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Molecular biomarkers for neuromuscular disorders – challenges and future perspectives

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Abstract

There is an ever-growing need for molecular biomarkers in assessing clinical course and diagnosing neuromuscular disorders, as well as in monitoring drug therapy. With the development of high throughput techniques, there has been an acceleration in the discovery of potential biomarkers. It is quite easy to find potential candidates, but difficult to validate them and translate into a clinical setting. Neuromuscular diseases (NMD) are a major challenge in terms of finding potential molecular biomarkers, mainly because of their heterogeneous aetiology and variability in phenotype, their as yet incompletely understood pathophysiology, and their slow clinical progression. Furthermore, it is challenging to assemble a large cohort of patients, as many NMDs are rare diseases.

In this literature review, we provide an update on the latest discoveries in DNA, RNA, miRNA, epigenetic, protein, metabolic and cellular biomarkers for NMD. The advantages and potential difficulties of clinical application and the role of identification of biomarker panels are discussed. We have especially sought to highlight translational biomarkers which can be easily transferred to the clinic, where they may eventually present possible future therapies related to molecular biomarker discoveries.

Key words: translational biomarkers, neuromuscular diseases, NMD, biomarkers

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Introduction

In the field of NMD (neuromuscular disorders) there is an ever-increasing need for new molecular biomarkers. The discovery of new biomarkers is essential, not only for diagnostic purposes, but also to help monitor disease course in clinical trials and changes in treatment. This type of biomarker, known as a pharmacodynamic biomarker, can indicate e.g. if a protein is restored in the course of treatment. Innovative therapeutic trials, which tend to be shorter and not always impact phenotype, require efficient biomarkers. Some molecular biomarkers can even replace functional outcome measures in clinical trials and therapy, i.e. surrogate biomarkers.

A good example of such a surrogate biomarker is dystrophin increase in skeletal muscle during therapy with eteplirsen [1]. In this case, an effective biomarker enabled accelerated approval by the United States FDA (Food and Drug Administration). However, despite much effort and countless developments in this field, finding a biomarker suitable for clinical use remains a challenge. The development of high throughput technologies such as NGS (next generation sequencing) and omic technologies for proteins have significantly speeded up

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the process of screening for molecular biomarkers, but have also at the same time created problems related to the interpretation of large amounts of data. As screening usually reveals multiple potential candidates, it is likely that only a few will prove to be effective in the course of clinical validation, which is demanding, time-consuming, and expensive. The application of a panel of molecular biomarkers may also be recommended in some cases. Furthermore, large clinical studies led by international consortia allow for surveys to be carried out on a large cohort of patients, something of great importance when dealing with rare diseases. In this article, we discuss recent advances in the field of molecular biomarkers for NMDs, analyse approaches to testing, and review the possible development directions and clinical applications in this field.

Criteria for useful biomarkers in NMD

According to the NIH (National Institutes of Health), a biomarker is defined 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" [2]. The most important characteristics of biomarkers for NMD are:

- analytical validity (the ability to accurately distinguish between normal and altered status as well as treatment response/non-response),
- clinical validity (the ability to reflect the features of the disease),
- non-invasiveness,
- feasibility (how simple it is to measure),
- cost-effectiveness (being quick and easy to use) [3].

According to the FDA, an ideal biomarker must be specifically associated with a particular disease or disease state, and be able to differentiate between similar physiological conditions [4–5]. Biomarkers should also correlate with clinical outcomes and radiological measures. In terms of accessibility, an ideal biomarker would be easily accessible e.g. from plasma [6]. However, due to the tissue-specific expression of a number of proteins, biomarkers from muscle and skin biopsies still play an important role. CK is still widely used as a biomarker in NMD despite its limitations (it is neither a specific nor a sensitive marker for neuromuscular disease). Reliable biomarkers for NMD in urine or saliva have yet to be identified. In this article, we will differentiate between seven types of biomarkers:

- diagnostic – to detect or confirm the presence of a disease or condition of interest, or identify an individual with a subtype of the disease,
- monitoring – to assess the status of a disease or to detect the effect of a medical product or biological agent,
- pharmacodynamic response - changes in response to exposure to a medical product,
- predictive – its presence or change predicts whether an individual will experience an effect from the exposure to a medical product,
- prognostic - identifies the likelihood of a clinical event in an individual,
- safety - indicates the likelihood, presence, or extent of a toxicity
- susceptibility/risk biomarkers – indicates potential for developing a disease in a healthy individual [7].

A surrogate biomarker is defined as 'a biomarker intended to substitute for a clinical endpoint'. Biomarkers used as an endpoint are often easier to assess and more cost efficient than clinical biomarkers. Translational biomarkers can be easily transferred between pre-clinical and clinical research.

Neuromuscular disorders

NMDs are heterogeneous groups of neurological diseases, including both myopathies as well as neuropathies. The term encompasses various conditions with a different pathophysiological and genetic aetiology, distinct pathophysiological pattern, and heterogeneous phenotypes, even within one disease [8]. There are 955 neuromuscular diseases associated with 535 different genes [9]. They affect directly, by causing pathology in a muscle, or indirectly, by affecting a neuromuscular junction or nerves. The onset varies from infancy to late adulthood. The most common symptoms include muscle weakness that can lead to twitching, cramps and pains, muscle atrophy and hypertrophy, ptosis, swallowing problems, skeletal deformities, fatigability, a waddling gait, and respiratory and cardiac dysfunction. In this article we will concentrate on muscular dystrophy (DMD, BMD), limb girdle muscular dystrophy (LGMD), amyotrophic lateral sclerosis (ALS), facioscapulohumeral muscular dystrophy (FSHD), Charcot-Marie-Tooth disease (CMT), spinal muscle atrophy (SMA), dystrophic myotonias (DM1 and DM2) and Pompe disease (MP). As some of these neuromuscular diseases are very rare, one of the main obstacles to discovering potential molecular biomarkers is the scarcity of samples. Rare neuromuscular conditions can often remain undiagnosed, which significantly impacts upon care.

Molecular biomarkers

We classified the biomarkers based on their chemical structure into:

- DNA
- RNA (including miRNA)
- proteins and peptides
- others (metabolic, cellular) [10].

The main advantages and disadvantages of each class of biomarker are set out in Table 1.

Genomic biomarkers

RNA, and DNA and DNA epigenetic modifications encompass a larger class of genomic biomarkers.

Table 1. Advantages and disadvantages of DNA, RNA and protein biomarkers

Type of biomarker	Advantages	Disadvantages
DNA	High stability High specificity Good accessibility	Demanding validation and discovery process Difficulties with data interpretation
RNA	Tissue specificity	Low stability Low accessibility
Protein	Good accessibility High specificity	Low stability

DNA

DNA biomarkers include SNP (single polymorphism variant) and CNV (copy number variant) as well as insertions, deletions and translocations. DNA biomarkers are easily accessible and repeatable, but their functional meaning is often difficult to assess. Next generation sequencing (NGS) has enabled the rapid discovery of new genes in one-gene disorders and genetic modifiers due to the possibility of finding statistical correlations between phenotypes and genetic variants.

SNP

A single-nucleotide polymorphism (SNP), which is a variation in a single nucleotide that occurs at a specific position in the genome, is a promising alternative biomarker. DMD is one of the neuromuscular conditions most often screened for SNP biomarkers, mainly because of the range of phenotypes among individuals who carry the same mutation. Several independent studies have identified genetic diagnostic and prognostic biomarkers belonging to pathways involved in inflammation, muscle regeneration and contraction (TGF-β), calcium homeostasis, fibrosis and macrophage infiltration (*Cd68)* and genetic modifiers (SPP1) [11]. Large studies, with more than 200 DMD patients enrolled, have shown that the following variants: V194I, T787A, T820A, and T1140M, form the VTTT and IAAM in LTBP4 haplotypes. LTBP4 encoding TGF-beta-binding protein acts as a genetic modifier. Steroid-treated DMD patients homozygous for IAAM remained ambulatory significantly longer (up to 12.5 ± 3.3 years) than similarly treated individuals heterozygotes or homozygotes for VTTT (ambulatory up to 10.7 ± 2.1 years under treatment) [12–13].

Another candidate biomarker for DMD is osteopontin (SPP1). SPP1 expression has been found to be downregulated approximately three-fold in DMD patients with a mild phenotype compared to those who are severely affected. SNP –66T>G in the promoter region of *SPP1* correlates with greater DMD phenotype severity [14]. *SMN2*, *PLS3* and *ZPR1* were rated as modifiers for SMN2 and *EPHA4* and *SMN* for ALS. The modifier effect is not yet fully understood, and further studies are needed [15]. Four single nucleotide SNPs in *SIPA1L2* were recently proved to correlate with foot dorsiflexion strength in Charcot-Marie-Tooth patients. This study was performed on more than 300 patients using genome-wide methods [16].

CNV

Another very promising alternative biomarker is a copy number variation (CNV). This refers to the structural variations – individually variable repeats in the genome. They have already found their diagnostic application in diseases such as CMT1A with *PMP22* duplication and SMA with *SMN1* duplication [17]. Some smaller studies regarding CMT showed that neuropathy-associated CNVs outside of the *PMP22* locus are rare [18]. In ALS, *EPHA3* deletion was defined as a potential protective factor (prognostic and monitoring biomarker), but here again the data was gathered only in a small study [19]. There are ongoing works for validating potential modifying CNVs in the SD region of *TTN* and the TRI region of *NEB* [20]. One big biomarker study failed to identify CNV biomarkers in DMD and COL6 myopathies [21].

Epigenetic biomarkers

Epigenetics is a heritable and acquired alteration in gene activity and expression via chromatin reorganisation without changes in DNA sequence. Epigenetic alterations can occur even in the differentiated cells in response to external factors. Examples of epigenetic changes are DNA methylation and histone modifications. FSHD is a neuromuscular disease associated with an impaired methylation pattern. FSHD is caused by a reduction in the number of 3.3 kb *D4Z4* units arrayed on chromosome 4 to fewer than 11 units. This epigenetic pattern results in transcription of the *DUX4*, which is normally repressed, but only in patients with permissive *D4Z4* haplotypes. The latest research indicates that other factors may be involved in *DUX4* repression and may therefore act as biomarkers [22]. These are *PRC2* as the complex primarily responsible for *DUX4* repression in FSHD, and H3K9 acetylation along with loss of H3K27me3 as key epigenetic events that result in *DUX4* expression [23]. Other examples of the NMD with impaired epigenetics pattern are Emery-Dreifuss muscular dystrophy (EDMD) and progeria. It is still not fully understood how the disease can manifest with two extremely diverse phenotypes. A decrease of the heterochromatin mark H3K9me3 in pericentric regions and a downregulation of the *PRC2* may act as a modifier and may be regarded as a prognostic biomarker in assessing the course of the disease [24].

RNA

RNA, and particularly miRNA, has been investigated as a biomarker in multiple recent studies. Measuring RNA as biomarkers is effective, as RNA provides the most direct route for biomarker validation and assay development. A significant bottleneck in advancing RNA biomarker discoveries to the clinic is the lack of standardised and robust technologies for

measuring RNA biomarkers *in situ* in clinical specimens, not to mention the fact that RNA is unstable and not easily accessible.

mRNA

The possibility of whole transcriptome analysis has contributed greatly to the diagnosis and discovery of RNA expression patterns in numerous NMDs. The limitation is the tissue-specificity of RNA.

In DM, aberrant alternative splicing plays a key role in the pathogenesis. The timing of the appearance of certain miss--spliced events correlates well with disease severity [25]. However, there is not always a direct link between phenotype and RNA level. An SMA study (copy variant dependent disease) surprisingly did not show a clear correlation between SMN RNA expression and motor function [26]. In hereditary neuropathies, recent studies have proved a correlation with several RNA biomarkers from skin biopsies (which can be regarded as prognostic and monitoring biomarkers): *PARG, GSTT2, CTSA, CDA, ENPP1* and *NRG1* in different metabolic pathways with disease progression over time [27]. One analysis addressing whole transcriptome in ALS detected more than 2,000 differentially expressed transcripts in whole blood transcriptome among nearly 400 ALS patients and a large group of healthy individuals. Nevertheless, data interpretation is challenging [28]. Whole transcriptome analysis of a DMD animal model showed different expression patterns in a substantial number of genes. A major obstacle to this study is data interpretation [29].

Non-coding RNA

In recent years, high throughput technologies have enabled an outline of how non- coding RNAs play a regulatory role, providing a potential biomarker for NMD.

miRNAs are non-coding nucleic acids which target mRNA. They are relatively stable and have high concentrations in the blood and the cerebrospinal fluid (CSF). MiRNAs are especially attractive as biomarkers because of their potential utility in experimental therapy, as some of them correlate with therapeutic outcomes. Several microRNAs showed dysregulated expression levels in NMD [30]. miR-1, miR-133a, and miR-206 can be used as powerful prognostic serum biomarkers, not only in DMD but also in myotonic dystrophy 1 (DM1), limb-girdle muscular dystrophy (LGMD), facioscapulohumeral muscular dystrophy (FSHD), Becker muscular dystrophy (BMD), and distal myopathy with rimmed vacuoles (DMRV). DMD is probably the most-investigated NMD in terms of miRNA. Not only three different miRNAs, also known as myomiRs (miR-1, miR-133a/b, and miR-206), showed increased expression in DMD, but also others, such as: miR-208b, miR-499 and miR-31, were overexpressed in DMD patients [31–32]. Studies with phosphorodiamidate morpholino oligonucleotide (PMO)-mediated dystrophin restoration therapy in mdx mice (animal model of DMD) showed the ability of the therapy to correct the dysregulation of the myomiRs (miR-1, -133a/b, -206) to normal wild type levels in serum [33]. These are especially attractive as potential surrogate biomarkers. Serum levels of miRNAs: miR-206, 143-3p and 374b-5p could differentiate a patient with ALS from healthy controls and from patients with other, similar, neuromuscular diseases [34]. MiR-9, miR-132, miR-206, miR-183 and miR-375 were identified as prospective biomarkers for SMA [35]. miR-133a was increased in patients with Pompe disease in a study of 52 patients, while in three newborns miR-133a decreased after starting a replacement therapy [36].

Piwi-interacting RNAs have only recently been linked to human diseases. piRNAs are of interest because they have established gene regulatory functions. Specific serum piRNAs also responded to exon skipping therapy. The role of Piwi--interacting RNAs needs further investigation [37].

Proteins and peptides

Proteomic biomarkers have rapidly developed in recent years, mainly because of the introduction of new discovery techniques. Thanks to mass spectrometry (MS)-based proteome profiling and affinity multiplexing assays, candidate proteins and peptide biomarkers can be identified faster and more effectively [38]. Protein and peptide biomarkers are easily accessible and relatively cost effective to test. Their limitations are instability and problems with repeatability.

CK

A protein diagnostic biomarker still widely used in clinical practice is creatinine kinase-M (CK-M). This is a diagnostic biomarker of muscle (sarcolemma) damage. CK can be applied as a screening parameter for NMD, but it does not correlate well with disease progression and treatment, and shows variability among individuals.

NfL as marker of nerve damage

Neurofilament is a major cytoskeletal protein, expressed in both CNS and PNS, which increases during neuronal damage both in the blood and in the CSF. It is composed of neurofilament light (NFL), medium (NFM), and heavy (NFH) chains. NfL is elevated in multiple neurodegenerative disorders, including Alzheimer's disease, multiple sclerosis, and frontotemporal dementia. A 20-year study revealed that NfL can be a reliable biomarker for ALS, particularly when measured in CSF [39]. Some recent studies have shown that NfL may find its application as a prognostic biomarker for CMT. NfL tested in the blood of patients with CMT was increased compared to healthy controls. This biomarker correlated with clinical disease severity assessed with Rasch modified CMT examination and neuropathy scores [40].

Candidate protein biomarkers

Recent studies, based on multi omics techniques, have identified multiple potential protein biomarkers, but very few of them have found an application in clinics. DMD is of great

interest regarding serum protein biomarkers. As potential candidates, the following proteins have been identified: carbonic anhydrase III (CA-III), MMP9, TIMP1, osteopontin, amyoglobin, myosin light chain-3 (MYL3), troponin T, fast skeletal muscle (TNNT3), plastin-2 (LCP1), protein phosphatase 1F (PPM1F) and electron transfer flavoprotein A (ETFA) [41–43]. They are tied to different pathobiochemical pathways indicative of muscle fibre leakage, inflammation, fibrosis and muscle degeneration/regeneration. MMP-9, a serum marker of degradation and remodelling of the extracellular matrix, was identified a few years ago as one of the most suitable serum biomarkers to monitor disease progression and therapy in DMD [44]. MMP-9 is not specific for DMD and is also elevated in patients with Bethlem and Ullrich myopathy compared to controls. The rise in MMP-9 levels in COL6-related myopathies was less pronounced than in DMD and BMD. Recent studies have revealed that MMP-9 is not a reliable marker to monitor disease course in antisense therapy of DMD [45]. High throughput technologies have highlighted myomesin 3 (MYOM3) as a potential proteomic biomarker, which correlates well with clinical course of both LGMD and DMD. MYOM3 is a myofibrillar structural protein, present in the sera of DMD and LGMD2D patients, as well as in their respective animal models [46]. No powerful candidate biomarker has been identified in the sera of patients with SMA: there was a trend correlation with clinical symptoms for a few candidates, but statistical significance was not reached [47, 48]. Some recent studies have indicated exosome-derived SMN protein as a promising biomarker for SMA [48]. For LGMD, troponin I (sTnI), myosin light chain 3 (Myl3) and fatty acid binding protein 3 (FABP3) have been proposed as potential protein serum biomarkers. These proteins reached statistical significance with some clinical endpoints [49]. In CMT patients, a decrease of some mitochondrial proteins in skin biopsies was noticed [50]. In a small study of CMT patients, several biomarkers were identified, including alterations to serum amyloid protein specific for CMT1 patients and serum transferrin. These results should be verified on a larger cohort of patients [52]. In ALS patients, the most promising protein biomarkers are p75 neurotrophin domain and neurofilaments subunit proteins (NfH and NfL). All these markers are released by neuron or Schwann cell injury and they are not specific for ALS only. p75, which regulates growth and cell differentiation, is high after birth, then decreases in later life and is released to the CSF by Schwann cell or axonal damage. p75 is also elevated in the urine of affected individuals and model animals, and increases with disease progression [53]. Another discussed candidate is one of the growth factors - progranulin (PGRN), which rises in CSF as ALS progresses (monitoring biomarker) [54].

Other biomarkers

Metabolites – These are the intermediate end product of metabolism and are usually various types of small molecules. A new metabolomics technology to study data from a large number of metabolites at the same time is called metabolomics [55]. For NMD there are only a few known metabolic biomarkers, as they are not always useful in this application. Prostaglandin D2 metabolite is increased in DMD patients compared to healthy controls [56]. Other studies have shown increased lipid profile and L-arginine/nitric oxide pathway imbalance in DMD patients [57, 58]. In omni metabolomics analysis, IBM (inclusion body miosities) and mitochondrial neuromuscular diseases clustered differently from other NMDs. Sorbitol, alanine, myoinositol, and cystathionine have been proposed as candidate metabolite biomarkers for mitochondrial myopathies [59].

Cellular biomarkers – for collagen disease in Bethlem myopathy and Ullrich myopathy, the infiltration of macrophages can serve as a biomarker. A difference between DMD

Table 2. Promising biomarkers for NMD in terms of possible clinical application

Biomarker	Disease	Chemical class	Comment
CK	NMD	protein	Not useful in monitoring disease course; great inter-individual variability
LTBP4	DMD	SNP	Haplotypes well characterised in a relatively large cohort of DMD patients
SPP1	DMD	SNP	Correlates well with time of ambulation loss
miR-1, -133a/b, -206	DMD	miRNA	Correlates with treatment outcomes
miR-133a	Pompe disease	miRNA	Assessed in a large cohort of patients afflicted with Pompe disease
NfL	ALS, CMT	protein	Large and long term studies on ALS patients; correlates well with ALS course especially when measured in CSF
PGRN	ALS	protein	
MMM-9	DMD	protein	Not suitable for monitor splicing therapy
Troponin I (sTnI), myosin light chain 3 (Myl3) and fatty acid binding protein 3 (FABP3)	LGMD	protein	Apart from LGMD expressed also in DMD, BMD, but presents a different pattern
Serum amyloid protein and serum transferrin	CMT	protein	Serum amyloid protein is specific to CMT genetic type

and healthy controls can be tested with a flow cytometer. In the case of SMA, the expression of SMN protein in CD3+, CD19+, and CD33++ cells from SMA patients was significantly reduced compared to that in cells from control subjects [60].

Conclusion

Future directions and clinical implications. High-throughput technologies have enabled fast screening for candidate biomarkers, but clinical validation remains a challenge. The integration of molecular biomarkers with clinical endpoints and MRI biomarkers is needed. In some cases, a panel of biomarkers can be much more sensitive than one. Because many NMDs are rare, the omics databases should be available for the researcher community. Another challenge is the interpretation of big data produced in high-throughput technologies and establishing a reliable pattern of biomarkers expression in healthy and affected individuals. Novel biomarkers such as miRNA and protein biomarkers open new therapy perspectives as they can correlate with disease course. A few attractive potential biomarkers have already been outlined, and their advantages and limitations are set out in Table 2. Many of them were investigated only in small studies, and validation on a larger cohort of patients is required. Due to the discovery of candidate biomarkers, molecular pathogenesis of NMDs is better understood and potential targets for future therapies can be identified. Some candidates are attractive options for surrogate biomarkers in future clinical trials. This could significantly speed up trials, thus making them more affordable. The efforts to deliver reliable and sensitive biomarkers have intensified in recent years. We hope that some of the achievements from basic science and pre-clinical trials can be successfully translated into clinical practice.

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