

# Serum Concentrations of Myostatin and Myostatin-Interacting Proteins Do Not Differ Between Young and Sarcopenic Elderly Men

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Sarcopenia is the loss of muscle size and function during ageing. The aim of this study was to test whether serum concentrations of myostatin and interacting proteins (GASP-1, FLRG, and follistatin) differed between young and elderly sarcopenic men. Isometric knee extensor maximal voluntary contraction and quadriceps cross-sectional area (magnetic resonance imaging measurement) were significantly higher in young ( $22 \pm 2$  years;  $266 \pm 54$  N/m;  $8,686 \pm 1,154$  mm<sup>2</sup>) than in mildly sarcopenic ( $69 \pm 3$  years;  $183 \pm 17$  N/m;  $6,621 \pm 718$  mm<sup>2</sup>) and severely sarcopenic men ( $76 \pm 6$  years;  $127 \pm 23$  N/m;  $5,846 \pm 591$  mm<sup>2</sup>), respectively ( $p \leq .01$  for all comparisons). There was a trend ( $p = .06$ ) toward higher FLRG in young ( $20 \pm 8$  ng/mL) than in mildly ( $15 \pm 6$  ng/mL) and severely sarcopenic men ( $17 \pm 8$  ng/mL). Myostatin, follistatin, GASP-1, tumor necrosis factor  $\alpha$ , and interleukin-6 did not differ significantly. Insulin-like growth factor-1 and free testosterone were both significantly lower in sarcopenic men ( $p < .001$ ). This suggests that altered serum concentrations of myostatin and myostatin-interacting proteins are not contributing to sarcopenia with the possible exception of FLRG.

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“SARCOPENIA” is defined as the loss of muscle mass and function that occurs during normal ageing (1,2). More recently, the term “dynapenia” has been suggested to specifically describe the age-associated loss of muscle strength (3). However, this term is rarely used, and in this article, we solely use the term “sarcopenia” to describe the age-related loss of both muscle size and function. Sarcopenia is often accompanied by fat infiltration or “marbling” of skeletal muscles (2,4). Complications associated with low muscle mass and function include a reduced ability to perform ambulatory tasks (5), old age disability (6), falls (7), poor recovery after hip fracture or major disease (8), and increased mortality (9).

Efforts to research, diagnose, and treat sarcopenia are hampered by the lack of universally accepted guidelines and cutoff points for diagnosing sarcopenia (10). Criteria that have been proposed to identify or diagnose sarcopenia are the ratio of appendicular skeletal muscle mass relative to height (11) or the knee extension strength/torque, handgrip strength, or calf muscle area at least 2 SDs below the mean of a young reference group (12). Recently, a European working group

on sarcopenia has recommended a diagnosis based on measurements of gait speed, grip strength, and muscle mass (13).

Sarcopenia is characterized by many alterations of which several stand out. First, muscle fibres are lost and type 2 fibres atrophy especially after 60 years of age (2,14). Second, muscle displays “anabolic resistance,” which is a decreased protein synthesis response to amino acids (15), insulin (16), and resistance exercise (17). Related to this, insulin also reduces proteolysis less in old muscle than in young (18). Third, satellite cells, as identified by Pax7, neural cell adhesion molecule, and M-cadherin positivity, are  $\approx 2$ -fold reduced in  $\approx 70$ -year-old human vastus lateralis muscle when compared with  $\approx 20$ -year-old controls (19). Old muscle also responds with diminished satellite cell proliferation to muscle injury, resulting in impaired muscle regeneration (19).

Many mechanisms have been proposed to cause the aforementioned sarcopenia alterations (2), but the overall etiology is still incompletely understood. A possible contributing cause is that ageing increases systemic myostatin activity. Myostatin inhibits muscle growth (20–22), and an increased serum concentration of myostatin in mice has

been reported to cause muscle atrophy (22). Moreover, prolonged absence of myostatin reduces sarcopenia in mice, implying that presence of myostatin contributes to sarcopenia in this species (23).

Until recently, it was impossible to test the hypothesis that serum myostatin was increased in humans with sarcopenia because serum myostatin levels could not be measured reliably (24). A well-validated human myostatin assay has now been developed (24). Using this assay, it has been reported that serum myostatin was significantly higher by 14% in men with an average age of  $27 \pm 5$  years and 77% lean body mass than in older men with an average age of  $66 \pm 5$  years and 70% lean body mass (24). The receptor-binding activity of myostatin, however, depends additionally on the concentration of myostatin-interacting proteins that can dimerize with myostatin to form a latent inactive complex that cannot bind to its receptor. These proteins include growth/differentiation factor-associated serum protein-1 [GASP-1; (25)], follistatin-related gene [FLRG; (26)], or follistatin (27).

Myostatin and myostatin-interacting proteins are not the only serum factors that have been shown to be associated with muscle size and function. Higher concentrations of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) were associated with 5-year declines in muscle mass and grip strength (28), whereas interleukin-6 (IL-6) concentrations predicted sarcopenia in women (29). Serum and muscle-specific insulin-like growth factor-1 (IGF-1) splice variants have been proposed to contribute to sarcopenia (30). IGF-1 concentrations are lower in elderly participants than in young (31,32), and in the Framingham heart study, higher IGF-1 concentrations were associated with a smaller loss of fat-free mass women (29). Serum testosterone is often decreased in elderly men (33), and because testosterone increases muscle protein synthesis (34), the decline of free testosterone during ageing is a potential cause of sarcopenia.

The main aim of the study was to test the hypothesis that the concentration of serum myostatin is higher and/or that the concentrations of myostatin-interacting factors such as GASP-1, FLRG, and follistatin are lower in older (>65 years) men with mild or severe sarcopenia when compared with young men, respectively. Sarcopenia was defined on the basis of reduced knee extensor torque when compared with young men (12). Quadriceps cross-sectional area (CSA) was measured using magnetic resonance imaging (MRI), isometric knee extensor torque during a maximal voluntary contraction (MVC) using an isokinetic dynamometer, and voluntary activation using the twitch interpolation technique (35). Serum TNF $\alpha$ , IL-6, IGF-1, and free testosterone were additionally measured to allow comparison with previous studies.

## METHODS

### *Participant Characteristics*

This study was approved by the North and South Scotland Research Ethics committees. All the volunteers gave

their written informed consent to take part in the study. A total of sixty-six men were recruited in Aberdeen ( $n = 52$ ) and Edinburgh ( $n = 14$ ). These included 20 young men ( $22 \pm 2$  years, body mass index [BMI]:  $24.0 \pm 2.5$  kg/m<sup>2</sup>) as well as 46 older men with mild sarcopenia ( $n = 20$ ,  $69 \pm 3$  years, BMI:  $25.2 \pm 2.0$  kg/m<sup>2</sup>) and severe sarcopenia ( $n = 26$ ,  $76 \pm 6$  years, BMI:  $24.7 \pm 2.8$  kg/m<sup>2</sup>; BMI did not differ significantly between groups,  $p = .30$ ) (11). Because muscle function measurements are easier and cheaper to perform than MRI measurements, we decided for practical reasons to select sarcopenic participants based on their maximal knee extension torque rather than their mid-thigh quadriceps CSA. Mild sarcopenia was defined as having an MVC between 1 and 2 *SD* below the mean for the young men, while reduction in MVC by 2 *SD* was used as a cutoff point for severe sarcopenia.

### *Exclusion Criteria*

The following exclusion criteria were applied to exclude individuals with conditions that affect muscle mass or the serum factors studied (36): obesity (BMI > 30 kg/m<sup>2</sup>) or being underweight (BMI < 18 kg/m<sup>2</sup>); history of myocardial infarction within the previous 2 years; cardiac illness: moderate/severe aortic stenosis, acute pericarditis, acute myocarditis, aneurysm, severe angina, clinically significant valvular disease, uncontrolled dysrhythmia, and claudication within the previous 10 years; major systemic disease within the last 2 years (cancer and rheumatoid arthritis); diabetes; uncontrolled metabolic disease (eg, thyroid disease); thrombophlebitis or pulmonary embolus within the previous 2 years; history of cerebrovascular disease; severe airflow obstruction; acute febrile illness within the previous 3 months; significant emotional distress, psychotic illness, or depression within the previous 2 years; and lower limb arthritis, classified by inability to perform maximal contractions of lower limbs without severe pain.

### *Prestudy Instructions and Blood Sampling*

Volunteers were asked not to exercise the day before and arrived in the laboratory in the morning after an overnight fast to control for exercise and nutrition. A BD Vacutainer 21 G multisample blood collection needle was used to obtain 15 mL of venous blood. After that, volunteers received a light breakfast and took part in assessment of muscle strength and size.

### *Measurement of Isometric Muscle Strength*

The dominant leg of volunteers was tested. MVC was measured as the isometric knee extensor torque at 90°. The measurement was performed using a Biodex isokinetic dynamometer (System 2; Biodex Medical Systems, Shirley, NY) similarly to our previous study (37). The volunteers sat upright, and MVC was determined as the best of three

Table 1. Experimental Detail for the Elisa Assays

Elisa Assay	Supplier	Catalog Number	Serum Dilution	Intra-Assay Precision (% CV)	Inter-Assay Precision (% CV)	Lower Limit of Quantification
Total myostatin*	n/a	n/a	Neat <sup>†</sup>	2.5–8.5	4.6–8.3	1172 pg/ml
Follistatin	R&D	DFN00	Neat	1.9–3.3	1.5–9.6	0.125 ng/ml
FLRG	R&D	DY1288	01:09	2.1–4.5	10.1–14.8	2.8 ng/ml
GASP-1	R&D	DY2070	01:15	2.4–4.4	2.9–15.1	468.8 pg/ml
IL-6	R&D	HS600B	Neat	6.7–8.1	14.1–19.4	0.16 ng/ml
TNF $\alpha$	R&D	HSTA00D	Neat	3.0–13.0	4.9–8.0	1.0 pg/ml
IGF-I	R&D	DG100	Neat <sup>‡</sup>	3.3–4.0	2.7–3.9	9.4 ng/ml
Free testosterone	DRG Europe	EIA-2924	Neat	6.8–15.3	10.7–12.5	0.2 pg/ml

Notes: CV = coefficient of variation; FLRG = follistatin-related gene; GASP-1 = GDF-associated serum protein-1; IGF-I = insulin-like growth factor-1; IL-6 = interleukin-6; n/a = not applicable; TNF $\alpha$  = tumor necrosis factor  $\alpha$ .

\* In-house (Pfizer) antibodies were used to detect myostatin. These were a capture (RK35) and detector (RK22) antibody.

<sup>†</sup> Neat samples underwent pretreatment prior to assay, resulting in a 1:13.3 dilution for the assay.

<sup>‡</sup> Neat samples underwent two pretreatment steps, resulting in a 1:100 dilution for the assay.

isometric knee extensions lasting approximately 5 seconds each; 2 minutes of rest was allowed between the attempts.

#### Measurement of Voluntary Activation

The twitch interpolation technique was used to estimate voluntary activation of knee extensors using similar methods as in our previous study (37). A constant current electrical stimulator (DS7A; Digitimer, Hertfordshire, UK) was used to apply a square-wave pulse of 0.5 ms in duration to the quadriceps muscle via two surface electrodes (6 × 12 cm). The intensity of electrical stimulation was selected individually by applying single stimuli to the tested muscles. During this procedure, the current was increased until no increment in single twitch torque could be detected by an additional 10% increase in current strength. Three 5-second MVCs were performed with a 2-minute rest in-between. At ~3 seconds of an MVC effort, an electrical pulse was superimposed on the voluntary contraction. The same electrical stimulus was repeated 1–2 seconds after the MVC. These single twitches were used to calculate voluntary activation of knee extensors using the following formula: voluntary activation index (%) = 100 – superimposed twitch torque/control twitch torque × 100%.

#### MRI Measurement of the Quadriceps Mid-Thigh CSA

Mid thigh, that is, the midpoint between trochanterion and tibiale laterale, was marked using a cod liver oil capsule, which is easy to detect on an MRI scan. Sequential MRI images were obtained using a Phillips 3 Tesla scanner. Dual-mode 80 mT/m gradient and parallel imaging systems were used to achieve high-resolution images. T1-weighted cross-sectional images of right and left thigh were obtained at a slice thickness of 3 mm with 12 mm interslice distance. The sum of the largest CSA of vastus lateralis, vastus medialis, vastus intermedius, and rectus femoris for the dominant leg was measured by two independent observers and was used for analysis.

#### Immunoassays

The venous blood samples were centrifuged to obtain serum, transported on dry ice, and stored at –80°C until further analysis. Enzyme-linked immunosorbent assays for the following analytes were performed on serum samples from all participants: total myostatin, follistatin, FLRG, GASP-1, IGF-I, TNF $\alpha$ , IL-6, and free testosterone (Table 1). The validation of the total myostatin assay has previously been published (24). The assays were validated for use in serum using either the manufacturer's or the published protocols with the following three exceptions: (a) For follistatin, an extra low calibrator point of 0.125 ng/mL was added; (b) for FLRG and GASP-1, Immunoglobulin-Inhibiting Reagent (Sera Laboratories International, West Sussex, UK) was routinely added to the standard and samples; (c) for myostatin, the calibrator matrix was 5% bovine serum albumin/phosphate-buffered saline rather than the serum-based calibrator used in the earlier study (24) as we found that the Belgian blue calf serum had high batch to batch variability.

For each of the assays, three validation samples corresponding to low, medium, and high analyte concentrations were used to determine assay precision. Intra- and inter-assay coefficient of variations for each assay were determined in six separate aliquots of low, medium, and high validation samples in five independent analytical runs (Table 1). Subsequently, these samples served as quality controls for each analyte on each assay plate.

#### Statistical Analysis

All data are given as mean  $\pm$  standard deviation. We tested the hypothesis that variables differed between the young, mildly sarcopenic, and severely sarcopenic groups by using a one-factorial analysis of variance. Homogeneity of variance was tested by Levene's test. If variance did not differ significantly, then a least significant difference test was used post hoc. If variances differed significantly then Dunnett's test was used post hoc as this test does not require equal variances. To correlate serum variables to muscle size and other

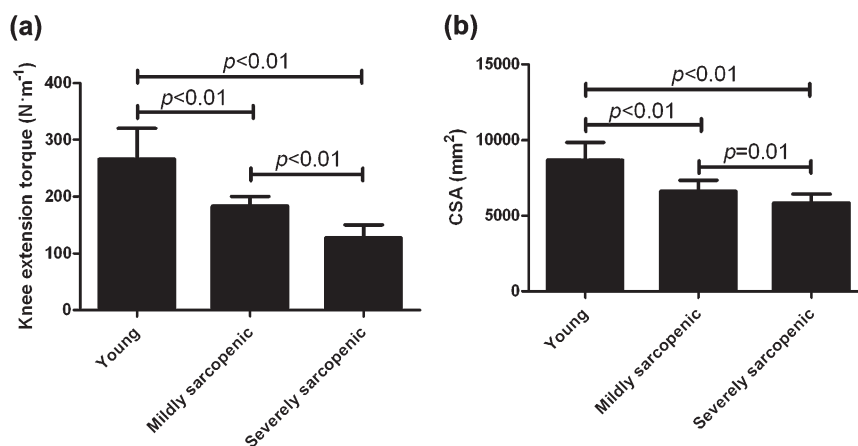


Figure 1 (a) Maximum voluntary contraction (MVC) measured as isometric knee extension torque and (b) quadriceps cross-sectional area (CSA) for the dominant leg in young mildly sarcopenic and severely sarcopenic men (mean  $\pm$  SD). For CSA, only 24 of 26 severely sarcopenic participants could be analyzed.

functional variables, Pearson's product-moment correlation coefficient was calculated for the pooled sarcopenic men. All statistical tests were computed using SPSS 17.0.

## RESULTS

### Muscle Function and Size

Data on muscle function and size are shown in Figure 1. For the mildly and severely sarcopenic groups, quadriceps CSA was reduced by 24% and 33%, while MVC was lowered by 31% and 52% when compared with young men, respectively (Figure 1). Voluntary muscle activation did not differ significantly between groups ( $p = .59$ ). It was  $94.9\% \pm 3.9\%$  in the young ( $n = 18$ ),  $95.3\% \pm 3.8\%$  ( $n = 21$ ) in the mildly sarcopenic, and  $93.4 \pm 7.0\%$  ( $n = 11$ ) in the severely sarcopenic men. Reduced participant numbers are because not all men volunteered to take part in the assessment of voluntary muscle activation. For the pooled sarcopenic men, the correlation between MVC and quadriceps CSA was  $r = .44$  ( $p = .03$ ), whereas MVC and voluntary activation did not correlate.

### Myostatin and Myostatin Interacting Factors

Serum concentrations of myostatin, follistatin, FLRG, and GASP-1 did not differ between the groups (Figure 2). However, there was a trend for decreased FLRG concentrations in the sarcopenic cohorts ( $p = .06$ ; Figure 2): FLRG was 26% lower in the mildly sarcopenic and 19% lower in the severely sarcopenic group than in the young, respectively. Myostatin did not correlate with either MVC ( $r = .01$ ;  $p = .97$ ) or quadriceps CSA ( $r = .11$ ;  $p = .48$ ), and there was also no correlation between follistatin, FLRG, and GASP-1 and MVC or quadriceps CSA in the pooled sarcopenic men, respectively.

### Other Serum Factors

IL-6 and TNF $\alpha$  did not differ significantly between groups (Figure 3). IGF-1 and free testosterone were both

significantly lower in the mildly and severely sarcopenic groups than in the young (Figure 3). However, IGF-1 did not correlate with isometric knee extension torque nor with quadriceps CSA in the pooled sarcopenic men. In contrast, free testosterone correlated with both isometric knee extension torque ( $r = .40$ ;  $p = .01$ ) and quadriceps CSA ( $r = .36$ ;  $p = .02$ ) in the pooled mildly and severely sarcopenic men.

## DISCUSSION

This is the first report where both serum myostatin and major myostatin-interacting proteins have both been measured in relation to human sarcopenia.

### Muscle Function and Size

MVC was reduced 7% and 19% more than quadriceps CSA in the mildly and severely sarcopenic groups when compared with young, respectively (Figure 1). The observed difference between MVC and CSA was not due to reduced voluntary activation. This suggests that specific muscle strength or muscle quality, defined as the ratio of MVC per CSA, is reduced in the sarcopenic participants compared with young (37,38). The limited correlation of  $r = .44$  ( $p = .03$ ) between MVC and quadriceps CSA and the lack of significant correlation between MVC and voluntary activation suggest that both CSA and muscle quality but not voluntary activation contribute to the variation of MVC seen in sarcopenic participants.

### Myostatin-Related Serum Factors

Earlier studies on serum myostatin in diseases with muscle atrophy have been considered unreliable (39) because of the antibodies and assays used (24). To our knowledge, this is only the second publication where myostatin has been measured in young and old men using a well-validated myostatin assay (24). However, serum myostatin concentrations were 40%–50% lower in our study (Figure 2) than

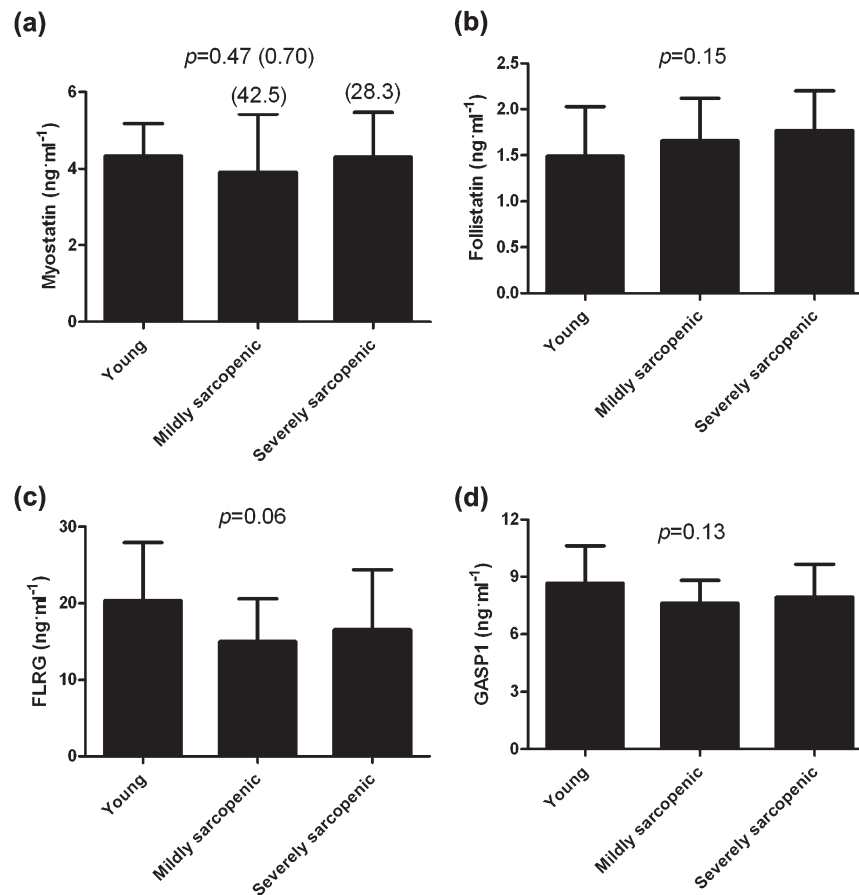


Figure 2 (a) Myostatin and myostatin-interacting proteins (b–d) in the serum of young ( $n = 20$ ), mildly sarcopenic ( $n = 20$ ), and severely sarcopenic ( $n = 26$ ) men (mean  $\pm$  SD). For myostatin, one outlier in the mildly sarcopenic (42.5 ng/mL) and another in the severely sarcopenic (28.3 ng/mL) group has not been included in this figure. For myostatin, an analysis of variance has been performed with the outliers included ( $p$  value without brackets) and excluded ( $p$  value inside brackets). FLRG = follistatin-related gene protein; GASP-1 = GDF-associated serum protein-1.

those measured in the first study (24). Also, myostatin was reported to be higher by 1 ng/mL in the young than in the old men (24), whereas we did not find any difference. The most likely explanation for the on average higher concentrations reported by Lakshman and colleagues is that these investigators used myostatin-null Belgian Blue cattle serum as a calibrator matrix for their myostatin assay. In our hand, the commercially Belgian blue calf serum had a high batch to batch variability, which is why we decided to use 5% bovine serum albumin/phosphate-buffered saline. This is the most likely explanation for the observed systematic difference in serum myostatin concentrations. A possible reason for the significant 1 ng/mL myostatin difference in Lakshman and colleagues (24) is that the BMI of the old men was 2.8 kg/m<sup>2</sup> higher in the old men than in the young men. In our study, the groups were more closely matched with respect to BMI and the largest difference between young and old men was 1.2 kg/m<sup>2</sup>.

To our knowledge, this is the first study where the myostatin and the myostatin-interacting factors GASP-1 (25), FLRG (26), or follistatin (27) have all been measured in human serum (Figure 2). Myostatin, GASP-1, FLRG, and

follistatin did not differ significantly between groups nor did they correlate significantly with MVC or quadriceps CSA in the pooled sarcopenic men. This suggests that at least the serum concentrations of myostatin and myostatin-interacting factors are unlikely to be a contributing cause of sarcopenia. This does not exclude the possibility that local or serum myostatin and myostatin-interacting factors respond differently to exercise, nutrition, or hormones between young and old men. The FLRG findings should be verified in future studies as they were on the borderline of significance ( $p = .06$ ) with a trend toward  $\approx 20\%$  lower FLRG concentrations in the sarcopenic men. Lower FLRG levels would result in a lower concentration of the latent inactive complex (26) and a higher concentration of free myostatin.

#### Cytokines

Neither IL-6 nor TNF $\alpha$  differed significantly between groups. Our data do not support previous studies showing that TNF $\alpha$  was a predictor of the decline of muscle mass and grip strength (28) and that IL-6 was a predictor of sarcopenia albeit only in women (29).

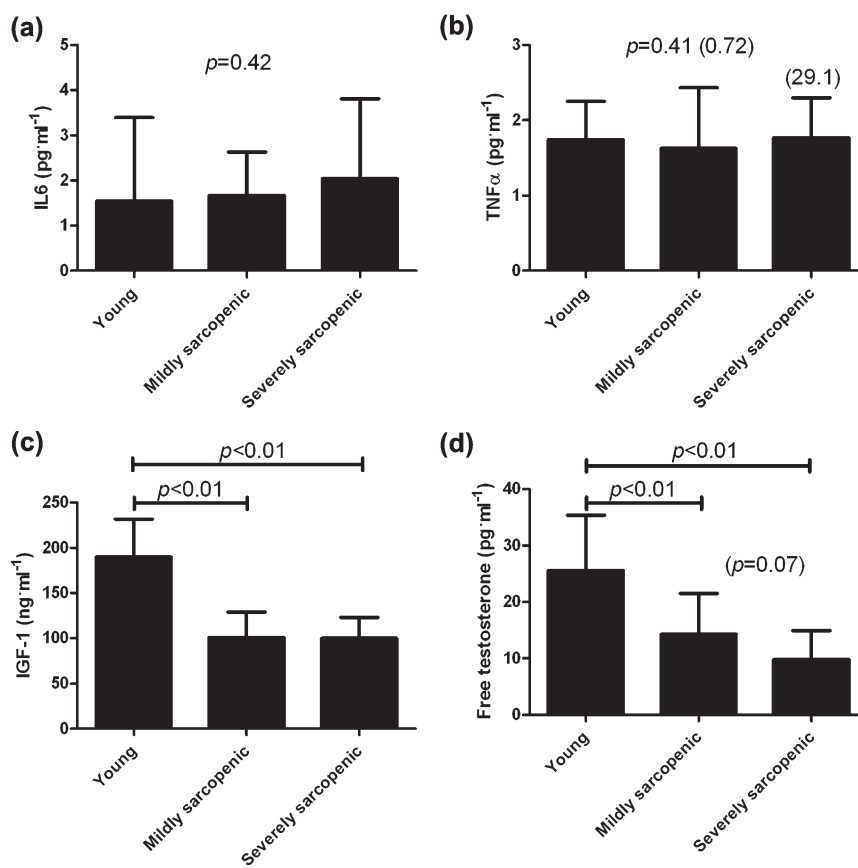


Figure 3 Muscle growth-related serum variables in the serum of young ( $n = 20$ ), mildly sarcopenic ( $n = 20$ ), and severely sarcopenic ( $n = 26$ ) men (mean  $\pm$  SD). For tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), an analysis of variance has been performed with the outliers included ( $p$  value without brackets) and excluded ( $p$  value inside brackets). For free testosterone, the difference between mildly and severely sarcopenic men was approaching significance ( $p = .07$ ). IL-6 = interleukin-6 and TNF $\alpha$  = tumor necrosis factor  $\alpha$ .

### IGF-1

IGF-1 was significantly lower in older men with mild and severe sarcopenia than in the young, confirming previous reports (31,32). However, IGF-1 did not correlate with isometric leg extension torque nor with quadriceps CSA in the older men. IGF-1 stimulates protein synthesis via the mammalian target of rapamycin system (mTOR); (40), but muscle protein synthesis does differ little or not at all between fasted young and old males (15) and certainly not by the magnitude by which IGF-1 differs. Thus, the contribution of lowered serum IGF-1 levels to sarcopenia is unclear.

### Free Testosterone

Free testosterone was significantly lower in the sarcopenic groups and was the only factor that correlated significantly with isometric leg extension strength and m. quadriceps CSA in the elderly and in all participants. This finding is in line with other studies that report decreased free testosterone in elderly men (33) and that free testosterone was a predictor of muscle mass, explaining 2.6% of muscle mass variation (41).

### Conclusions

This is the first study to report the measurement of four myostatin-related serum factors in young and older sarcopenic men. None of the myostatin-related factors differed in-between groups nor correlated with knee extension torque or quadriceps CSA in the pooled sarcopenic cohorts. However, mean FLRG was  $\approx 20\%$  lower in the sarcopenic groups and achieved borderline significance ( $p = .06$ ). Our findings suggest that therapies that aim at inhibiting myostatin in humans (42) do probably not directly target a human sarcopenia defect. This does not exclude that such therapies may be effective in increasing muscle mass and function in sarcopenic individuals.

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