AWARD NUMBER: W81XWH-21-1-0182

TITLE: Short TDP-43 Isoforms as Therapeutic Targets and Biomarkers for Amyotrophic Lateral Sclerosis

PRINCIPAL INVESTIGATOR: Veronique Belzil, PhD

CONTRACTING ORGANIZATION: Mayo Clinic, Jacksonville, FL

REPORT DATE: May 2022

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE
May 2022

2. REPORT TYPE
Annual

3. DATES COVERED
01Apr2021-31Mar2022

4. TITLE AND SUBTITLE

Short TDP-43 Isoforms as Therapeutic Targets and Biomarkers for Amyotrophic Lateral Sclerosis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-21-1-0182

5c. PROGRAM ELEMENT NUMBER
FY20 - ALSRP

5d. PROJECT NUMBER
AL200117

5e. TASK NUMBER

5f. WORK UNIT NUMBER

6. AUTHOR(S)

Veronique Belzil, PhD; Sami Barmada, MD, PhD; Paul Valdmanis, PhD

E-Mail: belzil.veronique@mayo.edu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Mayo Clinic Jacksonville
4500 San Pablo Road
Jacksonville FL, 32224

8. PERFORMING ORGANIZATION REPORT NUMBER

01

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command

Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Background: Even though the majority of amyotrophic lateral sclerosis (ALS) patients have no family history of the disease or defined genetic risk factor, almost all exhibit nuclear clearance and cytoplasmic accumulation of transactive response DNA binding protein 43 (TDP-43), an RNA-binding protein critical for RNA processing. However, the mechanisms responsible for TDP-43 mislocalization and their impact on neuronal survival remain elusive. We recently uncovered two uncommon splicing events predicted to generate TDP-43 isoforms carrying a cryptic nuclear export sequence in a 18 amino acid segment replacing the canonical C-terminus. The cytoplasmic distribution of these highly insoluble shortened (s)TDP-43 isoforms is upregulated by hyperactivity — another unexplained phenomenon in ALS — and downregulated by silencing neuronal activity. Importantly, these sTDP-43 isoforms retain the ability to interact with full-length (fl)TDP-43, and consequently recruit it to cytoplasmic aggregates. **Hypotheses:** As our preliminary studies demonstrate that sTDP-43 overexpression significantly increases the risk of death in isolated primary rodent neurons, we believe that therapeutic strategies capable of selectively targeting sTDP-43 isoforms will prevent nuclear clearance and cytoplasmic redistribution of flTDP-43, preserve its RNA regulatory functions, and prevent neurodegeneration in ALS. Moreover, we expect specific knockdown of shortened TAR DNA-binding protein (*sTARDBP*) to reduce the abundance of sTDP-43 isoforms in human cerebrospinal fluid (CSF) exosomes; these factors, in turn, may represent sensitive and specific biomarkers of target engagement. **Specific Aims:** First, we assess the pathophysiological significance and therapeutic potential of sTDP-43 isoforms in induced pluripotent stem cell (iPSC)-derived neurons and astrocytes and *in vivo*. Second, we determine the sensitivity and specificity of methods to detect *sTARDBP* transcripts and sTDP-43 isoforms from CSF exosomes, and examine the potential of exosomal *sTARDBP* transcripts and sTDP-43 isoforms to act as ALS biomarkers. **Study Design**: In Specific Aim 1, we design antisense oligonucleotides (ASOs) and short hairpin RNAs (shRNAs) targeting *sTARDBP* transcripts, test them in patient iPSC-derived neurons and astrocytes, and assess the effects of these perturbations on motor neuron and astrocyte survival. We determine the localization, function and regulation of flTDP43 after treatment, and assess for on- and off-target effects of *sTARDBP* knockdown. We then reduce sTDP-43 isoforms *in vivo* by delivering control and *sTARDBP* directed ASOs to vehicle-injected mice or those inoculated with adeno-associated virus (AAV) vectors encoding the chromosome 9 open reading frame 72 (*C9orf72*) hexanucleotide repeat expansion, and assess the effects of these perturbations upon molecular, pathological and behavioral outcomes in treated animals. In Specific Aim 2, we assess *sTARDBP* mRNA and sTDP-43 protein levels in exosomal fractions following ASO- and shRNA-mediated knockdown in human iPSC-derived neurons and astrocytes, and in mice injected with control or *sTARDBP* directed ASOs. We also extract exosomal RNA and proteins from the CSF of asymptomatic *C9orf72* cases, symptomatic *C9orf72* ALS patients, sporadic ALS patients, and control participants collected cross-sectionally and longitudinally, and measure *sTARDBP* mRNA and sTDP-43 protein levels within and across patients. **Innovation:** This proposal leverages *in vitro* and *in vivo* studies to test and develop selective knockdown strategies, in addition to a unique longitudinal bank of CSF samples from presymptomatic and symptomatic ALS patients, to explore sTDP-43 as both a therapeutic target and specific ALS biomarker. Our interdisciplinary team, including investigators with complementary expertise in disease modeling, TDP-43 biology and biomarker development, is uniquely suited for these studies, enabling an innovative and highly collaborative proposal focused on sTDP-43 and its potential consequences for ALS. **Impact:** Our studies center on a broadly applicable gene therapy approach with the potential to impact the large majority of ALS patients, and to be quickly translated from bench to bedside.

15. SUBJECT TERMS

Amyotrophic Lateral Sclerosis, antisense oligonucleotides, biomarkers, cerebrospinal fluid, shortened TDP-43 / *TARDBP*, therapeutic target.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRDC
a. REPORT	b. ABSTRACT	c. THIS PAGE	Unclassified	17	19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified			

TABLE OF CONTENTS

1. Introduction 5
2. Keywords 5
3. Accomplishments 5
4. Impact 14
5. Changes/Problems 14
6. Products 15
7. Participants & Other Collaborating Organizations 15
8. Special Reporting Requirements 17
9. Appendices 17

1. INTRODUCTION

Here we present the annual progress report of the project entitled ***'Short TDP-43 Isoforms as Therapeutic Targets and Biomarkers for Amyotrophic Lateral Sclerosis'***. The goal of this report is to show progress toward specific aims, achievements, stage of completion, as well as obtained HRPO, ORP forms, and IRB protocol approval. The report includes preliminary results, difficulties, and mitigation actions to ensure work continuation.

2. KEYWORDS

Amyotrophic Lateral Sclerosis, antisense oligonucleotides, biomarkers, cerebrospinal fluid, shortened TDP-43 / *TARDBP*, therapeutic target.

3. ACCOMPLISHMENTS

What were the major goals of the project?

Specific Aim 1: Establish gene therapy approaches to mitigate sTDP-43-associated neurotoxicity.

Major Task 1: Selective knockdown of *sTARDBP* transcripts in iPSC-derived neurons and astrocytes.

Subtask 1: Test *sTARDBP*-specific ASOs and shRNAs in HeLa and N2A cells (months 2-4).
Estimated completion: 75%

Milestone 1: Identify >3 ASOs and >2 shRNAs capable of reducing *sTARDBP* mRNA and sTDP-43 protein in HeLa cells and N2A cells.

Subtask 2: Test *sTARDBP*-specific ASOs and shRNAs in human iPSC-derived motor neurons and astrocytes (months 5-8).
Estimated completion: 50%

Milestone 2: Identify >2 ASOs and >1 shRNA capable of reducing *sTARDBP* mRNA and sTDP-43 protein in human iPSC- derived motor neurons and astrocytes.

Subtask 3: Determine if ASO/shRNA-mediated *sTARDBP* knockdown improves the survival of human iPSC-derived motor neurons and astrocytes (months 9-15).
Estimated completion: 25%

Milestone 3: Track the survival of human iPSC-derived motor neurons in isolation or co-cultured with astrocytes, with and without *sTARDBP* knockdown.

Major Task 2: Evaluate flTDP-43 localization, function, and autoregulation in response to *sTARDBP* knockdown.

Subtask 1: Examine flTDP-43 abundance and subcellular localization in human iPSC-derived motor neurons and astrocytes following *sTARDBP* knockdown (months 9-13).
Estimated completion: 25%

Milestone 4: Determine if *sTARDBP* knockdown affects flTDP-43 levels and localization in human iPSC-derived motor neurons.

Subtask 2: Evaluate Dendra2-TDP-43 abundance and subcellular localization in genetically modified human iPSC-derived motor neurons and astrocytes following *sTARDBP* knockdown (months 14-18).
Estimated completion: 0%

Subtask 3: Investigate and catalog flTDP43-mediated splicing events after *sTARDBP* knockdown, using next generation sequencing (months 19-24).
Estimated completion: 0%

Milestone 5: Evaluate if *sTARDBP* knockdown affects flTDP-43 splicing and autoregulation in human iPSC-derived motor neurons.
Milestone 6: Confirm the specificity of *sTARDBP*-targeting strategies in human iPSC-derived motor neurons.

Major Task 3: Delivery and validation of ASOs targeting *sTARDBP* transcripts in a mouse model of ALS.

Subtask 1: Obtain regulatory approval from local IRB, local IACUC, and DoD ACURO (months 1-3).
Estimated completion: 100%

Subtask 2: Preparation of adeno-associated viral vectors (months 1-2).
Estimated completion: 100%

Subtask 3: ICV injection of AAVs in mice followed by stereotactic delivery of ASOs (months 3-14).
Estimated completion: 0%

Subtask 4: Behavioral tests, biochemical analyses, and histology (months 12-21).
Estimated completion: 0%

Milestone 7: Improved Rotarod function.
Milestone 8: *sTARDBP* knockdown.
Milestone 9: Reduced pTDP-43 aggregates.

Specific Aim 2: Demonstrate that exosomal *sTARDBP* mRNA and sTDP-43 proteins are detectable in biofluids and are reliable biomarkers for ALS.

Major Task 1: Assess levels of exosomal *sTARDBP* mRNA and sTDP-43 proteins in CSF from pre-symptomatic cases, symptomatic ALS patients, and control participants.

Subtask 1: Obtained regulatory approval from local IRB and DoD HRPO (months 1-6).
Estimated completion: 100%

Subtask 2: Selection and preparation of CSF samples (months 6-7).
Estimated completion: 80%

Subtask 3: Testing of affinity and precipitation methods to purify CSF exosomes (months 6-7).
Estimated completion: 50%

Subtask 4: Purification of CSF exosomes. RNA and protein extractions. *flTARDBP*, *sTARDBP*, flTDP-43 and sTDP-43 quantification (months 7-11).
Estimated completion: 20%

Milestone 10: Consistent detection of upregulated *sTARDBP* mRNA and sTDP-43 proteins levels in c9ALS and sALS CSF exosomes, supporting the use of these factors as diagnostic biomarkers.
Milestone 11: Assessment of longitudinal changes in *sTARDBP* mRNA and/or sTDP-43 protein levels over the course of disease, indicating their utility for predicting disease outcome as prognostic biomarkers.

Major Task 2: Assess post-treatment levels of exosomal *sTARDBP* mRNA and sTDP-43 proteins from *in vitro* and *in vivo* studies.

Subtask 1: Collection of conditioned media from human iPSC-derived motor neurons and astrocytes at days 3, 5, and 7 after *sTARDBP* knockdown (months 5-9).
Estimated completion: 0%

Subtask 2: Purification of exosomal fractions. RNA and protein extractions. *flTARDBP*, *sTARDBP*, flTDP-43 and sTDP-43 quantification (months 10-14).

Estimated completion: 0%

Subtask 3: Statistical analyses of *in vitro* data (month 15).
Estimated completion: 0%

Subtask 4: Collection of postmortem CSF from mice sacrificed at 3, 6, and 12 months (months 12-21).
Estimated completion: 0%

Subtask 5: Purification of CSF exosomes. RNA and protein extractions. *flTARDBP*, *sTARDBP*, flTDP-43 and sTDP-43 quantification (months 17-21).
Estimated completion: 0%

Subtask 6: Statistical analyses of *in vivo* data (month 22).
Estimated completion: 0%

Milestone 12: Demonstrate that *sTARDBP* is a therapeutic target for ALS.
Milestone 13: Demonstrate that exosomal *sTARDBP* mRNA and/or sTDP-43 proteins can act as biomarkers of target engagement.

What was accomplished under these goals?

1) Major activities

A significant focus of the initial phase of the project involved **Specific Aim 1**, Major Task 1 (selective knockdown of *sTARDBP* transcripts in iPSC-derived neurons and astrocytes), specifically *Subtask 1* (test *sTARDBP*-specific ASOs and shRNAs in HeLa and N2A cells). We also developed the reagents and methodology for conducting studies in *Subtask 2* (test *sTARDBP*-specific ASOs and shRNAs in human iPSC-derived motor neurons and astrocytes) and *Subtask 3* (determine if ASO/shRNA-mediated *sTARDBP* knockdown improves the survival of human iPSC-derived motor neurons and astrocytes). Finally, we obtained regulatory approval from local IRB, local IACUC, and DoD ACURO (*Subtask 1*), allowing us to start the work described in Major Task 3 (delivery and validation of ASOs targeting *sTARDBP* transcripts in a mouse model of ALS).

Another significant focus of the initial phase of the project involved **Specific Aim 2**, Major Task 1 (assess levels of exosomal *sTARDBP* mRNA and sTDP-43 proteins in CSF from pre-symptomatic cases, symptomatic ALS patients, and control participants). We completed *Subtask 1* by obtaining regulatory approval from local IRB and DoD HRPO, and initiated *Subtask 2* by starting the selection and preparation of CSF samples. *Subtasks 3-4* (testing of affinity and precipitation methods to purify CSF exosomes & purification of CSF exosomes/ RNA and protein extractions/ *flTARDBP*, *sTARDBP*, flTDP-43 and sTDP-43 quantification) is ongoing.

2) Specific objectives

Our first goal was to receive all the necessary approvals to start our studies. We received approval for animal studies from the University of Washington IRB and IACUC on April 20th, 2021. We also submitted our animal use regulatory protocols and received approval from DoD ACURO on May 27th, 2021. Animal protocols are now in place for the conduction of mouse studies as outlined in **Specific Aim 1**, Major Task 3. We have the capacity to handle experiments stemming from ASOs deemed efficacious from Major Task 1. We also submitted an IRB protocol and HRPO forms to support our CSF work, and received approval from Mayo Clinic on July 14th, 2021 and September 17th, 2021, respectively. Mayo Clinic IRB approved the use of antemortem and postmortem CSF human samples using internal and external (collaborators) CSF repositories.

Our second goal was to identify >3 antisense oligonucleotides (ASOs) and >2 short-hairpin (sh)RNAs capable of reducing *sTARDBP* mRNA and sTDP-43 protein in HeLa cells and N2A cells (Milestone 1). We also sought to establish effective and accurate means of detecting sTDP43 protein in HeLa cells, N2A cells, human iNeurons and astrocytes.

Our third goal was to select postmortem and antemortem CSF samples from the Mayo Clinic Brain Bank and Biobank respectively, and secure antemortem CSF from the CReATe Consortium and the CRiALS Study.

Our fourth goal was to optimize our exosomes purification and characterization approach. After testing different techniques in brain tissues where exosomes are more abundant than CSF, we realized quickly that obtaining results from all three sizes of extracellular vesicles (EVs), exosomes being the smallest of the three, would provide additional information that is necessary to test our hypothesis. As such, we sought to establish effective and accurate means of purifying exosomes, microvesicles, and apoptotic bodies, characterize them, and extract RNA and proteins from each EV fraction.

3) <u>Significant results</u>

Specific Aim 1: We began by designing four separate shRNA constructs targeting *sTARDBP* transcript 1 (*sTARDBP-1*), *sTARDBP* transcript 2 (*sTARDBP-2*), and full-length (fl)*TARDBP*. We also created shRNA constructs against a negative control, the fluorescent protein mApple. All shRNAs were cloned into a mammalian expression vector (pLV-LTHM) enabling their introduction by transient transfection. The pLV-LTHM vector can also be used to generate lentiviral particles encoding shRNAs in the proper context for transduction into iPSC-derived neurons and/or astrocytes.

Following transient transfection of shRNA constructs into HeLa cells, the abundance of sTDP-43 protein was estimated by immunofluorescence (IF), taking advantage of antibodies that we have created previously, which are capable of selectively recognizing sTDP-43. These antibodies were raised against an 18-amino acid stretch that is common to sTDP-43-1 and sTDP-43-2, allowing us to detect changes in protein levels from shRNAs directed against either isoform. Simultaneously, we visualized changes in flTDP43, to ensure that the shRNA constructs are knocking down sTDP43, and not flTDP43. Unfortunately, despite effective knockdown of flTDP43 via *flTARDBP*-directed shRNAs, none of the eight shRNAs that were designed against *sTARDBP-1* or *sTARDBP-2* showed reliable effects on sTDP43 protein levels by IF (data not shown). Similar results were obtained in mouse N2A cells. Together, these data suggest that shRNA is not a tractable mechanism for targeted reduction of *sTARDBP* transcripts in cultured cells.

Subsequent efforts focused on the design and testing of ASOs against *sTARDBP* transcripts. All ASOs were composed of 20 phosphothiorate

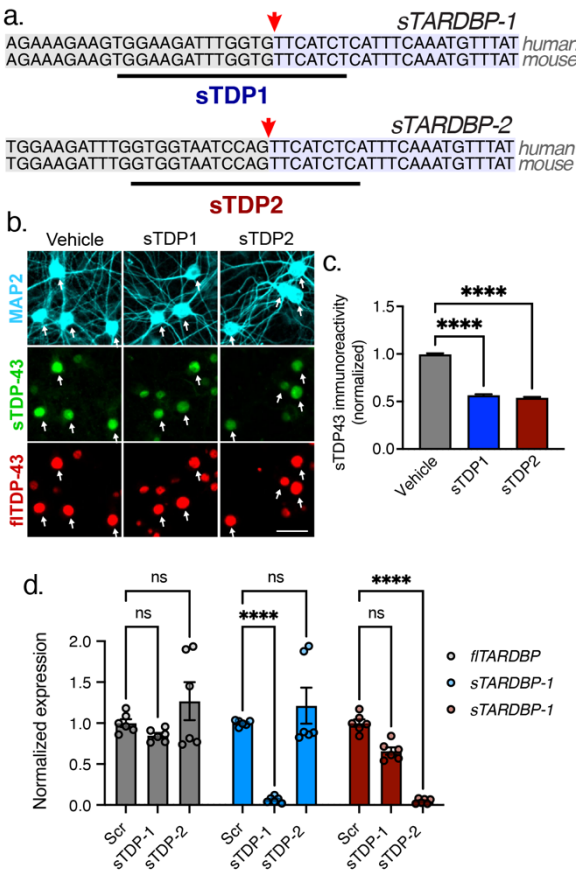

Figure 1: ASO-mediated knockdown of sTDP-43. (**a**) Schematic of splice junctions unique to *sTARDBP-1* or *sTARDBP-2*. ASO targeting regions are underlined. (**b**) 500nM ASOs were applied to rat primary cortical neurons. After 72h, cells were fixed and stained for the pan-neuronal marker MAP2 (cyan), sTDP-43 (green) or flTDP-43 (red). White arrows mark neurons. Scale bar, 25um. (**c**) sTDP1 and sTDP2 significantly reduced sTDP-43 protein compared to (d) HEK293T cells were treated with the indicated ASO (100nM each) for 30-40h, and *TARDBP* transcripts expression gauged by qRT-PCR. ****p<0.0001 two-way ANOVA with Dunnett's test. Error bars in (c-d), SEM. Data combined from >3 replicates/condition.

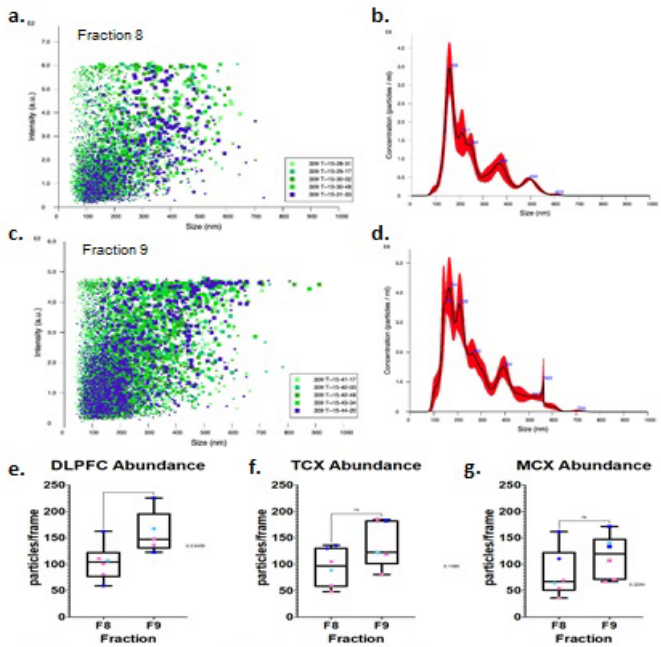

Figure 2. Fraction separation of EVs in brain tissue samples. (a) Fraction 8 NanoShight abundance/size and (b) concentration/size peaks outputs. (c) Fraction 9 NanoShight abundance/size and (d) concentration/size peaks outputs. Comparison of abundance of particles/fractions 8 and 9 isolated from (e) dorsolateral prefrontal cortex–DLFPC, (f) temporal cortex-TCX, and (g) motor cortex-MCX.

(PS)-modified nucleotides. Five bases on the extreme 5' were modified by 2'-O-methoxyethylation (2'-MOE), as were the 5 bases on the extreme 3' end. This 'gapmer' design allows for increased ASO stability and knockdown efficacy. ASOs directed against *sTARDBP-1* and *sTARDBP-2* (500nM each) significantly reduced sTDP-43 protein levels in rodent mixed primary cortical neurons, as judged by IF (**Figure 1a-c**). We also tested these ASOs in HEK293T cells, demonstrating a selective knockdown of *sTARDBP-1* and *sTARDBP-2* transcripts without affecting *flTARDBP* mRNA (**Figure 1d**). In ongoing experiments, we are applying candidate ASOs to human iPSC-derived motor neurons and astrocytes and evaluating *TARDBP* transcripts by IF, qRT-PCR and RNA-seq (Major Task 1: *Subtasks 2 and 3*, Major Task 2: *Subtasks 1-3*). These investigations will set the stage for *in vivo* studies described in Major Task 3.

Specific Aim 2: We isolated EVs from brain tissue homogenates of dorsolateral prefrontal cortex (DLPFC), temporal cortex (TCx) and motor cortex (MCx). Homogenates underwent filtration and four sequential centrifugation steps to separate the different EV sizes using size exclusion chromatography columns, or qEV isolation. After the isolation of the three fractions, we performed NanoSight Tracking analysis to profile the vesicles according to size and abundance. We obtained three fractions containing EVs: fraction 7, 8 and 9. The smallest particles were found in fraction 7, however in very small quantities, suggesting that the brain extraction protocol is established but still needs to be optimized. Of note, the abundance of EV fractions varied across brain regions, so we anticipate to also have a difference in abundance in postmortem and antemortem CSF (**Figure 2**).

Regarding CSF isolation, we successfully developed a protocol for high yield EV precipitation (1-8x10^{10} nanoparticles/mL of CSF). We have optimized the volume of CSF required to 750 µL. We used NanoSight Tracking analysis to quantify the concentration of EVs and analyze the size distribution. Our postmortem CSF results show significant differences between *C9orf72-* ALS, *C9orf72+* ALS, and controls participants. The EV profile of *C9orf72-* ALS patients show a high concentration of small nanoparticles (48-100 nm). In contrast, *C9orf72+* ALS patients and controls show prominent peaks between 130-180 nm. Furthermore, the concentration of EVs is increased in *C9orf72-* ALS patients compared to *C9orf72+* ALS patients and controls. Western blot analysis shows the presence of sTDP43 in EVs isolated from the CSF in most cases. Finally, we further characterized EVs using transmission electron microscopy (**Figure 3**).

4) Other achievements

In addition to the work described above, we also developed new means of tracking sTDP-43 protein levels *in vitro* and *in vivo*. At the time that the proposal was submitted, we had a single sTDP-43 polyclonal antibody that showed specific reactivity via immunofluorescence, but not immunoblotting. It also detected sTDP-43 deposits *in vivo* by immunohistochemistry, but its

sensitivity was limited. Since then, we generated novel recombinant antibodies capable of sensitive and specific detection of sTDP-43 by immunoblot, as well as immunofluorescence and immunohistochemistry (data not shown). These reagents now enable us to measure sTDP-43 reductions through multiple approaches. Furthermore, because of the recombinant nature of the antibodies, we can produce as much monoclonal antibody as needed without the need for hybridomas. One additional advantage is the flexibility to include the Fc region of choice when generating more recombinant antibody. Therefore, while our current sTDP-43 monoclonal antibodies carry a mouse Fc region, we can adjust this as dictated by the experiment to include rabbit, human, or goat Fc regions, or attach the sTDP-43 targeting variable regions to bispecific antibodies enabling penetration of the blood brain barrier. These technologies promise to be invaluable in designing *in vivo* biomarkers and/or therapeutics for the detection and targeting of sTDP-43.

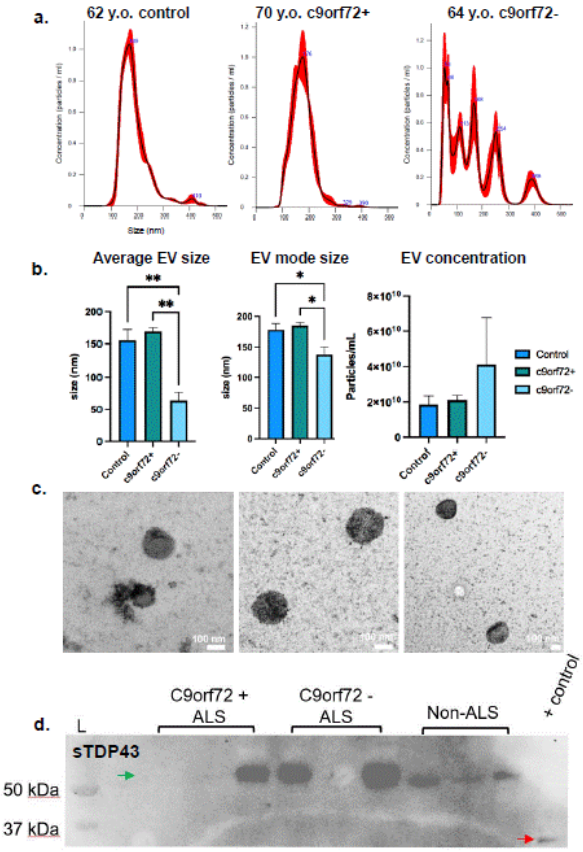

Figure 3. Characterization of CSF derived EVs in ALS patients. (**a**) Distribution of EVs isolated from non-ALS controls, c9orf72+ ALS patients, and c9orf72- ALS patients according to their size and concentration. (**b**) Statistical analysis shows a significantly higher size of EVs in non-ALS controls and c9orf72+ ALS patients compared to c9orf72- ALS patients (One-way ANOVA, *p value=0.5, **p value=0.1) (**c**) Electron microscopy characterization of CSF-derived EVs. (**d**) Western blot analysis for sTDP43 shows an intense labeling at 60kDa in CSF-derived EVs (green arrow) and a 30 kDa band in sTDP43 obtained from a HeLa cell lysate transduced with the human isoform of sTDP43 (red arrow).

Describe the Regulatory Protocol and Activity Status (if applicable).

Our DoD-funded activities include the purification and characterization of antemortem and postmortem CSF exosomes in pre-existing human samples. Our studies will also generate data from animal studies and human iPSC-derived neurons and astrocytes. No new iPSC lines will be generated or created under these DoD-funded activities. The Biospecimen IRB# 21-005248 protocol for this project was submitted to Mayo Clinic IRB, and we obtained approval on July 14th, 2021. Our studies utilize two different sources of antemortem CSF: the Mayo Clinic Biobank and the University of Miami CReATe and CRiALS Repositories. Postmortem CSF specimens are obtained from the Mayo Clinic Brain Bank, as described in the documentation submitted to the DoD HRPO and the DoD ORP. As our studies only use pre-existing biospecimens, no consent form is needed. We also submitted protocols and consent forms of all clinical trials under which these pre-existing samples were collected.

Below are all protocols related to this project. Since there was no defined section for human biospecimens, we added all protocols in section **(b)**, including all human antemortem and postmortem samples (Biospecimen IRB Protocols submitted to HRPO as Secondary Research Involving the Use of Data/Specimen and ORP as cadaver use submission).

(a) Human Use Regulatory Protocols

No human subjects research will be performed to complete the Statement of Work.

(b) Use of Human Cadavers for Research Development Test & Evaluation (RDT&E), Education or Training

- Human antemortem and postmortem CSF samples will be used to complete **Specific Aim 2**, Major Task 1.
  Mayo Clinic IRB# 21-005248
  Title: Biospecimen processing to investigate pathophysiological significance and therapeutic potential of TDP-43 isoforms in amyotrophic lateral sclerosis (ALS).

The Reviewer approved waiver of informed consent and HIPAA authorization in accordance with applicable regulations for data/specimens collected under Mayo Clinic IRB# 15-009452, IRB# 13-004314, IRB# 07-005711, IRB# 11-002025, IRB# 12-004718, IRB# 12-00795, IRB# 15-001187, IRB# 18-008270, and University of Miami IRB ePROST# 20160603 and #20101021. All consent forms were approved by the DoD HRPO on September 17th, 2021.

- Human iPSCs will be used to complete **Specific Aim 1**.
  University of Michigan IRB# HUM00028826
  Title: Epidemiologic Risk Factors and the Genetics of ALS

(c) Animal Use Regulatory Protocols
One animal protocol is required to complete the Statement of Work.

TOTAL PROTOCOL(S): 1

PROTOCOL (1 of 1 total):

Protocol ***AL200117.e001***

Title: RNAi as gene therapy

Target required for statistical significance: 298

Target approved for statistical significance: 298

SUBMITTED TO AND APPROVED BY:

Protocol Principal Investigator Paul Valdmanis, Ph.D.

STATUS: APPROVED BY ACURO AS OF 05/27/2021

Animal protocols are in place for the conduction of mouse studies as outlined in **Specific Aim 1**, Major Task 3. We have the capacity to handle experiments stemming from ASOs deemed efficacious from Major Task 1.

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

Specific Aim 1: Establish gene therapy approaches to mitigate sTDP-43-associated neurotoxicity.

Major Task 1: Selective knockdown of *sTARDBP* transcripts in iPSC-derived neurons and astrocytes.

Subtask 1: Test *sTARDBP*-specific ASOs and shRNAs in HeLa and N2A cells.

We intend to continue testing ASOs for their ability to selectively target sTDP-43 protein in HeLa cells and N2A cells. Once the optimal dosing and length of treatment has been confirmed, we will also evaluate *sTARDBP-1* and *sTARDBP-2* mRNA levels in response to ASO application.

Subtask 2: Test *sTARDBP*-specific ASOs and shRNAs in human iPSC-derived motor neurons and astrocytes.

We will culture iPSC-derived motor neurons and test the most efficient ASOs identified in Subtask 1 for their ability to reduce *sTARDBP-1* and *sTARDBP-2* mRNA, as well as sTDP-43 protein.

Simultaneously, we will differentiate astrocytes from human iPSCs and evaluate *sTARDBP* mRNA and sTDP-43 protein in response to ASO treatment.

Subtask 3: Determine if ASO/shRNA-mediated *sTARDBP* knockdown improves the survival of human iPSC-derived motor neurons and astrocytes.

We will culture iPSC-derived motor neurons and astrocytes, then apply the ASOs that proved most effective in reducing *sTARDBP* mRNA and sTDP-43 protein. Over the next 10-14d, the survival of neurons and astrocytes will be monitored by longitudinal fluorescence microscopy and automated survival analysis, highly sensitive and quantitative approaches for estimating any changes in survival due to *sTARDBP* knockdown.

Major Task 2: Evaluate flTDP-43 localization, function, and autoregulation in response to *sTARDBP* knockdown.

Subtask 1: Examine flTDP-43 abundance and subcellular localization in human iPSC-derived motor neurons and astrocytes following *sTARDBP* knockdown.

After applying the most effective ASOs identified in Major Task 1 (*Subtask 2*), we will visualize flTDP-43 by immunofluorescence and immunoblotting using antibodies specific for this variant. These studies will enable us to determine if *sTARDBP* knockdown interferes with flTDP-43 localization and autoregulation, an essential process for maintaining flTDP-43 homeostasis in neurons and other cell types.

Subtask 2: Evaluate Dendra2-TDP-43 abundance and subcellular localization in genetically modified human iPSC-derived motor neurons and astrocytes following *sTARDBP* knockdown.

These studies are similar to those described above in *Subtask 1*, but we will use novel lines of human iPSC-derived motor neurons and astrocytes that express TDP-43 that has been labeled at the N- or C-terminus with the fluorescent protein Dendra2. These lines allow us to determine if ASO-mediated *sTARDBP* knockdown affects the abundance and/or localization of native TDP-43 isoforms in living cells.

Subtask 3: Investigate and catalogue flTDP43-mediated splicing events after *sTARDBP* knockdown, using next generation sequencing.

flTDP-43 is crucial for RNA splicing, transport, stability and translation. We and others have characterized the changes in splicing that result from a relative reduction or increase in flTDP-43 abundance. Using this information, we will determine how or if *sTARDBP* knockdown affects the splicing function of endogenous flTDP-43 through next generation transcriptomics (RNA-seq). After application of control ASOs or those most effective at reducing *sTARDBP* transcripts, human iPSC-derived motor neurons will be collected, and RNA isolated for high-depth (minimum 100M reads/sample) sequencing. Subsequent bioinformatic analyses will be accomplished using LeafCutter or Majiq, methods that allow for detection of flTDP-43-dependent unannotated cryptic splicing events that might otherwise be overlooked by conventional approaches.

Major Task 3: Delivery and validation of ASOs targeting *sTARDBP* transcripts in a mouse model of ALS.

Subtask 2: Preparation of adeno-associated viral vectors.

rAAV viruses will be concentrated in sterile filtered phosphate buffered saline to 3x1013 vg/ml.

Subtask 3: ICV injection of AAVs in mice followed by stereotactic delivery of ASOs.

rAAV viruses will be delivered by intracerebroventricular (ICV) injection to postnatal day 0 or 1 mice. Control or *sTARDBP*-directed ASOs will be administered at week 5 by ICV stereotactic injections into the right ventricle to vehicle-injected mice or those receiving *C9orf72* (2 or 149 copies).

Subtask 4: Behavioral tests, biochemical analyses and histology.

We will test our mice behavioral function using Rotarod and wire hang tests starting at 7 weeks of age and continue until the last experimental endpoint (testing at 3, 6 and 12 months). We will also monitor *sTARDBP* mRNA and sTDP-43 protein levels by immunoblotting, immunohistochemistry and single-molecule in situ hybridization at 3, 6 and 12 months after ASO administration, and track neuron loss, neuroinflammation, flTDP-43 localization/function/phosphorylation, and markers of excitability.

Specific Aim 2: Demonstrate that exosomal *sTARDBP* mRNA and sTDP-43 proteins are detectable in biofluids and are reliable biomarkers for ALS.

Major Task 1: Assess levels of exosomal *sTARDBP* mRNA and sTDP-43 proteins in CSF from pre-symptomatic cases, symptomatic ALS patients, and control participants.

Subtask 2: Selection and preparation of CSF samples.

We will prepare the Material Transfer Agreement (MTA) and request CSF samples from the University of Miami. All CSF samples will be aliquoted and stored in our RLIMS system until ready to be processed.

Subtask 3: Testing of affinity and precipitation methods to purify CSF exosomes.

We will continue optimizing the approach in post-mortem CSF, and move on to using ante-mortem CSF.

Subtask 4: Purification of CSF exosomes. RNA and protein extractions. *flTARDBP*, *sTARDBP*, flTDP-43 and sTDP-43 quantification.

We recently acquire an ultracentrifuge for EV isolation using our own laboratory funds. This significantly increases our capability of optimizing the methodology and purify the three EV fractions from CSF. We will also work on further characterizing the three CSF EV fractions for all samples using electron microscopy and mass spectrometry, and we will extract RNA and proteins from these three fractions. We will use the nCounter NanoString Platform for RNA quantification, and the affinity capture (AC) method for proteomic profiling.

Subtask 5: Statistical analyses.

We will conduct statistical analyses of data obtained from *flTARDBP*, *sTARDBP*, flTDP-43 and sTDP-43 expression measures, ALSFRS-R and ALS-CBS scores, and other clinical and demographic information collected from study subjects.

Major Task 2: Assess post-treatment levels of exosomal *sTARDBP* mRNA and sTDP-43 proteins from *in vitro* and *in vivo* studies.

Subtask 1: Collection of conditioned media from human iPSC-derived motor neurons and astrocytes at days 3, 5, and 7 after *sTARDBP* knockdown.

We will treat iMNs and iAstrocytes with NT or *sTARDBP*-directed ASO or shRNA, and we will collect conditioned media after 3, 5 and 7 days.

Subtask 2: Purification of exosomal fractions. RNA and protein extractions. *flTARDBP*, *sTARDBP*, flTDP-43 and sTDP-43 quantification.

We will purify the three EV fractions from the media collected after 3, 5 and 7 days, we will extract RNA and proteins from each fraction, and we will quantify *flTARDBP*, *sTARDBP*, flTDP-43 and sTDP-43.

Subtask 3: Statistical analyses of *in vitro* data.

We will conduct statistical analyses of data obtained from *flTARDBP*, *sTARDBP*, flTDP-43 and sTDP-43 expression measures.

Subtask 4: Collection of postmortem CSF from mice sacrificed at 3, 6, and 12 months.

We will collect postmortem CSF from mice injected with either NT or *sTARDBP*-directed ASOs, and sacrificed at 3, 6, and 12 months.

Subtask 5: Purification of CSF exosomes. RNA and protein extractions. *flTARDBP*, *sTARDBP*, flTDP-43 and sTDP-43 quantification.

We will purify the three EV fractions from mice CSF sacrificed at 3, 6, and 12 months, we will extract RNA and proteins from each fraction, and we will quantify *flTARDBP*, *sTARDBP*, flTDP-43 and sTDP-43.

Subtask 6: Statistical analyses of *in vivo* data.

We will conduct statistical analyses of data obtained from *flTARDBP*, *sTARDBP*, flTDP-43 and sTDP-43 expression measures.

4. IMPACT

As TDP-43 pathology is a characteristic signature found in >95% of individuals with ALS, our studies have the potential to impact the large majority of ALS patients, including those without a known disease-associated mutation. Importantly, should our studies confirm *sTARDBP* transcripts as relevant therapeutic targets, and *sTARDBP* and sTDP-43 as reliable biomarkers for ALS, Mayo Clinic is committed to translating these advances as quickly as possible to the ALS Clinic.

5. CHANGES/ PROBLEMS

a. Actual Problems or delays and actions to resolve them

As described above in Section 1, none of the shRNAs we designed showed significant effects on the abundance of sTDP-43 protein isoforms in HeLa or N2A cells. Although these results were disappointing, we have identified candidate ASOs that effectively reduce sTDP-43 protein levels, and plan to focus our efforts on this strategy for all subsequent experiments. We do not expect these findings to affect the ultimate translatability of our findings, since ASOs have been successfully applied *in vivo* in rodent disease models, non-human primate models, and in human clinical trials. Furthermore, co-Investigator Dr. Valdmanis is experienced with the use of ASOs in animal models of ALS. Once our initial characterization of the specificity and efficacy of these ASOs is completed, we anticipate moving forward with the most promising candidate ASOs in animal models of ALS, as described in **Specific Aim 1**, Major Task 3.

We also experienced considerable delays due to the search and hiring of a research fellow at Mayo Clinic. Dr. Maria Jose Ulloa was hired in December 2021 and is currently working on extracellular vesicle methodology standardization, optimization, and characterization from CSF samples.

b. Anticipated Problems/Issues

Potential problem 1: We will be unable to identify a strategy for the selective knockdown of *sTARDBP* mRNA or sTDP-43 protein in cultured cells or animal models.

Mitigating actions: This concern is partially addressed by data presented in this report (**Figure 1**) showing effective knockdown of sTDP-43 protein in rodent primary neurons. Still, if subsequent data show inefficacy in human motor neurons or astrocytes, or within mouse CNS *in vivo*, we will employ an alternate strategy involving CRISPR to eliminate the 3' splice acceptor site required for *sTARDBP* generation. We may also utilize recombinant antibodies to elicit TRIM21-dependent reductions in sTDP-43 protein levels.

Potential problem 2: Despite effective knockdown of *sTARDBP* using ASOs in cells, we will be unable to achieve sufficient *sTARDBP* knockdown to ameliorate motor deficits in a mouse model of ALS bearing a pathogenic *C9orf72* repeat expansion.

Mitigating actions: We will increase doses and frequency of ASO delivery in mice, as outlined in alternatives to our mouse experimental plan. We may also consider various ASO delivery paradigms, such as intrathecal administration, which may lead to higher spinal motor neuron transduction levels but is limited by difficulties in delivery of a high volumes of ASOs. We may also consider alternative mouse models including a wildtype TDP-43 overexpression model or a regulatable TDP-43 model with mutations in the nuclear localization signal.

6. PRODUCTS

In addition to ASOs targeting *sTARDBP* transcript variants described above (Major Task 1), we generated recombinant antibodies against sTDP-43 protein variants that promise to be invaluable for these and other studies centering on the function and relevance of these isoforms to ALS detection, pathogenesis and treatment. We aim to establish CSF exosome profiling as a source of diagnostic biomarkers that can be quickly translated to the clinic.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Mayo Clinic Team

Name	**Veronique Belzil**
Project Role	Principal Investigator
Researcher Identifier (e.g. ORCID ID)	eRA commons ID: verobelzil
Nearest person month worked	1

Contribution to Project: Dr. Belzil oversees all aspects of the project, specifically those described in **Specific Aim 2**. She organizes monthly meetings with the three groups, ensures timeline is respected, and make sure milestones are achieved. She works closely with Drs. Oskarsson (Mayo), Dickson (Mayo), and Benatar (Miami) to obtain human CSF samples, and supervises the student and postdoctoral fellow in charge of purifying and characterizing EVs from human CSF, mouse CSF, and cell culture media. Dr. Belzil assists in designing experiments, interpreting results, assembling data for presentations and manuscripts, and presenting results at national/international meetings.

Name	**Bjorn Oskarsson**
Project Role	Consultant
Researcher Identifier (e.g. ORCID ID)	eRA commons ID: OSKARSSON.B
Nearest person month worked	1

Contribution to Project: Dr. Oskarsson works closely with Dr. Belzil to provide human CSF samples collected cross-sectionally or longitudinally from his patients, along with their respective de-identified demographic/clinical information.

Name	**Dennis Dickson**
Project Role	Consultant
Researcher Identifier (e.g. ORCID ID)	eRA commons ID: ddickson
Nearest person month worked:	1

Contribution to Project: Dr. Dickson works closely with Dr. Belzil to provide post-mortem CSF obtained from Mayo Clinic Brain Bank cases, along with their respective de-identified demographic/clinical information.

Name	**Luc Pregent**
Project Role	Senior technician
Researcher Identifier (e.g. ORCID ID)	eRA commons ID: NA
Nearest person month worked	1

Contribution to Project: Mr. Pregent helps with the processing and organizing of all samples used for these studies. He is also responsible for RNA and protein extractions.

Name	**Erica Engelberg-Cook**
Project Role	Research Scientist
Researcher Identifier (e.g. ORCID ID)	eRA commons ID: ERICACOOK
Nearest person month worked	1

Contribution to Project: Dr. Engelberg-Cook supports Dr. Belzil in all aspects of the project, specifically those described in **Specific Aim 2**. She helps with the organization of monthly meetings, with supervising laboratory work, and with other related administrative tasks necessary to ensure continuity of the studies. She is involved in all aspects of animal and human regulatory documentation, IRB protocol approvals, and DoD regulatory approvals.

Name	**Maria Jose Ulloa**
Project Role	Research Fellow
Researcher Identifier (e.g. ORCID ID)	eRA commons ID: NA
Nearest person month worked	0.5

Contribution to Project: Dr. Ulloa works on EVs methodology standardization, optimization, and characterization. She is also responsible for presentations and manuscript preparation.

Name	**Marissa Russo**
Project Role	Graduate student
Researcher Identifier (e.g. ORCID ID)	eRA commons ID: NA
Nearest person month worked	2

Contribution to Project: Ms. Russo worked on purifying EVs from brain/CSF. She was also in charge of analyzing the data generated and present results at our monthly meeting.

University of Michigan Team

Name	**Sami Barmada**
Project Role	Co-investigator
Researcher Identifier (e.g. ORCID ID)	eRA commons ID barmsam
Nearest person month worked	1

Contribution to Project: Dr. Barmada oversees studies described in **Specific Aim 1**, Major Tasks 1-2. He also assists in designing experiments, interpreting results, assembling data for presentations and manuscripts, and discussing data at regularly scheduled meetings of our consortium as well as national/international seminars.

Name	**Megan Dykstra**
Project Role	Graduate student
Researcher Identifier (e.g. ORCID ID)	eRA commons ID dykstram
Nearest person month worked	1

Contribution to Project: Ms. Dykstra conducts studies on ASOs and shRNAs in cultured cells (HeLa, N2A) and human iPSC-derived motor neurons and astrocytes. She is also responsible for interpreting and analyzing data and presenting at regularly scheduled meetings of our consortium.

Name	**Elizabeth Tank**
Project Role	Research investigator
Researcher Identifier (e.g. ORCID ID)	eRA commons ID: NA
Nearest person month worked	2

Contribution to Project: Dr. Tank is responsible for culturing human iPSCs and for their differentiation into motor neurons and astrocytes. She also assists with experimental design, data interpretation, survival studies of iPSC-derived motor neurons and astrocytes, and manuscript preparation.

Name	**Xingli Li**
Project Role	Laboratory Specialist

Researcher Identifier (e.g. ORCID ID)	eRA commons ID: NA
Nearest person month worked	2

Contribution to Project: Dr. Li assists with the preparation of HeLa and N2A cells for studies described in **Specific Aim 1**, Major Task 1.

University of Washington Team

Name	**Paul Valdmanis**
Project Role	Co-investigator
Researcher Identifier (e.g. ORCID ID)	eRA commons ID: VALDMANIS.PAUL
Nearest person month worked	1

Contribution to Project: Dr. Valdmanis oversees animal studies described in **Specific Aim 1**, Major Tasks 3. In addition, he aids in shRNA experimental design and troubleshooting (**Specific Aim 1**, *Major Task 1 and 2*) as well as interpreting results and discussing data at regularly scheduled meetings of our collective group.

Name	**Kathryn Gudsnuk**
Project Role	Research Scientist
Researcher Identifier (e.g. ORCID ID)	eRA commons ID: NA
Nearest person month worked	2

Contribution to Project: Ms. Gudsnuk is involved in all aspects of animal protocol approval through the University of Washington and alignment with the Department of Defense for ACURO approval. She assists with experimental design and implementation of mouse studies, animal ordering and husbandry.

Name	**Samuel Smukowski**
Project Role	Graduate student
Researcher Identifier (e.g. ORCID ID)	eRA commons ID: NA
Nearest person month worked	1

Contribution to Project: Mr. Smukowski designs strategies for mouse rAAV and ASO delivery as outlined in **Specific Aim 1**, *Major Task 3*. He contributes to discussions at our regularly scheduled consortium meetings.

8. SPECIAL REPORTING REQUIREMENTS

Quad Charts:

Not applicable.

9. APPENDICES

Not applicable.