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Measurement of myostatin concentrations in human serum: Circulating concentrations in young and older men and effects of testosterone administration☆

Kishore M. Lakshman[a], Shalender Bhasin[a,*], Christopher Corcoran[b], Lisa A. Collins-Racie[b], Lioudmila Tchistiakova[b], S. Bradley Forlow[c], Katie St. Ledger[c], Michael E. Burczynski[c], Andrew J. Dorner[b], Edward R. LaVallie[b]

[a] Section of Endocrinology, Diabetes, and Nutrition, Boston University School of Medicine, Boston Medical Center, 670 Albany Street, Boston, MA 02118, United States
[b] Department of Biological Technologies, Wyeth Research, 35 Cambridge Park Drive, Cambridge, MA 02140, United States
[c] Department of Clinical Translational Medicine, Wyeth Research, 500 Arcola Road, Collegeville, PA 19426, United States

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ABSTRACT

Methodological problems, including binding of myostatin to plasma proteins and cross-reactivity of assay reagents with other proteins, have confounded myostatin measurements. Here we describe development of an accurate assay for measuring myostatin concentrations in humans. Monoclonal antibodies that bind to distinct regions of myostatin served as capture and detector antibodies in a sandwich ELISA that used acid treatment to dissociate myostatin from binding proteins. Serum from myostatin-deficient Belgian Blue cattle was used as matrix and recombinant human myostatin as standard. The quantitative range was 0.15–37.50 ng/mL. Intra- and inter-assay CVs in low, mid, and high range were 4.1%, 4.7%, and 7.2%, and 3.9%, 1.6%, and 5.2%, respectively. Myostatin protein was undetectable in sera of Belgian Blue cattle and myostatin knockout mice. Recovery in spiked sera approximated 100%. ActRIIB-Fc or anti-myostatin antibody MYO-029 had no effect on myostatin measurements when assayed at pH 2.5. Myostatin levels were higher in young than older men (mean ± S.E.M. 8.0 ± 0.3 ng/mL *vs.* 7.0 ± 0.4 ng/mL, $P = 0.03$). In men treated with graded doses of testosterone, myostatin levels were significantly higher on day 56 than baseline in both young and older men; changes in myostatin levels were significantly correlated with changes in total and free testosterone in young men. Myostatin levels were not significantly associated with lean body mass in either young or older men.

Conclusion: Myostatin ELISA has the characteristics of a valid assay: nearly 100% recovery, excellent precision, accuracy, and sufficient sensitivity to enable measurement of myostatin concentrations in men and women.

1. Introduction

A substantial body of evidence from experiments of nature and man supports the view that myostatin is an important inhibitor of skeletal muscle growth (Grobet et al., 1997; Kambadur et al., 1997; McPherron et al., 1997; McPherron and Lee, 1997; Gonzalez-Cadavid et al., 1998; Reisz-Porszasz et al., 2003; Schuelke et al., 2004). Naturally occurring mutations of myostatin in cattle, sheep, mice, dogs, and humans are associated with hypermuscularity (Grobet et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997; Reisz-Porszasz et al., 2003; Schuelke et al., 2004; Mosher et al., 2007). Similarly, knockout mice carrying a targeted deletion of the myostatin gene (Lee, 2008) and transgenic mice that hyperexpress myostatin prodomain (Yang et al., 2001; Bhasin et al., 2005), or are unable to process myostatin into its active product have higher skeletal muscle mass than their wild type controls (Zhu et al., 2000). Conversely, transgenic mice which hyperexpress myostatin in the skeletal muscle have lower muscle mass compared to wild type controls (Reisz-Porszasz et al., 2003). Inhibition of myostatin action in adult animals by myriad strategies – administration of monoclonal antibodies, propeptide, follistatin, or soluble ActRIIB receptor – increases muscle mass, suggesting that myostatin also restrains skeletal muscle mass during adult life (Whittemore et al., 2003; Lee, 2004; Lee et al., 2005; Welle et al., 2007; Nakatani et al., 2008).

Myostatin is a secreted protein derived by proteolytic cleavage of its precursor protein at a dibasic site by a furin type protease;

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* Corresponding author. Tel.: +1 617 414 2951; fax: +1 617 638 8217.
E-mail address: bhasin@bu.edu (S. Bhasin).

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this proteolytic cleavage generates the mature myostatin protein and a propeptide, which binds myostatin and inhibits its action (Hill et al., 2002, 2003; Lee, 2008). Myostatin circulates in plasma bound to several proteins, including its propeptide, follistatin-related protein, and growth and differentiation factor-associated serum protein-1 (GASP-1) (Hill et al., 2002, 2003; Lee, 2008). Myostatin bound to these plasma proteins in a latent complex is unable to bind its receptor and activate signaling (Hill et al., 2002, 2003; Lee, 2008). The precise mechanisms leading to activation of myostatin in its target tissues are not fully understood. Proteolytic cleavage of the bound myostatin propeptide by members of bone morphogenetic protein (BMP)-1-tollloid family of metalloproteinases (Wolfman et al., 2003) or extracellular processing and maturation of pro-myostatin by furin (Anderson et al., 2008) are potential mechanisms for activation of myostatin.

Although several studies have reported the presence of myostatin protein in blood (Zachwieja et al., 1999; Gonzalez-Cadavid and Bhasin, 2004; Walker et al., 2004; Hosoyama et al., 2006), methodological problems have confounded the measurements of circulating myostatin concentrations. The earlier studies using direct radioimmunoassays preceded the recognition that myostatin circulates as a latent complex bound to plasma proteins (Gonzalez-Cadavid et al., 1998; Zachwieja et al., 1999; Walker et al., 2004; Hosoyama et al., 2006). Also, the cross-reactivity of other plasma proteins in the myostatin assays was not fully appreciated (Zachwieja et al., 1999; Walker et al., 2004; Hosoyama et al., 2006). Therefore, there has been a paucity of accurate data on circulating myostatin levels in healthy young and older individuals, and the sparse data available have been contradictory. For instance, some studies reported higher myostatin levels in older men and women while others found lower or unchanged myostatin expression in skeletal muscle of older men and women in comparison to young men and women (Zachwieja et al., 1999; Welle et al., 2002; Kim et al., 2005; Raue et al., 2006).

Here, we report the development and validation of an accurate assay for the measurement of serum myostatin levels in humans. The assay uses acid treatment to strip myostatin from its binding proteins. Serum from Belgian Blue cattle (naturally devoid of myostatin protein due to an inactivating mutation) was used as the matrix, and recombinant human dimeric myostatin protein as reference standard. Using this validated assay, we measured the circulating myostatin concentrations in healthy young and older men. We also determined the effects of testosterone treatment on circulating myostatin levels using stored samples from a testosterone dose response study. In that study, the details of which have been published (Bhasin et al., 2001, 2005; Storer et al., 2003), administration of graded doses of testosterone to healthy young and older men was associated with dose-dependent increases in skeletal muscle mass. We hypothesized that testosterone-associated increase in muscle mass would be associated with suppression of serum myostatin levels. However, we recognized that if myostatin serves as a chalone to regulate muscle growth, as has been proposed by Lee (2004) and others (Gaussin and Depre, 2005), myostatin levels would be expected to increase after testosterone administration as a counter-regulatory mechanism to restrain testosterone-induced increase in muscle mass.

2. Methods

Human subjects. Serum samples were derived from healthy young men, aged 18–35 years, and older men, aged 60–75 years, with normal testosterone levels, who were participants in a testosterone dose response study (Bhasin et al., 2001, 2005). The design and main findings of this study have been reported previously (Bhasin et al., 2001, 2005). The study protocols were approved by the institutional review boards of Charles Drew University and Harbor-UCLA Research and Education Institute. All participants provided informed consent. Exclusion criteria included history of prostate cancer, PSA >4 ng/mL, AUA lower urinary tract symptom score >7, hematocrit >48%, severe sleep apnea, diabetes mellitus, congestive heart failure, myocardial infarction in the preceding 6 months, androgen use in the preceding year, or participation in moderate to intense exercise training. After a 4-week control period, participants were randomized to one of five treatment groups to receive monthly injections of a GnRH agonist (leuprolide depot, 7.5 mg; TAP, North Chicago, IL) to suppress endogenous testosterone production. The participants also received weekly intramuscular injections of testosterone enanthate (TE, Delatestryl, 200 mg/mL; Savient Pharmaceuticals, Iselin, NJ) in one of five doses: 25, 50, 125, 300, or 600 mg (Bhasin et al., 2001, 2005). Treatment duration was 20 weeks. The Data and Safety Monitoring Board stopped the 600 mg TE dose group in December 2002 due to a number of adverse events in older men in this dose group. After this point, randomization was limited to one of four TE dose groups: 25, 50, 125, or 300 mg weekly.

Healthy and menopausal women. Serum samples of healthy, menstruating women ($n = 33$), 19–21 years of age, and postmenopausal women ($n = 37$), 67–87 years of age, were purchased from BioServe, Beltsville, MD. These participants had consented to participate in an IRB-approved Bioserve study. Surgically menopausal women were 18–55 years of age ($n = 24$), who had ovarian surgery at least 6 months before enrollment and serum FSH >30 U/L, BMI <35 kg/m^2, a normal PAP smear and mammogram in the preceding 12 months, and who had provided informed consent approved by the Boston University IRB.

2.1. Myostatin assay

Reagents. Monoclonal antibodies to myostatin were raised in myostatin-null mice (Wyeth Research). Antibodies that bind to distinct regions of myostatin (data not shown) were used as the capture (RK35) and detector (RK22) antibodies. RK22 was biotinylated using EZ®-Link Sulfo-NHS-LCBiotinylation Kit (Pierce, Rockford, IL). MYO-029 is a humanized murine monoclonal antibody that binds to ActRIIB receptor-interaction site on myostatin (Girgenrath et al., 2005; Wagner et al., 2008). Recombinant mature human myostatin, and ActRIIB extracellular domain fused to Fc portion of IgG (del Re et al., 2004), was expressed and purified from CHO cells (Wyeth BioPharma, Andover, MA). Myostatin-deficient serum from Belgian Blue cattle (Grobet et al., 1997; Kambadur et al., 1997), normal bovine serum, and cynomolgous monkey serum was obtained from Bioreclamation (Hicksville, NY). Serum from myostatin-null mice (C57BL/6 background) (McPherron et al., 1997) and normal littermates was obtained from Wyeth BioResources.

Myostatin assay method. Serum samples (or calibrator samples in Belgian Blue serum) were mixed with acid dissociation buffer (0.2 M glycine–HCl pH 2.5) at a ratio of 1:13.3. For non-dissociative assays, samples were mixed with THST buffer (50 mM Tris–HCl pH 8.0, 500 mM NaCl, 1 mM glycine, 0.05% Tween-20; pH 8.0). Assay plates were incubated with 2.0 μg/mL RK35 in coating buffer (100 mM sodium borate pH 9.1) overnight at 4 °C, washed, and blocked with 200 μL/well of SuperBlock-TBS (Pierce). Diluted serum samples (100 μL) were transferred to assay plate, incubated at room temperature for 90 min, washed 4 times with THST and 100 μL biotinlylated RK-22 secondary antibody (0.1 μg/mL) was added to each well for 90 min at room temperature. Plates were washed 4 times with THST, and 100 μL Streptavidin–HRP (SouthernBiotech) diluted 1:40,000 in THST buffer was added for 1 h at room temperature. Plates were washed again 4 times with THST, and developed by addition of 100 μL TMB substrate for 12 min. 100 μL 0.5 M H_2SO_4 was added per well, and ELISA plates were read at 450 nm with wavelength correction set at 540 nm. A calibration curve was generated by plotting the OD against calibrator concentration and using a 5-parameter logistic fit. The myostatin concentrations in human samples reported in the manuscript were generated in glycine buffer at pH 2.5.

Calibration curve range. A calibration curve consisting of 2-fold dilutions of recombinant human mature myostatin in Belgian Blue serum extending from 0.07 to 75.00 ng/mL was prepared. The intra- and inter-assay imprecision of read-back values for the 11 calibrators were determined from 6 analytical runs. The mean, S.D., CV, and bias of the extrapolated concentrations were calculated for each analytical run (to assess intra-assay imprecision) and for all analytical runs (to assess inter-assay imprecision). The lower and upper limits of quantitation were defined as the lowest (LLQ) and highest (ULQ) calibrator concentrations that could be measured with an intra-assay CV and bias ≤30%.

Validation samples. Three sets of validation samples corresponding to low, mid-range, and high serum concentrations of myostatin were prepared using serum samples from healthy subjects with endogenous myostatin concentrations in the lower end and mid-range of the calibration curve, respectively. The high validation sample was a serum sample from a healthy subject spiked with recombinant myostatin protein.

Intra- and inter-assay imprecision. Intra- and inter-assay CVs were measured in six separate aliquots of low, mid, and high validation samples in five independent analytical runs. The QC analytical run acceptance range (total assay variation mean ± 2 S.D.) was determined from myostatin concentrations measured in ≥3 separate aliquots of each of the three QC samples in ≥5 independent analytical runs. One aliquot of each of the three QC samples was analyzed in each analytical run of samples. An analytical run was accepted if the measured myostatin concentration in two out of three QC samples was within acceptance range.

2.2. Other assays

Serum total testosterone levels were measured by a specific radioimmunoassay that has been validated previously against liquid chromatography–tandem

mass spectrometry (LC–MS/MS) (Bhasin et al., 2005). The intra- and inter-assay CVs for total testosterone assay were 8.2% and 13.2%, respectively. Free testosterone, separated from serum by an equilibrium dialysis procedure, was measured by a sensitive radioimmunoassay that had a sensitivity of 8 pmol/L, and intra- and inter-assay CVs 4.2% and 12.3%, respectively (Sinha-Hikim et al., 1998). The radioimmunoassay and LC–MS/MS methods were compared by analyzing samples prepared in charcoal stripped serum to which known amounts of testosterone had been added. These measurements demonstrated a correlation of 0.99 between the radioimmunoassay and LC–MS/MS measurement. Serum sex hormone binding gobulin (SHBG) levels were measured by an immunofluorometric assay that has a sensitivity of 6.25 nmol/L. Body composition was assessed at baseline and during week 20 by dual-energy X-ray absorptiometry (DXA, Hologic 4500, Waltham, MA). A body composition phantom was used to calibrate the machine before each measurement.

2.3. Statistical analyses

All outcome variables were evaluated for distribution and homogeneity of variance; variables that did not meet the assumptions of homogeneity of variance or normal distribution were log-transformed. ANOVA was used to evaluate differences across dose groups stratified by age, younger *vs.* older, at a single time point; Tukey's multiple comparison test was used to determine which groups differed significantly if a difference was identified by ANOVA. Changes within groups from baseline to treatment were evaluated by using paired *t*-tests. Alpha was set at 0.05 for determining statistical significance. Data are presented as mean ± S.E.M. or mean percent change from baseline ± S.E.M., unless otherwise indicated.

3. Results

3.1. Myostatin assay characteristics

Linear range. The mean intra- and inter-assay imprecision was determined from 6 analytical runs for each of the 11 calibrators (0.07–75.00 ng/mL) in the standard curve. The inter-assay CV for the 0.07 ng/mL calibrator was 36.4%, which exceeded the acceptable limit (<30%). Therefore, the LLQ of the assay was determined by the next calibrator point (0.15 ng/mL) at which the inter-assay CV and bias were 19.7% and +3.4%, respectively. The inter-assay CV of 32.4% for the 75.00 ng/mL calibrator was also not within the acceptable limit (<30%), thereby defining the ULQ to the next calibrator point (37.50 ng/mL) at which the CV and bias were 3.6% and +0.8%, respectively. Thus, the quantitative range of the assay extended from 0.15 to 37.50 ng/mL in a biologically relevant matrix.

Intra- and inter-assay imprecision. The mean myostatin concentrations (ng/mL, ±S.D.) in the low, mid, and high validation samples in five analytical runs were 3.70 ± 0.15, 7.60 ± 0.13, and 18.27 ± 0.95, respectively. The measured intra-assay CV for the low, mid, and high validation samples ($n = 5$ for each validation sample) was 4.1%, 4.7%, and 7.2%, respectively, and the inter-assay CV was 3.9%, 1.6%, and 5.2%, respectively.

Assay specificity. The mature myostatin protein has a high degree of sequence conservation among mammalian species (McPherron and Lee, 1997), allowing use of the myostatin assay on many non-human samples, including mouse, rat, dog, cow, and monkey. Serum samples from myostatin-deficient cattle (Belgian Blue) and from mice with an inactivating mutation in the myostatin gene (mstn KO) were assayed under dissociative conditions, and compared to normal animals of the same species. Serum myostatin concentration in wild type mice averaged 113.00 ± 20.70 ng/mL (mean ± S.E.M.), and in normal cows 41.30 ± 1.10 ng/mL (mean ± S.E.M.). In contrast, myostatin protein was undetectable in sera of Belgian Blue cattle and the myostatin KO mice, confirming the specificity of the assay; mutations in these myostatin-null animals abolish the synthesis of myostatin protein (McPherron et al., 1997; McPherron and Lee, 1997).

Evaluation of binding protein dissociation after sample acidification. Acidification of serum samples dissociates specific myostatin binding proteins such as the myostatin propeptide and follistatin resulting in activation of receptor binding and signaling activity (Zimmers et al., 2002). The anti-myostatin mAb MYO-029 and the soluble myostatin receptor protein ActRIIB-Fc are both capable of neutralizing myostatin activity, and both bind to regions of myostatin that overlap with epitopes for RK22 and RK35 antibodies. Therefore, addition of these myostatin-binding proteins to serum would be expected to compete with the ELISA antibodies and block the myostatin-specific signal in the assay. The RK35 capture antibody is capable of binding to myostatin at both pH 2.5 and pH 8, while MYO-029 and ActRIIB-Fc binding to myostatin is abolished at pH 2.5 (data not shown). Increasing concentrations (0, 3, 30, and 300 μg/mL) of either MYO-029 or ActRIIB-Fc were added to non-human primate serum and assayed in myostatin ELISA at pH 8 (non-dissociative conditions, Fig. 1B) and at pH 2.5 (dissociative conditions, Fig. 1C). The standard curves under dissociative and non-dissociative conditions are shown in Fig. 1A. Without addition of binding proteins, the apparent myostatin concentration in the serum at pH 8 was 0.8 ng/mL; under dissociative conditions at pH 2.5 the measured myostatin concentration increased more than 7-fold to 6 ng/mL. Addition of up to 300 μg/mL of either MYO-029 or ActRIIB-Fc had no effect on myostatin measurements when assayed at pH 2.5; however, when assayed at pH 8 the presence of these binding proteins diminished the myostatin signal in a dose-dependent manner to values approaching the LLQ of the assay, providing further evidence that most or all of the signal in the ELISA reflects myostatin protein concentration. Studies in human serum provided similar results (data not shown).

To further characterize the effect of pH on assay performance, we diluted human serum with glycine buffer ranging from pH 6.0 to 0.5. Myostatin levels were estimated based on a calibrator curve of purified rGDF-8 dimer spiked into Belgian Blue serum. We obtained stable myostatin concentrations in the pH range 3.0–2.0 (data not shown). The measured myostatin immunoreactivity was lower at pH below 2.0 and above pH 3.5. Thus, myostatin binds the capture antibody RK35 robustly at pH 2.5.

Table 1
Baseline characteristics of the subjects (mean ± S.D.).

Characteristic	Young men ($n = 50$)	Older men ($n = 48$)
Age (years)	26.5 ± 4.6	66.4 ± 4.7
Height (cm)	176.3 ± 6.4	175.9 ± 5.7
Weight (kg)	75.1 ± 10.9	83.2 ± 11.7
BMI (kg/m^2)	24.1 ± 3.0	26.9 ± 3.5
Lean body mass (kg)	57.6 ± 7.2	57.9 ± 6.3
Percent fat mass (%)	18.0 ± 6.4	26.6 ± 5.4
Total testosterone (ng/dL)	578.4 ± 165.2	330.6 ± 96.1

3.2. Baseline characteristics of human subjects

The baseline characteristics of the young and healthy men in the parent study have been described (Bhasin et al., 2001, 2005). Fifty-two of the 61 randomized young men and 51 of 60 randomized older men completed the treatment phase. The causes of treatment discontinuation and loss to follow up have been described (Bhasin et al., 2001, 2005). Sufficient serum for myostatin assays and body composition data were available through week 20 for 50 young men and 48 older men; these subjects were included in this secondary analysis and their baseline characteristics are shown in Table 1. Drug compliance rate was >99%.

Baseline total and free testosterone, percent free testosterone, and SHBG concentrations, did not differ among the five groups at baseline in either the young or older groups. However, older men had lower total and free testosterone, and higher SHBG than younger men. Body weight, body mass index, and percent fat mass were greater in the older men than younger men, while height was similar in both.

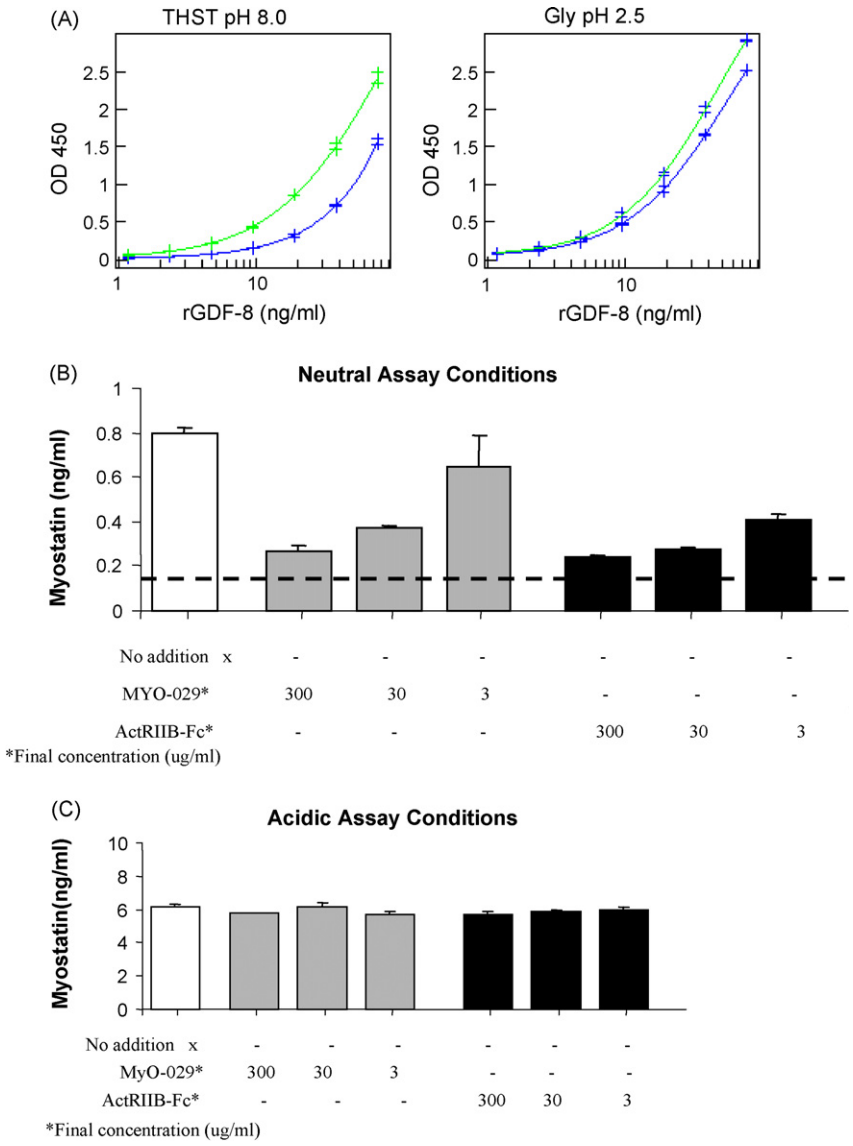

Fig. 1. (A) Serum myostatin calibrator curves under dissociative and non-dissociative conditions. Calibration curves were prepared by spiking recombinant myostatin into serum, and then serially diluting with THST +1% BSA buffer (green line) or Belgian Blue serum (blue line). Each calibrator curve was generated in either THST buffer pH 8.0 or glycine buffer pH 2.5 for non-dissociative or dissociating conditions, respectively. Standard curves of recombinant myostatin standard in buffer (not serum) and Belgian Blue serum at pH 8 are different, presumably due to the interference in the assay by myostatin-binding proteins in the Belgian Blue serum. Standard curves at pH 2.5 shows very little difference between buffer and Belgian Blue serum, and the lower limit of quantitation is lower at this pH. Each data point represents mean ± S.E.M. of three replicates. (B) and (C) Myostatin levels in cynomolgous monkey serum measured in the myostatin ELISA under non-dissociative (pH 8, panel (B)) or dissociative (pH 2.5, panel (C)) conditions following addition of increasing concentrations of anti-myostatin antibody MYO-029 or soluble myostatin receptor ActRIIB-Fc. Bars represent mean ± S.D. of three replicate samples. Dashed line indicates the LLQ. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

3.3. Myostatin levels in young and older men

Serum myostatin levels were normally distributed in both young and older men. Young men had significantly higher myostatin levels than older men (8.0 ± 0.3 ng/mL *vs.* 7.0 ± 0.4 ng/mL, $P = 0.03$) (Table 2A). Serum myostatin levels were not significantly correlated with lean body mass in either young or older men (Fig. 2A and B).

Table 2A
Serum baseline myostatin levels in young and older men.

Group	Myostatin (ng/mL)			
	Mean ± S.D.	Median	25th percentile	75th percentile
Young men ($n = 50$)	8.0 ± 2.3[a]	7.8	6.5	9.8
Older men ($n = 48$)	7.0 ± 2.5[a]	6.8	5.7	8.5

[a] $P = 0.03$ for t-test

Similarly, there was no significant correlation between myostatin levels and body weight, body mass index, or testosterone levels at baseline (not shown).

3.4. Myostatin levels in women

Serum myostatin levels in young women were not significantly different from those in young men. Myostatin levels in menstruating women, surgically menopausal and naturally menopausal women did not differ significantly (Table 2B).

3.5. Effects of testosterone administration on myostatin levels in men

Serum myostatin levels at baseline did not differ significantly across the five dose groups within either young or older men. Serum

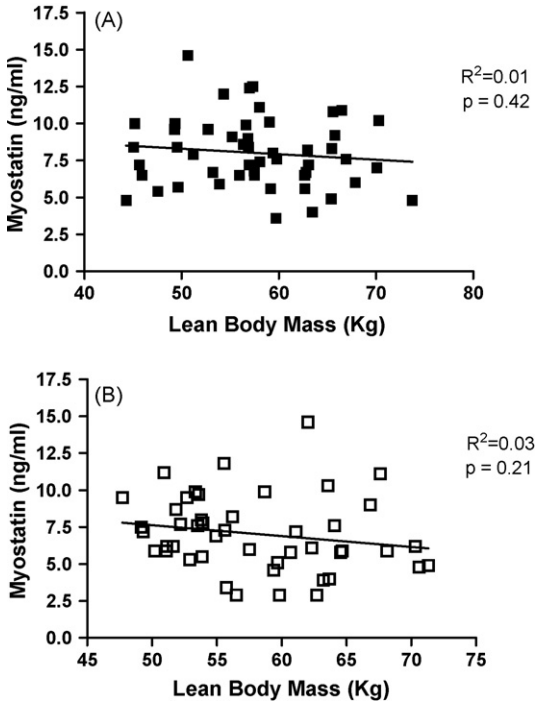

Fig. 2. (A) Regression plot showing correlation of baseline myostatin levels with lean body mass in young men. (B) Regression plot showing correlation of baseline myostatin levels with lean body mass in older men.

Table 2B
Serum myostatin levels in young menstruating women, surgically menopausal women, and in older women.

Group	Myostatin (ng/mL)			
	Mean ± S.D.	Median	25th percentile	75th percentile
Young menstruating women (n = 33)	7.0 ± 2.7[a]	6.1	5.0	9.2
Surgically menopausal women (n = 37)	6.7 ± 2.7[a]	6.2	5.5	7.4
Older women (n = 24)	6.7 ± 2.8[a]	6.2	5.1	8.2

[a] P = 0.86 for ANOVA.

myostatin levels were significantly higher on day 56 compared to baseline in both young and older men (Fig. 3A). Older men experienced a significantly greater percent increase in myostatin levels than young men (Fig. 3B). The increases in myostatin levels during testosterone therapy were not sustained; thus, serum myostatin levels on day 140 were not significantly different from those at baseline.

Changes in myostatin levels from baseline to day 56 were significantly positively correlated with changes in total (Fig. 4A) and free (Fig. 4C) testosterone concentrations in young men, but not in older men (Fig. 4B and D). As previously reported, testosterone treatment was associated with significant gains in lean body mass; the changes in lean body mass were significantly correlated with testosterone dose and testosterone concentration (Bhasin et al., 2001, 2005). However, changes in lean body mass

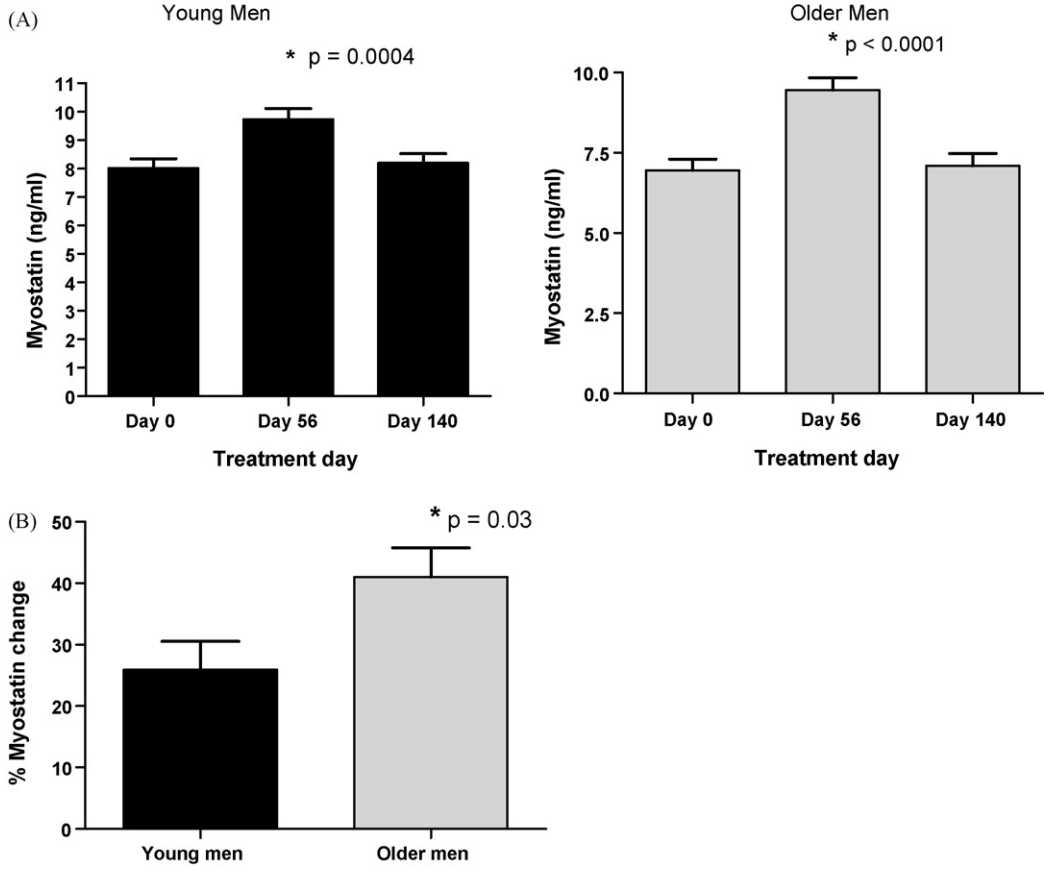

Fig. 3. Changes in myostatin levels in young men in response to administration of graded doses of testosterone (bar diagram showing mean ± S.E.M. levels at baseline, and days 56 and 140). Panel (A) shows the myostatin levels at baseline, treatment day 56, and 140 in young (left panel) and older men (right panel). The data are mean ± S.E.M. *P value as in comparison to baseline levels. Myostatin levels on day 140 were not significantly different from baseline levels. Panel (B) shows the percent change from baseline in serum myostatin levels from baseline to day 56 in young and older men. *P = 0.03.

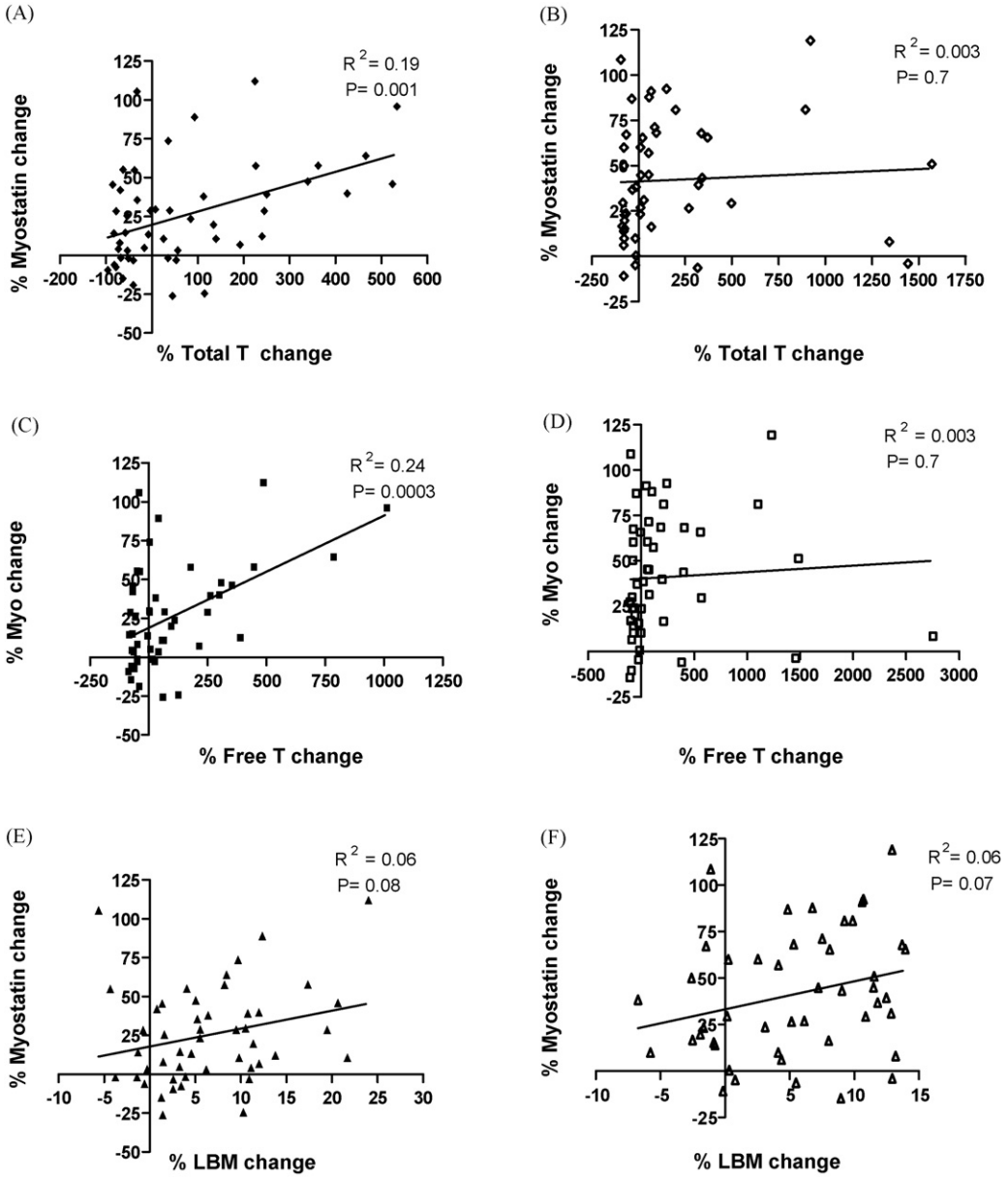

Fig. 4. Regression plots showing correlation of the change in myostatin levels from baseline to day 56 and changes in total and free testosterone concentrations and lean body mass in young and older men. Panel (A) shows the linear regression plot of percent change in myostatin levels from baseline to day 56 and percent change in serum total testosterone concentrations in young men. Panel (B) shows the linear regression plot of percent change in myostatin levels from baseline to day 56 and percent change in serum total testosterone concentrations in older men. Panel (C) shows the linear regression of percent change in myostatin levels from baseline to day 56 and percent change in serum free testosterone concentrations in young men. Panel (D) shows the linear regression of percent change in myostatin levels from baseline to day 56 and percent change in serum free testosterone concentrations in older men. Panel (E) shows the linear regression of percent change in myostatin levels from baseline to day 56 and percent change in lean body mass from baseline to day 140 in young men. Panel (F) shows the linear regression of percent change in myostatin levels from baseline to day 56 and percent change in serum lean body mass from baseline to day 140 in older men.

were not significantly correlated with either absolute or percent change (Fig. 4E and F) in myostatin concentrations.

4. Discussion

Previous data on circulating myostatin levels in healthy individuals and in individuals with disease are confounded by issues of specificity and the inability of these earlier assays to reliably take into account the binding of circulating myostatin to its binding proteins in plasma. In our assay, myostatin was stripped off its binding proteins by acid treatment that effectively dissociates myostatin from the binding proteins. Other serum proteins do not have significant cross-reactivity in our assays; even the highly homologous GDF-11 protein is not expected to cross-react in the assay because the RK-22 detector antibody in the sandwich ELISA does not bind to GDF-11. The assay has all the characteristics of a valid measurement system: nearly 100% recovery of spiked myostatin, excellent precision in the physiologic range, and accuracy. The assay also has sufficient sensitivity to be able to measure circulating myostatin concentrations in almost all men and women.

Using this validated myostatin assay, we report here the distribution of myostatin levels in healthy men and women. Although direct comparisons with previous assays are difficult because of the differences in calibrating standard, we find that the circulating concentrations in men and women are substantially lower than those reported previously. We show that myostatin levels are lower in older men than young men. Also, contrary to expectation, myostatin levels increased transiently in response to testosterone

administration, but returned to baseline by 20 weeks of treatment. The increments in myostatin levels were correlated with circulating testosterone concentrations. These observations support the hypothesis initially proposed by Lee (2004) and later by Gaussin and Depre (2005) that myostatin acts as a chalone – a counter-regulatory hormone – to restrain skeletal muscle growth in response to an anabolic stimulus.

The concept of chalones – inhibitors of cell growth that serve as counter-regulatory mechanisms to control the size of specific tissues – was introduced initially by Bullough. However, Lee (2004) was the first to articulate the hypothesis that myostatin functions as a chalone for skeletal muscle: it is produced and secreted by skeletal muscle and it circulates in plasma to restrain skeletal muscle mass. Our data support Lee's prescient prediction; myostatin levels are increased in response to the increase in skeletal muscle mass induced by testosterone administration. The increments in myostatin levels were correlated with testosterone concentrations. Thus, testosterone administration increases muscle mass resulting in increased myostatin production and secretion; it is possible that the increased circulating myostatin levels in turn restrain unlimited growth of skeletal muscle in response to continued testosterone administration. Similarly, older men with lower skeletal muscle mass have lower myostatin levels than young men; one could speculate that aging is associated with loss of skeletal muscle mass, leading to decreased myostatin secretion, which in turn brakes further muscle loss. In separate studies, Shyu et al. (2005) reported that cyclic mechanical stretch upregulates IGF-1 as well as myostatin expression. The stretch-induced myostatin increase in cardiomyocytes is mediated by IGF-1 in part through MAP kinase and MEF2 pathway. In an accompanying editorial, Gaussin and Depre (2005) suggested that myostatin represents a chalone of IGF-1 pathway in the heart. IGF-1 induces cardiac muscle hypertrophy resulting in increased myostatin production that then checks further hypertrophy of the cardiac muscle.

The availability of a reliable assay for accurate measurement of myostatin levels provides an excellent opportunity to examine the physiologic regulation of circulating myostatin levels in healthy humans and in patients with clinical disorders associated with loss of skeletal muscle mass. In this study, under basal steady-state conditions, serum myostatin levels were not correlated with lean body mass; thus, myostatin levels may not be a good biomarker of skeletal muscle mass. However, observations that myostatin levels rise early during the course of testosterone-induced muscle mass accretion raise the possibility that myostatin levels might serve as a useful early biomarker for the anabolic effects of promyogenic therapies such as testosterone. This speculation needs further investigation.

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