SHORT TERM SCIENTIFIC MISSION (STSM)
SCIENTIFIC REPORT

This report is submitted for approval by the STSM applicant to the STSM coordinator

Action number: CA17103 (Delivery of Antisense RNA Therapeutics)
STSM title: Assesment of side effects of antisense therapy on the transcriptional output in Duchenne Muscular Dystrophy (DMD)
STSM start and end date: 01-02-2019 – 30-04-2019
Grantee name: Raquel García Rodríguez

PURPOSE OF THE STSM

Duchenne muscular dystrophy (DMD) is a rare genetic disorder caused by mutations in the *DMD* gene encoding a structural protein called dystrophin. Lack of dystrophin is responsible for the instability of skeletal and heart muscles during contraction resulting in continuous muscle damage and weakness (1). Most of the mutations are out of frame deletions leading to absence of dystrophin. Nowadays, research has focused on the development of potential therapies able to restore dystrophin. One of these therapies is based on the use of antisense oligonucleotides (AONs) which has been shown to efficiently induce exon skipping of specific dystrophin exons. This leads to DMD reading frame restoration at the mRNA level and to the production of smaller but partially functional dystrophin (2). The efficacy of the therapy is however limited with dystrophin recovery below 1%. Previous observation suggested that oligonucleotides could interfere with the transcriptional process of the target thereby reducing the efficiency of the therapeutic approach. For that reason, the purpose of this STSM was to assess the side-effect of antisense therapy on the transcriptional output of the target locus.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSM

To achieve the proposed aim, I carried out different approaches:

1. Previous observation suggested that AONs could interfere with the transcriptional process decreasing mRNA levels of the target locus. To study this, immortalized myogenic cells obtained from DMD patients and healthy controls were transfected with different control oligonucleotides and 2'-*O*-methylphosphorothioate AONs (2'OMe AONs) able to skip different dystrophin exons. In particular, AONs targeting NOTCH3 and DYSF genes were transfected as controls in both cell lines. Healthy control cell line was transfected with AONs targeting exons 29 and 41 leading to in frame deletions in the *DMD* gene. On the other hand, DMD cell line was transfected with an AON targeting exon 51 to restore the open reading frame of the *DMD* gene. Twenty-four hours after transfection, total RNA was isolated and dystrophin expression levels were measured by ddPCR and qPCR.

2. Another approach to assess the transcriptional output in the presence/absence of AONs was based on the BruChase-seq technique (3). Both healthy control and DMD cell lines were transfected with an AON targeting the NOTCH3 gene as a control oligo; healthy control cells were transfected with an AON targeting exon 41 leading to an in frame deletion in the *DMD* gene, while DMD cells were transfected with an AON targeting exon 51 to restore the open reading frame. Transfection was followed by transient inhibition of RNAPII by adding 5,6-dichlorobenzimidazole1-β-D-ribofuranoside (DRB) to the medium. Following drug removal, RNA polymerases enter the elongation phase in a synchronized manner and nascent RNA was labeled by adding bromouridine (Bru) to the medium. Cells were incubated with Bru for 30 minutes and

Funded by the Horizon 2020 Framework Programme of the European Union

chased after that in uridine for different periods of time (6, 12 and 18h). Total RNA was isolated followed by specific capturing of Bru-labeled RNA using anti-BrdU antibodies conjugated to magnetic beads. Sequencing is currently ongoing.

3. Finally, to study possible post-transcriptional modifications including base alterations which can change the content of the mature transcript, we carried out direct RNA sequencing using nanopore arrays. Conventional RNA sequencing approaches lose information contained in biological RNA as some modifications are not carried forward during cDNA synthesis. Therefore, we carried out poly(A) RNA sequencing following the strategy developed by Oxford Nanopore Technologies (ONT) (4). Total RNA was isolated from quadriceps of 3 different mice: wildtype mouse, *mdx* mouse (main animal model of the disease) and *mdx* mouse treated with a morpholino oligomer able to skip exon 23 of *DMD* gene to restore the open reading frame. Poly(A) RNA was isolated using Dynabeads Oligo$(dT)_{25}$ and approximately 500 ng of the poly(A) isolate was adapted for nanopore sequencing.

DESCRIPTION OF THE MAIN RESULTS OBTAINED

1. Previous data obtained by ddPCR showed that AONs could reduce mRNA levels of the target locus but qPCR results did not confirm it. A new transfection round with different control oligonucleotides and AONs was carried out, total RNA was isolated and the ddPCR is currently ongoing.

2. RNA sequencing of the samples obtained in the BruChase experiment is currently ongoing. In particular, only RNA samples chased in uridine for 6 and 12 hours are being sequenced as long chases affect the ratio of Bru-labeled RNA to total RNA. Results of this experiment remain to be analysed.

3. Related to the study focused on poly(A) RNA sequencing from quadriceps of both wildtype mouse and *mdx* mice (treated or not with a morpholino oligomer), I sequenced a test sample of poly(A) RNA as it was the first time that this approach was carried out. Different runs changing some parameters in the workflow were tested and the best run generated 17,934 strand reads qualified as pass reads. These native RNA reads had an aligned N50 length of 1,345 bases and a maximum aligned length of over 10,000 bases. However, the mean read length was about 1,000 bases and most of the sequenced molecules were the smaller ones. To enrich the sample preparation with *DMD* mRNAs we performed size selection using different proportion of AMPureXP beads. Lab on a chip data showed a marginal improvement with small molecules still dominating the profile. A second approach was based on a pulsed-field gel electrophoresis. Pulsed-field gels work by switching the direction of the electric field in a gel. In this way, smaller molecules can change direction faster than larger molecules and more differentiated separation can be achieved. In addition, this kind of electrophoresis features the ability to collect in buffer the different fragment distributions separating largest molecules from smaller molecules in different fractions. Although only test samples of poly(A) RNA were analysed, some promising results were achieved using this approach. After running a poly(A) RNA sample on a pulsed-field gel, fractions with different size ranges were collected and analysed by qPCR followed by sequencing using the Illumina platform. qPCR results showed that *DMD* gene was only present in some fractions allowing its separation from the smaller molecules. Analysis of the Illumina data is currently ongoing to confirm the qPCR results. Future plans include using this approach before direct RNA sequencing to study both paired alternative splicing events and possible post-transcriptional modifications of *DMD* transcript.

FUTURE COLLABORATIONS (if applicable)

During my STSM a collaboration with the research group of Prof. Ljungman in the University of Michigan was achieved to analyse BruChase-seq data.

1. Mercuri, E. & Muntoni, F. Muscular dystrophies. *Lancet* (London, England). 381, 845-60 (2013).

2. Aartsma-Rus A, et al. Therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients. *Hum Mol Genet*. 12, 907–914 (2003).

3. Paulsen MT, et al. Use of Bru-Seq and BruChase-Seq for genome-wide assessment of the synthesis and stability of RNA. *Methods*. 67, 45–54 (2014).

4. Workman, R. E. et al. Nanopore native RNA sequencing of a human poly(A) transcriptome. Preprint at *bioRxiv*. https://doi.org/10.1101/459529 (2018).