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## 226<sup>th</sup> ENMC International Workshop: Towards validated and qualified biomarkers for therapy development for Duchenne muscular dystrophy 20–22 January 2017, Heemskerk, The Netherlands

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### Keywords

Duchenne muscular dystrophy; Biomarker; Dystrophin; MRI; Biobank

## 1. Introduction

Twenty-three participants from 6 countries (England; Germany; Italy; Sweden, The Netherlands; USA) attended the 226<sup>th</sup> ENMC workshop on Duchenne biomarkers “Towards validated and qualified biomarkers for therapy development for Duchenne Muscular Dystrophy.” The meeting was a follow-up of the 204<sup>th</sup> ENMC workshop on Duchenne muscular dystrophy biomarkers.

The workshop was organized with the support of Parent Project Muscular Dystrophy (PPMD) and Marathon Pharmaceuticals, which provided travel support for participants from the US via an unrestricted grant to PPMD in addition to ENMC support. It was attended by representatives of academic institutions, industry working in the Duchenne muscular dystrophy field and patient representatives.

### 1.1. Background to the workshop

**1.1.1. Biomarkers**—Biomarkers are defined as biological, measurable and quantifiable indicators of underlying biological processes. Different types of biomarkers can be distinguished: diagnostic biomarkers indicate the presence of disease, prognostic biomarkers correlate with predicted disease course, and therapeutic biomarkers are designed to predict

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or measure response to treatment [1]. Therapeutic biomarkers can indicate whether a therapy is having an effect. This type of biomarker is called a pharmacodynamics biomarker and can be used to e.g. show that a missing protein is restored after a therapy. Safety biomarkers assess likelihood, presence, or extent of toxicity as an adverse effect, e.g. through monitoring blood markers indicative of liver or kidney damage.

Sometimes biomarkers can also be used as primary endpoints in clinical trials instead of functional outcome measures, and these are termed “surrogate endpoints”. In Europe [2,3] biomarkers can only be used as surrogate endpoints after going through a rigorous regulatory process to officially qualify them for this purpose. Similar pathways exist in the US, where the Food and Drug Administration (FDA) also supplies a process for qualification of biomarkers for other contexts of use.

**1.1.2. Therapy development for Duchenne muscular dystrophy—**Duchenne muscular dystrophy (DMD) is a severe genetic disorder that leads to progressive muscle wasting and loss. Treatment is currently primarily symptomatic, and corticosteroids are used to slow down disease progression. Research into potential treatments is ongoing and many potential therapies have moved to the clinical trial phase (e.g. 203 trials were listed for DMD in [clinicaltrials.gov](https://clinicaltrials.gov) Feb 14 2017, of which 57 are currently recruiting). Notably, ataluren (stop codon read through, PTC therapeutics) has received conditional marketing authorisation from the European Medicines Agency (EMA) in 2014 and a marketing authorisation application for idebenone (antioxidant, Santhera) is pending in Europe. In the US, FDA granted accelerated approval to eteplirsen for the treatment of patients with eligible mutations (i.e. those where exon 51 skipping can restore the reading frame, to allow the production of a Becker muscular dystrophy like dystrophin protein). Emflaza (deflazacort) received full approval from FDA in 2017.

Therapy development for DMD is challenging [4]. Briefly, for a drug to be approved it is required to show clinical benefit and a positive benefit/risk ratio in a treated group of patients compared to a placebo group. Treatments currently in development for DMD aim to slow down disease progression. However, because DMD is a progressive disease spanning decades, it might be difficult to prove a clinical benefit during short trials. Indeed most clinical trials have durations of less than 48 weeks, which may prove too short in order to observe clear benefit (e.g. the FDA draft guidance for DMD therapy development suggests trials of longer duration (e.g. 96 weeks [5]). Consistent with this, Pfizer and Sarepta are currently conducting 96 week trials for an antimuscle atrophy drug and exon skipping compounds, respectively. Given the progressive, and age-dependent irreversible loss of muscle associated with DMD, time is of the essence and pharmacodynamic biomarkers that indicate a more rapid response that correlates with longer term functional improvement would accelerate and facilitate therapy development for DMD. These biomarkers need to be quantifiable, reproducibly measurable with small coefficients of variance, and be predictive of a therapeutic effect in a shorter timeframe than existing outcome measures.

Currently no qualified biomarkers exist for Duchenne muscular dystrophy (DMD). To align efforts, an ENMC workshop was organized on this topic and held in January 2014 [5]. This workshop was organized by Profs. Alessandra Ferlini, Peter ‘t Hoen, Kevin Flanigan, Hanns

Lochmuller, Francesco Muntoni and Elizabeth McNally and discussed DMD biomarker discovery, validation and interpretation. Given the rapid progress and scale of ongoing research in this area, the organizers and participants recognized the need to continue momentum in this area through another workshop.

The aims of this follow-up workshop were

- To discuss dystrophin quantification and skeletal muscle magnetic resonance imaging (MRI) as biomarkers to be able to prioritize and align the work that still needs to be done towards qualification.
- To compare the biomarkers detected in blood and urine to select the most suitable candidates and discuss future tests to confirm their usefulness
- To set up a way for collecting, storing and sharing blood and urine for biomarker identification and validation

## 2. Session 1: Setting the stage

### 2.1. 1-1 Introducing the 226th ENMC WS

Alexandra Breukel, Managing Director of ENMC, welcomed the participants underlining the role of ENMC in promoting research for the neuromuscular community. She encouraged applying for ENMC workshops as translational tools to bridge research and clinical applications.

Annemieke Aartsma-Rus introduced the aims of the workshop, working towards validated and qualified biomarkers for DMD. Indeed, the focus was on translational outputs of biomarker research. Following a period of intense discovery, now we need to prioritize biomarkers and implement their application in the clinic and clinical trial settings. Considering the chronic nature of DMD and the slow response to treatment with novel therapies or in clinical trials, biomarkers remain an ideal option to monitor the clinical course or outcomes in a shorter timeframe.

Alessandra Ferlini summarized the previous biomarker meeting achievements (204th ENMC workshop, held in [6] 2012). The workshop was quite ambitious in terms of deliverables, but this richness was encouraged by the EU BIO-NMD grant which supported many of the participants. The main deliverables and milestones were: i) sharing of data and setting up collaborations on new biomarker projects between Europe and the US; ii) alignment of biomarker discovery modalities in Europe and the US; iii) designing the best model for biomarker validation in larger cohorts to speed up translation in clinical practice; iv) biomarkers prioritization to facilitate the interaction with regulatory authorities.

Although the goals were many, general consensus was achieved especially on the identification of mandatory tools such as shared registries and biorepositories, the availability of clinical trial study samples, the use of dedicated technologies and platforms, often based on -omics approaches, and use of innovative and dedicated bioinformatics. The three conclusive breakout sessions (existing biomarkers; defining actions for combination of biomarker data in different cohorts and; issues related to the regulatory authorities) provided

a list of the technically/clinically validated biomarkers in DMD that could be taken further towards a qualification process with the regulators. The consensus was that dystrophin protein measurement and muscle quality assessment by MRI could be qualified as pharmacodynamic biomarkers, while CK measurement was debated and considered not appropriate for DMD monitoring.

Annemieke Aartsma-Rus then presented on interactions with regulators pertaining to DMD biomarker development. Regulatory agencies have a process in place to qualify biomarkers for a specific purpose ('context of use') [2,3]. Multiple interactions coordinated by patient organisations, the TREAT-NMD [7] alliance and a cooperation of science and technology (COST) Action (BM1207) [8] have taken place between the DMD field (academics, patient organisations and industry) and the regulators to discuss the specific challenges of DMD therapy development, including biomarkers where the focus thus far mostly has been on dystrophin quantification and magnetic resonance imaging (MRI) [4,9].

The first bilaterally educational effort took place in September 2009. It was hosted by EMA and served to raise awareness about DMD specific challenges with the regulators and regulatory requirements for outcome measures including biomarkers discussed with the DMD field [10]. In March, 2013 EMA published a draft guidance document for the clinical investigation of medicinal products for the treatment of Duchenne and Becker muscular dystrophies [11]. During the public consultation period, the patient community, TREAT-NMD and COST Action BM1207 organized a forum discussion again involving all stakeholders, to discuss the draft document in light of newly collected data. This resulted in aligned feedback from the field, which was implemented in the guidance document that was published in December 2015 [12]. The document outlines that no biomarkers existed at that time that could be used as primary or key secondary endpoint in phase 3 clinical trials. Markers of muscle damage, such as plasma creatine kinase levels, are useful for diagnosis, but have limitations in the clinical trial setting, since the creatine kinase levels tend to go down with age for DMD patients when muscle tissue is lost due to disease progression, and its levels are influenced by the degree of muscle activity. Dystrophin quantification is an obvious pharmacodynamic biomarker for therapies aiming to restore dystrophin expression. However, the guidelines indicate that current methodology to quantify dystrophin has debatable robustness and reproducibility especially for extremely low dystrophin levels. As such, dystrophin quantification can only be considered as an exploratory pharmacodynamic marker [13]. MRI is mentioned as well, and its use as an exploratory or secondary endpoint is encouraged to generate additional data.

Parent Project Muscular Dystrophy meanwhile set up a similar stakeholder meeting with FDA representatives in December 2013, and was the first patient organisation ever to coordinate the drafting of guidelines themselves. This document was submitted to the FDA in June 2014 [14]. Based on this document, FDA published draft guidelines a year later [5]. Public consultation ended in October 2015, but no final document has yet been published by the FDA. As of this workshop, no biomarker has been qualified for DMD in the US. Nevertheless, the accelerated approval of eteplirsen by the FDA was based on dystrophin quantification by Western blot.

### 3. Session 2: Different perspectives on biomarker development and qualification

Elizabeth Vroom (United Parent Project Muscular Dystrophy) introduced the patient perspective on biomarker development. DMD families hope the development of validated biomarkers will help to have shorter and less burdensome trials. Finding a biomarker which could ultimately be used as a surrogate endpoint would be very useful. Also it would be valuable to be able to test the effect of drugs in a wider range of DMD patients. Currently most trials are done in ambulant patients, while the majority of DMD patients are non-ambulant. Handling and optimal use of samples are a concern to families as well as ownership of left-over materials. One of the greatest concerns is the use of biopsies in placebo controlled trials. Before samples are taken, families need to know whether regulatory agencies have agreed with the proposed methods of analysis. There is a need to improve the knowledge about the role and choice of biomarkers in the DMD community.

Ellen Welch (PTC Therapeutics) gave the perspective from industry, underlining the need for biomarkers in clinical trials, but also outlining the need for robust and validated assays to assess biomarker levels, as was discussed in more detail at the previous ENMC workshop on DMD biomarkers [6].

Jane Larkindale (C-Path) presented on the Duchenne Regulatory Science Consortium (D-RSC), which was set up through a research agreement between Parent Project Muscular Dystrophy and the Critical Path Institute (C-Path) to support regulatory qualification of drug development tools for DMD to enable the earliest possible patient access to new treatments. C-Path has eleven years of experience in regulatory qualification of drug development tools, including qualification of multiple biomarkers with the FDA, EMA and the Pharmaceuticals and Medical Devices Agency (PMDA, Japan).

Due to muscle damage, serum levels of aspartate transaminase and alanine transaminase, AST and ALT, are respectively high in DMD patients. As these are typically used as liver safety biomarkers, AST and ALT serum elevation can lead to DMD patients being misdiagnosed as having liver damage but also prevents assessment of liver toxicity in drug development programs using these markers for DMD patients. A series of preclinical and clinical studies have demonstrated that glutamate dehydrogenase (GLDH) is consistently low in adults and children of both sexes with and without muscle damage. It consistently increases proportionally to liver damage caused in different ways as measured by existing biomarkers in both animals and humans. The Predictive Safety Testing Consortium of C-Path is seeking FDA and EMA qualifications for the biomarker as a measure of liver toxicity in patients with underlying muscle damage, and with D-RSC, they will seek *in vitro* diagnostic status for the assay and work with the community to ensure availability to DMD patients, and those developing new therapies for the disease.

C-Path's experience with biomarker qualification shows the importance of determining the context of use for biomarkers early in their development, and working with the agencies to agree on the level of evidence needed for the specific context. D-RSC has an FDA liaison engaged in discussions to ensure this. Levels of evidence for some contexts (e.g. surrogate

endpoints) can be very high, while evidence needed to qualify biomarkers for other uses (e.g. to define inclusion criteria) may require less data. Initial contexts can be expanded with additional data. C-Path is working with the FDA and others to define the evidentiary standards required for qualification across all disease areas.

C-Path recognizes the importance of using data standards to build integrated databases to support qualification efforts, so as to build scientifically valid databases representing the patient population as a whole. It has been found that assay validation and continuity in data collection between sites are critical to qualification efforts. Furthermore, it has been found that discussion of statistical plans with the authorities early in development of a tool is an advantage in assuring that all stakeholders are aligned, and the tools may be acceptable for qualification. D-RSC is open to working with others on qualification of additional biomarkers.

Pietro Spitali (Leiden University Medical Center) presented the early academic perspective on biomarker development and qualification. The presentation focused on molecular biomarkers and covered multiple aspects including the study design, differences among cohorts, discovery platforms and data analysis. The first point was the definition of the aims of the study; if the study aims to identify a diagnostic biomarker it is very important to have a sufficient number of cases and controls in the discovery cohort, to study the association in different replication cohorts, and to correctly identify the most meaningful controls (e.g. not only to study Becker muscular dystrophy (BMD) patients vs. healthy controls but also compare to other muscular dystrophies which may be misdiagnosed as BMD).

The cancer field is more advanced in terms of biomarker discovery and clinical use. Learning from the initiatives performed by other groups in the cancer field helps to identify the pros and cons and better tune studies for rare conditions such as DMD. Predictive biomarkers (which enable prediction of response to treatment) have been identified in both retrospective [15–18] and prospective [19–21] studies (for more detail we refer the reader to a recent review paper [22]). With a retrospective study design it is easier to have access to a higher number of samples (and follow-up samples), derived from patients receiving standard treatments. However, it is difficult to have homogeneous standards of care between countries and even between centres in one country, and to have clinical progression monitored homogeneously in the population. In a prospective study design the conditions are more controlled and it is easier to standardize sample collection, however these studies are normally associated with smaller samples sets.

For the identification of biomarkers, the LUMC uses a mixed strategy where samples are collected prospectively in order to collect the same type of clinical information for all patients and to standardize sample collection as much as possible. Once a sufficient number of samples (and follow-up samples) have been collected and the hypothesis has been generated based on the most recent literature, the analyses are performed. During the presentation the different types of samples (serum/plasma/PAXgene), tube type (heparin lithium or EDTA), sample processing (e.g. clotting time and globin depletion) and analysis platforms (e.g. targeted and untargeted proteomics) in relation to the sample types have been discussed. It was presented that studies performed in patients affected by different muscular

dystrophies using the same study design and proteomic platform result in very different output [23,24]. This makes proper powering of studies in different forms of muscular dystrophy very challenging.

One key element that was discussed was the need to perform small scale pilot studies in order to properly power the actual discovery study and collect independent samples to allow multiple layers of validation. Furthermore, there was discussion about the necessity of choosing the correct statistical test *a priori* and of validation of the findings using independent reliable, precise and accurate technologies. Finally, collaboration is necessary in order to obtain sufficient samples, including samples obtained during clinical trials. These samples have value, as they are collected during highly controlled clinical studies.

H. Lee Sweeney (University of Florida) provided the perspective on late stage biomarker development from an academic perspective (see next section).

#### **4. Session 3a: Towards biomarker validation and qualification**

H. Lee Sweeney reviewed the NIH sponsored Imaging DMD study that is being conducted across three sites in the US and is evaluating 150 DMD boys over 5 years. Dixon imaging has been found to be more effective than T2 with specific focus on measuring fat fraction of key muscle groups. For example, fat fraction of the vastus lateralis muscle is highly predictive for loss of ambulation in the near term. Challenges related to MRI in the DMD population relate to the limited ability to conduct these studies on very young boys due to the need to remain still during imaging.

Volker Straub (University of Newcastle) discussed progress using MRI methods to evaluate muscle disease. Imaging has been conducted in LGMD 2I patients (FKRP mutations) revealing that muscle which appears fairly impaired can still support ambulation in this disorder. He also reviewed the ongoing study of LGMD 2B (dysferlin) patients and the specific capacity to distinguish fat from protein and the use of the Myo-MRI website. It was highlighted that MRI is a highly useful method that can be adapted to multiple muscle groups.

#### **5. Session 3b: Towards biomarker validation and qualification—example of dystrophin**

Kristy Brown (Solid Biosciences) presented the mass spectrometry based assay developed at Children's National Health System to quantify dystrophin. Details about the procedure including gel separation, in gel digestion and mass spectrometry of peptides (endogenous and 15 standard peptides) were presented. Dystrophin quantity was calculated as the ratio between endogenous and standard peptides for each sample, showing overall low variation (CV between 10% and 20%). With the obtained results, Dr. Brown and colleagues applied for qualification for dystrophin as a biomarker at the FDA. The procedure started in 2013, but it was first put on hold in 2014 by the agency because there was no wide consensus over the use of dystrophin as biomarker in the field. When this was resolved in 2015 a new type of application was recommended by the FDA, as dystrophin as a surrogate endpoint was

deemed too broad. A new letter of intent for dystrophin as a pharmacodynamic biomarker was filed in August 2015 and was turned down in 2016, because data were not sufficient to initiate full review. At this time the FDA recommended applying for a letter of support, which “does not connote qualification of a biomarker and does not endorse a specific biomarker test or device. It is meant to enhance the visibility of the biomarker, encourage data sharing, and stimulate additional studies” (<https://www.fda.gov/drugs/developmentapprovalprocess/ucm434382.htm>). In November 2016, The FDA opted to not issue a letter of support to the applicants stating it was unnecessary because eteplirsen (Sarepta) was approved based on dystrophin restoration, and as such dystrophin was accepted in this application by the FDA as a surrogate endpoint. The overall process highlighted the hurdles of biomarker qualification within a defined context of use (e.g. dystrophin as surrogate for therapies aiming to restore dystrophin, not for DMD therapies in general) while drugs are being evaluated by the agency.

Diane Frank (Sarepta Therapeutics) presented on the dystrophin quantification strategy used by Sarepta. This involved the procedure to obtain the muscle biopsy, which has been optimized in order to obtain comparable biopsies across study sites. Western blot data (currently preferred by the FDA) are obtained by the analysis of 30 micrograms of total protein. A 5 point standard curve (0.25%, 0.5%, 1%, 2%, and 4% of healthy control) is used to interpolate the data. Equal amounts of protein amounts are loaded for all samples and a defined film exposure is used to avoid signal saturation. Quality checks for a successful experiment include a standard curve with  $R^2$  above 0.9 and the dystrophin intensity of DMD control samples below the intensity of the standard at 0.25%. Specific criteria are in place to evaluate whether a gel has sufficient quality to warrant downstream analysis. Alpha-actinin is used as a loading control even though this may cause dystrophin underestimation given that alpha-actinin is not a sarcolemmal protein and may not appropriately correct for the amount of sarcolemma present in that biopsy (which may be considered as the ability of that specific piece of muscle to produce dystrophin). It was discussed that dysferlin and spectrin might be good loading controls and work is ongoing to test these options.

Western blot data were also compared to data obtained by immunofluorescent analysis of dystrophin showing linearity between the 2 measurements across healthy controls, BMD and DMD patients. Even though the percentage of dystrophin positive fibres was perceived as subjective by the FDA, improvements are possible by automating the analysis after finding a shared consensus over the minimum requirements for dystrophin positivity.

Eric Hoffman (Reveragen Biopharma, AGADA Biosciences) presented assays available at AGADA Biosciences to quantify dystrophin. AGADA Biosciences is performing the dystrophin quantification in samples obtained in interventional clinical trials with antisense oligonucleotides (e.g. Nippon Shinyaku trial targeting exon 53). They provide video tutorials on how the procedure has to be performed and if that is not sufficient, support on site is available to show each step of the protocol to ensure high method reproducibility across sites. They optimized each step of the protocol starting with the muscle biopsy processing until data acquisition. Protocol instructions include tips on the freezing procedure, solubilization method and loading controls (multiple tested such as vinculin, alpha-actinin, spectrin and myosin heavy chain). The method includes details about accuracy, precision,



specificity, linearity, limit of detection, limit of quantification and stability. Dr. Hoffman reported dystrophin levels to be stable in frozen biopsies for 1–2 years at least.

AGADA Biosciences is not only providing western blot as a dystrophin assay but also other quantitative assays such as mass spectrometry which is less sensitive due to the absence of signal amplification, but it is characterized by low variation coefficient lying within the FDA guidelines. They also provide other services such as immunostaining and exon skipping quantification. All methods have standard operating procedures to be precisely followed.

Francesco Muntoni (University College London) presented the next steps to take in the collaborative effort of harmonizing dystrophin quantification. Different technologies to detect dystrophin in muscle biopsies are available. FDA prefers western blot information over the percentage of dystrophin positive fibres, or dystrophin intensity on immunocytochemistry, as they perceive the latter assays as more subjective. Efforts are however ongoing on methodology to quantify the dystrophin levels in individual muscle fibres using automated, unbiased techniques. This would provide reliable data if the technique is performed in an automated way, which is at the moment being tested in different labs (e.g. BioCruces Health Research Institute in Spain; Flagship Biosciences; Dubowitz Centre London) for DMD, BMD and intermediate phenotypes. It is challenging to have references of ‘normal dystrophin levels’ since there is variation in dystrophin expression between fibres within a muscle and between fibres of different muscles in healthy individuals.

Western blot data were presented in detail including a discussion of which loading marker would be more appropriate and vinculin was suggested as a good candidate as it is stable and not differentially expressed in dystrophic muscles compared to healthy controls. Finally, the data of a Cooperation of Science and Technology (COST) Action funded working group aiming to compare exon skipping quantification with different techniques across different European labs were presented (COST Action BM1207, manuscript in preparation).

Ellen Welch (PTC Therapeutics) discussed the nonsense read-through approach to treat patients carrying nonsense mutations. It was shown that premature stop codons are often the result of mutations of arginine encoding triplets into nonsense codons and that, often read-through of the stop codon reintroduced arginine in the correct position. The results of the phase 2a open label dose ranging study and the phase 2b study with ataluren were presented. In the phase 2a study boys were treated 3 times a day due to the short half-life of the compound and participants were divided into 3 groups: 6 boys were treated with 4–4–8 mg/kg, 20 boys with 10–10–20 mg/kg and 12 boys with 20–20–40 mg/kg daily. The treatment duration was 28 days, boys were 5 to 17 years of age, 4 were non-ambulatory and 11 were not treated with corticosteroids. Mutations were spread throughout the coding sequence. The change in dystrophin expression was presented as a percentage increase and seemed to follow a normal distribution with some patients showing an increase in dystrophin while others showed a decrease. There were no remarkable differences among the treatment groups. The phase 2b study was divided into 2 dose groups including 20 boys on 10–10–20 mg/kg and 12 boys on 20–20–40 mg/kg. Treatment duration was 48 weeks; the age range was 6–16 years of age and all 3 stop codons were represented in the study groups. Cultured

myotubes were obtained and treatment with ataluren showed dystrophin staining for all subjects.

## 6. Session 4a: Candidate biomarkers—results in DMD and BMD (serum)

Jessica Chadwick (SomaLogic) reviewed the proprietary aptamer technology used by SomaLogic. Currently, 1300 proteins per sample are quantified in a multiplex setting. Newer assays are anticipated to allow quantification of >3600 proteins per sample. A previous study using the smaller platform identified 44 proteins that differed between DMD and control serum [23]. The newer platform extends this finding to 163 proteins, including confirmation of the prior 44. The current list expands into proteins implicated in muscle development, metabolism, and mitochondrial function. Interestingly, immune and inflammatory proteins were reduced. The cohort in this study included both those treated with steroids as well as those not on steroids. A new DMD cohort is planned to evaluate how these components change over time in DMD, and a consortium is being developed to carry out this analysis.

Pietro Spitali discussed using the Somascan aptamer-based method to profile serum from 15 DMD subjects and 9 healthy controls. Of the DMD subjects, 11 were ambulant and all but one had been treated with steroids. With this aptamer approach, 111 proteins were decreased in DMD serum while 148 proteins were increased. A number of these proteins ( $n = 32$ ) were shared among other DMD serum profiling studies [23]. Furthermore, the same aptamer method was also used to study 14 patients who were followed for an average of 4 years. More than 400 proteins were observed to change over time. Metabolic profiling of a distinct DMD cohort (30 DMD and 10 controls, all fasted) identified 9 markers that differed between the two groups. A lipidomics analysis in 30 DMD patients identified two lipids that were altered including one that may correlate with performance on the North Star assessment tool.

Eric Hoffman discussed plans for vamorolone, a novel dissociative steroid selected for its ability to bind the glucocorticoid receptor (GR) with high affinity but not to dimerize the receptor [25,26]. The Phase IIA study is recruiting 48 DMD boys and Phase IIB is anticipated to start in the coming year. Dr. Hoffman discussed the challenges of assessing the effect of vamorolone on NF- $\kappa$ B since the standard assays monitor activity after lipopolysaccharides (LPS), an especially strong stimulus, which may not adequately reflect what occurs in DMD. At very high doses, adrenal suppression may occur with vamorolone. A proteomic scan was conducted on DMD and inflammatory bowel disease patients using aptamer technology to identify pharmacodynamic markers of glucocorticoid use [27]. This study identified MMP3, leptin, insulin, afamin and GH binding protein.

Christina Al-Khalili Szigyarto (KTH Royal Institute of Technology) reviewed the use of the Luminex platform which is used to assess protein content. She described the human protein atlas (<http://www.proteinatlas.org>) and the project to generate antibodies to all human proteins to more than 50,000 antigens. This website offers information where tissue specific, including muscle specific, proteins can be discerned. Protein biomarkers for muscular dystrophy were discussed including enolase 3 and carbonic anhydrase 3 (CA3) (which were also detected with the Somalogic's assay), confirming the utility of the method [23,24,28].

With this approach, antibody specificity for the target of interest is critical. Paired antibodies, where two or more antibodies target different regions of the same target protein, are highly useful to improve the robust, specific and quantitative nature of the findings. A new biomarker, nestin A, was discussed and the need for longitudinal studies was reinforced.

Graham McClorey (Oxford) is working within the group of Matthew Wood and focused on identifying serum micro RNA (miR) biomarkers for DMD using animal models like the *mdx* mouse. miR-1, 133 and 206 all decrease in early postnatal life in the mouse with an increase at 3–4 weeks of age [29]. Studies are being conducted using downhill running in order to determine the effect of acute exercise. Most miRs increase immediately and then again at day 7. A newer study is now focused on sequencing small RNAs from *mdx* mice. miR-483P was identified and is upregulated in DMD patients from the Newcastle Biobank.

An aptamer based serum study in *mdx* mice (1129 aptamers assayed) identified 75 proteins that were increased and 26 that were decreased in *mdx* mice. Among these ADAMTS5 was studied in patients with DMD, BMD, and FSHD; a positive association with age was found for DMD and FSHD while in BMD patients there was a negative correlation with age.

Jon Tinsley (Summit plc) described a larger aptamer analysis using the system with the capacity to detect nearly 4000 proteins in the serum. In this study, six DMD patients with 3–4 serum samples collected over 3 months were used. Serum levels varied up to 50% over several weeks for many of the target proteins. Carbonic anhydrase 3 (CA3) and CK showed the least variability among the samples, but these protein biomarkers were still felt to be highly variable. miR-133a and miR-133b were both up in early DMD. Given Summit plc's interest in upregulation of utrophin, Dr. Tinsley presented data on their approaches to accurately quantify utrophin upregulation in muscle biopsies from treated DMD patients.

## 7. Session 4b: Candidate biomarkers—results in DMD and BMD (urine)

Pietro Spitali discussed the utility of examining urine biomarkers in *mdx* mice. Urine is an attractive source since it is noninvasive to collect. PGDM (11,15-dioxo-9 $\alpha$ -hydroxy-,2,3,4,5-tetranorprostan-1,20-dioic acid (tetranor PGDM)) was shown to be elevated in DMD patients compared to healthy controls, especially for patients above 9 years of age [30]. Metabolic profiling of urine is ongoing in *mdx* mice. Prednisone was detected in the urine in steroid-treated DMD patients and *mdx* mice, providing a useful internal control. Issues surrounding urine biomarkers include variability in urine volume and the need to provide normalization.

Francesco Muntoni presented miRNAs as biomarkers in urine, since miRNAs have been studied by multiple groups in serum samples from animal models of muscle disease and DMD patients [31–36]. He also reviewed the challenges of profiling miRNAs from urine samples and the variability introduced by ambulatory status and activity as well as treatment status with respect to corticosteroids. He presented data acquired from 7 healthy controls and 30 DMD patients (18 ambulant and 12 non-ambulant). All miRNAs (178) were quantified with a TaqMan RT-PCR approach, and all miRNAs could be detected in the urine of healthy controls. miRNAs were identified that were differentially expressed between

healthy and DMD, while others were associated with either prednisone or deflazacort treatment.

Alessandra Ferlini (University of Ferrara) showed the use of urine-derived cells to generate cellular models of DMD [37]. Urine cells can be readily obtained and directly reprogrammed using MyoD. These cellular models are highly useful for testing antisense oligonucleotides for their ability to induce in frame production of dystrophin. As exon skipping moves to testing for rare DMD mutations, having a readily accessible cell model will be highly useful to e.g. study dystrophin restoration *in vitro*.

Elizabeth McNally (Northwestern University) also discussed the ease of working with urine-derived cells and their application to testing exon skipping. She reviewed progress in developing exon skipping to treat Limb Girdle Muscular Dystrophy type 2C, which is due to loss of function mutations in SGCG, encoding  $\gamma$ -sarcoglycan. In order to treat approximately half of the LGMD 2C patients, a uniform approach is planned that necessitates skipping exons 4, 5, 6 and 7. This would leave exons 2, 3 and 8 encoding a protein, referred to as Mini-Gamma. In order to assess whether Mini-Gamma is viable as a strategy, transgenes were created to test Mini-Gamma expression in both *Drosophila* and mouse models of LGMD 2C [38]. It was shown that transgenic expression of Mini-Gamma rescued many distinct features of muscular dystrophy. In order to test exon skipping in LGMD 2C, fibroblasts and urine cells were reprogrammed into myogenic lineages using MyoD to establish a cell based model of LGMD 2C [39]. Exon skipping was demonstrated in these models [38]. Furthermore, she showed that urine derived cells can be gene edited using CRISPR/Cas9 methods.

## 8. Session 4c: Genetic modifiers in DMD

Luca Bello (University of Padova) discussed the rs28357094 genetic variant present in the promoter of the *SPP1* gene, which has been linked to age of ambulation loss in DMD [40–42]. The *SPP1* gene encodes the protein osteopontin and is elevated after muscle injury and during regeneration. It was recently reported that the SNP exerts its function when patients are treated with corticosteroids [43]. In order to understand the mechanism underlying the SNP effect in the presence of corticosteroids, myoblasts of 11 DMD patients and 9 healthy controls were cultured with and without deflazacort to study the response of the *SPP1* promoter to corticosteroids. Gene expression and protein levels were used as promoter activity readouts. No change was detected in gene expression or protein levels from DMD and healthy control-derived myoblasts. An increase in gene expression of *SPP1* was observed in DMD myotubes but no difference was recorded at the protein level. Two main osteopontin protein isoforms were observed, 50 and 55 KDa, and these forms were differentially expressed in DMD in response to steroids [44]. Although a comprehensive mechanism is still needed to explain how this SNP regulates *SPP1*/osteopontin expression in DMD, it is clear that patients with the TT genotype respond better to corticosteroid therapy compared to patients carrying other genotypes.

## 9. Session 5: Sample and data sharing

The final session was on ways to facilitate sample and data sharing for biomarker discovery and validation. Several best practices were shared. First, Luca Bello outlined how identification and validation of genetic modifiers for DMD, such as variations in *SPP1* and *LTBP4*, benefited from an international collaboration. With rare diseases there are challenges to validate genetic modifiers. Generally, cohorts are small, and there are many confounders, such as steroid use, variation in care standards and ancestry. Currently there are two large cohorts available for modifier identification: the CINRG cohort and the Bio-NMD cohort. Through a reciprocal agreement modifiers identified in one cohort are validated in the other. Most recently this was used to validate a SNP in CD40 [45].

For MRI protocol development, there is a good cross talk between US and European initiatives, as was presented by Lee Sweeney and Volker Straub. In fact, the MRI work is becoming a global effort with common quality assurance protocols and standard operating procedures. Furthermore, using central data analysis, it has been confirmed that data collection and interpretation is reliable at different sites and over time. There is consensus on what to qualify as a biomarker with the regulators (fat fraction as measured by Dixon).

Pietro Spitali presented on an effort that was initiated after the 204th ENMC workshop to make an inventory of serum and plasma samples available for biomarker validation studies. The work was sponsored by a small grant from Duchenne Parent Project Netherlands. Samples have been identified, some of which are linked to functional data and some of which are longitudinal. It is clear that more effort is needed to collect additional samples and functional data in a controlled way. Samples collected in placebo arms of clinical trials would be ideal to validate candidate biomarkers.

Eric Hoffman presented from an industry perspective biomarker discovery in clinical trial samples that potentially poses a risk, because all drug related data is reportable and discoverable. In particular, unexpected findings in treatment arms may be difficult to interpret as to whether they are a safety concern. However, using a data analysis plan which outlines which part of the data will be analysed, unexpected findings may be avoided.

Eric Hoffman further presented on the issue of data comparability and the need for reference samples. Ideally datasets should become available after publication as a public resource. However, hosting and curating these datasets is laborious and expensive.

The topic of biobanking was then discussed during breakout sessions focusing on setting up, using and maintaining a virtual biobank of samples to be used to validate candidate biomarkers. First there was consensus on criteria that new therapeutic candidate biomarkers need to fulfil: they need to be specific, reproducible, reliable and robust. Ideally, markers found in animal models should be confirmed in humans, markers need to be responsive to treatment in animal models and need to be measurable in easily accessed samples (e.g. blood, urine, saliva).

The group agreed that a virtual biobank is needed, with a central management system and a central catalogue. This can make use of existing format such as Eurobiobank

([www.eurobiobank.eu](http://www.eurobiobank.eu)) and BB-MRI (biobanking and biomolecular research infrastructure). These are European initiatives, and would have to be set up on a more global scope for the DMD biobank. Standardized protocols for obtaining and collecting samples and informed consent are available at Eurobiobank and RD-Connect and should be used. Since for regulatory qualification the link to functionality is required, it will be crucial to also collect an agreed upon set of functional data in a standardized manner.

The group agreed that an oversight committee is required for custodianship of these samples. This committee should have representatives from patient advocacy groups, academia and industry, and should have access to a larger extended group when expertise is required for evaluations of requests.

Requests should be evaluated for several criteria, based on the model used by Telethon Italy. Both academic and industry groups would have access to the samples, provided they adhere to the selection criteria, which include items like the amount of sample (scaled to the proposed aims of the research), preliminary data (including information on the limit of detection and fold change of the biomarker(s)). Finally, risk assessment is included, where applicants have to show they have funding to achieve their aims, outline timelines and power calculations. Therapeutic biomarkers would be prioritized for DMD. There would be a publication obligation for everyone who uses samples from the biobank, including when results are negative and an obligation to share the results with the community in a timely fashion following the RD-Connect model.

Ideally the management part of this virtual biobank should be funded by patient organisations and industry. The patient representatives indicated that the patient community has played a large role in this thus far and will keep doing this. They also indicated that communication and feedback are important, e.g. the metrics of the biobank should be reported to the patients regularly.

## 10. Workshop participants

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