The effect of vitamin D on sarcopenia depends on the level of physical activity in older adults

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Abstract

Objective Sarcopenia in older adults is closely related to vitamin D deficiency and reduced levels of physical activity, but little has been reported on the interaction between physical activity and the positive effects of vitamin D. The purpose of this study was to explore the interactive effect of vitamin D and physical activity on muscle mass and function through animal experiments and population surveys.

Methods Male 4-week-old C57BL/6J mice were fed different purified diets: a vitamin D-deficient diet (with increased calcium and phosphorus to prevent the effects of abnormal mineral levels on muscle) or a 1,25-dihydroxyvitamin D3 (1,25D)-supplemented diet. After 24 weeks on the assigned diets, the mice were immobilized. The level of skeletal muscle atrophy in the mice was determined by grip strength, gastrocnemius (GA) muscle mass and muscle fiber cross-sectional area (CSA); additionally, the protein expression levels of FOXO3a and the E3 ubiquitin ligases MuRF1 and MAFbx were detected. A cross-sectional study included data from 4139 older adults (64.9% women, 67.9 ± 6.7 years) as part of a survey in Shenyang, Northeast China. The associations of serum 25(OH)D3 and physical activity with timed up and go test (TUG) performance, handgrip strength, calf circumference, and body muscle mass were assessed by a linear regression analysis that was adjusted for covariates.

Results In activity-limited mice, vitamin D deficiency accelerated the decrease in GA muscle weight, muscle fiber CSA, and grip strength and increased the protein expression of MuRF1, MAFbx, and FOXO3a (all $P < 0.05$). In addition, 1,25D supplementation may inhibit the grip-strength reduction induced by limited activity ($P = 0.069$). Serum 25(OH)D3 and physical activity were linearly related to TUG time ($P < 0.001$) and handgrip strength ($P < 0.05$) after adjustment for sex, age, body mass index (BMI), education level, smoking status, and serum calcium level. Serum 25(OH)D3 and physical activity had interactive effects on TUG ($P < 0.001$) and handgrip strength ($P < 0.05$) but not calf circumference or body muscle mass in older adults.

Conclusions The effect of vitamin D on muscle strength and physical performance depends on physical activity level in the elderly. It is recommended that older adults strive to avoid both physical inactivity and vitamin D deficiency. Because physical inactivity and vitamin D deficiency may exacerbate muscle atrophy, the biological mechanism may involve synergistic effects of vitamin D and physical activity on the promotion of muscle protein ubiquitination and degradation.

Keywords Sarcopenia; Interactive effect; Vitamin D; Physical activity; MuRF1; MAFbx

Introduction

Sarcopenia refers to the gradual age-related loss of muscle mass, muscle strength, and physical performance, which leads to reduced mobility and an increased risk of falls and is associated with premature death.[1–3] The prevalence of sarcopenia in people over 60 years old is approximately 5–10% in the general population and 14–33% in long-term care

populations.[3–5] The development of sarcopenia is related to many factors, such as aging, inactivity, nutritional deficiencies, metabolic disturbances and increased inflammation.[2,6]

Vitamin D and exercise are considered to be important factors associated with sarcopenia. Surveys have revealed that vitamin D levels are positively correlated with muscle mass, strength, and physical performance in older adults.[7–10] However, the beneficial effects of vitamin D supplementation on muscle mass, muscle strength, and physical performance are still controversial.[11] Other research has shown that vitamin D supplementation has little effect on muscle mass and has a positive effect on muscle strength and physical performance.[12,13] A meta-analysis conducted by Beaudart et al. showed that vitamin D supplementation is most effective for increasing muscle strength in people who have 25(OH)D levels < 30 nmol/L (approximately 12 ng/mL) and who are 65 years of age or older,[12] which suggests that the effect of vitamin D on sarcopenia may be affected by the baseline vitamin D level and by age-related factors. Resistance exercise has been widely accepted to prevent and reverse sarcopenia.[14,15] However, resistance exercise is less efficient in older adults than in young adults.[16–18] A meta-analysis by Antoniak and Greig indicated an additive effect of resistance exercise and vitamin D3 supplementation for the improvement of muscle strength in older adults.[19] Widespread vitamin D deficiency in elderly people may be an influential factor in their blunted responsiveness to resistance exercise.

Animal studies have shown that whole-body vitamin D receptor knockout mice or vitamin D deficiency in mice with or without adjusted mineral levels leads to decreased muscle mass and grip strength.[20,21] Studies have also shown that correction of serum calcium and phosphorus levels in a vitamin D deficiency model can be partially counteracted or reversed by muscle wasting or muscle force.[21,22] A recent study reported that deleting myocyte vitamin D receptor had important effects on muscle size and strength, demonstrating that vitamin D signaling is essential for myocyte function.[23] Immobilization of the hind limbs of mice leads to skeletal muscle atrophy, which is a good model for studying limited limb activity.[24–26] However, there is currently insufficient research on the effect of vitamin D deficiency/supplementation on skeletal muscle mass and function in mice with limited physical activity.

Therefore, considering the controversy regarding the effects of vitamin D on skeletal muscle mass and physical performance in population studies and the reduced efficacy of resistance exercise in the elderly, we hypothesize that the effects of vitamin D and physical activity on muscle strength and physical performance may interact with each other. The purpose of this study was to explore the interactive effect of vitamin D and physical activity on muscle mass and function through animal experiments and population surveys.

Materials and methods

Animal experiments

Thirty-two male C57BL/6J mice (3 weeks old) were purchased from Changsheng Biotechnology Co., Ltd. (Liaoning, China). The environmental temperature was set to 25 ± 1°C, and the light/dark cycle was 12/12 h. After 1 week of adaption, the mice were randomly divided into three groups, of which one contained 16 mice and the other two contained 8 mice per group. Each group received a control diet (with a standard quantity of vitamin D), a vitamin D-depleted diet [(VD (−)],[20] or a 1,25D-supplemented diet [VD(+)][27] (Table 1) for 24 weeks. The vitamin D-depleted diet contained no vitamin D, but calcium and phosphorus levels were increased to prevent abnormal mineral levels in association with vitamin D deficiency. All other components of the diets were identical. The feed was customized by Fubei Shiheng Biomedical Co., Ltd. (Shanghai, China). Then, the normal diet group was randomly divided into two groups, namely, the nonimmobilized group (NIM+N) and the immobilized group (IM+N), and the VD(-) and VD(+) groups were immobilized to form the IM+VD(-) and IM+VD(+) groups, respectively. All groups were immobilized for 7 days.

The immobilization method was performed as described previously.[24] Briefly, the left hindlimb was shaved and wrapped in gauze and surgical tape. The hindlimb was introduced into a 1.5 mL microcentrifuge tube while maintaining the foot in a plantar-flexed position to induce maximal gastrocnemius (GA) muscle atrophy. Due to accidental death and loss of fixation, one to two mice per group did not meet the model requirements; ultimately, six to seven mice remained in each group. After measurement of grip strength, the mice were euthanized by cervical dislocation. Ethical approval was obtained from the Animal Ethics Committee of China Medical University.

In vivo measurements in mice

Body weight and food intake were measured weekly in the study animals, and food and water were supplied ad

Table 1 Composition of diets administered in this study

AIN93M based diet	Normal	Vitamin D-depleted diet [VD (−)]	1,25D-supplemented diet [VD (+)]
Vitamin D3, IU/kg	1000	0	1000
Calcium, g/kg	5	10	5
Phosphorus, g/kg	2	4	2
1,25(OH)2D3, μg/kg	0	0	14.3

libitum for the entire duration of the immobilization or before grip strength was tested. The grip strength of the mice was measured before and after immobilization using a grip strength meter (47200, Ugo Basile, Italy). Grip strength was measured after the immobilization cast was removed. In all mice, the four limbs contributed equally to the measurement of grip strength.[24] Each mouse was measured three times at intervals of 30 min or more, and the average of the three measurements was calculated as the grip strength of the mouse.

Serum parameters of mice

Mouse serum 25(OH)D3 and 1,25D was detected using an enzyme-linked immunosorbent assay kit. Serum calcium and phosphorus were detected using the methyl thymol blue method and the phosphomolybdic acid method, respectively. The test kits were purchased from Walan Technology Co., Ltd. (Shanghai, China).

Muscle histology

One GA muscle from each mouse was dissected, placed in optimal cutting temperature compound, rapidly frozen in isopentane cooled in liquid nitrogen, and stored in a −80°C freezer. Frozen cross sections were cut from the midbelly of each muscle at a thickness of 10 mm using a cryostat (Leica, Germany) and placed on a glass slide. Sections were incubated with hematoxylin solution for 2 min, washed in deionized water, and then incubated with eosin solution for 2 min. Sections were then washed in deionized water, dehydrated in ethanol, and then mounted. The sections were observed using an inverted microscope, and images were captured. Photoshop was used to calculate the pixel count of the cross section of the muscle fiber, and this value was converted to an area. Muscle fiber cross-sectional area (CSA) was measured in 100 randomly selected fibers per mouse.

Western blotting

Approximately 40 mg of the GA muscle was placed in radioimmunoprecipitation assay lysis buffer (Beyotime, China) containing 1 mM phenylmethanesulfonylfluoride, and the tissue was homogenized using a grinder (OSE-Y50, Tiangen, China) and centrifuged at 12 000 *g* for 4 min at 4°C to obtain the protein lysate. The protein lysate (30 μg per lane) was subjected to 8–10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene fluoride membrane. After being blocked with 5% bovine serum albumin in Tris-buffered saline/Tween 20 (TBST) buffer (20 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h at room temperature, the membranes were incubated with primary antibodies overnight at 4°C in TBST buffer. The membranes were washed and incubated with a horseradish peroxidase-conjugated rabbit or mouse secondary antibody for 1 h in a 5% bovine serum albumin/TBST solution at room temperature. Then, protein expression levels were evaluated using an enhanced chemiluminescence substrate (34580, Thermo Scientific), and images were captured using a Tanon ChemiDoc MP Imager. Density measurements for the images were quantified using IMAGEJ software and were normalized to the loading control (glyceraldehyde 3-phosphate dehydrogenase). Primary antibodies against MuRF1 and MAFbx (sc-398608, sc-166806, Santa Cruz, CA, USA) were used at a 1:100 dilution, and other antibodies against FOXO3a (ab12162, Abcam, London, UK), phospho-FoxO3a (Ser253) (ABIN6255131, Zen Bioscience, Chengdu, China), and glyceraldehyde 3-phosphate dehydrogenase (2118, Cell Signaling Technology, MA, USA) and horseradish peroxidase-conjugated rabbit or mouse secondary antibodies (7074, 7076, Cell Signaling Technology, MA, USA) were used at a 1:1000 dilution. Three GA muscles were randomly selected from each group for western blot detection, which was repeated three times, and densitometry analysis was performed.

Study population

This study was conducted as part of a community management research programme on common chronic diseases among older adults in Shenyang, Northeast China in 2016. The data and blood were collected from April to October 2016, avoiding the November–March period of cold temperatures in Shenyang. A total of 6812 community-living older adults were involved in the study. The exclusion criteria were as follows: muscle disease, use of active vitamin D or treatment with non-active vitamin D at a dose of >800 U/d within the previous 6 months, and age under 60 years. In addition, individuals without complete information, such as timed up and go test (TUG) results, calf circumference, handgrip strength, body muscle mass, serum 25(OH)D3, and physical activity frequency, were excluded. Ultimately, a total of 4139 eligible participants were included in the study. This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University (Shenyang, China, ethical approval project identification code: AF-SOP-07-1.0-01). The original data were registered at the China Clinical Trial Center (Registration No. ChiCTR-ERC-17011100). Written informed consent was obtained from each participant.

General data collection

Face-to-face interviews were conducted using standardized questionnaires to collect general information about participants, such as sex, age, education level, history of medication use, history of smoking, use of vitamin D supplements, frequency of physical activity, and forms of physical activity. The categories for frequency of physical activity included almost no activity, one to two times per month, one to two times per week, three to four times per week, and more than five times per week. Forms of physical activity included leisurely walking, brisk walking, jogging, square dancing, and Tai Chi.

In addition, a physical examination was performed to measure each subject's height, weight, calf circumference, handgrip strength, body muscle mass, TUG performance, etc. Participants were asked to wear lightweight clothing and no shoes, and then height and weight were measured to the nearest 0.01 cm and 0.01 kg. Body mass index (BMI) was calculated as body weight divided by height in meters squared (kg/m^2). Calf circumference was measured with a non-elastic tape to the nearest 0.01 cm, and the calf circumference values presented here combine the results of left-foot and right-foot subjects, without consideration for lower body dominance. Body muscle mass was measured using a body fat meter (HBF-701, Omron). Dominant handgrip strength was measured with an electronic handgrip dynamometer (Wanqing, Shanghai, China) to the nearest 0.1 kg. For the TUG test, participants sat in a chair with armrests, stood, walked 3 m with a normal gait, turned 180°, walked back to the chair, and sat down; the time it took to complete the test was recorded with an electronic stopwatch to the nearest 0.01 s.[28]

Serum parameters of individuals

Fasting blood samples were collected in the morning and were subsequently centrifuged; serum samples were stored at −80°C. Serum 25(OH)D3 was determined by liquid chromatography-tandem mass spectrometry. Serum calcium was analyzed on a Roche module analysis system (Cobas602) using standard kits. All laboratory evaluations were performed by trained clinical laboratory technicians in accordance with standard operating procedures in hospital laboratories.

Statistical analysis

Statistical analysis was performed using SPSS v17.0 (Chicago, IL, USA). The participants were divided into four groups according to their serum 25(OH)D3 level: serious vitamin D deficiency (<10 ng/mL), vitamin D deficiency (10–20 ng/mL),

relative vitamin D insufficiency (20–30 ng/mL), and vitamin D sufficiency (≥30 ng/mL). The participants were also divided into three groups according to frequency of physical activity: *active* was defined as performing physical activity more than five times per week, *moderate* was defined as one to two or three to four times per week, and *inactive* was defined as one to two times per month or almost no activity, regardless of the form and duration of activity. For descriptive analysis, continuous variables are expressed as the mean ± standard deviation (SD), and categorical variables are expressed as frequencies (%). Continuous variables were analyzed by one-way analysis of variance (ANOVA) and Student's *t*-test, and categorical variables were analyzed by the χ^2 test. Animal data were analyzed by one-way or two-way ANOVA, and the Bonferroni method was used to compare the two groups. All results in the figures are presented as the mean ± SEM.

The associations of serum 25(OH)D3 and physical activity with TUG performance, handgrip strength, calf circumference, and body muscle mass were analyzed by linear regression analysis using two separate models. We entered TUG, handgrip strength, calf circumference, and body muscle mass as the dependent variables, and we entered serum 25(OH)D3 level and physical activity as independent variables. Two separate models were used for adjustment: Model 1 was adjusted for sex, age, and BMI; Model 2 was adjusted for the variables included in Model 1 + education level, smoking status, and serum calcium level. To detect the interaction of serum 25(OH)D3 with physical activity, we added the interaction terms from all linear regression equations. Statistical significance was indicated by a two-tailed α level of 0.05.

Results

Serum parameters and weight changes of mice

Serum 1,25D levels decreased significantly and serum 25(OH)D3 was not detectable in mice fed a vitamin D-deficient diet ($P < 0.05$), whereas there was no significant change in serum 1,25D or 25(OH)D3 levels in mice fed a 1,25D-supplemented diet. There were no significant differences in serum calcium or phosphorus levels between the three groups of mice, which excluded the effects of serum calcium and phosphorus on the experimental results (Supporting Information, *Table S1*).

The body weights of the mice fed vitamin D-removed diets decreased significantly ($P < 0.05$), whereas food intake per 24 h showed no difference (Supporting Information, *Figure S1*). There was no significant difference in the weight of the right hind limb GA muscle between the immobilized mice and the non-immobilized mice (Supporting Information, *Figure S2B*), indicating that the immobilization process had no significant effect on the right hind limb GA muscle weight. Therefore,

for the GA muscle weight of the immobilized mice, the normal active right hind limb can be compared as a control for the left hind limb with limited activity.[24]

mice ($P < 0.001$); the GA muscle fiber count showed no difference between groups (*Figure* 1C–1E).

Vitamin D deficiency accelerates gastrocnemius muscle atrophy induced by limited activity

Compared with the volume of the right hind limb GA muscle, the left hind limb GA muscle volume was reduced (Supporting Information, *Figure S2A*), and the muscle weight was significantly decreased (*Figure* 1A). Additionally, the vitamin D deficiency group had the largest decrease in GA muscle weight ($P < 0.05$) (*Figure* 1A). The GA muscle fiber CSA was reduced after activity limitation ($P < 0.01$), and the vitamin D deficiency group exhibited a more severe reduction than the control-fed

The effect of vitamin D on the grip strength of mice depends on activity

Regarding mouse grip strength, we found that vitamin D interacts with activity ($P = 0.020$) (*Figure* 1B). There was no significant difference in the grip strength of mice with different vitamin D levels before immobilization; after immobilization, the vitamin D-deficient mice had the greatest decline in grip strength ($P < 0.01$), and the grip strength of the 1,25D-supplemented mice was increased compared with that of the control-diet mice ($P = 0.069$).
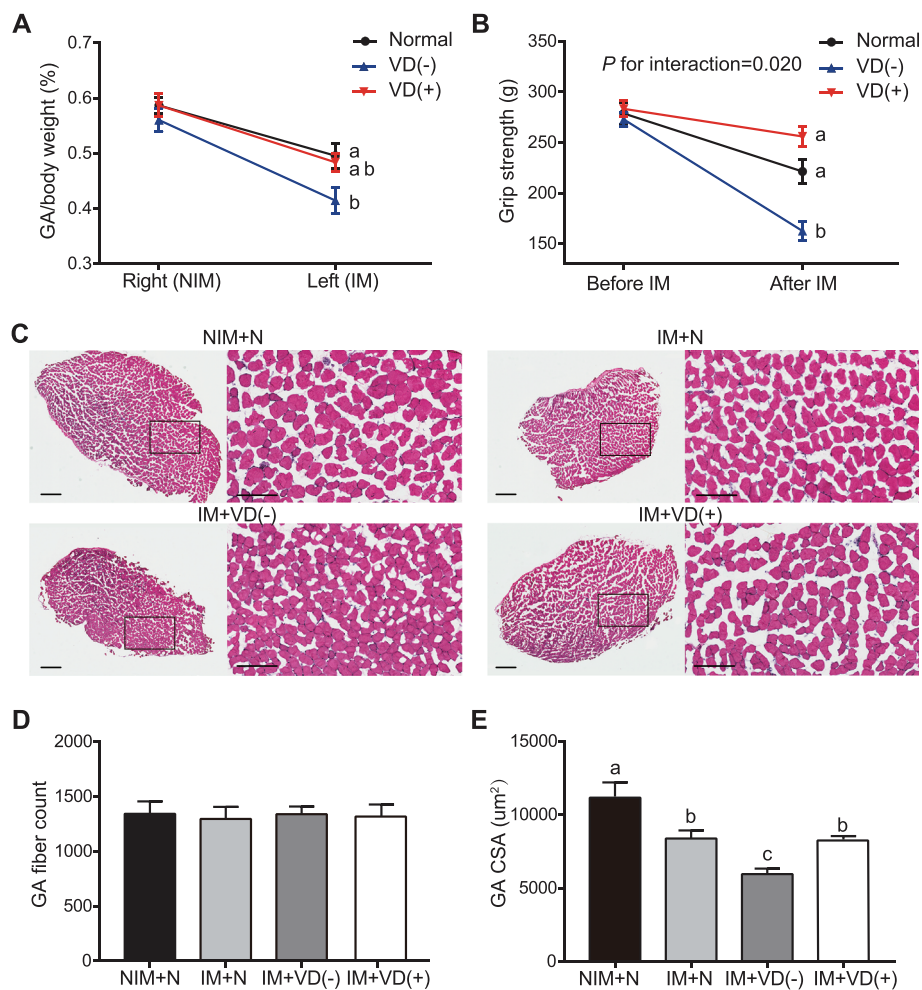

Figure 1 The gastrocnemius morphology and grip strength of mice with inactivity-induced skeletal muscle atrophy in the presence or absence of dietary vitamin D. Weight (A) of the left and right GA muscles after immobilization; grip strength (B) before and after immobilization. (C–E) Representative images of histological cross sections from the lateral head of the GA muscle, with GA fiber counts and fiber CSA statistics. Scale bar, 500 or 200 μm. The results are presented as the mean ± SEM (*n* = 6–7/group). a–c Values with different letters are significantly different ($P < 0.05$; one-way or two-way analysis of variance and Bonferroni test). CSA, cross-sectional area; GA, gastrocnemius.

Vitamin D deficiency increases the protein expression of E3 ubiquitin ligases and FOXO3a in the activity-limited gastrocnemius muscle

The expression levels of the E3 ubiquitin ligases MuRF1 and MAFbx in the activity-limited GA muscle increased significantly ($P < 0.01$) (*Figure* 2A–2C), and the vitamin D-deficient mice had the highest increase ($P < 0.001$). There was no significant difference in the expression of the phospho-FOXO3a protein. The expression of the FOXO3a protein increased significantly in the activity-limited GA muscle ($P < 0.01$), especially in the GA muscles from the vitamin D-deficient mice,

while the expression in the 1,25D-supplemented mice decreased significantly compared with the IM + N mice ($P < 0.05$) (*Figure* 2D–2F).

Cross-sectional study of older adults

The characteristics of the participants are shown in *Table* 2 according to serum 25(OH)D3 level. There were significant differences in the frequency distributions of sex, education level, physical activity, and smoking status (all $P < 0.001$): female individuals who had a low education level, had low
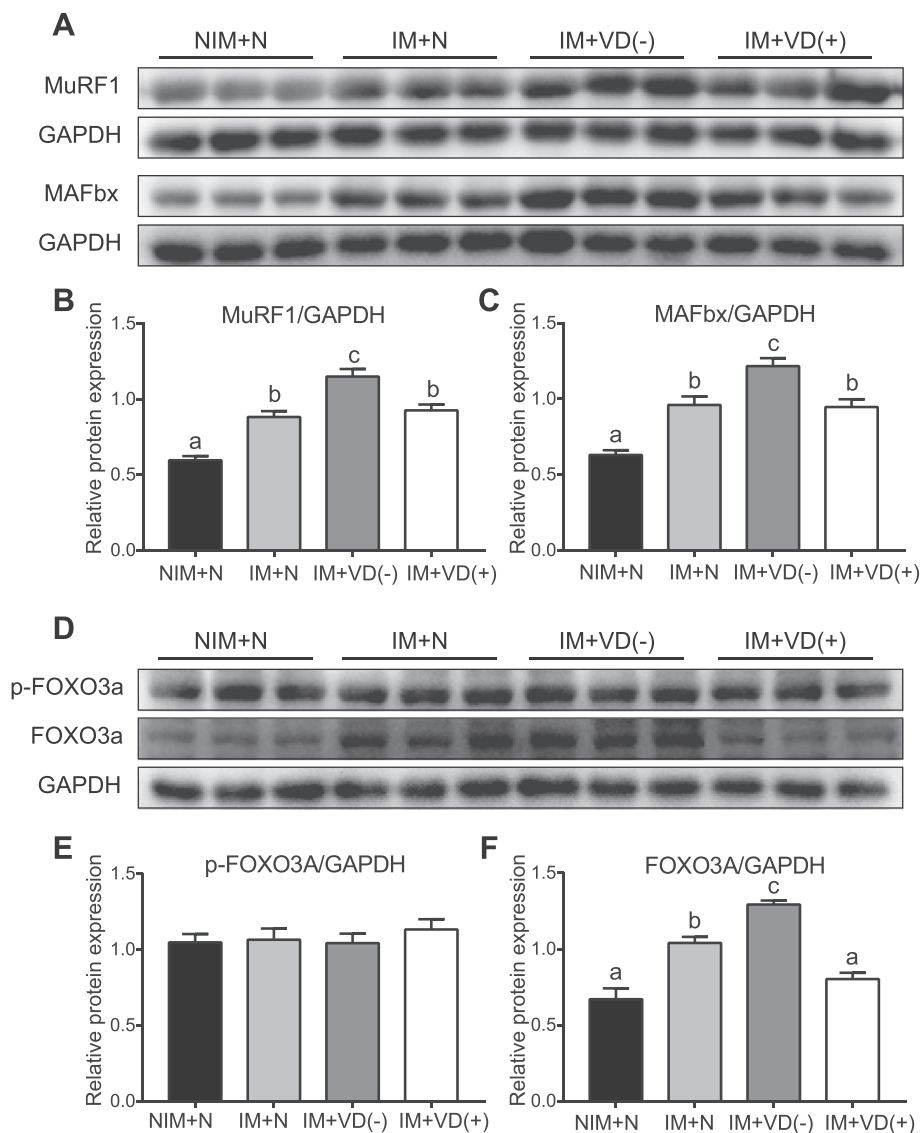
Figure 2 The expression of the FOXO3a-mediated protein degradation pathway in mice with limited activity-induced skeletal muscle atrophy with or without vitamin D in their diet. The protein expression levels of (A–C) MuRF1, MAFbx, (D–F) phospho-FOXO3a, and FOXO3a in gastrocnemius muscle. The results are presented as the mean ± SEM (*n* = 3/group). a–c Values with different letters are significantly different ($P < 0.05$; one-way analysis of variance and Bonferroni test).

Table 2 Descriptive characteristics of the study population stratified by serum 25(OH)D3 level

Characteristic	Serum 25(OH)D3, ng/mL					P for trend
	Total	<10	10–20	20–30	≥30	
N	4139	111	1539	1895	594	
Sex						0.000
Male, n (%)	1451 (35.1)	24 (21.6)	412 (26.8)	707 (37.3)	308 (51.9)	
Female, n (%)	2688 (64.9)	87 (78.4)	11275 (73.2)	1188 (62.7)	286 (48.1)	
Age, years	67.9 ± 6.7	71.3 ± 8.1^a	69.1 ± 7.1^b	67.0 ± 6.2^c	66.8 ± 6.2^c	0.000
Body mass index, kg/m^2	24.82 ± 3.31	24.14 ± 3.47	24.75 ± 3.48	24.97 ± 3.22	24.66 ± 3.09	0.083
Education level						0.005
Illiterate, n (%)	118 (2.9)	12 (10.8)	53 (3.4)	43 (2.3)	10 (1.7)	
Elementary school, n (%)	502 (12.1)	24 (21.6)	215 (14.0)	205 (10.8)	58 (9.8)	
Junior high school, n (%)	1950 (47.1)	39 (35.1)	680 (44.2)	929 (49.0)	302 (50.8)	
Senior high school, n (%)	880 (21.3)	23 (20.7)	321 (20.9)	410 (21.6)	126 (21.2)	
College, n (%)	689 (16.6)	13 (11.7)	270 (17.5)	308 (16.3)	98 (16.5)	
Physical activity						0.000
Inactive, n (%)	919 (22.2)	39 (35.1)	388 (25.2)	391 (20.6)	101 (17.0)	
Moderate, n (%)	296 (7.2)	5 (4.5)	110 (7.1)	143 (7.5)	38 (6.4)	
Active, n (%)	2924 (70.6)	67 (60.4)	1041 (67.6)	1361 (71.8)	455 (76.6)	
Smoking status, n (%)						0.000
Yes, n (%)	556 (13.4)	19 (17.1)	216 (14.0)	248 (13.1)	73 (12.3)	
No, n (%)	3583 (86.6)	92 (82.9)	1323 (86.0)	1647 (86.9)	521 (87.7)	
Timed up and go test, s	9.14 ± 3.29	11.56 ± 7.62^a	9.52 ± 3.99^b	8.83 ± 2.35^c	8.67 ± 2.02^c	0.000
Handgrip strength, kg	22.52 ± 8.80	19.35 ± 7.41^a	20.80 ± 8.07^a	23.16 ± 8.98^b	25.50 ± 9.13^c	0.000
Calf circumference, cm	34.52 ± 3.11	33.81 ± 3.14^a	34.28 ± 3.22^a	34.68 ± 2.99^b	34.75 ± 3.12^b	0.001
Body muscle mass, %	25.24 ± 3.92	24.11 ± 3.48^a	24.70 ± 4.12^a	25.43 ± 3.69^b	26.23 ± 3.88^c	0.000
Serum calcium, mmol/L	2.42 ± 0.09	2.39 ± 0.10^a	2.41 ± 0.08^b	2.42 ± 0.09^b	2.43 ± 0.09^c	0.000

Data are shown as the mean ± standard deviation for continuous variables and n (%) for categorical variables. [a-c] Data in the same row with different superscript letters are significantly different from each other (P < 0.05; one-way analysis of variance and Bonferroni test).

activity, and smoked had lower vitamin D levels. The mean values of age, TUG time, handgrip strength, calf circumference, body muscle mass, and serum calcium were significantly different among the different serum 25(OH)D3 levels (all P < 0.01). The TUG time was 33% higher, handgrip strength was 24% lower, calf circumference was 3% lower, body muscle mass was 8% lower, and serum calcium was 2% lower in participants with serious vitamin D deficiency than in vitamin D-sufficient participants. The changes in grip strength and TUG time were the most substantial.

The characteristics of the participants are shown stratified by physical activity area in *Table* 3. There were significant

Table 3 Descriptive characteristics of the study population stratified by physical activity

Characteristic	Physical activity				P for trend
	Total	Inactive	Moderate	Active	
N	4139	919	296	2924	
Sex					0.002
Male, n (%)	1451 (35.1)	285 (31.0)	99 (33.4)	1067 (36.5)	
Female, n (%)	2688 (64.9)	634 (69.0)	197 (66.6)	1857 (63.5)	
Age, years	67.9 ± 6.7	67.6 ± 7.2	67.3 ± 6.5	68.1 ± 6.6	0.059
BMI, kg/m^2	24.82 ± 3.31	25.12 ± 3.62^a	24.79 ± 3.21^{ab}	24.73 ± 3.21^b	0.002
Education level					0.000
Illiterate, n (%)	118 (2.9)	45 (4.9)	4 (1.4)	69 (2.4)	
Elementary school, n (%)	502 (12.1)	138 (15.0)	34 (11.5)	330 (11.3)	
Junior high school, n (%)	1950 (47.1)	476 (51.8)	123 (41.6)	1351 (46.2)	
Senior high school, n (%)	880 (21.3)	155 (16.9)	73 (24.7)	652 (22.3)	
College, n (%)	689 (16.6)	105 (11.4)	62 (20.9)	522 (17.9)	
Smoking status, n (%)					0.021
Yes, n (%)	556 (13.4)	144 (15.7)	41 (13.9)	371 (12.7)	
No, n (%)	3583 (86.6)	775 (84.3)	255 (86.1)	2553 (87.3)	
Timed up and go test, s	9.14 ± 3.29	9.88 ± 4.63^a	8.93 ± 2.42^b	8.92 ± 2.80^b	0.000
Handgrip strength, kg	22.52 ± 8.80	21.27 ± 8.82^a	22.63 ± 8.64^{ab}	22.90 ± 8.77^b	0.000
Calf circumference, cm	34.52 ± 3.13	34.46 ± 3.10	34.59 ± 2.90	34.53 ± 3.13	0.529
Body muscle mass, %	25.24 ± 3.92	24.81 ± 3.78 ^{a}	25.14 ± 3.31^{ab}	25.38 ± 4.00 ^{b}	0.000
Serum 25(OH)D3, ng/mL	22.57 ± 7.61	21.24 ± 7.45^a	22.79 ± 7.92^b	22.96 ± 7.59^b	0.000
Serum calcium, mmol/L	2.42 ± 0.09	2.41 ± 0.09^a	2.42 ± 0.10^{ab}	2.42 ± 0.09^b	0.013

Data are shown as the mean ± standard deviation for continuous variables and n (%) for categorical variables. [a,b] Data in the same row with different superscript letters are significantly different from each other (P < 0.05; one-way analysis of variance and Bonferroni test).

differences in the frequency distribution by sex, education level, and smoking status (all $P < 0.05$); female individuals who have low education level and who smoke were associated with a decreased level of activity. The mean values of BMI, TUG time, handgrip strength, muscle mass, serum 25(OH)D3, and serum calcium were significantly different among the different physical activity levels (all $P < 0.05$). Compared with active participants, the inactive participants' BMI was 2% higher, TUG time was 11% higher, handgrip strength was 7% lower, body muscle mass was 2% lower, serum 25(OH)D3 was 7% lower, and serum calcium was less than 1% lower; it can be seen that TUG time, handgrip strength, and serum 25(OH)D3 vary greatly.

The results of the linear regression analysis are shown in *Table* 4. Serum 25(OH)D3 and physical activity were linearly related to TUG time ($P < 0.001$) and handgrip strength ($P < 0.01$) in both Model 1 and Model 2, and serum 25(OH)D3 and physical activity had an interactive effect on TUG ($P < 0.001$) and handgrip strength ($P < 0.05$). Physical activity had a linear relationship with body muscle mass in both models ($P < 0.05$), but it had a linear relationship with calf circumference only in Model 1 ($P < 0.05$). Serum 25(OH)D3 levels were not associated with body muscle mass or calf circumference.

Figure 3 visually shows the interactive effect of serum 25(OH)D3 and physical activity on TUG time and handgrip strength. Linear regression analysis found that sex had no significant effect on TUG but had a significant effect on grip strength (*Table* 4); therefore, grip strength was expressed

hierarchically by sex. As shown in *Figure* 3B and 3D, as the serum vitamin D level decreases, TUG times gradually increase, and the strength gradually decreases. In particular, when the serum 25(OH)D3 level is less than 10 ng/mL, the changes in inactive older adults' TUG times and handgrip strengths are at their most obvious. In other words, in physically inactive older adults, vitamin D deficiency leads to increased TUG time and reduced handgrip strength. The interactive effect of vitamin D and physical activity on female handgrip strength appeared not to be notable.

Discussion

Vitamin D has direct and indirect effects on skeletal muscle.[29,30] To exclude the indirect effects of serum calcium and phosphorus, we appropriately increased the proportion of calcium and phosphorus in the vitamin D-deficient feed. The experimental results showed that there was no significant difference in serum calcium or phosphorus levels between groups, which excluded the possible indirect effects of serum calcium and phosphorus. Vitamin D deficiency accelerated the muscle atrophy induced by immobilization, which was reflected by the decreased GA muscle weight, muscle fiber CSA, and grip strength. 1,25D supplementation may inhibit the reduction in grip strength induced by immobilization; interestingly, vitamin D and activity have an interactive effect on grip strength.

Table 4 Linear regression analysis of timed up and go test, handgrip strength, calf circumference, and body muscle mass in older adults

Characteristic	TUG		HGS		CC		BMM	
	B (SEM)	*P* value	B (SEM)	*P* value	B (SEM)	*P* value	B (SEM)	*P* value
Model 1								
Sex	−0.019 (0.101)	0.851	−12.651 (0.204)	0.000	−1.258 (0.083)	0.000	−4.591 (0.106)	0.000
Age	0.167 (0.007)	0.000	−0.291 (0.015)	0.000	−0.048 (0.006)	0.000	−0.094 (0.008)	0.000
BMI	0.054 (0.014)	0.000	0.177 (0.029)	0.000	0.522 (0.012)	0.000	−0.122 (0.015)	0.000
Serum 25D3	−0.036 (0.006)	0.000	0.038 (0.013)	0.003	0.000 (0.005)	0.984	0.008 (0.007)	0.264
Phy−act	−0.429 (0.057)	0.000	0.488 (0.116)	0.000	0.106 (0.047)	0.024	0.155 (0.060)	0.010
25D3×Phy−act	0.037 (0.008)	0.000	−0.036 (0.015)	0.019	−0.003 (0.006)	0.590	−0.001 (0.008)	0.902
Model 2								
Sex	−0.220 (0.112)	0.050	−12.139 (0.226)	0.000	−1.257 (0.092)	0.000	−4.368 (0.118)	0.000
Age	0.155 (0.008)	0.000	−0.265 (0.015)	0.000	−0.048 (0.006)	0.000	−0.086 (0.008)	0.000
BMI	0.049 (0.014)	0.001	0.197 (0.029)	0.000	0.522 (0.012)	0.000	−0.117 (0.015)	0.000
Education								
Elementary school	−0.534 (0.310)	0.085	1.127 (0.626)	0.072	0.093 (0.256)	0.716	0.191 (0.326)	0.557
Junior high school	−1.193 (0.295)	0.000	2.198 (0.596)	0.000	0.236 (0.243)	0.331	0.684 (0.310)	0.028
Senior high school	−1.251 (0.304)	0.000	2.613 (0.613)	0.000	0.496 (0.250)	0.047	0.711 (0.319)	0.026
College	−1.775 (0.308)	0.000	3.221 (0.622)	0.000	0.617 (0.254)	0.015	1.216 (0.324)	0.000
Smoking status	−0.122 (0.151)	0.418	1.249 (0.305)	0.000	−0.293 (0.125)	0.019	0.298 (0.159)	0.060
Serum calcium	1.070 (0.546)	0.050	2.961 (1.103)	0.007	−0.740 (0.451)	0.101	−1.163 (0.574)	0.043
Serum 25D3	−0.037 (0.006)	0.000	0.034 (0.013)	0.008	0.001 (0.005)	0.849	0.009 (0.007)	0.169
Phy−act	−0.395 (0.057)	0.000	0.429 (0.116)	0.000	0.082 (0.047)	0.083	0.135 (0.060)	0.025
25D3 × Phy-act	0.035 (0.008)	0.000	−0.031 (0.015)	0.038	−0.003 (0.006)	0.682	0.000 (0.008)	0.997

Model 1 was adjusted for sex, age, and body mass index, whereas Model 2 was adjusted for the variables in Model 1 + education level, plasma calcium, and smoking status.
25D3, 25(OH)D3; 25D3×Phy-act, 25(OH)D3×physical activity; BMI, body mass index; BMM, body muscle mass; CC, calf circumference; HGS, handgrip strength; Phy-act, physical activity; TUG, timed up and go test.
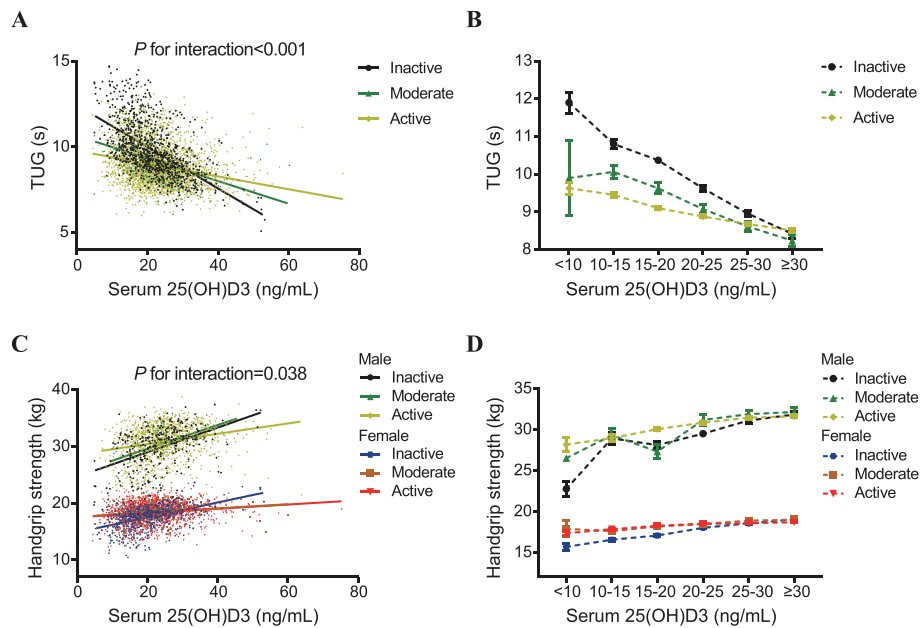
Figure 3 Diagram of the interactive effect of serum 25(OH)D3 level and physical activity on handgrip strength and TUG in older adults. Handgrip strength and TUG time were adjusted by sex (except in Figure C and D), age, body mass index, education level, serum calcium, and smoking status. The results are presented as the mean ± SEM (number of participants per group: inactive 919, male 285, female 634; moderate 296, male 99, female 197; active 2924, male 1067, female 1857). TUG, timed up and go test.

The main method of skeletal muscle protein degradation caused by immobilization is the ubiquitin-proteasome pathway.[31,32] Two important E3 ubiquitin ligases, MuRF1 and MAFbx, are highly expressed in a variety of disuse skeletal muscle atrophy models, and their expression levels are affected by time factors.[32–34] During the process of atrophy, MAFbx catalyzes the protein degradation that promotes protein synthesis and controls protein synthesis by regulating the eukaryotic translation initiation factor 3 subunit F (eIF3f).[35–37] MuRF1 selectively binds to and promotes the ubiquitination of fibrin during the process of muscle atrophy, thereby increasing the degradation of protein by the 26S proteasome.[38–40] In this experiment, vitamin D deficiency further increased the expression of MuRF1 and MAFbx protein in the activity-limited GA muscle, indicating that vitamin D deficiency resulted in more serious protein degradation of the GA muscle during immobilization.

In the skeletal muscle disuse atrophy model, FOXO3a regulates MuRF1 and MAFbx protein expression through transcription.[31,32,41,42] Our study showed that vitamin D deficiency led to increased FOXO3a protein in activity-limited GA muscle, which promoted the expression of MuRF1 and MAFbx proteins. Supplementation with 1,25D reduced the protein expression of FOXO3a, while the expression levels of MuRF1 and MAFbx proteins were not significantly reduced. One possible reason is that the regulatory factors of MuRF1 and MAFbx protein expression comprise not only FOXO3a but also p65/NF-κB and p38/MAPK.[31,32,43] The

decrease in FOXO3a protein expression induced by 1,25D did not cause a significant decrease in MuRF1 or MAFbx protein expression.

Animal experiments show that vitamin D deficiency accelerates GA muscle atrophy induced by limited activity via the FOXO3a-mediated E3 ubiquitin ligase pathway. More importantly, vitamin D and activity have an interactive effect on grip strength. Experimental mouse immobilization results in a model of disuse muscle atrophy, and its molecular mechanism may be different from that of sarcopenia. However, the experimental results still indicate that the role of vitamin D is affected by physical activity.

A typical feature of sarcopenia is the gradual loss of muscle strength. Grip strength is more effective than muscle mass as a predictor of adverse outcomes, such as falls, decreased physical performance, and mortality.[44–47] In their 2018 guidelines, the European Working Group on Sarcopenia in Older People 2 recommends the use of low muscle strength as the main parameter to identify sarcopenia.[48] The fall prevention guide recommends using the TUG for health screening of older adults,[28,49,50] and in the screening for sarcopenia, the TUG can be used as an alternative indicator of physical performance.[48,51] The TUG is used to obtain balance, gait speed, and functional ability information of older adults and is an important indicator to measure physical performance.

Our data analysis of cross-sectional studies in older adults supported the results of the animal experiments. In

elderly individuals over 60 years old, we found that vitamin D and physical activity had an interactive effect on TUG and handgrip strength, meaning that the effects of vitamin D may be affected by the level of physical activity, and the effects of physical activity may also be affected by vitamin D levels. A study by Wicherts et al., which included 1234 participants (51.4% women, 75.3 ± 6.5 years) from the Netherlands, also examined the interaction of physical activity with vitamin D, but the results were negative.[52] In contrast, our data included 4139 participants (64.9% women, 67.9 ± 6.7 years) from Shenyang, Northeast China. The difference in the number and characteristics of participants in the two studies may be the main reason for the different results.

As shown in our results, when the serum 25(OH)D3 level was lower than 10 ng/mL, the TUG was significantly increased, and the grip strength was significantly decreased, especially in inactive older adults. The results of our study may explain why the effects of vitamin D supplementation on the muscle strength and physical function of older adults vary widely, that is, the results of vitamin D supplementation therapy may be affected by differences in baseline physical activity levels and serum 25(OH)D3 levels in elderly populations.

There is little research on older adults receiving a single vitamin D supplement along with an exercise intervention. Uusi-Rasi et al.'s study of 70 to 80-year-old women found that exercise by itself can improve lower limb muscle strength and body function but that vitamin D does not enhance the impact of exercise on physical function.[53] The possible reason for this result was that the baseline level of serum 25(OH)D3 was higher in the participants, and the baseline mean (SD) of the vitamin D group was 25.1 (6.9) ng/mL, which increased to 37.0 (7.4) ng/mL at 24 months. A study conducted by Bunout et al. in older adults showed that vitamin D supplementation improves gait speed and TUG performance and that exercise improves muscle strength (including quadriceps strength and handgrip strength), while vitamin D supplementation has no significant effect on muscle strength.[54] The baseline mean (SD) serum 25(OH)D3 level in the vitamin D supplementation group increased from 12.4 (2.2) ng/mL to 25.8 (6.5) ng/mL at 9 months, with lower baseline levels and supplementation resulting in a high level, which had a significant beneficial effect on physical performance. In addition, although the study included men and women, the proportion of women was nearly 90%. The effect of vitamin D on muscle strength may be affected by sex. As shown in our study, the interactive effect of vitamin D and physical activity on female handgrip strength was not substantial.

The International Clinical Practice Guidelines for Sarcopenia do not recommend vitamin D supplementation in older adults with sarcopenia because the evidence is not sufficient.[55] Our study suggests that older adults should be encouraged to increase their physical activity. It is recommended that older adults with vitamin D deficiency take appropriate amounts of supplemental vitamin D and strive to avoid both physical inactivity and vitamin D deficiency. Because these factors may exacerbate the decline in muscle strength and physical performance, the related biological mechanism may involve a synergistic effect of vitamin D and physical activity in promoting the ubiquitination and degradation of skeletal muscle protein.

Acknowledgements

Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Mouse serum 1,25D, 25(OH)D3, calcium and phosphorus levels.
Figure S1. The body weight and food intake of mice consuming a diet with or without vitamin D. The results are presented as the mean ± SEM (n = 6-7/group). Values with different letters are significantly different ($P < 0.05$; one-way ANOVA and Bonferroni test).
Figure S2. The morphology and weight of the gastrocnemius muscle from the right hind limb of immobilized versus nonimmobilized mice. The results are presented as the mean ± SEM (n = 6-7/group). GA, gastrocnemius.

Conflict of interest

None declared.

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