248th ENMC International Workshop: Myotonic dystrophies: Molecular approaches for clinical purposes, framing a European molecular research network, Hoofddorp, the Netherlands, 11–13 October 2019

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Workshop report

248th ENMC International Workshop: Myotonic dystrophies: Molecular approaches for clinical purposes, framing a European molecular research network, Hoofddorp, the Netherlands, 11–13 October 2019

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1. Introduction and overview

A total of 27 participants including molecular biologists, clinicians, geneticists, and patient advocates from 13 countries convened from the October 11–13, 2019 in Hoofdorp, The Netherlands, for the 248th ENMC International Workshop, on the topic Myotonic dystrophies: molecular approaches for clinical purposes, framing a European molecular research network.

Following a welcome from Alexandra Breukel, and Ana Ferreiro, ENMC representative and director, and the chairpersons of the workshop, Geneviève Gourdon, Derick Wansink, Baziel van Engelen, and Benedikt Schoser, Benedikt Schoser gave an overview of the topic.

Myotonic dystrophy (DM) is a rare disorder, characterized by huge genetic and clinical variability. This makes fundamental research and translational therapeutic approaches more challenging and warrants close international collaboration between experts in Europe. To ensure optimal and efficient collaboration, it is required to share existing materials, models, protocols and partly unpublished data, and refinement of suitable and harmonized outcomes. Moreover, networking of the existing knowledge, infrastructure and personnel will facilitate appropriate progress and communication for basic scientists to patients and patient organizations.

The field of DM has continued to progress and expand in the past decade. Additional clinical cohort descriptions have been uncovered and subtypes that are more precise were associated with the DM1 and DM2 mutations. Given that the myotonic dystrophies are at a critical juncture, with significant changes and advances in the field as well as important unresolved issues and questions, our aims were:

Aim 1: The basic science of DM: what do we already know and what do we urgently need to understand further?
Aim 2: Therapy development for DM: where do we stand?

Session 1: summary and overview of former clinical DM workshops

Benedikt Schoser summarized the former 18 workshops on DM. Major topics since 1986 were the clinical characterization of DM1 and DM2, coordinating genetic testing, the clinical classification, standards of care and symptomatic therapy, harmonizing patient cohorts based on the genetic background, developing clinical registries and promoting clinical trial readiness across Europe. So far, none of the 18 workshops was solely dedicated to the molecular pathogenesis and molecular therapy of both disorders.

Despite his absence, for completeness, we included a brief summary of Giovanni Meola contribution. He summarized the former Marigold DM brain workshops. The Marigold Foundation (Calgary, Canada) funded three DM workshops with the purpose to create an international task force, including clinicians, scientists and patient representatives.

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The 1st DM CNS workshop with 24 participants (clinicians, scientists and patient representatives) was held in May 2012. Based on the meeting discussion, a short list of critical action items was suggested: 1) an urgent need for a centralized repository, or at the very least an updated database of available human tissues; 2) more longitudinal natural history studies with CNS measures to understand the pattern of progression and decline in DM; 3) the need to design better patient questionnaires and clinical neuropsychological tests to understand what cognitive domains of CNS are affected in DM. The 2nd workshop with 34 participants (clinicians, scientists and delegates of pharmaceutical industries) was held in Ferrere (Asti), Italy, May 2013. The workshop purpose was to stimulate research on CNS dysfunction in DM. Based on the discussion developed in 11 sessions, it was clear that a lot of information was lacking regarding CNS involvement. It was suggested to develop the following areas: a) the generation of subgroups to establish consensus protocols for neuroimaging, neuropathological studies and neuropsychological testing; b) the need of longitudinal studies; c) the generation of DM registries including paediatric patients. It was emphasized that DM should be considered also a brain disorder. The last workshop supported by the Marigold Foundation and the Fondazione Malattie Miotoniche (Milan, Italy) was held in Milan, December 2014. Here, 24 clinicians, scientists and delegates of pharmaceutical industries convened. During 13 sessions, the most recent clinical, molecular, neuroradiological and neuropsychological findings were presented and discussed. A consensus was reached on several biomarkers for cerebral involvement in DM [1–3].

Anne-Berit Ekström reported on congenital myotonic dystrophy (CDM). CDM is a challenging condition with variable clinical picture between individuals. Since 1999, multidisciplinary studies have been conducted on CDM in the western and southern parts of Sweden. These studies represent epidemiological data as they are population based. The studies have been both cross-sectional and prospective longitudinal. Individuals were divided into the severe and the mild congenital form and the same classification has been applied in all studies. Both forms present in the pre- or perinatal period. The difference between severe and mild CDM is that all children with severe CDM have a life-threatening disease at birth, with the need for resuscitation and/or respiratory assistance. In the Swedish cohort, there is a male preponderance in the severe group with approximate four times more males than females and with maternal inheritance in all individuals. Few patients with the most severe cognitive and behavioral impairment were excluded from some of the assessments, due to problems with cooperation. The longitudinal assessment of motor function demonstrated that motor function improves during the first decade, is most pronounced during the first six years, reaches a plateau during adolescence and starts to deteriorate in the beginning of the second decade. Further, all individuals with CDM had affected and reduced postural control. Also, all patients with CDM had an impact on the central nervous system. The longitudinal study on cognition and adaptation showed that there was no statistically significant decline in cognitive abilities and adaptive behavior, but the gap in cognitive and adaptive functioning in comparison to typically developing peers increased. There is also a high frequency of autism spectrum disorders in individuals with CDM as 67% of the individuals fulfilled the diagnostic criteria. At follow-up, the diagnosis of ASD was stable over time and even showed a tendency to increase, as the individuals grew older. Data from the remaining multidisciplinary studies regarding visual and ocular motor function, evaluation of daily-activity performance, orofacial dysfunction and speech characteristics, and oral hygiene aspects were also assessed by the research group and were presented as well. The experience gained from the Swedish studies has contributed to the preparation of the consensus-based care recommendations for congenital and childhood-onset DM1 [4–9].

Session 2: First European clinical trial in DM

On behalf of the OPTIMISTIC consortium, *Baziel van Engelen* and *Hans Knoop* presented the published trial data and elaborated on implementation issues [10]. DM1 leads to severe fatigue, significant physical functional impairment and restricted social participation. Treatment approaches should therefore preferably address multiple aspects. Cognitive behavioral therapy (CBT) is able to do so and has been shown to decrease fatigue and improve health status in other chronic diseases. In the four-center, prospective 16-month OPTIMISTIC trial, funded by the European Union Seventh Framework Program, 255 severely fatigued adult DM1 patients were randomly assigned to CBT or to standard care in a 1:1 ratio. The primary endpoint was the 10-month change on the DM1-Activ-C scale, a measure of activity and social participation. Secondary endpoints included objective physical activity, exercise capacity (as measured by the six-minute walk test), fatigue and daytime sleepiness, quality of life, depressive symptoms, and cognitive function. At 10 months, the DM1-Activ-C score in the intervention group had improved 1.53 points, whereas it had worsened 2.02 points in the standard care group, with a mean difference adjusted for baseline, MIRS, clinical site and involvement of the caregiver of 3.27 points. Secondary outcomes relating to objective physical activity, exercise capacity and experienced fatigue differed significantly between groups. Beneficial effects on primary and secondary outcomes were sustained at 16 months. Adverse and serious adverse events were distributed equally across groups, with the exception of falls occurring more frequently in the intervention group compared to the standard care group (160 *versus* 72 falls). CBT increased activity and social participation in DM1 patients at 10 months, with concurrent improvements in experienced fatigue, exercise capacity and objective physical activity. The main issue now is implementation of this treatment in the various healthcare systems. Based on OPTIMISTIC data, follow-up study ReCognitiON, investigates drug repurposing of the beneficial trial effect using a hypothesis-free approach.

Session 3: Recent advances in genetics in DM

Darren Monckton reported on new aspects of the DM1 genotype. As mentioned above, DM1 is an incredibly variable disorder that shows striking anticipation with age at onset typically decreasing by 20 to 30 years per generation and frequently progressing from the mild late onset form to congenital cases in only three generations. DM1 is an autosomal dominant disorder and is caused by the expansion of a polymorphic CTG repeat in the 3′-untranslated region of *DMPK*. Inherited disease-associated alleles range from 50–80 triplets in mild cases and to high hundreds or even thousands of repeat units in congenital cases. Once into the expanded disease-associated range, the repeat becomes highly unstable in the germline, with mutation rates approaching 100% and a major bias toward further increases in repeat length, concomitant with the pronounced anticipation. Smaller expanded alleles (<80 CTG triplets) are particularly unstable in the male germline, explaining the observed excess of transmitting grandfathers. Conversely, expanded alleles >80 CTG repeats are more prone to very large additional expansions when transmitted by females. The sex-dependent germline dynamics of the repeat, coupled with male-specific effects on fertility and fecundity, results in most congenital cases being born to affected mothers. The CTG repeat is also somatically unstable and expands throughout the lifetime of the individual, with the largest expansions observed in the primary affected tissues including skeletal muscle and brain. Somatic expansions contribute toward both the tissue-specificity and progressive nature of the symptoms. In addition, age-dependent somatic expansion of the repeat, in combination with the profound age at sampling bias mediated by anticipation, has grossly compromised genotype-phenotype correlations. Indeed, age at onset is most accurately predicted by estimating the inherited allele length and not the modal allele length at the time of sampling. Nonetheless, individual-specific differences in the rate of expansion have also been shown to drive variation in both age at onset and progressive phenotypes, with higher expansion rates associated with an earlier age at onset than expected, and more rapid disease progression. Importantly, although in the majority of DM1 patients the expansion appears to be comprised of pure CTG repeats, a subset of individuals, most frequently with adult onset disease, inherit alleles containing CCG, CGG, CAG or CTC variants interrupting the CTG array. Such alleles are more stable in both the germline and soma, and are associated with unusual inheritance patterns and reduced disease severity, further implicating somatic expansion as a therapeutic target. To this end, recent data have highlighted common polymorphisms in the DNA mismatch repair gene *MSH3* as mediating variation in the rate of somatic expansion and age at onset in both DM1 and the CAG repeat expansion disorder Huntington disease (HD). These data, and other data from recent HD genome-wide association studies, highlight *MSH3* and related DNA repair genes as therapeutic targets in DM, HD and likely other simple sequence repeat expansion disorders [11–19].

Annalisa Botta reported the Italian experience on DM2 genetic testing in 515 samples using a combination of SR-PCR, QP-PCR and LR-PCR methods. DM2 molecular diagnosis was confirmed in 175/515 samples analyzed (34%) and was mainly associated with the presence of myotonia, either clinical or instrumental. Sequencing of 100 unexpanded *CNBP* normal alleles revealed a unimodal distribution of 26 different alleles, with (CCTG)5–7(NCTG)3(CCTG)6–8 being the most common interruption motifs found. She also described five *CNBP* alleles with uninterrupted (CCTG)36–55 tracts in patients referred for DM2 genetic testing. However, the clinical significance of these *CNBP* premutated alleles is still unclear. Overall, these data are in accordance with previous reports on *CNBP* molecular analyses in Slovak and German population. A higher incidence of mutations in the *CLCN1* and *SCN4A* genes has also been reported in DM2 patients, leading to a more severe phenotype and earlier age of disease onset. Thus, either *CLCN1* or *SCN4A* can be considered as disease modifier genes in DM2 patients, since mutations in these genes may exaggerate the DM2 phenotype. Genetic screening of both genes is therefore recommended in DM2 patients with an unusual phenotype, even for a personalized pharmacological therapy. She then showed the results of a methylation study of DNA regions flanking the (CCTG)n expansion carried out in blood and muscle samples from a large cohort of DM2 patients. Pyrosequencing analysis of two CpG islands, upstream and downstream of the DM2 mutation, did not demonstrate any significant difference in the methylation profile between DM2 patients and controls in both tissues analyzed [20–23].

Rick Wansink presented an overview of previous and current work on RNA products generated from the DM1 locus. The unstable CTG•CAG repeat is located in a gene-dense region and is bidirectionally transcribed as part of the *DMPK* as well as the *DM1-AS* (DM1 antisense) gene. Moreover, the expansion is located in the promoter region of *SIX5*. *DMPK* RNA contains a CUG repeat and its expression shows a 30-fold variation between different cell and tissue types: highest in skeletal, cardiac and smooth muscle, significantly lower in brain. The *DM1-AS* gene encompassing a CAG repeat encodes a low-abundant, long noncoding RNA. *DMPK* expression peaks when myoblasts commit to fusion and during early myogenic differentiation. RNA-seq analysis of skeletal muscle biopsies of unaffected individuals and DM1 patients demonstrated that *DMPK* and *DM1-AS* expression increases with disease severity, up to 2–3 fold in severely affected muscle. In spite of this increase, *DM1-AS* expression remains very low, on average 50-fold lower than that of *DMPK* in muscle. In this respect, when compared to human skeletal muscle, it is important to realize that CUGexp RNA expression is relatively low in muscle in DMSXL mice, but very high in that of HSA^{LR} mice. *DMPK* pre-mRNA is subject to alternative splicing, particularly involving the 3′ end of the transcript, where the repeat is located. Recent work was dedicated to an underexamined splice mode that removes the CUG repeat as part of a cryptic intron located within final exon 15.

Splicing of this cryptic intron occurs in 1–10% of the cases, depending on cell type, differentiation and disease state. Also *DM1-AS* transcripts are alternatively spliced and the CAG repeat is frequently removed as part of an intron. A hallmark of DM1, the majority of expanded *DMPK* transcripts are retained in the cell nucleus and are detected as foci by RNA FISH. These foci disappear upon treatment with blocking-type and gapmer-type antisense oligonucleotides directed against *DMPK* transcripts. Careful quantification of *DMPK* expression and foci number resulted in the hypothesis that most foci represent one or only a few expanded transcripts, rather than an aggregate of many RNA molecules. Recent super-resolution microscopy experiments have shown that the size of CUGexp foci is easily overestimated. It was found that the focus diameter (max. width) depends on the repeat length and ranges from an average 0.26 μm (750 CTGs) to 0.39 μm (2600 CTGs). Finally, results were presented showing that the CTG•CAG repeat can be excised by CRISPR/Cas9-mediated gene editing. After excision, corrected cDM myoblasts do show recovery in their myogenic program, but CpG hypermethylation is not reversed [24–29].

Session 4: Basic science of the DM repeat mutations

Krzysztof Sobczak summarized data on the activity of muscleblind-like (MBNL) splicing factors and the mechanism of their sequestration by toxic RNA in DM. RNA-binding proteins play critical roles in RNA processing during tissue development and maintenance, but also in the pathogenesis of some genetic disorders. In DM1 and DM2, dominant-negative effect of toxic repeat RNA is driven by sequestration of MBNL proteins, which normally function as alternative splicing factors at multiple developmental stages [30]. Under physiological conditions, MBNL1, MBNL2 and MBNL3 bind equally and effectively to the same consensus sequences located within similar RNA structures *in vitro* and *in vivo*, through which they modulate alternative splicing of the same exons, but with different strength [31]. The recognized consensus sequence consists of three closely located YGCY motifs as a minimal target for efficient binding and functionality. The affinity and activity of all MBNL paralogs to RNA targets strongly depend on their structures. Similar structure determinants that modulate the effectiveness of protein binding and function may also partially explain sensitivity of specific transcripts to different MBNL concentrations in cells. The disruption of critical developmental transitions in alternative splicing occurs in DM since the RNA processing functions of MBNL proteins are compromised by genetic instability and the expression of CUG repeat expanded (CUGexp) RNAs. In DM, interactions between MBNL proteins and CUGexp RNA lead to the formation of nuclear complexes, detectable as microscopic foci using RNA FISH. It was shown that MBNL-CUGexp complexes are highly dynamic structures composed of tightly packed, although mobile, MBNL proteins that modulate RNA focus morphology and dynamics [32]. The nature of foci can be modulated by small compounds interacting with CUGexp, *e.g.* erythromycin [32]. Sequestration of MBNL proteins in DM results in aberrant processing of hundreds of pre-mRNAs not only at the level of alternative splicing [35] but also alternative polyadenylation [34]. These molecular abnormalities in RNA processing may serve as sensitive biomarkers in DM [33,35,36].

Geneviève Gourdon summarized her mouse models for DM1 and CDM. Mouse models are essential not only to study the mechanisms of diseases, but also to evaluate new therapeutic approaches in preclinical tests. The Gourdon Laboratory has developed from the 1990s onwards mouse models carrying the human DM1 locus including various CTG repeat lengths. Mice with 300 CTG triplets showed high levels or intergenerational and somatic instability and after breeding they eventually gave rise to DMSXL mice with >1000 CTGs. The *DMPK* transgene carried by DMSXL mice is expressed in various tissues with an expression pattern similar to the pattern observed in human tissues, *e.g.* higher expression in the heart and in muscles. However, the expression levels of the *DMPK* transgene are lower than for the murine *Dmpk* gene, except in the CNS where the transgene is expressed three times higher than the murine gene. In addition, it has been observed that the level of *DMPK* in DMSXL mice varies with age, with the highest expression around the neonatal period. The high levels of expression around the neonatal period in tissues such as the heart, muscles and the CNS could explain why homozygous DMSXL mice have a severe phenotype at young age. DMSXL homozygous mice have a multisystemic phenotype and can therefore be used in preclinical trials, especially in young mice. However, like all models, DMSXL mice have advantages (high level of instability, sense and antisense expression of human *DMPK*, multisystemic phenotype, newborn phenotype) and disadvantages (mild splicing defects in adult mice, high mortality during the first weeks of life, difficult reproduction, integration site in the *Fbxl7* gene, variable phenotype) which was all covered in the presentation [37–43].

Session 5: Disease models in DM

Denis Furling reported on immortalized muscle cells to assess therapeutic approaches for DM1. In muscle diseases including DM1, human primary myoblasts or fibroblasts are the most commonly used models. Due to limited access to DM1 patient biopsies and reduced proliferative capacity of myoblasts [44,45], the Furling laboratory has generated immortalized muscle cell lines displaying molecular hallmarks of the disease [46]. Thus, transdifferentiated DM1 immortalized fibroblasts and differentiated DM1 immortalized myoblasts carrying CTG expansions of >1000 triplets showed nuclear CUGexp RNA foci and splicing misregulation, two features commonly used as molecular readouts for DM1. These resources are shared with the DM community and many laboratories utilize the DM1 muscle cell lines to either screen compound libraries or test therapeutic strategies. In the attempt to increase the penetration of antisense

oligonucleotides in muscle, the Furling group tested cell-penetrating peptide Pip6a. Pip6a was conjugated to a (CAG)7 PMO sequence aimed to target the CUG expansion in mutant *DMPK* transcripts. *In vitro*, this peptide-conjugate was able to carry the oligonucleotide into DM1 myotubes without transfection agent. Pip6a-CAG7 displaced MBNL1 from nuclear CUGexp RNA foci, which resulted in the correction of splicing defects. In addition, degradation of mutant *DMPK* transcripts was observed, whereas normal *DMPK* transcripts were not affected. *In vivo*, systemic administration of low dose of Pip6a-CAG7 corrected myotonia and normalized splicing defects in skeletal muscle of HSA^{LR} mice supporting the development of the peptide-conjugate to increase oligonucleotide delivery in DM1 [47].

Peter Meinke reported on the characterization of DM myoblasts and myotubes. One cellular model system to investigate the molecular pathology of DM is the usage of primary human myoblasts. As any model, this one is having its strength and weaknesses. Limiting factors are the relatively limited passage number that can be achieved in tissue culture as well as the need to characterize properties like myogenic content and actual repeat length of the cultures used. The strength of the system is the possibility to compare cultures from different patients, thus giving the possibility to look at common effects in DM and excluding individual effects. Furthermore, there is no need to introduce DM repeats risking off-target effects. An advantage over immortalized cell lines is the option to investigate cell cycle effects. The comparison of proliferating myoblasts and differentiated myotubes of DM1, DM2, and unaffected controls revealed few differences between DM2 and controls, but did show clear defects in DM1, including reduced differentiation efficiency, increased senescence, and increased nuclear envelope (NE) morphological abnormalities. These abnormalities were an enrichment of NE invaginations correlating with DM1 repeat length: the longer the CTG repeat, the more nuclei carried invaginations [48]. These invaginations were present in DM1 myoblasts as well as in myotubes and coincided in myoblasts with cell cycle withdrawal. Quantification of NE proteins revealed down-regulation of nuclear lamins and LINC-complex proteins [49], thus providing a likely explanation for the clinical as well as molecular overlap to nuclear envelope-linked disorders. Analysis of mis-splicing events via RNA-seq did confirm the milder effects in DM2 compared to DM1: more mis-splicing was found in DM1 myoblasts and myotubes.

Ruben Artero reported on miRNA-based therapeutics for DM. Several lines of evidence support that DM1 can be regarded as an *MBNL1* and *MBNL2* loss-of-function disease. From the therapeutic perspective, this is a particularly favourable situation because depletion of MBNL proteins through sequestration by CUGexp RNAs can be compensated by activating the endogenous *MBNL* genes, which are normal in DM1 patients. Activation of gene expression can be achieved by blocking repressive miRNAs, which typically bind to partially complementary sequences in mRNAs. The therapeutic potential of enhancing Muscleblind expression by blocking miRNAs was first put to the test in a Drosophila model of DM1 that reproduces molecular, cellular and functional DM1 phenotypes. Drosophila miR-277 and miR-304 were found to repress Muscleblind directly. Making use of sponge constructs that act as decoys for these miRNAs, it was shown that upregulation of Muscleblind was concomitant to significant rescue of muscle atrophy and fly survival. In human cells, it was discovered that miR-23b can bind to MBNL1 and MBNL2 while miR-218 recognizes three specific sequences in MBNL2. Antisense oligonucleotides (so-called antagomiRs) proved efficient in blocking miR-23b and miR-218 in DM1 myoblasts and the HSA^{LR} mouse model, giving rise to enhanced levels of MBNL proteins and rescue of missplicing, myotonia, grip strength, and histopathological signs. More recently, they compared the activity of antagomiRs by subcutaneous and intravenous delivery and have addressed the biological response to a range of concentrations and the time course of antagomiR activity. Currently, they are addressing the improvement of these molecules and the use of antisense oligonucleotides specific for miRNA binding sites in *MBNL1* and *MBNL2* [50,51].

Mario Gomes-Pereira reported on mouse models to study the brain phenotype in DM1. The proper function of the central nervous system (CNS) relies on the complex crosstalk between different cell types, each with differing contributions in neurological disease. In spite of the increasing knowledge on the core molecular features of RNA toxicity in DM1, a major gap still exists in our understanding of the disease mechanisms behind the debilitating neuropsychological impairments. In particular, the molecular intermediates and pathways deregulated in individual cell types, and how they contribute to CNS dysfunction are unknown. To address this question, the DMSXL transgenic mouse model of DM1 is used as a source of homogeneous populations of brain cells. RNA sequencing and live cell imaging have been combined to investigate disease pathogenesis with cell-type resolution. The findings point to a deleterious effect of the DM1 repeat expansion on multiple cell types of the brain, indicating that brain dysfunction is not explained by neuronal pathology alone. In particular, the repeats appear to affect key molecular pathways and functions of glial cells, interfering with the neuroglia crosstalk that is essential for complex brain functions.

Session 6: Therapeutic approaches in cell and animal models for DM

Karen Sermon summarized the use of human pluripotent stem cells in DM1. The Vrije Universiteit and the Universitair Ziekenhuis Brussel in Belgium were pioneers in the development of preimplantation genetic diagnosis, in particular for DM1, allowing the study of the behavior of the trinucleotide repeat in gametes and embryos [51,52]. Human embryonic stem cells (hESC) were derived from the embryos carrying DM1 and were subsequently used for disease modelling [53–57]. In 2017, the Sermon lab published

in collaboration with the Pearson lab (Toronto, Canada), a paper studying DNA methylation around the DM1 triplet repeat in blood, chorionic villus samples, foetal samples and four hESC lines. It was shown that there is a strong correlation between the presence of the methylation upstream of the CTG repeat and congenital DM [57]. Currently, the hESC lines are used to further investigate the relationship between repeat instability and DNA methylation. Knock-out of DNA repair enzyme *MSH2* in two DM1 hESC lines showed a marked stabilisation of the repeat concurrently with disappearance of the upstream methylation. *MSH2* will be reintroduced in the KO lines and in parallel, a dead CAS9 fused to TET1CD together with a gRNA will be used to demethylate the site. These experiments will help to unravel the relation between repeat instability and DNA methylation. hESCs are also used as a model for muscle differentiation on normal and on DM1 lines. DM1 lines may possibly have a lower differentiation potential, but this needs confirmation. Samples for RNAseq are collected to search for differentially expressed genes and for missplicing.

Cecile Martinat reported her experiences with the use of human pluripotent stem cells for DM1. Human pluripotent stem cells, from embryonic origin or obtained by genetic conversion of patient's somatic cells, offer access to a large spectrum of disease-specific cell models. Disease-specific pluripotent stem cells capable of differentiation into the various tissues affected in each condition can provide new insights into the pathological mechanisms by permitting analysis in a human model system. Validating this concept, it was demonstrated that human pluripotent stem cells, expressing the causal mutation implicated in DM1, offer pertinent disease cell models, which are applicable for a wide systemic analysis ranging from mechanistic studies to therapeutic screening. Through a genome-wide analysis, two early developmental molecular mechanisms were identified, involved both in myogenesis as well as in neurite formation and the establishment of neuromuscular connections. These neuropathological mechanisms may bear clinical significance as they are related to the functional alteration of neuromuscular connections associated with DM1 [58–60]. In parallel to these functional pathological studies, they also identified new therapeutic strategies [61]. Also, these results identified the possibility for repurposing metformin, the most commonly prescribed drug for type 2 diabetes, for DM1 leading to a phase 2 clinical trial [62,63]. For the future, human pluripotent stem cells will be used to develop more complex cellular models, especially to better decipher the pathobiology in the nervous system.

Hans van Bokhoven reported on cell-based therapy in DM. Pericytes are multipotent, vessel-associated progenitors that exhibit high proliferative capacity, can cross the blood-muscle barrier, and can home to muscle tissue and contribute to myogenesis. Consequently, pericyte-based therapies hold great promise for muscular dystrophies. Pericytes may be a valuable cell source for a complex multi-system disorder like DM1. The aim is to develop an autologous cell therapy based on *ex vivo* genetically corrected pericytes. Pericytes were isolated and characterized from DMSXL mice and six DM1 patients, who vary with respect to gender, age, repeat length in blood and disease severity as measured by MIRS score. For that, muscle biopsies were used for explant cultures and cells emerging from the explants were sorted by FACS and used for further characterization. The pericyte identity of sorted $CD31^-$ ALP^+ cells was confirmed by Immunohistochemical analysis. The cells showed high proliferative capacity with no apparent differences between wt and repeat-carrying cells. The pericyte identity was maintained over 20 passages. Interestingly, mouse and human pericytes expressing an expanded repeat showed CUGexp RNA MBNL positive nuclear foci. Finally, the selected pericytes exhibited intrinsic myogenic capacity, and *in vitro* myotube formation of wild type/unaffected and mutant pericytes was indistinguishable with regard to percentage and morphology of myotubes. CRISPR-based editing is in progress to exchange the expanded repeat by a normal sized allele using a homology-directed repair strategy. This step may ultimately generate a barrier in the number of correctly-edited pericytes that can be obtained. Therefore, an intermediate step was established in which induced pluripotent stem cells (iPSCs), with unlimited proliferative capacity, were generated from pericytes, which after genetic correction will be redifferentiated into pericytes. Another line of investigation is directed at the neurological features of DM1. For that, iPSCs from patients are differentiated in cortical glutamatergic neurons (iNeurons). After neural induction of iPSCs, iNeurons will develop and form mature neuronal networks, characterized by spontaneous bursts of activity which can be measured non-invasively on micro-electrode arrays (MEAs). The bursting is highly reproducible in unaffected cells. It is known that cells from patients with a neurodevelopmental disorder show aberrant patterns, which occur in patterns specific for the underlying gene mutation. Currently, DM1-derived iNeurons are generated to study their neurophysiological and network properties in relation to repeat length and other parameters. After characterization of the MEA patterns of DM1 iNeurons, this platform will be used for testing candidate compounds for *in vitro* analysis of their ability to correct observed neurophysiological and network defects.

Thierry VandenDriessche summarized his experience with a CRISPR/Cas approach for DM1. Several therapeutic interventions are being developed that act at different levels to suppress DM1 pathogenesis. The 'downstream' mechanisms of DM1 pathogenesis are not fully understood, hampering the development of an effective cure. Hence, they developed an improved therapeutic approach for correcting the pathogenic DM1 mutation by gene editing. By targeting the mutation at the DNA level, it was hypothesized that this would consequently lead to a correction of known and unknown downstream pathogenic effects of DM1. Therefore, in the first part of the study, they established a DM1 cellular platform in order to test and develop their therapeutic approach. More specifically, they successfully derived DM1 patient-derived iPSCs and differentiated them

into myogenic cells (DM1-iPSCMyo) that resemble pericyte-like cells or mesoangioblasts, using a relatively robust myogenic differentiation protocol that requires expansion on extracellular matrix components (matrigel) and *de novo* expression of the myogenic switch *MyoD*. Culturing dissociated pluripotent cells on a matrigel-coated surface exposes them to laminin, collagen IV, heparan sulfate proteoglycans and entactin/nidogen, all components of the vascular and skeletal muscle basement membranes. The DM1-iPSC-Myo "disease-in-a-dish" model was scalable in culture, negating the limitation of restricted proliferative capacity of patient-derived primary cells for *in vitro* studies. This DM1 myogenic model was also an invaluable *in vitro* model mimicking the aberrant DM1 alternate splicing and biomarkers. Subsequently, a dual guide RNA-based CRISPR/Cas9 approach was developed to excise the expanded CTG repeat by simultaneously targeting upstream and downstream target sequences. Analysis of the results demonstrated a relatively robust correction of the DM1 mutation; up to 52% correction efficiency in DM1-iPSC-derived myogenic cells and primary DM1 myoblasts was achieved without any selection step, consistent with the disappearance of ribonuclear foci. The excision of the pathogenic CTG repeat was confirmed by triplet repeat PCR and single molecule real-time sequencing. The downstream biological effects of CRISPR/Cas9 correction were detected in the form of reversal in alternative splicing of *SERCA* and redistribution of MBNL1 proteins. These results are consistent with previous studies demonstrating CRISPR/Cas9-mediated excision of CTG repeats in immortalized myoblasts from DM1 patients. Keeping the ultimate goal of future clinical translation in mind, a "hit and go" strategy to correct the DM1-iPSCs would be preferred to overcome the potential risks associated with long-term expression of the CRISPR/Cas components. Based on transfection with CRISPR/Cas9 ribonucleoprotein complexes up to 90% correction efficiency could be attained, as confirmed by triplet repeat primed PCR, Southern blot analysis and ribonuclear foci analysis of individual iPSC clones, obviating the need for selective enrichment. Additionally, alternative splicing of the corrected DM1-iPSCs post cardiomyogenic differentiation was examined and showed a reversal in the splicing pattern in the corrected cells as compared to their non-corrected counterparts (Dastidar et al., unpublished observations). In conclusion, this study demonstrates that CRISPR/Cas9 can mediate relatively efficient correction of the pathogenic DM1 mutation. This represents an essential step towards development of novel therapeutic strategies that can ultimately be translated into the clinic in patients. However, concerns related to potential off-target genome editing and potential immunogenicity of Cas9 remains. In addition, there is a need to further augment the efficiency of *in vivo* editing in DM1 mouse models. The DM1 myogenic and cardiomyogenic disease models generated provide invaluable tools for developing novel therapies and to further study the pathophysiological mechanisms of DM1 [26,42,64,65].

Lise Ripken presented her work on the dose-response relationship - *i.e.* CUGexp RNA expression *versus* downstream toxic effects - for pathogenic *DMPK* transcripts in DM1. How should the dosage of CUGexp RNA be defined? Are ten CUG100 transcripts equally toxic as one CUG1000 transcript? Moreover, in a therapeutic setting, which reduction in expanded *DMPK* RNA level is required to obtain a clinical effect in patients? She showed pilot results obtained with a DM1 cell model in which expanded *DMPK* RNA expression was tuned by using the CRISPR (in)activation approach. Expanded *DMPK* RNA expression was measured by RT-qPCR and single cell RNA FISH. The development of patient cell lines with CRISPR-mediated, tunable *DMPK* expression, both down- and upregulated, appears a feasible concept and will provide valuable information on DM1 pathogenesis, interpatient variability and therapy development.

David Brook reported on cell-based assays to identify compounds that eliminate nuclear foci in DM1 and DM2 cells. Nuclear foci represent an important feature in both forms of DM. CUGexp transcripts accumulate in the nuclei of patient cells where they interact with MBNL proteins to form the distinctive foci. They reasoned that a small-molecule compound which eliminated nuclear foci could provide a possible treatment for DM. Thus, they devised a screening protocol based on the elimination of nuclear foci in a cell-based assay. They screened three different libraries: one consisting of 18,000 drug-like molecules, around 3,000 molecules from the NIH collection of approved drugs and a small collection of kinase and phosphatase inhibitors. High content imaging with a defined journal to allow the automatic scoring of foci. The screening efforts pointed towards small molecule kinase inhibitors as being particularly effective at eliminating nuclear foci. In collaboration with colleagues at Glaxo Smith Kline they screened the PKIS collection of characterized kinase inhibitors. This allowed them to narrow down the kinase target which, when inhibited, led to the elimination of nuclear foci. This approach was supported by additional analyses using Kino-beads, which are derivitised beads containing ATP mimetics on their surface. These beads can be used in plus/minus screens in conjunction with Mass spectrometry to identify the key kinase targets. This combination of molecular and cell biological screening in conjunction with target deconvolution has allowed them to pinpoint particular inhibitors targeting specific cyclin dependent kinases. Future medicinal chemistry programs should allow the development of suitable drug like molecules to target the specific kinases as treatments for DM1 and DM2 [66–69].

Michael Sinnreich reported his studies on small molecules in DM cell systems. In an effort to identify small molecules that liberate sequestered MBNL1 from (CUG)n RNA, they focused in a collaborative effort with the research group of Matthias Hamburger, Pharmazentrum Basel, specifically on small molecules of natural origin. They developed a DM1 pathomechanism-based biochemical assay and screened a collection of isolated natural compounds, as well as a library of over 2100 extracts from plants and fungal strains. HPLC-

based activity profiling in combination with spectroscopic methods were used to identify the active principles in the extracts. Bioactivity of the identified compounds was tested in a human cell model and in a mouse model of DM1. They identified several alkaloids, including the beta-carboline harmine and the isoquinoline berberine, which ameliorated certain aspects of the DM1 pathology in these models. These compounds may provide pharmacophores for further medicinal chemistry optimization. To investigate whether deregulation of central metabolic pathways such as AMPK/mTOR may also be implicated in the pathogenesis of DM1, they are currently examining human skeletal muscle biopsies, as well as human cell lines and DM1 mice challenged by different conditions. In parallel, they are analyzing whether DM1 muscle shows alterations in the autophagy flux and/or proteasome activity by biochemical and histological means. Lastly, they are testing the consequences of the modulation of these metabolic pathways on myotonia by ex-vivo electrophysiological methods. They found that AICAR, an agonist of AMPK, markedly reduces myotonia in DM1 mice; the underlying mechanisms are currently being investigated [70,71].

Nicolas Charlet-Berguerand presented data on splice regulators beyond MBNL. Mutant RNAs containing expanded CUG (DM1) or CCUG (DM2) repeats interfere with the metabolism of other RNAs through titration of MBNL proteins. In support of this mechanism, the size of the CTG repeat grossly correlates with the titration of MBNL1 and with the severity of the disease in DM1. However, DM2 is characterized by a much higher number of expanded repeats compared to DM1, but follows a more favourable clinical course than DM1. This paradox suggests that some mechanisms, yet to be identified, modulate the severity of DM pathologies. Searching for modifiers of DM2 severity, they identified that the rbFOX1, rbFOX2 and rbFOX3 RNA binding proteins bind to expanded CCUG RNA repeats that cause DM2, but not to expanded CUG repeats that cause DM1. Furthermore, rbFOX1 competes with the binding of MBNL1 to CCUG expanded repeats and partially reduces the amount of MBNL1 sequestered within CCUG RNA foci in muscle cells of individuals with DM2. Importantly, expression of rbFOX1 rescues muscle atrophy, climbing and flying defects caused by expression of expanded CCUG repeats in a novel Drosophila models of DM2. Overall, these data support that rbFOX RNA binding proteins may contribute, with other mechanisms, to the lesser toxicity of CCTG repeat expansion in DM2.

Jonathan Hall presented work from his group on molecules targeting a stem cell factor Lin28. This RNA binding protein interacts with conserved binding sites on numerous microRNA precursor molecules and inhibits their conversion to mature microRNAs by Drosha and Dicer processing enzymes. Several of the miRNAs affected regulate the expression levels of ion channels involved in myotonic dystrophies. Inhibition of Lin28 activity is expected to release the processing of these miRNAs and restore activity of these channels, eventually with a potential benefit for DM treatments. A FRET based high-throughput screen identified a small set of drug-like compounds that inhibit the binding of Lin28 to the let-7 miRNA precursors. Testing of one of these molecules in DM1 and DM2 cells indeed showed inhibition of Lin28 and an increase in selected miRNA levels. Future investigation will determine which miRNAs and ion channels are most strongly affected by the inhibitors, and in parallel, other cellular pathways affected by Lin28 inhibition.

Session 7: DM – the patient and family perspective

Alain Geille, also on behalf of *Bas Haasakker* and *Nathalie Loux,* shed light on the patientś perspective in DM. The purpose of the talk was to review unmet needs in DM and identify action items to fix them, viewed from the patient side. Starting from the previous meeting dedicated to DM in 2016, these needs concern the timescale of events, the patient expertise and the coherence of actions in Europe. Geographical and social isolation of patients in conjunction with their cognitive condition require to connect with dedicated patient groups and organizations from the very beginning of actions, to avoid loss of time. The patient groups have the deepest and most direct contacts with families to help clinicians and scientists in their studies or trials, but their size and knowledge of the disease is variable in Europe and need a shared effort of harmonization. A new patient association called Euro-DyMA is proposed to help solving the identified issues. Euro-DyMA is a federation of European patient organizations involved in DM. It is non-profit, fully dedicated to the disease, registered at the Institute of Myology in Paris, to be funded through adhesion fees and contracts with academic and industrial partners, excluding any sponsoring from the drug industry. Its main activity will be to stimulate cooperation, accelerate the availability of drugs, promote the interests of DM patients in European health policies and implement best practices. As of 1st of September 2019, eight associations registered from seven countries (France, Germany, Greece, The Netherlands, Belgium, Italy and Switzerland). U.K., Denmark and Cyprus are knocking at the door.

Session 8: Framing a European DM research network

During this workshop, we tried to identify the key unknowns, defined the missing tools needed to advance research, and we formulated plans for how the required reagents, materials and protocols can be generated and shared, and how the important unknowns can be addressed. We summarized current therapeutic strategies and their status in relation to clinical translation in the European laboratories. We explored the barriers to new therapy identification and established first steps towards an action plan fostering novel drug discovery in DM.

As consensus from this workshop, the group will proceed to establish a European DM Consortium that will share the diversity of expertise and material, methods, and patient data to inform therapy paradigms through a standardized

and harmonized data set. As first coordinated action, the group under the lead of *Ruben Artero* applied for an EU-funded Innovative Training Network entitled "ENTRY-DM – From toxic RNA to translational medicine in myotonic dystrophy" to ensure exchange and integration of young scientists dedicated to research in DM in Europe. More European collaborative grant attempts will certainly follow.

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