RESEARCH ARTICLE

Change in D$_3$Cr muscle mass in oldest old men and its association with changes in grip strength and walking speed

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Abstract

Background

The use of lean soft tissue (LST) mass as a surrogate measurement of skeletal muscle
mass (SMM) has led to the conclusion that muscle loss is poorly related to functional
decline. We hypothesized that when using a more accurate measure of SMM determined
by D$_3$-creatine dilution (D$_3$Cr), longitudinal changes in SMM will be similar in magnitude to
changes in strength and physical performance and that skeletal muscle mass will partially
mediate the relationship of age with these outcomes.

Methods

We measured change in D$_3$Cr muscle mass (kg), handgrip strength (kg), and 6m walk
speed (m/s) in 208 men from the Osteoporotic Fractures in Men Study (85.2 ± 4.3 years)
over an average of 6.1 years follow-up. Mixed linear effects models adjusted for potential
confounders were used to examine the relationship of changes in D$_3$Cr muscle mass with
changes in grip strength and walking speed.

Results

Annual losses of D$_3$Cr muscle mass, grip strength, and walking speed were 2.1%, 2.2%,
and 2.6%, respectively ($p < 0.001$). Each additional kg loss in D$_3$Cr muscle mass was asso-
ciated with a 0.55 kg loss in grip strength and a 0.01 m/s loss in walking speed indepen-
dent of changes in age ($p < 0.001$). 41.3% and 22.4% of the relationship between age and
loss of grip strength and walking speed, respectively, was attributed to loss of D$_3$Cr muscle
mass ($p < 0.001$).

Conclusion

Skeletal muscle mass may have a more important role than previously considered and should not be overlooked as a potentially modifiable determinant in the loss of strength and performance in older age.

Introduction

Sarcopenia was initially described as the age-associated loss of skeletal muscle mass (SMM) and strength that is associated with functional decline and adverse outcomes including falls, fractures, physical disability and mortality [1]. However, subsequent studies using fat-free mass (all lean components including bone mineral) or lean soft tissue mass (LST) (i.e., all lean components excluding bone mineral) as surrogate measurements of SMM demonstrated a greater age-related decline in strength and function than FFM or LST [2–6]. This lack of relationship resulted in a questioning of the role of SMM in the development of functional decline. Several cross-sectional and longitudinal studies on aging cohorts have suggested that the relationship between SMM as estimated by FFM or LST, functional capacity and health related outcomes to be either weak or negligible, while muscle strength had strong associations with these outcomes [6]. Moreover, a number of studies have indicated that low muscle strength was related to reduced physical function in older adults, irrespective of whether there were concurrent reductions in FFM or LST [6]. These findings have led to alterations in the working definitions of sarcopenia, which now place greater importance on low muscle strength, measured by grip strength, as a diagnostic criterion for sarcopenia [7], and for many to conclude that SMM is relatively unimportant in the declines in muscle function that accompany aging [2,8–10].

However, many of the previous studies have used LST assessed by dual energy x-ray absorptiometry (DXA) to approximate total body or appendicular SMM. The measure of LST includes not only SMM, but non-contractile components, such as fibrotic and connective tissue, organs, and body water [11]. Thus, we posit that the limited association previously reported between "SMM" and adverse health outcomes in older adults is due to the systematic measurement error in the approximation of SMM using DXA LST.

Recently, the use of the d3-creatine dilution (D_3Cr) method has been highlighted as being a feasible, accurate, and precise measure of total body functional SMM [11]. Low D_3Cr muscle mass has been found to be associated with poor physical performance [12–14], decreased physical function [3,13], and increased risk of incident fractures [15], falls [12], disability [16,17] and mortality [14,17] in older men and correlated with physical function in older women [18,19], with these relationships not being observed using DXA measures of LST or appendicular LST (ALST). Although these findings suggest that D_3Cr muscle mass is more highly related to important age-related health outcomes, there are limited data on changes in D_3Cr muscle mass. Preliminary data from a sample of 40 men from the Osteoporotic Fractures in Men Study (MrOS) demonstrated that over ~ 1.6 years D_3Cr muscle mass, grip strength and walking speed declined by about 5% over follow-up, whereas DXA LST did not change [3].

In this study we aimed to build upon these preliminary findings and assess the long-term changes in D_3Cr muscle mass over ~ 6 years and its association with concurrent changes in grip strength and walking speed in oldest old men from MrOS. Our aims were to 1) describe the relationship between age and changes in D_3Cr muscle mass, grip strength, and walking speed; 2) evaluate the association of changes in D_3Cr muscle mass with concurrent changes in grip strength and walking speed; and 3) assess if the association of age with declines in grip strength and walking speed are mediated by age-associated changes in D_3Cr muscle mass.

Methods

Study sample

Community-dwelling ambulatory men aged ≥65 years were enrolled in the multicenter cohort study of aging and osteoporosis (MrOS), which took place across six U.S. clinical sites (Birmingham, AL; Minneapolis, MN; Palo Alto, CA; Monongahela Valley near Pittsburgh, PA; Portland, OR; and San Diego, CA) (https://mrosonline.ucsf.edu/). Recruitment for MrOS took place between April 25, 2000 to March 21, 2002 across all clinical sites. Details of the MrOS study design and recruitment have been published elsewhere [20,21]. All men provided written informed consent, and the study was approved by the Institutional Review Board (IRB) at each center and a centralized Western-Copernicus Group (WCG) IRB and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments (IRB approval #20200148). Data for this analysis was accessed on 15/03/2024 and included surviving participants from follow-up Year 14 (2014–2016) and Year 20 (2020–2022) visits who had measures for D_3Cr muscle mass, physical performance, and strength. At the Year 14 visit, 711 men had complete measures for D_3Cr muscle mass, grip strength, and walking speed from the 3 clinical sites that were included in the Year 20 visit. Of the 243 men who returned for the Year 20 visit, 208 had complete measures for D_3Cr muscle mass, grip strength, and walking speed at both time-points. A flow diagram of the men included in the study is depicted in S1 Fig.

D_3Cr muscle mass

The D_3-creatine (D_3Cr) dilution method was used to estimate SMM as described previously [12]. In brief, this method involves a participant ingesting a 30-mg dose of stable isotope-labeled creatine (D_3-creatine), and providing a fasting, morning urine sample 72–144 hours (3–6 days) later in which D_3-creatinine, unlabeled creatinine, and creatine are measured using high performance liquid chromatography and tandem mass spectroscopy. These measures are then incorporated into an algorithm developed to determine total body creatine pool size and thus SMM [22]. D_3Cr muscle mass divided by body mass (kg) was calculated to account for variations in total SMM by body size and used in assessing the descriptive characteristics of the sample.

Physical performance and strength

Grip strength (kg) was assessed by analyzing the maximal value from two tests of each hand using Jamar handheld dynamometers [23]. Walking speed (m/s) was determined by timing the completion of a 6-meter course at the participant's usual pace and was used as an indicator of physical performance [24].

Other measures

Participants self-reported physical activity using the Physical Activity Scale for the elderly (PASE) [25] and a physician diagnosis of chronic disease (i.e., diabetes, hyper/hypothyroidism, chronic obstructive pulmonary disease, Parkinson's disease, dementia/Alzheimer's disease, congestive heart failure, angina, heart attack, transient ischemic attack, stroke, peripheral vascular disease, high blood pressure, rheumatoid arthritis, osteoarthritis, cancer, and kidney disease) [26]. For analyses, comorbidities were categorized as having 0, 1–2, or >2 physician diagnoses of chronic disease. The self-reported birthdate at baseline was used to calculate age at the Year 14 and Year 20 visits. Stature was measured by wall-mounted stadiometers and body mass by balance beam or digital scales. Body mass index (BMI) was calculated as body

mass (kg)/stature (m²). In a subsample of MrOS participants, usual protein intake was estimated by the Block 98.2 MrOS brief FFQ, whose validity and reliability have been previously documented [26].

Statistical analysis

Baseline characteristics were presented with mean and SD and n (%). Differences between men with and without follow-up data from Year 20 visit were assessed using t-tests for continuous variables and a chi square test for categorical variables.

Mixed effects linear regression models (PROC MIXED procedure in SAS Version 9.1.3, SAS Institute, Cary, NC, USA) were performed to quantify the rate of change in D_3Cr muscle mass, grip strength and walking speed for each additional year of age. Time was modeled as age at the time of each measurement and centered to the mean age of 86.3 yrs. Models were fit with a random intercept and included age as a fixed effect in the unadjusted model and stature, body mass, physical activity, clinical site, and presence of co-morbidities in the adjusted model. A quadratic term for age was considered to test whether losses in D_3Cr muscle mass, grip strength, and walking speed increased nonlinearly with increasing age. The Kenward and Roger method was used to estimate degrees of freedom for the tests of fixed effects and generate p-values [27]. Restricted maximal likelihood was used to estimate covariance parameters while assuming an unstructured covariance matrix.

We further used mixed effects linear models to assess if the change in D_3Cr muscle mass that occurs with age was associated with concurrent changes in grip strength and walking speed. For these models, we separated the within and between individual differences in D_3Cr muscle mass by including a term for D_3Cr muscle mass measured at the Year 14 visit to represent between person differences and a term for the change in D_3Cr muscle mass (i.e., Year 20 visit D_3Cr muscle mass minus Year 14 visit D_3Cr muscle mass) to represent longitudinal within person differences. All models were fit with a random intercept and included age in the crude model and the additional covariates of body mass, stature, physical activity, comorbidities, and clinical site as fixed effects in the fully adjusted model.

To assess if the associations between age and changes in grip strength and walking speed were mediated by age-induced changes in D_3Cr muscle mass, we performed a mediation analysis using a series of mixed linear effects regression models (See S2 Fig for path diagram):

Path 1: $Y_{ij} = \text{ß}_1 + cX_{ij} + \text{covariates} + \gamma_{1i} + \epsilon_{1ij}$

Path 2: $M_{ij} = \text{ß}_2 + aX_{ij} + \text{covariates} + \gamma_{2i} + \epsilon_{2ij}$

Path 3: $Y_{ij} = \text{ß}_3 + bM_{ij} + c'X_{ij} + \text{covariates} + \gamma_{3i} + \epsilon_{3ij}$

where Y is either grip strength or walking speed for subject *i* at observation *j*, X is age for subject *i* at observation *j*, M is D_3Cr muscle mass for subject *i* at observation *j*, γ is the random intercept for subject *i*, and ϵ is the residual error.

In path 1, *c* describes the total effect (i.e., reflects the sum of the direct effect of age on strength or walking speed and the mediated (indirect) effect of changes in D_3Cr muscle mass). In path 2, *a* describes the effect of increasing age on D_3Cr muscle mass. In path 3, *b* describes the unique effect of changes in D_3Cr muscle mass on strength or walking speed and *c'* represents the direct effect of age on strength or walking speed after accounting for the effect of D_3Cr muscle mass. The indirect effect is the impact of increasing age on strength or walking speed that is transmitted through changes in D_3Cr muscle mass and is expressed as *a* x *b*. The proportion mediated was calculated as the ratio between the indirect effect and the total effect. The mediation analysis was performed in R using the *lme4* and *mediation* packages [28,29].

Given the role of protein intake in muscle homeostasis, we evaluated whether adjusting for dietary protein intake affected the results in the subsample of men for whom this was measured. A sensitivity analysis was also performed with only men who had data for both time points.

Model assumptions for the mixed-effects linear models were assessed to ensure validity. Linearity was evaluated using residual plots, normality of residuals and random effects was checked with Q-Q plots, homoscedasticity was assessed by plotting residuals against fitted values, and the random effects structure was validated using likelihood ratio tests and AIC/BIC, with no violations in model assumptions being detected.

Results

Characteristics of the men at the Year 14 visit from the Palo Alto, Pittsburgh, and Portland clinical sites, who had measures of D_3Cr muscle mass, grip strength, and walking speed are described in Table 1. The average follow-up time to the Year 20 visit was 6.1 (0.6) years. Men who were able to attend and complete Year 14 and 20 measurements were on average younger, taller, more active, stronger (more grip strength and D_3Cr muscle mass), walked faster, and had fewer comorbidities compared to men without Year 20 data (p < 0.05).

As depicted in Fig 1, estimated mean D_3Cr muscle mass, grip strength, and walking speed decreased with increasing age, with an estimated annual change in D_3Cr muscle mass of −0.53 kg/yr (95% CI: −0.58, −0.48), change in grip strength of −0.78 kg/yr (95% CI: −0.87, −0.69), and change in walking speed of −0.03 m/s/yr (95% CI: −0.03, −0.02). These associations were slightly attenuated after adjusting for body mass, stature, physical activity, comorbidities, and clinical site (−0.40 kg D_3Cr muscle mass, 95% CI: −0.45, −0.35; −0.49 kg grip strength, 95% CI: −0.59, −0.40; −0.02 m/s walking speed, 95% CI: −0.03, −0.02). There was no indication of a curvilinear relationship of age with D_3Cr muscle mass, strength, and performance measures (β-coefficient for the age^2 (quadratic) term p > 0.05). Table 2 shows the

Table 1. Characteristics of the men at the Year 14 visit and comparison of Year 14 visit characteristics between men who were evaluated at Year 20 and those who were not.

	Full sample Year 14 visit (n = 711)	Sample with both follow-up time-points (n = 208)	Sample without Year 20 visit measures (n = 503)	p-value
Age, years	85.2 ± 4.3	83.3 ± 3.1	86.0 ± 4.4	<0.001
Died during follow-up, n(%)			280 (55.7)	
White race, n (%)	632 (88.9)	184 (88.5)	448 (89.1)	0.85
Body mass, kg	79.0 ± 12.2	79.5 ± 10.7	78.7 ± 12.8	0.42
Stature, cm	171.9 ± 6.8	172.9 ± 6.5	171.4 ± 6.9	0.01
BMI, kg/m²	26.7 ± 3.6	26.6 ± 3.2	26.8 ± 3.8	0.52
PASE	118.4 ± 63.6	142.8 ± 65.3	108.4 ± 60.1	<0.001
Number of comorbidities, n (%)				0.03
0	100 (14.1)	37 (17.8)	63 (12.5)	
1–2	472 (66.4)	141 (67.8)	331 (65.8)	
3 or more	139 (19.5)	30 (14.4)	109 (21.7)	
D_3Cr muscle mass, kg	23.9 ± 4.3	25.7 ± 4.0	23.2 ± 4.2	<0.001
D_3Cr muscle mass/wt, %	31.9 ± 4.9	32.0 ± 5.0	30.1 ± 4.0	0.22
Grip strength, kg	34.8 ± 8.8	38.4 ± 6.8	33.2 ± 9.1	<0.001
6m Walk speed, m/s	1.1 ± 0.3	1.2 ± 0.2	1.0 ± 0.3	<0.001
Dietary protein [a], g/d	61.6 ± 24.5	59.1 ± 22.3	62.7 ± 25.4	0.15

Note. PASE, Physical Activity Scale for the Elderly[22]; D_3Cr, D_3-creatine dilution

[a]Full sample Year 14 n = 425; Sample with both follow-up time-points n = 132; Sample without Year 20 n = 293
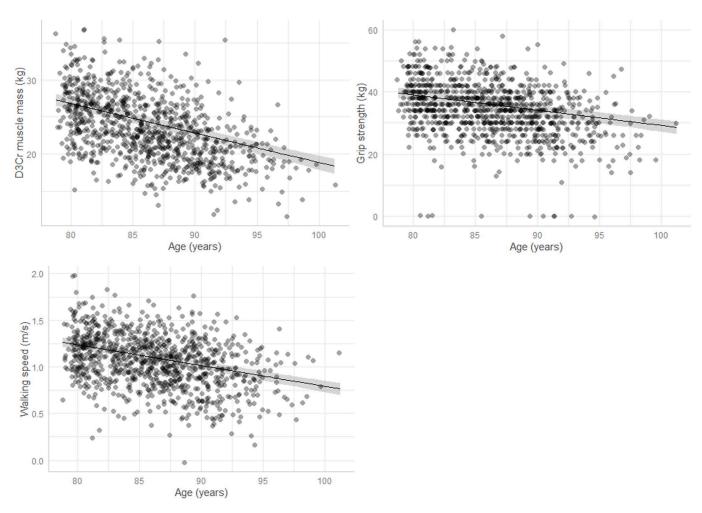
Fig 1. Relationship between increasing age and changes in a) D3Cr muscle mass, b) grip strength, and c) walking speed.

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Table 2. Estimated change in D_3Cr muscle mass, grip strength, and walking speed between follow-up Year 14 and Year 20 visits (Average Follow-Up, 6.1 yrs) for oldest old men [Mean (SD)].

	Absolute change	Percent change	Annualized change	Annualized percent change
D_3Cr muscle mass (kg)				
Unadjusted[a]	−3.25 (0.31)	−13.19 (1.57)	−0.53 (0.00)	−2.17 (0.16)
Adjusted[b]	−3.18 (1.02)	−12.86 (4.07)	−0.52 (0.16)	−2.12 (0.66)
Grip strength (kg)				
Unadjusted[a]	−4.74 (0.45)	−13.23 (1.58)	−0.78 (0.00)	−2.18 (0.16)
Adjusted[b]	−4.81 (1.96)	−13.21 (5.20)	−0.79 (0.32)	−2.17 (0.85)
Walking speed (m/s)				
Unadjusted[a]	−0.17 (0.02)	−15.25 (1.93)	−0.03 (0.00)	−2.51 (0.22)
Adjusted[b]	−0.18 (0.07)	−15.70 (5.83)	−0.03 (0.01)	−2.59 (0.97)

Note. D_3Cr, D_3-creatine dilution.

[a]Change estimated using linear mixed effects models.

[b]Adjusted for body mass, stature, physical activity, comorbidities, and clinical site

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estimated change in D_3Cr muscle mass, grip strength, and walking speed between follow-up Year 14 and Year 20 visits derived from the linear mixed effects models. The greatest percent changes were seen for walking speed (−15%), followed by similar declines in grip strength and D_3Cr muscle mass, which declined by a similar magnitude (−13%) over the 6-year timeframe. Estimated changes were similar after further adjusting for dietary protein intake (S1 Table).

Associations between longitudinal changes in D_3Cr muscle mass and changes in grip strength and walking speed are reported in Table 3. Each additional kg of within-person change in D_3Cr muscle mass was associated with a 0.44 kg change in grip strength and a 0.01 m/s change in walking speed independent of changes in age and additional confounders (i.e., body mass, stature, physical activity, comorbidities, and clinical site). Hence, a man with an average baseline grip strength and walking speed who lost 5 kg D_3Cr muscle mass over 6 years would be expected to have a grip strength of 32.6 kg and walking speed of 1.01 m/s, whereas a man who lost 1 kg D_3Cr muscle mass with a similar Year 14 D_3Cr muscle mass, age and other characteristics (i.e., body mass, stature, physical activity level, and medical conditions) would be expected to have a grip strength of 34.3 kg and walking speed of 1.06 m/s. Year 14 between-person differences in D_3Cr muscle mass were related to changes in both grip strength and walking speed ($p < 0.05$). Further adjustment for dietary protein intake did not alter associations (S2 Table).

Given the observed relationship between age and D_3Cr muscle mass, grip strength, and walking speed and the relationship of D_3Cr muscle mass with grip strength and walking speed, we further assessed if the age relationship with grip strength and walking speed was mediated by age-induced changes on D_3Cr muscle mass. Results of the mediation analysis are presented in Table 4. We observed a significant indirect effect of age on grip strength through D_3Cr muscle mass, such that 42% of the relationship between age and loss of grip strength can be attributed to loss of D_3Cr muscle mass. Similarly, but to a lesser magnitude, D_3Cr muscle mass mediated 22% of the relationship between age and slowing of walking speed. Hence, declines in grip strength and walking speed with age are partially due to the age-related decline of D_3Cr muscle mass. The percentage mediated did not substantially change after adjusting for dietary protein intake, although the value was slightly reduced for walking speed (18.2%) (S3 Table).

When including only men with complete data for both Year 14 and Year 20 visits (n = 208), we observed similar results for the estimated change in D_3Cr muscle mass, grip strength, and

Table 3. Association of longitudinal changes in D_3Cr muscle mass with concurrent changes in grip strength and walking speed.

	Minimally adjusted[a]		Multivariable adjusted[b]	
	β (95% CI)	p-value	β (95% CI)	p-value
Grip Strength (kg)				
Year 14 Visit D_3Cr muscle mass (between-person difference)	0.78 (0.64, 0.92)	<0.0001	0.56 (0.41, 0.71)	<0.0001
6-year change in D_3Cr muscle mass (within-person change)	0.55 (0.39, 0.71)	<0.0001	0.44 (0.28, 0.60)	<0.0001
Walking speed (m/s)				
Year 14 Visit D_3Cr muscle mass (between-person difference)	0.011 (0.007, 0.016)	<0.0001	0.013 (0.008, 0.017)	<0.0001
6-year change in D_3Cr muscle mass (within-person change)	0.013 (0.007, 0.019)	<0.0001	0.011 (0.006, 0.017)	<0.0001

Note. D_3Cr, D_3-creatine dilution.

[a]Adjusted for age.

[b]Adjusted for age, body mass, stature, physical activity, comorbidities, and clinical site

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Table 4. Mediation of D_3Cr muscle mass on age and grip strength and walking speed relationship.

	Total effect of Age on outcome (Path 1, β = *c*)		Effect of Age on D_3Cr muscle mass (Path 2, β = [a])		Unique effect of D_3Cr muscle mass on outcome (Path 3, β = *b*)		Direct effect of Age on outcome (Path 3, β = *c'*)		Indirect effect (β = a * b)		Proportion Mediated (%)
	β (95% CI)	p−value	β (95% CI)	p−value	β (95% CI)	p−value	β (95% CI)	p−value	β (95% CI)	p−value	
Grip strength, kg	−0.49 (−0.59, −0.38)	<0.0001	−0.40 (−0.45, −0.35)	<0.0001	0.51 (0.38, 0.64)	<0.0001	−0.28 (−0.40, −0.17)	<0.0001	−0.20 (−0.26, −0.15)	<0.0001	41.3 (29.0, 58.0)
Walking speed, m/s	−0.022 (−0.025, −0.020)	<0.0001	−0.40 (−0.45, −0.35)	<0.0001	0.012 (0.008, 0.016)	<0.0001	−0.017 (−0.021, −0.014)	<0.0001	−0.005 (−0.007, −0.000)	<0.0001	22.4 (13.9, 31.0)

Note. D_3Cr, D_3−creatine dilution

[a]All models adjusted for body mass, stature, physical activity, comorbidities, and clinical site

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walking speed with and without adjustment for body mass, stature, physical activity, comorbidities, and clinical site (S4 Table). Associations between longitudinal changes in D_3Cr muscle mass and changes in grip strength and walking speed were slightly weaker when removing men with missing data and were no longer significant for walking speed (S5 Table). Results from mediation analyses still showed that the D_3Cr muscle mass mediated the relationship of age with grip strength and walking speed, however the percent mediated was slightly greater for grip strength (45.4%) and less for walking speed (14.5%) (S6 Table).

Discussion

Our findings suggest that loss of SMM as measured using the D_3Cr method is an important contributor to loss of muscle strength and performance in oldest old men. In oldest old men, D_3Cr muscle mass, grip strength, and walking speed continued to decrease with age. Changes in D_3Cr muscle mass were associated with changes in grip strength and walking speed, such that the effect of age on grip strength and walking speed was partially explained by age−related losses in D_3Cr muscle mass.

There has been debate in the field as to the importance of SMM as a key characteristic in defining sarcopenia. While there is no disagreement among experts that SMM is reduced with advancing age, the degree to which this reduction is associated with loss of strength and performance has been questioned. The debate has arisen from observations of a dissociation between loss of surrogate measures of SMM (i.e., LST, muscle cross−sectional area (CSA), and FFM) with loss of strength, with much steeper declines in strength being observed compared to these surrogate measures with advancing age. For instance, annual losses in LST, muscle CSA, and FFM for men ≥70 years have been reported to be 0.80%, 0.98%, and 0.18%, respectively, with even steeper declines of 3−4% reported for muscle strength [30]. Moreover, despite increases in leg LST and CSA in men who gained body mass during 5−years follow−up, these gains did not prevent age−related loss of leg muscle isometric strength [2,31]. Our results, on the other hand, indicated that loses of SMM as assessed with D_3Cr dilution were related to and tracked linearly with losses in strength, with annual loses of ~2% among oldest old men.

The relationship between SMM and performance has also been inconsistent in the literature. In a meta−analysis of cross−sectional studies of older adults, low "SMM" estimated either by DXA (i.e., LST or ALST), bioelectrical impedance (BIA) (i.e., FFM), or computed tomography (CT) CSA of a single muscle group was found to be associated with poor physical performance for only 35% of the functional measures of disability tested [32]. When focusing on solely longitudinal studies, a meta−analysis assessing the relationship of LST, muscle CSA,

and FFM with measures of functional decline (mainly self−reported activities of daily living and/or walking speed) concluded that low SMM was not associated with the loss of physical performance with age, despite the fact that SMM was not in fact measured [10]. Contrarily, we observed that changes in SMM with age were associated with concurrent changes in walking speed in oldest old men, albeit modest in magnitude.

It is likely that part of the inconsistent results across previous studies can be attributed to the method used to estimate SMM. With aging, the intercellular gap between muscle fibers increases due to the infiltration of intramuscular adipose tissue and non−contractile fibrotic tissue [33], and the extracellular water relative to intracellular increases due to decreases in cell volume [34]. Previous studies have mainly relied on DXA, BIA, or CT to estimate SMM, however, none of these methods directly measure SMM [35]. For instance, DXA measures LST and not muscle, with intramuscular fat, connective and fibrotic tissue, water, and organ mass included as part of LST [11]. When comparing DXA and D_3Cr in a sample of older men from the MrOS population at the Year 14 visit (n = 1,376), Orwoll et al. found that D_3Cr muscle mass was only about 50% of LST [36]. Similarly, Cawthon et al. observed only a moderate correlation between D_3Cr muscle mass and DXA LST, with a substantial overestimation of SMM by DXA and no significant relationship between percent SMM and ALST/ht [12]. BIA also does not measure SMM. It measures the impedance of various tissues based on their water and ion concentration to an electrical current and incorporates this information into population−specific prediction equations, which are commonly validated against DXA, to obtain an estimate of FFM or LST [37]. Thus, BIA is highly sensitive to hydration status and electrolyte balance, factors known to vary in older adults, as well as to the equation used to predict FFM or LST [37]. CT usually measures the volume of individual muscle groups rather than total body SMM, which can alter results, given muscle−specific differences in the response to aging [38]. The D_3Cr dilution method measures the total body creatine pool size and, thus skeletal muscle contractile mass [39], the cellular site of muscle force−generating capacity [33]. When using this more accurate way to assess whole body SMM that can capture and account for age related changes occurring in muscle tissue, we found changes in SMM to be an important component of age−related loss of muscle strength and performance independent of changes in physical activity levels and age−associated chronic diseases. Other commonly used proxy measures of SMM (e.g., FFM or LST) were not assessed at the Year 20 follow−up visit, thus, although not comparable by nature as these other measures represent different constructs, we were unable to see how age−related changes in these measures differed from that of D_3Cr muscle mass. Nevertheless, the current findings extend previous cross−sectional findings demonstrating the association of decreased D_3Cr muscle mass with lower muscle strength and walking speed [13,18,36].

Although changes in SMM with age are related to the declines in the force−generating capacity of muscle, cellular and molecular factors at the single muscle fiber level and at the level of the neuromuscular system are also altered with age, and contribute to the loss of muscle strength and performance independent of changes in muscle size/mass. Such factors include weaker excitation−contraction coupling due to decreased calcium sensitivity and release, mitochondrial dysfunction and diminished respiratory capacity, lower myosin concentrations resulting in reduced force generation, decrease in the proportion of Type II muscle fibers, declines in motor unit recruitment, and reductions in corticospinal excitability [40−42]. Despite all these SMM independent factors, we still found that ~ 41% of the relationship between increasing age with changes in grip strength was mediated by age−related declines in D_3Cr muscle mass. We also observed that age−related declines in D_3Cr muscle mass were partially responsible for the slowing of walking speed with age, although to a slightly lesser degree than muscle strength (~22%). This is likely due to walking being a

more complex action affected by additional SMM independent factors, such as age−induced changes in sensorimotor and cognitive processes involved in balance, attention, and motivation [43]. Habitual walking speed has been found to be strongly associated with maximal aerobic capacity (VO_{2max}) in older men and women [44]. Previous data from the Baltimore Longitudinal Study on Aging demonstrated that a substantial component of the age−related decrease in VO_{2max} resulted from declining SMM [45]. Overall, deciphering the predominant driving mechanisms for the loss of strength and performance with advancing age is challenging, as they are multifactorial in nature. Nevertheless, these results highlight the classic biological concept of "function follows form", with physiological functions being mechanistically linked to their structural/anatomic source [46]. Therefore, SMM should not be overlooked as an important modifiable determinant in the loss of strength and performance in older age.

It is important to note that this study in not without limitations. As with any longitudinal study on aging, there will be survivor bias. For instance, men who survived but did not attend the follow−up visit most likely had greater declines in SMM, grip strength, and walking speed. We did not observe major differences in results when excluding men with missing data from the analyses, although the relationship between changes in D_3Cr muscle mass and changes in walking speed was no longer significant, perhaps reflecting leg power. This study also included only older men, restricting the generalization of our findings to women, which is of importance considering the well−established sex differences in body composition [47]. In addition, the MrOS cohort was composed of relatively healthy men representing a narrow age range (i.e., early 80s to early 90s), thus declines in SMM, strength, and performance may be lower and decline at different rates than that of the general population of that age. Although we did not observe non−linear declines in D_3Cr muscle mass, strength, and performance with age, it is possible that greater rates of decline with increasing age could have been observed if we had a cohort with a larger age range. Lastly, like all body composition assessment techniques D_3Cr dilution relies on underlying assumptions that may not always be met, leading to inherent errors and limitations in its accuracy and applicability [48]. First, a small variable amount of labeled D_3Cr is "spilled" into the urine, and although a correction factor has been incorporated into the estimation of D_3Cr muscle mass, it was validated for ages 20−81 years [22], and thus may not be fully valid for oldest−old adults, like in our study. Second, the concentration of creatine is assumed to be 4.3 g/kg wet weight of muscle, however, this number has been shown to vary between 3.8 to 5.4 g/kg depending on muscle fiber composition, age, and physical activity level, although these large deviations are mostly seen in young males, athletic populations, and certain wasting disease states [48,49]. Diet can also influence creatine concentration; however, dietary creatine intake is minimal and contributes only a small fraction to the total creatine pool, with significant increases in muscle creatine content only occurring with high−dose supplementation (>5g/d) [50]. We did not have data on supplemental creatine use among the MrOS participants; however, it is unlikely that most of the men were consuming high doses of creatine supplements.

Despite limitations, this is one of the few longitudinal studies to assess the relationship of D_3Cr muscle mass with the major sarcopenic outcomes of grip strength and walking speed in an older adult population. Using a subsample from the same MrOS cohort who had repeat measures for both DXA LM and D_3Cr muscle mass, Duchowny et al. observed weak to moderate correlations between changes in D_3Cr muscle mass with changes in grip strength and walking speed, respectively, however these correlations did not reach statistical significance [3]. These results, differed from our significant findings of changes in D_3Cr muscle mass with concurrent changes in grip strength and walking speed. It is likely that differences in results stem from lack of adequate power to detect significant associations (i.e., small sample size of n = 40) and short follow−up time (1.6 years) in the Duchowny et al. pilot study [3]. Moreover,

the analytical approach of using simple correlations between change in D_3Cr muscle mass and change in the outcomes does not consider the great variability between individuals over time. We were able to account for this with our use of mixed effects regression (an approach not possible in smaller samples), which allows for a random intercept. The random intercept accounts for the fact that each individual may start with a different baseline level of D_3Cr muscle mass, strength, and/or function. This allows the model to account for these baseline differences when assessing the relationship between changes in D_3Cr muscle mass and changes in strength/function over time. Nevertheless, future longitudinal studies that include women and with a large age range, as well as important confounders (e.g., objective PA assessment, supplementary Cr use) are needed to confirm our findings.

Conclusion

Men in the last decades of life experience previously unreported losses in SMM, strength, and habitual walking speed with similar annual losses of ~ 2% for D_3Cr muscle mass and grip strength and ~ 3% for walking speed. Our findings suggest that SMM itself plays a more important role in the loss of strength and performance with age than has been previously considered. Future studies should focus on longitudinal associations of D_3Cr muscle mass with not only strength and physical performance, but also functional limitations and disability, which could provide more insight into the clinical utility of D_3Cr muscle mass as a diagnostic criterion for sarcopenia.

Supporting information

S1 Fig. Flow diagram of study sample.
(TIFF)

S2 Fig. Mediation path diagram.
(PDF)

S1 Table. Estimated change in D_3Cr muscle mass, grip strength, and walking speed between follow−up Year 14 and Year 20 visits (Average Follow−Up, 6.1 yrs) for oldest old men after adjusting for dietary protein intake (g/d) (n = 434) [Mean (SD].
(DOCX)

S2 Table. Association of longitudinal changes in D_3Cr muscle mass with concurrent changes in grip strength and walking speed after adjusting for dietary protein intake (n = 434).
(DOCX)

S3 Table. Mediation of D_3Cr muscle mass on age and grip strength and walking speed relationship adjusted for dietary protein intake
(DOCX)

S4 Table. Estimated change in D_3Cr muscle mass, grip strength, and walking speed between follow−up Year 14 and Year 20 visits (Average Follow−Up, 6.1 yrs) for oldest old men with complete measures for both time points (n = 208) [Mean (SD]
(DOCX)

S5 Table. Association of longitudinal changes in D_3Cr muscle mass with concurrent changes in grip strength and walking speed in men with complete measures for the Year 14 and Year 20 visits (n = 208).
(DOCX)

S6 Table. Mediation of D3Cr muscle mass on age and grip strength and walking speed relationship in men with complete measures for the Year 14 and Year 20 visits (n = 208) (DOCX)

S1 STROBE checklist. Key reporting items for observational studies. (DOCX)

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References

1. Evans W. What is sarcopenia?. J Gerontol. 1995;50(A):5–8.

2. Goodpaster BH, Park SW, Harris TB, Kritchevsky SB, Nevitt M, Schwartz AV, et al. The loss of skeletal muscle strength, mass, and quality in older adults: the health, aging and body composition study. J Gerontol A Biol Sci Med Sci. 2006;61(10):1059–64. https://doi.org/10.1093/gerona/61.10.1059 PMID: 17077199

3. Duchowny KA, Peters KE, Cummings SR, Orwoll ES, Hoffman AR, Ensrud KE, et al. Association of change in muscle mass assessed by D3 –creatine dilution with changes in grip strength and walking speed. J Cachexia Sarcopenia Muscle. 2020;11(1):55–61. https://doi.org/10.1002/jcsm.12494 PMID: 31621207

4. Wang X, Miller GD, Messier SP, Nicklas BJ. Knee strength maintained despite loss of lean body mass during weight loss in older obese adults with knee osteoarthritis. J Gerontol A Biol Sci Med Sci. 2007;62(8):866–71. https://doi.org/10.1093/gerona/62.8.866 PMID: 17702878

5. Clark BC, Manini TM. Sarcopenia =/= dynapenia. J Gerontol A Biol Sci Med Sci. 2008;63(8):829–34. https://doi.org/10.1093/gerona/63.8.829 PMID: 18772470

6. Riviati N, Indra B. Relationship between muscle mass and muscle strength with physical performance in older adults: A systematic review. SAGE Open Med. 2023;11:20503121231214650. https://doi.org/10.1177/20503121231214650 PMID: 38033420

7. Bhasin S, Travison TG, Manini TM, Patel S, Pencina KM, Fielding RA, et al. Sarcopenia definition: the position statements of the Sarcopenia definition and outcomes consortium. J Am Geriatr Soc. 2020;68(7):1410–8. https://doi.org/10.1111/jgs.16372 PMID: 32150289

8. Hughes VA, Frontera WR, Wood M, Evans WJ, Dallal GE, Roubenoff R, et al. Longitudinal muscle strength changes in older adults: influence of muscle mass, physical activity, and health. J Gerontol A Biol Sci Med Sci. 2001;56(5):B209–17. https://doi.org/10.1093/gerona/56.5.b209 PMID: 11320101

9. Visser M, Deeg DJ, Lips P, Harris TB, Bouter LM. Skeletal muscle mass and muscle strength in relation to lower–extremity performance in older men and women. J Am Geriatr Soc. 2000;48(4):381–6. https://doi.org/10.1111/j.1532–5415.2000.tb04694.x PMID: 10798463

10. Schaap LA, Koster A, Visser M. Adiposity, muscle mass, and muscle strength in relation to functional decline in older persons. Epidemiol Rev. 2013;35:51–65. https://doi.org/10.1093/epirev/mxs006 PMID: 23221972

11. Evans WJ, Cawthon PM. D3Creatine Dilution as a direct, non–invasive and accurate measurement of muscle mass for aging research. Calcif Tissue Int. 2024;114(1):3–8. https://doi.org/10.1007/s00223–023–01124–w PMID: 37594505

12. Cawthon PM, Orwoll ES, Peters KE, Ensrud KE, Cauley JA, Kado DM, et al. Strong relation between muscle mass determined by d3–creatine dilution, physical performance, and incidence of falls and mobility limitations in a prospective cohort of older men. J Gerontol A Biol Sci Med Sci. 2019;74(6):844–52. https://doi.org/10.1093/gerona/gly129 PMID: 29897420

13. Cawthon PM, Blackwell TL, Kritchevsky SB, Newman AB, Hepple RT, Coen PM, et al. Associations Between D3Cr muscle mass and magnetic resonance thigh muscle volume with strength, power, physical performance, fitness, and limitations in older adults in the SOMMA study. J Gerontol A Biol Sci Med Sci. 2024;79(4):glae056. https://doi.org/10.1093/gerona/glae056 PMID: 38367212

14. Orwoll ES, Blackwell T, Cummings SR, Cauley JA, Lane NE, Hoffman AR, et al. CT Muscle density, D3Cr muscle mass, and body fat associations with physical performance, mobility outcomes, and mortality risk in older men. J Gerontol A Biol Sci Med Sci. 2022;77(4):790–9. https://doi.org/10.1093/gerona/glab266 PMID: 34529767

15. Cawthon PM, Peters KE, Cummings SR, Orwoll ES, Hoffman AR, Ensrud KE, et al. Association between muscle mass determined by D3 –creatine dilution and incident fractures in a prospective cohort study of older men. J Bone Miner Res. 2022;37(7):1213–20. https://doi.org/10.1002/jbmr.4505 PMID: 35253257

16. Zanker J, Patel S, Blackwell T, Duchowny K, Brennan–Olsen S, Cummings SR, et al. Walking speed and muscle mass estimated by the D3–creatine dilution method are important components of sarcopenia associated with incident mobility disability in older men: a classification and regression tree analysis. J Am Med Dir Assoc. 2020;21(12):1997–2002.e1. https://doi.org/10.1016/j.jamda.2020.03.017 PMID: 32381425

17. Cawthon PM, Blackwell T, Cummings SR, Orwoll ES, Duchowny KA, Kado DM, et al. Muscle mass assessed by the D3–creatine dilution method and incident self–reported disability and mortality in a prospective observational study of community–dwelling older men. J Gerontol A Biol Sci Med Sci. 2021;76(1):123–30. https://doi.org/10.1093/gerona/glaa111 PMID: 32442245

18. Zhu K, Wactawski–Wende J, Ochs–Balcom HM, LaMonte MJ, Hovey KM, Evans W, et al. The association of muscle mass measured by D3–creatine dilution method with dual–energy X–Ray absorptiometry and physical function in postmenopausal women. J Gerontol A Biol Sci Med Sci. 2021;76(9):1591–9. https://doi.org/10.1093/gerona/glab020 PMID: 33475725

19. Cegielski J, Brook MS, Phillips BE, Boereboom C, Gates A, Gladman JFR, et al. The Combined Oral Stable Isotope Assessment of Muscle (COSIAM) reveals D–3 creatine derived muscle mass as a standout cross–sectional biomarker of muscle physiology vitality in older age. Geroscience. 2022;44(4):2129–38. https://doi.org/10.1007/s11357–022–00541–3 PMID: 35303223

20. Blank JB, Cawthon PM, Carrion–Petersen ML, Harper L, Johnson JP, Mitson E, et al. Overview of recruitment for the osteoporotic fractures in men study (MrOS). Contemp Clin Trials. 2005;26(5):557–68. https://doi.org/10.1016/j.cct.2005.05.005 PMID: 16085466

21. Orwoll E, Blank JB, Barrett–Connor E, Cauley J, Cummings S, Ensrud K, et al. Design and baseline characteristics of the osteoporotic fractures in men (MrOS) study––a large observational study of the determinants of fracture in older men. Contemp Clin Trials. 2005;26(5):569–85. https://doi.org/10.1016/j.cct.2005.05.006 PMID: 16084776

22. Shankaran M, Czerwieniec G, Fessler C, Wong P–YA, Killion S, Turner SM, et al. Dilution of oral D3 –Creatine to measure creatine pool size and estimate skeletal muscle mass: development of a correction algorithm. J Cachexia Sarcopenia Muscle. 2018;9(3):540–6. https://doi.org/10.1002/jcsm.12278 PMID: 29663711

23. Härkönen R, Harju R, Alaranta H. Accuracy of the Jamar dynamometer. J Hand Ther. 1993;6(4):259–62. https://doi.org/10.1016/s0894–1130(12)80326–7 PMID: 8124439

24. Cawthon PM, Fullman RL, Marshall L, Mackey DC, Fink HA, Cauley JA, et al. Physical performance and risk of hip fractures in older men. J Bone Miner Res. 2008;23(7):1037–44. https://doi.org/10.1359/jbmr.080227 PMID: 18302496

25. Washburn RA, Smith KW, Jette AM, Janney CA. The Physical Activity Scale for the Elderly (PASE): development and evaluation. J Clin Epidemiol. 1993;46(2):153–62. https://doi.org/10.1016/0895–4356(93)90053–4 PMID: 8437031

26. Boucher B, Cotterchio M, Kreiger N, Nadalin V, Block T, Block G. Validity and reliability of the Block98 food–frequency questionnaire in a sample of Canadian women. Public Health Nutr. 2006;9(1):84–93. https://doi.org/10.1079/phn2005763 PMID: 16480538

27. Kenward MG, Roger JH. An improved approximation to the precision of fixed effects from restricted maximum likelihood. Comput Stat Data Anal. 2009;53(7):2583–95. https://doi.org/10.1016/j.csda.2008.12.013

28. Bates D, Mächler M, Bolker B, Walker S. Fitting linear mixed–effects models using lme4. J Stat Soft. 2015;67(1). https://doi.org/10.18637/jss.v067.i01

29. Tingley D, Yamamoto T, Hirose K, Keele L, Imai K. mediation: R Package for causal mediation analysis. J Stat Soft. 2014;59(5). https://doi.org/10.18637/jss.v059.i05

30. Mitchell WK, Williams J, Atherton P, Larvin M, Lund J, Narici M. Sarcopenia, dynapenia, and the impact of advancing age on human skeletal muscle size and strength; a quantitative review. Front Physiol. 2012;3:260. https://doi.org/10.3389/fphys.2012.00260 PMID: 22934016

31. Delmonico MJ, Harris TB, Visser M, Park SW, Conroy MB, Velasquez–Mieyer P, et al. Longitudinal study of muscle strength, quality, and adipose tissue infiltration. Am J Clin Nutr. 2009;90(6):1579–85. https://doi.org/10.3945/ajcn.2009.28047 PMID: 19864405

32. Manini TM, Clark BC. Dynapenia and aging: an update. J Gerontol A Biol Sci Med Sci. 2012;67(1):28–40. https://doi.org/10.1093/gerona/glr010 PMID: 21444359

33. Grosicki GJ, Zepeda CS, Sundberg CW. Single muscle fibre contractile function with ageing. J Physiol. 2022;600(23):5005–26. https://doi.org/10.1113/JP282298 PMID: 36268622

34. Boonyarom O, Inui K. Atrophy and hypertrophy of skeletal muscles: structural and functional aspects. Acta Physiol (Oxf). 2006;188(2):77–89. https://doi.org/10.1111/j.1748–1716.2006.01613.x PMID: 16948795

35. Yamada Y. Muscle mass, quality, and composition changes during atrophy and Sarcopenia. Adv Exp Med Biol. 2018;1088:47–72. https://doi.org/10.1007/978–981–13–1435–3_3 PMID: 30390247

36. Orwoll ES, Peters KE, Hellerstein M, Cummings SR, Evans WJ, Cawthon PM. The importance of muscle versus fat mass in sarcopenic obesity: a re–evaluation using D3–creatine muscle mass versus DXA lean mass measurements. J Gerontol A Biol Sci Med Sci. 2020;75(7):1362–8. https://doi.org/10.1093/gerona/glaa064 PMID: 32436565

37. Sergi G, De Rui M, Stubbs B, Veronese N, Manzato E. Measurement of lean body mass using bioelectrical impedance analysis: a consideration of the pros and cons. Aging Clin Exp Res. 2017;29(4):591–7. https://doi.org/10.1007/s40520–016–0622–6 PMID: 27568020

38. Siparsky PN, Kirkendall DT, Garrett WE Jr. Muscle changes in aging: understanding sarcopenia. Sports Health. 2014;6(1):36–40. https://doi.org/10.1177/1941738113502296 PMID: 24427440

39. Clark RV, Walker AC, O'Connor–Semmes RL, Leonard MS, Miller RR, Stimpson SA, et al. Total body skeletal muscle mass: estimation by creatine (methyl–d3) dilution in humans. J Appl Physiol (1985). 2014;116(12):1605–13. https://doi.org/10.1152/japplphysiol.00045.2014 PMID: 24764133

40. Tieland M, Trouwborst I, Clark BC. Skeletal muscle performance and ageing. J Cachexia Sarcopenia Muscle. 2018;9(1):3–19. https://doi.org/10.1002/jcsm.12238 PMID: 29151281

41. Miljkovic N, Lim J–Y, Miljkovic I, Frontera WR. Aging of skeletal muscle fibers. Ann Rehabil Med. 2015;39(2):155–62. https://doi.org/10.5535/arm.2015.39.2.155 PMID: 25932410

42. Dowling P, Gargan S, Swandulla D, Ohlendieck K. Fiber–type shifting in Sarcopenia of old age: proteomic profiling of the contractile apparatus of skeletal muscles. Int J Mol Sci. 2023;24(3):2415. https://doi.org/10.3390/ijms24032415 PMID: 36768735

43. Caetano MJD, Menant JC, Schoene D, Pelicioni PHS, Sturnieks DL, Lord SR. Sensorimotor and cognitive predictors of impaired gait adaptability in older people. J Gerontol A Biol Sci Med Sci. 2017;72(9):1257–63. https://doi.org/10.1093/gerona/glw171 PMID: 27573810

44. Fiser WM, Hays NP, Rogers SC, Kajkenova O, Williams AE, Evans CM, et al. Energetics of walking in elderly people: factors related to gait speed. J Gerontol A Biol Sci Med Sci. 2010;65(12):1332–7. https://doi.org/10.1093/gerona/glq137 PMID: 20679072

45. Fleg JL, Lakatta EG. Role of muscle loss in the age–associated reduction in VO2 max. J Appl Physiol (1985). 1988;65(3):1147–51. https://doi.org/10.1152/jappl.1988.65.3.1147 PMID: 3182484

46. Heymsfield SB, Prado CM, Gonzalez MC. Skeletal muscle–focused guideline development: hierarchical model incorporating muscle form, function, and clinical outcomes. Appl Physiol Nutr Metab. 2023;48(10):751–6. https://doi.org/10.1139/apnm–2023–0176 PMID: 37473448

47. Hughes VA, Frontera WR, Roubenoff R, Evans WJ, Singh MAF. Longitudinal changes in body composition in older men and women: role of body weight change and physical activity. Am J Clin Nutr. 2002;76(2):473–81. https://doi.org/10.1093/ajcn/76.2.473 PMID: 12145025

48. McCarthy C, Schoeller D, Brown JC, Gonzalez MC, Varanoske AN, Cataldi D, et al. D3 –creatine dilution for skeletal muscle mass measurement: historical development and current status. J Cachexia Sarcopenia Muscle. 2022;13(6):2595–607. https://doi.org/10.1002/jcsm.13083 PMID: 36059250

49. Sagayama H, Yamada Y, Kondo E, Tanabe Y, Uchizawa A, Shankaran M, et al. Skeletal muscle mass can be estimated by creatine (methyl–d3) dilution and is correlated with fat–free mass in active young males. Eur J Clin Nutr. 2023;77(3):393–9. https://doi.org/10.1038/s41430–022–01237–9 PMID: 36376405

50. Ostojic SM. Assessing dietary creatine intake in population studies: challenges and opportunities. Nutr Rev. 2024:nuae155. https://doi.org/10.1093/nutrit/nuae155 PMID: 39441715