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Review

Circulating Biomarkers for Duchenne Muscular Dystrophy

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Abstract.
Duchenne muscular dystrophy is the most common form of muscular dystrophy. Genetic and biochemical research over the years has characterized the cause, pathophysiology and development of the disease providing several potential therapeutic targets and/or biomarkers. High throughput – omic technologies have provided a comprehensive understanding of the changes occurring in dystrophic muscles. Murine and canine animal models have been a valuable source to profile muscles and body fluids, thus providing candidate biomarkers that can be evaluated in patients. This review will illustrate known circulating biomarkers that could track disease progression and response to therapy in patients affected by Duchenne muscular dystrophy. We present an overview of the transcriptomic, proteomic, metabolomics and lipidomic biomarkers described in literature. We show how studies in muscle tissue have led to the identification of serum and urine biomarkers and we highlight the importance of evaluating biomarkers as possible surrogate endpoints to facilitate regulatory processes for new medicinal products.

Keywords: Biological markers, biomarkers, Duchenne muscular dystrophy, gene expression, proteomics, metabolomics

Duchenne muscular dystrophy (DMD) is a severe muscle-wasting disease caused by genetic mutations in the *DMD* gene encoding a structural protein called dystrophin [1]. Mutations in the same gene are responsible for the milder form of the disease, which is called Becker muscular dystrophy (BMD) [2]. Protein truncating mutations cause the Duchenne form of the disease characterized by complete or almost complete absence of dystrophin, while BMD patients have in frame mutations and are partly protected from muscular degeneration by reduced levels of (smaller or semi-functional) dystrophins [3, 4]. During the years following the gene discovery, research focus has been on the development of potential therapies able to restore dystrophin and the dystrophin associated glycoprotein complex (dystrophin pre-mRNA splicing modulation with antisense oligonucleotides, dystrophin mRNA ribosomal read-through of non-sense mutations, gene therapy, allogenic or genetically corrected autologous stem cells, utrophin up-regulation and differential glycosylation of α-dystroglycan) or to reduce the secondary pathology caused by the absence of dystrophin (reducing oxidative stress or increasing muscle mass) [5–16]. These therapeutic strategies were optimized and proof of concept was shown in cellular and animal models [17–30]. In fact the development of therapeutic strategies was so fast that when the first clinical trials were designed it was clear which mutation specific drug was suitable for which patients, while it was not

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known which primary endpoint should be used. Furthermore, lacking detailed knowledge on the natural history of the disease and the outcome measures to be used in clinical trials, it was difficult to power studies properly and to identify biomarkers for therapeutic monitoring. Finally, while detailed knowledge was available about general genotype-phenotype relationships, studies linking different out-of-frame mutations to variation observed in natural history studies using outcome measures used in clinical trials were unavailable, nor was it known if and how genetic modifiers influenced disease progression as measured by these functional outcome measures [31]. Once the field realized this, clinical researchers started to evaluate how known and new functional scales could describe disease progression in patients with Duchenne [31–41]. These studies in natural history cohorts provided a baseline for clinical trials, but meanwhile clinical trials had already initiated. On occasion this made the interpretation of results difficult as natural history studies showed that individual differences in disease progression were due to genetic modifiers or differences in the mutation site [42–47]. Importantly, natural history studies also revealed that the progression of the disease over time was different depending on the age group and the walking skills at baseline as measured by the 6 minute walk test (6MWT) [48]. Molecular researchers in parallel continued to investigate the pathophysiological changes in patients' muscle biopsies and animal models by proteomic and gene expression studies. Even though studies in muscle biopsies have limitations such as the fact that they do not reflect the condition of the entire muscle nor of other muscles, this approach increased the understanding of which genes and proteins were driving the pathology in Duchenne as well as in other muscular dystrophies, showing common characteristics that could lead to a dystrophic phenotype [49]. Some of these genes were then identified as modifiers of disease progression or prognostic biomarkers [44]. We will however not discuss genetic modifiers as they have been recently reviewed by Lamar et al. in the first issue of this *Journal* [50]. The identification of muscle biomarkers lead the way to the identification of new therapeutic targets as well as biomarkers which could be used as surrogate endpoints or secondary endpoints. Even though the categorization of biomarkers is beyond the scope of this review we would like to spend a few words on the term surrogate endpoint to avoid confusion. The term "biomarker" has been defined by the Biomarkers Definitions Working Group of the National Institutes of Health (NIH) as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic response to a therapeutic intervention" [51]. This definition is quite ample and may include many types of possible biomarkers such as prognostic biomarkers, diagnostic biomarkers and biomarkers for response to a therapeutic agent. A surrogate endpoint is a "biomarker that is intended to substitute a clinical endpoint. A surrogate endpoint is expected to predict clinical benefit (or harm or lack of benefit or harm) based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence". Based on this definition it is clear that all surrogate endpoints are biomarkers but not all biomarkers can be surrogate endpoints. To qualify as surrogate endpoint a biomarker should correlate to, or predict clinical endpoints. As such, it is not sufficient to know that the levels of the biomarker differ between DMD patients and age-matched controls. Rather, natural history data of the biomarker should be available as well as information on how the biomarker levels correlate with disease progression and functional endpoints. Excellent papers further explaining the characteristics that a biomarker should have to classify as surrogate endpoint are available and we would like to refer the reader to these papers [52–54]. For DMD patients, dystrophin restoration is an obvious pharmacodynamic biomarker for DMD therapies aiming at dystrophin restoration; dystrophin analysis has been covered in depth in a review paper in the inaugural issue of this *Journal* [55] and will therefore not be discussed here.

As it frequently happens in science, the identification of candidate targets (in this case biomarkers that could be suitable surrogate endpoints) is based on the availability of enabling technologies. Several complementary approaches have been used to identify nucleic acids, proteins, peptides, metabolites and lipids that are able to discriminate between DMD patients and healthy controls. Discovery has been mostly driven by non-targeted high-throughput technologies even though several groups have identified biomarkers based on *a priori* hypotheses. We will here describe reported candidate biomarkers based on their chemical nature.

NUCLEIC ACIDS

Thanks to the development of micro-arrays first and next generation sequencing later, gene expression studies have been one of the major determinants for understanding the pathophysiological changes in

Duchenne patients' cell lines and muscles as well as in animal models [56–63]. The genes identified in several independent studies belong to pathways involved in energy production, muscle regeneration and contraction, inflammation, calcium homeostasis, fibrosis and macrophage infiltration. The TGF-β pathway has been central in many reports and recently it was shown that correct phasing of TGF-β signalling is crucial for successful muscle regeneration. Some genes belonging to the TGF-β and IGF-1 pathways have been considered as therapeutic targets for DMD (e.g. myostatin and Akt), others have led to the discovery of genetic modifiers (e.g. *SPP1* gene) and to the identification of elevated gene products in circulation (e.g. MMP-9). Interestingly some genes such as *Cd68* (macrophages marker), *Lgals3* and *Bgn* showed normalization towards control levels upon dystrophin restoration in the *mdx* mouse, thus qualifying as candidates biomarkers for the evaluation of therapeutic treatment [64]. It is important to mention that *Cd68* and *Lgals3* also correlated with disease severity at 6 weeks of age in 3 dystrophinopathy mouse models with different levels of utrophin expression [65]. Other studies identified differential expression of miRNAs in muscles of animal models and patients [66–70]. Among the most reported miRNAs are miR-206 (linked to muscle regeneration in DMD and in patients affected by amyotrophic lateral sclerosis [71]), miR-1 and miR-133 (highly expressed in skeletal muscle), miR-29c (linked to fibrosis), miR-31 (targeting dystrophin), miR-378 (myofiber enriched miRNA), miR-499 and miR-208 (cardiac enriched miRNAs). Multiple research groups investigated the potential of these and other miRNAs to act as peripheral biomarkers and they evaluated the presence of these miRNAs in sera of animal models and patients [72–76]. While dystromiRs remain a good tool to differentiate between cases and controls, not only for DMD but also for other muscular dystrophies [68, 73], the initial correlation with disease severity [72] has not been confirmed in a larger study [77].

PROTEINS AND PEPTIDES

Creatine kinase (CK) is an enzyme that is abundant in muscle, which leaks into the bloodstream upon muscle damage. As such serum CK activity has been used to diagnose muscle damage and muscular dystrophy for more than 50 years and for this special issue dedicated to the launch of the *John Walton Muscular Dystrophy Research Centre* we would like to cite a paper that underlines the contribution of Lord Walton to these findings, which paved the way to biomarker discoveries in Duchenne over time [78]. Serum CK activity has been extensively studied and even though CK activity in serum has several limitations (such as seasonal variation [79] and intra/inter individual variability [80]), it remains still one of the first evidences that could lead to a diagnosis of muscular dystrophy. Reports in literature showed however that CK activity is mainly useful as a diagnostic biomarker as it peaks between 1 and 6 years of age and decreases with age as the disease progresses [81]. This decrease reflects the replacement of muscle tissue by fibrotic and adipose tissues. Therefore, CK activity is of little use for therapeutic monitoring, since lower CK levels can mean both that the disease progressed further (muscle quality further decreased) or that the muscle quality improved (less leakage of CK). As for gene expression studies, researchers initially focused their attention on muscle tissue, with the intention to better understand the pathophysiology and identify therapeutic targets and biomarker candidates. Proteomic studies in the *mdx* mice, especially in the diaphragm muscle, have provided a number of candidate proteins that are elevated or reduced in dystrophic muscles. Results showed alteration in nucleotide metabolism, luminal and cytosolic calcium handling, glycolytic enzymes, mitochondrial energy metabolism, oxidative stress, cytoskeletal proteins and proteins present in the extracellular matrix [82–87]. Experiments in aged *mdx* mice provided further evidence that proteins of the extracellular matrix are elevated with age and that myofibrillar proteins decrease with age [88, 89]. Further comparison of heart muscle tissue between young and old *mdx* mice helped to distinguish between differences due to aging and differences due to dystrophin deficiency, highlighting again impaired mitochondrial metabolism, contractile function and cell signalling [90]. More studies in muscle of mdx-4cv mice showed an increase in extracellular matrix and cytoskeletal proteins and a reduction in contractile proteins [91]. Similar findings have been obtained in the canine model of muscular dystrophy [92]. Interestingly studies of muscles that are relatively spared in Duchenne patients and animal models, such as extra-ocular and sartorius muscles, provided potential evidence that the dystrophin paralog utrophin and muscle hypertrophy could exert a protective effect on affected muscles [93, 94]. Several studies exist where the obtained knowledge was used to propose therapeutic targets, but less effort has been put in trying to translate the muscle findings to serum/plasma biomarkers. Studying the serum proteome is still an analytical challenge

even with the newest technologies. The high dynamic range and the presence of high abundant proteins has so far reduced the successful identification of serum biomarkers using mass spectrometry based approaches. Nevertheless researchers have been able to identify and replicate associations for a number of proteins in the recent years using unbiased approaches such as mass spectrometry and targeted approaches such as antibody and aptamers based assays. Alagaratnam et al. identified a peptide belonging to factor XIII as serum discriminator between *mdx* and wild type mice [95]; Colussi et al. reported increased fibrinogen and glutathione peroxidase (GPX3) and reduced levels of gelsolin and leukemia inhibitory factor receptor (LIFr) [96]. Notably, GPX3 and LIFr normalized after treatment with a histone deacetylase inhibitor, suggesting these biomarkers might be used to monitor therapy. Fibrinogen was also found in two other studies [97, 98] and it has been linked to the formation of fibrotic tissue in mice [99]. Nadarajah et al. reported serum MMP-9 levels to be elevated in DMD patients and to increase with age [100]. This finding was later on confirmed by an independent group [98]. Martin et al. found elevated levels of fibronectin in DMD sera [101]. Ayoglu and colleagues performed a comprehensive study using a customized antibody array where hundreds of proteins were quantified in sera and plasma of Duchenne and Becker patients [49]. The most interesting candidates were carbonic anhydrase 3, myosin light chain 3, malate dehydrogenase 2, electron transfer flavoprotein subunit alpha (mitochondrial) and beta and troponin T. Hathout and co-workers identified other glycolytic enzymes such as glycogen phosphorylase and fructose-bisphosphate aldolase A, myofibrillar proteins such as myomesin-3 and titin [98]. Titin has also been found in DMD patients' urine [102]. Recently an aptamer-based study quantified 1125 proteins in serum of DMD and healthy controls and replicated the findings in an independent cohort: 44 proteins were found to be differentially represented between DMD patients and controls [103]. The authors classified the proteins into 4 groups among which the muscle derived proteins show a CK like behaviour (elevated compared to controls, but down-trending with age). These results show that the muscle degeneration processes ongoing in patients' muscles are indeed reflected in the circulation. Notably the other 3 groups showed other characteristics that are more interesting from a surrogate endpoint perspective. They contained markers that are elevated or decreased at all ages, or markers for which the levels are comparable between young patients and controls, but levels change in opposite direction with age (and presumably resemble disease severity). Most of the markers in these groups were not enriched in muscle. Since the patterns vary between DMD and control individuals of different ages, they may have the potential to monitor treatment effect in clinical trials. However, longitudinal studies in individual patients are needed to elucidate the natural history of these markers and to assess whether these markers are indeed candidate biomarkers to act as surrogate endpoints, i.e. whether their levels correlate or anticipate functional outcome measures used in clinical trials. Notably, the co-linearity between age and disease progression (assessed by e.g. 6MWT) may make it difficult to interpret changes as age remains one of the best predictors of disease progression. Finally, studies are needed to understand how biomarkers levels respond to therapeutic intervention, e.g. by analysing serum samples of DMD patients participating to clinical trials. This process is laborious and therefore in our opinion should focus on candidate biomarkers that have been reported to be elevated compared to healthy controls and rapidly increase with age or the ones that are reduced compared to controls and rapidly decreasing with age. Some examples of these are Metalloproteinase-9, ETFA/ETFB, Adiponectin, Persephin, Prolyl endopeptidase FAP, Osteomodulin, Proto–oncogene tyrosine–protein kinase receptor Ret, Complement decay–accelerating factor, Growth/differentiation factor 11, Gelsolin and Tumor necrosis factor receptor superfamily member 19L [98, 100, 103].

METABOLITES AND LIPIDS

There is less evidence in literature of a muscular metabolic signature in circulation in patients with DMD. However early evidence was published in 1984 by Shapira and colleagues that a vitamin D metabolite (24,25(OH)2D3) was less abundant in DMD patients' sera compared to healthy controls [104]. The authors stressed in the article how the findings were probably related to muscle ATP and calcium homeostasis and in those years the link between calcium metabolism, muscle contraction and vitamin D was suggested [105]. It took fifteen years before other groups could study in more detail metabolic perturbations *in vivo* since in those days animal models for DMD were barely available (the *mdx* mouse had just been published [106]). McIntosh et al. described the association between taurine levels in muscle and muscle regeneration [107]. Griffin et al. obtained metabolic profiles for skeletal muscle, heart, cortex and cerebellum in dystrophic

mice and identified taurine and creatine as strong classifiers [108, 109]. The same group showed that utrophin could partially restore the metabolic signature in diaphragm of dystrophic mice, showing the potential of metabolic data to act as biomarker for therapeutic treatment [110]. Franciotta and colleagues tested whether creatinine concentration in 24 hours urine could be used as an indirect measure of skeletal muscle mass in DMD patients but they could not find a significant association [111]. Recently a large study reported that a prostaglandin D2 metabolite was elevated in the urine of DMD patients over controls and that the concentration further increased in patients above 8 years of age, making it a good candidate biomarker also for patients who are in the declining ambulatory phase [112]. In 2010 it was reported that DMD patients have higher serum concentrations of triglycerides, phospholipids, free cholesterol, cholesterol esters and total cholesterol compared to healthy controls and that the ratio between phospholipids and cholesterol had the highest discriminant power [113]. Very recently Hörster and colleagues reported that the L-arginine/nitric oxide pathway regulating endothelial function is affected in DMD patients urine and plasma and that treatment with corticosteroids reduced the intensity of this signature [114].

WHAT IS MISSING

Very little effort has been put in the integration of the many datasets available. There are only a couple of examples in literature where metabolomic and proteomic data have been combined and in these studies taurine could be linked to oxidative phosphorylation and mitochondrial metabolism [115, 116]. More effort should be put in the integration of datasets to have a complete understanding of the pathophysiology and to identify molecular targets which can serve as biomarkers [117]. Evidence from other fields shows how important it is to integrate datasets to have a good understanding of the biology, plan interventions and facilitate therapy [118]. Datasets should be available in public repositories and bioinformatic tools should be developed to enable the comparison of different datasets, for example at the pathway level. The integration of different datasets on such a higher level would reduce the number of pathways, ontologies or concepts to be tested thus reducing the number of tests and increasing power to identify significant pathways and molecules that can serve as therapeutic targets or biomarkers. This is particularly important for rare diseases such as DMD because of the low numbers of samples available. Once pathways and molecular candidates are identified, they should be replicated in large cohorts to identify biomarkers that can predict or correlate to clinical endpoints. The association of a biomarker with clinical endpoints is a key point to enable the translation of a candidate biomarker into a surrogate endpoint. To date there are no surrogate endpoints for DMD but natural history studies and placebo arms of clinical trials represent a unique opportunity because longitudinal samples are available and biomarker data could be associated with known and newly developed clinical endpoints. These and other studies should also try to understand how confounders such as age, progression, biological and environmental co-factors and physical activity affect the robustness, sensitivity and specificity of individual biomarkers. The analysis of biomarkers in trials for corticosteroid use (such as the FOR-DMD trial - http://for-dmd.org) would also enable to quantify the effect of different steroid regimens on biomarker levels. Furthermore, when biomarkers respond to steroid treatment, this is important knowledge because most of the current therapies are tested in trials on top of prednisone or deflazacort treatment. Biomarkers should be also linked to specific aspects of the disease such as the amount of fibrofatty infiltration in muscle and cardiac function. These aspects can be evaluated by magnetic resonance imaging, which in itself is also a promising candidate biomarker for DMD [119, 120]. The possibility to closely monitor heart function with circulating biomarkers is needed not only for DMD patients, but also in Becker patients where severity of skeletal muscle involvement is not a predictor of cardiac involvement. Some candidates have already been identified with the identification of cardiac specific dystrophin binders [121]. Last but not least a link between dystrophin levels (in case of dystrophin restoring drugs) and clinical outcome needs to be established to make dystrophin not only a pharmacodynamic biomarker but also a surrogate endpoint. So far this association has only been studied in Becker patients and in patients with an exclusive heart involvement [122, 123]. The high variability in functional performance and the wide age range of Becker patients combined with the small sample size have so far hampered a complete understanding of this relation [55]. For other drugs (e.g utrophin up-regulation), the target levels (utrophin or the dystrophin associated glycoprotein complex levels) should be reliably quantified to consider these readouts as pharmacodynamic biomarkers. The available assays were not set up

to consistently quantify these outcomes (e.g. distribution and quantification of utrophin along the myofibers membrane) since standards of quantification are not available and the available assays have been mainly designed for research purposes. Standard operating procedures should be developed and the quantified levels should be connected to clinical performance to consider the readouts surrogate endpoints.

CONCLUSIONS

Enabling technologies have driven the discovery of deregulated pathways in DMD. These studies have produced lists of candidate therapeutic targets and biomarkers. During the last 5 years many candidates have been evaluated for their potential to serve as non-invasive biomarkers by measuring their concentration in body fluids. The potential of these candidates as surrogate endpoints needs to be evaluated in *ad hoc* studies where molecular and clinical outcomes can be compared. The availability of surrogate endpoints has the potential to facilitate regulatory approval of medicinal compounds for patients affected by Duchenne muscular dystrophy.

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CONFLICT OF INTEREST

Pietro Spitali and Annemieke Aartsma-Rus declare being employed by the LUMC and receiving salary from the LUMC. LUMC has patents on exon skipping, some of which Dr. Aartsma-Rus is co-inventor on. On sublicensing some of these patents to Prosensa Therapeutics and GSK, Dr. Aartsma-Rus has received a share of royalty payments from LUMC.

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