Research Article

Fibronectin is a serum biomarker for Duchenne muscular dystrophy

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Purpose: To identify and validate serum biomarkers for the progression of Duchenne muscular dystrophy (DMD) using a MS-based bottom-up pipeline.

Experimental design: We used a bottom-up proteomics approach, including a protein concentration equalization step, different proteolytic digestions, and MS detection schemes, to identify candidate biomarkers in serum samples from control subjects and DMD patients. Fibronectin was chosen for follow-up based on the differences in peptide spectral counts and sequence coverage observed between the DMD and control groups. Subsequently, fibronectin levels were determined with ELISA in 68 DMD patients, 38 milder Becker muscular dystrophy patients, 33 patients with other neuromuscular disorders, and 15 age-matched adult and child controls.

Results: There was a significant increase in fibronectin levels in DMD patients compared to age-matched controls. Fibronectin levels in patients with Becker muscular dystrophy, Bethlem myopathy, or myasthenia gravis were comparable to control levels. Progressive elevation in fibronectin levels was observed in longitudinal samples from 22 DMD patients followed up for a period of 6 months up to 4 years.

Conclusion and clinical relevance: This study suggests that serum fibronectin levels may constitute a promising biomarker to monitor disease progression in DMD patients.

Keywords: Biomarker / Duchenne muscular dystrophy / Fibronectin / Spectral counting

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1 Introduction

Biomarker discovery efforts have intensified over the past years, inspired by the utility of biomarkers for diagnosti-
Biomarkers are particularly important for monitoring disease progression and therapeutic efficacy in rare disorders, where cohort sizes are small and clinical phenotypes can be highly variable. This is true for Duchenne muscular dystrophy (DMD), which is a lethal X-linked muscular disease associated with muscle wasting and weakness due to muscles that are more sensitive to damage during contraction [6]. A milder form of the disease, Becker muscular dystrophy (BMD), has a later onset and a much longer survival [7]. Lacking molecular biomarkers, the 6-min-walk test is a commonly used clinical outcome measure to assess disease progression in individual boys and to assess a therapeutic effect in clinical trials in DMD [8–10]. High CK (creatine kinase) levels are an early sign of the disease. Unfortunately, CK is not a good biomarker to monitor disease progression, since CK levels vary largely from day to day, are influenced by activity, and decrease in later stages of the disease due to significant muscle loss [11].

There is no cure for DMD and corticosteroids represent the only palliative treatment able to slow down disease progression [12]. To evaluate new therapeutic compounds, non-invasive biomarkers correlating with disease progression are highly desired, since for most clinical trials invasive muscle biopsies are required to perform histological assessment and molecular analyses. Additionally, a single biopsy may not be representative of the general condition of the muscles. Blood biomarkers that give a general picture of the pathology, correlate with disease progression, and allow therapeutic monitoring, would therefore be very valuable for future trials in DMD patients. We described earlier matrix metalloproteinase (MMP)-9 as a biomarker for DMD disease progression [13]. However, it is unlikely that a single biomarker would give sufficient insight in the complex spectrum of pathophysiological events in a multifaceted disease like DMD. Therefore, unbiased biomarker discovery approaches are invaluable. Mouse models for DMD have been exploited for serum biomarker discovery [14, 15], but the relevance of the identified biomarkers for human patients is yet unclear.

Protein sequencing by MS/MS has evolved into a widely applied and standardized method for biomarker discovery [16, 17]. The bottom-up approach involves the digestion of proteins by specific enzymes followed by MS/MS-analysis of the resulting peptides, usually after online separation with LC. Most fragmentation studies of peptides are based on CID, in which neutral gas molecules induce dissociation of the amide bonds in the peptide backbone (b/y-fragmentation) [18]. There is a growing interest in electron-based approaches to fragment peptides in general, and great benefits have been reported for those carrying PTMs [19–21]. The most popular one is electron transfer dissociation (ETD), in which fragmentation is induced after “addition” of an electron to a multiply charged peptide through a fluoranthene charge carrier [20]. The resulting peptide radical is unstable and undergoes fragmentation with preferred peptide backbone dissociation between the nitrogen and the alpha-carbon (c/z-fragmentation). Application of two complementary fragmentation methods may result in improved peptide confidence levels and detection of increased numbers of peptides and numbers of distinct proteins [22].

Comparative discovery MS-based proteomics pipelines comparing disease and control samples are powerful tools for the identification of new disease biomarkers [23]. MS/MS-based approaches allow for reliable protein identification. This is of particular importance in biomarker research for clinical and diagnostic applications, where selected proteins subsequently need to be analyzed with validated assays across large patient cohorts. In biomarker discovery studies, it is also important to quantify relative differences between samples, for which several protein-labeling approaches are available [24]. However, label-free approaches are an alternative to costly labeling techniques and involve a less tedious sample work-up, allowing measurement of significant changes within complex mixtures in a single experiment. In label-free approaches, the spectral count for a protein, that is, the sum of all MS/MS spectra for a protein, has been shown to be a reliable semiquantitative estimate of the relative abundance of a protein [25–27].

The top hit from our semiquantitative biomarker discovery approach, comparing serum samples from DMD patients and control individuals, was fibronectin. Fibronectin was further validated by targeted immunoassays in larger and longitudinal DMD cohorts. To address its specificity for DMD, we studied fibronectin levels in patients affected by other myopathies.

2Materials and methods

2.1Study participants

Blood was taken from DMD, BMD, Bethlem myopathy (BM), myasthenia gravis (MG), and control groups. Three to five microliters of venous blood was drawn and placed in BD-Vacutainer (367614) tubes. Blood was allowed to clot at room temperature for 30 min to 8 h before spinning at 2800 × g at room temperature for 10 min. Serum supernatant was carefully removed, aliquotted, and stored at −80°C pending use. Sixty-eight male patients with a genetic diagnosis of DMD (mean age 12.5, age range between 2.9 and 31.2 years) were recruited for this study in the academic hospitals of Newcastle and Leiden. Twenty-two DMD boys were followed up in a longitudinal study involving blood collection at various time intervals. Thirty-eight BMD patients older than 18 years were selected from Newcastle and Leiden (mean age 42.4, age range between 19 and 67 years). We also included in the study 13 BM patients (mean age 28.4, age range 0.4–62.2 years) and 19 MG patients (mean age 31.7, age range 11.3–43.1 years). As part of the study, six adult male volunteers (mean age 33.3, age range 29.5–35.2 years), and ten male child controls (evaluated for urinary infection by renal isotope imaging; affected subjects were excluded from our study; mean age 8.7, age range 6.5–11.1 years) were recruited. None of the controls had significant medical disorders or systemic
medication. The study was approved by the local Research Ethics Committee prior to commencement. For the MS-based screens, we created four pools of four samples (adult controls, child controls, ambulant DMD, and nonambulant DMD).

2.2 MS analysis of serum samples

Mentioned pools of four samples were utilized for the bottom-up proteomics approach (Fig. 1). ProteoMiner™ (Bio-Rad, Hercules, CA, USA) beads were used to reduce the high dynamic range of the serum proteome. A mixture of 50 µL of serum and 100 µL of MilliQ water from Millipore (Bedford, MA, USA) was loaded on to 20 µL of beads in the ProteoMiner kit and further instructions from the manufacturer’s protocol were followed. The ProteoMiner eluates from the four different groups were fractionated on a Mini-Protean Tris-Glycine (TGX) 4–20% gradient precast gel (Bio-Rad) for which a prestained protein ladder 170 – 10 kDa (Fermentas, Germany) was used. The gel was stained for 1 h with 25 mL of SimplyBlue™ SafeStain (Invitrogen, USA). Lanes were cut into ten equidistant pieces. The ten gel pieces from each group were washed with a solution of 25 mM ammonium bicarbonate: 50% ACN (mixed 1:1 v/v) and repeated until the gel pieces were destained. Then, the pieces were alkylated with 50 µL of 50 mM DTT (Sigma-Aldrich, Steinheim, Germany) at 56°C for 45 min and reduced with 50 µL of 50 mM iodoacetamide (Sigma-Aldrich) in the dark for 1 h. The reduced gel pieces were kept for overnight digestion with 20 ng/µL of trypsin in 25 mM NH₄HCO₃ (Promega, USA) or with 12.5 ng/µL Lys-N in 50 mM NH₄HCO₃ (U-Protein Express BV, The Netherlands) in a water bath set to 37°C. The peptides were extracted from the gel pieces by addition of 15 µL extraction buffer (50% ACN, 0.1% formic acid) and agitation for 15 min. The last step was repeated three times. Each time the supernatant was collected and pooled and lyophilized. The lyophilized fractions were suspended in 20 µL of 0.1% formic acid (Sigma-Aldrich and separated using a reverse phase NanoLC-Ultra 2D plus UHPLC (Eksigent, CA, USA), equipped with Eksigent analytical columns (ChromXP C18, 0.3 mm id× 150 mm, 3 µm particle size), and precolumns from LC Packings (Acclaim Pepmap 300 C18, 300 µm id × 55 mm, 5 µm particle size). The mobile phases consisted of (A) 0.05% v/v formic acid in MilliQ water (Millipore) and (B) 0.05% v/v formic acid, 95% v/v ACN in MilliQ water (LC-MS grade, Fisher Scientific, USA). Peptide separations were carried out using a 120 min linear gradient, starting with 96% A and 4% B, and ending with 68% A and 32% B at a flow rate of 4 µL/min. The UHPLC was coupled to an Amazon ETD ion trap instrument (BrukerDaltonics). All samples were measured in positive ion mode. The LC system was controlled by HyStar software (version 3.4, Bruker Daltonics) with a plugin from the LC-manufacturer. The ion trap was controlled by EsquireControl software (version 7.0, BrukerDaltonics). The parameters used for measurements were capillary voltage 4500 V; plate offset 500 V; dry gas 3.0 L/min; dry temperature 220°C; Nebulizer 10 psi. Independent measurements for CID and ETD were obtained from each trypsin or Lys-N digested fraction. Exclusion limits were automatically placed on previously selected mass-to-charge ratios for 0.5 min. The ion trap MS/MS datasets acquired with CID and ETD fragmentation methods were processed using DataAnalysis software (version 3.3, BrukerDaltonics), that is, find compounds (AutoMS(n)) with deconvolution options. The peak lists were exported as a mascot generic file (mgf) and searched against the Human Swissprot protein sequence database using the Mascot search engine. The search parameters included carbamidomethylation as a fixed modification, with enzyme specificity either trypsin or Lys-N. Mass tolerances were set at 0.5 Da for MS and 1 Da for MS/MS; three missed cleavage sites were allowed and number of hits set to auto. For the Mascot search, “ETD trap” was used as “instrument type” to search ETD spectra and “ESI trap” to search CID spectra. Methionine oxidation was allowed as a variable modification. Proteins were assembled on the basis of peptide ion score >30 and probability p < 0.05. Decoy database option was considered to filter out false-positive hits. The Mascot search results were exported as dat files and then converted to pepXML file format using the MASCOT2XML tool in the Trans Proteomic Pipeline (TPP) [28]. PeptideProphet and ProteinProphet were used as a tool for identification and validation [28–31]. Next to the “standard” protein searches using the SwissProt database, spectral library searches were performed in which spectra were compared against a library of previously identified spectra categorized in PeptideAtlas [32]. Spectral library searching, advantageous for matching relatively low quality spectra in comparison to sequence search engines [33], was performed using the SpectraST tool within the TPP. The raw MS/MS datasets were converted into mzXML file format using CompassXport 3.0.4 (BrukerDaltonics). The mzXML files were searched against a consensus human plasma library of tryptic digest constructed from the 40 public datasets from PeptideAtlas. Only CID spectra from tryptic digests were searched against the human plasma library since no library is yet available for ETD datasets and Lys-N datasets. PeptideProphet software (TPP) was used to validate peptide identifications by calculating probabilities for correct peptide-to-spectrum matches. In this model deviations between observed masses and calculated masses were evaluated, as well as search scores, number of enzymatic termini, number of missed cleavages, and retention times. Peptide identifications were further validated using iProphet software (TPP). This algorithm assesses repeated discoveries of the same peptide ion or the same peptide sequence with a different charge state or modification, thereby rewarding or penalizing the peptide-to-spectrum matches. Model parameters includes the number of sibling peptides, the number of replicate spectra and the number of sibling ions. As a final step in the data analysis pipeline, protein-level validations were performed using ProteinProphet software (TPP). Here, protein identification probabilities were calculated based on the number
of unique peptides per protein where peptides with multiple siblings are given a higher probability while penalizing peptides without siblings. A protein inference analysis was performed to create the simplest list of proteins that can explain all the peptide observations [28]. Each protein is assigned a probability of being present in the sample. Proteins with probability between 1 and 0.8 were chosen for comparison. The spectral counting approach was used to compare relative protein abundance in the four different groups. This was achieved by comparing the number of identified MS/MS spectra from the same protein in each of the LC-MS/MS datasets.

2.3 Determination of fibronectin levels in serum samples

Levels of fibronectin were determined in independent cohorts using a commercially available ELISA kit (catalog number ECM300) purchased from Millipore. This kit uses the competitive enzyme immunoassay technique where the labeled antigen competes with the sample antigen for the binding to the primary antibody. All experiments were carried out in duplicates using the manufacturer’s protocols. For each individual 2 µL of serum were diluted in PBS at 1:10 000 ratio. The unknown concentrations were determined from the standard curve by plotting the log of concentrations on the X-axis and the mean of 450 nm absorbance on the Y-axis.

2.4 Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS), version 20 (IBM). For comparisons between means, a 2-tailed t-test was applied. Pearson correlation analysis was used to determine the correlation between serum fibronectin levels and clinical parameters like age and corticosteroid therapy. A linear mixed model was used to study the effect of disease progression on serum fibronectin levels. Individuals were treated as the random effect and follow-up time as the fixed effect.

3 Results

3.1 MS workflow

Serum samples pooled from adult controls, child controls, ambulant and nonambulant DMD patients were used to
screen for DMD candidate biomarkers. A summary of the biomarker discovery workflow is given in Fig. 1. Each serum pool was processed with a ProteoMiner™ protein enrichment kit aiming at equalization of the large range in protein concentrations [34]. Previously, it was shown that by applying a combinatorial hexapeptide library attached to ProteoMiner™ beads, the large variation in protein concentrations was decreased in complex biological samples, thus resulting in a relative enrichment of medium- and/or low-abundant proteins [34, 35]. After treatment of the samples with ProteoMiner™, the serum pools were fractionated on a 1D SDS gel, as shown in Supporting Information Fig. 1A. The reproducibility of ProteoMiner is shown in Supporting Information Fig. 1B. Comparison of the eluates with the corresponding flow-through fractions shows the removal of the majority of albumin (Supporting Information Fig. 1A).

3.2 LC-MS/MS identifications

As a next step, each lane was cut into ten equidistant pieces (shown in Supporting Information Fig. 1). Then, proteins were reduced and alkylated, and subjected to overnight proteolysis with either trypsin or Lys-N, and finally peptides were extracted. Two replicate gels were used to obtain 80 digest samples in total, namely ten times four tryptic and ten times four Lys-N digests of adult controls, child controls, ambulant and nonambulant DMD patients, respectively. All digests were analyzed on a nanoUHPLC CID/ETD ion trap system and MS/MS peak lists were analyzed and validated using TPP. CID and ETD fragmentation methods resulted in approximately 250–300 validated peptide IDs in each sample group at a false discovery rate of 1% at the peptide level. An overview of the number of peptide IDs is given in Fig. 2. The number of tryptic peptides identified was always higher from CID spectra. Most identified peptides were in the mass range of 1000–1500 Da, and second between 1500 and 2000 Da. The number of Lys-N peptide identifications with a mass higher than 1500 Da, was higher in ETD than in CID spectra in all samples (Fig. 2). On the other hand, the smaller Lys-N peptides were well fragmented by CID. There is clear complementarity of ETD and CID in the nature of the identified peptides (Supporting Information Table 1).

3.3 Evaluation of confidence of protein identifications

For validation of the peptide identifications iProphet probability values were determined. These values were summarized for all four sample pools and are plotted in Supporting Information Fig. 2, for tryptic and Lys-N peptides separately. In general, a probability value of 0.95 corresponds to a false discovery rate of 1% [29]. Plots show that the majority of values are within this confidence level. No differences were observed between the probability scores of CID and ETD identified peptides, which is in agreement with a previous report comparing Mascot scores of CID and ETD data [36]. ProteinProphet was used to build the simplest list of proteins sufficient to explain all observed peptides. Subsequently, the number of all MS/MS spectra for this list of proteins were determined (spectral counting) and used for semi-quantitative evaluation of protein abundance in the different pools.

3.4 Difference in spectral counts

Through trypsin digestion in combination with CID fragmentation, fibronectin peptides and protein were confidently identified in all four groups with high probability scores (with PeptideProphet probability scores of >0.9 and ProteinProphet probability scores of >0.99 for peptides and proteins, respectively). From the list of quantified proteins, fibronectin was the only protein with consistent and > threefold differences between both control and both DMD groups (Supporting Information Table 2). Moreover, the higher spectral counts in the more severe, nonambulant over ambulant DMD patients, suggest that fibronectin levels increase during the progression of the disease.

3.5 ELISA-based quantification of fibronectin serum levels

Fibronectin was further validated as a biomarker for DMD by ELISA measurements in independent DMD sample cohorts using age-matched controls. In accordance with the MS-based findings in the discovery cohort, fibronectin levels in DMD patients were found elevated compared to child controls, as shown in Fig. 3A (492 ± 192 and 86 ± 23 µg/mL, respectively, p < 0.001). There was no significant difference in serum fibronectin levels between DMD patients from Leiden and Newcastle. We did not find a significant difference between ambulant and nonambulant DMD patients. Fibronectin levels were not significantly associated with age (Pearson correlation coefficient 0.119, p = 0.334, Supporting Information Fig. 3), nor with years on corticosteroid treatment (correlation coefficient of 0.114, p = 0.354).

3.6 Longitudinal analysis of fibronectin levels in DMD patients

Longitudinal serum samples were collected from 22 DMD patients, who were followed up for 6 months to 4 years. The observed trends in fibronectin levels are plotted in Fig. 4. To determine whether fibronectin levels increased during progression of the disease, longitudinal data were analyzed using a linear mixed model. The levels of fibronectin increased significantly with the progression of the disease (p < 0.01).
Figure 2. Number of peptide identifications categorized in tryptic and Lys-N digests, and for different mass ranges. (A) and (B) represent the peptides from the adult control group (E1 in Supporting Information Fig. 1), (C) and (D) represent the peptides from child control group (E2 in Supporting Information Fig. 1), (E) and (F) represent the peptides from the DMD ambulant group (E3 in Supporting Information Fig. 1), and (G) and (H) represent the peptides from DMD nonambulant group (E4 in Supporting Information Fig. 1).
Clinical Relevance

DMD is a devastating neuromuscular disorder. Non-invasive tests to evaluate disease progression are lacking. The only serum biomarker currently used for early detection of muscular dystrophy is serum CK, the levels of which are markedly increased in early stages of DMD. However, serum CK levels decrease with the progression of the disease and they do not correlate with clinically assessed muscle performance. Therefore, they cannot be used for therapeutic monitoring during, for example, clinical trials. The aim of the study was to identify serum protein biomarkers that correlate with disease progression and may show an earlier response to treatments than functional parameters. From a panel of potential serum biomarkers identified by MS, fibronectin was selected and validated as a potential biomarker correlated with DMD disease progression.

Figure 3. ELISA-based measurements of serum fibronectin levels. (A) Fibronectin levels in DMD patients compared with child controls. (B) Fibronectin levels in adult BMD patients and adult controls. The median levels are indicated by the horizontal lines bisecting the boxes showing the interquartile range. The upper and lower limit of the bars show the maximum and minimum values considered. A t-test was used to test for statistically significant differences between groups. * indicates significance at \( p < 0.001 \).

Figure 4. Serum fibronectin levels for the longitudinal DMD patient cohort. The serum fibronectin levels of 22 DMD patients over 2–4 time points (depending on sample availability) are plotted. The time points are represented as the patient's age.

There was, however, a downward trend in the two oldest non-ambulant DMD patients. In contrast, CK levels decreased significantly with age (\( p < 0.01 \), Supporting Information Fig. 4).

3.7 Measurements in other muscular dystrophies

Evaluation of fibronectin levels in less severely affected BMD patients was restricted to 38 adult patients. We noted that the levels in adult controls were significantly higher than in child controls (\( p < 0.001 \)). However, there was no significant difference in serum fibronectin levels between BMD patients and adult controls (272 ± 205 and 307 ± 59 μg/mL, respectively, Fig. 3B). Likewise, fibronectin levels in BM and MG patients were not significantly different from controls (117 ± 81, and 170 ± 104 μg/mL, for BM and MG, respectively).
4 Discussion

Exploration of fibronectin as a potential biomarker to monitor DMD disease progression was hinted at by protein identifications and spectral counting of MS/MS data. The TPP analysis workflow revealed a difference in the number of spectral counts between the four different analyzed groups (adult control, child control, DMD ambulant, and DMD nonambulant). The increased spectral counts in adult versus young controls and DMD versus controls were confirmed by the absolute ELISA-based quantifications. The increased spectral counts in nonambulant versus ambulant DMD patients were not reflected by the ELISA experiments, likely due to the pooling of only a limited number of subjects for the MS-based screen.

Fibronectin levels have been described to be comparable in children and adults and to remain constant from the age of 1 year onwards [37, 38]. Our observations demonstrate that levels in control children are lower than those in adults. Therefore, it is important to include age-matched controls. Fibronectin levels did not correlate significantly with the age of DMD patients, likely due to relatively large interindividual variability in such a cross-sectional study. Still, we found a positive correlation of fibronectin levels with age in the analysis of longitudinal samples taken at different intervals. This suggests that fibronectin levels increase as the disease progresses and would therefore be suitable as biomarker to monitor disease progression and response to therapy in clinical trials, as long as the baseline levels from the patients have been evaluated. It will be better suited as a biomarker for monitoring therapeutic response than CK, since a drop in CK may either be due to undesired progressive loss of muscle mass [11] or (desired) improvements in muscle integrity.

Fibronectin has been linked to fibrosis, which is one of the hallmarks of muscular dystrophies and contributing to muscle dysfunction and the lethal phenotype of DMD [39]. Collagen is the most predominant extracellular matrix component of fibrotic tissue. Several studies showed that excessive deposition of fibronectin precedes collagen deposition [40, 41]. Moreover, gene expression profiling studies on muscles from DMD patients during the early phases of the disease revealed fibronectin gene expression to be upregulated by almost twofold [42]. Our studies in longitudinal patient cohorts also show that serum fibronectin levels are elevated in young DMD patients with significant increase over time, at least until approximately the age of 15. This warrants further studies correlating fibrotic content in skeletal or heart muscles with fibronectin levels in serum, validating serum fibronectin as a surrogate biomarker for fibrosis. Moreover, more extensive studies into the correlations of fibronectin levels with functional measures for skeletal or cardiac muscle performance would be highly informative.

In DMD patients, fibrosis has also been associated with increased numbers of activated macrophages [43, 44] that secrete proteases such as MMPs. Serum levels MMP-9 and its specific inhibitor tissue inhibitor of metalloproteinase-1 have been investigated in DMD patients. Serum MMP-9 levels have been suggested before as a biomarker for monitoring disease progression in patients with DMD [45]. Fibronectin acts as one of the substrates of MMP-9. A study by Opdenakker et al. [46] suggests that fibronectin inhibits secretion of proMMP-9 and monocyte migration, whereas its fragmented form increases secretion. Monocytes contribute to ECM remodeling by degrading its proteins via secretion of proteases such as MMP-9 on the one hand and by depositing ECM proteins such as fibronectin on the other hand. A feedback loop exists between MMP-9 and fibronectin, as MMP-9 degrades fibronectin, and binding of fibronectin upregulates the expression of MMP-9 [46–48]. We could not find a correlation between the MMP-9 levels and fibronectin levels measured in the cross-sectional study of 68 DMD patients nor in the longitudinal study of 22 DMD patients (Spitali, manuscript in preparation). This may be explained by the presence of the described feedback mechanisms.

In a study of skin atrophy in mice, fibronectin levels were found to be increased by corticosteroid therapy [49]. Another study demonstrated that corticosteroids significantly reduced peribronchial fibrosis in a mouse model of airway remodeling [50] and a study by Tomic et al. [51] showed that corticosteroids reduced lung tissue remodeling by lowering the levels of fibronectin. In our cross-sectional study of 68 DMD patients, there was no correlation of fibronectin levels with the duration of corticosteroid therapy or age. In contrast, the increase of fibronectin levels with age was significant in the longitudinal study that included patients on continuous corticosteroid therapy. To dissect the effect of corticosteroids on fibronectin levels in DMD, it would be necessary to sample steroid-naive patients at baseline and at regular levels after commencement of corticosteroid therapy.

Fibronectin levels were not elevated in the other patient cohorts evaluated. These diseases generally have lower (BMD and BM) or no (MG) muscle fibrosis, supporting the association of fibronectin serum levels with progressive muscle fibrosis.

Antifibrotic therapies, such as those inhibiting transforming growth factor beta1 [52, 53] or myostatin [54] and antisense oligonucleotide exon skipping [55], are investigated as a potential treatment approach for DMD. Fibronectin may constitute a promising molecular biomarker to monitor response towards antifibrotic and other DMD therapies that induce muscle tissue remodeling.

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