212th ENMC International Workshop: Animal models of congenital muscular dystrophies, May 29th-31st, 2015 in Naarden, The Netherlands,

M. Saunier, C.G. Bönnemann, M. Durbeej, V. Allamand

Title

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Authors

Saunier M[a], Bönnemann CG[b] *, Durbeej M[c] *, Allamand V[a] *[$], on behalf of the CMD Animal Model Consortium

[a] Sorbonne Universités, UPMC Univ Paris 06, Inserm UMRS974, CNRS FRE3617, Center for Research in Myology, Institut de Myologie, GH Pitié-Salpêtrière, F-75013 Paris, France

[b] National Institutes of Health, Neuromuscular and Neurogenetic Disorders of Childhood Section, Bethesda, MD, USA.

[c] Department of Experimental Medical Science, Lund University, Lund, Sweden.

* Co-last authors

[$] Corresponding author. Tel.: +33 1 42 16 57 07; Fax: +33 1 42 16 57 00.

Email address: v.allamand@institut-myologie.org (V. Allamand).

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Introduction

The 212th ENMC workshop, « Animal models of Congenital Muscular Dystrophies » took place in Naarden, The Netherlands on May 29-31, 2015 and was attended by 13 participants from France, Israël, Italy, Japan, Sweden, Switzerland, United Kingdom and the USA, including clinical and basic science researchers, as well as one PhD student who received support from the ENMC Young Scientist Program.

Congenital muscular dystrophies (CMD) constitute a heterogeneous group of rare genetic muscle disorders, often but not exclusively inherited in an autosomal recessive fashion [1]. Over the past decade, our understanding of their molecular basis has expanded greatly [2], with close to 20 genes known to be involved to date. The corresponding proteins can be classified according to their localization, thereby highlighting three main groups of CMD, with the first two being related to the extracellular matrix and its connection to the muscle, *i.e.* those caused by the primary deficiency of components/receptors of the extracellular matrix (ECM), and those due to defective glycosylation of alpha-dystroglycan (α-DG), the major matrix receptor on muscle [3, 4]. These forms account for the majority of CMD cases [5, 6]. The remaining group encompasses forms of CMD that involve proteins of the nuclear envelope (L-CMD), and other intracellular compartments (*SEPN1*-related myopathies).

The development and characterization of animal models of all these CMD subgroups has subsequently shed some light on the pathophysiological mechanisms involved, and provided invaluable in vivo models for assessing therapeutic options in the preclinical space. The diversity of animal models now available (mouse, zebrafish, cat, dog) enables complementary studies to model different aspects of the human condition, which often are not all covered in a single model. Hence detailed knowledge about strength and limitations of each of these

models clearly enriches the understanding that we have on the function(s) of genes, proteins and pathways involved both in physiological and diseased muscle.

This workshop focused on the three most common forms of CMD for which animal models are available, with sessions dedicated to each of the following:

- alpha-dystroglycanopathies, caused by an abnormal glycosylation of α-DG, a central protein of the dystrophin-glycoprotein complex (DGC),
- *LAMA2*-related muscular dystrophies, due to a deficiency of laminin-211, one of the main components of the basal lamina, which binds α-DG and integrins,
- *COL6*-related muscular dystrophies, due to a deficiency or dysfunction of collagen type VI (COLVI), a major extracellular matrix protein in many tissues,

Additional sessions reviewed Standard Operating Procedures (SOPs) and the translational link to the clinic, respectively.

The objectives of the workshop were (i) to present the different animal models currently available, review their value and whether some aspects of the human disease features need further modelling, (ii) to assess and define the most adequate tools; (iii) to discuss and identify relevant SOPs and outcome measures so that the data emanating from the various models can be compared.

For each form of CMD discussed during the workshop, **Carsten Bönnemann** presented a brief introduction of the pathophysiology and symptoms in humans.

1. Animal models of dystroglycanopathies

Dystroglycan is encoded by the *Dag1* gene, whose transcript gives rise to a precursor protein that is post-translationally cleaved into two subunits, alpha and beta. The latter spans

the sarcolemma, enabling ECM proteins to recognize and bind the special glycan structures of α-DG. To date, mutations in up to 18 genes encoding putative or demonstrated glycosyltransferases have been shown to cause altered O-mannose α-DG glycosylation, including LARGE, fukutin, FKRP, POMT1, POMT2 and POMGnT1 as the most prevalent ones [7-12].

A number of animal models have been generated for the alpha dystroglycanopathies with a spectrum of phenotypes. Although considerable progress has been made, the entire pathway leading to the functional glycosylation of α-DG still is only incompletely understood, while it also became clear that reagents (*e.g.* the most widely used IIH6 antibody) and methods for identifying the diversity of glycosylated α-DG remain a challenge. Several mouse models of alpha dystroglycanopathies were presented and discussed by **Susan Brown**, **Kevin Campbell**, and **Tatsushi Toda**. These models recapitulate deficits in some of the proteins involved in α-DG glycosylation (FKRP, LARGE, Fukutin).

Work performed in **Susan Brown's** laboratory on muscle development in the FKRP knock-down mouse (originally generated in 2009), showed that the specific glycosylation of α-DG was reduced by embryonic day 15.5 in this model [13]. Whilst this did not appear to alter myotube differentiation as determined by myosin heavy chain distribution, there was some indication that the number of Pax-7 positive satellite cells in the *tibialis anterior* muscle was decreased relative to wild type. Similar differences were seen in the *extensor digitorum longus* although they did not achieve statistical significance suggesting that the impact of a defect in α-DG is first manifested during the later stages of muscle development and that the timing of this defect may differ between muscles. Overall these findings imply that postnatal muscle growth and regeneration may be compromised in the absence of properly glycosylated α-DG.

With regards to therapeutic options for alpha dystroglycanopathies, compensatory up-regulation of the final α-DG glycoepitope mediated by the LARGE bifunctional glycosyltransferase, encoded by the causative gene for MDC1D, is currently pursued as an important therapeutic strategy across models, raising important questions regarding the need for biomarker(s), specific endpoints, target tissues and therapeutic windows. Recently published studies from the laboratory of **Kevin Campbell** demonstrated the importance of LARGE2-mediated modification of α-DG, a paralog of LARGE (like-acetylglucosaminyltransferase), that also has xylosyltransferase (Xyl-T) and glucuronyltransferase (GlcA-T) activities and the ability to polymerize Xyl-GlcA repeats. Using a newly developed assay for endogenous glucuronyltransferase (GlcA-T) activity, the Campbell lab demonstrated that normal mouse and human cultured cells have endogenous LARGE GlcA-T, and that this activity is absent in cells from the Large^{myd} (Large-deficient; [14]) mouse model of muscular dystrophy, as well as in cells (lymphoblasts, myoblasts) from CMD patients with mutations in the *LARGE* gene [15]. This assay, which could be used as a tool in the diagnosis of patients with mutations in the *LARGE* gene, also provided a good correlation between enzymatic activity and the tissue expression patterns of the 2 Large paralogs in mice. Interestingly, Large2-deficient animals do not display any kidney disease due to the functional compensation by Large. Overall, this body of work highlights the importance of both LARGE and LARGE2 for effective functional modification of α-DG *in vivo*, and raises the question of potential novel substrate(s) for laminin-binding glycan in kidney. Additionally, **Tatsushi Toda** and co-workers have demonstrated that myofiber-selective expression of LARGE *via* systemic AAV gene transfer ameliorated dystrophic pathology of Large-deficient mice even when the intervention occurred after disease manifestation. However, the same strategy failed to ameliorate the dystrophic phenotype of fukutin-conditional knockout mice. Furthermore, forced expression of Large in fukutin-

deficient embryonic stem cells also failed to recover α-DG glycosylation, however coexpression with fukutin strongly enhanced α-DG glycosylation. Together, these data demonstrated that fukutin is required for LARGE-dependent rescue of α-DG glycosylation, with important implications for LARGE-utilizing therapy targeted to myofibers [16,17].

Another promising therapeutic approach being developed in **Toda**'s laboratory relies on the introduction of 3 cocktailed (*i.e.* mixed) antisense oligonucleotides (AONs) to prevent aberrant pathogenic splicing in FCMD patient cells and model mice, due to the founder SVA retransposal element in the *fukutin* gene [10]. This strategy normalized protein production and function of Fukutin as well as *O*-glycosylation of α-DG. Optimization of the best, single AON for clinical trial, was carried out by re-designing AONs precisely around the splice sites, and their efficacy for exon trap inhibition was assessed in FCMD patient cells and model mice [18]. By testing normal Fukutin production and functional analysis, the most promising candidate AON was finally selected. *In silico* analysis of this AON was performed to identify off-target or on-target effect on other sites in human genome. These studies show the promise of splicing modulation therapy as the first gene mutation based clinical treatment for FCMD in the near future.

2. Animal models of *LAMA2*-related muscular dystrophies

Laminin-211 (Lm-211) is a cell adhesion molecule that is strongly expressed in the basement membrane surrounding skeletal muscle fibres, whose binding to the sarcolemma is important for the structural stability of muscle. The muscle basement membrane consists of Lm-211, collagen type IV and several associated components including nidogen and perlecan. By binding to the cell surface receptors dystroglycan and integrin α7β1, laminin-211 is believed to protect the muscle fibre from damage under the constant stress of contractions and to influence signal transduction events from the matrix to the muscle. Lm-211 is a

heterotrimer formed by the association of the α2, β1 and γ1 chains. Mutations in the gene encoding the α2 chain leads to congenital muscular dystrophy type 1A (LAMA2-CMD, MDC1A, MIM 607855), characterized by severe hypotonia, progressive muscle weakness and wasting, joint contractures, severely impaired motor ability, and respiratory failure, which causes great difficulty in daily life and often leads to premature death [19, 20]. There is also an associated motor neuropathy, which however in human patients may be less relevant compared to the mouse model of the disease.

Several mouse models for laminin α2 chain deficiency exist (*dy/dy, dy^{2J}/dy^{2J}, dy^{W}/dy^{W}, dy^{3K}/dy^{3K} and dy^{nmj417}/dy^{nmj417}*), which overall adequately model human disease and confirm the relationship between laminin α2 chain expression and severity of disease [21]. Surprisingly, although the *dy/dy* model was reported in 1955 [22], the causative mutation remains elusive. The most studied models, discussed during this workshop by **Markus Ruegg**, **Madeleine Durbeej**, **Yoram Nevo** and **Dean Burkin**, are the *dy^{W}/dy^{W}*, *dy^{3K}/dy^{3K}* and *dy^{2J}/dy^{2J}* lines, the former 2 modelling complete protein deficiency and displaying severe phenotypes, while the latter displays partial deficiency and a truncated form of laminin α2 chain that lead to a longer lifespan and milder phenotype. There are also cat, dog and zebrafish models of MDC1A [23, 24]. Analyses of the various laminin α2 chain-deficient animal models have led to a significant improvement in our understanding of MDC1A pathogenesis. Additionally, these animal models have been valuable tools for the preclinical development of novel therapeutic approaches for laminin α2 chain deficiency highlighting a number of important disease driving mechanisms that can be targeted by pharmacological approaches.

Markus Ruegg and **Dean Burkin** presented data on the *dy^{W}/dy^{W}* mouse model, demonstrating functional benefit by expressing proteins that can compensate for the loss of Lm-211. Previous proof-of-principle studies in *dy^{W}/dy^{W}* mice have shown that transgenic

expression of mini-agrin, which binds to Lm-411 and to α-dystroglycan, ameliorates the dystrophic phenotype [25, 26] The data presented by Markus Ruegg showed that introduction of a transgene designed to allow self-polymerization of Lm-411, which is normally increased in Lm-211 deficient muscle but cannot self-assemble and binds only weakly to the sarcolemma, has some, although rather weak, therapeutic activity in dy^W/dy^W mice. However, when combined with mini-agrin, this construct shows a very strong synergistic effect. The improvements were seen on all levels, including histology of skeletal muscles, muscle force, weight gain and survival. Moreover, analysis of the basement membrane composition by differential extraction revealed restoration of the stability of the basement membrane, providing strong evidence that the myopathy in dy^W/dy^W mice is caused by defects in myomatrix assembly and its connection to the sarcolemma. Functional compensation can also be obtained by laminin-111 (Lm-111; α1, α1, α1), the predominant laminin isoform in embryonic skeletal muscle that supports normal skeletal muscle development in Lm-211 deficient muscle but is absent from adult skeletal muscle. Dean Burkin and coworkers have recently shown that Lm-111 protein can be systemically delivered to the muscle of laminin-α2 deficient mice to prevent muscle pathology, maintain muscle strength and dramatically increase life expectancy [27]. More recently they performed an RNA-Seq analysis on the diaphragm muscle of dy^W mice to identify transcriptional changes that occur during disease progression and after Lm-111 treatment. Overall, 828 transcripts changed significantly in the diaphragm muscle of untreated dy^W animals compared to wild-type mice. Of these, 206 transcripts changed between untreated and Lm-111 treated mice and changes of 81 transcripts were specific only to Lm-111 treatment. This study demonstrated that during disease progression at 2- and 5 weeks of age, dy^W mice had increased transcript levels of Tenascin C and Galectin-3 compared to control animals. Analysis of disease-specific and Lm-111-specific transcript changes in dy^W mice and LAMA2-CMD patient muscle biopsies are

currently ongoing. Together these results will provide information about transcript changes during LAMA2-CMD disease progression and the on-target and off-target activity of Lm-111 protein therapy. Overall, these data will have significant impact in future attempts to develop new treatment strategies for MDC1A.

The other Lm-211 deficient model, the *dy^{3K}/dy^{3K}* mouse, was generated by Shin'ichi Takeda and co-workers and displays a very severe muscular dystrophy with a median postnatal survival of three weeks [28]. It is completely deficient in laminin α2 chain and skeletal muscle presents with early massive inflammation, apoptosis and extensive fibrosis [21]. **Madeleine Durbeej** reported on the phenotype of this model and disease driving mechanisms that have been targeted by transgenic and pharmacological approaches using this mouse model. Aberrant expression of muscle miRNAs has been demonstrated in *dy^{3K}/dy^{3K}* muscle and plasma [29]. Additionally, the *dy^{3K}/dy^{3K}* mouse displays non-muscle defects such as peripheral neuropathy, blood-brain-barrier defects and impaired spermatogenesis [21, 30]. Durbeej's team has previously demonstrated that transgenically expressed laminin α1 chain significantly ameliorates muscular dystrophy and reduces peripheral neuropathy in *dy^{3K}/dy^{3K}* mice [21]. Also, gene and protein expression analyses and proteasome activity analysis demonstrated an overactive ubiquitin-proteasome system and dysregulated autophagy in *dy^{3K}/dy^{3K}* muscle as well as in laminin α2 chain-deficient human muscle cells. Separate treatment with proteasome inhibitors and an autophagy inhibitor significantly improved the dystrophic phenotype of *dy^{3K}/dy^{3K}* mice [31-33]. More recently, Durbeej and co-workers have performed comparative proteomic analyses of affected muscles from *dy^{3K}/dy^{3K}* mice in order to obtain new insights into the molecular mechanisms underlying MDC1A. A large number of differentially expressed proteins in diseased compared to normal muscles were identified. A majority of the downregulated proteins were involved in different metabolic processes and mitochondrial metabolism while upregulated proteins were related to inflammation and

fibrosis [34]. These data imply that metabolic alterations could be novel mechanisms that underlie MDC1A and might be targets that should be explored for therapy. Indeed, preliminary data indicate defective metabolism in laminin α2 chain-deficient muscle cells.

Yoram Nevo presented work carried out in his laboratory using the spontaneous dy^{2J}/dy^{2J} model with partial laminin α2 deficiency, initially reported in the early 1970's. It was then backcrossed onto C57BL/6J. The dy^{2J}/dy^{2J} mouse has a G>A splice donor site mutation in exon 2 of chromosome 10 of the *Lama2* gene, resulting in several transcripts including 165 and 171 bp in-frame deletions. The mutation results in a new *Nde*I restriction site, which enables diagnosis of the mice before onset of symptoms. Truncation of the α2 chain in the N-terminal domain VI disrupts the formation of the laminin network and binding to α-dystroglycan. Mutant mice are sterile, however, heterozygous mice are easily bred making maintenance of a colony quiet easy. Nevertheless, due to the inbred nature of the colony, it may need to be renewed every few years. dy^{2J}/dy^{2J} mice share similarity with human MDC1A in clinical and histological features. Clinically, the weakness starts at 3-4 weeks. There is progressive unremitting muscle weakness and reduced life span to about 6 months. As expected there is some variability in the clinical phenotype, but it is milder than other *dy* subtypes. The dy^{2J}/dy^{2J} mice have a clinically significant peripheral neuropathy. Histologically there is continuous muscle fiber necrosis, inflammation, ineffective regeneration and progressive severe fibrosis. Y Nevo presented previously published data regarding losartan (LS) treatment in the dy^{2J}/dy^{2J} [35, 36]. Losartan is an angiotensin II type I receptor antagonist, commercially available and extensively used as a medication for hypertension. LS was approved by the FDA for the treatment of hypertension, cardiac hypertrophy and renal disease in 1995. Losartan can be safely used in children and has a low side effect profile. LS shows anti-fibrotic effects in many tissues including lung, kidney, liver, heart and muscle. Following LS treatment there was no effect on mouse weight. Losartan

improves muscle pathology and significantly reduces fibrosis in dy^{2J}/dy^{2J} mice. Reduced vimentin and fibronectin were noted in treated dy^{2J}/dy^{2J} mice. Losartan treatment inhibited TGF-β and MAPK signaling pathways and increased SMAD7 [35]. Recently significantly increased serum tumor necrosis factor alpha (TNF-α) level, p65 nuclei accumulation, and a decreased muscle IκB-β protein level, indicating NFκB activation, were found in the dy^{2J}/dy^{2J} mouse following LS treatment. NFκB anti-apoptotic target genes TNF receptor-associated factor 1 (TRAF1), TNF receptor-associated factor 2 (TRAF2), cellular inhibitor of apoptosis (cIAP2), and Ferritin heavy chain (FTH1) were increased following treatment. Muscle apoptosis reduction was confirmed using terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay demonstrating that LS treatment promotes survival via NFκB pathway in the dy^{2J}/dy^{2J} mouse model of MDC1A [36].

3. Animal models of *COL6*-related muscular dystrophies

Deficiency in collagen type VI myopathies as the result of mutations in three genes encoding collagen VI (COLVI), *COL6A1*, *COL6A2* and *COL6A3*, leads to a group of clinically heterogeneous neuromuscular disorders: Ullrich congenital muscular dystrophy (UCMD, MIM 254090), Bethlem myopathy (BM, MIM 158810), phenotypes of intermediate severity, myosclerosis and an autosomal dominant form of limb-girdle muscular dystrophy (reviewed in [37-39]).

COLVI plays a number of biological roles, notably in maintaining tissue integrity by providing a structural link between different components of connective tissue, basement membranes and cells. Available mouse models were discussed by **Paolo Bonaldo**, **Valérie Allamand** and **Carsten Bönnemann**.

The first animal model of COLVI deficiency was created in the laboratory of **Paolo Bonaldo** in1996 by targeted inactivation of the *Col6a1* gene, coding for the α1(VI) subunit, which represents a well-characterized model of human CMD linked to COLVI deficiency [40]. *Col6a1*$^{-/-}$ mice, originally generated in the CD1 strain, were subsequently bred into the C57Bl/6 background and during the last two decades this knockout mouse line was made available to several teams worldwide. Since assembly and secretion of COLVI strictly requires the presence of three different alpha subunits, ablation of the α1(VI) chain completely prevents the deposition of COLVI in the ECM. Since then, additional mouse lines have been generated to model mutations identified in human patients, such as the dominant negative exon 16 skipping mutation in *Col6a3* presented by **Carsten Bönnemann** on behalf of Mon-Li Chu and co-workers [41], and *Col6a2* nonsense mutation creating a premature termination codon (PTC) and lack of the α2(VI) chain (**Valérie Allamand**'s group; unpublished data). All these mouse models of collagen VI disease, compared to the *LAMA2* models, display a relatively mild disease, more closely resembling the milder Bethlem variant rather than the severe Ullrich type. Initial histological and functional analyses showed that *Col6a1*$^{-/-}$ mice display an early onset myopathic phenotype affecting diaphragm and other skeletal muscles, with myofiber degeneration, significantly decreased muscle strength, lower locomotor activity and limb contractures [40, 42]. Similarly, heterozygous del16 *Col6a3* mice as well as homozygous KI-*Co6a2* have reduced body weight and reduced weight of selected muscles, with signs of degeneration – regeneration. The deficits present in the heterozygote del16 *Col6a3* mice are consistent with the prediction of a dominant-negative action of the mutation, while homozygous animals had an even more pronounced force deficit. Over the past 20 years, the *Col6a1*$^{-/-}$ mice have been highly valuable for elucidating pathophysiological mechanisms of *COL6*-related disorders, opening new unexpected venues for treatments and therapy. In particular, electron microscopy of *Col6a1*$^{-/-}$ muscles revealed

organelle alterations, with marked dilation of sarcoplasmic reticulum and abnormally swollen mitochondria with tubular cristae. *Ex vivo* studies in isolated myofibers derived from these mice uncovered a latent mitochondrial dysfunction linked to higher open propensity of the permeability transition pore (mPTP) and associated with a markedly increased incidence of apoptosis [43]. Further mechanistic studies demonstrated that the persistence of abnormal organelles and spontaneous apoptosis of *Col6a1*$^{-/-}$ muscles are caused by a failure of the autophagic machinery. Indeed, lack of COLVI has a remarkable impact on molecules involved in the regulation of autophagy, with decreased Beclin 1 and Bnip3 levels and constitutive activation of the Akt/mTOR pathway. Skeletal muscles of *Col6a1*$^{-/-}$ mice display an impaired autophagic flux, which matches the lower induction of Beclin 1 and Bnip3 and the lack of autophagosomes after starvation. Unpublished data generated in collaboration between Mon Li Chu's team and the Bonaldo lab suggested increased opening of the mitochondrial permeability transition pore with oligomycin treatment in the del16 *Col6a3* mice, similar to the original *Col6a1* knock-out model in which these defects were completely recovered by plating *Col6a1*$^{-/-}$ myofibers onto purified native COLVI or by addition of cyclosporin A (CsA), a desensitizer of mPTP opening [43]. Studies from Bonaldo and colleagues on muscle biopsies and cell cultures from patients affected by *COL6*-related myopathies showed remarkably similar defects to those displayed by *Col6a1*$^{-/-}$ mice. Interestingly, treatment of *Col6a1*$^{-/-}$ mice for 5 days with CsA was capable to recover the structural and functional muscle defects, leading to a pilot clinical study with CsA in Bethlem and Ullrich patients [44, 45]. Consistent with the data obtained in *Col6a1*$^{-/-}$ mice, analysis of muscle biopsies from Ullrich and Bethlem patients showed reduced amounts of Beclin 1 and Bnip3. Notably, reactivation of the autophagic flux by either dietary approaches (i.e., prolonged starvation, low protein diet, nutraceutical supplementation) or pharmacological means (i.e., rapamycin, CsA) was able to clear the abnormal organelles and ameliorate the

myofiber defects of *Col6a1*$^{-/-}$ mice, with also a significant recovery of muscle strength [46]. Based on the studies performed in *Col6a1*$^{-/-}$ mice, a phase II pilot study was recently carried out in 8 Bethlem and Ullrich patients to assess the efficacy, safety and tolerability of a 12-month normocaloric low protein diet aimed at stimulating autophagy (http://clinicaltrials.gov/ct2/show/NCT01438788). While these strategies have not been tested in the KI-*Col6a2* or del16 *Col6a3* mice, these models add useful genotypes to model the common dominant mechanism in the human disease, on the one hand, and recessive PTC mutations that lead to the severe Ullrich phenotype in patients. Interestingly, the human del16 *COL6A3* mutation is also the target of a recently published siRNA-mediated allele-specific mutation knock down approach [47]. Additional phenotypes displayed by both the *Col6a1*$^{-/-}$ and del16 *Col6a3* mice include abnormal tendon formation, evidenced by ultrastructure [48, 49]. Moreover, given the distinct microfilamentous network formed by COLVI in close contact with myofiber sarcolemma and its abundant distribution in muscle endomysium, further studies were carried out in *Col6a1*$^{-/-}$ mice to assess whether this ECM molecule plays any role for satellite cells, the major stem cell population of adult skeletal muscles. These experiments revealed that COLVI is a component of the satellite cell niche and plays a pivotal role in the proper self-renewal of satellite cells both in physiological conditions and during muscle regeneration [50]. Moreover, they also established that COLVI is an important determinant of the biomechanical properties of skeletal muscle, which in turn are critical for the self-renewal of satellite cells. Indeed, *in vivo* and *in vitro* studies in *Col6a1*$^{-/-}$ mice demonstrated that the fine regulation of muscle stiffness is the main mechanism through which COLVI influences the maintenance of satellite cell stemness. Notably, *in vivo* restoration of COLVI deposition in *Col6a1*$^{-/-}$ muscles after grafting with wild-type fibroblasts led to recovery of muscle stiffness and rescue of satellite cells self-renewal [50]. These findings strengthen the concept that COLVI is a fundamental protein for skeletal muscles, and

besides influencing myofiber homeostasis it also plays a critical and unanticipated role in the regulation of satellite cell activities.

In addition to mouse models of COLVI deficiency, zebrafish models have been generated by transient morpholino-mediated exon skipping in all *col6a* genes. Carsten Bönnemann briefly presented the published zebrafish model generated by Jim Dowling, recapitulating both dominant (targeting exon 9) and recessively (targeting exon 13) acting mutations in the *col6a1* gene [51]. Larva presented with dose-dependent defective escape reflex and altered myoseptal and mitochondrial structure, which were more severe in the exon 9 target. There also was evidence for increased apoptosis that - similar to the work shown in the Bonaldo mouse model - diminished by the addition of cyclosporine A. However, while this treatment increased motor performance it did not rescue the loss of birefringence of muscle, indicating that eliminating apoptosis may not address the full muscle phenotype in this model. On the contrary, *col6a2* morphants created in Valérie Allamand's group did not display increased apoptosis, although they presented altered muscle structure and impaired motility, as well as vertical myosepta (equivalent to myotendinous junctions) that are misshapen and abnormally ramified. Her group also recently identified 2 novel COLVI long chains in zebrafish that are most homologous to the mammalian α4 chain, encoded by the *col6a4a* and *col6a4b* genes. These orthologs represent ancestors of the mammalian *Col6a4-6* genes, which were recently reported [52-54] and the functions of which remain poorly understood. The corresponding morphants clearly displayed muscle phenotypes reminiscent of those reported in the *col6a1* and *col6a2* models, but they also presented some specific phenotypes, suggesting only partial functional redundancy, which may provide important clues for potential human phenotypes associated with their deficiency [55].

Very recently, COLVI-deficiency was also reported in a Labrador retriever dog [56], which will constitute a very valuable large animal model for this group of diseases.

4. Standard operating procedures

The various talks presented during the workshop clearly illustrate the need for common operating procedures and comparable outcome measures so that the data emanating from the various models can be compared and preclinical work using different interventions can be more directly compared. To accelerate such preclinical development, the patient advocacy CureCMD and the European network of excellence TREAT-NMD posted a listing of animal models and SOPs for selected models as a resource on their websites (http://curecmd.org/scientists/sop; www.treat-nmd.eu/research/preclinical). **Markus Ruegg** has been responsible for establishing several SOPs for histological analyses of dy^W muscle within the TREAT-NMD network (http://www.treat-nmd.eu/research/preclinical/cmd-sops/).

Raffaella Willmann, who worked on the development of several standard procedures in the context of TREAT-NMD, presented one of her studies illustrating perfectly the difficulty of working on mouse models seeing the disparity of the results observed in the literature. Indeed, focusing only on the currently used mammalian animal models for Duchenne muscular dystrophy, she stressed the importance/need of SOPs to reduce the inter-lab variability [57]. Indeed, it is very important for researchers to think forward during study design about genetic background, age, gender, and sample size, food regimen, as well as experimental setup, experimental protocol and to strictly report each of those information in publications.

The discussion that ensued led to the conclusion that the 49 SOPs already in place regarding biochemical, physiological, functional, histological readouts on the CureCMD and

TREAT-NMD websites should be used, and completed, in order to minimize variability thereby increasing comparability, reproducibility and/or robustness of the studies, to further ameliorate the predictability for translation to human.

An example of a noninvasive biomarker was given by **Mahasweta Girgenrath** whose team uses the PLY 421 (Buxco Research), an unrestrained plethysmograph that uses pressure differentials to calculate various respiratory parameters to investigate respiratory function in dy^W and dy^W-IGF-1 overexpressing mice. Indeed, acute respiratory failure resulting from weak respiratory muscles and progressive scoliosis constitutes a very significant contribution to patients with LAMA2-CMD and other CMD patients' survival. While respiratory insufficiency is an important component of this pathology, it has been largely unexplored. The key parameter that Girgenrath and colleagues focus on in this study is enhanced pause, or PenH. This is an indirect measure of airway resistance, calculated by the formula: PenH = (Te/RT-1) * PEF/PIF, where Te is total expiratory time, RT is relaxation time, PEF is peak expiratory flow, and PIF is peak inspiratory flow. Longitudinal PenH measurements for untreated dy^W and dy^W-IGF-1 overexpressing groups showed a two-fold increase in PenH values immediately prior to death. While this trend holds true for both untreated and the IGF-1 overexpressing group, respiratory decline seems to be delayed significantly in dy^W-IGF-1 group, which reflects the increased lifespan of these transgenic animals. Interestingly, broncheoalveolar lavage (BAL) fluid cytospins and lung H&E staining of dy^W and dy^W-IGF samples that exhibited high PenH values also have high amounts of infiltrating neutrophils and other immune cells. Cryosections of paraformaldehyde fixed lungs reveal that elevated PenH correlates with hypertrophied goblet cells and a higher number of infiltrating cells in the interstitium of lungs. Finally, expression analyses show that TNF-α and osteopontin (OPN) expression drastically increases in lungs of mice with high PenH. M Girgenrath's team results suggest that increased PenH is accompanied with increased lung inflammation and also show

a strong correlation with morbidity in these dystrophic mice. Based on these findings they propose that plethysmography likely can be used as a predictive non-invasive biomarker for laminin-211 deficiency.

5. Translational link to the clinic

Carsten Bönnemann raised several points for each disease group models, some of which were common across genotypes studied, in particular regarding the window of intervention. Numerous emerging therapeutic questions in the CMD to be addressed in the shorter term were discussed and led to the following conclusions:

- In the dystroglycanopathies prednisone seems to be ready for a clinical trial, and should be tested in fukutin and FKRP mouse models. Further questions to address are the applicability of LARGE upregulation, as well as its limitations since its transgenic upregulation on disease backgrounds has been shown to be deleterious [58, 59], modelling cardiac disease in the mouse model, and the question whether the CNS diseases can be influenced by therapeutic interventions. The applicability of AAV-mediated gene transfer therapy could be assessed in various genetic models.
- For LAMA2-CMD, the critical question remaining unanswered is to identify the different driving mechanisms at different stages of the disease. According to M. Girgenrath's work, apoptosis is involved early on. TGF beta over-activity could be a suitable therapeutic target based on several completed preclinical studies, so that understanding its importance at different disease states becomes particularly relevant.
- One of the main challenges with regards to COL6-RD lies in the difference in phenotype severity between the mouse models available and the patients, as well as in identifying the relative importance of the various disease drivers for therapeutic intervention. Allele-

specific knock down for the dominant mutations needs to be optimized for oligo chemistries that can be used in clinical applications.

Of note, a phase 1 pharmacokinetic study of omigapil, an anti-apoptotic molecule, is currently being assessed in paediatric and adolescent patients with *LAMA2*- and *COL6*-related CMD, along with its safety and tolerability (CALLISTO study; NCT01805024) at the NIH. Omigapil has been found to ameliorate apotosis and disease burden in the dy^w mouse model by Markus Ruegg's group – hence it is a good example of using mouse model to select therapeutic approaches and molecules for translation into clinical trials.

6. Future perspectives

The last morning of the workshop was dedicated to open discussions in order to identify unanswered questions that could be resolved in a collaborative manner. For example, it was felt that it would be very useful to have the most used model for dystroglycanopathies, the *myd* mice, readily available live to the community through the Jackson Laboratory (www.jax.org), and also to be able to breed as many models as possible on different genetic backgrounds, a task generally too expensive for academic laboratories that could possibly be achievable at the Jackson Laboratory. There was also a consensus on the need to compare models within disease groups (natural history, lethality, phenotype variability, secondary deficiencies). To this end, the development of a registry cataloguing the different CMD animal models available and all known/relevant information was encouraged.

Finally, some aspects that need further modelling or characterizing were identified, such as brain abnormalities in *Lama2*- and COLVI-deficient animals. With regards to pre-clinical

studies, a crucial point is the importance of knowing which outcome measures are the most appropriate for each model.

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PARTICIPANTS

Valérie Allamand (Paris, France);

Paolo Bonaldo (Padova, Italy);

Carsten Bönnemann (Bethesda, USA);

Susan Brown (London, UK);

Dean Burkin (Reno, USA);

Kevin P Campbell (Iowa City, USA);

Madeleine Durbeej-Hjalt (Lund, Sweden);

Mahasweta Girgenrath (Boston, USA);

Yoram Nevo (Petach Tikva, Israël);

Markus Ruegg (Basel, Switzerland);

Margot Saunier (Paris, France);

Tatsushi Toda (Kobe, Japan);

Raffaella Willmann (Zürich, Switzerland).

ENMC representatives

Alexandra Breukel (The Netherland);

Annelies Zittersteyn (The Netherland);

Denise Dute (The Nertherland).

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