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: April 2023

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
April 2023	Annual	01Apr2022-31Mar2023

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

6. AUTHOR(S)

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

E-Mail: belzil.veronique@mayo.edu

5d. PROJECT NUMBER
AL200117

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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 an 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. event neurodegeneration in ALS. Moreover, we expect specific knockdown of shortened TAR DNA-binding protein (*sTARDBP*)

15. SUBJECT TERMS
Amyotrophic Lateral Sclerosis, antisense oligonucleotides, biomarkers, biofluids, shortened TDP-43 / *TARDBP*, therapeutic target.

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

TABLE OF CONTENTS

1. INTRODUCTION

The goal of this annual progress report is to show progress and achievements obtained for our DoD funded project entitled ***'Short TDP-43 Isoforms as Therapeutic Targets and Biomarkers for Amyotrophic Lateral Sclerosis'*** and describe activities completion and resources use.

2. KEYWORDS

Amyotrophic Lateral Sclerosis, antisense oligonucleotides, biomarkers, biofluids, 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: 100%

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: 90%

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: 75%

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: 50%

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: 50%

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: 20%

Subtask 4: Behavioral tests, biochemical analyses, and histology (months 12-21).
Estimated completion: 10%

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: 100%

Subtask 3: Testing of affinity and precipitation methods to purify CSF exosomes (months 6-7).
Estimated completion: 100%

Subtask 4: Purification of CSF exosomes. RNA and protein extractions. *flTARDBP*, *sTARDBP*, flTDP-43 and sTDP-43 quantification (months 7-11).
Estimated completion: 50%

Milestone 10: flTDP-43 detection in CSF, no sTDP-43 detected. Neither flTDP-43, nor sTDP-43 were detected in EVs. We used saliva and detected both proteins.
Milestone 11: We detected sTDP-43, flTDP-43 and pTDP-43 in saliva and secretory cell types of salivary glands.

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

We initially focused on **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). A large part of our effort was devoted to verifying the magnitude, consistency, and specificity of *sTARDBP* knockdown strategies. We also conducted studies for *Subtask 2* (test *sTARDBP*-specific ASOs and shRNAs in human iPSC-derived motor neurons and astrocytes) and began performing investigations describe under *Subtask 3* (determine if ASO/shRNA-mediated *sTARDBP* knockdown improves the survival of human iPSC-derived motor neurons and astrocytes). Additionally, we made significant progress on Major Task 2 (flTDP-43 localization, function, and autoregulation in response to *sTARDBP* knockdown), in particular *Subtask 1* (flTDP-43 abundance and subcellular localization in human iPSC-derived motor neurons and astrocytes following *sTARDBP* knockdown) and *Subtask 2* (Dendra2-TDP-43 abundance and subcellular localization in genetically modified human iPSC-derived motor neurons).

Major Task 3 (delivery and validation of ASOs targeting *sTARDBP* transcripts in a mouse model of ALS). We successfully performed intrathecal injections of ASOs targeting *sTARDBP* in male and female mice and assessed mice for their ability to tolerate ASO delivery and robustness of *sTARDBP* knockdown.

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 Major task 1. However, we could not find any TDP-43 isoforms in extracellular vesicles isolated from CSF. Therefore, we moved on to study another biofluid, saliva. Using this fluid, we successfully detected sTDP-43, flTDP-43, and pTDP-43.

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. We also received approval for the collection of patient's saliva on October 6th, 2022. We have started collecting longitudinal saliva samples from ALS patients at Mayo Clinic, Jacksonville, and we are working on the logistics to collect samples at Mayo Clinic Rochester.

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). Following this, we aimed to knock down *sTARDBP* in human iPSC-derived neurons and astrocytes, then measure relevant phenotypes, including flTDP-43 levels and localization, RNA processing (via next generation RNA sequencing) as well as cellular survival.

Our third goal was to test efficaciousness of *sTARDBP* ASOs *in vivo* as outlined in **Specific Aim 1**, Major Task 3. We also sought to assess the ability of *sTARDBP* ASOs to prevent ALS-associated motor phenotypes and improve survival in a *C9orf72* repeat expansion mouse model that harbors concomitant TDP-43 pathology.

Our fourth 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 fifth 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) Significant results

Specific Aim 1: We initially tested four separate shRNA constructs targeting *sTARDBP* transcript 1 (*sTARDBP-1*), *sTARDBP* transcript 2 (*sTARDBP-2*), and full-length (fl)*TARDBP*, but failed to identify any candidate that effectively and specifically reduced *sTARDBP* in rodent or human cell lines. In contrast, we noted significant knockdown of *flTARDBP* RNA using shRNA, confirming the overall utility of this strategy. We suspect that the similarities between *flTARDBP* RNA and *sTARDBP* transcripts limit our ability to selectively target the latter without affecting the former using shRNA. We then focused our efforts on developing ASOs against *sTARDBP* transcripts. These 'gapmer' ASOs were composed of 20 phosphothiorate (PS)-modified nucleotides, with 2'-O-methoxyethylation (2'-MOE) of the first 5 and last 5 bases, maximizing ASO stability and knockdown efficacy. Initial studies of these ASOs in human cell lines (HEK293T) and human iPSC-derived neurons were promising (**Fig. 1**), setting the stage for *in vivo* experiments in Major Task 3.

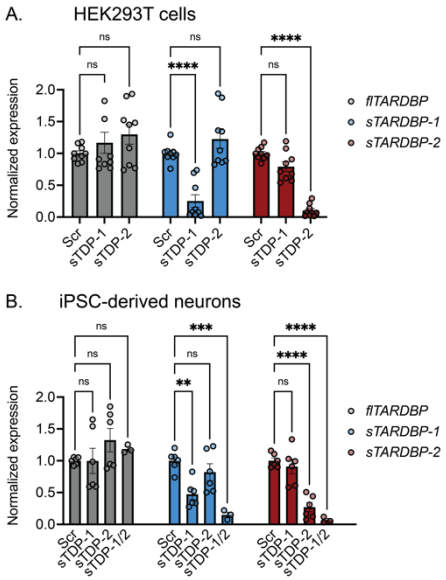

Figure 1: Selective knockdown of *sTARDBP* using ASOs. RT-qPCR of HEK293T cells (**A**) or human iPSC-derived neurons (**B**) treated with 100nM ASO for 48h. ***p<0.001, **p<0.01 by 2-way ANOVA, combined among 3 biological replicates, each containing 3 technical replicates

In performing these experiments, however, we noted considerable variability in the degree and extent of *in vitro* ASO-mediated *sTARDBP* knockdown. We modified several parameters, including ASO concentration, incubation times, and delivery methods. Despite confirming efficient uptake of ASOs, knockdown efficiency remained variable. Therefore, we sought a different means of selectively eliminating *sTARDBP* expression, turning to proteolysis targeting chimeras (PROTACs). These compounds are comprised of two functional heads, one of which binds to the protein of interest, while the other recruits a ubiquitin ligase. Upon recognition of the protein, PROTACs trigger its ubiquitination and subsequent degradation via the proteasome. To target TDP-43 variants, we took advantage of CRISPR/Cas9 genome editing to insert an open reading frame encoding HaloTag just downstream of the *TARDBP* start codon, creating HaloTag-TDP-43 (**Fig. 2A**). Based on the known splicing of the *TARDBP* locus, we expect HaloTag-TDP-43 to be comprised of both flTDP-43 as well as sTDP-43 variants. We then differentiated iPSCs into neurons and applied PROTACs, resulting in the rapid and selective degradation of HaloTag-TDP-43 (**Fig. 2B, C**). While these approaches are not specific to sTDP-43, we plan to repeat these studies in cell lines in which the HaloTag open reading frame has been inserted just upstream of the *sTARDBP* stop codon via CRISPR/Cas9, thereby specifically labeling sTDP-43 with HaloTag. In preliminary experiments, we confirmed the effectiveness of our targeting strategy by selectively labeling sTDP-43 in HEK293T cells with mNeonGreen, an ultra-bright fluorescent protein (data not shown). Future studies will knock in HaloTag in place of mNeonGreen in iPSCs, enabling the manipulation of sTDP-43 levels by PROTACs in human iPSC-derived neurons.

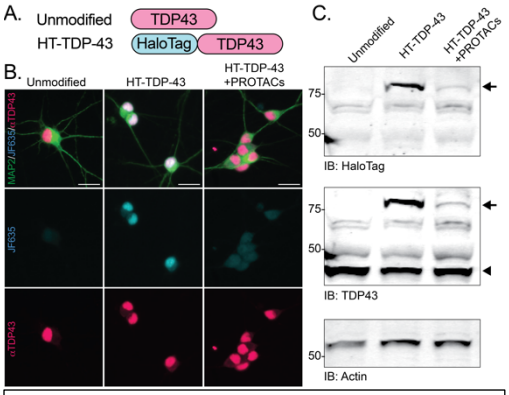

Figure 2: Elimination of TDP-43 in human neurons using PROTACs. (**A**) Schematic of endogenous TDP43 and HaloTag-fused TDP-43 in human iPSC-derived neurons. (**B**) Unmodified and HT-TDP-43 neurons treated with PROTACs for 48h, then immunostained for MAP2 (a pan-neuronal marker), TDP43, and HT-TDP43 using JF635. (**C**) Unmodified and HT-TDP-43 neurons treated with PROTACs for 48h, followed by lysis, SDS-PAGE and immunoblotting (IB) using HaloTag, TDP43 and Actin antibodies. Arrow, HT-TDP-43; arrowhead, endogenous TDP-43.

Related to the *in vivo* studies, we commenced testing ASOs in mice. Male and female C57Bl/6J mice were purchased from the Jackson Laboratory (strain # 000664). The top ASOs targeting *sTARDBP* isoforms 1 and 2 were mixed in a 1:1 ratio and diluted in sterile filtered PBS for a final volume of 10ul. Intrathecal injection was performed between vertebrae L4 and L5. A total of 350ug was administered of the ASO mix or a scramble ASO. Successful administration of the ASO was visually confirmed by a robust "tail flick" after injection (mice that did not demonstrate this tail flick were sacrificed). We monitored mice for acute injection stress and recovery from the procedure. A subset of mice were sacrificed two weeks after surgery, and brain, spinal cord and liver (as a control non-injected organ) were collected after perfusion with 4% paraformaldehyde. RNA was extracted using an RNA-easy lipid kit and converted to cDNA. Primers spanning the *sTARDBP* specific junction were used to assess the amount of knockdown in the brain and spinal cord. Additional mice were monitored longitudinally for signs of toxicity associated with ASO injection and effects of *sTARDBP* knockdown. We observed stable weight gain and no signs of distress in mice over the course of 14 weeks, thereby crucially demonstrating safety and efficacy of this technique in ALS mouse models. We further acquired rAAV vectors with short and long hexanucleotide expansions in *C9orf72* and have successfully performed saline ICV injections in P1 mice. This preliminary work is critical for demonstrating the ability of *sTARDBP* ASOs to prevent motor deficits in the rAAV-C9orf72 administered mice – steps we are now prepared to undertake.

Specific Aim 2: We started harvesting organs with secretory functions, specifically those producing biofluids of interest to study the expression of TDP-43 isoforms. We were able to show that there are several cell types in the body such as the ependymal cells in the choroid plexus, the renal tubules in the kidney, and the striated ducts and acinar cells in the salivary glands that express sTDP-43 and flTDP-43 **(Figure 3)**. All these cell types display secretory features, and the molecules they express can be measured in their corresponding biofluids (**Fig. 3**).

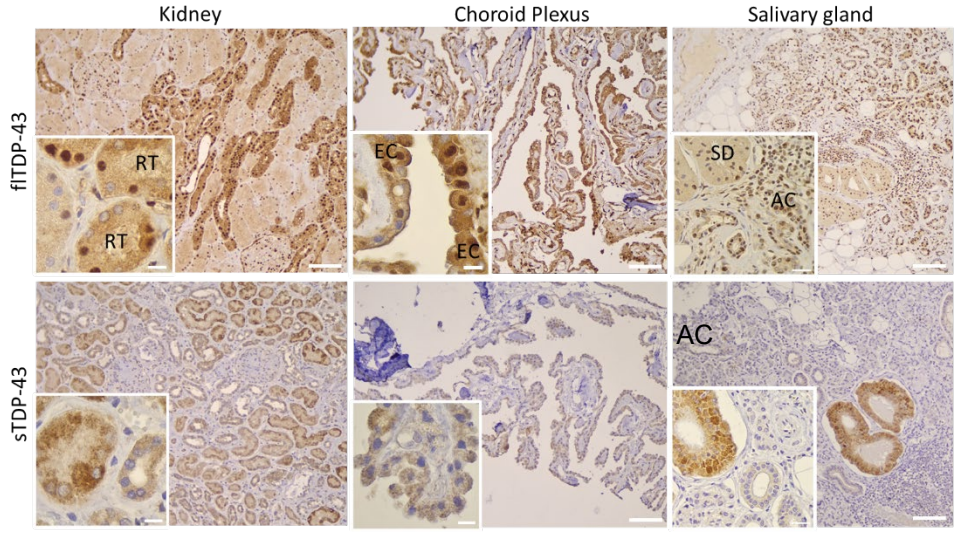

Figure 3. flTDP-43 and sTDP-43 expression in secretory cell types of the kidney, choroid plexus and salivary glands. IHC shows that flTDP-43 is expressed in the cytoplasm and nuclei of renal tubules (RT) of the kidney, ependymal cells (EC) of the choroid plexus, striated ducts (SD) of the salivary gland, as well as in the cytoplasm of acinar cells (AC). sTDP43 is expressed in apparent granules in the cytoplasm of RTs, ECs, and ACs. Scale bars: 100 um, insets 25 um

We also developed a protein precipitation protocol specific for saliva. In this fluid, we detected the presence of sTDP-43, flTDP-43 and phospho-TDP43 (pTDP43) using western blot **(Fig. 4)**. Interestingly, these proteins were present in different phases of the saliva, in both patients and control individuals. While sTDP43 was detected in the supernatant, flTDP43 was detected in the dense pellet that spontaneously precipitates in saliva. Finally, pTDP43 was detected in both

supernatant and pellet. We also standardized saliva processing and adapted it to a commercially available kit to detect flTDP-43 through ELISA. The concentrations obtained for control samples were (3.272 ng/mL ± 0.536 ng/mL, N=4).

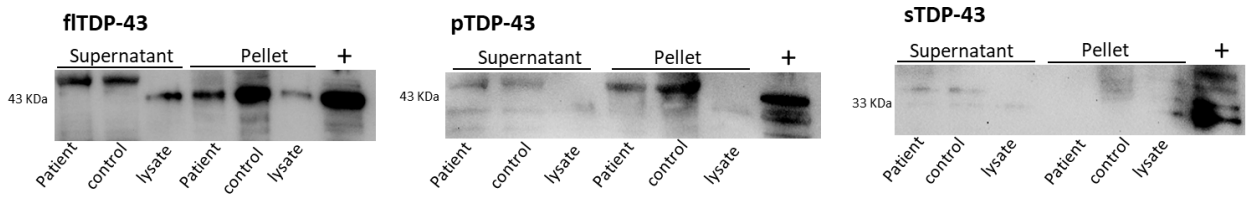

Figure 4: flTDP-43, pTDP-43 and sTDP-43 expression in saliva after precipitation. Western blot showing the expression of different isoforms of TDP-43 in different phases of saliva. While flTDP-43 is exclusively in the pellet, sTDP-43 is detected in the supernatant only. pTDP-43 is detected in both. We did not detect differences between patients and controls; therefore, a fine qualitative analysis is required. Control fibroblast lysates were used as controls for processing.

Our results show that TDP-43 isoforms are present in the salivary glands and in saliva. sTDP-43 is expressed in acinar cells (fluid/mucus secretory cells) of the salivary glands and in the saliva supernatant. flTDP43 is expressed in the cytoplasm of striated ducts (organic molecules' secretory cells) and in the dense pellet formed spontaneously in saliva. Our results highlight the necessity to perform more experiments to understand the secretion pathway for both isoforms, and to develop fine characterization and quantification for biomarker validation.

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.

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. During the studies, we also included the use of saliva samples. Therefore, a modification to the Biospecimen protocol (IRB# 21-005248) was submitted to Mayo Clinic IRB and was approved on October 6th, 2022. Regarding the use of antemortem CSF samples, a material transfer agreement (MTA) was established with the University of Miami CReATe and CRiALS Repositories and 18 samples were received at Dr Belzil Lab (6 controls, 6 C9ALS, and 6 sporadic ALS. Postmortem CSF specimens were obtained from the Mayo Clinic Brain Bank (N=24).

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.

We received approval for our animal use regulatory protocols from our local animal use and care committee at the University of Washington on April 20th, 2021, and from DoD ACURO on May 27th, 2021.

(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 under Mayo Clinic IRB protocol # 21-005248 entitled "*Biospecimen processing to investigate pathophysiological significance and therapeutic potential of TDP-43 isoforms in amyotrophic lateral sclerosis (ALS)*".
- Human iPSCs will be used to complete **Specific Aim 1** under University of Michigan IRB protocol # HUM00028826 entitled "*Epidemiologic Risk Factors and the Genetics of ALS*".

(c) Animal Use Regulatory Protocols

An animal model will be used to complete **Specific Aims 1** under the University of Washington protocol # AL200117.e001 entitled "*RNAi as gene therapy*". Details are below:

- Target required for statistical significance: 298
- Target approved for statistical significance: 298
- Submitted to and approved by 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 undertaken safety and efficacy 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 successfully identified 2 ASOs that effectively reduce *sTARDBP-1* and *-2* (subtask complete).

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

We verified the ability of ASOs to reduce *sTARDBP-1* and *-2* in human iPSC-derived forebrain and motor neurons. Future studies will focus on the effect of ASOs in iPSC-derived astrocytes.

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

We are currently testing the effect of *sTARDBP*-targeting ASOs in human iPSC-derived neurons carrying ALS-associated mutations in *TARDBP* and *C9ORF72*.

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.

We have begun testing flTDP-43 levels and localization upon *sTARDBP* knockdown in human iPSC-derived neurons. Initial strategies focused on ASOs, but we will also verify changes through additional methods, such as PROTACs targeting sTDP-43-HaloTag.

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

These investigations are similar to studies in Subtask 1 and are being performed concomitantly.

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

We will perform next generation RNA sequencing in human iPSC-derived neurons and astrocytes upon *sTARDBP-1* and *-2* knockdown. We will also verify changes in PROTACs-mediated sTDP43-HaloTag cells.

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 1 mice. Control or *sTARDBP*-directed ASOs will be administered at week 5 by intrathecal injections into the lumbar spinal cord in 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 flTDP-43, sTDP-43 and pTDP-43 proteins in saliva from ALS patients, and control participants.

Subtask 2: Protein precipitation

We will develop a method to precipitate the proteins for further TDP-43 analysis and quantification through Western Blot and ELISA

Subtask 3: Structural protein characterization

We will characterize flTDP-43, sTDP-43 and pTDP-43 structure through immunoelectron microscopy in protein precipitates using recombinant proteins as controls.

Subtask 4: characterization and quantification of flTDP-43, sTDP-43 and pTDP-43.

We will immunoprecipitate the proteins and analyze them through mass spectrometry at the Mayo Clinic Proteomics core.

Subtask 5: Statistical analyses.

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

Major Task 2: Assess levels of flTDP-43, sTDP-43, pTDP-43, *flTARDBP and sTARDBP* in salivary glands.

Subtask 2: Protein detection and quantification

We will isolate the proteins from controls and patients' salivary glands and perform Western Blot to detect flTDP-43, sTDP-43 and pTDP-43.

Subtask 3: mRNA detection and quantification

We will isolate RNA and perform qPCR for *flTARDBP and sTARDBP.*

Subtask 4: Statistical analyses

We will conduct statistical analyses of data obtained from *flTARDBP*, *sTARDBP*, flTDP-43, pTDP43 and sTDP-43 expression measures.

Subtask 5: Characterization of flTDP-43, sTDP-43 and pTDP-43 secretion pathway.

We will perform immunoelectron microscopy in salivary glands to detect the intracellular localization on the proteins and their pathway of secretion.

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. 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. Notably, the approaches described here have the potential to identify disease-relevant proteins in highly accessible biofluids such as saliva, considerably facilitating the acquisition of longitudinal biomarkers from pre-symptomatic subjects with disease associated mutations, symptomatic patients, and those at different disease stages. Such an advance will transform our current concept of disease onset as well as progression and could open new avenues for patient stratification and target engagement.

5. CHANGES/ PROBLEMS

a. Actual Problems or delays and actions to resolve them.

None of the original shRNAs we designed effectively reduced *sTARDBP* isoforms in HeLa or N2A cells. Although we subsequently identified *sTARDBP*-targeting ASOs with prominent effects of *sTARDBP*-1 and -2 (**Fig. 1**), our *in vitro* studies uncovered unexpected variability in *sTARDBP* knockdown. Therefore, we adopted a separate strategy for eliminating sTDP-43, utilizing PROTACs (**Fig. 2**). Preliminary studies confirmed that addition of PROTACs can effectively degrade HaloTag-labeled TDP-43. To make this a viable strategy, we are using CRISPR/Cas9 to fuse HaloTag with the C-terminus of sTDP-43. Because they share the same C-terminus, this approach will enable the simultaneous elimination of both sTDP-43-1 and sTDP-43-2 variants. Control lines expressing HaloTag-TDP-43 as well as free HaloTag (expressed under control of the *GAPDH* locus) have already been generated.

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 could not detect flTDP-43, sTDP-43 and pTDP-43 in CSF. As such, we considered testing another biofluid. This change caused delays resulting from protocol modification approvals and new sample type collections, and an overall change in the original research plan. However, the plan modification resulted in a promising approach, since saliva samples are easier to collect and very stable. We were also able to enroll and collect a significant number of patients in a short period of time.

We encountered a personnel-related obstacle when the animal technician in the Valdmanis lab left to pursue another position at the Allen Brain Institute. We therefore needed to hire and train a new research scientist, Eli Kaufman, to acquire all the skills associated with the animal techniques and protocols. He was able to successfully complete the animal safety trainings and demonstrate successful independent intrathecal and intracerebroventricular surgeries as described in **Specific Aim 1**, Major Task 3.

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 addressed by data presented in this report (**Figs. 1, 2**) showing effective knockdown of sTDP-43 and TDP-43 in human iPSC-derived neurons using ASOs and PROTACs. 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 also created iPSC lines modified by CRISPR/Cas9 such that endogenous TDP-43 is labeled at the N- or C-terminus with HaloTag, as well as lines in which sTDP-43 is labeled by HaloTag at its C-terminus, and control lines expressing HaloTag from the *GAPDH* locus. All lines will be shared with interested investigators upon publication. We aim to establish saliva profiling as a source of diagnostic biomarkers that can be quickly translated to the clinic.

- ### Publications, conference papers, and presentations

Invited talks, S. Barmada

1. Barmada, S. University of California, San Francisco, Medical Center, Memory and Aging Center Grand Rounds, June 2022, San Francisco, CA
2. Dykstra, M. Regulation of Alternative TDP43 Isoform in ALS and FTD. Michigan Neuroscience Conference, May 2022, Ann Arbor, MI
3. Barmada, S. Indiana University School of Medicine, Stark Neurosciences Research Institute, November 2022, Baltimore, MD
4. Barmada, S. Western Michigan University School of Medicine, Seminars in Investigative Medicine, Kalamazoo, MI, November 2022
5. Barmada, S. ALS Translational Research Meeting, Massachusetts General Hospital, January 2023
6. Barmada, S. University of Pittsburgh Children's Hospital, Rangos Seminar Series, University of Pittsburgh Medical Center, Pittsburgh, PA, February 2023
7. Barmada, S. RNA stability in ALS and FTD. Columbia University Medical Center Department of Neurology, New York NY, February 2023

Poster presentations, S. Barmada and M. Dykstra

1. Dykstra, M. Regulation and Toxicity of Shortened TDP43 Isoforms. Michigan Alzheimer's Disease Research Center Beyond Amyloid Research Symposium, May 2022, Ann Arbor, MI.

2. Dykstra, M. Regulation and Toxicity of Truncated TDP43 Isoforms in ALS & FTD. Michigan Neroscience Graduate Program Annual Retreat, September 2022, Maumee Bay, OH.
3. Barmada, S. Regulation and Toxicity of Truncated TDP43 Isoforms in ALS & FTD. Annual Meeting of the American Neurological Association, October 2022, Chicago, IL (Poster S232)
4. Dykstra, M. Regulation and Toxicity of Truncated TDP43 Isoforms in ALS & FTD. Protein Folding Diseases Initiative Annual Symposium, October 2022, University of Michigan, Ann Arbor, MI
5. American Society of Cell Biology Annual Meeting, December 2022, Washington, DC
6. Dykstra, M. Regulation and Toxicity of Truncated TDP43 Isoforms in ALS & FTD. Robert Packard Center for ALS Research Annual Meeting, February 2023, Baltimore, MD`
7. RNA Center for Biomedicine Annual Meeting, March 2023, University of Michigan, Ann Arbor, MI

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), Staff (Mayo), Dickson (Mayo), and Benatar (Miami) to obtain human CSF and saliva samples, and supervises the student and postdoctoral fellow in charge of purifying and characterizing human and mouse biofluids 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 and saliva samples collected cross-sectionally or longitudinally from his patients, along with their respective de-identified demographic/clinical information.

Name	**Nathan Staff**
Project Role	Consultant
Researcher Identifier (e.g. ORCID ID)	eRA commons ID: STAFF12
Nearest person month worked	1

Contribution to Project: Dr. Staff works closely with Dr. Belzil to provide human saliva 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	**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 biofluid processing methodology standardization, optimization, and characterization. She is also responsible for presentations and manuscript preparation.

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

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

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.