REVIEW ARTICLE

Biological biomarkers in muscle diseases relevant for follow-up and evaluation of treatment

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Abstract

Muscle diseases cover a diverse group of disorders that in most cases are hereditary. The rarity of the individual muscle diseases provides a challenge for researchers when wanting to establish natural history of the conditions and when trying to develop diagnostic tools, therapies, and outcome measures to evaluate disease progression. With emerging molecular therapies in many genetic muscle diseases, as well as biological therapies for the immune-mediated ones, biological biomarkers play an important role in both drug development and evaluation.

In this review, we focus on the role of biological biomarkers in muscle diseases and discuss their utility as surrogate endpoints in therapeutic trials. We categorise these as either 1) disease unspecific markers, 2) markers of specific pathways that may be used for more than one disease or 3) disease-specific markers. We also propose that evaluation of specific therapeutic interventions benefits from biological markers that match the intervention.

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Introduction

Muscle diseases cover a diverse group of disorders that in most cases are hereditary. The rarity of the individual muscle diseases provides a challenge for researchers when wanting to establish natural history of the conditions and when trying to develop diagnostic tools, therapies, and outcome measures to evaluate disease progression. The last decades have shown major diagnostic improvements for muscle diseases, largely driven by improvements in genetic testing. With emerging molecular therapies in many muscle diseases, targeting the genetic defects or abnormal proteins, biological biomarkers play an important role in both drug development and evaluation. This has been a focus for the European Medical Agency through the "Qualification of Novel Methodologies for Medicine Development",[1] as well as the American Food and Drug Administration (FDA) through the "Biomarker Qualification Program".[2] To advance understanding of the utility of biological biomarkers, natural history studies in muscle diseases are needed to better understand the natural progression of the diseases and the corresponding changes in biological biomarkers. To that end, several natural history studies have been carried out[3–9] or are under way,[10–12] but natural history data are still insufficient for most muscle diseases.

The FDA and NIH co-authored "Biomarkers, Endpoints and Other Tools (BEST)", which is a resource that defines a biomarker as "*a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions*".[13] This resource categorises biomarkers into 7 groups.

1) **Diagnostic biomarkers** used to detect or confirm the presence of a (subtype of) disease. An example is the disease-specific antibodies in myasthenia gravis.

2) **Monitoring biomarkers** measured repeatedly over time to evaluate the rate of disease progression. These biomarkers are valuable tools in trials of progressive diseases such as the muscular dystrophies, where a change in the rate of disease progression might be used to evaluate treatment response.

3) **Response biomarker** is used to show that a biological response has occurred in an individual after an exposure. These biomarkers can be used as surrogate endpoints in clinical trials when sufficient scientific evidence supports their ability to predict future

clinical outcomes. An example is the direct demonstration of a gene product after gene therapy in monogenic diseases.

4) **Predictive biomarkers** are used to predict if an individual is more or less likely to gain an effect from an exposure such as antibodies against the vector used in a gene therapy.

5) **Prognostic biomarkers** are used to identify the likelihood of a specified clinical event in patients with a specific disease. An example could be antibodies indicative for a risk of cancer in myositis.

6) **Safety biomarker** measured before/after exposure are used to indicate the extent of an adverse effect. An example is the monitoring of liver transaminases to measure liver toxicity after an exposure such as for instance gene therapy.

7) **Susceptibility biomarkers** indicate the potential for developing a condition in a healthy individual. In muscle diseases, this could be elevated serum creatine kinase (CK) in an asymptomatic carrier of an inherited myopathy.

Currently, biological biomarkers are rarely used as primary endpoints in clinical trials for muscle diseases. A few phase 2 trials have used different biological biomarkers as primary endpoints, such as DUX4 activity in Facioscapulohumeral Muscular Dystrophy , splice correction in Myotonic dystrophy type 1 and dystrophin levels in Duchenne muscular dystrophy .[14–18] However, biological biomarkers used as surrogate endpoints could be used to either 1) detect disease progression or improvement before this can be detected clinically, 2) used instead of a clinical observation because it is easier, quicker, or less expensive to measure, or 3) used to give a more exact quantitative estimate. For a response biomarker to be useful, change in the biomarker must parallel changes in the disease state within a reasonable timeframe. Ideally, measurements of the biomarker can be done across different laboratories with high reproducibility and little variability among labs, secured by participation in an ongoing quality control program.[13] A deeper understanding of biological biomarkers could pave the way for their use as surrogate endpoints in therapeutic trials in muscle diseases.

In this position review, we limit our coverage and discussion strictly to muscle diseases. We will not discuss MRI as a biomarker, as this has been done in a recent review by Dahlqvist et al.[19] For

muscle diseases, biological response biomarkers can be categorised as either 1) disease unspecific markers, 2) markers of specific pathways that may be used for more than one disease or 3) markers of specific diseases. An overview of the muscle-derived biomarkers discussed in this paper is seen in figure 1.

Biological biomarkers common to more than one disease

These biomarkers typically reflect similar pathomechanisms, for instance leakage of intracellular proteins with muscle damage.

Biological biomarkers of muscle damage

Most trials use the well-known markers creatine kinase and/or myoglobin as a biomarker for muscle damage. Both CK and myoglobin are mainly found in the cytosol of striated muscle cells. Elevation of these biomarkers is seen when there is a leakage through the damaged cell membrane.[20] CK and myoglobin exhibit qualities of good biomarkers in the form of ease of measurement, low testing costs, and reproducibility among laboratories. However, they have several shortcomings. CK is found not only in skeletal muscle, but also in other tissues with high energy demand such as heart and brain. CK from these tissues can be distinguished by measuring the isoform of the CK-dimer (MM found in skeletal muscle, BB in the brain, and MB in heart muscle, and macro-CK type 1 and 2),[21] though the standard measurements of CK are not isoenzyme specific. In a similar way, myoglobin is not specific to skeletal muscles, but is also found in heart muscle. CK and myoglobin depend on total muscle mass (in Duchenne muscular dystrophy, CK is very elevated in young boys, but can reach normal limits in adults when residual muscle mass is low),[22] recent exercise, and muscle trauma, which collectively lead to a significant inter-subject variability.[23] Thus, a single measurement of CK and myoglobin is unreliable as a biomarker for disease state.[24,25] As CK and myoglobin are primarily located in the cytosol, an increase of plasma levels primarily indicates increased cell membrane permeability. By contrast, troponin I is a predominantly structural protein connected to the thin contractile filament of the sarcomeric structure. Troponin I is a more specific marker for muscle damage, as it is connected to the contractile filaments in striated muscles and allows differentiation between skeletal and cardiac isoforms as well as distinguishing between different fiber types by measuring the slow-

twitch (TNNI1) or fast-twitch (TNNI2) isoforms. Elevated skeletal troponin I indicates structural damage to the muscle fiber in addition to disruption of the cell membrane. This has been used to show that mainly TNNI2 is elevated in both the exercise response of healthy persons, and in baseline samples of Becker Muscular Dystrophy patients.[26,27] An ideal biomarker for disease state would be independent of recent muscle strain and show disease progression independent of or relative to muscle mass. In Duchenne muscular dystrophy, disease severity has shown a direct correlation with plasma creatine/creatinine ratio and an inverse relationship with plasma myostatin levels.[28,29] A similar pattern was seen in Limb-Girdle type R1 (Calpain-3-related) and Limb-Girdle type R2 (Dysferlin-related), and as such could possibly be used as a response marker in trials and could potentially be applied to other muscle dystrophies as well.[28] CK level does not represent a good biomarker of treatment response as a decrease (outside of physiological fluctuations) can be caused by either progression of the disease with a decrease of muscle mass, or by a treatment response and thus less muscle injury and leakage to the blood.

Large scale biomarker discovery

Circulating biomarkers have been examined in a few unbiased large-scale studies of muscle diseases, summed up in table 1. Although both methodology and the specific disease differ, the biomarker candidates show a large degree of overlap, with CK, carbonic anhydrase III (CA3), Myosin Light Chain 3 (MYL3), and TNNI2 being the standouts. The large overlap between different muscular dystrophies suggests that these are not markers of the unique diseases, but rather common circulating biomarkers for the pathology of striated muscle tissue.

MicroRNAs

A group of muscle-specific microRNAs, named myomiRs have been investigated across several myopathies.

In the dystrophinopathies, both Duchenne muscular dystrophy, Becker Muscular Dystrophy as well as female carriers show elevated levels of myomiRs compared to healthy controls.[36–38] While Duchenne muscular dystrophy -patients seem to have higher levels of all myomiRs than Becker

Muscular Dystrophy, the levels of miR-133b correlated with disease severity in both groups, and were age-dependent.[39–41] Cacchiarelli et al[42] found increasing levels of miR-133 with higher age in young Duchenne muscular dystrophy patients (age 3-6 years). The opposite age correlation was found by Zaharieva et al[40] where lower levels of miR-133 were found in those who had lost ambulation in a cohort of older Duchenne muscular dystrophy patients (age 4-13 years). Restoration of myomiRs in response to treatment show some promise, as exon-skipping in dystrophin-deficient mdx mice caused a dose-dependent lowering of both myomiR levels (1a, miR-133a, miR-206, miR-483) and CK.[43] A single study has showed a trend towards normaliation of miR-1, miR-206, miR-133a and miR-133b levels with exon skipping therapy in Duchenne muscular dystrophy patients, though this failed to reach statistical significance, possibly due to low sample size (12 subjects) and a short follow up period (12 weeks).[40] Lastly, miR-379 has been shown to be modulated by glucocorticoid treatment in Duchenne muscular dystrophy patients and could constitute a biomarker of response to steroid treatment.[44]

A recent study analysed miRNA transcriptomic profiles in muscle biopsies from well identified Idiopathic Inflammatory Myopathy subtypes and compared them to healthy controls.[45] Their findings showed that specific groups of miRNA are exclusively expressed in dermatomyositis, anti-synthetase syndrome and inclusion body myositis. This corresponds to a subtype signature, which can be potentially interesting in a research setting, to understand the epigenetic regulation of immunological pathways related to different Idiopathic Inflammatory Myopathy subtypes' pathogenesis. Interestingly, the study found no specific miRNA profile associated to immune-mediated necrotizing myopathy.

In Myotonic dystrophy type 1, elevated miR-133a/b levels as well as a compound miRNA score averaging levels of miR-1,-133a, -133b and -206 have been shown to be inversely correlated with muscle strength and the Muscular Impairment Rating Scale,[46] while levels of miR-1, miR-133a, miR133b and miR-206 were higher in Myotonic dystrophy type 1 patients with progressive muscle wasting compared to stable patients.[47]

Besides Duchenne muscular dystrophy, Idiopathic Inflammatory Myopathy and Myotonic dystrophy type 1, elevated myomiR levels have also been found in Facioscapulohumeral Muscular Dystrophy (miR-1, -133a, -133b and -206)[48] and Pompe disease (miR-133a, with a normalisation in response to enzyme replacement therapy).[49] Besides these myopathies, dysregulated myomiR levels are also seen with muscle denervation in patients with Amyotrophic lateral sclerosis (elevated levels of miR-206), Spinal-Bulbar Muscular Atrophy (elevated levels of miR-206),[50] Charcot-Marie-Tooth disease type 1A (eleveated levels of miR-1, miR-133a/b and miR-206),[51] and Spinal muscular atrophy (in which miR-133a reduction predicted response to therapy),[52] and also in non-muscle cancer (both reduced and elevated levels are seen)[53,54] and sarcopenia (reduced levels of miR-133b and miR-206 associated with sarcopenia).[55] As the same set of myomiRs (mainly miR-1, miR-133a, miR-133b and miR-206) are elevated across a range of both muscle- and non-muscle diseases, we categorise these myomiRs as unspecific markers of a common muscle pathomechanism in the same way as CK/myoglobin and their usefulness as biomarkers should be discusses in this context. The main advantage of myomiRs compared to more established biomarkers is that both individual myomiRs and myomiRs panels seem to correlate better with disease state and/or treatment response across several diseases. A major disadvantage of CK is the intra-subject variability. Such variability can also occur for microRNAs. Extreme changes were seen in elite athletes after a 24 hours run, in which circulating myomiRs increased 18 to 124,723 fold from baseline to the end of the run,[56] while other studies report a 2-7-fold increase dependent on the type of acute exercise.[57,58] This variability is probably linked to the sarcolemmal rupture caused by the extreme exercise, and will most likely also be present in muscle diseases where sustained rupture is happening. Investigations of the intra-subject variability of myomiR levels in myopathic subjects would be an important step before they can be fully used as trial endpoints.

Markers of specific pathways

These biomarkers are specific to diseases sharing a common pathogenic pathway.

Acquired myopathies: idiopathic inflammatory myopathies

In the last decade, clinico-biological correlations have revolutionised the field of idiopathic inflammatory myopathies, stratifying phenotypes into four main disease groups: dermatomyositis, anti-synthetase syndrome, immune-mediated necrotising myopathies and sporadic inclusion body myositis. The latter will be treated in a separate paragraph. Overlap myositis (myositis with another systemic rheumatic disease) is not usually treated by neuro-myologists and will not be considered in this paper. "Polymyositis" is a poorly defined phenotype, widely overlapping with the aforementioned entities and in particular anti-synthetase syndrome, rendering this term progressively obsolete in modern scientific literature. Different biological mechanisms are involved in Idiopathic Inflammatory Myopathy pathogenesis, impacting follow-up and treatment, especially with new strategies such as complement inhibition. Interferon (IFN) activation plays a key role in Idiopathic Inflammatory Myopathy.[59] In blood samples from dermatomyositis and polymyositis patients, IFN-1 signature has been shown to correlate with disease activity and decrease in response to immunomodulatory treatment. The levels of the highest differently expressed IFN-1 transcripts, *IFI44L* and *RSAD2*, could easily be analysed with quantitative real-time PCR and become accessible biomarkers of disease progression and response to treatment in clinical trials.[60] In anti-Jo1 anti-synthetase syndrome, the IFNγ-induced chemokines CXCL9 and IP-10 are increased in serum, although any correlation with muscle disease activity remains to be defined.[61] Expression of IFN2-inducible genes seems to be more important in immune-mediated necrotizing myopathies pathogenesis,[62] although studies focusing on measurable blood transcripts are lacking. A study focusing on immune-mediated necrotizing myopathies found a group of upregulated cytokines and chemokines. Among these molecules, the levels of the cytokines IP-10 and MIP-1α have been shown to significantly decrease after immunosuppressive treatment,[63] thus representing potential biomarkers for future pharmacological trials.

Centronuclear Myopathy

Centronuclear myopathies are a group of congenital disorders characterised by an increased centralisation of the nuclei of muscle cells, where mutations in several genes can lead to either X-linked, autosomal recessive, or dominant inheritance. Several studies have suggested a common

pathway for centronuclear myopathies caused by variants in *MTM1* (X-linked), *DNM2* (autosomal dominant) and *BIN1* (autosomal recessive) that are all involved in membrane remodeling and -trafficking.[64–66] All three myopathies share elevated DNM2 expression in muscles. Antisense oligonucleotide (ASO) therapy targeting DNM2 mRNA has shown promising results in preclinical studies of both DNM2, MTM1 and BIN1 deficient mice.[64,67,68] Using ASO therapy in patients was attempted in the DYN101 phase 1/2 trial, however, the trial was discontinued due to tolerability issues prior to dose escalation.[69(p101)] Potential biomarkers common for this pathway could be either mRNA (as used in the DYN101 trial) or protein levels of DNM2 in muscle. No studies have yet investigated the correlation between DNM2 levels and clinical state of the disease. Another potential biomarker for this pathway could be miR-133a, a regulator of DNM2 expression in skeletal muscle of mice.[70] A large-scale multi-omics approach was done by Djeddi et al,[71] who explored both the disease and therapeutic signatures of DNM2, MTM1, and BIN1 deficient mice. They found both a disease and a therapy-sensitive signature shared among the three mouse models, suggesting plasma levels of MSTN and ANXA2 as potential biomarkers for disease progression. In addition to these biomarkers showing promising potential in pre-clinical models, the levels of circulating myostatin has also been correlated with a better clinical condition in X-linked centronuclear myopathy patients.[72]

Mitochondrial myopathies

Mitochondrial myopathies are caused by many different genetic variants in mitochondrial or nuclear DNA, and they give rise to a diverse range of phenotypes. Elevated levels of Growth differentiation factor-15 (GDF15) and Fibroblast growth factor-21 (FGF21), along with the lactate and pyruvate concentrations and ratios in serum have been used as diagnostic biomarkers.[73–77] Several studies have explored them as potential response/monitoring biomarkers, with conflicting findings. Montano et al[78] found no relation between FGF21 or GDF15 and disease severity, and no relation between lactate levels and functional test scores in 118 patients. Domínguez-González et al[79] measured GDF15 in 7 patients with Thymidine Kinase 2–Deficient Myopathy undergoing deoxynucleoside treatment, and found a decline in GDF15 in parallel with the clinical improvement during the treatment. Koga et al[80] found that pyruvate therapy lowered plasma levels of lactate, pyruvate, alanine and GDF15, but not FGF21 in 11 patients with various myopathies.

Madsen et al[81] used lactate as an exploratory endpoint in the evaluation of omaveloxolone treatment, showing a significant reduction of lactate levels with treatment despite no effect on oxidative capacity. Pirinen et al[82] found restoration of elevated plasma alanine, but no change in FGF21, GDF15 or their RNA levels in skeletal muscles in 5 patients with adult-onset mitochondrial myopathy when treated with niacin, despite an improvement in muscle performance. In conclusion, no suitable response biomarkers have yet emerged for the mitochondrial myopathies. A challenge in finding biomarkers in the mitochondrial myopathies, it their large heterogeinity caused by differences in genetic variants, heteroplasmia and involvement of other tissues which might lead to a broader range of biomarkers that potentially can respond differently to treatments.

Markers of specific diseases

These biomarkers are unique for a single disease and often relate directly to the pathogenic mechanisms of the disease. They are typically used in follow-up of disease-modifying therapies that target the primary defect of the disease. For most of the above-mentioned biomarkers, a high correlation between the level of the biomarker and the evolution of disease severity is desirable. This is not necessarily the case in specific inherited myopathies, where a biomarker might be persistently missing (as the lack of dystrophin in Duchenne Muscular Dystrophy) or focally and transiently increased (as the misexpression of DUX4 and its targets in Facioscapulohumeral Muscular Dystrophy) throughout the progressive disease course. In trials aiming to express dystrophin in Duchenne muscular dystrophy-muscles or supress DUX4 expression in Facioscapulohumeral Muscular Dystrophy, direct measures of these would be relevant response biomarkers and have indeed been used in such trials.

Idiopathic inflammatory myopathies

Myositis-specific and myositis-associated antibodies are immune biomarkers especially useful for diagnosis and phenotype stratification. Emerging evidence has shown a correlation between some antibodies and disease progression, turning them into potential biomarkers for

pharmacological trials. In anti-synthetase syndrome, a correlation between anti-Jo1 antibodies and disease activity has been demonstrated.[83] Furthermore, the antibody levels decreased after rituximab treatment,[84] highlighting its potential role in clinical trials. In dermatomyositis, both anti-transcription intermediary factor 1 γ and anti-Mi-2 antibodies levels correlated with clinical severity (albeit weaker then anti-Jo1) and decreased after rituximab treatment.[85] However, since all IgG antibodies decrease after anti-CD20 treatment, this does not prove either the pathogenicity of these myositis-specific antibodies related to dermatomyositis nor their validity as response biomarkers. Hence, more studies are needed for this subgroup of Idiopathic Inflammatory Myopathy. In immune-mediated necrotizing myopathies, anti-3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) antibodies are pathogenic and correlate with clinical severity and CK levels.[86] Hence, they represent a useful biomarker for monitoring clinical progression and response to treatment, as demonstrated in recent studies evaluating the effect of modified human IgG1 antibody Fc-fragment (Efgartigimod)[87] and C5 complement inhibitors,[88] in an animal model of immune-mediated necrotizing myopathies. Similarly, anti-signal recognition particle (SRP) antibody levels correlated with clinical severity and CK levels, and coherently decreased after immunosuppressive treatment.[89]

Facioscapulohumeral muscular dystrophy

Facioscapulohumeral Muscular Dystrophy is caused by misexpression of the double homeobox 4 (*DUX4*) transcription factor in skeletal muscle. Most of the current therapeutic strategies for Facioscapulohumeral Muscular Dystrophy are targeting DUX4 suppression.[90] Measuring the change in muscle *DUX4* expression in response to an intervention may serve as a biomarker to predict clinical benefit. In support of this, several studies have provided a link between upregulation of DUX4 target genes and early myodegenerative changes identified on muscle MRI.[91–93] However, *DUX4* expression has some limitations as a biomarker in that

1) It requires a muscle biopsy.
2) *DUX4* is expressed dynamically in bursts in cultured cells of Facioscapulohumeral Muscular Dystrophy patients, where a small population of DUX4-positive nuclei (around 1/200-1000 nuclei expressing *DUX4* transcripts) are thought to drive pathogenesis for the

whole muscle.[94–97] This transient and sporadic distribution of *DUX4*-inappropriate transcription makes acquiring predictive measures in a single biopsy challenging and potentially not representative of the overall status.

3) Detection of DUX4 is not trivial and several studies have failed to reliably detect DUX4 expression in biopsies[98] or single cell RNA sequencing experiments in cell culture,[95] therefore assessing the expression of its targets is thought to be a preferable option.

4) A newly conducted phase II trial using losmapimod, which is supposed to repress DUX4 expression, failed to show changes in DUX4 target gene expression, despite some changes in functional assessments and patient-reported outcomes,[18] further highlighting the difficulties in performing such an assay.

Besides tissue-derived biomarkers, research is devoted to the identification of less invasive blood-based (circulating) biomarkers that reflect the status of the affected muscles as well as disease pathophysiology. Along with the unbiased approaches listed above, targeted approaches have been undertaken mostly focusing on inflammatory mediators upregulated in Facioscapulohumeral Muscular Dystrophy patients' muscles showing early signs of muscle degeneration. Pilot studies have identified possible candidate molecules such as chemokines,[99] mediators linked to specific inflammatory response pathways such as S100-A8[100,101] and complement factors.[102] Interestingly, interleukin-6, which is a cytokine used as a non-disease specific biomarker in several systemic inflammatory conditions, was elevated in Facioscapulohumeral Muscular Dystrophy sera compared with controls and correlated with disease severity in a single cohort,[103] thus holding some promise as a monitoring biomarker. Recently, a refined transcriptomic signature based on of PAX7 target gene repression was identified in Facioscapulohumeral Muscular Dystrophy muscles as a possible circulating biomarker correlating with disease severity.[104] Notably, all these circulating molecules are at the current stage candidates requiring validation in multicentric studies and linkage with other more established prognostic and monitoring biomarkers, such as those derived from muscle imaging studies,[105] before being implemented as surrogate endpoints in clinical trials.

Dystrophinopathies

The *DMD* gene exceeds the packaging capacity of viral vectors currently used for gene therapy, which has led to the use of truncated versions of the gene in what is called micro-dystrophin gene therapy.[106] In the Duchenne muscular dystrophy population, the expression of even small amounts of truncated dystrophin seems to be associated with a milder phenotype.[107] Duchenne muscular dystrophy patients with exon 44 skippable mutations, who present with a milder phenotype, express a higher level of truncated dystrophin than other Duchenne muscular dystrophy patients.[108] In Becker Muscular Dystrophy, mild or asymptomatic patients express more truncated dystrophin than severe patients.[109] Truncated dystrophin can be expressed in revertant fibers by naturally occurring exon skipping or driven by ASO or AAV-mediated gene therapy. The expression of truncated dystrophin either naturally expressed by the patients or secondary to treatment has been used or considered as an acceptable biomarker. Recently the FDA has approved 4 ASO-therapies based on the exon skipping principle for skipping exons 45 (Casimersen), 51 (Eteplirsen) and 53 (Golodirsen and Viltolarsen).[110] The approvals were all based on the expression of small amount of truncated dystrophin, considered by US regulators as an acceptable surrogate endpoint. However, the relationship between micro-dystrophin and functional improvement is weak both in animals[111] and in humans. Indeed, micro-dystrophin or truncated dystrophin have several flaws as a response biomarker. First, the function of micro-dystrophin depends on the structure of the truncated protein.[112,113] In studies with micro-dystrophin gene therapy, all subjects will have the same micro-dystrophin for a single therapy, whereas in exon-skipping trials, the structure of the protein depends on the subject-specific mutation. This makes the biomarker difficult to compare between studies and for exon skipping therapy, also among participants in the same trial. In addition, it is unknown how much truncated dystrophin is needed for a clinical effect; the amount most likely differs for the various micro-dystrophins, the different muscles, and the functional status of the patient. There is also a time-sensitive issue in the possible relation between dystrophin expression and functional improvement. Restoring truncated dystrophin in already damaged muscles may differ from having truncated dystrophin from birth, such as in Becker Muscular Dystrophy. Finally, the effect of truncated or mini-dystrophin is entirely dependent on a correct localisation within the muscle. To that end, micro-dystrophin has a non-homogeneous expression across muscle fibers, which makes a single muscle biopsy unreliable as a marker for the total expression of the whole muscle.[113] Thus, a combination score including both the quality, amount,

and localisation of dystrophin should be optimal. Studies of the correlation of such a marker to clinical changes are needed to assess dystrophin expression as a clinically meaningful endpoint.

Limb Girdle Muscular Dystrophies

Limb-Girdle Muscular Dystrophy type R9is caused by mutations in the fukutin-related protein (*FKRP*) gene, which contributes to glycosylation of α-dystroglycan in skeletal muscles. The effect of introducing *FKRP* to skeletal muscles by gene therapy can be evaluated by measuring FKRP expression and glycosylation of α-dystroglycan in muscle biopsies, which inform about successful grafting and transcription of the gene. Another therapeutic strategy is to give ribitol, which acts as a substrate for FKRP.[114–116] Potential biomarkers for this approach would be to assess functional glycosylation of α-dystroglycan in muscle. Alhamidi et al[117] found no clear correlation between self-reported walking ability and α-dystroglycan glycosylation levels in vastus lateralis biopsies of 25 Limb-Girdle Muscular Dystrophy type R9patients. On the other hand, animal studies have found that restoration of functionally glycosylated α-dystroglycan is associated with muscle regeneration and functional improvement in FKRP-mice.[118,119] Sarcoglycanopathy is caused by deficiency of either α-, β-, λ-, or δ-sarcoglycan (corresponding to Limb-Girdle Muscular Dystrophy types R3, R4, R5 and R6), which are transmembrane proteins of the dystrophin-glycoprotein-complex acting as a link between the muscle cytoskeleton and the extracellular matrix. In gene therapy trials of the sarcoglycanopathies, measures of gene expression along muscle levels of the specific sarcoglycan have been used as secondary outcomes.[120–122] Unfortunately, none of these studies evaluated relevant functional outcomes. Thus, the relationship between biomarker and disease outcomes remains unknown. A potential serum marker was explored by Rouillon et al,[123] who found that elevated MYOM3 levels was restored in mice in parallel with restoration of α-sarcoglycan. Limb-Girdle Muscular Dystrophy type R2 is caused by deficiency of the dysferlin protein, which is linked to membrane repair of muscle cells. A large natural history study investigated 76 Limb-Girdle Muscular Dystrophy type R2 patients.[9] Cross-sectional analysis of these patients found lower myostatin levels to correlate with lower functional ability as well as higher fat-fraction and lower contractile cross-sectional area measured on MRI. The following longitudinal assessment found no correlation between change in myostatin and change in functional and MRI-assessments, which indicate a limited use of myostatin as a response

biomarker. Further exploration of biological biomarkers and functional outcomes in Limb-Girdle Muscular Dystrophies are currently being explored.[124,125] In general, the use of either the protein or the product of a relevant enzymatic process are likely biomarkers in inherited diseases with loss of function.

Myotonic Dystrophy type 1

Myotonic Dystrophy type 1 is caused by a CTG-expansion in the dystrophia myotonica protein kinase (*DMPK*) gene, which results in abnormal splicing of several genes.[126] An aim is to correct this spliceopathy by ASO therapy directed towards *DMPK*.[127,128] The level of missplicing may function as a biomarker for Myotonic dystrophy type 1 severity and also as a response biomarker in therapeutic trials, and is currently set as the primary outcome in phase I studies.[126] Measures of splicing reversal have been used in both patient-derived cell cultures and mice showing splicing reversal and improvement of myotonia in the mouse model.[129,130] Kurkiewicz et al[131] used machine learning to create a model for treatment effect based on alternate splicing in muscle tissue. A supporting power analysis was used to estimate the required size of a clinical trial using this model as outcome. Using splicing biomarkers of disease severity in Myotonic dystrophy type 1 can therefore be used to correlate disease severity and progression and to monitor treatment effects of drug interventions quantifying multiple splicing reversal effects. However, to measure splicing reversal in myotonic dystrophy, a muscle biopsy is necessary at multiple time points. A needle biopsy is the preferred choice, and this is usually obtained from the tibialis anterior muscle, one of the early and most affected muscles in the disease. As disease progresses, and with multiple biopsies being required, repeated needle biopsies may not provide sufficient muscle tissue and adipose tissue may be found limiting the biomolecular analysis. How the splicing index in the tibialis anterior correlates to muscle weakness in this muscle and in other muscles involved in the disease is being explored in both natural history trials and ongoing randomized controlled trials using ASOs targeting the abnormal splicing pathways.[132–134]

Pompe disease

Enzyme replacement therapy (ERT) has been used to treat Pompe disease for 16 years. Besides recent advancement in ERTs, other therapeutic strategies involve gene therapy of skeletal muscle and liver as well as chaperone therapy and substrate reduction therapy.[135–139] Several biomarkers are used in therapeutic trials, including levels of α-glucosidase activity in muscle and urine levels of Glc4, all shown to correlate with glycogen levels and response to treatment.[140–142] Another potential biomarker in Pompe disease is linked to the secondary accumulation of autophagic build-up in the skeletal muscle fibers of these patients.[143] Spampanato et al[144] found transcription factor EB to trigger lysosomal exocytosis and promote cellular clearance in both isolated muscle fibers and whole muscle of a Pompe mouse model, thereby decreasing the glycogen levels. Thus, either direct measurements of autophagic bodies or indirect measurements of transcription factor EB could be a promising response biomarker in Pompe disease. Chien et al[145] measured serum levels of MSTN, IGF1, and CK in 10 patients with Pompe disease and 10 gender- and age-matched controls. The participants were retested after an average of 12 months, in Pompe patients on ERT-treatment. The levels of IGF1 and MSTN were lower in the pre-treatment Pompe samples than in the controls but were within the normal range after treatment. The biomarkers may reflect muscle regeneration after ERT and might be used as response biomarkers in future trials. An understudied biomarker in Pompe disease is the muscle glycogen. The primary target of ERT is to increase the degradation of glycogen, but how muscle glycogen content changes in response to therapy has only been examined in a single study using muscle biopsies.[146] They used muscle biopsies, in which a small sample of tissue is evaluated, which may not be representative of the whole muscle, and most biopsies were from vastus lateralis, which is relatively unaffected in Pompe disease.[147,148] A recent study by Beha et al[149] used high field carbon-13 magnetic resonance spectroscopy to show that glycogen levels in the hamstrings and lumbar muscles, muscles, which are prone to degeneration, were much higher in young Pompe patients compared to controls. Muscles that are preserved for a long time in Pompe disease, such as the calf and anterior thigh muscles, had glycogen content comparable to healthy controls. The benefit of this approach is the evaluation of whole muscle groups in a non-invasive way as opposed to a small, and maybe not representative, needle biopsy procured invasively. Evaluating how glycogen levels in hamstrings and lumbar

muscles change in response to ERT may provide evidence for the use of glycogen content as a biomarker for treatment response in Pompe disease.

McArdle disease

McArdle disease is caused by deficiency of the muscle-specific enzyme myophosphorylase, which causes patients to experience exercise intolerance and the pathognomonic second wind phenomenon in which the exercise intolerance improves after 8-10 minutes of continued exercise.[150] Patients are unable to increase blood lactate levels in response to exercise.[151] Potential therapies involve gene therapy, read-through of the common R50X* mutation or activation of the liver/brain isoform (Pygb/Pygl).[152] These therapies can be evaluated by measuring myophosphorylase activity in muscle biopsies. Other biomarkers measure restored metabolic effects, such as the lactate response to exercise or abolishment of the second wind has beed used in a few trials[153,154].

Inclusion Body Myositis

No effective treatment is available for Inclusion Body Myositis. In recent years, some therapies, such as bimagrumab and arimoclomol, have been explored but phase 3 clinical trials failed to demonstrate drug efficacy.[155,156] Specific biological biomarkers were not included in these two studies. However, a few serum biomarkers have been proposed over the years, although most of them have focused on diagnostic purposes, without investigating correlation with disease severity. As discussed before, some miRNA, such as miR-206 and -133b, are known to be increased in muscular and non-muscular conditions. Data on these unspecific miRNAs in the sera of Inclusion Body Myositis patients are contrasting, with one study showing normal[157] and another upregulated expression.[45] Moreover, other miRNAs where found to be specifically changed in the sera of Inclusion Body Myositis patients compared to controls, as miR-299-5p and miR-150-5p were upregulated in one study[45] while another study found decreased levels of hsa-miR-192-5p and increased levels of hsa-miR-372-3p.[157] All these miRNAs are probably related to the degenerative and inflammatory pathways of Inclusion Body Myositis. Serum cytokines were investigated in a cohort including 59 Inclusion Body Myositis patients; 10 cytokines (TRAIL, IL-8, MIF, MCP-1,

LIF, IP-10, IFN-α2, MIG, bNGF and IL-3) were able to discriminate Inclusion Body Myositis patients from healthy controls.[158] More interestingly to the purposes of our review, specific cytokines appeared to be useful as biomarkers of disease severity or response to treatment. Indeed, IP-10 and Eotaxin decreased significantly upon methotrexate treatment and development of muscle weakness was negatively associated with changes in IL-8 and SDF1A levels.[158] Conversely, IFN-γ levels correlated with survival of Inclusion Body Myositis patients, but only before correction for multiple comparisons. A further study[159] including acquired and genetic muscle disorders, showed that interferon gamma-protein 10 (IP-10) induced chemokine, was significantly elevated in the sera of Inclusion Body Myositis patients compared to healthy controls, hereditary myopathies, and immune-mediated necrotizing myopathy; furthermore, mononuclear cells surrounding and invading nonnecrotic muscle fibers in Inclusion Body Myositis patients markedly expressed IP-10, suggesting that the increased circulating levels reflect inflammatory activity in the muscle tissue. Mitochondrial dysfunction is one of the main pathways in Inclusion Body Myositis.[160] In this regard, increased level of GDF-15, a biomarker of mitochondrial disease, was increased in a small cohort of Inclusion Body Myositis patients.[161] In the same study, a new mitochondria-homing drug, mitochonic acid-5 (MA-5), was able to ameliorate mitochondrial dysfunction in Inclusion Body Myositis myoblasts and reduced the expression of GDF-15, which was suggested as possible biomarker for drug efficacy. Overall, circulating biomarkers have been poorly investigated in Inclusion Body Myositis and further studies are needed to establish serum biomarkers for Inclusion Body Myositis disease activity and therapeutic effects.

The future of biological biomarkers in muscle diseases

Therapies in muscle diseases aim to stop or even improve disease progression, but clinical changes are not always noticeable within a reasonable timeframe. Biomarkers have the potential to show relevant changes in disease state long before any clinically detectable change. Therefore, they are of high value in the evaluation of therapies. Validation of surrogate endpoints is needed across the whole spectrum of muscle diseases to improve therapeutic trial design, as the current biological biomarkers have yet to be accepted by regulatory bodies. As discussed in this review, there are many promising biomarkers within the field of muscle diseases that show correlation between serum levels and functional status. The most important knowledge gap is to understand the

correlation between levels of a biological biomarker and changes in disease state, whether it be naturally or after an intervention. Many studies have shown a correlation between biomarker levels and disease severity, but lowering of CK, correct splicing in Myotonic dystrophy type 1, suppression of DUX4 expression in Facioscapulohumeral Muscular Dystrophy, etc. do not *necessarily* translate into future clinical stabilisation or improvements. An example is the matrix metallopeptidase 9 (MMP-9) enzyme, that was found to be elevated in Duchenne muscular dystrophy and with an increased concentration over time,[162] thus being a potential marker of disease progression.[163] However, Lourbakos et al[164] performed further exploration of this enzyme in 1704 samples by combining data from two natural history studies and 3 independent clinical trials, and found no support of MMP-9 as a predictive biomarker in Duchenne muscular dystrophy. Large studies with longitudinal evaluations are needed to verify potential biomarkers as clinically relevant surrogate endpoints. Combining biological biomarkers with functional, and/or imaging biomarkers into panels could potentially give better information. Most of the biological biomarkers for specific diseases have clear links to the disease mechanism, which add to their validity as biomarkers. An important aspect of biomarker development of the biomarkers shared between several diseases, is to better understand the link between the biomarkers and the pathophysiological mechanisms of the diseases.

We propose that evaluation of specific therapeutic interventions would benefit from biological biomarkers that match the intervention. For therapies that specifically target the pathogenic mechanism of a disease (e.g., gene therapy), biological biomarkers are of particular value. For therapies targeting general mechanisms of muscle damage, evaluation benefits from less specific markers of muscle damage.

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Supplementary material

Supplementary material is available at *Brain* online.

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Figure legend

Figure 1 Muscle derived biomarkers. α-DG = α-Dystroglycan, β-DG = β-Dystroglycan, MYL3 = Myosin Light Chain 3, MTM1 = Myotubularin 1, DNM2 = Dynamin 2, BIN1 = Bridging Integrator-1, CA3 = Carbonic Anhydrase III, Glc4 = Glucose Tetrasaccharide, LDHB = Lactate Dehydrogenase B, MDH2 = Malate Ddehydrogenase 2, EFTA = Electron Transfer Flavoprotein A, TFEB = Transcription factor EB, *DUX4* = Double Homeobox 4.

Table 1 Summary of large-scale biomarkers studies

Subjects	Method (number of testet proteins)	Main findings	Study
22 FSHD patients and 23 matched controls	Myriad, Human Discovery MAP 250, v2.0 (243)	A moderate correlation between FSHD severity and CKMB, PLAT and EGF	Statland et al[30]
Two independent cohorts. Total of 42 FSHD patients and 35 controls.	SOMAScan 1K (1129)	CKMB, CKMM, CA3 and TNNI2 could reliably predict disease state and correlated with STIR brightness on MRI.	Petek et al[31]
Two independent DMD cohorts. Total of 93 patients and 45 controls.	SOMAScan 1K (1125)	44 proteins significantly different between DMD and healthy including TNNI2, CA3, CKMM.	Hathout et al[32]
285 DMD, 30 asymptomatic female carriers and 37 healthy individuals.	Bead array platform (118)	CA3, MDH2, MYL3, TNNT3, ETFA, NES, LDHB, COL1A1 and MAP4 together with CK founds to be associated with disease progression.	Strandberg et al[33]
130 DMD, 33 BMD, 16 female carriers and 66 healthy individuals.	Bead array platform (384)	Nine protein profiles were found to correlate with disease severity. Proteins in these profiles include CA3, MYL3, MDH2, EFTA, TNNT3 and CK.	Ayoglu et al[34]
9 BMD, 9 LGMDR12, 8 LGMDR9 and 9 healthy controls.	SOMAScan 7K (7288)	34 proteins found to be significantly different in all three myopathies compared to controls. This includes CKMM, CA3, MYL3 and TNNI2.	Stemmerik et al[35]

BMD = Becker Muscular Dystrophy, CA3 = Carbonic Anhydrase III, CK = Creatine Kinase, CKMB = Creatine kinase M:Creatine kinase B heterodimer, CKMM = Creatine Kinase M-type, COL1A1 = Collagen Alpha-1(I) chain, DMD = Duchenne Muscular Dystrophy, ETFA = Electron Transfer Flavoprotein A, EGF = Epidermal Growth Factor, FSHD = Facioscapulohumeral Muscular Dystrophy, LDHB = Lactate Dehydrogenase B, LGMDR9 = Limb-Girdle Muscular Dystrophy R9, LGMDR12 = Limb-Girdle Muscular Dystrophy R9, MAP4 = Microtubule Associated Protein 4, MDH2 = Malate Dehydrogenase 2, MYL3 = Myosin Light Chain 3, NES = Nestin, PLAT = Tissue-type Plasminogen Activator, TNNI2 = Fast-Myofiber Troponin I, TNNT3 = Troponin T Type 3.

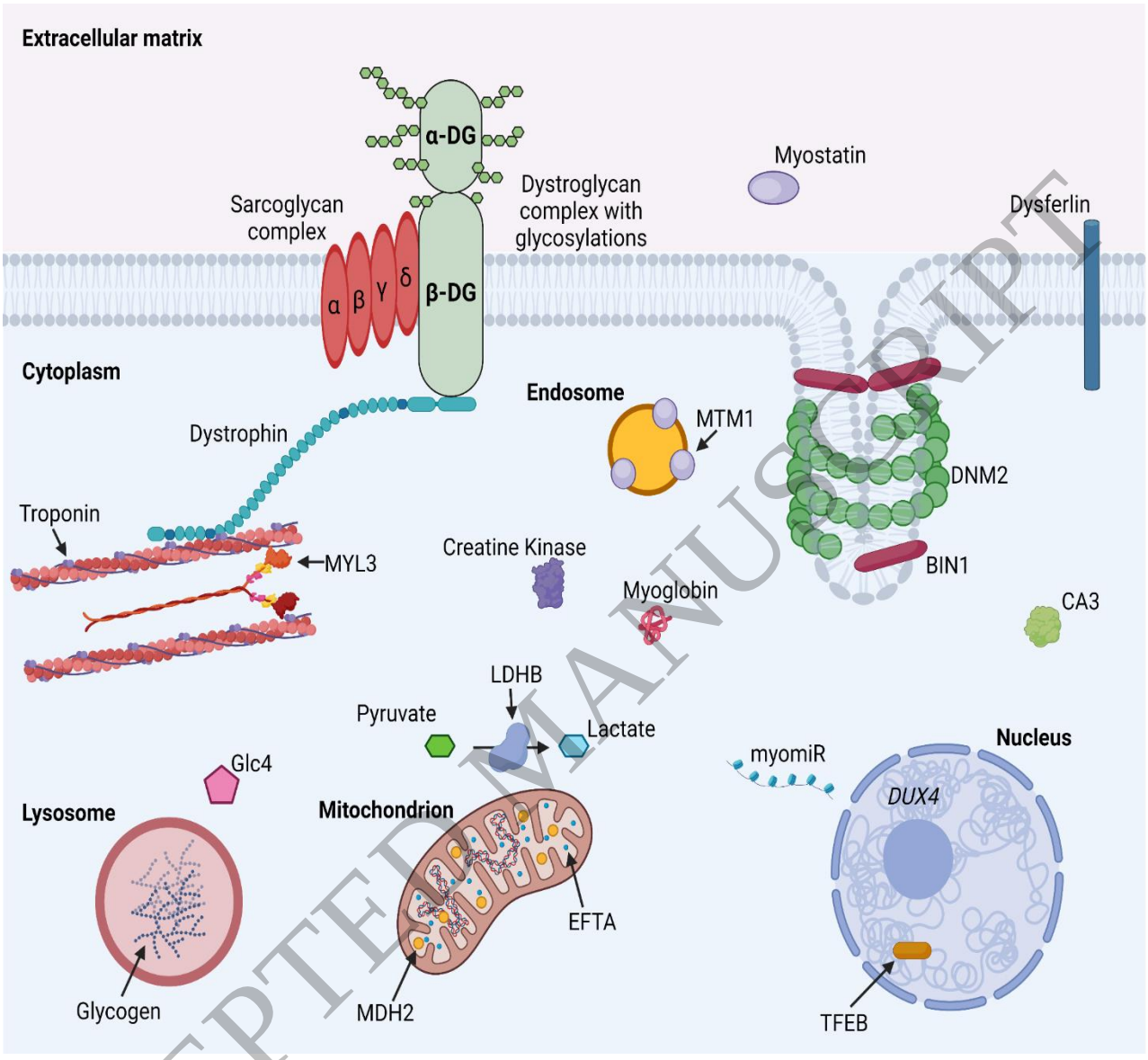

Figure 1
254x203 mm (x DPI)