Large-scale serum protein biomarker discovery in Duchenne muscular dystrophy

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Serum biomarkers in Duchenne muscular dystrophy (DMD) may provide deeper insights into disease pathogenesis, suggest new therapeutic approaches, serve as acute read-outs of drug effects, and be useful as surrogate outcome measures to predict later clinical benefit. In this study a large-scale biomarker discovery was performed on serum samples from patients with DMD and age-matched healthy volunteers using a modified aptamer-based proteomics technology. Levels of 1,125 proteins were quantified in serum samples from two independent DMD cohorts: cohort 1 (The Parent Project Muscular Dystrophy–Cincinnati Children's Hospital Medical Center), 42 patients with DMD and 28 age-matched normal volunteers; and cohort 2 (The Cooperative International Neuromuscular Research Group, Duchenne Natural History Study), 51 patients with DMD and 17 age-matched normal volunteers. Forty-four proteins showed significant differences that were consistent in both cohorts when comparing DMD patients and healthy volunteers at a 1% false-discovery rate, a large number of significant protein changes for such a small study. These biomarkers can be classified by known cellular processes and by age-dependent changes in protein concentration. Our findings demonstrate both the utility of this unbiased biomarker discovery approach and suggest potential new diagnostic and therapeutic avenues for ameliorating the burden of DMD and, we hope, other rare and devastating diseases.

proteomics | muscular dystrophy | biomarkers | SOMAscan | SOMAmer

There is an urgent need for a reliable surrogate biomarker or set of biomarkers for Duchenne muscular dystrophy (DMD), ideally based on readily accessible and measurable molecules (1). DMD is a severe form of myopathy with an incidence of about 1 in 3,600–9,337 boys worldwide (2, 3), and is a result of different types of mutations in the X-linked *DMD* gene that abolish the expression and biological activity of dystrophin, an essential protein for muscle-fiber plasma membrane integrity and myofiber function (4, 5). Clinically, the disease is characterized by progressive muscle wasting, leading to loss of ambulation by 8–15 y of age and early death from complications from respiratory, orthopedic, and cardiac problems (2, 6).

Several current drug-development programs are focused on slowing or preventing the progressive muscle loss in DMD either in conjunction with the standard of care treatment or as standalone therapies. Standard of care is currently chronic high-dose glucocorticoids, which are able to prolong ambulation by 3–4 y (7, 8) and slow disease progression, but are associated with a significant array of side effects (2, 6, 9, 10). Promising therapeutic approaches for DMD include restoring expression of the dystrophin gene via exon-skipping strategies (11–13), viral-based gene therapies (14, 15), and nonsense suppression/read-through strategies (16). Other genetic approaches include delivering minidystrophins, up-regulation of utrophin to compensate for the missing dystrophin, and many others (17). Pharmacological strategies in development include dissociative steroid drugs, which offer the potential of greater efficacy and lesser side effects (18), other anti-inflammatory therapies, and effectors of signaling pathways (19). The current primary clinical endpoint used for determining efficacy in the majority of these therapeutic approaches for ambulatory boys with DMD is the "six-minute walk test" (20, 21), although it is not ideal (22).

Blood provides a circulating protein representation of all body tissue in both normal and pathological conditions, and serum proteins are emerging as useful biomarkers for diagnosis and prognosis of a growing number of diseases (23, 24). Mass spectrometry (MS)-based proteomic screens recently have proved successful at de novo biomarker identification in DMD (25). However, verification and validation of MS-discovered serum biomarkers remain challenging (24). Other approaches, such as multiplexed antibody or aptamer-based assays, are being considered for proteome screens because of their potential for higher throughput and better sensitivity, which may help overcome the validation challenges of identified biomarkers. For example, a

Significance

Duchenne muscular dystrophy (DMD) is a rare and devastating muscle disease caused by mutations in the X-linked *DMD* gene (which encodes the dystrophin protein). Serum biomarkers hold significant potential as objective phenotypic measures of DMD disease state, as well as potential measures of pharmacological effects of and response to therapeutic interventions. Here we describe a proteomics approach to determine serum levels of 1,125 proteins in 93 DMD patients and 45 controls. The study identified 44 biomarkers that differed significantly between patients and controls. These data are being made available to DMD researchers and clinicians to accelerate the search for new diagnostic, prognostic, and therapeutic approaches.

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recent study using an antibody-based array against 384 target proteins identified 11 protein biomarkers of disease across different muscular dystrophies from patient samples gathered from four different clinical sites (26). In addition, a modified aptamer-based technology (the SOMAscan assay) is emerging as another highly sensitive and multiplexed assay for biomarker discovery and validation (27–29). Based on novel reagents (Slow Off-rate Modified Aptamers, or SOMAmer reagents) that recognize specific conformational epitopes of native 3D proteins with high specificity and high sensitivity (30–32), the SOMAscan assay measures levels of 1,125 analytes in only 65 μL of serum over a wide dynamic range (>8 logs of concentration). Because the SOMAscan assay relies on the availability of the protein epitopes (i.e., the epitopes are not blocked by other protein binding, posttranslational modifications, and so forth), what is measured in the assay and the actual protein concentration in the sample being interrogated is frequently but not always correlated. In the same manner, ELISAs for the same proteins also are frequently but not always correlated.

Because blood is the preferred diagnostic clinical material, and biomarkers in the blood can differ by several orders-of-magnitude in abundance, the SOMAscan assay may be a path forward to identify and verify key blood-based biomarkers for DMD and other diseases. We used the SOMAscan technology to screen for protein biomarkers associated with DMD using serum samples from two independent cohorts collected in different locations and run at different times (cohort information in *Demographics, Characteristics, and Enrollment Criteria of the PPMD-C and CINRG Cohorts* and Dataset S1). The first cohort analyzed was from The Parent Project Muscular Dystrophy–Cincinnati Children's Hospital Medical Center (hereafter PPMD-C), which included the goal of identifying alternative treatment paths (i.e., nondystrophin-centric) for patients with DMD. The second cohort analyzed was from The Cooperative International Neuromuscular Research Group, Duchenne Natural History Study (hereafter CINRG) (33), which included the goal of identifying changes in biomarkers with age in patients with DMD. In the present study, we compared the data from these two independent studies. This process enabled us to identify 44 biomarkers in the blood associated with DMD: 24 that are significantly increased and 20 that are significantly decreased in patients with DMD.

These data suggest new protein targets and biomarkers for further DMD studies. The data also may facilitate future clinical studies designed to identify new therapeutics for DMD, as well as further demonstrating the utility of the SOMAscan assay technology for identifying protein biomarkers for both rare and common diseases. We are making our data fully available to the DMD research community to enable further studies that may be suggested by these findings.

Results

Independent SOMAscan Assay Analyses on Two DMD Cohorts. Two independent DMD natural history cohorts were used in this study. The PPMD-C cohort comprised 42 DMD patients (2–27 y old) and 28 healthy male volunteers (4–28 y old, most often from the DMD male sibling pool). The CINRG cohort comprised 51 DMD patients (age range 4–29 y old) and 17 healthy male volunteers (age range, 6–18 y old). The demographics, characteristics, and enrollment criteria of the two cohorts are summarized in *Demographics, Characteristics, and Enrollment Criteria of the PPMD-C and CINRG Cohorts* and Dataset S1. In the initial analysis, the PPMD-C study design included steroid treatment for a subset of patients and the CINRG study included ambulatory status. Steroid treatment had no statistically significant effect on the 44 protein biomarkers described below, and ambulatory status was relevant only insofar as it related to increasing age but had no statistically significant effect on the results. Our standard quality-control protocols detected no significant difference in the samples from the two cohorts.

Serum samples were tested using the SOMAscan protein biomarker discovery assay (SomaLogic), which detects 1,125 proteins simultaneously using 65 μL of serum. At a 1% false-discovery rate (FDR) (*Materials and Methods*), based on SOMAscan assay data from a total of 93 DMD patients and 45 age-matched controls from the two cohorts, we identified 44 proteins that consistently differed in the serum in both cohorts when comparing DMD patients vs. controls. The UniProt names and a measure of differential expression [the signed Kolmogorov–Smirnov (KS) distance] for these 44 proteins in each cohort are shown in Table 1, along with an indicator of each protein's known enrichment in muscle tissue. The entire 1,125 protein SOMAscan assay results for each cohort independently are listed in Dataset S2.

Of the 44 protein biomarkers that were significantly different between DMD and controls, detected levels increased for 24 and decreased in 20 in DMD patients compared with normal controls. Fig. 1 shows the empirical cumulative distribution functions (CDFs) for six representative proteins from the combined cohort analysis [three proteins that are increased are troponin 1 fast skeletal muscle (TNNI2), myoglobin (MB), heat-shock protein 70 (HSPA1A); and three that are decreased are proto-oncogene tyrosine-protein kinase receptor Ret (RET), gelsolin (GSN), bone sialoprotein 2 (IBSP) in DMD patients vs. controls]. These

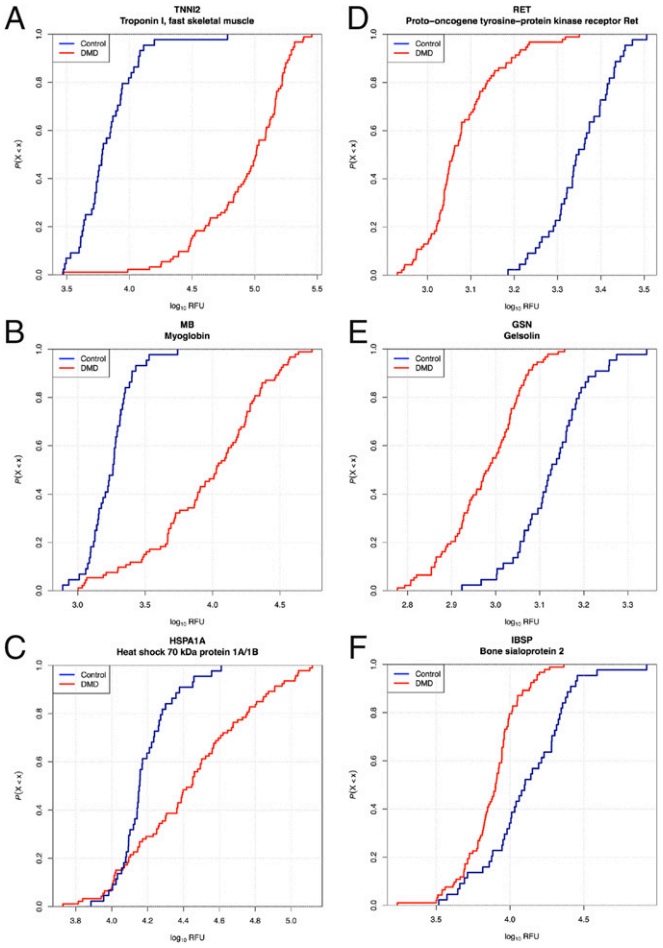

Fig. 1. Representative CDFs of proteins that are up or down in DMD patients vs. controls from both cohorts. Up proteins: (*A*) Troponin I, fast skeletal muscle, (*B*) myoglobin, (*C*) heat-shock protein 70. Down proteins: (*D*) RET, (*E*) gelsolin, (*F*) bone sialoprotein 2.

examples range from the highest KS distance (near 1 or −1) to the lowest significant (near 0.5 or −0.5) for both the "up" and "down" groups, respectively. The CDFs of all 44 proteins identified in both cohorts are provided in Fig. S1.

Correlation Between Biomarker Levels and Age of DMD Patients. In this DMD study, age is a proxy for disease severity, because older patients have more advanced disease. Because multiple biological samples over time from individual patients were not available, we

Table 1. Proteins that increase (positive KS distance) or decrease (negative KS distance) significantly in DMD patients vs. controls in both PPMD-C and CINRG cohorts

Protein name (UniProt)	Gene name (UniProt)	PPMD-C signed KS distance	CINRG signed KS distance	Average KS	Rank	Muscle enriched	Age-related change group no.
Troponin I, fast skeletal muscle	*TNNI2*	1.000	0.918	0.959	1	Yes	1
Carbonic anhydrase 3	*CA3*	0.964	0.938	0.951	2	Yes	1
Fatty acid-binding protein, heart	*FABP3*	1.000	0.882	0.941	3	Yes	1
Troponin I, cardiac muscle	*TNNI3*	0.917	0.961	0.939	4	Yes	1
Creatine kinase M-type	*CKM*	0.976	0.839	0.908	5	Yes	1
Mitogen-activated protein kinase 12	*MAPK12*	1.000	0.797	0.898	6	Yes	1
Alanine aminotransferase 1	*GPT*	0.738	0.941	0.840	7	No	1
Myoglobin	*MB*	0.857	0.820	0.838	8	Yes	1
Fibrinogen	*FGA FGB FGG*	0.810	0.784	0.797	9	No	1
Phospholipase A2, membrane associated	*PLA2G2A*	0.762	0.800	0.781	10	No	3
Acidic leucine-rich nuclear phosphoprotein 32 family member B	*ANP32B*	0.821	0.706	0.764	11	No	1
Hepatoma-derived growth factor-related protein 2	*HDGFRP2*	0.738	0.691	0.715	12	No	3
40S ribosomal protein S7	*RPS7*	0.690	0.734	0.712	13	No	1
Glucose-6-phosphate isomerase	*GPI*	0.774	0.604	0.689	14	Yes	1
Heparin cofactor 2	*SERPIND1*	0.560	0.813	0.686	15	No	3
Persephin	*PSPN*	0.595	0.757	0.676	16	No	3
Calcium/calmodulin-dependent protein kinase II α	*CAMK2A*	0.738	0.586	0.662	17	Yes	1
Malate dehydrogenase, cytoplasmic	*MDH1*	0.595	0.706	0.651	18	Yes	1
L-lactate dehydrogenase B chain	*LDHB*	0.631	0.608	0.619	19	Yes	1
Aminoacylase-1	*ACY1*	0.643	0.577	0.610	20	No	1
Proteosome subunit α type-2	*PSMA2*	0.571	0.600	0.586	21	No	3
C-X-C motif chemokine 10	*CXCL10*	0.560	0.600	0.580	22	No	3
cAMP-dependent protein kinase catalytic subunit α	*PRKACA*	0.560	0.570	0.565	23	No	1
Heat-shock 70 kDa protein 1A/1B	*HSPA1A*	0.476	0.600	0.538	24	Yes	1
Proto-oncogene tyrosine-protein kinase receptor Ret	*RET*	−0.917	−0.961	−0.939	1	No	2
Growth/differentiation factor 11	*GDF11*	−0.667	−0.941	−0.804	2	No	4
Complement decay-accelerating factor	*CD55*	−0.762	−0.745	−0.754	3	No	4
Cadherin-5	*CDH5*	−0.821	−0.675	−0.748	4	No	2
Tumor necrosis factor receptor superfamily member 19L	*RELT*	−0.786	−0.706	−0.746	5	No	4
Gelsolin	*GSN*	−0.750	−0.718	−0.734	6	Yes	4
Wnt inhibitory factor 1	*WIF1*	−0.679	−0.714	−0.697	7	No	2
Contactin-5	*CNTN5*	−0.655	−0.702	−0.678	8	No	2
Prolyl endopeptidase FAP	*FAP*	−0.643	−0.659	−0.651	9	No	2
Jagged-1	*JAG1*	−0.679	−0.613	−0.646	10	No	2
Netrin receptor UNC5C	*UNC5C*	−0.560	−0.718	−0.639	11	No	2
Kunitz-type protease inhibitor 1	*SPINT1*	−0.667	−0.597	−0.632	12	No	2
Protein SET	*SET*	−0.500	−0.722	−0.611	13	No	2
Disintegrin & metalloproteinase domain-containing protein 9	*ADAM9*	−0.595	−0.600	−0.598	14	No	2
Cell adhesion molecule L1-like	*CHL1*	−0.583	−0.589	−0.586	15	No	2
Osteomodulin	*OMD*	−0.452	−0.718	−0.585	16	No	2
WAP, Kazal, Ig, Kunitz and NTR domain-containing protein 1	*WFIKKN1*	−0.464	−0.699	−0.581	17	No	4
Bone sialoprotein 2	*IBSP*	−0.476	−0.613	−0.544	18	No	2
Interleukin-34	*IL34*	−0.488	−0.558	−0.523	19	No	2
Neurogenic locus notch homolog protein 3	*NOTCH3*	−0.488	−0.550	−0.519	20	No	2

Signed KS distances are given for each protein in both cohorts, along with their average value to emphasize consistency in the two cohorts. Proteins known to be enriched in muscle tissue are indicated as such. The last column lists the "group" number for each protein based on their concentration as a function of age (see *Results*, *Discussion*, and Fig. 2 and Fig. S2).

instead examined the age-dependence in protein levels across the whole cohort. Proteins were screened using a single protein linear regression model to identify candidates where patient age was a useful predictor of protein concentration. We identified four general groupings of differential protein changes for the 44 biomarkers identified in this study (Fig. 2, Table 1, and Fig. S2).

Group 1 has protein biomarkers that were at their highest levels in young patients with DMD—far higher than in normal controls—and then decreased as a function of age in DMD while remaining relatively unchanged or increasing slightly with age in controls (18 proteins, represented by creatine kinase) (Fig. 2*A*).

Group 2 has proteins that changed with age in DMD and controls, but which were significantly lower in patients at most ages (15 proteins, represented by RET) (Fig. 2*B*).

Group 3 has protein biomarkers that changed with age in DMD and controls, but which were significantly higher in patients at most ages (six proteins, represented by phospholipase A2) (Fig. 2*C*).

Group 4 has protein biomarkers whose concentrations were very similar between DMD and controls at an early age, but then decreased with age in DMD patients while increasing in controls [five proteins, represented by growth differentiation factor 11 (GDF11)] (Fig. 2*D*).

Age-related regression plots for all 44 proteins are available in Fig. S2.

Discussion

Using the SOMAscan assay, we identified 44 circulating serum biomarkers associated with DMD patients vs. healthy controls from two independent cohorts with a 1% FDR-corrected significance level. Although some of us are experts in this field, in the following discussion we have tried to minimize hypothesizing about the potential meaning of the markers discovered in this study so as to provide the wider DMD community an unbiased opportunity to pursue these results following their own interpretations.

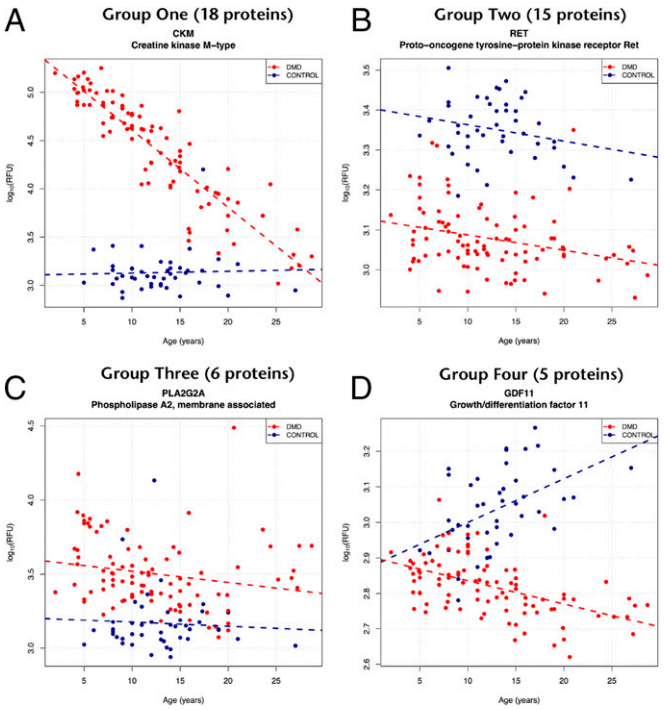

Fig. 2. Example proteins from the four "types" of age-related changes in protein signal levels seen in DMD patients (red) vs. controls (blue) from both cohorts. (*A*) Group 1, creatine kinase; (*B*) group 2, RET; (*C*) group 3, phospholipase A2; (*D*) group 4, growth-differentiation factor 11.

The most striking differences between DMD patients and controls were observed in the young age range (4–10 y old), where the most significant biomarkers were elevated up to two orders-of-magnitude in serum samples of DMD patients relative to healthy volunteers (group 1 proteins). These biomarkers then declined with age and disease progression. These "creatine kinase-like" proteins (Fig. 2*A*) are mostly of muscle origin and their early elevation in blood is likely associated with muscle damage/cell death and inflammation at an early early age, and their subsequent decline with age is most likely the result of loss of muscle mass in the DMD patients.

The high-to-low change in concentration of these creatine kinase-like proteins likely reflects high myofiber membrane instability/damage, necrosis, and leakage of cytoplasm into the extracellular space. This group includes muscle-enriched proteins such as creatine kinase M-type (CK-M) itself, fatty acid binding protein 3 (FABP3), myoglobin (MB), carbonic anhydrase III (CA3), malate dehydrogenase (MDH1), lactate dehydrogenase B (LDHB), glucose phosphate isomerase (GPI), Hsp70 (HSPA1A), troponin I, fast skeletal muscle (TNNI2), troponin I, cardiac muscle (TNNI3), mitogen-activated protein kinase 12 (MAPK12), and calcium-calmodulin–dependent protein kinase IIα (CAMK2A). Most of these muscle leakage proteins have been previously reported by others to be elevated in DMD boys relative to healthy volunteers (25, 26), except for Hsp70, MAPK12, and CAMK2A, which are novel to this study.

We also identified several proteins (all group 2) that are associated with connective tissue remodeling, including prolyl endopeptidase FAP (FAP), protein jagged-1 (JAG1), bone sialoprotein 2 (IBSP), ADAM metallopeptidase domain 9 (ADAM9), cadherin-5 (CDH5), neural cell adhesion molecule L1-like protein (CHL1), osteomodulin (OMD), and contactin-5 (CNTN5). Each of these proteins was found to be significantly lower in DMD patients than in controls at all ages. These proteins may regulate connective tissue remodeling in skeletal muscle.

Several other proteins identified in this study are functionally associated with inflammation and innate immune pathways, including: group 2 protein interleukin-34 (IL-34); group 3 proteins C-X-C motif chemokine 10 (CXCL10), phospholipase A2 (PLA2G2A), and hepatoma-derived growth factor-related protein 2 (HDGFRP2); and group 4 proteins CD55/complement decay-accelerating factor (CD55) and RELT tumor necrosis factor receptor (RELT). These proteins do not show significant change as a function of age, with the two exceptions of CD55 (decreases with age in DMD and increases with age in controls) and fibrinogen (increases with age in both DMD and controls). Two of the above group 3 proteins (PLA2G2A and CXCL10) are of particular interest because they could be useful pharmacodynamic biomarkers to monitor efficacy of anti-inflammatory agents in DMD patients. Phospholipase A2 activity has been reported to be dramatically increased (10-fold) in the skeletal muscle of DMD patients relative to controls and is associated with muscle inflammation (34), consistent with the high serum levels reported here. CXCL10 is an extracellular chemokine and its elevation in serum could be associated with increased T-cell infiltration in inflamed skeletal muscle (35).

Another intriguing protein that emerged from our studies is the group 3 protein persephin, a member of the GDNF family of neurotrophic factors. Persephin signals through the RET receptor tyrosine kinase-mitogen–activated protein kinase pathway, and is known to be expressed in skeletal muscle, motor neurons and, perhaps, Schwann cells (26). Although its role in motor neurons is uncertain, persephin may be involved in the reinnervation process, as it has been observed to stimulate neurite outgrowth in oculomotor neurons (36). Thus, the increased detection of persephin and decreased detection of RET (group 2) levels in DMD patients vs. controls (Table 1) could be a marker of the ongoing denervation/reinnervation that is occurring. In terms

of biomarkers, lower concentrations of persephin and increased concentrations of RET may be biomarkers for therapeutic approaches that stabilized the muscle fibers and stabilized innervation. Although the significance of these particular data must first be addressed in animal models of DMD, it is exciting to think of the possibilities for these biomarkers for diseases and therapies.

The group 4 proteins from this study are also worth noting [CD55, growth differentiation factor-11 (GDF-11), gelsolin (GSN), RELT, and WAP, Kazai, Ig, Kunitz, and NTR domain-containing protein (WFIKKN1)]. All five of these proteins are initially at similar levels at a young age between DMD patients and controls, but then decrease significantly with age in DMD while increasing with age in controls, although the meaning of these changes in concentration is unclear (see below).

GDF-11 is of particular interest, given recent studies that have suggested that exogenous GDF-11 can reverse age-related cardiomyopathy (37) and skeletal muscle deterioration (38) in mice. Our data would be consistent with the hypothesis that GDF-11 is a candidate for potentially ameliorating the cardiomyopathy as well as skeletal muscle deterioration seen in patients with DMD. However, there are two significant questions that must be addressed. First, it is not clear that we are measuring GDF-11 specifically and not its close homolog GDF-8 (myostatin). To that end, experiments are underway using new and highly specific GDF-11 and GDF-8 SOMAmer reagents we recently developed. Second, there are several published preclinical and clinical studies aimed at inhibiting GDF-8 for the treatment of muscular disorders and it is likely that these approaches inhibit GDF-11 as well as GDF-8, with no discernible detrimental effects, or even with positive effects (39–42). Perhaps the clearest thing that can be said is that the relative benefits of inhibiting GDF-8 vs. increasing GDF-11 (and the biological interplay of those two proteins) requires further study.

Thus, it is important to keep in mind three issues as one contemplates SOMAscan data: epitope counting, causality, and directionality. The X-ray structures for SOMAmers bound to their protein targets (29) make clear that SOMAmers recognize conformational protein epitopes, and (as noted above) any component of the sample (other proteins bound to the target, posttranslational modifications, and so forth) that alters epitope availability or shape may be reflected as an "up" or a "down" in the SOMAscan data. In that sense, MS provides a complementary measure for the absolute protein concentration (usually as peptides after proteolysis). When a value does go up or down the temptation is to ascribe causality to that change, when in fact correlation is more likely than causality. "Elevator science" must be followed by experimental tests of causality, which will be influenced by directionality. Biological networks and homeostatic regulation allow two opposite interpretations of the same data. If a protein (epitope) is elevated in a disease condition, for example, one might ascribe causality to that elevation and counter the elevation with an antagonist, such as an antibody or other drug. Alternatively, the elevated biomarker might reflect homeostatic regulation and the proper intervention could be to provide more of the protein that was elevated. This distinction is not trivial: separate biotech companies have often pursued biologics and antagonists for the same protein for the same disease until clinical data decided the directionality. Directionality decisions always require data.

The data for the proteins that are very high early in life for DMD patients and that diminish in blood as muscle mass decreases (group 1 in Fig. 2*A* and Fig. S2) suggest that significant muscle cell death is occurring very early in life, perhaps even during embryonic development. However, it is striking that the total absence of dystrophin does not cause abrupt muscle cell death: the decrease we see in these proteins suggests that the number of muscle cells in DMD patients decreases by a median half-life of ~7.2 y. This observation suggests that the balance between muscle stem cell-derived muscle mass preservation and dystrophinless-derived muscle loss is a slow battle. This relative "slowness" of muscle cell death may provide an opportunity for a novel nondystrophin-centric treatment option for DMD patients that tips the balance in favor of muscle preservation, at least for a longer period. Cell culture studies and a mouse study in either the dystrophin-negative mouse or the utrophin-dystrophin double-knockout mouse could be designed to test all secreted proteins to determine, in an unbiased manner, if GDF-11, GDF-8, or any other growth factor (or even anti-inflammatory or membrane stabilization small molecule compounds) or antagonists of those proteins can slow the loss of muscle mass over time, independent of dystrophin restoration. We also hope there is a role for small oral drugs that will work intracellularly to extend muscle cell survival in DMD patients.

Finally, we have recently been given access to an unpublished SOMAscan study on the *mdx* mouse model with confirmation of some mouse biomarker data with human samples. That study provides additional novel data, including responses of the identified biomarkers to treatment.

Using the SOMAscan assay, we have discovered a rich set of protein biomarkers that change with age in serum from two different cohorts of patients with DMD and age-matched controls. We are planning to extend these findings by running these and many additional DMD and control samples, as well as samples from the full spectrum of Becker muscular dystrophy patients, in an imminent new version of the SOMAscan assay that will measure several thousand additional proteins. However, it is our hope that research and clinical experts in DMD can use the markers described here to pursue potential improvements in clinical trial designs, and to generate new diagnostic and therapeutic approaches to this devastating disease. We also believe that SOMAscan can be applied with equal success to many different rare diseases; when proteomic changes are large, as they are in DMD, even small clinical studies can be informative.

Materials and Methods

PPMD-C and CINRG Cohort Samples.

PPMD-C cohort. Samples and clinical and demographic data were from DMD patients ($n = 42$) and healthy age-matched volunteers ($n = 28$). Institutional approval came from the Cincinnati Children's Hospital Medical Center Institutional Review Board and informed consent was obtained from patients or their parent or legal guardian.

CINRG cohort. For the CINRG cohort, sera samples and clinical and demographic data from DMD patients ($n = 51$) and age-matched healthy volunteers ($n = 17$) were collected through the Cooperative International Neuromuscular Research Group Duchenne Natural History Study. The study protocol was approved by Institutional Review Boards at all participating institutions, and informed consent was obtained from patients or their parent or legal guardian.

Demographics, characteristics, and enrollment criteria of the two cohorts are summarized in *Demographics, Characteristics, and Enrollment Criteria of the PPMD-C and CINRG Cohorts* and Dataset S1.

SOMAscan Assay. The SOMAscan proteomic assay is described more extensively elsewhere (27–29). In brief, each of the 1,125 proteins measured in serum by the version of the SOMAscan assay performed in this study has its own targeted SOMAmer reagent, which is used as an affinity binding reagent and quantified on a custom Agilent hybridization chip.

DMD and control samples were randomly assigned to plates within the each assay run along with a set of calibration and normalization samples. No identifying information was available to the laboratory technicians operating the assay.

Intrarun normalization and interrun calibration were performed according to SOMAscan v3 assay data quality-control procedures as defined in the SomaLogic good laboratory practice quality system. Samples from the PPMD-C and CINRG cohorts were assayed independently and data from all samples passed quality-control criteria and were fit for analysis.

Analysis of SOMAscan Assay Results. SOMAscan proteomic data are reported in relative fluorescence units (RFU), as previously described (27). RFU data were log-transformed before statistical analysis to reduce heteroscedasticity. The nonparametric KS test was used to identify differentially expressed proteins between DMD and controls. The KS test statistic is an unsigned quantity; here we include a sign to indicate the direction of the differential expression, with a

positive test statistic indicating higher protein levels in DMD patients than in controls. We show the empirical CDF of the protein levels as an accurate representation of the underlying signals in the two patient populations. In all cases the ordinant represents the fraction of patients with signal levels below the corresponding abscissa reported in $\log_{10}$ RFU. In statistical tests we account for multiple comparisons by reporting the FDR computed using the BH method (43) in the *p.adjust* function in the R base package, *stats* (44). All statistical analysis performed with the R language for statistical computing v3.1.2 (2014-10-31).

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