Impact of Above-Average Proanabolic Nutrients Is Overridden by High Protein and Energy Intake in the Muscle-Tendon Unit Characteristics of Middle- to Older-Aged Adults

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Abstract

Background: The impact, within a single cohort, of independent modulators of skeletal muscle quality, including age, adiposity and obesity, habitual nutritional intake, and physical activity (PA), is unclear.

Objective: We examined the bivariate associations between age, adiposity, habitual nutritional intake, and PA against 11 key intrinsic muscle-tendon unit (MTU) characteristics to identify the strongest predictors. We also compared overall profile differences between MTU categories with the use of z scores shown in radar graphs.

Methods: Fifty untrained independently living men ($n = 15$) and women ($n = 35$) aged 43–80 y (mean $\pm$ SD: 64 $\pm$ 9 y) were categorized by adiposity [men: normal adiposity (NA) <28%, high adiposity (HA) $\geq$28%; women: NA <40%, HA $\geq$40%] and body mass index [BMI (in kg/m^2); normal: 18 to <25; overweight: $\geq$25 to <30; and obese: $\geq$30]. Group differences were examined by body composition assessed with the use of dual-energy X-ray absorptiometry, habitual nutritional intake through a 3-d food diary, PA (work, leisure, sport) using the Baecke questionnaire, 14 serum cytokine concentrations using multiplex luminometry, and 11 MTU characteristics of the gastrocnemius medialis using a combination of isokinetic dynamometry, electromyography, and ultrasonography.

Results: Interestingly, classification by BMI highlighted differences between normal and obese individuals in 6 of 11 MTU characteristics ($P < 0.001$ to $P = 0.043$). No significant differences were reported in serum cytokine concentrations between adiposity and BMI classifications. BMI predicted 8 of 11 ($r = 0.62–0.31$, $P < 0.001$ to $P = 0.032$), daily energy intake predicted 7 of 11 ($r = 0.45–0.34$, $P = 0.002–0.036$), age predicted 5 of 11 ($r = -0.49–0.32$, $P < 0.001$ to $P = 0.032$), work-based PA predicted 5 of 11 ($r = 0.43–0.32$, $P = 0.003–0.048$), and adiposity predicted 4 of 11 ($r = 0.51–0.33$, $P < 0.001$ to $P = 0.022$) MTU characteristics. Mathematical z scores and radar graphs showed how endocrine and dietary profiles, but not PA, differed between the top and bottom $\sim$20% of muscle unit size and specific force.

Conclusions: Given the number of factors associated with MTU, education should be targeted to both adequate food quantity and quality (especially protein intake) and increasing habitual moderate to vigorous PA while decreasing sedentary behavior. Specific endocrine variables are also proposed as key pharmaceutical targets.   *J Nutr* 2018;148:1776–1785.

Introduction

Obesity in old age affects both the structural and functional characteristics of skeletal muscle (SM), culminating in decreased physical performance (1) and an increased risk of disability (2). This is primarily the result of lower relative (strength and body mass) muscle strength (3), lower muscle activation capacity (3), increased intramuscular fat infiltration (4), and a decrease in SM specific force (4). However, to our knowledge, the impact of environmental factors such as nutritional intake (both quantity and quality), physical activity (PA) levels, endocrine factors, and obesity on SM properties has not been extensively studied in aging adults.

Adequate habitual nutrition is a key positive modulator of successful aging, with identified essential nutrients positively associated with SM, including protein (5), vitamin D (6,

7), vitamin E (8), vitamin C (8), omega-3 and ω-6 FAs (9, 10), calcium (6), and vitamin B-12 (11). Protein intake is the main protagonist in the maintenance and development of SM through increasing muscle protein synthesis (MPS) rates via the mammalian target of rapamycin pathway (12). Vitamin D supplementation in older adults is reported to both increase neuromuscular function and decrease the risk of falls (7) in deficient individuals (25-hydroxyvitamin D $\leq$12 mg/L), while additionally stimulating MPS rates (13). Current understanding of any interaction between habitual nutrition, muscle function, and obesity is limited, yet questions remain whether foodstuffs may negate or even accelerate the loss of muscle mass witnessed in the obese elderly (14).

Although the positive influence on either upregulation of MPS or improvements in neuromuscular function through diet alone is a beneficial outcome, aging and obesity are associated with an increase in proinflammatory cytokines such as IL-6 (15, 16) and TNF-α (17). Greater inflammation as we age is hypothesized to be a driver in frailty, through the SM catabolic effect of high concentrations of proinflammatory cytokines (18). However, EPA has been reported to possess anti-inflammatory properties, as noted by the reduction in both TNF-α (19) and IL-6 (20) after supplementation. Therefore, the inclusion of high amounts of ω-3 FAs within a habitual diet may benefit both aging and obesity due to low-grade systemic inflammation being associated with both conditions (21), through creating a proanabolic environment for SM (22).

Although nutritional strategies may aid in both the maintenance and development of muscle mass with aging, an individual's daily activity profile is equally important in determining SM strength, and it is understood to interact with an individual's diet (the concept of metabolic balance). The current activity guidelines of 150 min moderate activity/wk (23) is achieved by $\sim$55% of 54- to 64-y-olds and by $\sim$36% of those aged >75 y (24). Therefore, the lower levels of both PA and nutritional intake and quality in older compared with younger persons (25) could directly affect MPS, translating to muscle loss and a reduction in muscle function. Research conducted by Chastin et al. (26) confirmed an association between lower fat-free mass, leg extension power, and muscle quality with sitting time. Yet, current research has not conclusively shown how these factors influence the determinants of strength capability, including neuromuscular activation, muscle architecture [fascicle pennation angle, fascicle length, and muscle volume (MV)], and muscle quality (specific force) of weight-bearing SM, and how any decline in these factors is either accelerated or maintained by aging, nutritional intake, and adiposity.

Therefore, the aim of the present study was to take a multifactorial approach to examining the factors that potentially negatively influence SM function with aging, ranging from habitual nutritional intake to obesity, and to identify key modulators that may aid in counteracting the deleterious changes that aging has on SM. It was hypothesized that the following would positively affect muscle size and strength: *1*) high calorie and protein intake, *2*) low adiposity and high BMI, *3*) high amounts of proanabolic nutrients, and *4*) increased PA.

Methods

Participants. Fifty untrained men ($n = 15$) and women ($n = 35$) aged 43–80 y volunteered to take part in this study and were categorized by adiposity [men: normal adiposity (NA) <28%, high adiposity (HA) $\geq$28%; women: NA <40%, HA $\geq$40%) and BMI [in kg/m^2; normal weight (NW): 18 to <25; overweight: $\geq$25 to <30; and obese: $\geq$30]. The principal exclusion criteria highlighted in a health questionnaire before undertaking the study were issues with lower limb muscles and joints affecting mobility or the ability to exert maximum plantar flexion and dorsiflexion (DF) strength, any specific nutritional needs, and having had to drastically alter their habitual diet or PA levels in the past 12 mo. Participants gave their written informed consent before undertaking any assessment, and all of the procedures in this study had approval from the local university ethics committee.

Measurement of body composition. Body composition was determined with the use of a DXA scanner (Hologic Discovery; Vertec Scientific Ltd.) after an overnight 12-h fast, with scan results processed using the Hologic APEX software (version 3.3). A detailed description of the methodology was reported by Tomlinson et al. (14).

Muscle strength. Plantar flexion (PF) maximal voluntary contraction (MVC) torque was assessed in the participant's dominant limb using an isokinetic dynamometer (Cybex Norm; Cybex International). Participants were seated with a hip angle of 85° and their dominant leg fully extended. The dominant foot was secured to the footplate of the dynamometer using unyielding rigid straps, while extraneous movements were limited. After a warm-up procedure, participants performed 3–4 isometric PF MVCs with their ankle positioned at 0°, (anatomically neutral). The highest recorded PF MVCs were used for subsequent analysis.

Muscle activation capacity was calculated by using the interpolated twitch technique (27) after the assessment of PF MVC. Two stimulation pads (50 mm × 100 mm; American Imex) were placed transversely distal to the popliteal crease and at the myotendinous junction of the soleus. The amplitude of the elicited supramaximal stimuli doublets (DSV Digitimer Stimulator; Digitimer) during PF MVC was determined before interpolation while the participant was in a relaxed state. Then, a twitch stimulus-response curve was established. Supramaximal doublets were subsequently superimposed during and immediately after a maximal PF MVC.

$$\text{Muscle Activation (\%)} = [1 - (\text{superimposed doublet torque/}$$
$$\text{resting doublet torque})] \times 100 \quad (1)$$

Coactivation of the tibialis anterior (TA) was calculated using surface electromyography (sEMG) during a PF MVC. Two Ag-AgCl electrodes (size: 30 mm × 22 mm; Neuroline; Medicost) were placed centrally in the proximal third of the TA (1- to 2-mm gap), with a reference electrode placed on the head of the fibula. Raw sEMG signal was recorded at 2000 Hz, band-pass filtered at 10–500 Hz, and notch at 50 Hz. TA coactivation (%) was calculated using the raw sEMG signal (root mean square of 500 ms on either side of peak torque) during the PF MVC and divided by the sEMG signal during a DF MVC at 0° ankle joint angle. Coactivation torque was consequently calculated using the participants' percentage of coactivation and maximal DF torque through the assumed linear relation between DF sEMG and DF torque (28).

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Abbreviations used: DF, dorsiflexion; G-CSF, granulocyte-colony–stimulating factor; GM, gastrocnemius medialis; HA, high adiposity; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MPS, muscle protein synthesis; MTJ, muscle-tendon junction; MTU, muscle-tendon unit; MV, muscle volume; MVC, maximum voluntary contraction; NA, normal adiposity; NW, normal weight; PA, physical activity; PCSA, physiological cross-sectional area; PF, plantar flexion; RANTES, regulated on activation, normal T-cell expressed and secreted; sEMG, surface electromyography; SM, skeletal muscle; TA, tibialis anterior; TV, television.

Muscle size. The 7.5-MHz linear probe of a B-mode sonographer (AU5 Harmonic; Esaote Biomedica) was used to measure muscle anatomic cross-sectional areas (at 0%, 25%, 50%, 75%, and 100% of muscle length) and length, hence informing the subsequent calculation of the MV of the gastrocnemius medialis (GM) in each participant's dominant leg with the use of the truncated cone method. A detailed description of the methodology is reported by Tomlinson et al. (29).

Muscle architecture. Muscle architecture of the GM was measured using B-mode ultrasonography (AU5 Harmonic; Esaote Biomedica) during a 6-s ramped PF isometric MVC at 0°. Participants sat in the isokinetic dynamometer as detailed in the protocol above. The ultrasound probe (7.5 MHz) was positioned at 50% of GM muscle length, centrally along the midsagittal line. Participants then performed a ramped MVC over 6 s, with the change in SM pennation angle and fascicle length simultaneously recorded. Three fascicles were analyzed, and the mean value was used. Therefore, physiological cross-sectional area (PCSA) was calculated as follows:

$$\text{PCSA} = \text{GM MV} \div \text{GM fascicle length} \qquad (2)$$

Moment arm. Achilles tendon moment arm length was calculated using the tendon excursion method (30). Participants were seated in the isokinetic chair following the setup detailed above. Primarily, the insertion of the GM was located using B-mode ultrasonography (AU5 Harmonic; Esaote Biomedica) and marked using micropore tape (3M; Bracknell) as an echo-absorptive reflective marker. The ultrasound probe (7.5 Hz) was then placed over the marker on the GM muscle-tendon junction (MTJ). The participant's ankle was passively rotated at 1°/s between 10° and −5° PF, which was synchronized with torque outputs using a square wave signal generator to distinguish ankle joint position. The displacement of the MTJ between 10° and −5° PF was calculated by using the reflective marker as a distance indicator when using analysis software (version 1.45s; NIH). The Achilles tendon moment arm was subsequently calculated using the displacement of the MTJ divided by the change in the ankle angle during the passive rotation (31).

Steps in the calculation of GM specific force.

$$\text{Tendon force} = \text{PF MVC torque (corrected for muscle activation}$$
$$\text{and co-activation)} \div \text{Achilles tendon moment arm.}$$
$$(3)$$

Calculation of the GM contribution toward maximum total PF tendon force was assumed to be 25% of the total force (14).

$$\text{GM fascicle force} = \text{GM tendon force} \div \cos\left(\text{GM pennation angle}\right)$$
$$(4)$$

$$\text{GM specific force} = \text{GM fascicle force} \div \text{GM PCSA} \qquad (5)$$

Nutritional intake and analysis. Detailed food and drink intakes were assessed with the use of a 3-d food diary recorded over 2 weekdays and 1 weekend day (32). Participants were asked to maintain their normal eating habits over the 3-d period. Dietary analysis was conducted with the use of Nutritics software (version 1.8; Nutritics Ltd. Co.), with one researcher completing all analyses. Participants' total nutritional intake and identified proanabolic nutrients were scored against recommended daily values (33–35) (as shown in **Supplemental Table 1**). The Harris-Benedict equation (36), which was previously shown to be valid and accurate in the elderly (37), was utilized to calculate basal metabolic rate and give an indication of the participants' metabolic balance accounting for PA and sedentarism.

PA questionnaire. Participants' PA status was ascertained with the use of the Baecke PA questionnaire (38). The questionnaire is divided into 3 sections that delineate work, sports, and leisure-based PA and additionally gives a combined score classified as a global index (38). Work scoring focused on the physical intensity of working and factored in time spent sitting, whereas leisure scoring focused on leisure-based nonstructured PA and factored in time spent watching television (TV). Sports scoring denoted structured PA and classified the intensity, repetition, and duration of the activity.

Serum inflammatory cytokine concentration. Thirty-three participants provided a 10-mL overnight fasting (12 h) blood sample, without having performed vigorous exercise for 48 h before sampling. Blood was centrifuged (IEC CL31R; Thermo Scientific) for 10 min at $2700 \times g$ (4°C), and serum was extracted and stored at −20 °C until subsequent analysis. Blood samples were unavailable for 17 participants (sampling failure or consent withheld).

Multiplex luminometry was used to measure the serum concentrations of 9 inflammatory cytokines [proinflammatory: IL-1β, IL-6, TNF-α, granulocyte-colony–stimulating factor (G-CSF), IFN-γ; anti-inflammatory: IL-10 and TGF-β1, -β2 and -β3] and 5 chemokines [IL-8, monocyte chemoattractant protein (MCP) 1, macrophage inflammatory protein (MIP) 1α, MIP-1β, and regulated on activation, normal T-cell expressed and secreted (RANTES)]. A 3-plex panel was used to measure TGF-β1, TGF-β2, and TGF-β3 concentrations (R&D Systems Europe Ltd.), and a Bio-Plex Pro Human Inflammation Panel Assay (Bio-Rad Laboratories Ltd.) was used to measure the remaining 11 cytokines, following the manufacturers' instructions. Samples were analyzed with the use of a Bio-Plex 200 system (Bio-Rad Laboratories Ltd.).

Statistical analyses. Statistical analyses were carried out with the use of SPSS (version 22; SPSS, Inc.). To determine parametricity, Kolmogorov-Smirnov (whole sample) or Shapiro-Wilk (BMI and adiposity) and Levene's tests were utilized. If parametric assumptions were met, between-group differences for participant characteristics, muscle-tendon unit (MTU) characteristics, and endocrine profile were examined by independent *t*-tests (adiposity) or 1-factor ANOVA and ANCOVA (BMI), with Bonferroni-corrected post hoc pairwise comparisons. However, if parametric assumptions were breached, between-group differences were examined by Mann-Whitney *U* test (adiposity) or a Kruskal-Wallis test (BMI), with post hoc Mann-Whitney *U* tests as appropriate. A repeated-measures ANOVA and Friedman's ANOVA (if parametric assumptions were breached) were utilized to assess any differences in nutritional intake over the 3-d period. Pearson (*r*; or Spearman ρ if parametric assumptions were breached) correlations were used to define any relations or associations between MTU characteristics and age, PA scores, adiposity, BMI, and nutrition variables. It should be noted that some MTU characteristics were not recorded due to a fault during data capture; therefore, the data on some correlations utilize fewer samples than the complete cohort of 50 participants. Radar graphs (Microsoft Excel, version 2013) of participants' muscle size (PCSA) and muscle quality (GM specific force) segregated into the top and bottom 10 participants (i.e., ~20%), used *z* scores [*z* score = (mean of group − mean of sample population) ÷ SD of sample population]. Comparison of composite *z* scores (mean unit weighted and accounting for the direction of the difference) was through converting these into percentages with the use of a *z*-score comparison table to determine a holistic picture of the differences in physical behavior, endocrine profile, and food intake of the participants exhibiting the top and bottom 20% values of selected MTU variables. Data are reported as means ± SDs, and significance was accepted when $P < 0.05$. Study power (β) and effect size ($p\varepsilon^2$) are also reported.

Results

Impact of adiposity and BMI on musculoskeletal characteristics. When participants were classified by adiposity, an independent *t*-test showed HA individuals to have 16.9% lower relative muscle strength than their NA counterparts ($P = 0.011$). This, however, was the only significantly different

TABLE 1 Participant and musculoskeletal characteristics grouped by adiposity and BMI[1]

	Adiposity		BMI		
	Normal adiposity, $n = 18$	High adiposity, $n = 32$	Normal weight, $n = 15$	Overweight, $n = 19$	Obese, $n = 16$
Participant characteristics					
Age, y	60 ± 11	$66 \pm 8^*$	64 ± 8	66 ± 9	60 ± 10
Height, cm	164 ± 9	165 ± 6	163 ± 7	166 ± 8	164 ± 7
Body mass, kg	65 ± 16	$81 \pm 13^*$	58 ± 8^c	75 ± 7^b	92 ± 11^a
BMI, kg/m^2	23.6 ± 3.9	$30.0 \pm 4.7^*$	21.9 ± 1.8^c	27.0 ± 1.2^b	34.1 ± 3.4^a
Body fat, %	31.6 ± 5.2	$41.1 \pm 6.7^*$	31.7 ± 5.4^c	$38.1 \pm 6.8^{a,b}$	42.9 ± 6.8^a
Fat mass, kg	19.8 ± 5.2	$32.6 \pm 8.1^*$	18.0 ± 3.7^c	27.3 ± 3.8^b	38.1 ± 7.4^a
Lean mass, kg	40.7 ± 11.0	$44.0 \pm 7.9^*$	36.7 ± 5.9^b	$42.8 \pm 8.4^{a,b}$	48.6 ± 9.2^a
Leg lean mass, kg	6.6 ± 1.8	7.0 ± 1.5	5.9 ± 1.0^b	$6.9 \pm 1.7^{a,b}$	7.7 ± 1.5^a
Arm lean mass, kg	2.2 ± 0.8	2.2 ± 0.6	2.0 ± 0.5	2.1 ± 0.7	2.4 ± 0.7
Muscle characteristics					
PF MVC, Nm	114 ± 46	117 ± 31	101 ± 26	114 ± 36	132 ± 43
PF MVC (corrected), Nm	131 ± 49	136 ± 32	113 ± 26^b	$134 \pm 35^{a,b}$	153 ± 44^a
PF MVC/body mass, Nm/kg	1.7 ± 0.4	$1.4 \pm 0.4^*$	1.7 ± 0.4	1.5 ± 0.4	1.4 ± 0.4
Muscle activation, %	86.5 ± 14.1	83.9 ± 13.3	88.1 ± 11.1	83.5 ± 14.2	83.4 ± 14.9
GM pennation angle, °	28.2 ± 5.0	31.2 ± 5.5	26.9 ± 4.1^b	29.0 ± 4.7^a	34.4 ± 4.9^a
GM fascicle length, cm	3.9 ± 0.7	3.8 ± 0.6	3.8 ± 0.6	3.9 ± 0.8	3.8 ± 0.6
GM muscle volume, cm^3	220 ± 49	225 ± 57	192 ± 45^b	$224 \pm 44^{a,b}$	249 ± 59^a
GM PCSA, cm^2	57 ± 14	58 ± 16	51 ± 14^b	$56 \pm 13^{a,b}$	65 ± 15^a
Tendon force, kN	3.89 ± 1.45	4.16 ± 1.02	3.42 ± 0.82^b	$4.06 \pm 1.17^{a,b}$	4.66 ± 1.24^a
GM fascicle force, kN	1.12 ± 0.47	1.22 ± 0.32	0.97 ± 0.26^b	$1.14 \pm 0.35^{a,b}$	1.42 ± 0.40^a
GM specific force, N/cm^2	19.9 ± 6.0	21.9 ± 6.4	20.1 ± 7.5	20.7 ± 5.6	22.6 ± 5.8

[1]Values are means ± SDs. Labeled BMI classification means in a row without a common superscript letter differ, $P < 0.05$. *Different from normal adiposity, $P < 0.05$. GM, gastrocnemius medialis; kN, kiloNewton; MVC, maximum voluntary contraction; N, Newton: Nm, Newton meter; PCSA, physiological cross-sectional area; PF, plantar flexion.

variable between NA and HA individuals, because there were no significant differences reported in PF MVC, GM MV, and GM specific force (Table 1). Interestingly, when conducting a linear regression, HA individuals exhibited an apparent larger rate of GM MV loss with age compared with NA individuals [$\beta_1 = -4.38$ ($P = 0.001$) compared with $\beta_1 = -2.46$ ($P = 0.03$)]. However, neither slope differences (Student's t-statistic $= 1.25$, $P > 0.05$) nor correlation coefficients ($z = -0.236$) differed significantly.

When participants were classified by BMI, a 1-factor ANOVA showed PF MVC corrected for antagonist coactivation and agonist activation to have a main effect of BMI ($P = 0.014$, $\beta = 0.759$, $\eta_{p^2} = 0.166$). Pairwise comparisons showed that obese individuals had 35% greater corrected PF MVC ($P = 0.011$) than their NW counterparts. Interestingly, the significant difference remained when conducting a 1-factor ANCOVA comparing the influence of BMI on corrected PF MVC while controlling for energy intake ($P = 0.05$, $\beta = 0.585$, $\eta_{p^2} = 0.122$). This pattern was followed in SM structural characteristics by GM pennation angle, GM MV, and GM PCSA, all showing a main effect of BMI ($P < 0.001$, $P = 0.013$, and $P = 0.043$, respectively), whereby obese individuals had a 22% ($P = 0.010$) greater pennation angle, 29.6% ($P < 0.001$) had a greater GM MV, and 26.6% ($P = 0.041$) had a greater GM PCSA than their NW counterparts. A 1-factor ANCOVA comparing the influence of BMI on GM PCSA while controlling for energy intake removed the significant group difference ($P = 0.09$, $\beta = 0.476$, $\eta_{p^2} = 0.112$). A 1-factor ANOVA showed tendon force to have a main effect on BMI ($P = 0.016$, $\beta = 0.744$, $\eta_{p^2} = 0.175$). Pairwise comparisons showed that obese individuals had 36% greater tendon force ($P = 0.013$) than their NW counterparts. Interestingly, the significant difference remained when conducting a 1-factor ANCOVA comparing the influence of BMI on tendon force

while controlling for energy intake ($P = 0.038$, $\beta = 0.627$, $\eta_{p^2} = 0.144$). This pattern was additionally followed by GM fascicle force, which showed a main effect of BMI ($P = 0.003$, $\beta = 0.891$, $\eta_{p^2} = 0.244$), whereby obese individuals had a 47% ($P = 0.003$) greater GM fascicle force than their NW counterparts. Again, the significant difference remained when conducting a 1-factor ANCOVA comparing the influence of BMI on GM fascicle force while controlling for energy intake ($P = 0.008$, $\beta = 0.824$, $\eta_{p^2} = 0.216$).

Finally, a 1-factor ANOVA showed no significant effect of BMI on PF MVC, relative muscle strength, GM fascicle length, or GM specific force (Table 1).

Comparison of habitual nutritional intake and PA levels between adiposity and BMI classifications. A repeated-measures ANOVA with a Greenhouse-Geisser correction showed no statistical differences in daily energy and carbohydrate intake between the 3 d in the pooled population. In addition, a Friedman's ANOVA showed no statistical differences in total fat, protein, saturated fat, monounsaturated fat, polyunsaturated fat, ω-3 FAs, ω-6 FAs, *trans* fat, cholesterol, vitamin D, and sugar intake between the 3 d in the pooled population.

There were no differences in total calorie, carbohydrate, or fat intakes between NA and HA individuals; yet, HA individuals had 16.9% ($P = 0.028$) greater absolute protein intake. Surprisingly, there were no group differences in metabolic balance with participants classified by adiposity. Yet, with participants classified by BMI, a 1-factor ANOVA showed a main effect of BMI group ($P = 0.032$, $\beta = 0.653$, $\eta_{p^2} = 0.136$), with obese individuals remarkably having 291-kcal lower relative calorific intake than NW ($P = 0.032$). No between-group differences (either by adiposity or by BMI) or associations were reported for potential proanabolic nutrients,

TABLE 2 Participants' nutritional characteristics and physical activity scores grouped by adiposity and BMI[1]

	Adiposity		BMI		
	Normal adiposity, $n = 18$	High adiposity, $n = 32$	Normal weight, $n = 15$	Overweight, $n = 19$	Obese, $n = 16$
Daily nutritional intake					
Energy intake, kcal/d	1942 ± 371	2021 ± 402	1866 ± 298	2013 ± 368	2087 ± 473
Metabolic, balance, kcal/d	−6 ± 263	−38 ± 352	105 ± 249[b]	5 ± 362[a,b]	−186 ± 273[a]
Carbohydrate, g/d	222 ± 49	234 ± 60	216 ± 46	238 ± 59	233 ± 62
Protein, g/d	77 ± 17	91 ± 21*	77 ± 15	90 ± 22	89 ± 22
Fat, g/d	71 ± 16	73 ± 19	70 ± 15	70 ± 17	76 ± 22
Saturated fat, g/d	26 ± 7	26 ± 9	27 ± 7	24 ± 8	28 ± 10
Monounsaturated fat, g/d	25 ± 7	26 ± 9	24 ± 6	24 ± 7	27 ± 10
Polyunsaturated fat, g/d	12 ± 5	13 ± 4	11 ± 4	13 ± 4	13 ± 5
trans Fat, g/d	0.8 ± 0.4	0.8 ± 0.4	0.8 ± 0.4	0.7 ± 0.4	0.9 ± 0.4
ω-3 FAs, g/d	1.8 ± 2.1	1.2 ± 0.9	1.6 ± 2.0	1.2 ± 0.8	1.6 ± 1.7
ω-6 FAs, g/d	5.7 ± 2.4	6.1 ± 3.9	5.3 ± 2.1	6.5 ± 4.6	5.9 ± 2.7
Vitamin D, μg/d	7.1 ± 12.1	4.4 ± 3.4	7.2 ± 12.8	4.8 ± 4.0	4.3 ± 4.1
Vitamin E, mg/d	9.5 ± 4.1	11.1 ± 4.0	10.4 ± 3.9	11.0 ± 4.8	10.2 ± 3.4
Calcium, g/d	0.96 ± 0.26	1.05 ± 0.33	0.95 ± 0.21	0.11 ± 0.38	0.99 ± 0.27
Zinc, mg/d	9.0 ± 1.9	10.6 ± 2.8	9.6 ± 2.5	10.0 ± 2.5	10.5 ± 3.0
Vitamin B-12, μg/d	5.6 ± 2.9	6.5 ± 6.3	5.3 ± 2.2	6.8 ± 7.7	6.2 ± 4.0
Physical activity score					
Work	2.6 ± 0.5	2.6 ± 0.3	2.5 ± 0.4	2.5 ± 0.3	2.8 ± 0.4
Sport	2.4 ± 0.7	2.3 ± 0.5	2.4 ± 0.6	2.4 ± 0.4	2.2 ± 0.7
Leisure	2.8 ± 0.5	2.8 ± 0.5	2.6 ± 0.4	3.0 ± 0.6	2.8 ± 0.5
Global index	7.8 ± 0.8	7.7 ± 0.8	7.5 ± 0.7	7.9 ± 0.6	7.8 ± 1.1

[1]Values are means ± SDs. Labeled BMI classification means in a row without a common superscript letter differ, $P < 0.05$. *Different from normal adiposity, $P < 0.05$.

such as ω-3 and ω-6 FAs, calcium, zinc, and vitamins D, B-12, C, E, and K (Table 2).

There were no significant differences reported in work, sport, and leisure PA scores between sexes, adiposity, or BMI classifications, showing that the study population was equally matched across PA-grouping variables (Table 2).

Impact of adiposity and BMI on serum cytokine concentrations. Interestingly, a Mann-Whitney U test showed

no significant difference between NA and HA individuals for 5 proinflammatory cytokines (IL-1β, IL-6, TNF-α, G-CSF, and IFN-γ), 4 anti-inflammatory cytokines (IL-10, TGF-β1, TGF-β2, and TGF-β3), and 5 chemokines (IL-8, MCP-1, MIP-1α, MIP-1β, and RANTES). These results were mirrored when participants were classified by BMI, because a Kruskal-Wallis test showed no significant differences between BMI classifications (Table 3). In addition, Spearman correlations showed that age was additionally not correlated with

TABLE 3 Serum cytokine and chemokine concentrations in 33 participants classified by adiposity and BMI[1]

	Adiposity		BMI		
	Normal adiposity, $n = 12$	High adiposity, $n = 21$	Normal weight, $n = 8$	Overweight, $n = 14$	Obese, $n = 11$
Cytokines					
Proinflammatory					
IL-1β, pg/mL	3.14 ± 2.31	2.78 ± 2.85	2.78 ± 2.27	3.73 ± 3.09	1.98 ± 2.09
IL-6, pg/mL	2.53 ± 1.51	16.6 ± 57.9	2.33 ± 1.12	23.9 ± 70.6	2.28 ± 1.48
TNF-α, pg/mL	10.2 ± 10.6	11.5 ± 15.2	6.31 ± 5.98	15.9 ± 17.5	8.32 ± 10.1
G-CSF, pg/mL	94 ± 143	102 ± 168	59 ± 153	150 ± 181	62 ± 116
IFN-γ, pg/mL	2.8 ± 3.7	54 ± 145	1.3 ± 1.5	81 ± 173	1.72 ± 2.67
Anti-inflammatory					
IL-10, pg/mL	6.5 ± 3.4	25.2 ± 67.2	7.7 ± 4.7	32.5 ± 81.9	8.3 ± 9.2
TGF-β1, ng/mL	39.3 ± 47.6	27.0 ± 31.4	31.7 ± 32.6	43.6 ± 50.6	15.8 ± 14.8
TGF-β2, pg/mL	276 ± 176	311 ± 253	316 ± 680	334 ± 205	221 ± 184
TGF-β3, pg/mL	140 ± 125	282 ± 354	92 ± 55	252 ± 301	305 ± 372
Chemokines					
IL-8, pg/mL	42.0 ± 31.8	41.0 ± 17.4	36.7 ± 12.8	50.1 ± 31.0	33.59 ± 12.7
MCP-1, pg/mL	89.8 ± 127	69.2 ± 81	59 ± 57	102 ± 135	56 ± 64
MIP-1α, pg/mL	13.1 ± 6.9	6.9 ± 5.1	8.55 ± 9.82	8.86 ± 6.43	9.99 ± 8.03
MIP-1β, pg/mL	712 ± 1010	400 ± 278	348 ± 181	613 ± 930	507 ± 410
RANTES, ng/mL	78.3 ± 53.6	91.8 ± 44.9	83.9 ± 52.2	95.3 ± 42.6	78.3 ± 53.7

[1]Values are means ± SDs. No differences were reported between adiposity or BMI classifications. G-CSF, granulocyte-colony–stimulating factor; MCP-1, monocyte chemoattractant protein 1; MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T-cell expressed and secreted.

TABLE 4 Bivariate Pearson (r) and Spearman (ρ) correlations between age, physical activity scores, adiposity, BMI, and habitual nutritional intake against 11 musculoskeletal characteristics[1]

Muscle characteristics	Age	Physical activity score				Adiposity	BMI	Nutrition		
		Work	Sport	Leisure	Global			DI score	Proanabolic score	Total energy
PF MVC ($n = 50$)	−0.22†	0.32*†	0.15†	−0.07†	0.18†	0.16†	0.31*†	−0.11†	−0.25†	0.34*†
PF MVC (corrected) ($n = 50$)	−0.19†	0.16†	0.07†	−0.05†	0.09†	0.20†	0.37**†	−0.08†	−0.23†	0.43**†
PF MVC/body mass ($n = 50$)	−0.24	0.26	0.2†	−0.05†	0.19	−0.47**	−0.37**	−0.03†	−0.13†	0.08
Muscle activation ($n = 48$)	−0.13†	0.34*†	0.18†	−0.10†	0.17†	−0.19†	−0.19†	0.02†	−0.13†	−0.05†
GM pennation angle ($n = 47$)	−0.42**	0.19	0.17†	−0.01†	0.13	0.51***	0.62***	−0.07†	−0.24†	0.31*
GM fascicle length ($n = 47$)	0.13	0.01	−0.19†	0.11†	0.01	−0.08	−0.07	0.09†	−0.06†	0.00
GM muscle volume ($n = 47$)	−0.49***	0.13	0.05†	−0.04†	0.10	0.33*	0.45**	−0.11†	−0.31*†	0.43**
GM PCSA ($n = 44$)	−0.49**	0.10	0.19†	−0.07†	0.08	0.34*	0.42**	−0.18†	−0.26†	0.42**
Tendon force ($n = 46$)	−0.32*	0.39**	0.08†	0.04†	0.23	0.11	0.33*	−0.14†	−0.25†	0.41**
GM fascicle force ($n = 44$)	−0.40**	0.45**	0.06†	0.06†	0.23	0.20	0.43**	−0.20†	−0.35*†	0.42**
GM specific force ($n = 44$)	0.08	0.30*	−0.09†	0.09†	0.07	−0.04	0.11	0.01†	−0.18†	0.12

[1] †Spearman (ρ) correlations. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. DI, daily intake; GM, gastrocnemius medialis; MVC, maximum voluntary contraction; PCSA, physiological cross-sectional area; PF, plantar flexion.

serum cytokine concentrations of individuals in our sample ($P > 0.05$).

Bivariate correlations. Table 4 shows bivariate correlation coefficients between MTU characteristics and age, PA scores, adiposity, BMI, and nutritional intake to determine the strongest predictors of SM structural and functional characteristics. BMI was the most prolific predictor of 11 MTU variables, with 8 (range: $r = 0.62$–0.31; $P < 0.001$ to $P = 0.032$) significant associations, followed by daily energy intake with 7 ($r = 0.43$–0.34; $P = 0.002$–0.036) significant associations, both age ($r = −0.49$–0.32; $P < 0.001$ to $P = 0.032$) and work-based PA score ($r = 0.45$–0.32; $P = 0.002$–0.048) with 5 significant associations, adiposity with 4 ($r = 0.51$–0.33; $P < 0.001$ to $P = 0.022$) significant associations, and proanabolic nutrient score with 2 ($r = −0.35$–0.31; $P = 0.021$–0.037) significant associations. Additional correlations of particular interest were absolute protein intake being positively correlated with lean mass ($\rho = 0.30$, $P = 0.036$), yet GM MV was negatively correlated with relative protein intake (grams per kilogram of body mass; $r = −0.36$, $P = 0.013$). Interestingly, when controlling for BMI, a partial correlation removed the statistical association ($P = 0.36$). Finally, leisure-based PA was negatively correlated with daily energy intake ($\rho = −0.29$, $P = 0.04$).

z-Score comparisons of participants' nutritional profile, physical characteristics, and endocrine profile classified by the top and bottom 20% of muscle characteristics. Figure 1 graphically summarizes the overall dietary habits, participant characteristics, and endocrine profiles of participants' PCSA. The unit-weighted PCSA z score for all nutrients produced a 2% higher score in the low-PCSA group (Figure 1A), showing the similarity in overall "hypertrophic" nutritional profiles. The unit-weighted score for age, body composition, and physical behavior was 92% lower in the low-PCSA group (Figure 1B), and the unit-weighted score for endocrine profile was 13% lower in the low-PCSA group (Figure 1C).

Figure 2 graphically summarizes the overall dietary habits, participant characteristics, and endocrine profiles of participants' specific force. The unit-weighted z score for all nutrients was only 6% higher in the low-specific-force group, again confirming the similarity in overall "hypertrophic" nutritional profiles (Figure 2A). The unit-weighted score for age, body

composition, and physical behavior was 67% lower in the low-specific-force group (Figure 2B), and the unit-weighted score for endocrine profile was 37% lower in the low-specific-force group.

Discussion

The current study identified key factors known or projected to influence MTU characteristics, notably daily energy intake, protein intake, PA, BMI, and adiposity, as we age. Daily energy intake was found to positively correlate with both GM MV and PF MVC and 5 other MTU characteristics, thus partially supporting our first hypothesis. However, this trend was not continued between absolute protein intake and either GM MV or leg lean mass; yet, absolute protein intake did positively correlate with total lean mass. Surprisingly, relative protein intake was found to negatively correlate with GM MV; yet, this association was removed when controlling for BMI, potentially showing the loading effect that total mass has on SM. However, it should be noted that 48 participants consumed the minimum recommended daily protein intake for adults ($\sim$0.8 g · kg body mass^{-1} · d^{-1}), raising the question at what age should protein intake be increased, or is it related to additional factors such as functional status or low dietary intake? Equally surprising was the lack of bivariate associations between proanabolic nutrients and MTU characteristics, hence refuting our second hypothesis. There were no differences reported in work, sport, and leisure PA scores between adiposity and BMI classifications (a strength of the current study design), yet significant positive correlations were found between work-based PA and PF MVC, muscle activation, and GM tendon, fascicle, and specific force, partially supporting our final hypothesis and showing the important role of PA in healthy aging. Finally, the endocrine profile of participants was not influenced by either adiposity or BMI and was not exacerbated with increasing age, supporting the hypothesis that adequate nutrition and PA are important factors in maintaining endocrine profile as we age.

The effect of obesity on SM in middle-aged to older-aged adults remains unclear; thus, the aim of this study was not only to investigate intrinsic factors, such as adiposity and BMI loading on SM, but also the combined impact of extrinsic factors (e.g., nutritional intake and PA). We report that after controlling for average daily energy intake, any effect of BMI
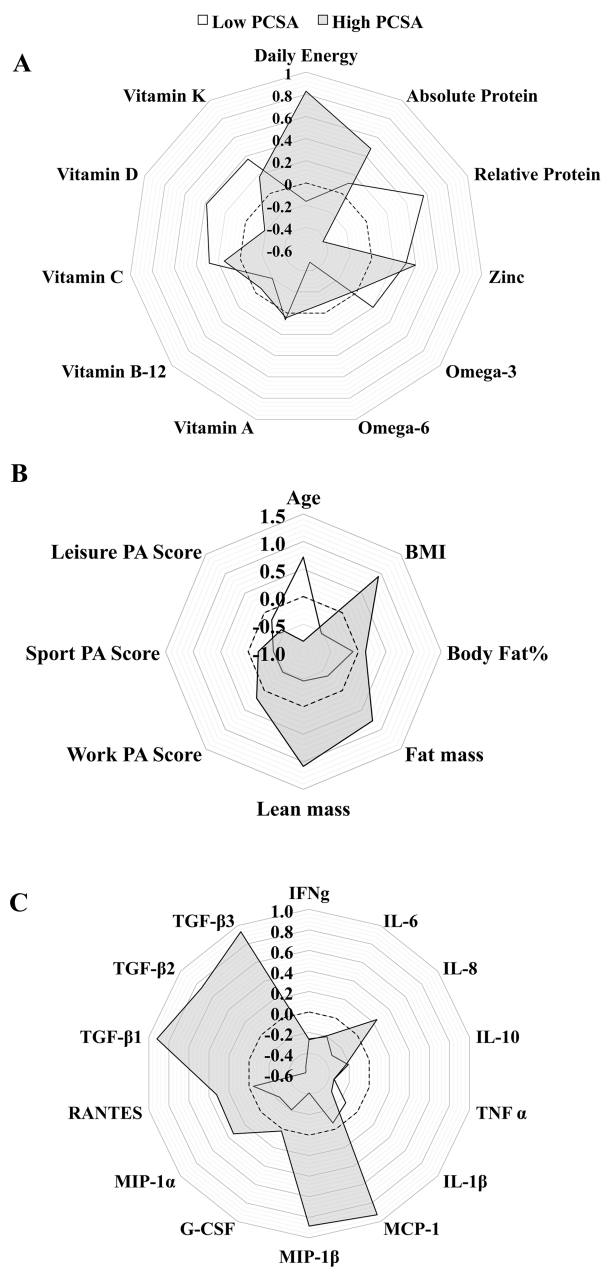
FIGURE 1 Radar graphs representing *z* scores calculated by using the study sample mean (*n* = 44) focusing on the top (high) and bottom (low) 10 participants' (~20% of total sample) PCSA of the study sample defined by their nutritional profile (A), characteristics (B), and endocrine profile (C). (A) A high PCSA is associated with 36% greater total energy intake, 14% greater absolute protein intake, and 15% greater ω-6 intake *z*-score differences. However, an opposite pattern was shown for relative protein and vitamin D intakes, with 38% and 23% *z*-score differences, respectively. (B) A low PCSA was associated with a 55% greater *z*-score difference for age and a 9% difference for leisure-based PA, but a 43% lower *z*-score difference for fat mass, 54% for lean mass, 26% for work-based PA, 10% for sport-based PA, 53% for BMI, and 9% for body fat percentage. (C) A high PCSA was associated with a 22% greater *z*-score difference for IL-8, 54% for TGF-β1, 48% for TGF-β2, 52% for TGF-β3, 14% for RANTES, 35% for MCP-1, 23% for MIP-1α, 47% for MIP-1β, 0.5% for IL-6, 0.1% for TNF-α, and 9% for G-CSF, but a lower *z*-score difference of 0.3% for IFN-γ, 1% for IL-10, and 7% for IL-1β. The dashed circles denote a *z* score of 0. G-CSF, granulocyte-colony–stimulating factor; MIP, macrophage inflammatory protein; PA, physical activity; PCSA, physiologic cross-sectional area; RANTES, regulated on activation, normal T-cell expressed and secreted.
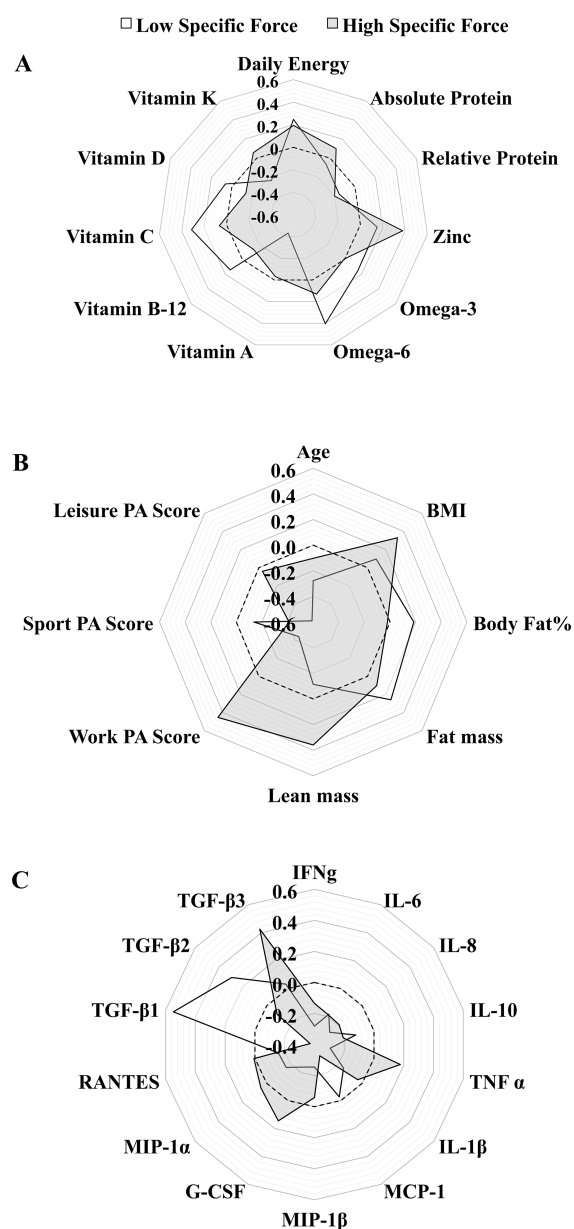
FIGURE 2 Radar graphs representing *z* scores calculated by using the study sample mean (*n* = 44) focusing on the top (high) and bottom (low) 10 participants' (~20% of total sample) SF of the study sample defined by their nutritional profile (A), characteristics (B), and endocrine profile (C). (A) A high SF was associated with a 6% greater absolute protein intake, 16% greater vitamin A intake, and 12% greater vitamin K intake. However, an opposite pattern was shown for total energy intake, relative protein intake, and vitamin D intake, with 2%, 2%, and 8%, respectively. (B) A low SF was associated with an 8% higher *z*-score difference for body fat percentage, an 11% higher sports-based PA, and a 6% greater fat mass, but a 19% lower lean mass, a 34% lower work-based PA, a 21% lower leisure-based PA, and a 9% lower BMI. (C) The pattern of *z*-score differences showed high SF to be associated with a 3% higher IL-8, 15% higher TGF-β3, 7% higher RANTES, 5% higher IL-1β, 8% higher MIP-1α, 8% higher MIP-1β, 0.5% higher IL-6, 19% higher TNF-α, 6% higher IFN-γ, and 15% higher G-CSF, but a 35% lower TGF-β1, 15% lower TGF-β2, 12% lower MCP-1, 0.2% lower IL-6, and 3% lower IL-1. The dashed circles denote a *z* score of 0. G-CSF, granulocyte-colony–stimulating factor; MCP-1, monocyte chemoattractant protein 1;MIP, macrophage inflammatory protein; PA, physical activity; PCSA, physiologic cross-sectional area; RANTES, regulated on activation, normal T-cell expressed and secreted; SF, specific force.

on GM PCSA was removed. These results suggest that 11% of the differences in GM PCSA between BMI classifications in middle- to older-aged adults are, at least in part, due to differences in nutritional intake. Thus, statistically separating out (through an ANCOVA) the effects of daily energy intake removed the apparent BMI group differences, hence supporting the proposal that loading differences between individuals is a key factor in the hypertrophic potential of obesity. In fact, contrary to our expectation that obese individuals would exhibit decreased PA (39), our sample of obese persons was recreationally physically active. However, we cannot exclude the possibility that differences in PA would normally be expected to explain the effect that obesity has on SM strength and size whereby high levels of adiposity may instigate even greater mechanical loading during higher PA. This possibility is supported by work by Rolland et al. (40), who reported similar results to the current study in that, when classifying individuals by PA levels, no differences were reported in MVC between sedentary individuals, yet active obese individuals had greater absolute strength than their active NW counterparts. Therefore, interpretation of previous literature in conjunction with the current study would not rule out the role that excess adiposity elicits on loading SM; however, nutritional intake was perceived to play an important role in increasing MTU characteristics irrespective of BMI or adiposity classification (Table 4).

It was also hypothesized that higher protein intake would have a substantial effect on muscle structure due to its ability to stimulate MPS independently of overloading the muscle (41). What we, in fact, observed was that, although the relation between total lean mass and daily protein intake was positive, there was a negative relation between total lean mass and protein intake normalized to body mass (i.e., relative protein intake). This latter observation was reflected in an inverse relation between GM MV and relative protein intake. Interestingly, this association was removed when controlling for BMI. Yet, within the literature, there have been numerous studies that have investigated the optimum relative protein intake for both men and women, with it being accepted that protein intake <0.8 g · kg body mass^{-1} · d^{-1} is a modifiable risk factor for sarcopenia (42). The recommended daily intake for adults aged >18 y is set above the threshold of 0.8 g · kg body mass^{-1} · d^{-1}. Deutz et al. (43), from the European Society for Clinical Nutrition and Metabolism expert group, recommended this value to be increased to 1.0–1.2 g · kg body mass^{-1} · d^{-1} in healthy older adults, and increased to 1.2–1.5 g · kg body mass^{-1} · d^{-1} for older adults with acute or chronic illness, with Phillips et al. (44) more recently recommending levels of 1.2–1.6 g · kg body mass^{-1} · d^{-1}. The mean relative protein intake of the present study sample was 1.17 g · kg body mass^{-1} · d^{-1}, with only 2 individuals being marginally below the recommended limit for adults. Therefore, the negative association between relative protein intake and GM MV may be a moot point given that our study sample habitually ingested adequate daily protein. This possibility is additionally supported by obese individuals who consume lower relative amounts of protein due to greater total mass, yet who are still above the threshold of 0.8 g · kg body mass^{-1} · d^{-1}. Additional explanations may include protein intake per meal compared with total daily protein ingestion (30 g/meal) (45), the wide age ranges of the total study sample (43–80 y), and the good general health of the study sample who were both independently living and recreationally active. Future research should examine the above proposal using a longitudinal study design while systematically recording dietary habits (week-to-week fluctuation). Arguably, a limitation of our current study is that we only took a 3-d snapshot of the individuals' nutritional intake. Future longitudinal studies of dietary habits on the rate of aging may also increase our knowledge of any associations between proanabolic nutrients that have been shown to improve muscle function in the elderly, such as vitamin D (46) and ω-3 FAs (22). Indeed, our current data were surprising in that they did not highlight any advantage to taking elevated amounts of these nutrients in terms of MTU size or function.

As for the projected effect of PA on SM characteristics, given that there were no group differences in PA scores, it was interesting to find positive correlations in the pooled study sample between work-based PA and PF MVC, muscle activation, GM tendon force, fascicle force, and specific force. Work-based PA in the Baecke PA questionnaire quantifies a person's general PA (standing, walking, and lifting heavy loads), intensity of activity during this period (how much individuals sweat, comparison of work physicality against their own age), and their sedentary behavior (time spent sitting) profile. These associations seem to confirm previous research that associated high sedentary behavior (TV viewing and computer use) with decreased muscle strength (47) in 6228 elderly men and women. Interestingly, the association of decreased muscle activation and GM specific force is associated with aging independently (27, 48). The suggested mechanism is through the de-innervation of motor units (preferentially type II) and physical inactivity (27), as suggested from our current data. Therefore, further research is needed to examine the "PA-independent effects" of sedentary behavior on muscle activation and specific force. Indeed, research has previously shown the importance of maintaining optimal status in these variables from young adulthood through later life because of the importance of muscle strength in maintaining functional independence in old age. Interestingly, the negative association reported between leisure activity and daily energy intake may be related to time spent watching TV (sitting behavior) as screened for in leisure-based PA scoring. TV watching and poor diets have been associated in both children and adolescents (49) but also in young and older adults (50). This finding shows the link between inactivity and decreased muscle strength. It also highlights that inactivity may potentially be a driver to increased calorie intake, ultimately leading to obesity or sarcopenic obesity (51) in older individuals.

Surprisingly, there was no relation between adiposity, BMI, or age affecting the endocrine profile of participants within this study. Notably, within the literature, both obesity (52) and aging (53) are associated with an increase in low-grade inflammation, which is additionally linked to increasing the risk of developing comorbidities [e.g., coronary heart disease (54)] and accelerating muscle aging (55), thus negatively affecting SM function. A potential explanation for these findings may be the participants' habitual nutritional intake (both quality and quantity) and their habitually active PA profile. Although no associations were observed in nutrients associated with anti-inflammatory properties [i.e., ω-3 FAs (19)], the overall dietary profile of participants was good (see Table 2), with 48 of 50 participants consuming the recommended amounts of protein and 50 of 50 consuming the recommended amounts of vitamins B-12 and E. Therefore, taking into account both the nutritional and PA profiles, and given the positive relation between total body mass and muscle structure and function (3, 14, 16, 29), any deleterious effects of low-grade inflammation in this population appears to be outweighed (55). This, however, needs further investigation, because a limitation of the current

study was the small sample size of the adiposity and BMI groups with regard to the highly variable endocrine profile data. Therefore, future, adequately powered research should sample 51 participants/adiposity group and 53/BMI group to detect significant differences between the various cytokines and chemokines.

In parallel, the endocrine profile of the top and bottom 20% PCSA of participants would suggest that TGF-β1–3, MIP-1β, and MCP-1 are good indicators of the hypertrophic potential, whereas TGF-β1–3, G-CSF, and TNF-α appeared to be good indicators of the intrinsic muscle functional capacity (specific force) potential of an individual. Our data support the body of work linking the TGF-β superfamily with an important role in directing the characteristics of the MTU (56). Similarly, the emergence of cytokines as key contrasting factors for high compared with low MTU characteristics is a novel finding that closes the loop between the widely described impact of inflammatory makers in muscle cell models and what we now present as exhibited in vivo in humans. Thus, notwithstanding the fact that cytokines are highly regulated, by adipocytes among others (57), we present how a snapshot of these, regardless of concurrent adiposity, can provide an insight into intrinsic MTU characteristics in free-living middle- to older-aged individuals.

In conclusion, our study showed that age, total energy intake, work-based PA activity, BMI, and adiposity were significant predicators of MTU characteristics in middle- to older-aged adults. We propose that with no difference in the endocrine profile of participants classified by obesity (i.e., either through adiposity or BMI), this suggests the independent influence of adequate PA on SM structure and function properties in older age. In addition, suboptimal body composition, specifically HA, was associated with increased risk of functional disabilities shown by its negative relation with relative strength and increased loss of muscle mass with increased age. In parallel, the endocrine profiles suggest a number of reliable indicators of either or both the hypertrophic (PCSA) and the intrinsic muscle functional capacity (SF) potential of an individual. Also of note, in relation to diet, our data show that, whereas a number of nutrients can be theoretically associated with muscle hypertrophy in free-living middle-aged to older individuals, any advantage of greater-than-average intakes of vitamins C, D, or K and ω-3 FAs or even relative protein intake is overridden by a greater-than-average total protein and high daily energy intake. Future research should look to apply interventions to confirm (or refute) this proof-of-principle in relation to the impact of various combinations of "hypertrophic nutrients."

Acknowledgments

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