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Ursula Moore
Newcastle University

Alan Pestronk
Washington University School of Medicine in St. Louis
et al.

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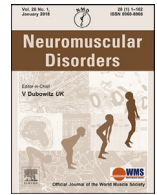
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Myostatin and follistatin as monitoring and prognostic biomarkers in dysferlinopathy

Ursula Moore^a, Esther Fernández-Simón^a, Marianela Schiava^a, Dan Cox^a, Heather Gordish-Dressman^{b,c}, Meredith K. James^a, Anna Mayhew^a, Ian Wilson^d, Michela Guglieri^a, Laura Rufibach^e, Andrew Blamire^d, Pierre G. Carlier^f, Madoka Mori-Yoshimura^g, John W. Day^h, Kristi J. Jonesⁱ, Diana X. Bharucha-Goebel^{j,k}, Emmanuelle Salort-Campana^l, Alan Pestronk^m, Maggie C. Walterⁿ, Carmen Paradas^o, Tanya Stojkovic^p, Elena Bravver^q, Elena Pegoraro^r, Jerry R. Mendell^s, Kate Bushby^a, Jordi Diaz-Manera^{a,t,u}, Volker Straub^{a,*}, The Jain COS Consortium

^a The John Walton Muscular Dystrophy Research Centre, Translational and Clinical Research Institute, Newcastle University and Newcastle Hospitals NHS Foundation Trust, Newcastle upon Tyne, UK

^b Center for Translational Science, Division of Biostatistics and Study Methodology, Children's National Health System, Washington, DC, USA

^c Pediatrics, Epidemiology and Biostatistics, George Washington University, Washington, DC, USA

^d Magnetic Resonance Centre, Translational and Clinical Research Institute, Newcastle University, Newcastle upon Tyne, UK

^e Jain Foundation, Seattle, WA, USA

^f University Paris-Saclay, CEA, DRF SHF, Orsay France

^g Department of Neurology, National Center Hospital, National Center of Neurology and Psychiatry, Tokyo, Japan

^h Department of Neurology and Neurological Sciences, Stanford University School of Medicine, Stanford, CA, USA

ⁱ The Children's Hospital at Westmead and The University of Sydney, Sydney, NSW, Australia

^j Department of Neurology, Children's National Health System, Washington, DC, USA

^k National Institutes of Health (NINDS), Bethesda, MD, USA

^l Service des maladies neuromusculaire et de la SLA, Hôpital de La Timone, Marseille, France

^m Department of Neurology, Washington University School of Medicine, St. Louis, MO, USA

ⁿ Friedrich-Baur-Institute, Department of Neurology, LudwigMaximilians-University of Munich, Munich, Germany

^o Neuromuscular Unit, Department of Neurology, Hospital U. Virgen del Rocío/Instituto de Biomedicina de Sevilla, Sevilla, Spain

^p Centre de reference des maladies neuromusculaires, Institut de Myologie, AP-HP, Sorbonne Université, Hôpital Pitié-Salpêtrière, Paris, France

^q Neuroscience Institute, Carolinas Neuromuscular/ALS-MDA Center, Carolinas HealthCare System, Charlotte, NC, USA

^r Department of Neuroscience, University of Padova, Padua, Italy

^s The Abigail Wexner Research Institute at Nationwide Children's Hospital, Columbus, OH, USA

^t Neuromuscular Disorders Unit, Neurology Department, Hospital de la Santa Creu I Sant Pau, Barcelona, Spain

^u Centro de Investigación Biomédica en Red en Enfermedades Raras (CIBERER), Madrid, Spain

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ABSTRACT

Myostatin is a myokine which acts upon skeletal muscle to inhibit growth and regeneration. Myostatin is endogenously antagonised by follistatin. This study assessed serum myostatin and follistatin concentrations as monitoring or prognostic biomarkers in dysferlinopathy, an autosomal recessively inherited muscular dystrophy. Myostatin was quantified twice with a three-year interval in 76 patients with dysferlinopathy and 38 controls. Follistatin was quantified in 62 of these patients at the same timepoints, and in 31 controls. Correlations with motor function, muscle fat fraction and contractile cross-sectional area were performed. A regression model was used to account for confounding variables. Baseline myostatin, but not follistatin, correlated with baseline function and MRI measures. However, in individual patients, three-year change in myostatin did not correlate with functional or MRI changes. Linear modelling demonstrated that function, serum creatine kinase and C-reactive protein, but not age,

* Corresponding author at: John Walton Muscular Dystrophy Research Centre, Translational and Clinical Research Institute, Newcastle University, Centre for Life, Newcastle upon Tyne, NE1 3BZ, UK.

E-mail address: volker.straub@newcastle.ac.uk (V. Straub).

were independently related to myostatin concentration. Baseline myostatin concentration predicted loss of ambulation but not rate of change of functional or MRI measures, even when relative inhibition with follistatin was considered. With adjustment for extra-muscular causes of variation, myostatin could form a surrogate measure of functional ability or muscle mass, however myostatin inhibition does not form a promising treatment target in dysferlinopathy.

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1. Introduction

Dysferlinopathy, also known as limb girdle muscular dystrophy R2 (LGMDR2) and Miyoshi Myopathy (MM), is a recessively inherited form of muscular dystrophy characterised by progressive muscle weakness and wasting [1–3]. Despite always being caused by mutations in the *DYSF* gene, the age of onset of muscle weakness (symptom onset) and rate of deterioration of muscle strength varies significantly between patients. In general, patients first present with weakness late in the 2nd or early 3rd decade, but onset can be as late as the 7th decade of life [4]. While some patients progress rapidly, requiring a wheelchair after 10 years of symptoms, others remain ambulant throughout their life [5].

This variability creates challenges for both monitoring and predicting disease progression. Detecting change over time in the more slowly progressing patients requires either very sensitive outcome measures, large cohorts or long periods of follow-up. Functional outcome measures in dysferlinopathy are capable of detecting change over a 6 months period, but require large cohorts of patients to do so [6]. Quantitative muscle MRI potentially forms a more sensitive measure of disease progression, but comes with its own limitations of cost and difficulty in standardisation across multiple sites [7].

Serum proteins which correlate with function or muscle pathology could theoretically be used as biomarkers of disease progression, while those that predict subsequent functional decline could be used for prognosis [8]. As quantitative measures, serum biomarkers could provide both sensitivity and reproducibility and therefore be a useful outcome measure for multi-site clinical trials. Biomarkers have been investigated in dysferlinopathy previously, with markers of muscle damage such as serum creatine kinase M-type (CK), being higher in patients than controls [9]. However, these markers did not perform well in monitoring disease progression, showing a poor correlation with function in patients [9].

Myostatin, also known as growth and differentiation factor 8 (GDF8), is part of the TGF- β family of cytokines [10] and has been proposed as a biomarker for disease progression [11] or for the increase in muscle in response to emerging pharmacological therapies [12]. Myostatin is predominantly produced by skeletal muscle [13] and is reduced in patients with several inherited muscle wasting diseases including Duchenne muscular dystrophy (DMD), LGMDR2 and spinal muscular atrophy [14,15]. In cross-sectional analysis, serum myostatin levels correlate with functional ability in muscular dystrophies, [14,15] and with muscle mass in sarcopenia [14,15]. However, correlation with function and muscle mass has not previously been assessed longitudinally in dysferlinopathy. Although a lower muscle mass is generally associated with lower myostatin levels, [16] a longitudinal correlation of deteriorating muscle mass or function with decreased myostatin concentration is not a given, because myostatin production is not solely correlated with muscle mass. Myostatin is also reduced acutely in response to injury [17] or long term exercise [18], allowing muscle growth, repair and exercise

adaptation, and increased as a short term response to exercise, [18] high glucose and insulin levels [10], and in long term systemic conditions [10,19,20].

Theoretically, myostatin also holds promise as a prognostic biomarker in muscular dystrophies. Myostatin binds to activin type IIB and TGF- β type 1 receptors in muscle and leads to inhibition of muscle growth through downregulation of structural genes, and increased muscle degradation by increasing activity of the ubiquitin-proteasome pathway (UPP) [10]. Exogenous myostatin leads to muscle wasting in animal models, while a complete lack of myostatin results in excessive hypertrophy in both animal and human muscles [21,22]. Inhibition of myostatin, including using exogenous follistatin, results in muscle growth and improved functional ability in animal models of muscular dystrophy [23–26]. In clinical trials in muscular dystrophy, myostatin inhibition has led to increased muscle mass, although this has not translated to stabilisation in functional ability [23]. If extended to endogenous myostatin levels, it is plausible that individuals with a high endogenous myostatin concentration, relative to others of the same functional ability or muscle mass, may lose muscle and deteriorate functionally more rapidly.

Follistatin is the endogenous antagonist of myostatin and, as such, influences the relative myostatin activity within muscle [27]. While synthetic forms of follistatin have been investigated as myostatin inhibitors, [23–26] endogenous follistatin has been less widely studied. Mariot et al. demonstrated an upregulation of follistatin expression in patients with DMD but not Becker or fascioscapulohumeral muscular dystrophy (FSHD), [14] but follistatin has not previously been quantified in patients with dysferlinopathy.

This study reports a longitudinal analysis of circulating myostatin and follistatin concentration in 76 and 62 patients with dysferlinopathy respectively. Linear mixed modelling of myostatin concentration with functional outcome measures, leg muscle fat fraction and contractile cross-sectional area is performed, including covariates of serum CRP, CK levels and age. The utility of myostatin concentration in predicting loss of ambulation and magnitude of functional progression is also assessed.

2. Methods

2.1. Subjects

Patient samples were collected as part of the Jain Clinical Outcome Study in Dysferlinopathy (Jain COS), [5] where they were donated to The John Walton Muscular Dystrophy Research Centre (JWMDRC) Biobank [28]. This study included annual blood draws over three to five years, although these were not mandatory. Clinical patient information including ambulation status, objective functional outcome measures of the North Star assessment for limb girdle type muscular dystrophies (NSAD score) [29] and time to walk/run 10 m (10MWR), T1-weighted (T1w) and Dixon muscle MRI, CRP and CK levels obtained at the time of the study visit were accessed from study records. Not all patients completed every assessment at each visit and numbers (n) are listed in Tables. There

was no significant difference in age, sex or baseline NSAD score between those who had or had not completed all assessments (supplementary Table 1). Ambulant patients were defined as those able to complete the 10MWR, with usual walking aids or orthotics.

Subjects were included in this biomarker study if they had sufficient stored serum from two study visits at a three-year interval. As some patients had limited samples available, this allowed paired myostatin quantification in all 76 patients, but paired follistatin quantification in only 62 of these.

Control samples were matched for gender and age at first visit (\pm five years) with patient samples in a ratio of one control: two patients giving 38 myostatin controls and 32 follistatin controls.

2.2. Samples

Serum samples were obtained from the JWMDRC biobank [28] for 76 subjects with genetically confirmed dysferlinopathy and 38 age and gender matched healthy control subjects. Two samples, drawn at a three-year interval, were obtained for each patient and a single sample was obtained for each control subject. After collection, local samples were centrifuged and then serum was aliquoted and stored at -80°C until analysis. Samples obtained at international sites were aliquoted locally and then couriered to the JWMDRC biobank in frozen transport.

2.3. Ethical approval

All participants in the Jain COS project provided informed consent and the study was approved by ethical review boards in each country. The JWMDRC biobank [28] has ethical approval for storing and conducting research on collected samples (NHS Health Research Authority; North East – Newcastle & North Tyneside 1 Research Ethics Committee REC reference: 19/NE/0028). Access to samples was approved for patient and control samples by the Jain COS steering committee and the biobank access committee respectively.

2.4. Myostatin and follistatin quantification

Myostatin and follistatin levels were quantified using commercially available enzyme linked immunosorbent assays (ELISA) – myostatin/GDF8 (#DGDF80; R&D Systems Europe) and follistatin (#DFN00; R&D Systems Europe). A microplate reader was used to measure optical density (Varioskan™ LUX multimode microplate reader, Thermo Scientific™). A common control was measured on each plate to allow for data standardisation between plates.

2.5. MRI

Muscle fat fraction (FF) and muscle cross sectional area (CSA) were calculated from regions of interest (ROIs) within the following muscles of the thigh and leg bilaterally: adductor magnus, vastus lateralis, vastus intermedius, vastus medialis, semitendinosus, semimembranosus, biceps femoris, gracilis, sartorius, extensor digitorum, gastrocnemius lateralis, gastrocnemius medialis, peroneus, soleus, tibialis anterior and tibialis posterior as previously reported [30]. In summary, using a free software tool (www.itksnap.org), ROIs for each muscle were drawn manually by a single investigator (I.W.) avoiding other muscles, subcutaneous and intermuscular fat, tendons and major blood vessels. Each ROI was drawn in the same five central slices on the shortest echo time (TE) image of the MSE series. Dixon images were used to compute the FF and CSA values as previously described [30].

A mean FF value for the lower limb was calculated by summing the FF of each muscle bilaterally and dividing by 30 (the total number of muscles imaged). Contractile CSA (cCSA) was calculated using the FF and CSA values using $cCSA = (1 - FF_{\text{mean}}) * CSA$. This was then summed to give a ‘total cCSA’ value representative of the contractile area of the thigh and leg.

Muscle MRI was not a mandatory component of the Jain COS project and complete lower limb FF and cCSA data were available for 41 of the myostatin patients at baseline and 20 at both baseline and year 3.

2.6. Statistical analysis

The 10MWR was converted to a velocity in m/s for all analysis.

2.6.1. Cross-sectional analysis

Myostatin and follistatin concentrations were not normally distributed. Concentration comparisons between controls, ambulant and non-ambulant patients at the first time point (baseline) were therefore compared using a Wilcoxon-Mann-Whitney test. Concentrations in patients at baseline were assessed for Spearman correlation with age, disease duration, NSAD score, 10MWR, FF, cCSA, CK and CRP. Correlations were considered significant if the p value was less than 0.05. Correlation coefficient (r) is provided for each correlation for the cohort as a whole and split into male and female subgroups. Correlations were considered strong if $r > 0.8$ and moderate if $r > 0.6$. In controls, correlations between concentration and age were calculated while gender differences were compared using a Wilcoxon-Mann-Whitney test.

2.6.2. Longitudinal analysis

The influence of age and disease duration on changes in myostatin concentration were assessed using a linear model of the form myostatin concentration \sim functional score + age/disease duration + sex + 1|person. This demonstrated that age, disease duration and sex were not independently related to myostatin concentration when function was also considered. This was repeated with FF and cCSA in place of functional scores. Therefore, functional and MRI measures, but not age and disease duration were included in further modelling.

Outcome measures and myostatin concentration were first assessed to determine if they changed significantly over the 3-year follow up period in this cohort. A linear model of the form NSAD score \sim visit number + 1|person was fitted and B value estimates of the difference in score between visits were reported, along with their p values. This was repeated for 10MWR, FF and cCSA. Accuracy of modelling was checked using a mixed model with robust errors to account for minor departures from normality. Results between the two models did not differ significantly and results from the original model are presented. For each participant, change scores for each assessment were calculated using: year 3 result – baseline result. Myostatin change scores were correlated with change in NSAD score, change in the 10MWR, change in FF and change in cCSA, CK and CRP using a Spearman correlation coefficient. Correlations were performed for the cohort as a whole and individually for each sex. In order to assess the cause of variability in longitudinal myostatin concentration, a linear mixed model of the form myostatin concentration \sim NSAD score + Creatine kinase + CRP + sex + 1|person was fitted. Accuracy of modelling was again assessed using a mixed model with robust errors.

2.6.3. Prognostic capability

Two approaches were used to assess the use of myostatin as a prognostic marker.

Survival analysis for time to loss of ambulation: In patients who were ambulant at the baseline visit ($n = 56$), the baseline myostatin concentration with the optimum sensitivity and specificity for distinguishing between those who retained vs lost ambulation over the next three-years was identified using receiver operating characteristic (ROC) curves. Survival analysis was then performed in these patients and the time to loss of ambulation was calculated for those with baseline myostatin concentrations A: above and B: below the ROC curve identified thresholds. In order to compare to outcome measures currently in use, this methodology was also completed to determine the performance of the baseline NSAD score in predicting loss of ambulation.

Linear model for predicted functional deterioration: Myostatin concentration is expected to decline with falling muscle mass and therefore comparing disease progression of those with a higher or lower than mean myostatin concentration risks simply comparing those early vs late in their disease course. As myostatin inhibits muscle growth and promotes degradation, [10] we hypothesised that individuals with a relatively high myostatin concentration for their muscle mass may progress more rapidly than those with the same muscle mass but a lower myostatin concentration. To investigate this, a linear model was created with baseline total cCSA against baseline myostatin concentration, thus giving the predicted myostatin concentration for any cCSA value. Baseline myostatin concentrations were then classified as being higher or lower than the value predicted by the linear model for the corresponding total cCSA in each individual. The magnitude of change in functional scores, FF and cCSA over 3 years was then compared between those with higher vs lower than predicted myostatin concentrations using a Wilcoxon-Mann-Whitney test.

All statistical analysis was conducted using RStudio version 3.6.2 (www.rstudio.com).

2.6.4. Sub-cohort analysis

Initial analysis identified that those with a symptom duration of 20 years or less showed greater variability in their myostatin concentration between visits, than those with symptoms for longer than 20 years (Fig. 2). It is possible that the relationship between myostatin concentration and function or MRI outcomes may be more evident in sub-cohorts showing larger variations in myostatin concentration. Therefore, longitudinal change and prognostic capability was also analysed separately in a sub-cohort consisting of 51 patients with symptom duration of 20 years or less at both visits.

3. Results

3.1. Subjects

Myostatin quantification was performed in 76 patients (32 male, 75% ambulant at baseline) with a median age of 36 years (range 16 – 67 years) and 38 controls (16 male) with a median age of 37 years (15 – 65 years). Follistatin quantification was performed in 62 of these patients (26 male, 77% ambulant at baseline) with a median age of 36 years (range 16–67) and 31 controls (13 male), with a median age of 38 years (range 15–65). Myostatin:follistatin ratio was therefore available in 62 patients and 31 controls. There was no significant difference (Wilcoxon-Mann-Whitney test >0.05) in age (37.5 years vs 35.5 years), symptom duration (13 years vs 15 years) or functional score (30.5 vs 20.5), fat fraction (34% vs 40%) or cCSA (7382mm² vs 8107mm²) at baseline between males and females.

3.2. Cross-sectional analysis

In a cross-sectional analysis at the first timepoint (baseline), serum myostatin concentration was lower in non-ambulant (median myostatin 540 pg/ml, IQR 374–736 pg/ml) than ambulant patients (median 1475 pg/ml, IQR 886 pg/ml – 1899pg/ml, $p <0.001$), and lower in ambulant patients than in controls (median, 2053 pg/ml, 1574 – 2529 pg/ml, $p <0.001$). Follistatin concentrations did not differ between groups (Fig. 1).

Myostatin concentrations were weakly negatively correlated with age in patients ($R = -0.41$, $p <0.001$), but not in controls ($R = -0.09$, $p > 0.05$). In controls, median myostatin concentrations were significantly higher in male than female participants (2271 pg/ml vs 1777 pg/ml, $p <0.05$), while in patients this sex difference was not statistically significant (male 1317pg/ml, female 899pg/ml, $p >0.05$). In patients, lower myostatin levels correlated moderately with lower functional ability (as measured by NSAD score and 10MWR), higher FF and lower cCSA (Table 1). When examined by sex, the correlation between myostatin and FF and cCSA was significant in females but not males (supplementary Table 2), although modelling using sex as a covariate showed no significant influence of sex on the relationship between myostatin concentration with FF or cCSA ($p >0.05$).

Follistatin concentrations at baseline did not correlate with age in patients ($R = 0.03$, $p >0.05$) or controls ($R = 0.30$, $p >0.05$) and did not correlate with functional or MRI outcome measures in patients (Table 1). Follistatin concentration did not differ between male and female controls or patients.

Myostatin:follistatin ratio at baseline correlated with declining functional ability, cCSA and increasing FF in patients, although with lower correlation coefficients than for myostatin alone. (Table 1)

3.3. Longitudinal assessment

All outcome measures, apart from cCSA, changed significantly in this cohort over the three-year follow up period (Table 2). When assessed by each sex group, changes in functional outcomes remained significant but changes in fat fraction were no longer significant in females, likely due to the smaller sample sizes (supplementary Table 4).

Myostatin concentrations did not fall significantly between baseline and year three either in the whole cohort (baseline median 1036 pg/ml, year 3 median 952pg/ml, $p > 0.05$) or in the subset of patients with symptoms for 20 years or less (1475pg/ml vs 1262pg/ml $p >0.05$). Variability in concentrations between visits was high (range –1472pg/ml to + 999pg/ml), particularly in patients with symptoms for 20 years or less (Fig. 2).

Change in myostatin concentrations between visits correlated with change in serum CK levels ($P = 0.34$ $p <0.01$) but not with change in functional assessments (10MWR and total NSAD score), muscle MRI (FF and cCSA) or with changing CRP levels (Table 2).

In a subgroup of patients with symptoms for 20 years or less, change in myostatin concentrations between visits did positively correlate with change in NSAD scores and CK levels, although correlation coefficients were low. Change in myostatin concentrations did not correlate with change in FF, cCSA, 10MWR or CRP in this subgroup (Table 2).

Changes in follistatin concentrations and in myostatin:follistatin ratios did not correlate with changes in any variables (supplementary Table 3).

Linear mixed modelling in the whole cohort demonstrated that the difference in myostatin concentration between time points in each individual was independently related to function as measured by total NSAD score, CK and CRP concentrations but not biological sex. A 1-point decrease in NSAD score was associated with a 23pg/ml decrease in myostatin concentration (standard error 4,

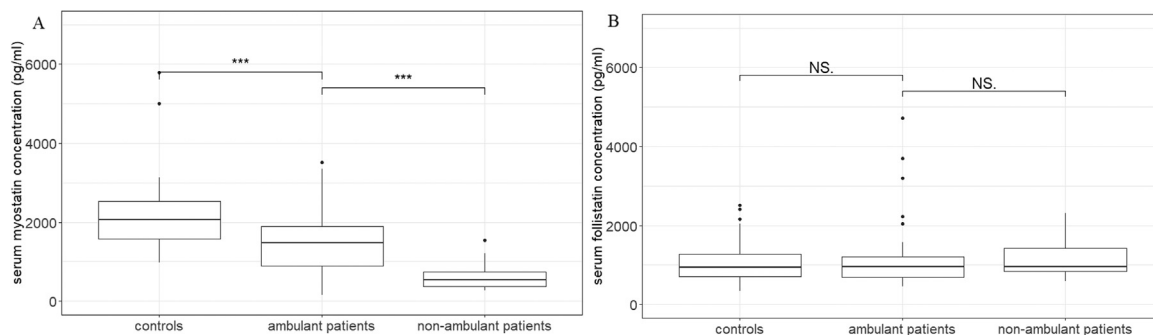


Fig. 1. Box plots displaying serum myostatin (a) and follistatin (b) concentrations at baseline in controls vs ambulant vs non-ambulant patients. Box plots show median concentration (central line), interquartile range (within the box), range (whiskers) and outliers (single points).*/NS denote if there is a significant difference between myostatin concentration using Wilcoxon-Mann-Whitney test. ****p* < 0.001, NS - not significant.

Table 1

Table shows the median baseline value and the spearman correlation with the quantified biomarkers (myostatin, follistatin and myostatin:follistatin) for functional, biochemical and MRI parameters in dysferlinopathy patients. NSAD score – North star assessment for limb girdle type muscular dystrophy, 10MWR – velocity to run or walk 10 m, CRP – C-reactive protein, CK – creatine kinase, cCSA – contractile cross-sectional area, FF – fat fraction. Correlation coefficient is the Person correlation coefficient. Bold values are significant correlations and p-value is denoted using ns = not significant, * < 0.05, ** < 0.01, *** < 0.001. *n* = the number of patients involved in each analysis.

Variable	Median value	Correlation with baseline myostatin (pg/ml)		Correlation with baseline follistatin (pg/ml)		Correlation with baseline myostatin:follistatin	
		Correlation coefficient	<i>n</i>	Correlation coefficient	<i>n</i>	Correlation coefficient	<i>n</i>
baseline variables							
Myostatin (pg/ml)	1036	1.00***	76	−0.21ns	62	0.81***	62
Follistatin (pg/ml)	955	−0.21ns	62	1.00***	62	−0.68***	62
Myostatin:follistatin (pg/ml)	1.3	0.81***	62	−0.68***	62	1***	62
Age (yrs)	36	−0.41***	76	0.03ns	62	−0.16ns	62
symptom duration (yrs)	14	−0.56***	71	−0.01ns	59	−0.37**	59
Total NSAD score	27.5	0.56***	56	−0.14ns	47	0.44**	47
10MWR (m/s)	1.24	0.55***	52	−0.21ns	43	0.46**	43
CRP (mg/l)	5	−0.35**	70	0.35**	57	−0.38**	57
CK (IU)	3176	0.65***	70	0.06ns	58	0.37**	58
Mean FF (%)	35	−0.63***	41	0.44**	38	−0.66***	38
Lower limb cCSA (mm ²)	7773	0.64***	41	−0.35*	38	0.64***	38

Table 2

Table shows the median values at baseline and year 3 for biochemical, functional and MRI imaging parameters for patients where paired assessments were available (*n*). This is fewer patients than had baseline assessments alone and therefore baseline median values in this group differ slightly from Table 1. Spearman correlation between 3-year change in functional, biochemical and MRI parameters and 3-year change in myostatin concentration. NSAD score – North star assessment for limb girdle type muscular dystrophies, 10MWR –velocity to run or walk 10 m, CRP – C-reactive protein, CK – creatine kinase, cCSA – contractile cross-sectional area, FF – fat fraction. Bold values show a significant change between baseline and year 3 assessments or show significant correlations between change in myostatin concentration and change in listed variable. p-value is denoted using * < 0.05, ** < 0.01, *** < 0.001. *n* = the number of patients involved in each analysis.

variable	Baseline median	Year 3 median	Correlation between change in variable and change in myostatin concentration over 3 years	<i>n</i>
All patients				
myostatin (pg/ml)	1036	952	1.00***	76
follistatin (pg/ml)	955	1079	−0.05	62
NSAD score	28	21***	0.22	53
10MWR (m/s)	1.37	1.24***	0.01	43
cCSA (mm ²)	9457	8845	0.18	20
FF (%)	30	37***	−0.38	20
CRP (mg/l)	5	5	0.02	67
CK (IU)	3162	2729**	0.39**	67
Symptom duration ≤ 20 years				
Myostatin (pg/ml)	1475	1262	1.00***	
follistatin (pg/ml)	1003	1061	−0.01	46
NSAD score	30	21.5***	−0.33*	44
10MWR (m/s)	1.35	1.12***	0.13	43
cCSA (mm ²)	9946	9458	0.23	17
FF (%)	30	37**	−0.33	17
CRP (mg/l)	5	5	−0.02	46
CK (IU)	4191	3596*	0.39**	45

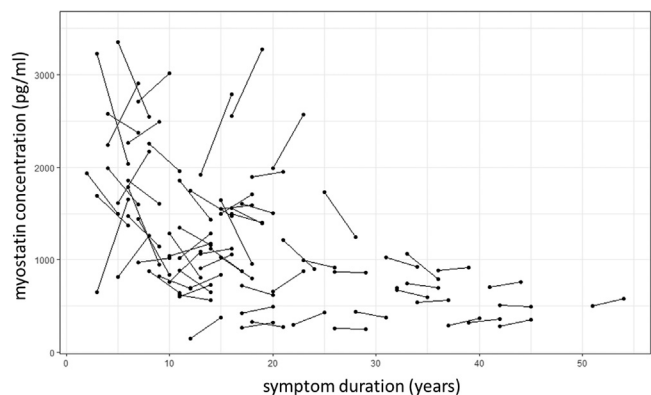


Fig. 2. Change in myostatin concentration over time
This graph displays each patient's myostatin concentration at baseline assessment and 3 years later plotted against symptom duration. Samples from the same patient are connected by a solid line. Symptom duration was calculated as: patient age at time of assessment – patient recalled age of symptom onset (i.e. age at onset of muscle weakness).

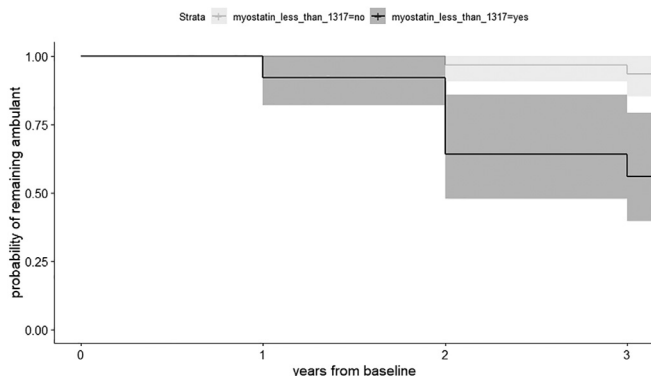


Fig. 3. Loss of ambulation based on baseline myostatin ($n = 56$)
Chart shows a survival analysis of patients who were ambulant at their baseline visit ($n = 56$). The cohort is split into those with a myostatin concentration of more than or equal to 1317pg/ml at baseline (in red, $n = 31$) or less than 1317 pg/ml at baseline (blue, $n = 25$). The coloured boxes around the survival line denote the confidence intervals of the survival probability. At 3 years, 11 of the lower myostatin group (blue) have lost ambulation but only 2 of the higher myostatin group (red) have lost ambulation. Significant difference between groups is shown by separation of the 95% confidence intervals (red and blue areas) and log rank chi squared 11.3, $p < 0.001$.

$p < 0.001$), 1 U/L decrease in CK with a decrease of 55pg/ml myostatin (se 16, $p < 0.001$) and 1 mg/l increase of CRP with a decrease of - 32pg/ml myostatin (se 14, $p < 0.05$).

3.4. Predicting loss of ambulation

Fifty seven patients were ambulant at the baseline visit, with 13 of these patients losing ambulation by the year 3 visit. ROC analysis demonstrated an optimum myostatin concentration of 1317pg/ml (sensitivity 88%, specificity 73%, ROC AUC 0.75, CI 0.61 – 0.90) for predicting loss of ambulation over three years. Of the 32 patients with myostatin levels below this threshold, 11 lost ambulation before the year 3 follow up visit, while only 2 of the 25 patients with myostatin concentration above this threshold lost ambulation in this time period (log rank chi squared 11.3, $p < 0.001$, Fig. 3). In one of these two patients with a myostatin concentration of over 1317pg/ml who lost ambulation by year 3, the myostatin concentration had fallen to below the 1317pg/ml threshold by the year 3 visit. When each sex was considered in turn, 1317pg/ml remained the optimum concentration in males (sensitivity 80%, specificity 78%, ROC AUC

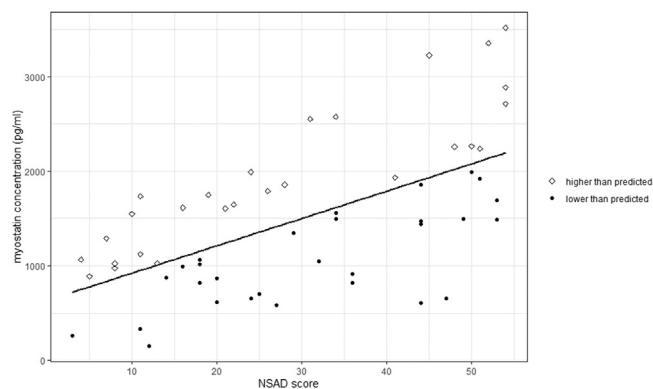


Fig. 4. Myostatin concentration at baseline correlates with NSAD score.
Figure shows the correlation between north star assessment for limb girdle type muscular dystrophy (NSAD) and myostatin concentration (pg/ml). The Spearman correlation coefficient is 0.56, $p < 0.001$. Individual patients whose myostatin concentration falls below this correlation line are shown in black and classified as having a 'lower than predicted' myostatin concentration for their NSAD. Those above the line are classed as 'higher than predicted'.

while the optimum concentration in females was 1254pg/ml (sensitivity 88%, specificity 60%, ROC AUC 0.72) (supplementary Figs. 3 and 4). However, although ROC analysis demonstrated a slightly lower optimum concentration in females, using a value of 1317pg/ml still achieved the same sensitivity and specificity as the 1254pg/ml value (88% and 60%) showing there was no significant difference in optimum values between each sex. Myostatin was less sensitive but similarly specific to baseline NSAD scores for predicting loss of ambulation, where ROC analysis demonstrated an optimum NSAD score of 27 (sensitivity 100%, specificity 74%) for predicting loss of ambulation over three years (Supplementary Fig. 1).

Six individuals crossed down and one individual crossed up over this threshold (1317pg/ml) at the year 3 visit. Compared to the rest of the cohort those crossing down over the threshold did not show greater changes in NSAD (median change -5 in these 6 individuals vs -5 in whole cohort, $p > 0.05$), 10MWR (-0.20 m/s vs -0.27 m/s, $p > 0.05$), FF (+12% vs +8%, $p > 0.05$) or cCSA (-2230 vs -794, $p > 0.05$) between visits. The individual who crossed up over the threshold had only been symptomatic for 3 years at the baseline assessment and was relatively strong with an NSAD score of 47 at baseline and 44 at year 3.

3.5. Predicting functional decline

Myostatin concentrations correlated consistently with NSAD score in both male and female patients in a cross-sectional analysis (Table 1). This correlation line was used to predict the estimated myostatin concentrations at a given NSAD score (Fig. 4).

Myostatin concentrations at baseline were higher than predicted based on NSAD score in 27 and lower than predicted in 29 of the 56 patients for whom baseline NSAD data was available (Fig. 4).

Patients with a higher than predicted myostatin concentration did not differ in disease progression over three years compared to those with a lower concentration, with no significant difference ($p > 0.05$) between median deterioration in NSAD score (-5 points in higher myostatin group and -4.5 in lower myostatin group), velocity to walk 10 m (-0.28 m/s vs -0.34 m/s), FF (+6.2% vs + 8.5%) or cCSA (-193mm² vs -854mm²) between groups (supplementary Fig. 2). When repeated for male and female sub cohorts individually there was still no significant difference between median deterioration in NSAD, velocity to walk 10 m,

FF or cCSA between higher and lower than predicted myostatin groups (supplementary Table 5).

Myostatin:follistatin ratios at baseline were higher than predicted based on NSAD score in 21 and lower than predicted in 26 of the 47 patients for whom baseline NSAD score and follistatin was available. Patients with a higher than predicted myostatin:follistatin ratio did not differ in disease progression over 3 years compared to those with a lower ratio, with no difference between median deterioration in NSAD score (-5.5 vs -5), velocity to walk 10 m (-0.36 m/s vs -0.22 m/s), FF ($+9\%$ vs $+8\%$) or cCSA (-194mm^2 vs -1261mm^2) between groups. When repeated for male and female sub cohorts individually there was still no significant difference between median deterioration in NSAD, velocity to walk 10 m, FF or cCSA between higher and lower than predicted myostatin:follistatin groups (supplementary Table 5).

4. Discussion

This study assessed circulating myostatin and follistatin concentrations in patients with dysferlinopathy and demonstrates that myostatin holds promise as a monitoring biomarker.

Myostatin concentrations fell with functional and muscle MRI measures of disease progression. This allowed myostatin to differentiate between healthy controls, ambulant and non-ambulant patients and to identify those patients whose motor function had fallen to such a degree that they would soon lose ambulation. These findings build on previous work showing lower myostatin concentrations in patients with muscular dystrophies [14,15] and positions myostatin as one of the strongest monitoring biomarkers so far identified in dysferlinopathy. While multiple other proteins have been identified as elevated in patients with dysferlinopathy compared to healthy controls, [9,31] these biomarkers have either not been assessed with [31] or do not correlate with functional scores such as the North Star ambulatory assessment (NSAA), [9] with the exception of a weak correlation ($r = 0.36$, $p = 0.045$) between myosin light chain 3 and the 10 m walk/run test [9].

Importantly, we also demonstrated that change in paired myostatin concentration correlated with change in NSAD score in a subgroup of patients with symptoms for less than 20 years. This longitudinal correlation with change in motor function on an individual patient level is necessary for using a biomarker as a surrogate endpoint, but it has so far been elusive in other biomarker investigations in muscular dystrophies generally. In the more extensively investigated DMD for example, while multiple biomarkers have been identified which correlate with function in cross-section, these have not been shown to correlate within individual patients longitudinally [9,31–33].

The changes in myostatin concentration which we report were not uniform, with those early in their disease course (symptoms of 20 years or less) showing large changes in myostatin concentrations between visits, while myostatin concentrations plateaued in those with a longer duration of symptoms. Indeed, significant correlations between paired myostatin and paired NSAD were only observed in a sub-cohort of patients with a shorter disease duration, perhaps because this is when patients have more muscle mass to lose. This suggests that myostatin concentration would be most useful as a biomarker early in the disease and mimics other protein biomarkers in muscular dystrophies, which display a particular ‘window’ in which they correlate with functional ability [32].

However, myostatin was by no means a perfect monitoring biomarker and correlations between paired assessments and paired myostatin concentrations were weak, even in the sub-cohort of patients with symptom durations of 20 years or less. This suggests that there is variability in myostatin concentrations which is

not related to muscle mass or function. We demonstrated a negative association between CRP and myostatin concentration, which has also been described previously [34,35]. Myostatin is also reduced acutely in response to injury [17] and long term exercise [18]. Myostatin has previously been demonstrated to be increased as a short term response to exercise, [18] in response to high glucose and insulin levels [10] and is increased in long term systemic conditions including obesity and metabolic syndrome, [19] cancer cachexia, [10] advanced chronic obstructive pulmonary disease, [20] liver [36] and renal disease [37]. In this study, some of these variables were controlled for in the Jain COS protocol – with blood samples being collected before physiotherapy assessment and exclusion of patients with significant known comorbidities such as advanced cancer. However, patients were not required to have fasting blood samples or follow a specific exercise or rest regimen in the days before sampling and were not excluded on grounds of obesity or more minor co-morbidities. Further longitudinal research in patients and in healthy controls assessing myostatin concentration at different timepoints under different environmental conditions could help to elucidate the influence of these confounding variables more specifically. Similarly, investigation of myostatin concentrations in mouse models could allow a controlled manipulation of these environmental stressors to further improve our knowledge of these mechanisms. With additional understanding, more stringent inclusion criteria or statistical modelling may minimise inter-visit variability in a research setting, although this would not be readily generalisable into daily clinical practice.

We observed a different relationship between sex and myostatin concentrations in controls and patients. In the healthy controls in our study, males had higher myostatin concentrations than females. However, myostatin concentration was not different between male and female patients, even when controlling for function, CK and CRP nor did the relationship between myostatin and muscle function or mass differ between sexes. The male patients in our cohort did trend towards having a lower muscle mass and functional ability than female counterparts and trend towards a higher myostatin concentration, perhaps suggesting a tendency to relatively higher myostatin levels as we saw in the healthy control patients. However, it is also difficult to know what is expected of myostatin in healthy muscle and previous research investigating the relationship between gender, myostatin concentration and muscle mass has produced conflicting reports, with some reporting higher concentrations in males, and others in females and inconsistent reports of sex specific relationships with muscle mass [18,38]. Understanding the role of sex on myostatin, and specifically in disease conditions such as muscular dystrophy, may require more controlled laboratory based research.

We have shown that myostatin concentrations fall as disease progresses, which is likely related in part to the loss of muscle able to synthesise myostatin [15]. However, work by Mariot et al. has demonstrated reduced myostatin and myostatin receptor mRNA in the muscle of patients with DMD, leading to the hypothesis that the myostatin pathway is specifically downregulated in muscular dystrophies as a compensatory mechanism to limit muscle loss [14]. Mariot et al. suggest that myostatin inhibition therapies may so far have failed to succeed in the clinic [23,39–41] because endogenous levels of myostatin are already too low [14]. They suggest that some individuals with residual higher myostatin concentrations might form a subgroup of patients in whom these therapies may be effective. However, here we demonstrated that neither a lower than predicted myostatin concentration or relative myostatin antagonism (a lower baseline myostatin:follistatin ratio) was associated with slower disease progression in this cohort, suggesting that endogenous concentrations are not linked to differences in disease progression in dysferlinopathy. While it

is possible that samples taken on a single day were not representative enough of the average myostatin concentration to accurately classify into high or low myostatin groups, our results suggest that additional inhibition in those with higher myostatin concentrations would not be beneficial.

Myostatin concentrations were capable of predicting loss of ambulation and were equally specific (although less sensitive) as clinical assessments using a validated scoring system (NSAD score [29]). This suggests that myostatin quantification could conceivably be used to remotely screen for a cohort of patients likely to lose ambulation during the course of a 3-year clinical trial. Such a cohort may be appealing to trial planners, allowing a successful treatment to demonstrate a delay to loss of ambulation. However, it would first be important to further understand the non-muscle related variability to ensure appropriate sampling procedures.

6. Conclusion

This study quantified myostatin and follistatin in patients with dysferlinopathy. We demonstrated that myostatin could be useful surrogate measure for functional ability or muscle mass in dysferlinopathy and can be used to predict loss of ambulation, although clinical use would require further understanding of extra-muscular causes of variation. However, neither myostatin concentrations nor relative myostatin inhibition (myostatin:follistatin ratios) were predictive of subsequent rate of disease progression suggesting that myostatin inhibition would not be an effective treatment for dysferlinopathy.

Declaration of Competing Interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nmd.2023.01.001.

References

- [1] Bashir R, Strachan T, Keers S, Stephenson A, Mahjneh I, Marconi G, et al. A gene for autosomal recessive limb-girdle muscular dystrophy maps to chromosome 2p. *Hum Mol Genet* 1994;3(3):455–7.
- [2] Bejaoui K, Hirabayashi K, Hentati F, Haines JL, Hamida CB, Belal S, et al. Linkage of Miyoshi myopathy (distal autosomal recessive muscular dystrophy) locus to chromosome 2p12–14. *Neurology* 1995;45(4):768–72.
- [3] Moore U, Gordish H, Diaz-Manera J, James MK, Mayhew AG, Guglieri M, et al. Miyoshi Myopathy and Limb Girdle Muscular Dystrophy R2 are the same disease. *Neuromuscul Disord* 2021.
- [4] Klinge L, Dean AF, Kress W, Dixon P, Charlton R, Müller JS, et al. Late onset in dysferlinopathy widens the clinical spectrum. *Neuromuscul Disord* 2008;18(4):288–90.
- [5] Harris E, Bladen CL, Mayhew A, James M, Bettinson K, Moore U, et al. The Clinical Outcome Study for dysferlinopathy: an international multicenter study. *Neurol Genet* 2016;2(4):e89–e.
- [6] Moore U, Jacobs M, James MK, Mayhew AG, Fernandez-Torrón R, Feng J, et al. Assessment of disease progression in dysferlinopathy: a 1-year cohort study. *Neurology* 2019;92(5):e461–e474.
- [7] Carlier PG, Marty B, Scheidegger O, Loureiro de Sousa P, Baudin P-Y, Snehzko E, et al. Skeletal Muscle Quantitative Nuclear Magnetic Resonance Imaging and Spectroscopy as an Outcome Measure for Clinical Trials. *J Neuromuscul Dis* 2016;3(1):1–28.
- [8] Szigartyo CA, Spitali P. Biomarkers of Duchenne muscular dystrophy: current findings. *Degener Neurol Neuromuscul Dis* 2018;8:1–13.
- [9] Burch PM, Pogoryelova O, Goldstein R, Bennett D, Guglieri M, Straub V, et al. Muscle-derived proteins as serum biomarkers for monitoring disease progression in three forms of muscular dystrophy. *J Neuromuscul Dis* 2015;2(3):241–55.
- [10] Sharma M, McFarlane C, Kambadur R, Kukreti H, Bonala S, Myostatin SS. Expanding horizons. *IUBMB Life* 2015;67(8):589–600.
- [11] Koch C, Buono S, Menuet A, Robé A, Djeddi S, Kretz C, et al. Myostatin: a circulating biomarker correlating with disease in myotubular myopathy mice and patients. *Mol Ther Methods Clin Dev* 2020;17:1178–89.
- [12] Mariot V, Le Guiner C, Barthélémy I, Montus M, Blot S, Torelli S, et al. Myostatin is a quantifiable biomarker for monitoring pharmacogene therapy in Duchenne muscular dystrophy. *Mol Ther Methods Clin Dev* 2020;18:415–421.
- [13] atlas Hp. The Human Protein Atlas - MSTN 2021 [Available from: <https://www.proteinatlas.org/ENSG00000138379-MSTN/tissue>].
- [14] Mariot V, Joubert R, Houdé C, Féasson L, Hanna M, Muntoni F, et al. Downregulation of myostatin pathway in neuromuscular diseases may explain challenges of anti-myostatin therapeutic approaches. *Nat Commun* 2017;8(1):1859.
- [15] Burch PM, Pogoryelova O, Palandra J, Goldstein R, Bennett D, Fitz L, et al. Reduced serum myostatin concentrations associated with genetic muscle disease progression. *J Neurol* 2017;264(3):541–53.
- [16] Alexopoulos T, Vasilieva L, Kontogianni MD, Tenta R, Georgiou A, Stroumpouli E, et al. Myostatin in combination with creatine phosphokinase or albumin may differentiate patients with cirrhosis and sarcopenia. *Am J Physiol Gastrointest Liver Physiol* 2021;321(5):G543–G51.
- [17] Åkerfeldt T, Helmersson-Karlqvist J, Gunningberg L, Swenne CL, Larsson A. Postsurgical acute phase reaction is associated with decreased levels of circulating myostatin. *Inflammation* 2015;38(4):1727–30.
- [18] Baczek J, Silkiewicz M, Wojszel ZB. Myostatin as a biomarker of muscle wasting and other pathologies-state of the art and knowledge gaps. *Nutrients* 2020;12(8):2401.
- [19] Carvalho LP, Basso-Vanelli RP, Di Thommazo-Luporini L, Mendes RG, Oliveira-Junior MC, Vieira RP, et al. Myostatin and adipokines: the role of the metabolically unhealthy obese phenotype in muscle function and aerobic capacity in young adults. *Cytokine* 2018;107:118–24.
- [20] Ju CR, Chen RC. Serum myostatin levels and skeletal muscle wasting in chronic obstructive pulmonary disease. *Respir Med* 2012;106(1):102–8.
- [21] Schuelke M, Wagner KR, Stolz LE, Hübner C, Riebel T, Kömen W, et al. Myostatin mutation associated with gross muscle hypertrophy in a child. *N Engl J Med* 2004;350(26):2682–8.
- [22] McPherron AC, Lawler AM, Lee SJ. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* 1997;387(6628):83–90.
- [23] Rybalka E, Timpani CA, Debruin DA, Bagaric RM, Campelj DG, Hayes A. The Failed Clinical Story of Myostatin Inhibitors against Duchenne Muscular Dystrophy: exploring the Biology behind the Battle. *Cells* 2020;9(12).
- [24] St Andre M, Johnson M, Bansal PN, Wellen J, Robertson A, Opsahl A, et al. A mouse anti-myostatin antibody increases muscle mass and improves muscle strength and contractility in the mdx mouse model of Duchenne muscular dystrophy and its humanized equivalent, domagrozumab (PF-06252616), increases muscle volume in cynomolgus monkeys. *Skelet Muscle* 2017;7(1):25.
- [25] Bogdanovich S, Krag TO, Barton ER, Morris LD, Whittemore LA, Ahima RS, et al. Functional improvement of dystrophic muscle by myostatin blockade. *Nature* 2002;420(6914):418–21.
- [26] Iskenderian A, Liu N, Deng Q, Huang Y, Shen C, Palmieri K, et al. Myostatin and activin blockade by engineered follistatin results in hypertrophy and improves dystrophic pathology in mdx mouse more than myostatin blockade alone. *Skelet Muscle* 2018;8(1):34.
- [27] Follistatin PK. *Int J Biochem Cell Biol* 1998;30(10):1087–93.
- [28] Reza M, Cox D, Phillips L, Johnson D, Manoharan V, Grieves M, et al. MRC Centre Neuromuscular Biobank (Newcastle and London): supporting and facilitating rare and neuromuscular disease research worldwide. *Neuromuscul Disord* 2017;27(11):1054–64.
- [29] Jacobs MB, James MK, Lowes LP, Alfano LN, Eagle M, Muni Lofra R, et al. Assessing dysferlinopathy patients over three years with a new motor scale. *Ann Neurol* 2021.
- [30] Reyngoudt H, Smith F.E., Araujo E.C.A., Wilson I., Torron R.F., James M., et al. Three-year quantitative MRI and 31P MRS study in lower limb muscle in dysferlinopathy. under review. 2021.
- [31] Choi YC, Hong JM, Park KD, Shin HY, Kim SM, Park HJ. Proteomic analysis of the skeletal muscles from dysferlinopathy patients. *J Clin Neurosci* 2019.
- [32] Strandberg K, Ayoglu B, Roos A, Reza M, Niks E, Signorelli M, et al. Blood-derived biomarkers correlate with clinical progression in Duchenne muscular dystrophy. *J Neuromuscul Dis* 2020;7(3):231–46.
- [33] Signorelli M, Ebrahimipour M, Veth O, Hettne K, Verwey N, García-Rodríguez R, et al. Peripheral blood transcriptome profiling enables monitoring disease progression in dystrophic mice and patients. *EMBO Mol Med* 2021;13(4):e13328.
- [34] Planella-Farrugia C, Comas F, Sabater-Masdeu M, Moreno M, Moreno-Navarrete JM, Rovira O, et al. Circulating irisin and myostatin as markers of muscle strength and physical condition in elderly subjects. *Front Physiol* 2019;10:871.
- [35] Skladany L, Koller T, Molcan P, Vnencakova J, Zilincan M, Jancekova D, et al. Prognostic usefulness of serum myostatin in advanced chronic liver disease: its relation to gender and correlation with inflammatory status. *J Physiol Pharmacol* 2019;70(3).

- [36] García PS, Cabbabe A, Kambadur R, Nicholas G, Csete M. Brief-reports: elevated myostatin levels in patients with liver disease: a potential contributor to skeletal muscle wasting. *Anesth Analg* 2010;111(3):707–9.
- [37] Zhang L, Rajan V, Lin E, Hu Z, Han HQ, Zhou X, et al. Pharmacological inhibition of myostatin suppresses systemic inflammation and muscle atrophy in mice with chronic kidney disease. *FASEB J* 2011;25(5):1653–63.
- [38] Choi SJ, Lee MS, Kang DH, Ko GJ, Lim HS, Yu BC, et al. Myostatin/Appendicular Skeletal Muscle Mass (ASM) ratio, not myostatin, is associated with low handgrip strength in community-dwelling older women. *Int J Environ Res Public Health* 2021;18(14).
- [39] Lee YS, Lehar A, Sebald S, Liu M, Swaggart KA, Talbot CC Jr, et al. Muscle hypertrophy induced by myostatin inhibition accelerates degeneration in dysferlinopathy. *Hum Mol Genet* 2015;24(20):5711–19.
- [40] Kramerova I, Marinov M, Owens J, Lee SJ, Becerra D, Spencer MJ. Myostatin inhibition promotes fast fibre hypertrophy but causes loss of AMP-activated protein kinase signalling and poor exercise tolerance in a model of limb-girdle muscular dystrophy R1/2A. *J Physiol* 2020;598(18):3927–39.
- [41] Leung DG, Bocchieri AE, Ahlawat S, Jacobs MA, Parekh VS, Braverman V, et al. A phase Ib/IIa, open-label, multiple ascending-dose trial of domagrozumab in fukutin-related protein limb-girdle muscular dystrophy. *Muscle Nerve* 2021;64(2):172–9.