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H. 知的財産権の出願・登録状況
(予定を含む)

1. 特許取得
（出願予定）ジストロフィン陽性線維の計測方法

2. 実用新案登録
なし

3. その他
なし

1厚生労働科学研究委託費
（障害者対策総合研究開発事業（神経・筋疾患分野））
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バイオマーカーの変動に影響を与える因子の検討

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研究要旨

DMD患者のバイオマーカーに影響を与える因子の一つとして，エクソン・スキップ治療に起因する免疫反応，即ち治療薬自身に対する抗体，及び発現したジストロフィンに対する抗ジストロフィン抗体の出現が懸念されていることから，抗ジストロフィン抗体を検出する手法の検討を行った。抗ジストロフィン抗体の出現が想定される被験者の血清を一次抗体，さらに標識された抗ヒトIgG抗体を二次抗体とする系を構築し，正常ヒト骨格筋に含まれるジストロフィンが検出されるかを検討したところ，正常ヒト由来血清を用いた場合，構築した系ではジストロフィンは検出されなかった。抗ジストロフィン抗体そのものの検出は技術的に克服すべき点が多いが，本項目については目的とする解析系の構築についてほぼ達成できたと考えられる。

A. 研究目的

DMD 患者のバイオマーカーに影響を与える因子の一つとして，エクソン・スキップ治療に起因する免疫反応，即ち治療薬自身に対する抗体，及び発現したジストロフィンに対する抗ジストロフィン抗体の出現が懸念されている。これは正常人には存在するジストロフィンがDMD患者では存在しないため，エクソン・スキップ治療によりジストロフィンの発現が回復した場合，これらは被験者にとっては異物と認識される可能性が指摘されているためである。そのため先行するエクソン51スキップによるDMD治療薬の第1相試験においては，Sarepta therapeutics 社のモルフォリノ製剤 eteplirsen，及び Prosensa therapeutics 社の2'-OMe製剤 drisapersen のいずれも，被験者血清中の抗ジストロフィン抗体の測定を行っている。このような状況を踏まえ，本研究で開発を進めているエクソン53スキップによる DMD 治療薬 NS-065/NCNP-01 の早期探索的臨床試験においても，治験薬の安全性評価項目の一つとして抗ジストロフィン抗体の測定を設定したところである。しかしこれまでのところ，ウイルスベクターによるジストロフィン遺伝子導入及びエクソン・スキップを目的としたアンチセンス投与など，ジストロフィン発現の回復を目的とした治療を受けたヒトにおいて，抗ジストロフィン抗体の出現は確認されていない。唯一確認されているのは，心不全の DMD 患者へ健常人の心臓移植を行った際に，レシピエントの血清から抗ジストロフィン抗体が検出された事例である[(1)]。本例では抗ジストロフィン抗体の出現が確認された1回目の心臓移植後に，この心臓が生着せず再移植を行なっているが，これが臓器移植一般に見られる拒絶反応であるのか，あるいは抗ジストロフィン抗体の影響かは明らかでない。また抗ジストロフィン抗体の検査手法としての問題点は，陽性対照となるヒト由来抗ジストロフィン抗体がいまのところ存在しないことである。ヒト化モノクローナル抗体を作成する

ことは技術的には可能と思われるが，今のところ成功した報告はないため，これを新たに作製して今回の早期探索的臨床試験に用いることは，測定系の構築に要する費用や期間の面から現実的ではないと考えられた。そこで，これまでに文献[2]で報告されている測定方法に基づいて，被験者血清に存在するIgG型の抗ジストロフィン抗体の検出を想定した，抗ジストロフィン抗体測定法の構築を本研究において試みたので報告する。

B. 研究方法

本研究では最初にドットブロット法により，測定方法の予備検討を行った。方法は以下のとおりである。

(1) 市販の正常ヒト血清（Normal Human Serum ,Sigma-Aldrich）を被験者血清とみなし，一次抗体として用いる。
(2) ２× ３ｃｍ程度にカットしたImmobilon-P（ミリポア）を用意する。
(3) メタノールに１分間浸したあと，ミリQ水に浸し５分間振盪して親水化する。
(4) メンブレンを１スポット毎にハサミで切り離す（１ｘ１ｃｍ）
(5) レトロウイルスベクターによりMYOD-IRES-ZsGreen1を導入後，FACSでZsGreen1陽性細胞のみを回収し，筋分化を誘導した正常由来線維芽細胞（TIG-119, ヒューマンサイエンス研究資源バンク）をRIPAバッファーで溶解したライセートを，間隔をあけて4 uLずつメンブレンに滴下し乾燥させる。
(6) それぞれを細胞培養用の６穴プレートに入れる
(7) PBST(0.05% tween-20入りPBS)で溶解した2% ECL Prime Blocking Agent（GE healthcare）で，室温1時間インキュベートする。
(8) Can Get Signal 1（TOYOBO）で希釈した一次抗体を，メンブレンに 50 uL/cm2で載せ，室温で1時間反応させる。一次抗体には抗ジストロフィンマウスモノクローナル抗体 NCL-DYS1（Leica biosystems），または正常ヒト血清を用いる。
(9) PBST で15分間2回洗浄する。
(10) Can Get Signal 2（TOYOBO）で希釈した二次抗体を，メンブレンに 50 uL/cm2で載せ，室温で10分間反応させる。二次抗体として，一次抗体にNCL-DYS1を用いたメンブレンには抗マウスIgG抗体（Bio-rad）を，一次抗体に正常ヒト血清を用いたメンブレンには抗ヒトIgG抗体（Life technologies）を用いる。
(11) PBST で15分間2回洗浄する。
(12) ECL Prime（GE healthcare）のプロトコールに従いメンブレンと反応させる。
(13) ImageQuant LAS 4000mini (GE Healthcare)で撮影する。

次に治験における被験者検体を想定した方法として，ウェスタンブロット法による検討を行った。方法は以下のとおりである。

(1) 市販の正常ヒト血清（Normal Human Serum ,Sigma-Aldrich）を被験者血清とみなし，一次抗体として用いる
(2) Promega muscle lysate (3.75 ug/uL)をT-PERで希釈し，0.25 ug/uLとする。希釈したライセート と 等量のEzApplyを混合し，95度5分間加熱する。
(3) 筋ライセートとして0.5 ug (EzApply混合液 4 uL)をレーンにアプライする．ブランクにはT-PERとEzApplyを等量で混合したものを4 uLアプライする。またマーカーとしてKaleidoscope (bio-rad)をアプライする。以上のとおり一つの組み合わせあたり3レーンを使用する。NuPAGE Novex 3-8% Tris-Acetate Gels, 1.5 mm, 15 wellを用いて，ゲル1枚あたり5つの組み合わせをアプライする。
(4) XCell SureLock Mini-Cell Electrophoresis System (life technologies)のプロトコール

に従い，ゲルをセットする。

(5) 下部泳動槽を NuPAGE Tris-Acetate SDS Running Buffer (20X) (life technologies)を希釈した running buffer 600mL で満たす。上部泳動槽は，この running buffer 200 mL に対し NuPAGE Antioxidant (life technologies) 0.5 mL を加えた buffer で満たす。

(6) POWER PAC 300 (BIO-RAD)に接続し 150 V 定電圧で 75 分間泳動する。

(7) 泳動後，ゲルキャストの 1 枚を剥がした状態で，ゲルのサイズが 8.0 x 6.5 cm となるように不要部分をカットする。

(8) 8.0 x 6.5 cm にカットした Immobilon-P メンブレン をメタノール に浸し，その後 EzBlot (ATTO) blotting buffer B で 30 分以上振盪して親水化させる。

(9) 8.0 x 6.5 cm にカットした，Extra Thick Blot Paper (BIO-RAD)を 2 枚ずつ，EzBlot blotting buffer A, B, C それぞれに浸す。

(10) Blotting buffer B のトレイでは，Extra Thick Blot Paper の上に Immobilon-P を載せ，この上にゲルを丁寧に載せる。

(11) BE-330 セミドライ・トランスファー装置 (バイオクラフト)に，陽極側から blotting buffer A, blotting buffer B, Immobilon-P, ゲル, blotting buffer C となるように重ねる。

(12) POWER PAC 300 に接続し 2 mA/cm2 定電流で 60 分間転写する。

(13) TBS-T でメンブレンを洗浄後，TBS-T に ECL Prime Blocking Reagent を 1%濃度で溶解し，ブロッキング溶液を調製する。

(14) メンブレン 1 枚あたり 50 mL のブロッキング溶液に浸し，i) 40 回／分で振盪しながら，室温で 1 時間反応，または ii)2-8℃，オーバーナイトで反応させる。

(15) キムタオルの上に清潔なパラフィルムを敷いて，この上にメンブレンを置く。ブランクのレーンと Kaleidoscope のレーンの境界を目安にメンブレンをカットし，メンブレンストリップを 5 枚作製する。

(16) パラフィルム (Pechiney Plastic Packaging Company)上に，お互いが接しないようにメンブレンストリップを配置し，550 µL の一次抗体溶液を載せ，室温で 1 時間反応させる。一次抗体には抗ジストロフィンマウスモノクローナル抗体 NCL-DYS1（Leica biosystems），または正常ヒト血清を用いる。

(17) 30 mL の TBS-T で，60 回／分の速度で振盪しながら，10 分間 3 回洗浄する。

(18) パラフィルム上に，お互いが接しないようにメンブレンストリップを静置し，550 µL（50 µL/cm2）の二次抗体溶液を載せ，室温で 10 分反応させる。二次抗体として，一次抗体に NCL-DYS1 を用いたメンブレンには抗マウス IgG 抗体（Bio-rad）を，一次抗体に正常ヒト血清を用いたメンブレンには抗ヒト IgG 抗体（Life technologies）を用いる。

(19) TBS-T で，60 回／分の速度で振盪しながら，15 分間 3 回洗浄する。

(20) ECL Western Blotting Detection Reagent をメンブレンストリップあたり 550 µL 調製し 5 分間反応させる。

(21) メンブレンを ImageQuant LAS 4000mini (GE Healthcare)で撮影する。

（倫理面への配慮）

本研究は当センター倫理委員会において，ヒト検体を用いたジストロフィン発現解析に関する研究として承認を受けた実験計画に従って実施した。

C. 研究結果

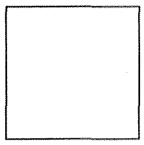
1. ドットブロットによる抗ジストロフィン抗体測定法の条件検討

最適な血清希釈濃度について予備検討を行うために，はじめにドットブロットによる検討を行った。まず陽性対照として一次抗体にマ

Fig 1.1

一次抗体
NCL-DYS1 1:200
↓
二次抗体
抗マウス IgG　1:2500

露光１分



↓
露光５分

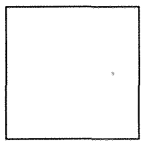
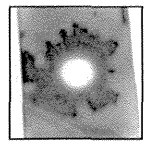


Fig 1.3

一次抗体
正常ヒト血清　1:50
↓
二次抗体
抗ヒト IgG　1:600

露光１分

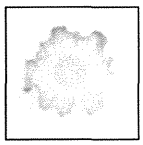


↓
露光５分
飽和

Fig 1.2

一次抗体
血清なし
↓
二次抗体
抗ヒト IgG　1:600

露光１分



↓
露光５分

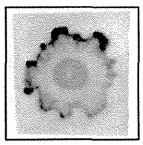
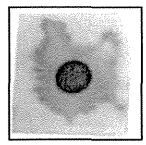


Fig 1.4

一次抗体
正常ヒト血清　1:200
↓
二次抗体
抗ヒト IgG　1:600

露光１分



↓
露光５分
飽和

ウス IgG 抗ジストロフィン抗体，二次抗体に抗マウス IgG 抗体を用いた系で測定を行った。

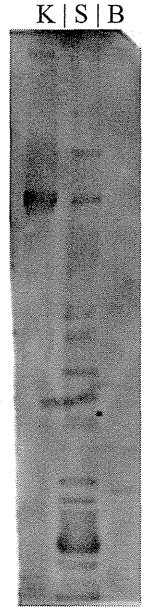
Fig.1 ドットブロット法による抗ジストロフィン抗体測定系の予備検討

その結果露光５分後に筋細胞ライセートを滴下した位置に淡いシグナルが観察され，この系において筋ライセート内ジストロフィンを検出できていることが示された（Fig 1.1）。次に一次抗体を用いずに，二次抗体に抗ヒト IgG 抗体のみを用いた系で測定を行ったところ，露光１分の時点ですでにライセートの位置に一致したシグナルが観察された。この解釈については，筋ライセート内の非特異的反応により，抗ジストロフィン抗体の非存在下でも抗 IgG 抗体が陽性を呈することを示しており，いわば本測定系におけるバックグラウンドと考えられた（Fig 1.2）。さらに一次抗体として正常ヒト血清を 1:50 で用いた場合，露光１分の時点ですでにライセート滴下部位のシグナルは飽和しており，おそらく反応基質の枯渇により，すでにシグナル陰性になったものと考えられた（Fig 1.3）。この推測は，正常ヒト血清を 1:200 まで希釈した系では，露光１分の時点においてまだライセート滴下部位で強いシグナルが検出されたことから（Fig 1.4），1:50 という希釈率に起因するものであることが裏付けられた。ただしヒト血清を用いた場合，露光 5 分ではいずれもメンブレン全体が強いシグナルを呈し，全体として飽和に至った（Fig 1.3-1.4）。以上の結果から，ヒト血清にもともと含まれる IgG 抗体の影響により，抗 IgG 抗体を用いた検出系では極めて強いバックグラウンドを検出することがわかった。またヒト血清を一次抗体として用いる場合には，少なくとも数百倍の希釈倍率とし，さらに二次抗体の希釈倍率は数千倍程度が妥当であることが示唆された。またドットブロットでは検出されるタンパクの分子量は考慮されないため，抗ジストロフィン抗体としてのシグナルの妥当性は，ウェスタンブロットによる評価に基づく必要があることから，以上の予備検討の結果を踏まえ，ウェスタンブロットによる検討を次に行った。

2.　ウェスタンブロットによる抗ジストロフィン抗体測定系の構築

まずマウス抗ジストロフィン抗体を用いた系で検討を行ったところ（Fig 2.1），正常骨格筋ライセートをアプライしたサンプル（S）レーンには分子量 427kDa に相当する位置に明瞭

なジストロフィンのシグナル (*) が検出され

Fig 2.1	Fig 2.2
一次抗体 NCL-DYS-1 1:200 ↓ 二次抗体 抗マウス IgG 1:2500	一次抗体 正常ヒト血清 1:200 ↓ 二次抗体 抗ヒト IgG 1:2500
K | S | B 	K | S | B 
いずれも露光 5 分 K: Kaleidoscope S: Sample B: Blank	

た。もし被験者由来の IgG 型抗ジストロフィ

Fig. 2 ウェスタンブロット法による抗ジストロフィン抗体測定系の検討

ン抗体が出現すれば，被験者血清を一次抗体に用いたレーンにおいて，同じ位置にシグナルが検出されるはずである。一方正常ヒト血清を一次抗体に用いた（S）レーンでは（Fig 2.2），ジストロフィンよりも低い位置にいくつかのシグナルが検出されたが，ジストロフィンに相当する位置にはシグナルは観察されなかった。観察されたシグナルはライセートに含まれる何らかの IgG あるいは非特異的反応と考えられた。メンブレンストリップのバックグラウンドは，一次抗体にヒト血清を用いた方で全体的に高いが，これは精製モノクローナル抗体と生体由来血清の違いに由来するものと考えられた。このバックグラウンドを低減することは技術的に困難と考えられたが，シグナルとの識別が不可能となる程度ではないために許容範囲内と考えられた。以上の結果より，被験者由来血清を用いた場合メンブレンストリップのバックグラウンドは全体的に高くなることを許容する必要があるものの，適切な希釈濃度の選択などによりシグナル／ノイズ比を向上させることは可能と考え，抗ジストロフィン抗体測定に係る基本的要件について一定の知見を得ることができた。

D. 考　察

今回の検討過程においては，ウェスタンブロットによる抗ジストロフィン抗体測定においては，測定に供するメンブレンストリップをどのように構成するかについても課題となった。これは今回検討した系が一般的なウェスタンブロットと異なり，同一のサンプル（骨格筋ライセート）を複数の抗体で反応させて評価する手法であるため，抗原抗体反応系をそれぞれ独立させておく必要が生じるためである。そのため 1 枚のゲルから作製したメンブレンは，複数枚のメンブレンストリップに分割して，それぞれを独立させて抗原抗体反応を行うこととなる。我々が用いるゲルは 15 レーンであるため，最大では 15 枚まで分割することが可能であるが，実際には表裏を判別するための標識及び分割する際の切り代も考慮する必要があり，その結果サンプル，マーカー，切り代となるブランクを含め，3 レーンを 1 枚のメンブレンストリップとして扱うことが最適との結論になった。よってゲル 1 枚から作製できるのは最大 5 枚のメンブレンストリップとなる。この 5 枚をどのように用いるかについて検討したところ陽性対照，陰性対照，バックグラウンド評価，被験者由来血清（治験薬投与前，及び投与後）

の合計5枚で構成することとなった。この配置によって，1枚のメンブレンあたり1名の被験者について，治験薬の投与前後における抗ジストロフィン抗体の有無について評価が可能になると考えられた。

なお抗ジストロフィン抗体の測定は，前述したとおり陽性対照となるヒト由来抗ジストロフィン抗体がいまのところ存在しないことが課題の一つである。ヒト化モノクローナル抗体を作成は技術的に可能と考えられるが，今回の早期探索的臨床試験における実施内容の範囲を大きく超えるものと考えられたため，見送ることとした。また Sarepta therapeutics 社が開発中の eteplirsen についても，局所投与による第1相試験において，複数の被験者で交差反応と見られる抗ジストロフィン抗体弱陽性の反応があったとの報告がなされている[3]。その測定方法については詳細には示されていないが，抗ジストロフィン抗体の測定手法は未だ発展途上段階にあり，治験における選択・除外基準に採用可能な水準とは言いがたいことを示唆する事例と言える。しかしながら先行するアンチセンス薬の臨床試験では，抗ジストロフィン抗体測定は一般的に実施されている。これは上述のとおり，当該抗体に関する知見が充分ではないことから，安全面に加え，科学的な観点からこれらに関しての知見を集積する趣旨からと考えられ，NS-065/NCNP-01 の早期探索的臨床試験においても，探索的なバイオマーカーの一種として慎重な評価を行う必要があると考えられた。

E. 結　論

抗ジストロフィン抗体を検出する手法の検討を行った。抗ジストロフィン抗体の出現が想定される被験者の血清を一次抗体，さらに標識された抗ヒト IgG 抗体を二次抗体とする系を構築し，正常ヒト骨格筋に含まれるジストロフィンが検出されるかを検討したところ，正常ヒト由来血清を用いた場合，構築した系ではジストロフィンは検出されなかった。抗ジストロフィン抗体そのものの検出は技術的に克服すべき点が多いが，本項目については目的とする解析系の構築についてほぼ達成できたと考えられる。

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F. 健康危険情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得
   なし

2. 実用新案登録
   なし

3. その他
   なし

様式第１９

学　会　等　発　表　実　績

委託業務題目「デュシェンヌ型筋ジストロフィーに対するエクソン・スキップ治療薬の臨床開発に資するバイオマーカーの探索」

機関名：独立行政法人国立精神・神経医療研究センター

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
Gene Therapy in DMD, Updates and Future Prospects	Takeda S	13th Annual Asean Oceanian Myology Center (AOMC) and 20th Philippine Neurological Association (PNA) Midyear convention	5.16, 2014	国外
デュシェンヌ型筋ジストロフィーに対するエキソン・スキップ誘導療法	武田伸一	第55回日本神経学会学術大会	5.23, 2014	国内
Molecular mechanism of muscle hypertrophy; our current attempt to do exon skipping clinical trial	Takeda S	Seminar at The Ohio State University College of Medicine	5.27, 2014	国外
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DMDの遺伝子治療の現状と未来	武田伸一	The third International Conference of DMD Rehabilitation and Treatment 2014年 第3回 筋ジストロフィー（DMD）リハと治療国際セミナー	8.24, 2014	国外
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ここまできた難治性筋疾患の治療法	武田伸一	平成26年度 愛知県医師会治験講演会	9.23, 2014	国内
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筋ジストロフィーに対する新たな治療へ『筋ジストロフィー医療50周年記念シンポジウム「筋ジストロフィー医療の転換点をむかえて」』	武田伸一	第68回国立病院総合医学会	11.15, 2014	国内
筋ジストロフィーに対するアンチセンス核酸医薬品の開発を目指して	武田伸一	日本DDS学会創立30周年記念シンポジウム，遺伝子医療・核酸医薬品とDDS	12.15, 2014	国内
国立精神・神経医療研究センター トランスレーショナル・メディカルセンターにおける医師主導治験の実際	武田伸一	革新的医療研究開発で挑む神経変性疾患―プリオン病治験体制の確立に向けて―	2.14, 2015	国内

Assessment of the Dystrophin Gene Exon 53 Skipping Using DMD Patient-Derived Fibroblasts for Exploratory Clinical Trial of Antisense Drug NS-065/NCNP-01	Saito T, Nagata T, Masuda S, Tanihata J, Ohata M, Tamaura A, Kanazawa M, Minami N, Goto K, Hayashi Y, Iwasawa K, Tatezawa K, Fukuda K, Mizutani T, Shimizu R, Suzuki M, Yamaguchi K, Tachimori H, Nishino I, Goto Y, Komaki H, Takeda S	American society of gene & cell therapy 17th Annual meeting	5.21, 2014	国外
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Truncated dystrophin with exon 45–55 deletion induced muscle atrophy and fiber type change through the hyper-nitrosylation of the ryanodine receptor type-1 and constant release of Ca2+ to the cytosol	Tanihata J, Nagata T, Saito T, Ito N, Aoki Y, Nakamura A, Takeda S	19th International Congress of the World Muscle Society	10.8, 2014	国外
Duchenne 型筋ジストロフィーに対するエクソン53スキップ治療薬の早期探索的臨床試験	永田哲也, 武田伸一	第55回日本神経学会学術大会	5.24, 2014	国内
Exon45-55を欠失した短縮型dystrophinはRyR1をニトロシル化し、細胞内Ca2＋濃度を上昇させる	谷端　淳, 永田哲也, 齊藤　崇, 伊藤尚基, 中村昭則, 武田伸一	第69回日本体力医学会大会	9.20, 2014	国内

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
Discovery of serum protein biomarkers in the mdx mouse model and cross-species comparison to Duchenne muscular dystrophy patients	Hathout Y, Marathi RL, Rayavarapu S, Zhang A, Brown KJ, Seol H, Gordish-Dressman H, Cirak S, Bello L, Nagaraju K, Partridge T, Hoffman EP, Takeda S, Mah JK, Henricson E, McDonald C	Hum Mol Genet	2014 Dec	国外
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Discovery of serum protein biomarkers in the *mdx* mouse model and cross-species comparison to Duchenne muscular dystrophy patients

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It is expected that serum protein biomarkers in Duchenne muscular dystrophy (DMD) will reflect disease pathogenesis, progression and aid future therapy developments. Here, we describe use of quantitative *in vivo* stable isotope labeling in mammals to accurately compare serum proteomes of wild-type and dystrophin-deficient mdx mice. Biomarkers identified in serum from two independent dystrophin-deficient mouse models (mdx-Δ52 and mdx-23) were concordant with those identified in sera samples of DMD patients. Of the 355 mouse sera proteins, 23 were significantly elevated and 4 significantly lower in mdx relative to wild-type mice (*P*-value < 0.001). Elevated proteins were mostly of muscle origin: including myofibrillar proteins (titin, myosin light chain 1/3, myomesin 3 and filamin-C), glycolytic enzymes (aldolase, phosphoglycerate mutase 2, beta enolase and glycogen phosphorylase), transport proteins (fatty acid-binding protein, myoglobin and somatic cytochrome-C) and others (creatine kinase M, malate dehydrogenase cytosolic, fibrinogen and parvalbumin). Decreased proteins, mostly of extracellular origin, included adiponectin, lumican, plasminogen and leukemia inhibitory factor receptor. Analysis of sera from 1 week to 7 months old mdx mice revealed age-dependent changes in the level of these biomarkers with most biomarkers acutely elevated at 3 weeks of age. Serum analysis of DMD patients, with ages ranging from 4 to 15 years old, confirmed elevation of 20 of the murine biomarkers in DMD, with similar age-related changes. This study provides a panel of biomarkers that reflect muscle activity and pathogenesis and should prove valuable tool to complement natural history studies and to monitor treatment efficacy in future clinical trials.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most common and severe form of childhood muscular dystrophy affecting 1 in 3500 boys worldwide (1). Currently, there is no cure for the disease, except corticosteroid treatment that has been shown in several clinical trials to improve muscle strength and respiratory function (2–5), and prolong ambulation for 2 or more years (6–9). These beneficial effects are offset, in part, by side effects, such as weight gain, growth stunting and bone health issues (5).

Fortunately, promising therapeutic strategies for DMD are increasingly under development, with many now in clinical trials (10–13). Perhaps one of the most interesting ones is those aiming to restore the missing dystrophin protein (10,14–16). These include exon skipping strategies with antisense oligonucleotide

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(17–19), a stop codon read through strategy using the drug Ataluren, also known as PTC124 (11), and viral gene therapy using the adeno-associated virus as a micro dystrophin gene carrier (15,20). VPB15, a novel steroid dissociative drug, has been recently shown to be also a promising anti-inflammatory agent since it improves muscle physiology without side effects (21). As these new-generation therapies move into human clinical trials in DMD, it becomes important to develop minimally invasive outcome measures that can give an early readout of potential drug efficacy.

Currently, most clinical trials for DMD rely on physical assessments such as the 6 min walk test, which is the walking distance a patient can cover in 6 min (22), the North Star Ambulatory assessment (23,24), as well as quantitative muscle strength tests (25). Even though useful, these physical tests are limited to ambulatory patients, often challenging to implement, and could be confounded with patient's willingness, especially in young children. Defining biochemical markers that correlate with the clinical phenotype and the above outcome measures will aid assessment of treatment efficacy in DMD clinical trials.

Serological levels of creatine kinase (CK) have long been used to aid diagnosis of DMD and other muscular dystrophies (26) and remain an important laboratory test for diagnosis. Unfortunately, because CK serum levels can be easily influenced by muscle exertion or trauma, and because of its high abundance in serum of DMD patients, it might not prove sensitive to early treatment response.

Recently, with advances in 'omics' technologies (e.g. genomics, proteomics and metabolomics), valuable sets of biomarkers are being discovered in muscle tissue and serum of both DMD patients and animal models. While major biomarkers and molecular mechanism have been discovered by proteomics and genomics studies of dystrophic muscle tissue in the past few years (27), effort toward large-scale biomarkers discovery in body fluids only started emerging in the last 3 years. Indeed, recent studies have shown a clear correlation between specific serum miRNAs and DMD severity in both human patients and animal models (28–30). Other molecular markers that correlated with disease progression include matrix metalloproteinase-9 (MMP9), which was found highly elevated in DMD patients relative to controls and increased with disease severity (31). And more recently, the same group identified fibronectin as potential biomarker for muscular dystrophy (32). Although these preliminary studies were conducted with targeted markers, they clearly support the hypothesis that serum circulating biomarkers can act as indicators of DMD disease progression and potentially as biomarkers to monitor treatment efficacy.

In this study, we carried out comprehensive serum proteome profiling using a combination of stable isotope labeling in mammals (SILAM) strategy (33,34) and high precision mass spectrometry to identify serum circulating protein biomarkers in dystrophin-deficient *mdx* mice. These were tested across the age range of *mdx* mice to correlate their association with muscle disease progression. Several of the biomarkers identified in the dystrophin-deficient mouse model also exhibited higher levels in human serum samples collected from DMD patients compared with age-matched healthy controls. Development of a DMD biomarker panel, as opposed to a single biomarker, may prove robust

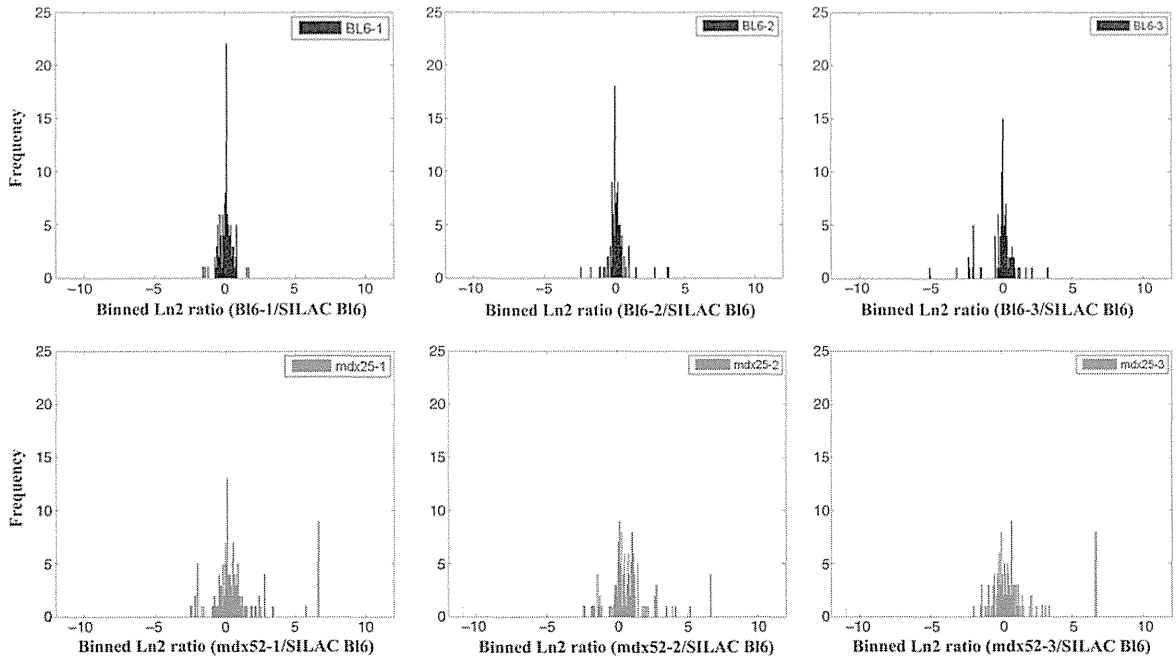

Figure 1. Distribution of protein ratios identified in proteome profiling of sera samples of mdx-52 mice and wild-type BL6 mice spiked at 1:1 ratio with serum aliquots from SILAC-labeled BL6 mouse. Each serum aliquot containing 50 μg of total proteins from 3 weeks old mdx-52 mice ($n = 3$) or 3 weeks old BL6 mice ($n = 3$) was spiked with 50 μg serum aliquots from 13C6-Lys labeled BL10 serum and processed for proteome profiling and quantification as described in Materials and Methods. Top panel shows the overall log ratio distribution of all identified unlabeled and labeled peptide pairs in 1:1 serum mixtures of unlabeled BL10 mice relative to $^{13}C_6$-Lys labeled BL6 internal standard and the bottom panel show similar ratios distribution in 1:1 serum mixture of unlabeled mdx-52 mice relative to $^{13}C_6$-Lys labeled BL6 internal standard. All distributions showed a normal Gaussian shape with values centered around 1 in BL6 versus SILAC BL6 pairs and wilder distribution in mdx-52 versus SILAC BL6 pairs analyzed. An average of 180–200 proteins were identified and quantified in each analysis.

and non-invasive readout tool for muscle tissue, its current disease state and potentially its response to therapy.

RESULTS

Serum protein biomarker discovery using two independent dystrophin-deficient mouse models (mdx-52 and mdx-23)

Biomarker discovery was first performed on serum samples from 3-week-old mdx-52 mice ($n = 3$) and age-matched wild-type controls BL6 ($n = 3$). Each *mdx* sample was spiked with an equal amount of $^{13}C_6$-Lys BL6 labeled serum. Three-week-old mdx mice were chosen because the initial onset of muscle pathogenesis in these mice usually occurs at 3 weeks of age (35). Supplementary Material, Figure S1 shows mass spectra of an elevated protein (e.g. CK M), unchanged protein (e.g. albumin) and decreased protein (e.g. leukemia inhibitory factor receptor) in serum of mdx-52 mouse relative to its wild-type BL6 mouse. This initial experiment led to the identification and quantification of 214 proteins in all samples combined. Keratins and multiple isoform immunoglobulins were removed from the analysis, leaving 192 proteins. These are listed in Supplementary Material, Table S1 with average ratios to internal SILAC standard, spectral count and standard deviation in the mdx-52 mouse versus BL6 groups. Ratio distributions of all quantified proteins in serum of the two mouse groups relative to the internal SILAC-labeled serum proteins are plotted in Figure 1 as log2 of the ratio. While the distribution of protein log2 ratios was around zero (ratio of 1:1) in the serum of all three BL6 to SILAC BL6 pairs, it was wider in the mdx-52 versus SILAC BL6 pairs. This clearly indicates strong alterations in the levels of some proteins in the serum of mdx-52 mice relative to its wild-type counterpart BL6 mice.

Similarly, biomarker discovery was performed on serum of the spontaneous allele mdx-23 mice ($n = 3$) and age-matched wild-type controls BL10 ($n = 3$). In this second discovery set, ^{15}N labeled wild-type mouse sera were used as a spike-in standard instead of $^{13}C_6$-Lys labeled serum. The ^{15}N labeling strategy allowed identification and quantitation of 355 proteins from six serum samples combined. Omitting again keratins and the multiple interchangeable immunoglobulin isoforms, 305 proteins are retained for analysis, and these are listed in Supplementary Material, Table S2 with ratios to internal SILAC standard, spectral count and standard deviation in the mdx-23 mice and BL10 mice groups. In general, ^{15}N based proteome profiling led to the identification and quantification of more proteins, 181 more proteins than $^{13}C_6$-Lys based proteome profiling strategy. This is mainly due to the fact that only Lys containing peptides are quantifiable in $^{13}C_6$-Lys proteome profiling experiment, while every peptide is quantifiable in ^{15}N based proteome profiling experiments.

Examples of protein biomarkers whose levels were increased, unchanged or decreased in mdx-23 mice versus wild-type BL10 are shown in Figure 2. Fructose-bisphosphate aldolase A (ALDOA), somatic cytochrome C (CYC) and myoglobin (MYG) were significantly increased in mdx-23 sera relative to wild-type BL10 mice. Adiponectin (ADIPO) and LUM were significantly decreased in serum of mdx-23 mice, while transthyretin remained unchanged between the two groups. Similar analyses of all 305 identified proteins were performed at the peptide level. Proteins that were concordant between the mdx-23 and mdx-52 studies are listed in Table 1 with average fold change relative to wild-type mice, an average peptide spectral count used to quantify each protein and *P*-values. Elevated proteins in the serum of the dystrophin-deficient mice included myofibril proteins such as titin (TITIN), myosin light chain 1/3 (MYL1), myomesin-3 (MYOM3), myosin-4 (MYH4), myosin-1,

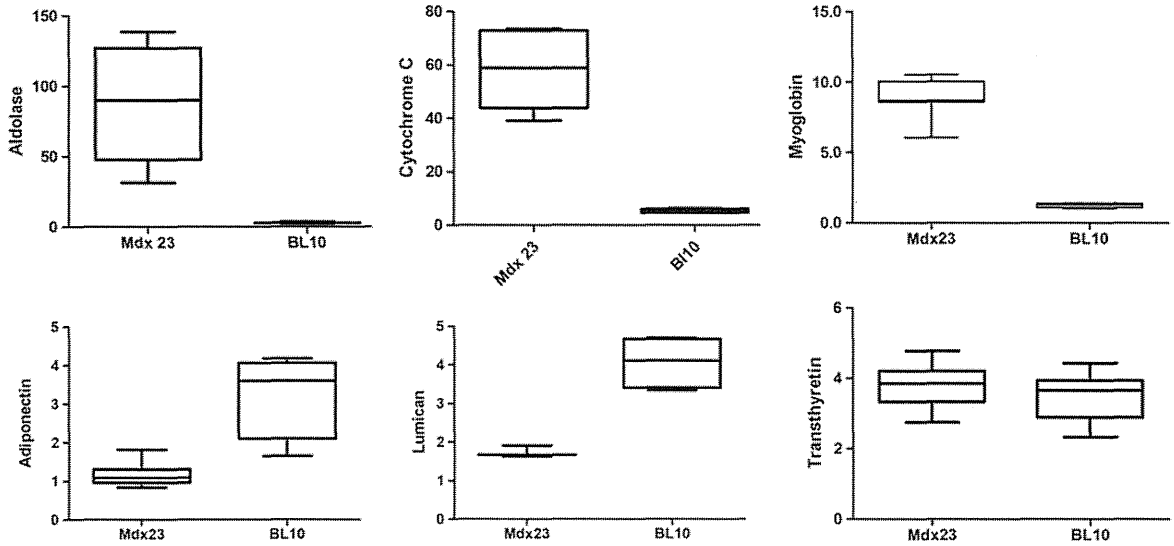

Figure 2. Box plots showing levels of six representative proteins in sera samples of mdx-23 mice compared with its healthy counterpart wild-type BL10 mice. Serum samples from 3 weeks old mdx-23 mice ($n = 3$) and age-matched wild-type BL10 mice ($n = 3$) were each spiked with fixed amount of ^{15}N-labeled serum samples from wild-type mice as internal standard. Sample mixtures were processed for protein identification and quantification as described in Materials and Methods. Tope panel shows an example of three proteins, aldoalse, cystochrom-c somatic and myoglobin whose level was significantly (P-value < 0.001) higher in serum of mdx-23 mice group compared with the wild-type BL10 mice group. Bottom panels show an example of two proteins, adiponectin and lumican whose level was significantly decreased in serum of the mdx-23 mice group compared with wild-type Bl10 mice (P-valued < 0.001) and transtherytin protein that remained unchanged between the two groups.

Table 1. List of candidate protein biomarkers whose levels were found significantly altered in the serum of dystrophin-deficient mdx-23 mice ($n = 3$) versus wild-type BL10 mice ($n = 3$) and in mdx-52 ($n = 3$) versus wild-type BL6 ($n = 3$)

Accession N°	Locus	Protein name	mdx-23 relative to BL10 (fold change)	mdx-52 relative to BL6 (fold change)	PSC average	*P*-value
Myofibrillar proteins						
A2ASS6	TITIN	Titin	Unique in mdx-23	Unique in mdx-52	25	0.0000
P05977	MYL1	Myosin light chain 1/3	Unique in mdx-23	Unique in mdx-52	4	0.0000
Q5SX39	MYH4	Myosin-4	Unique in mdx-23	Unique in mdx-52	3	0.0000
A2ABU4	MYOM3	Myomesin-3	Unique in mdx-23	Unique in mdx-52	3	0.0000
Q8VHX6	FLNC	Filamin-C	Unique in mdx-23	Unique in mdx-52	7	0.0000
P68134	ACTS	Actin, alpha skeletal muscle	+1.5-fold	+2.93-fold	16	0.0002
Glycolytic enzymes						
P05064	ALDOA	Fructose-bisphosphate aldolase A	+23-fold	+21-fold	5	0.0479
P16858	GAPDH or GP3	Glyceraldehyde-3-phosphate dehydrogenase	Unique in mdx-23	+4.43-fold	5	0.0000
P52480	KPYM	Pyruvate kinase isozymes M1/M2	Unique in mdx-23	Unique in mdx-52	12	0.0000
P06151	LDHA	L-lactate dehydrogenase A	+3.27-fold	+3.2-fold	3	0.0000
P17751	TPIS	Triosephosphate isomerase	Unique in mdx-23	+3.1-fold	3	0.0000
Q9WUB3	PYGM	Glycogen phosphorylase	Unique in mdx-23	Unique in mdx-52	16	0.0000
P21550	ENOB	Beta-enolase	Unique in mdx-23	nd	7	0.0000
O70250	PGAM2	Phosphoglycerate mutase 2	Unique in mdx-23	Unique in mdx-52	5	0.0000
Transport proteins						
P04247	MYG	Myoglobin	+11-fold	+2.9-fold	7	0.0000
P11404	FABPH	Fatty acid-binding protein, heart type	Unique in mdx-23	nd	3	0.0000
P62897	CYC	Cytochrome c, somatic	+55-fold	+20-fold	5	0.0109
Other muscle-specific proteins						
P07310	CK	Creatine kinase M-type	Unique in mdx-23	Unique in mdx-52	8	0.0000
P14152	MDHC	Malate dehydrogenase, cytoplasmic	+57-fold	+1.46-fold	7	0.0001
Q9Z1T2	TSP4	Thrombospondin-4	+7-fold	+1.53-fold	3	0.0000
P32848	PRVA	Parvalbumin alpha	+13.5-fold	+15-fold	6	0.0000
P05367	SAA2	Serum amyloid A-2 protein	+4.2-fold	nd	12	0.0000
Q8VCM7	FIBG	Fibrinogen gamma chain	+4.16-fold	+3.15-fold	1	0.0000
P20918	PLMN	Plasminogen	−1.5-fold	−0.2-fold	28	0.0000
Q60994	ADIPO	Adiponectin	−1.5-fold	Unchanged	9	0.0000
P51885	LUM	Lumican	−1.5-fold	−0.2-fold	4	0.0006
P42703	LIFR	Leukemia inhibitory factor receptor	−3.3-fold	−3-fold	25	0.0000

PSC, peptide spectral count.

filamin-C (FLNC) and actin alpha skeletal (ACTS); glycolytic enzymes such as ALDOA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH or GP3), pyruvate kinase isozymes M1/M2 (KPYM), glycogen phosphorylase (PYGM), L-lactate dehydrogenase A (LDHA), triosephosphate isomerase (TPIS), beta enolase (ENOB), phosphoglycerate mutase 2 (PGAM2); transport proteins such as MYG, CYC and fatty acid-binding protein heart type (FABPH). Other highly elevated proteins in the serum of mdx mice included malate dehydrogenase (MDHC), CK M, thrombospondin-4 (TSP4), serum amyloid A-2 protein, fibrinogen gamma chain (FIBG), parvalbumin (PRVA) and superoxide dismutase. Several IgG kappa and gamma chains were also found significantly increased in the serum of mdx-23 relative to BL10. These were not included in Table 1 but reported in Supplementary Material, Tables S1 and S2.

On the other hand, only a few proteins were found moderately decreased in serum of mdx-23 mice relative to wild-type BL10 mice. These included mostly proteins involved in extracellular matrix remodeling and cell proliferation, such as plasminogen (PLMN), ADIPO, lumican (LUM) and leukemia inhibitory factor receptor (LIFR). Perhaps, the most significant decrease was observed for LIFR which was concordant in both mdx-23 and mdx-52 model and decreased by 3-folds in both mouse models relative to their wild-type control.

Age-dependent changes in the level of serum biomarkers in the mdx-23 mouse model

The mdx-23 mouse model shows age-related pathology, with no overt pathology before the age of 3 weeks followed by an acute onset of muscle necrosis at 3 weeks of age, with subsequent successful regeneration and relatively mild dystrophic pathology in most muscle groups in older mice (35). To determine whether the identified candidate biomarkers above are associated with age-dependent muscle pathology in the dystrophin-deficient mdx-23 mouse, we performed quantitative proteome profiling using the ^{15}N SILAM strategy on serum samples of mdx-23 mice at 7 days, 3 weeks, 2 months, 4 months and 7 months of age, focusing on the levels of the biomarkers identified in the discovery set above listed in Table 1. Data were visualized by unsupervised hierarchical clustering of serum biomarkers in mdx-23 and wild-type BL10 mice as a function of age (Fig. 3). A dendrogram of age showed co-clustering of newborn wild-type and mdx mice, co-clustering of 3 weeks and 2 months old mdx mice and co-clustering of 4 months and 7 months old mdx apart from the wild-type mice groups. This clustering by age corresponded well to the established age-related histological patterns. Most of the biomarkers identified above were undetectable in the sera of new born mdx-23 mice then acutely rose in

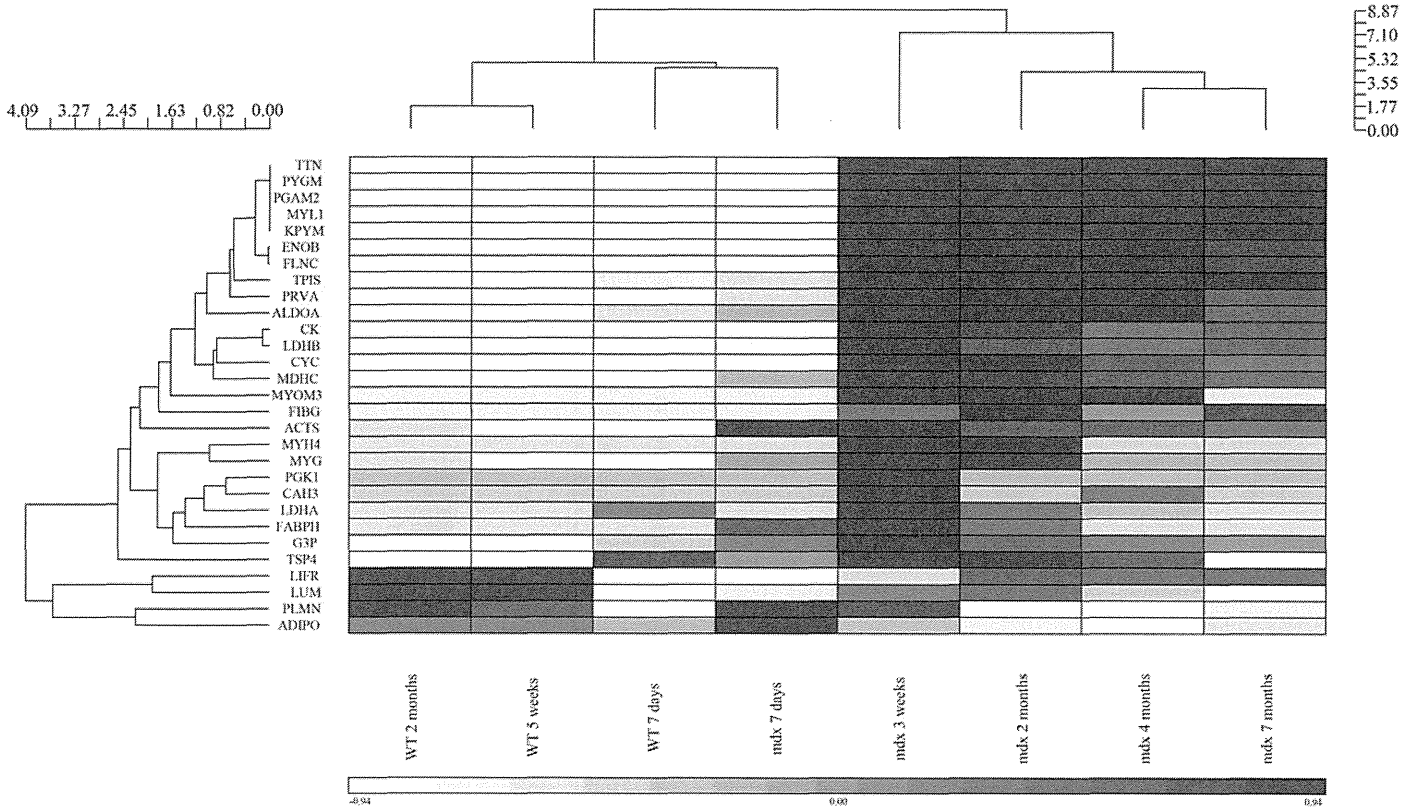

Figure 3. Hierarchical clustering of selected candidate protein biomarkers in serum samples of mdx-23 mice across different age group. Serum samples from mdx-23 mice at 7 days, 3 weeks, 2 months, 4 months and 7 months of age ($n = 3$ per group) were spiked with fixed amount of ^{15}N-labeled serum from the wild-type mouse as internal standard. Similarly serum samples from wild-type Bl10 mice at 7 days, 5 weeks and 2 months of age were spiked with ^{15}N- labeled serum and used as controls. Sample mixtures were processed for proteomes profiling as described in Materials and Methods. Ratios of candidate biomarkers were measured in serum samples across different age groups and then uploaded into Partek software for clustering analysis. SILAC ratio values were natural log transformed, and the color scale is based on how many standard deviations each value deviates from the mean (white for values below the mean, and black for values above the mean). Clustering by columns shows that a select group of biomarkers is able to discriminate wild-type BL10 from mdx-23 mice, and age within each group. Clustering by rows shows consolidation of biomarker 'classes' described in the text.

mdx-23 at 3 weeks of age correlating with the active necrotic phase (35). In addition to age-dependent clustering of mdx mice, the heat map revealed three major clusters of biomarkers. A first cluster consisted of biomarkers that acutely rose in sera of the 3 weeks old mdx-23 mouse (necrotic stage) and remained increased throughout the age studied, up to 7 months of age. These included seven glycolytic enzymes (e.g. PYGM, PGAM2, TPIS, KPYM, LDHB, ALDOA and ENOB), three myofibrillar proteins (e.g. TITIN, MYL1 and FLNC) and four other muscle-derived proteins (e.g. CK, MDHC, PRVA and CYC). The second cluster consisted of biomarkers that also acutely rose in blood of 3 weeks old mdx-23 then gradually decreased with age. These included three glycolytic enzymes (e.g. LDHA, PGK1 and GPADH), three myofibriallar proteins (MYOM3, ACTS and MYH4) and three muscle-derived proteins (CAH3, FABPH, MYG and TSP4). Finally, the third cluster consisted of biomarkers that decreased with age in mdx-23 and these were mostly of extracellular origin (e.g. LIFR, LUM, ADIPO, FIBG and PLMN). Even though the majority of these biomarkers rose in blood of mdx mice at 3 weeks of age few of them were, however, found moderately elevated in serum of 7 days old mdx mice, prior to the onset of muscle necrosis (e.g. ACTS, MYH4, ALDOA, GAPDH, MDHC, TSP4 and MYG) and could be classified as pre-necrotic biomarkers.

Biomarkers discovery in human serum samples from DMD and healthy controls subjects

The protein biomarker panel discovered in serum of dystrophin-deficient mouse models was examined in serum samples of DMD ($n = 15$) and healthy volunteers ($n = 6$), using a combination of label-free proteome profiling and western blot analysis. Immunoblot analysis was complicated by the serum biomarker typically being a truncated fragment of the full-length protein. Thus, availability of commercial antibodies for the specific fragment was challenging. Of the five antibodies tested against five target candidate biomarkers (FLNC, TITIN, MYOM3, PGAM2 and PGYM), only the antibody against PGAM2 provided clear results. PGAM2 was detected as full-length protein around 30 kDa in all serum samples of the DMD group but at low or undetectable levels in the control group (Fig. 4A). However, unlike in the mdx mouse model, the serum level of PGAM2 rapidly decreased with age in the DMD patient sera. It was significantly higher in younger DMD patients, between the age of 4 and 8 years old, compared with older DMD patients between the age of 11 and 15 years old. PGAM2 was almost undetectable in serum samples of DMD patients 11–15 years of age (Fig. 4B).

To identify additional biomarkers in DMD patients, label-free proteome profiling was performed on serum samples collected

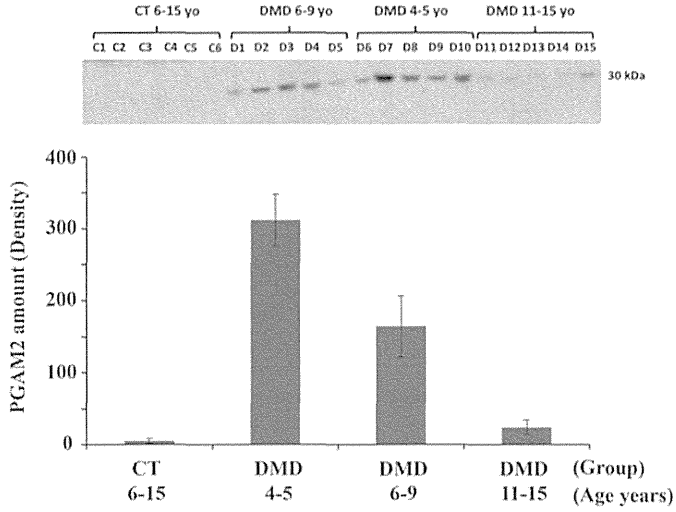

Figure 4. Western blot analysis showing levels of PGAM2 in sera samples of DMD patients and healthy controls. Serum aliquots containing 30 μg total proteins from DMD patients with different age groups (4 to 5, 6–9 and 11–15 years of age, $n = 5$ per group) and from healthy controls ($n = 6$) with age ranging from 6 to 15 years old were processed for western blot analysis against PGAM2 as described in Materials and Methods. Top panel shows the actual western blot results of PGAM2 which was detected around its expected molecular mass of 30 kDa and it was present only in serum samples of DMD patients (D1 to D15) and undetectable in serum of healthy controls (CT1 to CT6). The bottom panel shows the density histogram plot of the PGAM2 band across different age groups. The data clearly show the rapid age-dependent decrease in the serum levels of PGAM2 in DMD patients.

from DMD patients ($n = 15$) (4–15 years old), and healthy volunteers ($n = 3$) (6–15 years old). Importantly, of the 23 elevated biomarkers identified in the mdx-23 mouse model, 20 were also found significantly increased, based on spectral count, in sera of DMD patients compared with healthy volunteers (Fig. 5). As in the dystrophin-deficient mouse model, the serum biomarkers identified in DMD patient sera included glycolytic enzymes such as ALDOA, LDHA, LDHB, ENOB and PYGM (Fig. 5A); myofibril proteins such as MYOM3, TITIN, ACTS, FLNC and MYL1 (Fig. 5B) and other muscle-derived protein such as MYG, CAH3, ADIPO, MDHC, CYC, FIBG, TSP4, FABPH and MMP9 (Fig. 5C). All biomarkers, except for ADIPO and MMP9, were highest in younger DMD patients (4–8 years old), then decreased as a function of age in a manner similar to CK while ADIPO and MMP9 increased with age in serum of DMD patients (Fig. 5C).

DISCUSSION

Differential proteome profiling of sera samples of dystrophin-deficient mouse models and DMD patients were conducted using both the SILAM mouse strategy and label-free proteome profiling strategy, respectively. This enabled identification of 20 serum biomarkers that were concordant in sera samples of two independent dystrophin-deficient mouse models (e.g. mdx-23 and mdx-52) and DMD patients. Furthermore, analysis of serum samples across different age groups revealed correlation between the serum levels of these biomarkers and disease stage in both dystrophin-deficient mouse models and in DMD patients.

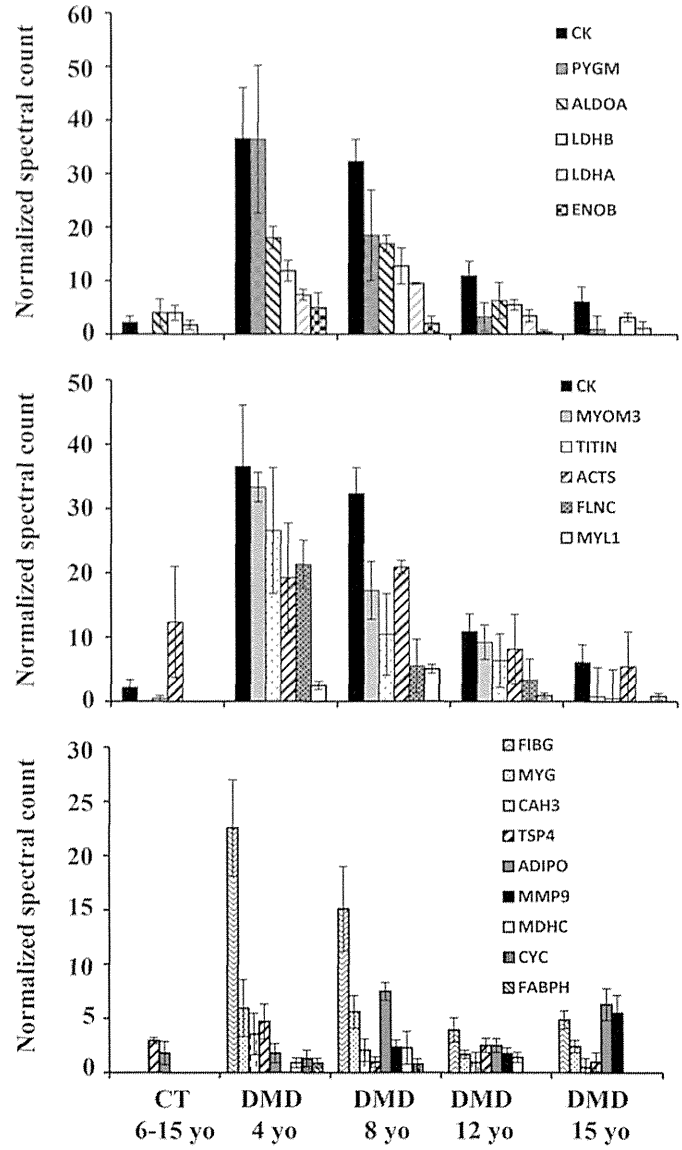

Figure 5. Serum levels of candidate biomarkers discovered by label-free proteome profiling in serum samples of DMD patients and healthy control across different age groups. Serum aliquots containing 100 μg total proteins from 12 DMD patients at 4, 8, 12 and 15 years of age ($n = 3$ per age group) and from 3 healthy controls at 6, 8 and 12 years of age were processed for label-free proteome profiling as described in Materials and Methods. (A) Levels of five glycolytic enzymes in DMD groups and control group. PYGM, glycogen phosphorylase; ALDOA, fructose-bisphosphate aldolase A; LDHB, lactate dehydrogenase B; LDHA, lactate dehydrogenase A; ENOB, beta enolase. (B) Levels of five myofibrillar proteins, in DMD groups and control group. MYOM3, myomesin-3; TITIN, titin; ACTS, alpha skeletal muscle actin; FLNC, filamin-C; MYL1, myosin light chain 1/3 in DMD groups and control group. (C) Levels of nine other muscle-specific proteins in DMD groups and control group. MYG, myoglonin; CAH3, carbonic anhydrase III; ADIPO, adiponectin; TSP4, thrombospondin-4; MDHC, cytosolic malate dehydrogenase; CYC, somatic cytochrome C; MMP9, matrix metalloproteinase-9; FIBG, fibrinogen gamma chain; FABPH, fatty acid-binding protein heart type. A CK level was added along with glycolytic enzyme in (A) and myofibrillar proteins in (B) for trends comparison. It was omitted in the other muscle-specific protein group in (C) due to the large dynamic range between the levels of CK and this class of proteins. Error bars represent standard error obtained from triplicate biological sample.

Most, if not all, the biomarkers identified in the serum of the mdx mouse models were muscle derived. These biomarkers

were undetectable in serum of pre-symptomatic young mdx mice (e.g. 7 days of age) and then acutely increased in 3-week-old mdx mouse serum, correlating with the onset of muscle necrosis (35), thereby confirming their validity as a muscle disease state biomarker panel. Interestingly, some proteins including ACTS, ALDOA, GAPDH, MDHC, TSP4, MYG and FABPH were found to be elevated in the serum of mdx mice at 7 days of age, even before the onset muscle necrosis. Presence of these proteins in newborn mdx sera is intriguing. At this time, we could only speculate that this is due to disturbance of an integral mechanism, perhaps via compromised control of permeability of the sarcolemma or exosomal shedding, rather than leakage from catastrophically damaged necrotic muscle fibers. Nevertheless, according to our experience, careful handling is required when collecting blood from newborn mice especially for biomarkers studies. We compared decapitation and heart puncture collection methods using healthy wild-type newborn mice and found that decapitation resulted in contamination with several muscle-derived proteins such as CK and several other glycolytic enzymes. In our hands, the heart puncture method was preferred, giving minimal or no contamination, and was therefore used to collect all mouse sera samples in this study.

These pre-necrotic biomarkers further increased in the blood of mdx mice at 3 weeks of age (necrotic phase). In general, the majority of biomarkers, especially glycolytic enzymes, myofibrillar proteins and other muscle-derived enzymes, acutely increased in serum of mdx mice during the necrotic phase. In DMD patients, where there is no marked separation between pre-necrotic and necrotic phases, this panel of biomarkers was detected in both young and old DMD patients (4–15 years old in this study) with the highest levels seen in 4-year-old DMD patients. In a future study, it would be interesting to examine the level of these biomarkers in newborn DMD patients to determine their value in neonatal screening.

Overall in this study, we identified concordant biomarkers associated with dystrophinopathy between two independent mdx mouse models and DMD patients, further confirming the validity and specificity of this biomarker panel. These mouse/human shared biomarkers can be classified into three categories as described below.

The first category of biomarkers consisted of six muscle-derived proteins (CK, CAH3, MYG, MDHC, FABPH and CYC) and three proteins of extracellular origin (ADIPO, FIBG and TPS4). CK, MYG and CAH3 are well-known DMD markers and were identified long ago to be elevated in sera of DMD patients (26,36,37), while MDHC, TSP4, FABH, CYC, FIBG and ADIPO are relatively novel. As a proof of principle, CK was undetectable in the pre-necrotic phase mdx mice and then acutely increased at 3 weeks of age in agreement with previous studies (38). Its level, however, decreased moderately with age and was still elevated in 7-month-old mdx mice while in DMD patients it decreased sharply with age. Similarly, the level of CAH3 was also historically suggested as a serum biomarker for muscular dystrophy (37) but was not revisited. The CAH3 serum level correlated well with that of CK and also decreased with age in DMD patients.

FABPH, heart type fatty acid-binding protein, has been previously used as a biomarker to monitor the cardiac status in DMD and BMD patients (39) and its level correlated well with that of CK. Even though this protein is named heart type FABPH, it may actually be of skeletal muscle orgin since it was found highly elevated in younger mdx mice and DMD patients whose cardiac muscle should be still healthy. Similarly, CYC was found elevated in 3 weeks old mdx mice and DMD boys and it release into blood circulation could be the result of increased apoptosis due to inflammation and necrosis.

FIBG was also reported to be elevated in serum of DMD patients relative to healthy controls (40) and in mdx mice relative to wild-type mice (41). Here, we further confirm its increased level in serum of both mdx-23 mice and DMD patients relative to their healthy control counterparts. Even though its level decreased with age in DMD patients, it remained relatively elevated in serum of older DMD patients compared with age-matched healthy controls where it was undetectable by mass spectrometry. Fibrinogen is a hexamer composed of two sets of three different subunits (e.g. α, β and γ). It is normally synthesized by liver and is involved in coagulation cascade during blood clot. But it has been also shown to play a major role in inflammation (42). Furthermore, alteration of the inflammatory function of FIBG subunit via missense mutation rescued muscle pathology in the mdx mouse model (43) making it an attractive biomarker and therapeutic target for future investigations.

As for the MDHC, there is only one single publication in 1975 reporting its elevated activity in serum of DMD patients that also decreased with age. At that time, it was not possible to distinguish between mitochondrial isoform (MDHM) and cytosolic isoform (MDHC), on the basis of enzymatic activity. However, the sequence homology between these two isoforms is only 18%, permitting accurate identification by mass spectrometry herein, to reveal MDHC and not MDHM, as the isoform elevated in the serum of dystrophin-deficient mice. Indeed, MDHC was identified with 6 unique tryptic peptides with no overlapping sequence homology with MDHM tryptic peptides. MDHC is highly expressed in skeletal and cardiac muscle (44,45). MDHC is involved in aerobic energy production during muscle contraction by converting malate to oxaloacetate and transporting the resulting NADH equivalent across the mitochondrial membrane (44) and could be a good marker for dystrophinopathies.

TSP4 is an extracellular glycoprotein that is involved in extracellular matrix remodeling. To the best of our knowledge, this is the first time that TSP4 levels have been shown to be elevated in the serum of dystrophin-deficient mouse models, and its increase may be associated with muscle degeneration and regeneration. Indeed, an earlier study by our group showed that expression levels of TSP4 mRNA are significantly increased in the skeletal muscle of DMD and limb-girdle muscular dystrophy patients, compared with normal controls (46). The increased expression of TPS4 in dystrophic muscle is most likely associated with its role in fibrosis and extracellular remodeling, as has been shown in cardiac muscle of the TSP4 knockout mouse model (47). However, the levels of TSP4 were only moderately elevated in serum of younger DMD patients compared with age-matched healthy controls and decreased rapidly with age. Further analyses are needed to confirm its utility as a potential biomarker in DMD patients.

All biomarkers in this first category decreased with age in serum of DMD patients except for ADIPO and MMP9, which actually increased with age. This increase was not related to aging since their levels did not increase in healthy controls with 6, 8 and 15 years of age. ADIPO is a hormone that is exclusively secreted

by adipose tissue (48). Thus, its increase in the sera of older DMD patients may reflect the progressive replacement of muscle by fat that is often seen in older DMD patients (49). MMP9, on the other hand, is an extracellular protease and has been shown to be involved in extracellular matrix remodeling, inflammation and fibrosis in a number of diseases, including DMD (50,51). In this study, increased levels of MMP9 in blood circulation of DMD patients became more apparent in older patients. This finding is an agreement with previous study where it has been shown that serum levels of MMP9 increased with age in DMD patients and correlated very well with disease severity, making it an attractive candidate biomarker to monitor disease progression and perhaps response to therapies (31). The fact that MMP9 was not detected or quantified in serum of the mdx mouse model could relate to the less inflammatory status of muscle tissue seen in the mdx mouse relative to DMD patients. Nevertheless, MMP9 was found increased in mdx mouse muscle and it is believed to be involved in muscle pathogenesis (50).

Other biomarkers previously identified in sera samples of mdx mouse and that could belong to this first class of biomarkers included an N-terminal peptide fragment of coagulation factor XIIIa which was reported to be elevated in serum of the mdx mouse relative to the wild-type mouse (52) and the LIFR which was reported to be decreased in serum of mdx mouse relative to the wild-type mouse (41). While the decreased level of LIFR was confirmed in both mdx-23 and mdx 52 mouse models in this study, we were not able to confirm coagulation factor XIIIa perhaps due to the fact that SDS–PAGE was used and low molecular mass fragments were overlooked.

The second category of biomarkers found elevated in serum of mdx and DMD patients consisted of glycolytic enzymes and included PYGM, ALDOA, LDHA, LDHB, ENOB and PGAM2. These enzymes are known to be highly abundant in skeletal muscle and their release into blood seems to correlate with that of CK. The enzyme activities of LDHs, ALDOA, ENOB and PGAM2 were previously reported to be significantly elevated in sera samples of DMD patients but never revisited for further evaluation (26,53,54). Furthermore, the level of PGAM2 was found to be elevated in the serum of DMD patients as early as the fetal stage, thus making it a candidate biomarker for neonatal screening (53). In this study, we identified an additional glycolytic enzyme biomarker, PYGM, whose level was as high as CK in younger DMD boys and then decreased with age. PYGM is one of the major glycolytic enzymes in skeletal muscle that breaks down glycogen to monomeric glucose molecules and controls glycogen metabolism (55) adding it to the panel of glycolytic enzyme biomarkers for DMD.

While the level of these glycolytic enzymes decreased with age in DMD patients, the levels remained elevated in aging mdx mouse, up to 7 months of age. The difference seen between mdx mouse models and DMD patients is perhaps attributable to the maintenance of muscle mass in mdx mice over the age range relative to human patients. In humans, the decrease correlates with a drastic loss of muscle mass, and to some extent reduced exercise because of severely impaired motor function. In the murine model, the maintained high level of these biomarkers is perhaps due to the successful and subsiding degeneration/regeneration cycles (56,57).

The third category of biomarkers consisted of relatively novel myofibrillar proteins and included MYL1, TITIN, MYOM3, FLNC and ACTS. These proteins were undetectable in serum of newborn mdx-23 mice, but rose acutely in the serum of 3-week-old mdx-23 mice. Except for MYOM3, these myofibrillar biomarkers remained elevated in serum of older mdx mice studied herein. Detection of these myofibrillar proteins in serum of 7 months old mdx mice was intriguing and suggests continuous disease activity in old mdx mouse model. However, further serum analysis of older mdx mice as well as age-matched wild-type healthy mice is needed to confirm this hypothesis and to rule out aging effect.

These biomarkers were also confirmed by mass spectrometry analysis as being elevated in serum of DMD patients compared with age-matched healthy subjects. However, their serum levels decreased rapidly with age in DMD patients. The high molecular mass myofibril proteins such as TITIN (3816 kDa molecular mass), FLNC (291 kDa molecular mass) and MYOM3 (161 kDa molecular mass) were all detected as fragments in the serum of dystrophin-deficient mice and DMD patients, with molecular masses ranging between 198 and 260 kDa for TITIN, 110 and 160 kDa for FLNC and around 96 kDa for MYOM3. Their detection in blood circulation seems likely to indicate severe skeletal muscle damage. Both TITIN and MYOM3 are components of the sarcomeres and interact with myosins and MYL1 in striated muscle (58), while FLNC is an actin cross linker protein and is located in the sarcolemma as well as in the sarcomeres (59). The release of these myofibrillar protein fragments into blood circulation is likely a result of proteolytic activity during muscle inflammation and necrosis. This hypothesis is further supported by the fact that their release into the bloodstream was not detected in the mdx mice until 3 weeks of age. In DMD patients however, TITIN and MYOM3 were already present in the serum of DMD patients at 4 years old, probably also being a reflection of the florid inflammation and necrosis at this age (60,61). This is further supported by an earlier study performed on skeletal muscle tissue that revealed dramatic degradation of the titin protein in DMD patients after 5 years of age (62) and is in agreement with a more recent study where it has been shown that specific N-terminal and C-terminal fragments of TITIN were detected in urine samples of both the mdx mouse model and DMD patients (63). In this recent study, it was suggested that TITIN could have been cleaved at its N-terminal and C-terminal extremities by calpains and matrix metalloproteinases during muscle necrosis. However, these low molecular mass TITIN fragments were not detected in DMD sera in this study, probably due to their rapid renal clearance leaving the large middle TITIN fragment in blood circulation. ACTS and MYL1, on the other hand, were detected by mass spectrometry in gel bands corresponding to expected molecular masses of 40 and 20 kDa, respectively, thus indicating their release as intact proteins. This is in agreement with our previous *in vitro* study showing that cultured dystrophin-deficient myotubes release intact MYL1 via vesicular process into the cultured secretome (64). However, further investigations are needed to verify if MYL1 is also released via vesicles *in vivo*, by performing proteome profiling on exosomal fractions prepared from serum of dystrophin-deficient mouse models and DMD patients.

To detect these biomarkers in serum of mdx mouse model and DMD patients required further fractionation of serum samples by SDS–PAGE to separate highly abundant proteins such as

albumin, transferrin and IgGs from low abundant proteins. Nevertheless, it is possible that other low abundant and valuable biomarkers were still overlooked due the large dynamic range and complex nature of the serum proteome. Thus, new methodologies are needed to perform in-depth serum proteome profiling and discover additional novel biomarkers in the future.

Conclusion

This study identified a valuable panel of serological biomarkers associated with dystrophin deficiency and age-related muscle pathology in two independent dystrophin-deficient mouse models: the naturally occurring mdx-23 and genetically engineered mdx-52. Many of these biomarkers were confirmed in sera of DMD patients, strongly supporting their validity as markers for dystrophinopathies. In our cross-sectional DMD study, all identified biomarkers declined with age along with CK; however, we argue that the behavior of a panel of proteins of different cellular functions better reflects the range of underlying pathological processes. According to this interpretation, we would expect individual biomarkers from this panel will respond differently to different therapies. So, instead of monitoring a single biomarker at a time, we propose, as the ideal, a protocol to monitor a panel of biomarkers simultaneously. In the future, we will pursue this hypothesis, making use of this DMD biomarker panel to monitor and predict clinical outcomes. To this end, we will evaluate and test these biomarkers for their value in monitoring disease progression and response to therapies in DMD in longitudinal prospective studies.

MATERIALS AND METHODS

Animal experiments

Mouse strains used in this study included the spontaneous dystrophin-deficient allele (mdx-23; splice site mutation in exon 23) on C57BL/10 background (C57BL/10ScSn-Dmdmdx/J) and wild-type C57BL/10 mice, and the induced deletion of exon 52 allele (mdx-52) on the C57BL/6 background (65) and corresponding wild-type background control (C57BL/6). Mdx-23 and wild-type background strains were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mdx-52 mice were provided by Dr Shin'ichi Takeda, NCNP, Kokaira, Japan, and bred and maintained at Children's National Medical Center. All mice were handled under an approved protocol according to the Institutional Animal Care and Committee guidelines at the Children's National Medical Center.

Generating $^{13}C_6$-Lys and ^{15}N-labeled mouse colonies

A fully labeled $^{13}C_6$-Lys BL6 mouse colony with more than 96% labeling efficiency was previously generated in our laboratory (34) using mouse-Express food containing 'heavy' L-lysine ($^{13}C_6$, 99%) from Cambridge Isotope Laboratories (Andover, MA, USA). Serum from these mice was collected, frozen at $-80°C$ and used for subsequent experiments.

^{15}N metabolic labeled mice were generated by feeding newly weaned 3-week-old wild-type C57/BL6 mice ^{15}N (98% +) enriched Spirulina food (Cambridge Isotope Laboratories, Andover, MA, USA). The mice-tolerated Spurilina food well, showing no difference in body weight and overall health from mice that were kept on standard laboratory chow (Supplementary Material, Fig. S2A). Labeling efficiency was monitored via analysis of the urinary proteome every week from the start of the diet. Sufficient urinary proteome labeling with ^{15}N (more than 95% labeling efficiency) was reached by 12 weeks of feeding (Supplementary Material, Fig. S2B). By 12 weeks, all organs and body fluids showed stable isotope labeling efficiencies of proteins greater than 90%. An example of labeling efficiency of the serum proteome is shown in Supplementary Material, Figure S2C. All identified serum proteins ($n = 153$) were enriched by 92–100% with ^{15}N isotope. Sera collected from each ^{15}N-labled mouse were pooled to make one ^{15}N serum labeled stock which was aliquoted in small volumes and stored at $-80°C$ for subsequent use.

Mouse serum collection

Each mouse used in this study was anesthetized by intraperitoneal injection of ketamine and xylazine (80 mg/kg ketamine and 10 mg/kg xylazine). Fully anesthetized animals (no response to toe or ear pinch, reduced heart rate and rate of breathing) were opened along the centerline of the chest, from the caudal end of the rib cage to the clavicle with small dissecting scissors. Ribs were then reflected laterally and stabilized with a retractor. Blood was immediately collected via heart puncture into an Eppendorf tube and allowed to clot at room temperature prior to centrifugation and serum collection. Typically 200 μl of blood could be collected per mouse aged from 3 weeks to 7 months, while only 10–20 μl of blood could be collected per mouse from 2 to 7 days old. This yielded about 80 μl of serum from each older mouse and about 10 μl of serum per young mouse. Serum samples including ^{15}N and $^{13}C_6$-Lys labeled sera were assayed for protein concentration using BCA assay (Thermo Scientific, Rockford, IL, USA). Each sample was then aliquoted in 10 μl volumes and stored at $-80°C$ for proteome profiling as described below. After blood collection, all other organs were harvested and flash-frozen in liquid nitrogen-chilled isopentane for other studies.

Serum sample preparation and mass spectrometry analysis

The overall experimental workflow is shown in Supplementary Material, Figure S3. Initial biomarker discovery was performed on serum of 3-week-old mdx-52 mice ($n = 3$) and age-matched BL6 mice ($n = 3$). Serum aliquots containing 50 μg total protein from each of mouse were spiked with an equal amount of serum protein from SILAM $^{13}C_6$-Lys labeled BL6 mouse serum stock.

Independent biomarker discovery was performed, using ^{15}N labeled mouse strategy, on the naturally occurring mdx-23 mouse ($n = 3$) and its wild-type control BL10 mice ($n = 3$). Serum samples, containing 50 μg of total protein, from 3 months unlabeled mdx-23 mice ($n = 3$) and its age-matched wild-type BL10 mice ($n = 3$) were spiked with ^{15}N labeled mouse serum stock. Unlike $^{13}C_6$-Lys labeled mouse strategy which permits quantification of Lys containing peptides only, the ^{15}N labeling strategy enables quantification of every tryptic peptide.

To test whether the serum levels of the identified biomarkers are associated with age-dependent muscle pathogenesis in mdx-23 mice, a longitudinal study was performed on serum samples