Characterization of the muscular and cardiac diseases of the DMSXL mouse model, a transgenic mouse model for Myotonic Dystrophy type 1

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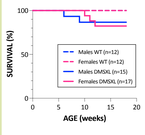
Context of the study

Myotonic Dystrophy type 1 (DM1) is an autosomal dominant, progressive, and multi-systemic genetic disorder affecting at least 900,000 individuals worldwide. It is primarily characterized by myotonia, muscular weakness, and muscle atrophy. Other clinical manifestations include cardiac conduction defects, cardio-respiratory problems, cataracts, endocrine dysfunction and frequent neurological manifestations. The disease is caused by unnaturally expanded repeats of CTG trinucleotide in the 3'-untranslated region of the *DMPK* (dystrophia myotonica protein kinase) gene. In DM1 patients, the number of CTG repeats within the mutant *DMPK* allele ranges from 50 to 5000. The number of repeats roughly correlates with disease onset, severity and life expectancy (more repeats correlated with earlier onset, more severe symptoms and shorter lifespan). CTG-containing mutant DMPK transcripts are toxic. They aggregate as nuclear *foci* and impact the expression and function of RNA-binding proteins (such as MBNL1 and CELF1), resulting in spliceopathy of downstream effector genes, which accounts for much of the disease phenotype. Several therapeutic approaches either pharmacological or gene-therapy based, are under investigation to address this unmet medical need. One current limitation for the efficient evaluation and development of therapeutic products is the lack of DM1 animal models that ideally recapitulate the symptoms and the complex pathophysiology of this disease. Among available animal models, one of the most relevant remains the DMSXL mouse model, which carries a 45-kb human genomic fragment including the *DMPK* gene with more than 1200 CTG repeats. The human *DMPK* transgene is under the control of its own promoter and has been shown to have an almost ubiquitous expression. Initial characterization studies demonstrated that homozygous DMSXL mice display several manifestations of the human DM1 pathology, including growth retardation, muscle defects, cognitive impairments, nuclear *foci,* and splicing abnormalities. After establishing a colony in our own facility, our goal was to define in our hands the most relevant and sensitive readouts that characterize this animal model, especially for its muscular and cardiac diseases, in both genders.

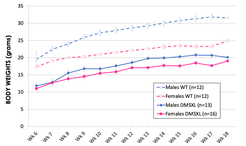
STUDY DESIGN

Different groups of male and female homozygous DMSXL mice and littermate wild-type (WT) controls were followed and evaluated at 4 months of age.
Tissue samples were also evaluated at 1 month of age.
To avoid bias in the analysis, all experiments were done in a blinded fashion.

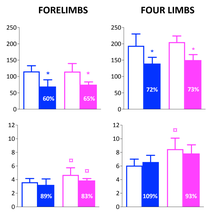
CLINICAL FOLLOW-UP



No WT control mice died during the course of the study, whereas we observed 5 **sudden deaths** (mice were found dead in their cage, without any preliminary symptom) out of the 32 DMSXL animals (15%), between 1.5 and 3 months of age. Chronic pneumonia was identified as one of the cause of death.
Clear **weight** reductions were observed in females (whatever the genotype), and in DMSXL mice (whatever the sex).
Finally, no modification of **hematology nor clinical chemistry parameters** (urea, creatinine, ALP, ALT, AST, CK, total proteins, glucose, calcium) were observed at 4 months of age).



MUSCLE FUNCTION

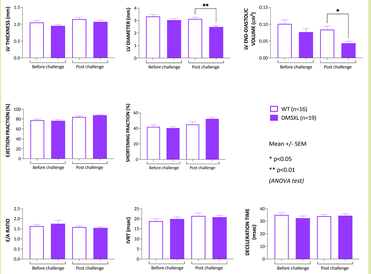


Muscular strength was assessed using forelimb and four limbs **grip test** test at 4 months of age. Force was measured over 5 successive trials with a short latency between each test. Results were expressed as the mean of 3 median values obtained during the 5 tests. Results showed that DMSXL males and females exhibited significant and similar decreases of absolute force developed by forelimbs and by four limbs. However, these differences were no longer significant after body mass normalization for the calculation of relative force.
Muscle resistance was also evaluated at using **treadmill** at 4 months of age The time and the total distance to exhaustion were determined. No significant difference were observed between WT and DMSXL mice (data not shown).

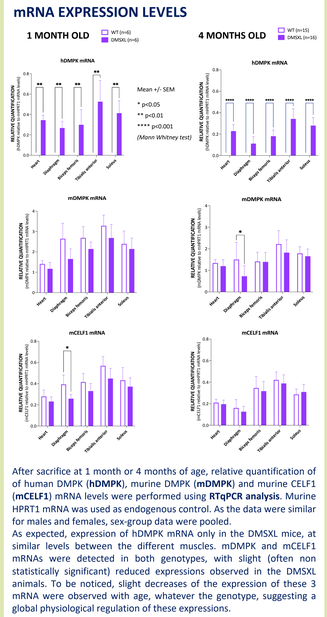
CARDIAC FUNCTION

At 4 months of age, conventional measurement of the **electrocardiography (EKG)** parameters was performed on anesthetized animals. Classical intervals and segments (P wave, RR interval, PR interval, QT interval, QRS interval) were measured, and compared between the different experimental groups. After basal measurements, animals were submitted to challenge with Flecainide (20mg/kg) which acts as a pharmacological stressor of conduction disorders. As the data were similar for males and females, sex-group data were pooled to explore the effect of the genotype. No significant difference were observed between WT and DMSXL mice, whether before or after the Flecainide challenge (data not shown).

During the same anesthesia, before and after Flecainide challenge, **2D-echocardiography and pulsed Doppler** was also performed to look for possible structural remodeling (left ventricular thickness, diameter and end-diastolic volume), as well as systolic function (ejection and shortening fractions) and diastolic function (E/A ratio, isovolumetric relaxation time =IVRT, and deceleration time). Again, as the data were similar for males and females, sex-group data were pooled. No modification of the systolic and diastolic functions were overserved, whatever the experimental group and condition. However, significant reduction of the left ventricle diameter and end-diastolic volume were observed in the DMSXL animals, suggesting initiation of a structural remodeling in these animals at 4 months old.

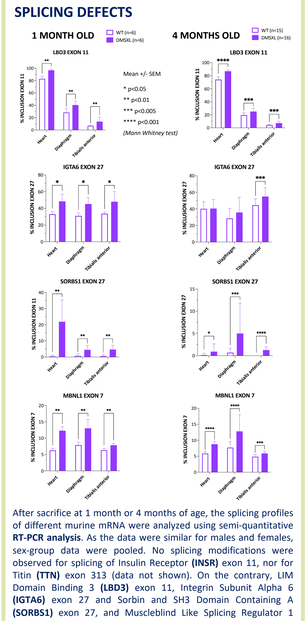


mRNA EXPRESSION LEVELS



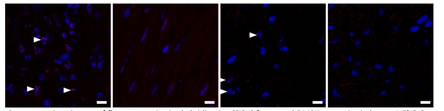
After sacrifice at 1 month or 4 months of age, relative quantification of human DMPK (**hDMPK**), murine DMPK (**mDMPK**) and murine CELF1 (**mCELF1**) mRNA levels were performed using **RTqPCR analysis**. Murine HPRT1 mRNA was used as endogenous control. As the data were similar for males and females, sex-group data were pooled.
As expected, expression of hDMPK mRNA only in the DMSXL mice, at similar levels between the different muscles. mDMPK and mCELF1 mRNAs were detected in both genotypes, with slight (often non statistically significant) reduced expressions observed in the DMSXL animals. To be noticed, slight decreases of the expression of these 3 mRNA were observed with age, whatever the genotype, suggesting a global physiological regulation of these expressions.

SPLICING DEFECTS

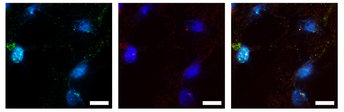


After sacrifice at 1 month or 4 months of age, the splicing profiles of different murine mRNA were analyzed using semi-quantitative **RT-PCR analysis**. As the data were similar for males and females, sex-group data were pooled. No splicing modifications were observed for splicing of Insulin Receptor **(INSR)** exon 11, nor for Titin **(TTN)** exon 313 (data not shown). On the contrary, LIM Domain Binding 3 **(LBD3)** exon 11, Integrin Subunit Alpha 6 **(IGTA6)** exon 27 and Sorbin and SH3 Domain Containing A **(SORBS1)** exon 27, and Muscleblind Like Splicing Regulator 1 (MBNL1) exon 7 showed clear mis-splicing in DMSXL muscles.

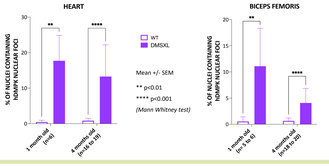
hDMPK NUCLEAR FOCI & MBNL1 COLOCALIZATION



Representative pictures of fluorescence in situ hybridization (FISH) for sense hDMPK transcript in heart. A 5'-Cy3-labeled (CAG)$_7$ probe detected sense RNA foci (red) in some nucleus (arrowheads) in samples from DMSXL mice. Nuclear DAPI counterstaining (blue). Scale=10 µm



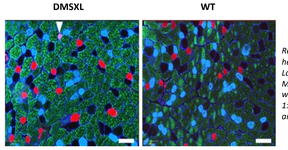
Representative picture of the colocalization of DMPK mutant transcripts with the MBNL1 protein in biceps femoris muscle of a 4 months old DMSXL mouse. Some nuclear MBLN1 aggregates also displayed CUG transcript accumulation that resulted in signal colocalization (arrowhead). Scale bar=10 µm



After sacrifice at 1 month or 4 months of age, we investigated the presence of ribonuclear inclusions of sense hDMPK mRNA carrying CTG expansions (thereafter designed as **nuclear foci**) in heart and biceps femoris muscles using using **fluorescence *in situ* hybridization (FISH)** with a (CAG)$_5$ fluorescent probe. Comparing to WT tissues in which no specific signal was detected, DMSXL muscles showed some nuclear foci, ranging from 1 up to 7 foci per nucleus, occasionally forming large ones. The number of nuclei with nuclear foci were counted using an automatized method, and showed a decrease from 1 to 4 months of age, especially in biceps femoris. As the data were similar for males and females, sex-group data were pooled.

In DMSXL skeletal muscles and heart, **IHC** using an antibody specific for MBNL1 followed by the FISH technique showed **sequestration of the MBLN1 protein** next to the hDMPK nuclear foci.

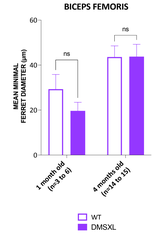
MUSCLE FIBER TYPES



Representative picture of immunolabelling for myosin heavy chain (MyHC) isoforms in biceps femoris muscles. Labeling of MyHC type 1 (red), MyHC type 2A (cyan) and MyHC type 2B (green) enables muscle fiber typing. Fibers with no labelling are type X (black). Some few hybrid type 1-2A fibers (arrowhead) were only observed in DMSXL animals. Scale=100 µm

After sacrifice at 1 month or 4 months of age, type of muscle fibers was determined based on their **expression of myosin heavy chain (MyHCI, IIa and IIb) isoforms** in biceps femoris muscle. The percentage of each fiber type were compared between the experimental group. For all analyzed fiber types (i.e. type 1, 2A, X and 2B), no group effect was demonstrated and no difference in fiber type distribution could be identified (data not shown), maybe secondary to the high level of inter-individual variations.

MUSCLE FIBER SIZE



After sacrifice at 1 month or 4 months of age, muscle fibers size was measured in biceps femoris muscle using **minimal Ferret diameter** and a laminin immunolabelling.

As the data were similar for males and females, sex-group data were pooled. Smaller fibers were identified in DMSXL mice at 1 month of age, but this difference was not statistically different (probably secondary to the high level of inter-individual variations), and was no more visible at 4 months of age.

Conclusion

The data obtained in the frame of this study confirm that the DMSXL mouse model exhibits several features of the DM1 pathology at the muscular and cardiac levels. Expression of the mutated human DMPK messenger, which contained CUG-repeats, was detected in skeletal muscles and heart at both 1 and 4 months of age. This mutant transcript aggregates as foci in the nuclei of the muscle cells, where sequestration of the MBNL1 protein was also observed. As a consequence, and even if expression of the murine CELF1 mRNA was not modified, splicing defects of several downstream messengers was shown. Due to high level of inter-individual variations, no clear skeletal muscle histological abnormalities were observed. However, grip force was found reduced in 4 months old DMSXL animals. At the same age, the heart exhibited no EKG abnormalities, but an initiation of structural remodeling. Except for body weights, no gender effect was raised during this study.
All these data will be of importance to design future preclinical studies for the evaluation of the efficacy and safety of different therapeutic products designed to treat DM1 at the muscular and cardiac levels, and using this unique DMSXL mouse model.

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