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CONTRACTING ORGANIZATION: Massachusetts General Hospital

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Scientific objective: Our goal is to use urine and/or blood samples from Duchenne patients to identify markers of muscle cell dysfunction that can be used to determine whether new treatments are working.
Rationale: Small particles called extracellular vesicles (EVs) are released from many different cell types into the urine and blood. EVs contain molecules called extracellular RNAs (exRNAs). In urine and blood, EVs contain exRNA biomarkers of cancers and other disease states. Recently, our group was the first to demonstrate that EVs in urine also contain a certain type of exRNA that can serve as specific biomarkers of Duchenne and other muscular dystrophies (Antoury, et al., 2018; Antoury, et al., 2019). Our data suggest that exRNAs are a rich and renewable biomarker source for Duchenne muscular dystrophy that could enable monitoring of the disease state and the molecular response to a new treatment.
Focus Area: Assessment of clinical trial tools and outcome measures, specifically the discovery and qualification of pharmacodynamic and predictive biomarkers.

15. SUBJECT TERMS
Duchenne muscular dystrophy; antisense oligonucleotides; exon-skipping; extracellular vesicles; extracellular RNA; biomarkers; PCR; mdx mouse model; lentiviral vectors

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1. Introduction: Our long-term goal is to develop minimally invasive biomarkers to help evaluate disease severity and the efficacy of different treatment paradigms in DMD. In this project, we will evaluate the capacity of extracellular vesicles (EVs) to serve as biomarkers following their release into the urine or blood circulation. By the term EVs we include exosomes, microvesicles and other membrane encased particles released and taken up by cells as a form of extracellular communication. EVs in serum and urine contain mRNA and non-coding RNAs, collectively termed extracellular RNAs (exRNAs), which are released from different tissues and can serve as genetic biomarkers of cancers and other disease states. While potentially valuable, to date relatively little is known about the constituency of exRNA in DMD, including the relationship of exRNA markers to the underlying disease state, rate of progression, or response to therapies. This project uses two approaches to identify exRNA biomarkers of DMD: 1) identify and characterize the exRNA profile in urine and/or blood of DMD patients, and 2) establish a relationship between the exRNA profile and ASO treatment in mdx mice and in human DMD cells.

2. Keywords: Duchenne muscular dystrophy; antisense oligonucleotides; exon-skipping; extracellular vesicles; extracellular RNA; biomarkers; PCR; mdx mouse model; lentiviral vectors.

3. Accomplishments:

Major goal (task) 1: Examine exRNA in urine and blood from DMD patients.

Major activities: We obtained local IRB approval for both performance sites during Month 3 and HRPO approval during Month 5. We subsequently enrolled N = 5 subjects for biofluid collection, examined extracellular vesicles using nanoparticle tracking analysis, isolated exRNA, examined exRNA using capillary gel electrophoresis, and prepared samples for RNA sequencing. Two of the samples were obtained from individuals that are being treated with the commercially available exon skipping antisense oligonucleotide (ASO) drug eteplirsen. We designed and tested new primer probe sets for monitoring exon skipping ASO activity by droplet digital PCR (ddPCR).

Significant results or key outcomes:

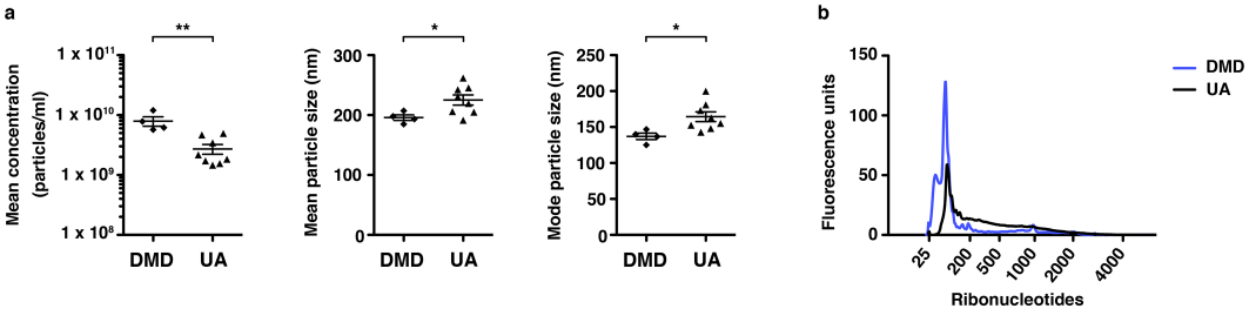

Figure 1. Characterization of urine EVs and exRNA in DMD and controls. a) We used particle imaging and tracking analysis software to measure extracellular particle concentration (left), mean size (center), and mode size (right) in urine of DMD (N = 4) and unaffected (UA) subjects. ** $P = 0.002$; * $P = 0.04$ (mean) and = 0.02 (mode); two-tailed *t* tests. b) We used capillary gel electrophoresis to examine the size distribution of exRNA recovered from urine. Representative electropherogram traces of exRNA size in nucleotides are shown.

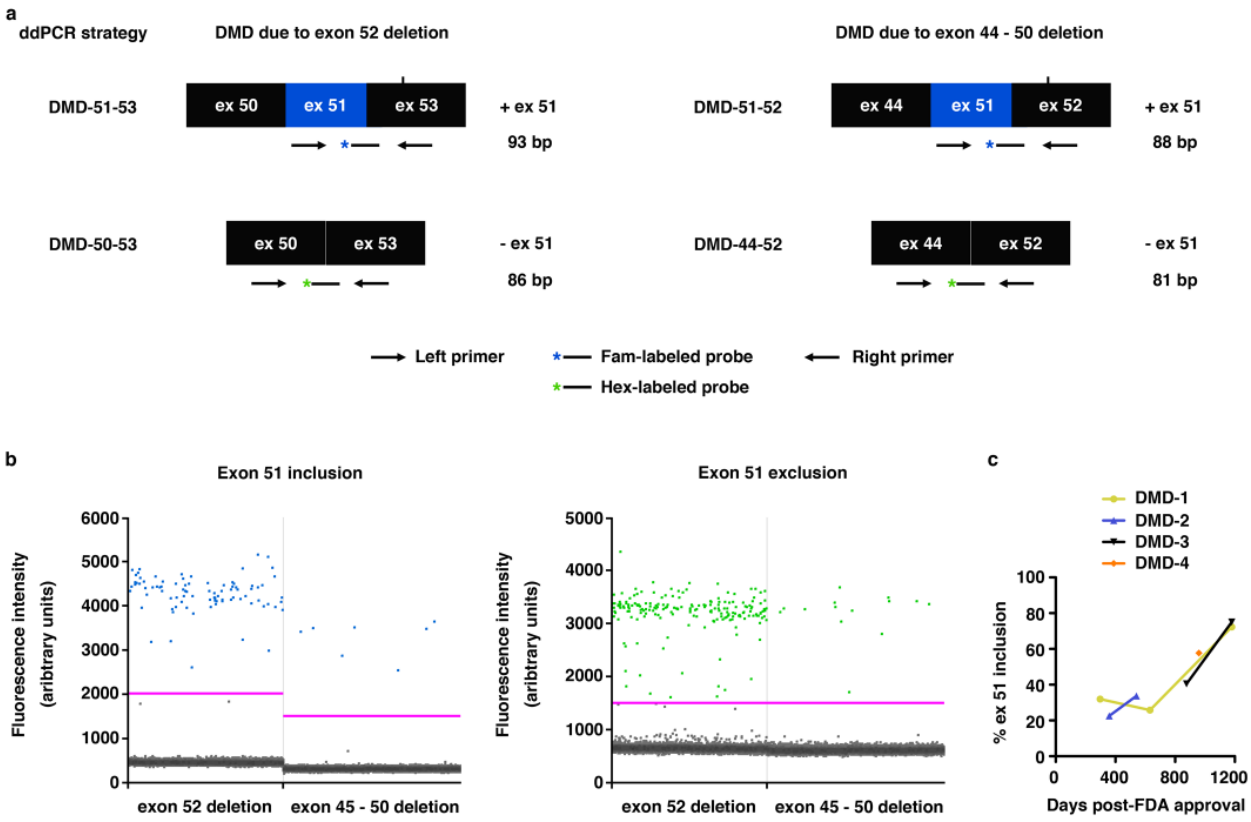

Figure 2. Longitudinal monitoring of eteplirsen drug activity. We examined urine exRNA from DMD individuals (N = 4) while on weekly eteplirsen treatment. Two have an exon 52 deletion and two have an exon 45 - 50 deletion. a) ddPCR strategy to identify exon 51 inclusion for both genotypes. b) Droplet populations showing exon 51 inclusion (left) and exon 51 exclusion (right). c) Exon 51 inclusion over time in three of the four individuals.

Major goal (task) 2: Develop a standard protocol for collecting and processing exRNA from biofluid of DMD patients.

Major activities: We tested exRNA isolation methods that include removal of urine cells by low speed centrifugation, passing the supernatant through a 0.8 um filter to remove any remaining cells or cell debris, and ultracentrifugation of the filtered supernatant. This work will continue throughout the funding period and will include comparison between ultracentrifugation and commercially available exosome isolation kits for exRNA yield and quality.

Major goal (task) 3: Examine exRNA from cultured human cells.

Major activities: We purchased from Coriell Research Institute DMD fibroblasts that were obtained from an individual with an exon 45 - 50 deletion in the *DMD* gene, which is amenable to therapeutic skipping of exon 51 using the commercially available ASO eteplirsen. To convert the fibroblasts to muscle cells, we used an Addgene lentiviral construct for the expression of a tamoxifen-inducible muscle transcription factor MyoD, prepared lentiviral vectors expressing this construct, treated the human DMD fibroblasts, and successfully produced muscle cells. Lentiviral transfection efficiency ranged from 88% to 98% based on immunolabeling of MyoD in treated cells. We collected EVs from the tissue culture medium of cells +/- MyoD treatment and began analysis.

Major goal (task) 4: Treat human cells with ASOs.

Major activities: We are waiting to receive research aliquots of eteplirsen and a peptide-conjugated

preparation of eteplirsen from Sarepta. In the meantime, we obtained a lentiviral micro-dystrophin construct from Addgene and prepared lentiviral vectors expressing micro-dystrophin for the purpose of measuring therapeutic activity in EVs released into the tissue culture medium.

Major goal (task) 5: Treat DMD mouse models with ASOs.

Major activities: We obtained local IACUC approval before the funding period began and ACURO approval during Month 4. We obtained ASOs targeting mouse *Dmd* exon 23 from Sarepta. We designed and tested novel primer probe sets for the ddPCR monitoring of exon 23 inclusion in the mouse *Dmd* gene. The first systemic delivery of ASO treatment of mdx mice is underway.

Significant results or key outcomes:

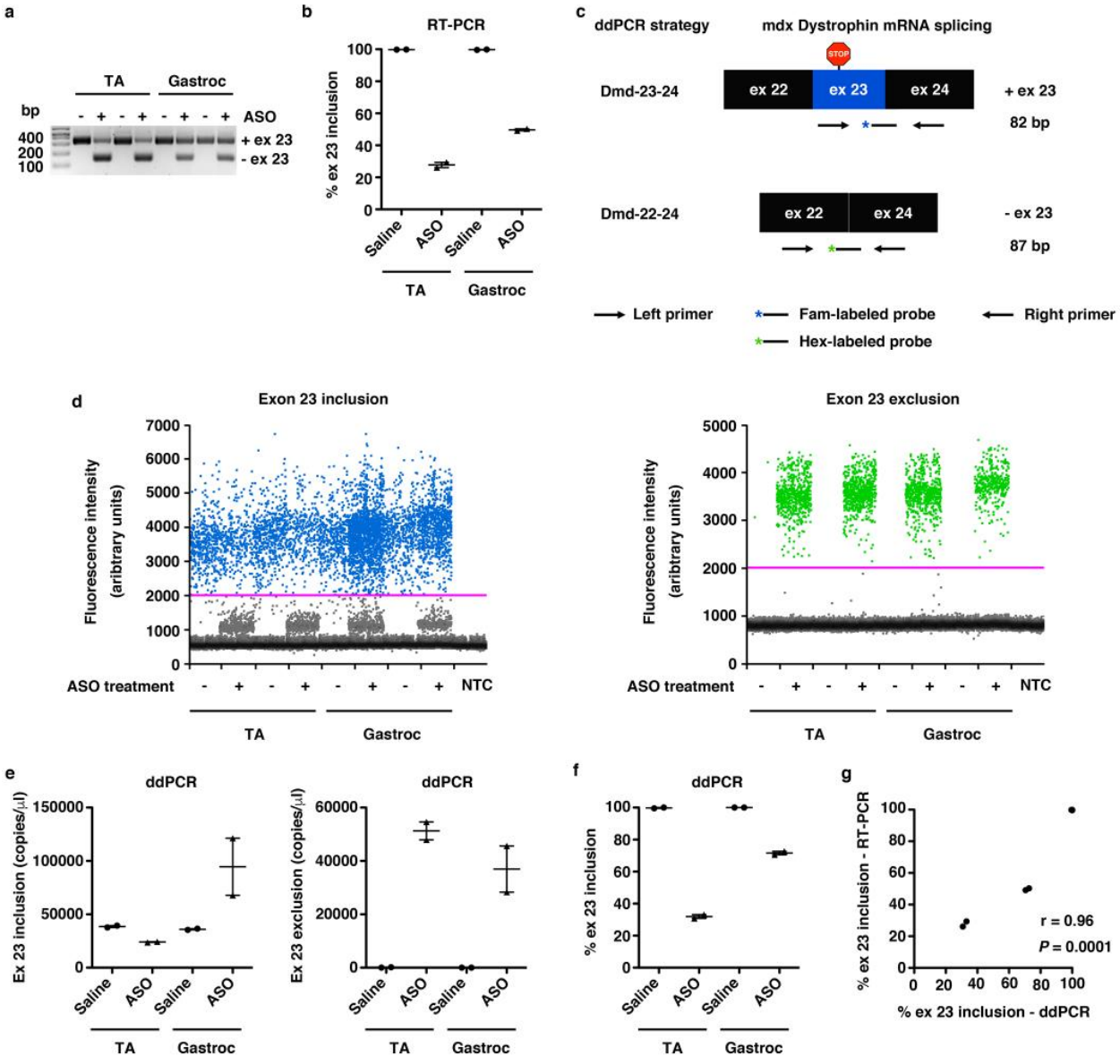

Figure 3. Design and validation of a ddPCR assay to measure exon 23 skipping in mdx mice. We treated tibialis anterior (TA) and gastrocnemius (gastroc) muscles of mdx mice by intramuscular injection of saline or an ASO design to inducing skipping of *Dmd* exon 23. Tissues were collected two weeks after treatment. a) RT-PCR and gel electrophoresis analysis of exon 23 inclusion. b) Quantification of exon 23 inclusion using the data in a). c) Assay design for ddPCR measurement of exon 23 inclusion. d) ddPCR analysis of exon 23 inclusion. Representative droplet populations using a fam-labeled probe specific for exon 23 inclusion (left; blue) and a hex-labeled probe specific for exon 23 exclusion (right; green) are shown. NTC = no template control. e) Quantification of exon 23 inclusion and exclusion splice products as copies/ul cDNA. f) % exon 23

inclusion in each group. g) Correlation of exon 23 inclusion by ddPCR and RT-PCR. The Pearson correlation coefficient r and *P* value are shown.

What opportunities for training and professional development has the project provided?

Nothing to report.

How were the results disseminated to the communities of interest?

The abstract that we presented at the American Society of Gene and Cell Therapy (virtual) meeting in May 2020 (see below) was highlighted by the CureDuchenne Foundation:
https://www.cureduchenne.org/blog/updates-from-asgct-2020/

What do you plan to do during the next reporting period to accomplish the goals?

As the clinics begin to re-open, we will obtain more clinical samples and compare exRNA recovery using ultracentrifugation vs. commercially available exosome isolation kits. We will complete RNA sequencing of several clinical samples and begin to analyze the results. Candidate biomarkers identified by RNA sequencing will be examined by RT-PCR and/or ddPCR for confirmation.

We will continue to use lentiviral vectors expressing the muscle transcription factor MyoD to convert human DMD fibroblasts to muscle cells and examine the exRNA profile in extracellular vesicles released from these cells into the culture medium with and without treatment.

We will treat additional cohorts of mdx mice with ASOs and correlate exon skipping in mouse urine with exon skipping in muscle tissue using ddPCR.

4. Impact:

Nothing to report.

5. Changes/problems:

Actual or anticipated problems or delays and actions or plans to resolve them.

The COVID-19 pandemic has eliminated in-person visits for DMD patients at MGH and BCH for the past five months. This has resulted in lower-than-expected recruitment of subjects for sample collection. In September 2020, in-person visits are scheduled to resume at approximately 50% capacity. We anticipate that recruitment will resume at this time but may remain below target for the next several months until full clinic capacity is restored.

6. Products:

Publications, conference papers, and presentations.

Sullivan LS, Hu N, Belanger-Deloge R, Darras BT, and Wheeler TM, Extracellular vesicles for monitoring therapeutic antisense oligonucleotide drug activity in Duchenne muscular dystrophy, *Mol Ther*, 28(4S1):459, 2020.

We presented an abstract at the American Society of Gene and Cell Therapy (ASGCT) annual meeting (virtual) in May 2020.

7. Participants and other collaborating organizations:

Which individuals have worked on the project?

Name: Thurman Wheeler
Project role: PI
Nearest person month worked: 2
Contribution to project: direct and supervise all aspects of this project, including experimental design, identification and recruitment of subjects at MGH, obtain relevant clinical information from muscular dystrophy subjects, and data interpretation. When needed, will assist with obtaining informed consent, maintaining of confidential records of all subjects from all sites, processing of biospecimens, isolation of extracellular vesicles and RNA, nanoparticle tracking, measuring quality, quantity, and size of exRNA recovered, cDNA synthesis, RT-PCR, qPCR, and droplet digital PCR.

Name: Ningyan Hu
Project role: Research technologist
Nearest person month worked: 6
Contribution to project: Obtain informed consent from subjects at MGH, maintenance of confidential records of all subjects from all sites, biospecimen processing, isolation of biofluid RNA, isolation of tissue RNA, cDNA synthesis, reverse transcription, PCR, and droplet digital PCR splicing analysis.

Name: Lauren Sullivan
Project role: Research technologist
Nearest person month worked: 6
Contribution to project: biospecimen processing, isolation of extracellular RNA, isolation of tissue RNA, cDNA synthesis, reverse transcription, PCR, droplet digital PCR, splicing analysis, mouse urine collection, injection of antisense oligonucleotides, and cell culture studies.

Name: Basil Darras
Project role: Subaward PI
Nearest person month worked: 1
Contribution to project: Direct responsibility for work performed at BCH related to this project, including the identification and recruitment of subjects, assist in obtaining informed consent, provide relevant medical information from each subject, and supervise the study coordinator activities.

Name: Raymond Belanger-Deloge
Project role: Subaward research coordinator
Nearest person month worked: 1
Contribution to project: Subject recruitment, sample collection and processing, maintenance of confidential records, and arrange delivery of samples from BCH to Dr. Wheeler's lab at MGH.

Has there been a change in the active other support of the PI/PD or senior key personnel since the last reporting period?

PI Wheeler
Previously active grant now closed:
Muscular Dystrophy Association
Extracellular RNA biomarkers of myotonic dystrophy
The goals of this project were to 1) identify and characterize the extracellular RNA profile in myotonic dystrophies, 2) establish a relationship between the extracellular RNA profile and clinical parameters of myotonic dystrophy, and 3) work with the FDA toward biomarker qualification for myotonic dystrophy.
Grants Management Specialist: Karen L. Smith
Muscular Dystrophy Association
Chicago, IL

Closed January 31, 2020.
Overlap: none

Previously pending grants now active:
W81XWH-20-1-0145 12/15/19 - 12/14/21 1.8 CM
Department of Defense-Congressionally
Directed Medical Research Programs $100,000/year
PI: Thurman Wheeler
Extracellular vesicles as therapeutic vehicles for myotonic dystrophy
The major goals of this project are to determine the feasibility and therapeutic activity of exo-AAV encoding CRISPR/Cas9 constructs in a mouse model of myotonic dystrophy type 1.
Grant management specialist: Jamie Shortall
U.S. Army Medical Research Acquisition Activity
Fort Detrick, MD 21702-5014
Overlap: None

W81XWH-20-1-0293 01/15/20 - 01/14/23 2.4 CM
Department of Defense-Congressionally
Directed Medical Research Programs $400,000/year
PI: Thurman Wheeler
Extracellular RNA biomarkers of myotonic dystrophy
The major goals of this project are, 1) identify and characterize the extracellular RNA profile in myotonic dystrophy, and 2) determine the natural history of extracellular RNA biomarkers and quantitative measurements of muscle function in myotonic dystrophy.
Grant management specialist: Jamie Shortall
U.S. Army Medical Research Acquisition Activity
Fort Detrick, MD 21702-5014
Overlap: None

W81XWH-20-1-0492 01/15/20 - 01/14/23 1.8 CM
Department of Defense-Congressionally
Directed Medical Research Programs $400,000/year
PI: Thurman Wheeler
Exosomes as therapeutic vehicles for Duchenne muscular dystrophy
The major goals of this project are to develop a novel approach to micro-dystrophin gene transfer therapy that is designed to avoid vector degradation by neutralizing antibodies.
Grant management specialist: Jamie Shortall
U.S. Army Medical Research Acquisition Activity
Fort Detrick, MD 21702-5014
Overlap: None

Co-I Das
Previously active grant now closed:
National Institutes of Health
Understanding the protective effect of an APOE epsilon4-linked haplotype using integrative bioinformatics analysis
The goal of this project were to understand the protective effect of an APOE epsilon4-linked haplotype using integrative bioinformatics analysis
Grants management specialist: Nina Silverberg
Overlap: none

IOS Press Publishing House
IOS Press neuroscience and biology communications platform
The goal of this project was to develop and enhance the community website of the *Journal of Alzheimer's*

Disease and other IOS journals.
Grants management specialist: Popke Huizinga
Overlap: none

Previously pending grants now active:
W81XWH-20-1-0293 01/15/20 - 01/14/23 1.2 CM
Department of Defense-Congressionally
Directed Medical Research Programs $400,000/year
PI: Thurman Wheeler
Extracellular RNA biomarkers of myotonic dystrophy
The major goals of this project are, 1) identify and characterize the extracellular RNA profile in myotonic dystrophy, and 2) determine the natural history of extracellular RNA biomarkers and quantitative measurements of muscle function in myotonic dystrophy.
Grant management specialist: Jamie Shortall
U.S. Army Medical Research Acquisition Activity
Fort Detrick, MD 21702-5014
Overlap: None

Which other organizations were involved as partners?

Organization name: Boston Children's Hospital
Location of Organization: Boston, MA
Partner's contribution to the project: Collaboration, facilities