Serum concentrations of myostatin and myostatin-interacting proteins do not differ between young and sarcopenic, elderly men
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1 Introduction

‘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 \(^2, 3\). Complications associated with low muscle mass and function include a reduced ability to perform ambulatory tasks \(^4\), old age disability \(^5\), falls \(^6\), poor recovery after hip fracture or major disease \(^7\) and increased mortality \(^8\).

Efforts to research, diagnose and treat sarcopenia are hampered by the lack of universally accepted guidelines and cut off points for diagnosing sarcopenia \(^9\). Criteria that have been proposed to identify or diagnose sarcopenia are the ratio of appendicular skeletal muscle mass relative to height \(^10\) or the knee extension strength/torque, handgrip strength or calf muscle area at least two standard deviations below the mean of a young reference group \(^11\). Recently, an European working group on sarcopenia has recommended a diagnosis based on measurements of gait speed, grip strength and muscle mass \(^12\).

Sarcopenia is characterised by many alterations of which several stand out. Firstly muscle fibres are lost and type 2 fibres atrophy especially after 60 years of age \(^2, 13\). Secondly, muscle displays ‘anabolic resistance’ which is a decreased protein synthesis response to amino acids \(^14\), insulin \(^15\) and resistance exercise \(^16\). Related to this, insulin also reduces proteolysis less in old muscle than in young \(^17\). Thirdly, satellite cells, as identified by Pax7, neural cell adhesion molecule (NCAM) and M-cadherin positivity, are \(\approx\)2-fold reduced in \(\approx\)70 year old human vastus lateralis muscle when compared to \(\approx\)20 year old controls \(^18\). Old muscle also responds with diminished satellite cell proliferation to muscle injury resulting in impaired muscle regeneration \(^18\).
Many mechanisms have been proposed to cause the aforementioned sarcopenia alterations but the overall aetiology is still incompletely understood. A possible, contributing cause is that ageing increases systemic myostatin activity. Myostatin inhibits muscle growth and an increased serum concentration of myostatin in mice has been reported to cause muscle atrophy. Moreover, prolonged absence of myostatin reduces sarcopenia in mice implying that presence of myostatin contributes to sarcopenia in this species.

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. A well validated human myostatin assay has now been developed (Lakshman et al., 2009). Using this assay it has been reported that serum myostatin was significantly higher by 14% in men with an average age of 27±5 years and 77% lean body mass than in older men with an average age of 66±5 years and 70% lean body mass. The receptor binding activity of myostatin, however, depends additionally on the concentration of myostatin-interacting proteins that can dimerise with myostatin to form a latent, inactive complex that can not bind to its receptor. These proteins include GDF-associated serum protein-1 (GASP-1), follistatin-related gene (FLRG) or follistatin.

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 tumour necrosis factor α (TNFα) were associated with 5-year declines in muscle mass and grip strength whereas interleukin-6 (IL-6) concentrations predicted sarcopenia in women. Serum and muscle-specific insulin-like growth factor-1 (IGF-1) splice variants have been proposed to contribute to sarcopenia. IGF-1 concentrations are lower in elderly subjects than in young and in the Framingham heart study higher IGF-1 concentrations were associated with a smaller loss of fat free mass women. Serum testosterone is often decreased in elderly men and because
testosterone increases muscle protein synthesis \(^{33}\) 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 to young men, respectively. Sarcopenia was defined on the basis of reduced knee extensor torque when compared with young men \(^{11}\). Quadriceps cross sectional area (CSA) was measured using MRI, isometric knee extensor torque during a maximal voluntary contraction (MVC) using an isokinetic dynamometer and voluntary activation using the twitch interpolation technique \(^{34}\). Serum TNF\(\alpha\), IL-6, IGF-1 and free testosterone were additionally measured to allow comparison with previous studies.
2 Methods

2.1 Subject 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±2 years, body mass index (BMI) 24.0±2.5 kg m⁻²) as well as 46 older men with mild sarcopenia (n=20, 69±3 years, BMI 25.2±2.0 kg m⁻²) and severe sarcopenia (n=26, 76±6 years, BMI 24.7±2.8 kg m⁻²; BMI did not differ significantly between groups, \( p=0.30 \)) \(^{11} \). Because muscle function measurements are easier and cheaper to perform than MRI measurements we decided for practical reasons to select sarcopenic subjects based on their maximal knee extension torque rather than their mid thigh quadriceps CSA. Mild sarcopenia was defined as having a MVC between 1 and 2 SD below the mean for the young men while reduction in MVC by 2 SD was used as a cut off point for severe sarcopenia.

2.2 Exclusion criteria

The following exclusion criteria were applied to exclude individuals with conditions that affect muscle mass or the serum factors studied \(^{35} \): Obesity (BMI>30 kg/m²) or being underweight (BMI<18 kg/m²); history of myocardial infarction within the previous 2 years; cardiac illness: moderate/ severe aortic stenosis, acute pericarditis, acute myocarditis, aneurism, severe angina, clinically significant valvular disease, uncontrolled dysrhythmia, claudication, within the previous 10 years; major systemic disease within the last 2 years (cancer, rheumatoid arthritis); diabetes; uncontrolled metabolic disease (e.g. 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; lower limb arthritis, classified by inability to perform maximal contractions of lower limbs without severe pain.
2.3 Pre-study instructions and blood sampling
Volunteers were asked not to exercise the day before and arrived in the lab in the morning after an overnight fast to control for exercise and nutrition. A BD Vacutainer 21 G multi sample 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.

2.4 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, New York, USA) similarly to our previous study. The volunteers sat upright and MVC was determined as the best of three isometric knee extensions lasting approximately 5 s each; 2 min of rest was allowed between the attempts.

2.5 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. A constant current electrical stimulator (DS7A; Digitimer, Hertfortshire, U.K.) was used to apply a square-wave pulse of 0.5 ms in duration to the quadriceps muscle via two surface electrodes (6 x 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 s MVCs were performed with a 2 min rest in-between. At ~3 s of a MVC effort an electrical pulse was superimposed on the voluntary contraction. The same electrical stimulus was repeated 1–2 s 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 x 100%.
2.6 MRI measurement of the quadriceps mid-thigh CSA

Mid thigh, i.e. the midpoint between trochanterion and tibiale laterale, was marked using a cod liver oil capsule which is easy to detect on a 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 cross sectional areas (CSA) of vastus lateralis, vastus medialis, vastus intermedius and rectus femoris for the dominant leg was measured by two independent observers and the was used for analysis.

2.7 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 subjects: total myostatin, follistatin, FLRG, GASP-1, IGF-I, TNFα, IL-6 and free testosterone (Table 1). The validation of the total myostatin assay has previously been published. The assays were validated for use in serum using either the manufacturers or published protocols with the following three exceptions: 1) For follistatin an extra low calibrator point of 0.125 ng/ml was added; 2) For FLRG and GASP-1 Immunoglobulin Inhibiting Reagent (IIR, Sera Laboratories International, West Sussex, UK) was routinely added to the standard and samples; 3) For myostatin the calibrator matrix was 5% BSA /PBS rather than the serum-based calibrator used in the earlier study 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 CVs for each assay was 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.

2.8 Statistical analysis

All data are given as mean ± standard deviation (SD). We tested the hypothesis that variables differed between the young, mildly sarcopenic and severely sarcopenic groups by using an one-factorial ANOVA. Homogeneity of variance was tested by Levene’s test. If variance did not differ significantly then a least significant difference (LSD) 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 compare serum variables to muscle size and functional variables, Pearson’s product-moment correlation coefficient was calculated. All statistical tests were computed using SPSS 17.0.
3 Results

3.1 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 depressed by 31% and 52% compared with young men, respectively (Figure 1). Voluntary muscle activation did not differ significantly between groups \((p=0.59)\). It was 94.9±3.9\% in the young \((n=18)\), 95.3±3.8\% \((n=21)\) in the mildly sarcopenic and 93.4±7.0\% \((n=11)\) in the severely sarcopenic. Reduced subject numbers are because not all men volunteered to take part in this experiment.

3.2 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 \((p=0.06; \text{Figure 2})\): FLRG was 26\% lower in the mildly sarcopenic and 19\% lower in the severely sarcopenic group than in the young, respectively. None of the myostatin-related serum factors correlated significantly with either isometric knee extension torque or quadriceps CSA in older men.

3.3 Other serum factors
IL-6 and TNF\(\alpha\) did not differ significantly between groups but 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 mildly and severely cohorts. In contrast, free testosterone correlated negatively with both isometric knee extension torque \((r=-0.40; p=0.01)\) and quadriceps CSA \((r=-0.36; p=0.02)\) in the pooled mildly and severely sarcopenic cohorts.
4 Discussion

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

4.1 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 muscle force to CSA, is reduced in the sarcopenic subjects compared with young \(^{37,38}\).

4.2 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 \(^{23}\). 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 \(^{23}\). However, serum myostatin concentrations were 40%-50% lower in our study (Figure 2) than those measured in the first study \(^{23}\). Also, myostatin was reported to be higher by 1 ng ml\(^{-1}\) in the young than in the old men \(^{23}\) whereas we did not find any difference. The most likely explanation for the on average higher concentrations reported by Lakshman et al. (2009) 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% BSA/PBS. This is the most likely explanation for the observed, systematic difference in serum myostatin concentrations. A possible reason for the significant, 1 ng ml\(^{-1}\) myostatin difference in Lakshman et al. (2009) is that the BMI of the old men was 2.8 kg m\(^{-2}\) 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\(^{-2}\).
To our knowledge this is the first study where the myostatin and the myostatin-interacting factors GASP-1\textsuperscript{24}, FLRG\textsuperscript{25} or follistatin\textsuperscript{26} have all been measured in human serum (Figure 2). Myostatin, GASP-1 and FLRG and follistatin did not differ significantly between groups nor did they correlate with isometric knee extension torque or m. quadriceps CSA. This suggests that at least the serum concentrations of myostatin and myostatin-interacting factors are unlikely to be a contributing cause for 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 ($p=0.06$) with a trend towards $\approx20\%$ lower FLRG concentrations in the sarcopenic men. Lower FLRG levels would result in a lower concentration of the latent, inactive complex (Hill \textit{et al.}, 2002) and a higher concentration of free myostatin.

4.3 Cytokines

Neither IL-6 nor TNF\textsubscript{\alpha} differed significantly between groups nor did these serum factors correlated with isometric knee extension strength or m. quadriceps cross sectional area. Our data contradict previous studies showing that TNF\textsubscript{\alpha} was a predictor of the decline of muscle mass and grip strength\textsuperscript{27} and that IL-6 was a predictor of sarcopenia albeit only in women\textsuperscript{28}.

4.4 IGF-1

IGF-1 was significantly lower in older men with mild and severe sarcopenia than in the young, confirming previous reports\textsuperscript{30, 31}. 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 mTOR system\textsuperscript{40} but muscle protein synthesis does differ little or not at all between fasted young and old males\textsuperscript{14} and certainly not by the magnitude by which IGF-1 differs. Thus the contribution of lowered serum IGF-1 levels to sarcopenia is unclear.
4.5 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 subjects. This finding is in line with other studies that report decreased free testosterone in elderly men \(^32\) and that free testosterone was a predictor of muscle mass explaining 2.6% of muscle mass variation \(^41\).

4.6 Conclusion

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 \((p=0.06)\). Our findings suggest that therapies that aim at inhibiting myostatin in humans \(^42\) do probably not directly target a sarcopenia defect. This does not exclude that such therapies may be effective in increasing muscle mass and function in sarcopenic individuals.
Figure legends

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

**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±SD). For myostatin one outlier in the mildly sarcopenic (42.5 ng ml⁻¹) and another in the severely sarcopenic (28.3 ng ml⁻¹) group has not been included in this figure. For myostatin an ANOVA has been performed with the outliers included (p value without brackets) and excluded (p value inside brackets). Abbreviations: FLRG Follistatin-Related Gene Protein; GASP-1 GDF-associated serum protein-1.

**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±SD). For TNFα an ANOVA has been performed with the outliers included (p value without brackets) and excluded (p value inside brackets). Abbreviations: IL-6 Interleukin-6; TNFα Tumour necrosis factor α.
5 References

(1) Rosenberg IH. Sarcopenia: origins and clinical relevance. J Nutr 1997 May;127(5 Suppl):990S-1S.


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Figure 1. (a) Knee extension torque and (b) quadriceps cross-sectional area for the dominant leg (CSA) in young, mildly sarcopenic and severely sarcopenic men (mean±SD). For CSA only 24 out of 26 severely sarcopenic subjects could be analysed.
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177x169mm (300 x 300 DPI)
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Abbreviations: IL-6 Interleukin-6; TNFα Tumour necrosis factor α.

177x174mm (300 x 300 DPI)
Table 1. Experimental detail for the Elisa assays.

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<th>Elisa assay</th>
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<th>Inter assay precision (%CV)</th>
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^In house (Pfizer) antibodies were used to detect myostatin. These were a capture (RK35) and detector (RK22) antibody.

*Neat samples underwent pretreatment prior to assay, resulting in a 1:13.3 dilution for the assay.

#Neat samples underwent two pretreatment steps, resulting in a 1:100 dilution for the assay.