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ORIGINAL ARTICLE

Biomarkers of muscle quality: N-terminal propeptide of type III procollagen and C-terminal agrin fragment responses to resistance exercise training in older adults

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Abstract
Background N-terminal peptide of procollagen type III (P3NP) and C-terminal agrin fragment (CAF) are circulating biomarkers that are related to lean body mass in older adults. P3NP is a circulating marker reflective of muscular structural remodeling while CAF is a circulating marker of neuromuscular remodeling. As resistance exercise is an established intervention that can effectively improve muscle quality, we sought to evaluate circulating biomarker changes corresponding to a resistance exercise intervention in older adults.
Methods Twenty-three older adults (aged 61 to 85 years) were randomized into an intervention (6-week resistance training) or control group. Resting circulating P3NP, CAF, lean body mass (LBM), muscle cross-sectional area (CSA), muscle strength, and muscle quality were determined at baseline and after the intervention or control period by enzyme-linked immunosorbent assay, dual-energy X-ray absorptiometry, ultrasound, leg extension, and relative strength, respectively. Changes in circulating biomarkers and measures of muscle mass and quality were evaluated with repeated-measures analysis of variance; clinical interpretations were made with magnitude-based inferences, and relationships between variables were evaluated with bivariate correlations.
Results The short-term resistance exercise intervention was effective at improving muscle quality by 28 % ($p<0.001$) despite no significant changes in lean body mass. Baseline circulating P3NP was somewhat lower in older women (4.15 ±1.9 ng/mL) compared with older men (4.81±2.1 ng/mL). The exercise intervention tended to increase circulating P3NP (baseline=4.53±1.80 to post=4.88±1.86) and was significantly correlated with changes in LBM ($r=0.422$, $p=0.045$). At baseline, women (3.91±1.12 pg/mL) had somewhat higher circulating CAF than men (3.47±1.37 pg/mL). Circulating CAF increased by 10.4 % (3.59 to 4.00 pg/ml) in older adults following 6 weeks of resistance exercise training. Changes in circulating CAF were significantly related to changes in CSA of the vastus lateralis ($r=0.542$, $p=0.008$).
Conclusions Assessment of P3NP and CAF from blood samples may provide minimally invasive and clinically informative measures of skeletal muscle status in older adults. Circulating CAF appears to increase in response to short-term resistance exercise training in older adults to a clinically meaningful magnitude. Changes in circulating P3NP in response to the intervention were less clear but appear to reflect muscle hypertrophy. Further research is needed to elucidate whether P3NP, CAF, or other biomarkers can reflect muscle qualitative adaptations with larger and longer studies.

Keywords Sarcopenia · Muscle quality · Biomarkers · Resistance exercise · Neuromuscular

1 Introduction

Sarcopenia is a progressive, debilitating musculoskeletal condition, affecting as many as half of the older adult population in the United States [1]. Despite its high prevalence, no accepted clinical criteria to diagnose its presence or pharmaceutical interventions to treat its symptoms presently exist. While rigorous attempts to define sarcopenia are ongoing, the

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unfolding intricacies of skeletal muscle changes that occur with aging have convoluted our understanding of the condition and complicated progress toward treatment options. Research has shown that measures of muscle mass give an incomplete picture of the functional scope of sarcopenia and may not be sensitive to changes. Functional and performance assessments may lack consistency and not be feasible for clinical practices. Alternative strategies like muscle biopsies are invasive. Hence, as reported by the International Working Group on Sarcopenia [2], circulating objective biomarkers may provide an alternative and informative clinically feasible measure of skeletal muscle quality.

During muscle remodeling, the structural framework of the muscle, comprised largely of collagen, provides the support for the alignment and growth of the myoblasts [3]. Type III collagen, a sub-type of collagen located in skeletal muscles, is synthesized from the larger procollagen III molecule through the cleavage of N- and C-terminal peptide ends [4]. During its synthesis, the N-terminal propeptide (P3NP) is cleaved and released into circulation in direct proportion to type III collagen synthesis. The cleaved P3NP resulting from the proteolytic cleavage in the synthesis of skeletal muscle that can be measured in the blood. Since it is a product of synthetic pathways, P3NP is reflective of actual tissue remodeling. Contrarily, other anabolic measures (e.g., testosterone, GH, IGF-1) present in circulation reflect only the hormonal milieu which may or may not translate to actual anabolic responses.

Previous research has indicated that older adults have reduced levels of circulating P3NP (4.51 ng/mL in community-dwelling men) [5], and sarcopenic muscle is lower in contractile proteins, higher in connective tissue, and lower in satellite cells, presenting a hampered capacity to remodel. Additionally, changes in serum P3NP levels have been previously been shown to be predictive of changes in lean body mass and strength [5]. Exercise promotes muscle remodeling and lean body mass and strength accrual through mechanical strain on the intramuscular connective tissue. Strain on the intramuscular connective tissue increases satellite cell activity [6], proteolytically driven degradation of collagen (and other proteins), and collagen synthesis 1–3 days after exercise [7–9]. The rate of collagen synthesis in skeletal muscle has been shown to increase with exercise, in a time-dependent manner that follows the increase in myofibrillar protein synthesis with exercise [8].

In healthy muscle, neuromuscular junctions are maintained by the nerve-derived protein agrin [10]. During neuromuscular remodeling, agrin is cleaved by the enzyme neurotrypsin into a 22 kDa C-terminal agrin fragment (CAF). By cleaving and inactivating agrin, neurotrypsin regulates the strength of communication between nerve and muscle cells. Recent discoveries have shown that excessive cleavage of agrin by neurotrypsin into a CAF leads to functional disintegration at the neuromuscular junction [10] and may consecutively cause sarcopenia [11]. During the cleavage of agrin, CAF is released into the blood in levels proportionate to increased neurotrypsin activity and reduced neuromuscular junction strength. In fact, animal models have shown that overexpression of neurotrypsin in motor neurons results in sarcopenic muscle characterized by fewer muscle fibers, more variable fiber thickness, centralized nuclei, fiber-type grouping, and proportion of type I fibers [11]. CAF is detectable in human serum and can be quantified by enzyme-linked immunosorbent assay (ELISA). In humans, serum CAF concentrations have recently been reported to be inversely related ($p = -0.524$) to appendicular lean mass in men [12] where lower appendicular lean mass was associated with higher CAF. Interestingly, no significant relationships were observed in women [12]. Similarly, serum CAF concentrations have been shown to be elevated in older adults with sarcopenia compared with aged-matched controls (4.7 ng/mL versus 2.64 ng/mL) [13]. Even higher concentrations have been reported in pre-frail men (5.4±2.9 ng/mL in older pre-frail men aged 79.4±7.6 years) and women (4.5±2.2 ng/mL in older pre-frail women aged 76.0±6.1 years) [12].

Previous research has implicated N-terminal peptide of procollagen type III (P3NP) and CAF as two potential biomarkers that may inform muscle quality status. Thus, careful examination of their circulating changes in response to a stable and known intervention may provide the fundamental evidence toward novel approaches to sarcopenia diagnosis and treatments. Accordingly, the purpose of this pilot study was to examine P3NP and CAF changes in response to a short-term resistance exercise program to determine if changes reflect muscle qualitative adaptations.

2 Methods

2.1 Study overview

A 6-week, randomized, wait-list controlled pilot study was conducted to evaluate changes in hypothesized biomarkers of sarcopenia in older adults. Participants randomized to the intervention group completed 6 weeks of supervised resistance exercise training. Participants randomized to the control group were instructed to maintain their normal daily activities during the 6-week "wait-list control" and began the exercise program following post-testing. Outcome assessments were conducted at baseline (pre-intervention) and follow-up (post-intervention). Testing occurred over two non-consecutive days within 1 week. On the first testing day, participants reported to the laboratory in the morning following an overnight fast and donated a blood sample and underwent skeletal muscle imaging. Performance tests were performed on the second day of testing where participants were not required to fast but instructed to duplicate their prior breakfast for follow-

up testing. Follow-up testing occurred in the same order at the same time of day to reduce the influence of prior tests or diurnal variations on testing measures.

2.2 Participants

Twenty-three ($n=23$) healthy older men and women (aged 61 to 85 years) volunteered to participate in the study. Interested participants completed an initial scripted phone screening to ensure eligibility and determine whether physician clearance was required prior to exercise participation. All participants were considered low to moderate risk according to the American College of Sports Medicines risk stratification criteria. Participants over the age of 69 years or who were considered moderate risk based on screening criteria were required to receive written physician's clearance for exercise participation. All participants were not currently participating in a resistance exercise program and were encouraged to maintain their normal daily activities throughout the study. All participants were free from current active malignant disease, immunodeficiency disorders, and cardiovascular, pulmonary, or metabolic disease or symptoms. Additionally, participants had not undergone major surgery in the 4 months preceding the study. Descriptive characteristics of the study participants can be viewed in Table 1. Prior to enrollment in the study, all participants were informed of the protocols, risks, and benefits of all study procedures and provided written informed consent. The study protocol involving human subjects was approved by the University's Institutional Review Board for the protection of Human and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

2.3 Resistance training program

Participants randomized to the resistance training group completed a short-term, 6-week strength training program. Prior to beginning the program and following pre-testing, all participants completed two days of familiarization to become acquainted with the exercises and to establish resistance for each of the exercises. The program consisted of two workouts per week with sessions lasting 1 to 1 1/2 h. Each workout was separated by at least 48 h to allow for adequate recovery. The training program consisted of an individualized, periodized, full-body program including exercises of varying progressions of all of the major muscle groups. Exercises included progressions of the squat, split squat, leg curl, leg extension, push-up, triceps extension, calf raise, lat pull down, seated low row, biceps curl, shoulder press, abdominal plank, and reverse crunch. Acute program variables varied progressively throughout the 6 weeks but generally consisted of three sets of 8 to 15 repetitions of seven to eight exercises at submaximal intensity (perceived exertion not to exceed 5–6 on the ten-point Omni scale) (~70 to 85 % of RM) with 60 s of rest allotted between sets and exercises. Resistance was adjusted to allow for the completion of the designated repetition range and to ensure participants were challenged to the specified perceived exertion rating. Each workout session began with a standardized dynamic warm-up consisting of body weight squats, high knee walking, and limb rotations and terminated with an appropriate cool down. The exercise program followed the recommended guidelines for older adults by the American College of Sports Medicine and the National Strength and Conditioning Association and was supervised by a Certified Strength and Conditioning Specialist.

2.4 Anthropometrics

Body mass and stature were measured following standardized anthropometric protocols on a digital scale and an upright standiometer during each testing occasion. Body mass index was calculated from body mass and height as kilograms per square meter at each time point.

2.5 Skeletal muscle ultrasound

Cross-sectional area and echo intensity of the vastus lateralis and rectus femoris were determined by ultrasonography using a 12 MHz linear probe scanning head (General Electric LOGIQ P5, Wauwatosa, WI, USA). Measures were taken using B-mode ultrasound with gain set to 50 and dynamic range set to 72. Image depth was fixed to 5 cm. Images of the rectus femoris were taken on the sagittal plane at a distance marked as half way from the anterior, inferior iliac to the proximal border of the patella. Images of the vastus lateralis

Table 1 Descriptive characteristics of study participants at baseline

	Exercise			Control		
	Men ($n=7$)	Women ($n=5$)	Total ($n=12$)	Men ($n=5$)	Women ($n=6$)	Total ($n=11$)
Age (years)	67.3±5.0	74.4±7.5	70.5±6.9	68.4±4.3	70.7±6.6	69.6±5.5
Body mass (kg)	88.0±21.2	73.1±11.0	81.8±18.7	89.0±19.8	69.6±11.1	78.5±17.9
BMI (kg/m^2)	28.1±5.4	27.7±4.5	27.9±4.8	30.0±6.0	26.6±4.8	28.2±5.4
Body fat (%)	32.4±7.4	42.2±10.0	36.5±9.6	28.0±9.4	40.0±6.3	35.2±9.5

were taken at a distance marked as half of the distance from the most prominent point of the greater trochanter to the lateral condyle. Echo intensity was determined by grayscale analysis of the region of interest [14]. Echo intensity was measured in arbitrary units on a 0–255 scale (0: black; 256: white). Higher echo intensity values are reflective of greater intramuscular connective tissue and adipose infiltration. The echo intensity intraclass correlation coefficients (ICCs) were 0.91 (SEM= 3.47 au) for rectus femoris (RF) and 0.93 (SEM=5.1 au) for vastus lateralis (VL). Prior to imaging, all participants lay supine for 15 min to allow any fluid volume shifts to occur. No vigorous physical activity was permitted for 72 h prior to ultrasound imaging. Images were analyzed using ImageJ (National Institutes of Health, USA, version 1.45 s). The ICC for RF cross-sectional area (CSA) was 0.99 (SEM=0.46 cm^2). The ICC for VL CSA was 0.99 (SEM=1.26 cm^2).

2.6 Dual energy X-ray absorptiometry (DEXA)

Total and regional body composition was evaluated using dual energy X-ray absorptiometry (DEXA) technologies. Measures of total body composition, absolute and relative muscle, adipose, and bone mass were obtained on a whole body scan. Regional measurements of lean quadriceps muscle mass were computed using the region of interest function on the DEXA software package. Reliability coefficients (ICC) for lean quadriceps muscle mass were 0.98 (SEM=0.261 kg). Participants having any procedure with iodine, barium, or nuclear medicine isotopes within 7 days (computed tomography and positron emission tomography scans are examples) were not be permitted to complete the body composition test. All DEXA scans were ordered by a licensed physician in the state of Florida and were performed in the Body Composition Laboratory by a technician licensed in the state of Florida. A single trained investigator performed all body composition analyses.

2.7 Muscle strength

Muscle strength was assessed as maximal voluntary dynamic leg extension strength estimated from a multiple repetition trial using the validated Brzycki prediction equation [load in kilograms/(1.0278−0.0278×repetitions)]. Leg extension trials were performed on a PLLE Power Lift ® knee extension machine (Conner Athletic Products, Inc., Jefferson, IA). After a warm-up set of ten repetitions at a relatively light load, participants were given 3 min of rest before performing the first testing trial. For the testing trial, the load was increased and participants were instructed to complete as many repetitions as possible. If more than ten repetitions were performed, the trial was terminated, the load was increased, and the trial was repeated until a load that could be lifted for ten or fewer repetitions was determined. The number of repetitions and load in kilograms were used to calculate maximal strength per the Brzycki prediction equation. Perceived exertion was monitored using the OMNI scale. This method has been previously validated in clinical populations yielding a typical error of 4 % (± 3.4 kg) [15].

2.8 Muscle quality

Muscle quality was determined as relative strength (Leg extension strength in kilograms/lean quadriceps muscle mass in kilograms).

2.9 Blood sampling and preparation

Resting blood samples were collected in the morning following an overnight fast. Blood was drawn from a forearm vein into serum separator tubes for serum sample preparation. Serum tubes were allowed to clot for 30 min prior to centrifugation. Serum samples were then separated by centrifugation for 10 min at 1,000×*g* at −4 °C. Serum was immediately aliquot into designated preservative tubes and stored samples at −80 °C until analysis. Prior to analysis, samples were thawed only once, centrifuged to remove particulates, and mixed completely by vortex.

2.10 Serum P3NP

Serum P3NP was measured in duplicate by commercially available ELISA (antibodies-online GmbH Catalog No: ABIN366578) following procedures as described by the manufacturer. The sensitivity of this assay is 0.31 ng/ml, and coefficient of variation was <10 %.

2.11 Serum CAF

Serum CAF was measured in duplicate by a commercially available ELISA kit (Neurotune Catalog No: NT1001) following procedures as described by the manufacturer. The measuring range of this assay is 0.4 to 8 ng/ml and coefficient of variation was <3 %.

2.12 Statistical analysis

Changes in dependent variables with the intervention were analyzed using two-way [group (exercise versus control)× time (pre- versus post-)] repeated-measures analysis of variance (ANOVA). When appropriate, post hoc *t* tests were used to evaluate pairwise differences. Baseline differences between the groups were evaluated using independent-sample *t* tests. Significance for this study was set a priori at $p \leq 0.05$. All analyses were performed using PASW version 18.0 (SPSS, Inc., Chicago, IL). Data are presented as mean ± SD unless otherwise stated. Relationships between circulating

biomarkers and measures of muscle strength, size, and quality were examined with bivariate correlations.

Magnitude-based inferences were used to identify clinical differences in the circulating biomarkers between the exercise and control groups. Magnitude-based inference statistics provide a complementary tool for null hypothesis testing to reduce errors in interpretation and to provide more clinically meaningful results [16, 17]. Additionally, magnitude-based inference statistics avoids the problem of interdependence of observations by using within-subject modeling, where repeated measures are combined into a single measure (unit of change) for analysis. The precision of the magnitude inference was set at 90 % confidence limits, using a p value derived from an independent t test. Threshold values for positive and negative effects were calculated by multiplying standard deviations of baseline values by 20 % [18]. Inferences on true differences between the exercise and control group were determined as positive, trivial, or negative according to methods previously described by Batterham and Hopkins [17]. Inferences were based on the confidence interval range relative to the smallest clinically meaningful effect to be positive, trivial, or negative. Unclear results are reported if the observed confidence interval overlaps both positive and negative values. The probability of the effect was evaluated according to the following scale—<0.5 %, most unlikely; 0.5–5 %, very unlikely; 5–25 %, unlikely; 25–75 %, possibly; 75–95 %, likely; 95–99.5 %, very likely; >99.5 %, most likely [18].

3 Results

The short-term resistance exercise training intervention was effective in increasing muscle strength and quality in the total training group (Table 2) and in men and women (Table 3). Leg extension muscle strength increased by 29 % from 39.7±16.5 to 51.1±18.3 kg in the exercise training group (p <0.001). Similarly, muscle quality (as relative strength) increased by 28 % (p <0.001) from 3.64±0.85 to 4.67±0.81 in the exercise training group. No significant changes in muscle strength or quality were observed in the control group. Total group mean lean body mass did not significantly change with the exercise intervention. However, individual lean body mass changes ranged from −2.2 kg to 1.4 kg over the 6 weeks. Cross-sectional area of the vastus lateralis muscle significantly increased in the exercise training group (14.5±4.1 to 15.7±4.8 cm^2). No significant changes in cross-sectional area of the rectus femoris was observed in the training group.

Baseline circulating P3NP was somewhat, albeit not significantly, lower in older women (4.15±1.9 ng/mL) compared with older men (4.81±2.1 ng/mL). While circulating P3NP concentrations increased by 7.9 % in the exercise group (baseline=4.53±1.80 to post=4.88±1.86) compared with a 1.9 % observed mean change in the control group, the change as a result of the exercise intervention was not statistically significant or clinically meaningful (Fig. 1). Magnitude-based inferences on the change scores revealed "unclear" clinical interpretations of the effect (Table 4). Further examination of the intervention revealed a positive correlation between baseline P3NP and changes in lean body mass (r =0.422, p = 0.045), where those with higher baseline P3NP tended to gain lean body mass over the 6-week training period, while those with lower baseline levels, tended to lose lean body mass (Fig. 2). No significant relationships between circulating P3NP and muscle strength or quality change were observed.

Baseline circulating CAF was somewhat, albeit not statistically higher in older women (3.91±1.12 pg/mL) than older men (3.47±1.37 pg/mL). While circulating CAF concentrations increased by 10.4 % (3.59±1.45 to 4.00±1.20 pg/ml) in older adults following 6 weeks of resistance exercise training compared with a 0.3 % observed mean change in the control group, the change as a result of the exercise intervention was not statistically significant (Fig. 3). Magnitude-based inferences on the change scores revealed a "likely beneficial" clinical interpretation of the effect of the intervention on circulating CAF. Magnitude-based inference computations revealed a 92.3 % positive, 0.9 % negligible, and 6.8 % negative effect of the intervention (Table 4). Further examination of the intervention revealed a positive correlation between change in circulating CAF and change in cross-sectional area of the vastus lateralis (r =0.542, p =0.008), where those who

Table 2 Muscular strength, functional, and structural changes from 6 weeks resistance exercise training in older adults

	Exercise (n =12)		Control (n =11)	
	Pre	Post	Pre	Post
Leg extension strength (kg)	39.7±16.5	51.1±18.3*	31.4±12.4	34.2±15.1
Muscle quality (relative strength)	3.64±0.85	4.67±0.81*	2.92±1.05	3.10±1.10
LBM (kg)	48.4±10.8	48.5±10.7	48.6±13.6	48.5±13.4
Rectus femoris CSA (cm^2)	7.7±2.5	8.1±2.9	7.8±2.8	7.9±2.6
Vastus lateralis CSA (cm^2)	14.5±4.1	15.7+4.8*	14.8±5.0	14.8±5.0
P3NP (ng/mL)	4.53±1.80	4.88±1.86	4.44±2.25	4.52±2.41
CAF (pg/mL)	3.59±1.45	4.00±1.20	3.77±1.06	3.79±1.02

Data are presented as mean±SD

*p <0.05 is indicated from pre-exercise

Table 3 Muscular strength, functional, and structural changes from 6 weeks resistance exercise training in older men and women

	Exercise				Control			
	Pre	Post	Mean change	(%)	Pre	Post	Mean change	(%)
Men		(n=7)				(n=5)		
Leg extension strength (kg)	48.5±16.0	61.0±17.9	12.5±4.5	(25.7 %)	37.3±6.6	44.7±8.1	7.4±5.2	(19.7 %)
Muscle quality (relative strength)	3.91±0.66	4.93±0.70*	1.02±0.33	(25.9 %)	2.78±0.50	3.33±0.43	0.55±0.35	(19.7 %)
LBM (kg)	52.4±12.4	52.4±12.5	0.0±1.2	(0.0 %)	57.4±15.1	57.3±15.2	−0.1±0.6	(−0.3 %)
Rectus femoris CSA (cm^2)	8.2±3.1	8.8±3.5	0.6±0.9	(7.6 %)	10.4±1.9	10.4±1.4	0.0±0.5	(−0.5 %)
Vastus lateralis CSA (cm^2)	16.4±3.8	17.4+4.7	1.0±1.4	(6.0 %)	18.7±4.8	18.7±4.9	0.0±0.2	(−0.1 %)
P3NP (ng/mL)	5.28±1.30	6.00±1.64	0.2±1.6	(3.1 %)	4.26±2.85	4.35±3.22	0.09±0.93	(2.2 %)
CAF (pg/mL)	3.78±1.76	4.19±1.54	0.1±0.8	(10.6 %)	3.02±0.38	3.14±0.39	0.12±0.44	(3.8 %)
Women		(n=5)				(n=6)		
Leg extension strength (kg)	27.2±5.7	37.3±5.7*	10.0±4.5	(36.8 %)	26.4±14.4	25.5±14.3	−0.9±6.5	(−3.4 %)
Muscle quality (relative strength)	3.24±1.02	4.31±0.89*	1.07±0.43	(32.9 %)	3.03±1.42	2.90±1.48	−0.13±0.84	(−4.3 %)
LBM (kg)	42.8±4.8	43.1±4.4	0.4±0.5	(0.9 %)	41.1±6.6	41.3±6.1	0.1±0.6	(0.3 %)
Rectus femoris CSA (cm^2)	7.1±1.3	7.1±1.5	0.0±0.7	(−0.2 %)	5.6±0.5	5.8±0.5	0.1±0.3	(2.1 %)
Vastus lateralis CSA (cm^2)	11.7±2.9	13.2+4.2	1.5±2.4	(12.4 %)	11.5±1.8	11.5±1.6	−0.1±0.2	(−0.5 %)
P3NP (ng/mL)	3.63±2.04	3.55±1.11	−0.08±1.02	(−2.2 %)	4.59±1.88	4.66±1.81	0.08±1.21	(1.7 %)
CAF (pg/mL)	3.33±1.01	3.74±0.46	0.41±0.91	(12.4 %)	4.39±1.05	4.32±1.08	−0.07±0.24	(−1.6 %)

Data are presented as mean±SD and percent change

*p <0.05 is indicated from pre-exercise

increased circulating CAF tended to increase cross-sectional area of the vastus lateralis over the 6 weeks, while those with lower baseline levels, tended to lose muscle size (Fig. 4). No significant relationships between circulating CAF and muscle strength or quality change were observed.

4 Discussion

The main findings of this investigation indicate that short-term resistance exercise training may result in clinically meaningful changes in circulating biomarker concentrations. As a pilot study designed to provide guidance on future research, we observed for the first time that circulating CAF may increase in response to short-term resistance exercise training in older adults to a clinically meaningful magnitude. Changes in circulating P3NP in response to the intervention were less clear but may still reflect of hypertrophic muscular adaptations. Our results reveal preliminary insights into circulating biomarker responses to an intervention and support further elaborative research efforts to better elucidate biomarker responses and potential clinical applications.

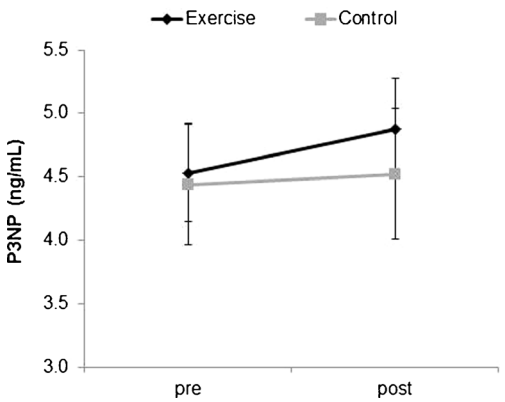

Fig. 1 Circulating P3NP response to resistance exercise training. *Black diamonds* represent exercise group. *Grey squares* represent control group

The 6-week resistance exercise training intervention was effective at improving muscle strength and quality by 29 % and 28 %, respectively. The observed strength gains are in agreement with strength [19–26] and muscle quality [26, 27] improvements achieved by older adults in prior short-term resistance training studies. While our muscle quality improvements were somewhat higher than those observed in the 9-week study by Tracy and colleagues [26], differences can likely be attributable to different training programs. Our exercise program included multiple exercises to strengthen the leg extensor muscles including progressions of modified squats, split squats, and seated leg extensions while the 9-week study by Tracy et al. [26] trained strictly with the seated leg press exercise. Thus, the greater overload created by multiple exercises likely explained the relatively greater muscle quality gains achieved in the present study. Nevertheless, the resistance exercise intervention was effective in eliciting muscle quality improvements, as intended.

Table 4 Magnitude-based clinical inferences on changes in biomarkers with resistance exercise training

Biomarker	Exercise Mean change (% change)	Control Mean change (% change)	% Positive	% Negligible	% Negative	Clinical inference
P3NP (ng/mL)	0.36±0.93 (7.9 %)	0.09±1.04 (1.9 %)	63.8	18.2	18.0	Unclear
CAF (pg/mL)	0.41±0.79 (10.4 %)	0.01±0.34 (0.3 %)	92.3	0.9	6.8	Likely beneficial

Interestingly, the significant muscle strength and quality increases observed in response to resistance exercise training over 6 weeks occurred despite no significant changes in total lean body mass. The lack of detectable muscle hypertrophy observed in the present study was expected for several reasons. First, this study was intentionally designed as a short term 6-week intervention in an attempt to detect muscle quality changes prior to significant hypertrophy. Prior studies show that longer duration training of at least 4 to 5 weeks is required before muscle hypertrophy is apparent [28] as strength gains achieved during the first several weeks of resistance exercise training are due primarily to neural adaptations [28–30]. However, in older adults, increases in strength are predominantly attributed to neural factors even up to 8 weeks into training [29]. Moreover, prior work by Wells et al. [27] reported that aging can attenuate the hypertrophic response of skeletal muscle to resistance training, while not impairing training-induced increases in muscle quality [27]. Muscle quality and strength increases observed in the present study are likely due to neural adaptations such as increases in voluntary activation of the agonists and reductions in the antagonist coactivation as previously described by Hakkinen and colleagues [24].

Although neural adaptations are commonly attributed to muscle strength and quality gains achieved early in a resistance training program, recent evidence by our lab suggests that some muscle structural adaptations can occur early on in a training program that cannot be detected by DEXA [31]. The process of muscle fiber remodeling begins as soon as myofibers are damaged during the acute mechanical strain of the exercise [32]. Hence, even in the absence of whole muscle hypertrophy, muscle is remodeled acutely during a resistance training intervention which may also contribute somewhat to muscle quality and strength improvement. As previously reported [31], cross-sectional area of the vastus lateralis muscle as measured via ultrasonography significantly increased in the exercise training group, despite no changes in total lean body mass or cross-sectional area of the rectus femoris. Hence, although some muscle structural adaptations can be detected early on in response to resistance training, non-invasive methodologies indicative of myofiber remodeling may yield a better understanding of muscular adaptations underlying strength and quality improvements.

The effects of the resistance exercise intervention on changes in circulating P3NP were clinically "unclear." Based on the work by Bhasin et al. [5], who reported significant increases in circulating P3NP after 4 weeks of anabolic treatments of testosterone and recombinant GH in older men, we had hypothesized that muscle qualitative adaptations to a resistance exercise intervention could be reflected in circulating concentrations of P3NP. Although our exercise group increased circulating P3NP by 7.9 %, compared with a 1.9 % increase in the control group, our results were not statistically significant or clinically meaningful. The null findings may be attributed to several factors. First, the relatively small sample size in this exploratory study may have been insufficient to detect statistical or meaningful changes. As the first study to evaluate changes in circulating P3NP in response

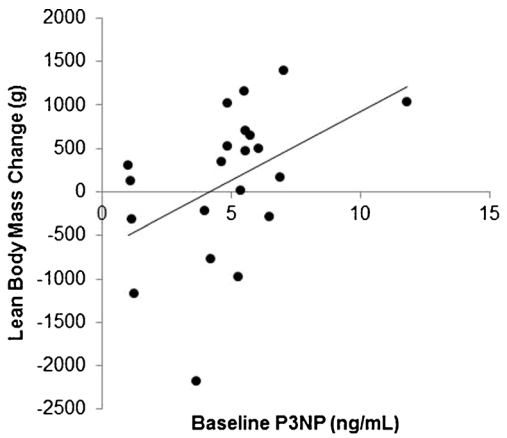

Fig. 2 Relationship between baseline P3NP (nanograms per milliliter) and change in lean body mass (grams) in all participants ($r=0.422$, $p=0.045$)

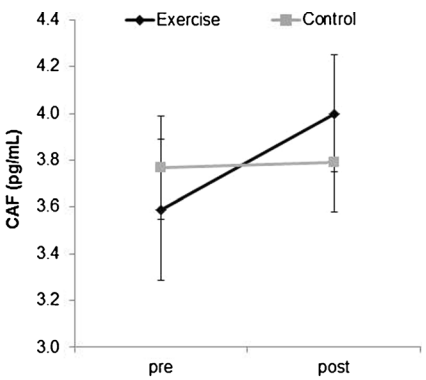

Fig. 3 Circulating CAF response to resistance exercise training. *Black diamonds* represent exercise group. *Grey squares* represent control group

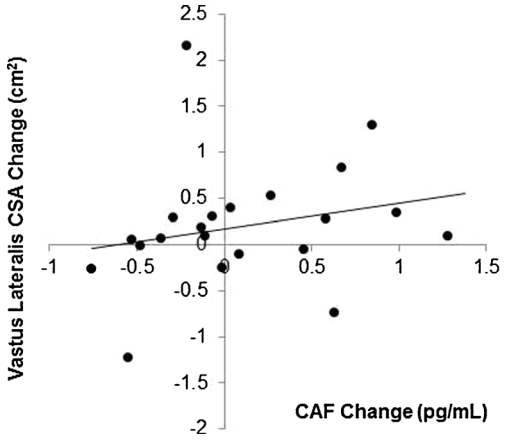

Fig. 4 Relationship between baseline CAF change (pictograms per milliliter) change in CSA of the vastus lateralis (square centimeters) in all participants (r=0.542, p=0.008)

to a resistance exercise intervention, power analysis could not be accurately performed a priori until expected variance could be determined from the results of the present study. Post hoc power analyses based on the variance observed in the present study yielded a sample size of approximately 80 to achieve statistical significance in this measure. Nevertheless, the direction of our findings would support the investment in a larger study to investigate changes in circulating P3NP in response to an anabolic intervention.

Another possible explanation for our null findings may be attributed to the lack of change in lean body mass during the resistance exercise intervention. The significant increase in circulating P3NP with anabolic treatments of testosterone and recombinant GH in the prior study by Bhasin and colleagues [5] were significantly correlated to changes in lean body mass. Our resistance exercise intervention did not result in significant group mean changes in lean body mass during the 6 weeks. However, individual lean body mass changes ranged from −2.2 kg to 1.4 kg over the 6 weeks and were positively correlated to baseline P3NP, where those who increased circulating P3NP also increased lean body mass. Thus, it may be that significant muscle hypertrophy is required to alter circulating P3NP concentrations.

Circulating CAF increased by 11.4 % in older adults following 6 weeks of resistance exercise training which translated to a "likely beneficial" clinical interpretation of the effect of the intervention. Based on the work by Bolliger et al. [10], Bütikofer et al. [11], and Hettwer et al. [13], we had hypothesized that muscle qualitative neuromuscular adaptations to an intervention could be reflected in circulating concentrations of CAF. As Hettwer et al. [13] found elevated circulating levels of CAF in older adults with sarcopenia and Drey et al. [12] found that physical exercise was significantly associated with a reduction in CAF concentration in pre-frail older adults [12], we had hypothesized that the resistance exercise intervention would reduce circulating CAF. Contrary to our hypothesis, circulating CAF increased rather than decreased in response to the resistance exercise intervention. However, it is important to emphasize that this result is based on magnitude-based inference statistics, a complementary statistical tool for null hypothesis testing to reduce errors in interpretation and to provide more clinically meaningful results [16, 17]. While repeated-measures ANOVA was also used to evaluate the changes, statistical significance was not achieved with this test due to the insufficient power of the small exploratory sample size. As our first study to evaluate changes in circulating CAF in response to a resistance exercise intervention, power analysis could not be accurately performed a priori until expected variance could be determined from the results of the present study. Nevertheless, this clinically relevant [16, 17] finding is potentially indicative of positive neuromuscular remodeling occurring as a result of the intervention.

It is well known that considerable neuromuscular adaptations occur during the early stages of resistance exercise training [24, 33–35]. As CAF is a biomarker of degradation at the neuromuscular junction where agrin is cleaved by the enzyme neurotrypsin [10], it may be that the elevated circulating concentrations of CAF are reflective of neuromuscular remodeling. Earlier research has shown that agrin is involved in the signaling that induces the clustering of nicotinic acetylcholine receptors on the muscle membrane of the neuromuscular junction [36, 37], and the transport of neurotrypsin to synapses and release from presynaptic terminals are dependent on muscle activity [38]. While it is plausible that the increase in circulating CAF is reflective of neuromuscular remodeling, the reason for why the direction of our results conflicted with those previously reported by Drey et al. [12] is less clear. It may be that participants in the present study were healthy older adults, compared with those in the study by Drey et al. [12] which recruited pre-frail older adults who were older and had higher baseline CAF. Interestingly, this prior study noted that the association of a decline in circulating CAF corresponded to the sub-group with elevated CAF. Hence, it may be that CAF like other biomarkers is indicative of disease or acute response to exercise or stress. Nevertheless, we observed an interesting clinically meaningful change in circulating CAF in response to the resistance exercise intervention, which warrants future mechanistic studies to examine whether circulating CAF is evidence of positive or negative neuromuscular remodeling.

Changes in circulating CAF were significantly related to changes in CSA of the vastus lateralis. Individuals who increased circulating CAF tended to increase cross-sectional area of the vastus lateralis over the 6 weeks, while those with lower baseline levels, tended to lose muscle size. The relationship between circulating CAF and muscle size has been previously reported by Drey et al. [12] who reported a relationship between appendicular lean mass and circulating CAF concentration in pre-frail men but not women [12]. While

CAF is a biomarker of neuromuscular remodeling, the reason for the association with changes in cross-sectional area is less clear. It may be that those who experienced greater adaptations were overall better "responders" due to differing genetic potential. Some recent evidence has attributed differing microRNA abundance to greater phenotypic changes in those who experience more pronounced adaptations to resistance exercise [39]. Whether or not genetic potential influences the observed results requires further study.

While the present study included both older men and women and was not statistically powered to detect gender differences, the potential of gender to influence results should be considered. At baseline, circulating P3NP was somewhat lower in older women compared with older men while, to the contrary, baseline circulating CAF was somewhat higher in older women than older men. However, as depicted in Table 3, there were no apparent trends in directionality of changes between men and women. Nevertheless, it is feasible that inclusion of both men and women in the present study convoluted our results. It is well known that skeletal muscle adaptations differ between men and women, where women have an attenuated myofiber hypertrophic capacity even at older ages [40]. The attenuated hypertrophy in older women has attributed strength gains in women to neurological adaptations predominantly [40]. Furthermore, we recently observed gender differences in contributions of muscle quality to physical performance in older adults [41]. As gender may influence skeletal muscle adaptations in older adults, further studies should examine potential gender differences in biomarker responses to muscle improving interventions.

Contrary to our hypothesis, changes in circulating biomarkers did not appear to relate to changes in muscle strength or quality. While the relatively small sample size in this pilot study may somewhat account for the null finding, it is also possible that circulating P3NP and CAF are not indicative of muscle strength or quality and are instead more indicative of muscle hypertrophy and strength via muscle hypertrophy. This possibility is somewhat supported by Bhasin et al. [5] who reported only a modest relationship between circulating P3NP and strength, despite the somewhat stronger relationship between circulating P3NP and lean body mass. Similarly, Drey et al. [12] did not observe a relationship between hand grip strength and circulating CAF concentration in frail older adults. Nevertheless, further study is needed to determine the relationship between circulating P3NP and muscle strength and quality.

5 Conclusion

Short-term resistance exercise training significantly improves muscle strength and quality in older adults and may result in clinically meaningful changes in circulating biomarker concentrations. Circulating CAF appears to increase in response to short-term resistance exercise training in older adults to a clinically meaningful magnitude. Changes in circulating P3NP in response to the intervention were less clear but appear to reflect muscle hypertrophy. Results support the potential of biomarkers reflective of skeletal muscle status to enhance clinical practice by informing muscular conditions without the need for more invasive or time consuming tests. Our results reveal preliminary insights into circulating biomarker responses to an intervention and support further elaborative research efforts to better elucidate responses and potential clinical applications. The present study provides preliminary evidence to justify further research to further understand how P3NP and CAF as potentially novel biomarkers of skeletal muscle status and sarcopenia are affected by interventions.

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Conflict of Interest Maren Fragala, Adam Jajtner, Kyle Beyer, Jeremy Townsend, Nadia Emerson, Tyler Scanlon, Leonardo Oliveira, Jay Hoffman, and Jeffrey Stout declare that they have no conflict of interest.

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