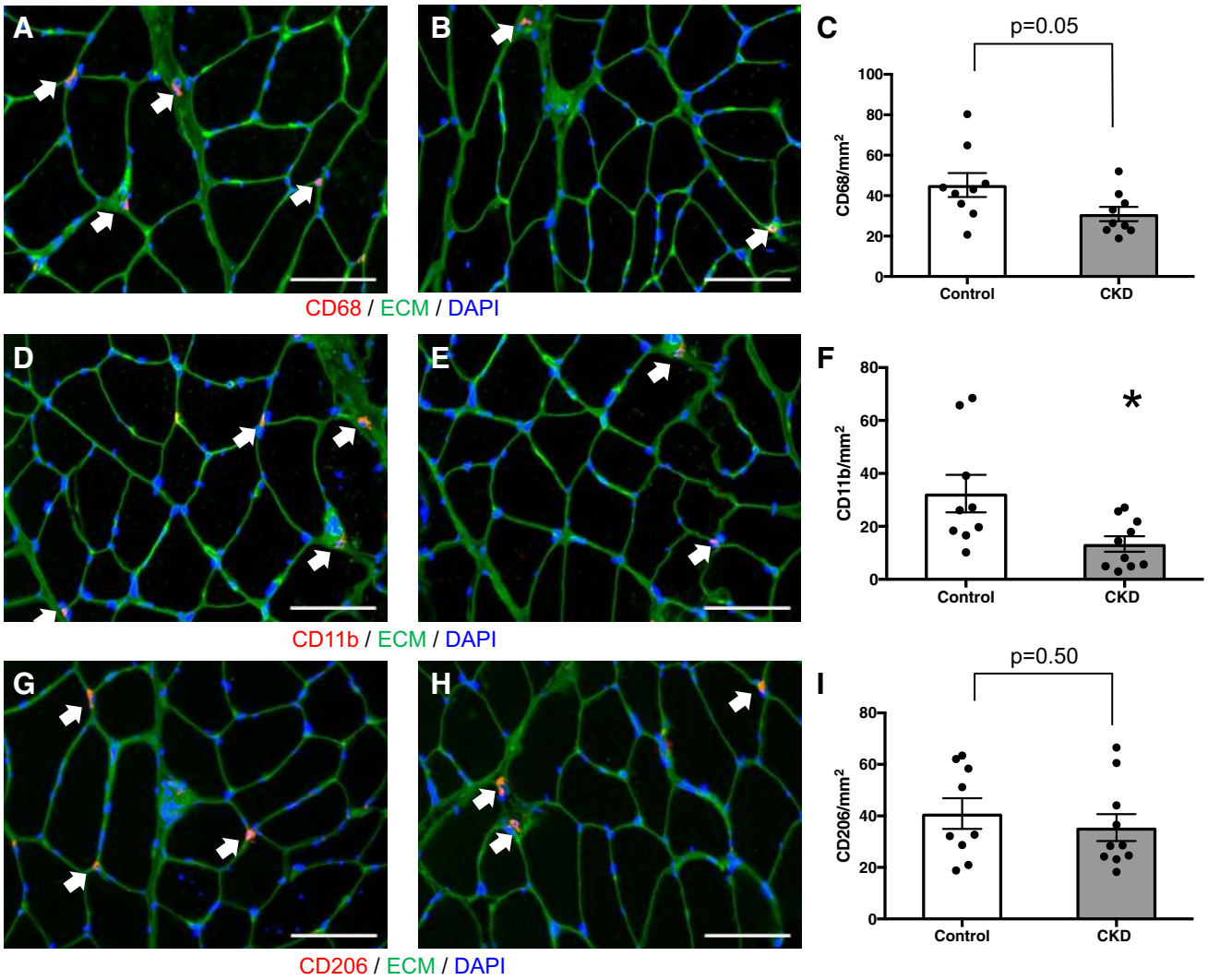

Fig. 5. Macrophage marker immunofluorescence within the m. vastus lateralis muscle of CKD patients and controls. *A* and *B*: representative immunohistochemical image demonstrating CD68+ muscle macrophages (white arrows, red), extracellular matrix (ECM; green), and DAPI (blue) in control (*A*) and CKD (*B*) muscle biopsies. Scale bar = 100 μm. *C*: quantification of CD68+ macrophage content within the muscle represented as mean number of CD68+ macrophages per total muscle area ± SE. *D* and *E*: representative immunohistochemical image demonstrating CD11b+ muscle macrophages (white arrows, red), ECM (green), and DAPI (blue) in control (*D*) and CKD (*E*) muscle biopsies. Scale bar = 100 μm. *F*: quantification of CD11b+ macrophage content within the muscle represented as mean number of CD11b+ macrophages per total muscle area ± SE. *G* and *H*: representative immunohistochemical image demonstrating CD206+ muscle macrophages (white arrows, red), ECM (green), and DAPI (blue) in control (*G*) and CKD (*H*) muscle biopsies. Scale bar = 100 μm. *I*: quantification of CD206+ macrophage content within the muscle represented as mean number of CD206+ macrophages per total muscle area ± SE. $^*P < 0.05$.

our cohort, this seems a less likely explanation for the lower muscle inflammation that we observed. However, as PEW is strongly associated with inflammation, we might have observed increased inflammation in skeletal muscle had we studied patients with symptomatic uremia and severe muscle wasting. If true, this would imply that the well-described manifestations of inflammation and muscle wasting are preceded during earlier stages of CKD by a relative paucity of inflammation in skeletal muscle and by the development of muscle fibrosis.

Several limitations of our study should also be considered. There were important differences between the CKD and control groups in terms of comorbidities and medication use. We accounted for these differences using stratified analyses and adjusted regression models, which indicated that our findings were not explained by these factors. The modest sample size of our cohort precluded more detailed statistical analyses. As this was a cross-sectional study, our findings do not prove a cause-effect relationship, and we lack temporal data on the development of fibrosis and the cellular abnormalities we have characterized. We cannot rule out reverse causality: for example, the possibility that the differences in FAPs and macrophages in CKD muscle were a response to fibrosis. Nevertheless, our findings agree with animal model data demonstrating the central role of macrophages and FAPs in the repair of muscle ECM and prevention of fibrosis (35). We used validated markers of pro- and anti-inflammatory macrophages to define macrophage subsets (66). However, in vivo there is a continuum of macrophage subsets with overlapping but relatively polarized gene expression profiles (77). Future studies are needed to characterize the effects of CKD on muscle macrophages in more detail.

In summary, skeletal muscle fibrosis is a previously unrecognized muscle pathology in patients with CKD and a potentially important contributor to physical function impairment. Increased FAP abundance, possibly caused by insufficient macrophage-mediated TNF-α secretion, may contribute to this pathology. The disparity between systemic and local inflammation highlights the need to study human muscle tissue directly rather than extrapolating from systemic measurements. These data provide a framework for further investigations into the mechanisms causing this pathology in CKD.

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DISCLAIMERS

Its contents are solely the responsibility of the authors and do not necessarily represent the official views or policies of the NCRR or NIH.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.K.A., H.K., J.E.P., M.H., and C.S.F. conceived and designed study; M.K.A., W.P., K.Z., C.R.B., J.N.N., H.K., M.C., R.S.B., H.F., B.Z., R.P., and C.S.F. performed experiments; M.K.A. and C.S.F. analyzed data; M.K.A., J.E.P., M.H., and C.S.F. interpreted results of experiments; M.K.A. and C.S.F. prepared figures; M.K.A. drafted manuscript; M.K.A., W.P., K.Z., C.R.B., J.N.N., H.K., M.C., R.S.B., H.F., B.Z., R.P., J.E.P., M.H., and C.S.F. edited and revised manuscript; M.K.A., W.P., K.Z., C.R.B., J.N.N., H.K., M.C., R.S.B., H.F., B.Z., R.P., J.E.P., M.H., and C.S.F. approved final version of manuscript.

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