

Workshop report

Developing standard procedures for pre-clinical efficacy studies in mouse models of spinal muscular atrophy

Report of the expert workshop “Pre-clinical testing for SMA”, Zürich, March 29–30th 2010

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1. Introduction

A workshop was held in Zürich (Switzerland) to develop standard procedures and protocols for pre-clinical efficacy studies in mouse models of spinal muscular atrophy (SMA).

The workshop was organized by the Network of Excellence TREAT-NMD (Translational Research in Europe – Assessment and Treatment of Neuromuscular Disorders), launched 2007, that addresses the fragmentation currently existing in the evaluation, diagnosis and care of neuromuscular diseases on one side, and in the procedures of drug development from research to the design of clinical trials on the other side. Within this frame, one aspect that needs particular care is the design and performance of pre-clinical animal studies that should predict the efficacy of a new treatment in patients. In the absence of commonly accepted guidelines and standardized protocols, data from different laboratories are often not comparable and the result is a duplication of efforts. The workshop was attended by 30 key researchers from Europe and North America, as well as industry representatives (Santhera Pharmaceuticals, Psychogenics, Genzyme, Trophos) and patient organisations (SMA Foundation, AFM-Association Française contre les Myopathies). The goals of the workshop were (1) to select endpoints that best assess drug efficacy in mouse

models and/or that best reflect human outcome measures and (2) for each endpoint, to form a working group of experts (consisting of a main author and contributing members), who were assigned the responsibility of preparing a selected SOP. The workshop was opened by Stefanie Possekkel, who, following an introductory round of all participants, described the structure and aims of the Network of Excellence TREAT-NMD in general and of Activity 7 in particular. She pointed out some of the key problems in animal research which included the large variability in animal species and strains, the difficulty in comparing different dose regimen used, the choice of appropriate controls, methods of animal randomization, group size (usually small) and the inadequate power of some experiments. She then summarized the success achieved in the harmonization of pre-clinical research for Duchenne Muscular Dystrophy [1] and presented an example of an existing SOP. Stefanie pointed out the efforts previously made in standardizing procedures in SMA pre-clinical research [2], to which Meg Winberg added that, as several mouse models are actually available and in use, it is particularly important to agree on common endpoints and methodologies to improve comparability across laboratories.

2. Session 1: endpoints in SMA mouse models (1)

The session was opened by Charlotte Sumner who addressed some of the key issues to be considered when planning experiments with SMA mice. The variation of

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phenotype encountered in SMA mouse colonies may depend on several extrinsic factors that can be controlled: background strain, litter size, temperature, frequency of handling, maternal diet and nutritional support for pups to name but a few. She also pointed out that it would be useful to reach a consensus on the definition of ‘survival’, which at the moment is quite variable between laboratories, since mice may have to be sacrificed due to institutional regulations versus being “found dead”. However, discussions showed that this may be difficult as local animal protection laws dictate to some extent how death is defined. It is therefore a clear wish of this audience that in future, when survival data is presented, the appropriate definition is always supplied. Arthur Burghes presented some recent results in his SMA mice (*SMN2*^{+/+}; *Smn*^{-/-}; *SMNΔ7* [3]) mouse model which also contained a doxycycline inducible *SMN* transgene. Survival was considered the best end point. The mice could be rescued by switching on the inducible *SMN* gene, with postnatal induction giving a very significant extension in survival. He addressed the optimal time point of initiation of drug therapy (the later *SMN* is ‘switched on’, the lower the responder rate) with induction at 5–6 days postnatally giving minimal survival extension. In addition, the Burghes lab has found that when dosing animals with drug compounds, mice from the same litter should all receive the same treatment, as cross contamination of neonatal mice is a major issue if two treatments are administered to the same litter. Danilo Tiziano reviewed the biomarkers currently used in patients (compound motor action potential, motor unit number estimation, dual-energy X-ray absorptiometry, dosage of the *SMN* protein and transcript with several methods). He pointed out strengths and weaknesses of these measurements and compared their effectiveness and feasibility with biomarkers used in mice. He finally concluded that none of these measurements can be considered as a ‘golden standard’ and that pre-clinical studies are indispensable to establish which should be tested in clinical trials. Some observations were made regarding the possible bias introduced in *hSMN2* full length transcript quantification by absolute real-time PCR, due to the presence of *mSmn* gene in heterozygous mice and likely related to the competition of primers for murine and human transcripts. However, no data are available and further studies are necessary to elucidate this aspect. Finally, it was pointed out that is crucial to gain comparative data on protein and transcript levels. Regarding this aspect, Karen Chen informed the audience that a new test kit (ELISA) optimized for detection of *SMN* protein in human peripheral blood mononuclear cells (but which probably cross-reacts with mouse *SMN* and could therefore be used also to detect mouse protein) is now available at the SMA Foundation. The SMA Foundation biomarker discovery study, the BforSMA study, was also briefly described by Karen. Bassem El-Khodori presented some of the issues involved in performing neuromuscular tests in SMA mice. Ideally, the test used to assess the efficacy of a drug should be reflex-based, be able

to differentiate between knock-out and wild type mice, be valid as early as postnatal day 2 (and for at least until 2 weeks of life) and resistant to side-effects. Care should be taken not to induce hypothermia (avoid separating the pups from their mothers for too long) and always perform the test at the same time of day (am or pm). The geotaxis test is considered a useful tool to evaluate neuromuscular function in neonatal mice in an easy, non-invasive, sensitive and quick way. It was remarked, however, that for this test it is key to define the surface material of the inclined plane. This test has its limitations as it is not entirely specific and may show up a drug’s side-effects (for instance drugs that increase alertness but not muscle function will have a positive effect on geotaxis), and cannot be performed before postnatal day 4. Therefore this test should be used as complementary to other assessments. In the next presentation Jean-Marc Raymackers discussed the practical details of the running wheel method, which can be used for adult mouse models to assess muscle function. The use of inclined wheels, the critical point of wheel resistance, the number of mice needed to account for the individual variability and the number of wheels necessary to test mice in parallel were discussed. The rotarod test and the pen test (also known as the balance beam test), that assess coordination and balance, were presented by Rashmi Kothary. The value of the rotarod test in the pre-clinical assessment of drugs in SMA was questioned as animals require training so can only be applied to mouse strains with a longer survival. It was agreed that the pen test was more suitable due to its simplicity and speed of performance and because no training was required. Therefore an SOP for the pen test would be prepared rather than for the rotarod test. Stefania Corti presented three different methods for grip strength measurements: the mesh grip test, the wire test and the automated grip strength test. Also here, the high variability and the degree of animal collaboration (motivation) represent the main disadvantages of this assay. For SMA, hind-limb strength may be more relevant than fore-limb strength but this is the more difficult parameter to measure reliably. Therefore a measure of all four limbs would be preferable as an alternative. In the final presentation of this session Brian Kaspar discussed the open field test for the recording of spontaneous locomotor activity. As for the geotaxis test, drugs that improve awareness and/or motivation could produce false positive results. From a practical point of view it was noted that the beam height of commercially available equipment may need to be adjusted (lowered) to suit the size of the young SMA mice and that the general well-being of the animals taking part in an assessment should be visually inspected for necrosis of the hind-limbs as this would also affect their ability to complete a task.

3. Session 2: endpoints in SMA mice models (2)

The afternoon session was kicked off by Christine DiDonato, who presented the righting reflex as a simple

and quick test which is particularly useful to assess the efficacy of a drug in severe SMA mouse models. This test can only be used for neonatal mice. Repetition of the assay during the day can cause fatigue and it would be important for across-lab comparisons to clearly define what is a valid 'righting' (i.e. how does one evaluate an animal that rights itself but then falls over again). Bassem El-Khodor then presented the tube test, a method developed to evaluate the neuromuscular function that considers three parameters: latency to fall from the edge of the tube, number of pulls and the hind-limb score. Together these parameters give an overall impression of performance (scores 1–5). The correlation of this test with others and some technical issues such as number of test repeats, effect of fatigue and influence of rate of growth on the tube test outcome were discussed. Brunhilde Wirth confirmed the usefulness and reliability of the test.

Ke Ning proposed a protocol for cell body counts in the ventral horn regions of the spinal cord, as this reflects the motor neuron loss observed in patients and represents a useful readout for gene therapy. It was observed that cell body loss is typically a late phenomenon which occurs shortly before death. However, it can also start earlier (e.g. from 3 to 5 days postnatal) or may not occur at all. Also, several technical hurdles have to be taken into account that may lead to false positives, for instance the specificity of common markers like choline-O-acetyltransferase in SMA-affected neurons.

George Mentis presented techniques to evaluate the motor neuron loss that occurs as early as postnatal day 4 and can be traced precisely if certain experimental conditions are followed. This technique is, however, highly sophisticated and not a method with widespread use. The following talk, by Thierry Bordet, focused on axon counts and showed the correlation with other endpoints such as the number of spinal motor neurons and muscle fibre area in the new SMA mouse line N11/N46 [4]. Despite the relevance to the disease and the ease of automation, this method has only limited value for assessing drug effects due to the lack of historical data, very small difference between knock-outs and wild types and the fact that axon loss is most often a late event. Therefore, this method is considered as probably not appropriate as a primary outcome in pre-clinical testing for SMA. Thomas Gillingwater presented methods for neuromuscular junction imaging with optical microscopy and electron microscopy, showed the pathological signs of neuromuscular junctions in SMA mice and addressed key points to achieve high imaging quality. Simon Parson presented several techniques for ex-vivo nerve-muscle preparations that can be used for targeted application of compounds to test efficacy and also to test for differential vulnerability of individual muscle phenotypes. These techniques should be more widely considered. The issue of SMN quantification at protein and RNA level was addressed by Giorgio Battaglia, who showed a collection of published data and concluded that very little data are available on subcellular localization of

SMN protein and transcript and no data are known on SMN protein fate and post-translational modifications. A discussion followed regarding the efficiency of N-terminal or C-terminal targeted antibodies. Michael Sendtner showed how isolated motor neurons in cell culture can be used to study drug candidates and how accurate measurements of calcium fluxes in motoneurons can predict dysfunction in the neuromuscular junction. He concluded that these methods are suitable for drug screening but less for efficacy tests. An overview of electrophysiological properties of the neuromuscular junction (synaptic transmission) was given by Lucia Tabares, who described spontaneous and evoked responses in wild type mice and SMA mice. Veronica Setola illustrated the measurement of compound muscle activated potentials as a sensitive method to evaluate functionality of motor units and showed data obtained in SMA mice as compared to wild type. And finally, Chien-Ping Ko concluded the session by presenting the use of *in vitro* muscle tension measurements, achieved by stimulating either the nerve or the muscle, as a functional readout that estimates neuromuscular junction transmission as well as motor unit numbers and sizes.

4. Relevance of mouse endpoints for translation into clinical trials

The first session of the second day was opened by Rudolf Korinthenberg, who gave an overview of SMA Type I, II and III pathology in human patients and of the clinical outcome measures used to assess impairment and performance. The authorities require a clinical benefit to be shown by a potential new drug and not just, for example, an increase in strength. The issue of motivation and investigator's training was addressed, and some questions were raised regarding the testing procedures used in the 6-min-walk test. This presentation was followed by a lively discussion, conducted by Thomas Gillingwater, which aimed at finding a consensus as to which endpoints in mouse models should be regarded as primary to evaluate efficacy of drugs and/or should be standardized with SOPs. Everyone agreed on the need to include measurement of body weight daily and survival analysis in every study. One main point that required consensus was the handling of spontaneous deaths that occur within the first 1–2 days of birth (i.e. are these animals included or excluded from the survival curves). Charlotte Sumner pointed out that exclusion criteria should be defined for mice as they are defined for patients in clinical trials. Arthur Burghes volunteered to prepare a proposal on this issue together with other points to consider when planning an experiment to test a new therapy (such as dosing schedule, times of testing and diet, as well as procedures to plot survival and body weight curves as endpoints). A second document on general SMA mouse housing and handling considerations will be authored by Charlotte Sumner, which would include maternal diet and nutritional support for pups. Among

the behavioural tests, there was agreement to write SOPs for the tube test/clasping response, geotaxis, open field testing, righting reflex, grip strength, running wheel and pen test. Several biochemical and histological SOPs were regarded as relevant for testing drug efficacy in mice: cell body/motor neuron counts, axon counts, neuromuscular junction imaging, SMN levels on western blot and ELISA, SMN immunocytochemistry, real-time PCR, small nuclear ribonucleoproteins (snRNP) assay, genotyping. Finally, the electrophysiological assays for synaptic transmission, compound muscle activated potential and electromyography, and muscle tension were considered worth being standardized through SOPs.

5. Creation of standardized operating procedures for endpoints in SMA mouse models

Judith Dubach opened the last session illustrating the advantages of implementing SOPs to optimize reproducibility both within a single institution over time and across institutions. She described key aspects of SOP writing and mentioned guidance documents (<http://www.epa.gov/QUALITY/qs-docs/g6-final.pdf>). She underlined the need of creating working groups that collaborate with authors with regards to the SOPs content, and of implementing an approval procedure (by the author and a named reviewer). An updating procedure for each SOP and a wide distribution to the appropriate work areas would increase acceptance and the overall use of the SOPs. Finally, she introduced a template for SOP writing and proposed deadlines for draft and final SOPs. In the plenary discussion that followed, the list of desired SOPs was circulated and workshop participants signed up in the working groups of interest. For SOPs not addressed during the workshop, authors were proposed and it was agreed to also contact other researchers for authorship and/or taking part in working groups according to their expertise.

The SOP-template was distributed to the SOP authors shortly after the workshop.

Once finalized, SOPs will be uploaded on the TREAT-NMD website on a web link that allows download of the documents as well as contact with the author should any questions or comments arise (www.treat-nmd.eu/SOPSMA).

6. Workshop participants

- Giorgio Battaglia (Istituto Neurologico C. Besta, Milan, Italy).
- Thierry Bordet (Trophos, France).
- Arthur Burghes (The Ohio State University, USA).
- Karen Chen (SMA Foundation, USA).

- Stefania Corti (University of Milan, Italy).
- Christine DiDonato (Northwestern University, USA).
- Judith Dubach-Powell (Santhera Pharmaceuticals Ltd., Switzerland).
- Bassem El-Khodor (Psychogenics Inc., USA).
- Thomas Gillingwater (University of Edinburgh, UK).
- Natalie Kayadjanian (AFM, France).
- Brian Kaspar (The Ohio State University, USA).
- Chien-Ping Ko (University of Southern California, USA).
- Rudolf Korinthenberg (Pediatric University Hospital Freiburg, Germany).
- Rashmi Kothary (Ottawa Hospital Research Institute, Canada).
- George Mentis (Columbia University, USA).
- Ke Ning (University of Sheffield, UK).
- Simon Parson (University of Edinburgh, UK).
- Marco Passini (Genzyme, USA).
- Stefanie Possekkel (Santhera Pharmaceuticals, Switzerland).
- Jean-Marc Raymackers (Université Catholique de Louvain, Belgium).
- Daniel Schümperli (University of Bern, Switzerland).
- Michael Sendtner (Institute for Clinical Neurobiology, Würzburg, Germany).
- Veronica Setola (EPFL Lausanne, Switzerland).
- Charlotte Sumner (Johns Hopkins School of Medicine, USA).
- Lucia Tabares (University of Seville, Spain).
- Danilo Tiziano (Catholic University, Rome, Italy).
- Raffaella Willmann (University of Basel, Switzerland).
- Meg Winberg (SMA Foundation, USA).
- Brunhilde Wirth (University Hospital of Cologne, Germany).
- Rafael Yáñez-Muñoz (Royal Holloway–University of London, UK).

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