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14. ABSTRACT

A well-known feature of amyotrophic lateral sclerosis (ALS) is the presence and activation of inflammation and inflammatory signaling pathways within the brain and spinal cord. While increased inflammation is present in ALS patients, drugs that target inflammation have not proven effective in clinical trials. One reason is that most anti-inflammatory drugs target general features of inflammation and not key factors of inflammation that drive neuronal death. One key factor of inflammation are a family of proteins called chitinases that are expressed by cells of the immune system. We and others discovered that chitinases are expressed and released by specific glial cell types during inflammation and present in high levels in cerebrospinal fluid of ALS patients. In this project we will develop novel viral based vectors (adeno-associated virus or AAV) that target specific cell populations to either turn on or turn off chitinase gene expression. Through this method we will regulate the "bad" chitinase signals while preserving and expanding the "good" chitinase signals, and will test this therapeutic approach in the mutant $SOD1^{G93A}$ mouse model. We will also further develop biomarker tests that measure each chitinase protein in human blood and cerebrospinal fluid. These chitinase biomarker tests will enable us to target our approach to ALS patients with high levels of chitinases and to monitor the therapeutic impact of our viral vector based treatments in biofluids collected after treatment.

15. SUBJECT TERMS
None listed.

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1. INTRODUCTION

Chitinases and chitinase-like proteins are members of the glycoside hydrolase family 18 and function to degrade chitin, modulate innate immune responses, cell migration and differentiation, and modulate inflammation in the progression of many human diseases. Chitinases are secreted by activated astrocytes and microglia in the central nervous system, and we and others have shown increased Chit-1 and CHI3L1 in the CSF of ALS patients. We recently demonstrated that CHI3L1 is expressed by a subset of activated astrocytes in ALS motor cortex and spinal cord, whereas Chit-1 is expressed by a subset of activated microglia in ALS motor cortex and spinal cord. Our data suggests that chitinase proteins function in modulating white matter pathology and neurodegeneration in ALS patients, representing a new therapeutic target. In this project we will elucidate the role of chitinase proteins in ALS and develop adeno-associated virus (AAV) vectors to regulate their expression in specific cell types in order to slow or halt ALS disease progression. This AAVs will be tested in the $SOD1^{G93A}$ mouse model of ALS. We propose that the CSF levels of chitinases are a pharmacodynamic biomarker to demonstrate target engagement of our novel therapy.

2. KEYWORDS

Adeno-associated virus (AAV); Chitinase; Biomarker; Inflammation; Astrocytes; Microglia

3. ACCOMPLISHMENTS

Each Aim is listed below and accomplishments during the first year described in the text.

Aim 1 Progress: Define chitinase expression in both the human brain and spinal cord.

The study design builds upon earlier data we published describing Chit-1 and CHI3L1 expression in the human brain and spinal cord (Vu et al., *JNNP* 91:350-258, 2020). We have performed immunohistochemistry for Chit-1 or CHI3L1 in 12 cases of sporadic ALS (sALS), 10 cases of C9orf72 ALS (familial form of ALS – C9ALS), 10 cases of frontotemporal dementia (FTD), 10 cases of Alzheimer's disease (AD), and 5 non-neurologic disease controls (NNDC). The regions of interest were the motor cortex, frontal cortex, hippocampus, and lumbar spinal cord. Slides were scanned and chitinase protein immunoreactivity was separately quantified in both the white matter and grey matter of all sections using software and results standardized using cytoplasmic area or percent positive cells. We determined that both Chit-1 and CHI3L1 immunoreactive glia were most numerous in the white matter of all regions in sALS, C9-ALS and FTD patients, whereas these same glial markers were most abundant in the grey matter of AD patients. Representative data is shown below in Figure 1.

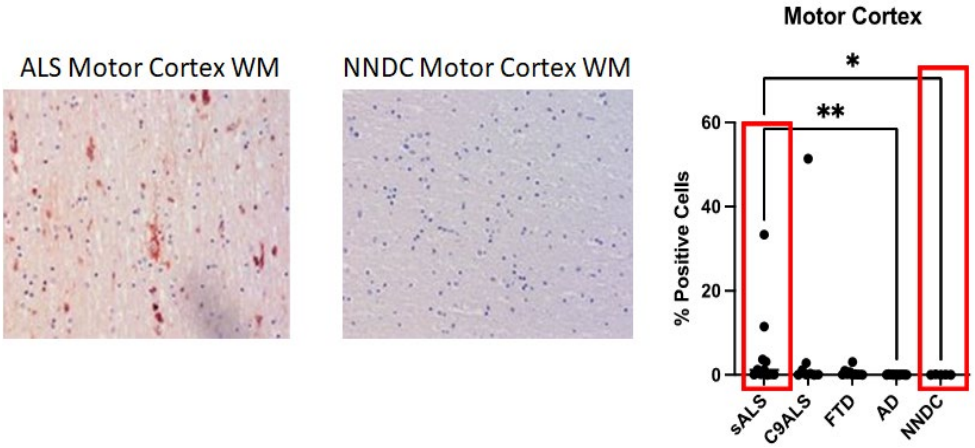


Figure 1: Chit-1 staining in the white matter (WM) of sALS and non-neurologic disease control (NNDC) motor cortex (Left and Middle panels). Quantification of IHC in the motor cortex WM of all cases (Right panel).

There was a significant increase in Chit-1 labeled glia in the motor cortex white motor of sALS patients when compared to AD or NNDC cases. We also determined the level of Chit-1 immunoreactivity within the total cytoplasmic area of the white matter and observed similar findings (Figure 2). When Chit-1 immunoreactivity is compared across different tissue regions we did not observe statistical significance between disease groups but the highest levels of Chit-1 immunoreactivity was consistently detected in the spinal cord (Figure 2).

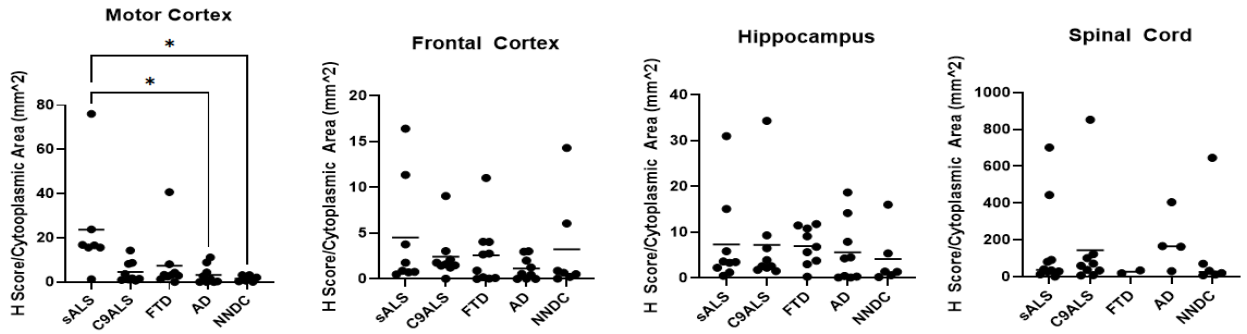


Figure 2: Chit-1 positive cells across all brain and spinal cord regions and in all disease groups. sALS motor cortex exhibited the highest levels compared to other disease groups. No statistically significant differences were observed in other regions but the spinal cord exhibited the highest levels of Chit-1 immunoreactive glia.

Similarly, we also observed statistically significant increased CHI3L1 immunoreactive astrocytes in the motor cortex white matter of sALS cases compared to AD and NNDCs (Figure 3).

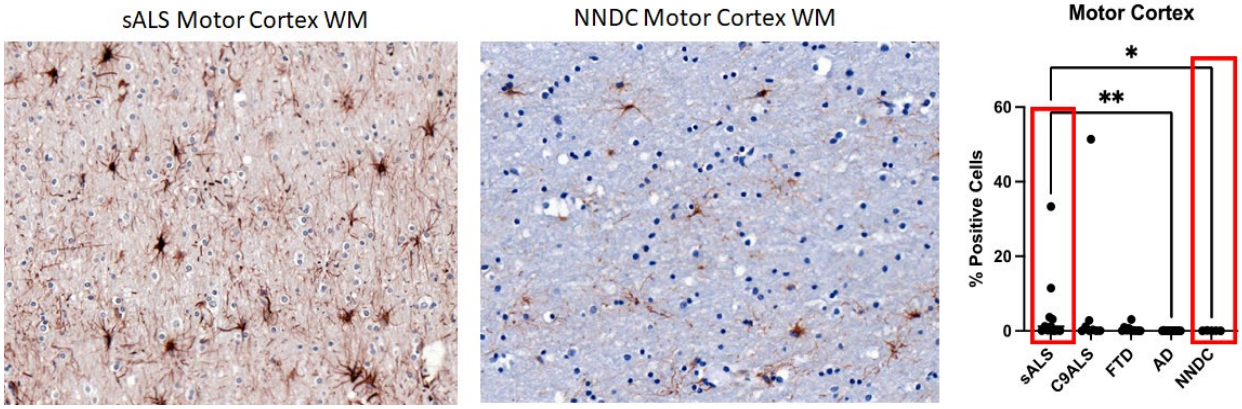


Figure 3: CHI3L1 immunoreactivity in the white matter (WM) of sALS and NNDC motor cortex (Left and Middle panels). Quantitation revealed statistically significant increases in sALS compared to AD or NNDC (Right panel).

We are also interested in the distribution of chitinase protein expression with respect to the underlying neuropathology typically observed in most ALS patients, namely inclusions of TDP-43. We have initiated studies to perform both double-label immunohistochemistry and double-label confocal microscopy to determine if chitinase containing glia are located near neurons containing TDP-43 pathology or if chitinase expressing glia also contain TDP-43 pathology. While these studies have only been initiated, we do not detect CH13L1 immunoreactive glia localized near pTDP-43 inclusions within the white matter of the motor cortex in sALS patients (Figure 4).

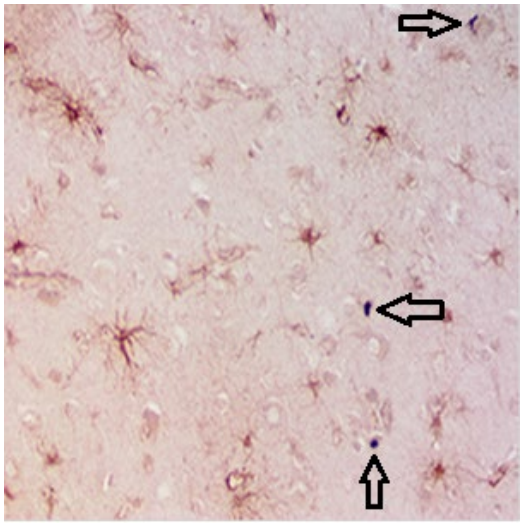


Figure 4: Double-label immunohistochemistry for CHI3L1 (red) and pTDP-43 (blue). Arrows denote pTDP-43 inclusions yet lack CHI3L1 immunoreactivity surrounding the pathology.

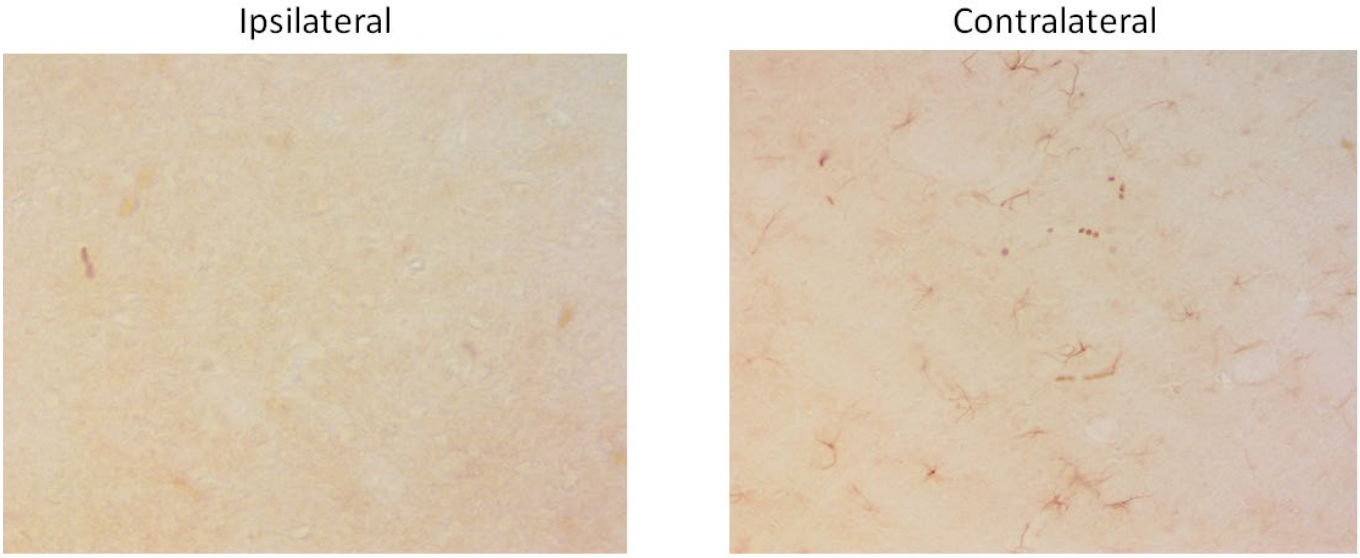
The following conclusions are made from Aim 1 results to date. Chit-1 mRNA levels are significantly increased in the motor cortex and spinal cord of sALS and C9ALS cases when compared to NNDC cases (prior progress report). Chit-1 immunoreactivity is highest in the white matter of sALS, C9ALS, and FTD cases, whereas it is highest in the grey matter of AD cases. The highest levels of Chit-1 immunostaining were in the lumbar spinal cord when compared to other brain regions. Similar patterns of immunoreactivity were observed for CHI3L1. Finally, we have not detected a co-localization of CHI3L1 immunoreactive glia near neurons containing cytoplasmic pTDP-43 inclusions, suggesting the neuropathology is not stimulating CHI3L1 expression in neighboring astrocytes.

Aim 2 Progress: AAV mediated regulation of chitinases to modulate inflammation in SOD1^{G93A} mice
This aim is designed to generate novel adeno-associated virus (AAV) mediated regulation of chitinase gene expression and determine the impact on neuroinflammation. Initial studies are to design and engineer the AAVs, generate vector stocks in cultured cells (HEK293) and then inject either vector into the motor cortex or spinal cord of mice to demonstrate efficiency and specificity of gene modulation. We will then scale production and test ability of each AAV to modulate neuroinflammation in the SOD1^{G93A} transgenic mouse model of ALS. The goal is to test whether increased or decreased chitinase gene expression will exasperate or impede disease progression in the G93A SOD1 mouse model of ALS.

We inserted the rodent cDNA for the CHI3L1 gene (Brp-39) or a miRNA that mediates repression of the Brp-39 gene (miR1059) downstream of the GFAP promoter. In rodents, the Brp-39 gene is expressed in astrocytes, and hence we are further regulating expression of our constructs to the astrocyte lineage. These constructs were independently co-transfected with a helper plasmid and the AAV5 or AAV6 capsid plasmid into HEK293 cells. Recombinant AAVs were purified and then used to inject into the motor cortex of mice. These two AAVs will either over express Brp-39 or knockdown Brp-39 gene expression.

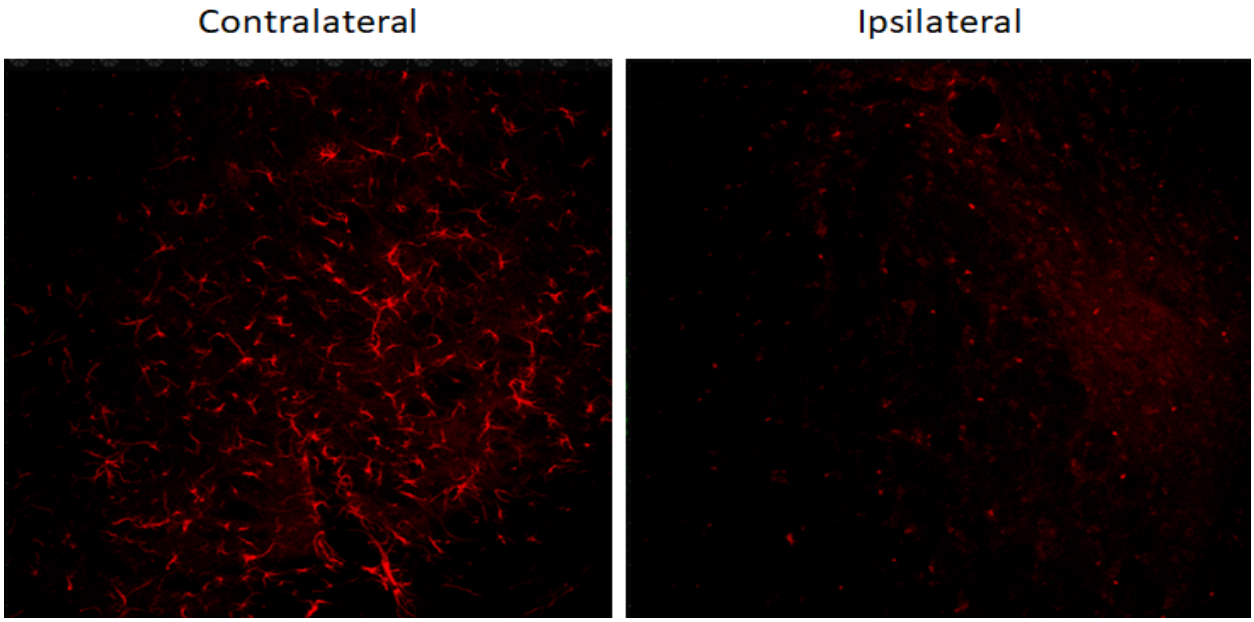
We first injected the recombinant AAV5 that impedes expression of Brp-39 into the cortex of wildtype mice and immunostained for Brp-39 protein by light microscopy in both the ipsilateral and contralateral hemispheres (Figure 5). Results demonstrate decreased Brp-39 immunoreactivity in the side injected with the AAV5 (ipsilateral) versus that level of Brp-39 immunoreactivity in the corresponding contralateral cortex of the same animal (Figure 5).

Figure 5: Brp-39 immunohistochemistry of the ipsilateral (left) and contralateral (right) in mice injected with the rAAV6-pGFAP_miR1059



We also performed similar experiments to inject an AAV5 vector containing the miRNA to down regulate Brp-39 expression. As shown in Figure 6, we detect reduced Brp-39 immunostaining in the ipsilateral versus the contralateral cortex. This *in vivo* data supports the conclusion that we have specific AAVs that either decrease Brp-39 (CHI3L1) in murine astrocytes.

Figure 6: Brp-39 immunostaining (red) in the ipsilateral (right) and contralateral (left) side of mice injected with rAAV5-miR1059 in the motor cortex.



There is clearly reduced Brp-39 immunostaining in the ipsilateral side that received either the rAAV5-miR1059 and rAAV6-miR1059 vectors. These results have been replicated in three mice with both vectors.

Finally, we expect that optimal delivery of the viral vectors will require introduction of a newer injection system into the subpial space, which has been shown to greatly facilitate delivery of the AAV and modulation of gene expression in the spinal cord and cortex of mice. Therefore, this year our technician and graduate student have learned the spinal cord subpial injection method and have been practicing the technique. They have recently mastered the technique and in the upcoming year will be performing spinal cord subpial injections to deliver our vectors into mice.

Aim 3 Progress: Biomarker assay development for chitinase proteins

The goal of this aim is to optimize and validate immunoassays for Chit-1, CHI3L1, and CHI3L2 proteins in human biofluids. These assays may be useful as pharmacodynamic biomarkers for downstream clinical studies that modulate chitinase expression to regulate neuroinflammation. Such biomarkers will be quite valuable for measuring neuroinflammation in ALS and other neurodegenerative diseases.

We have created immunoassays for each chitinase protein on the Meso Scale Discovery (MSD) platform, but selecting and optimizing antibodies, antibody conditions and buffer conditions for each assay. The capture and detection antibody for each assay are the following:

Chit-1:

Capture antibody – Goat polyclonal anti-human CHIT-1 (R&D Cat#AF3559)
Detection antibody – Mouse monoclonal anti-human CHIT-1 (R&D, Cat#MAB3559)

CHI3L1:

R&D Systems DuoSet (Cat#DY2599), that includes both Capture and Detection antibodies specific to CHI3L1

CHI3L2:

Capture antibody – Rabbit polyclonal anti-human CHI3L2 (Sigma cat#SAB1405615)
Detection antibody – Mouse monoclonal anti-human CHI3L2 (Sigma cat#HPA005443)

We have initiated assay validation studies prior to using each of the assays with large numbers of patient derived samples. The assay validation studies are performed using CSF or blood samples already in the Bowser laboratory and previously obtained from the NEALS biorepository, a national biorepository for ALS biofluids samples.

We provided validation results for the Chit-1 immunoassay in the last progress report. Below are data for the initial steps in the CHI3L1 immunoassay validation.

Intra-Assay Precision

Intra-assay precision was determined using 3 Serum, 3 plasma or 3 CSF samples. Samples were tested 8 times in duplicate in the course of one experiment. It was expected that the intra-assay precision (%CV) among the results for each level would be ≤ 20%. Results are as follows:

Intra-Assay Precision: CHI3L1 in Human Serum			
Replicate Number	**CHI3L1 Serum1 (ng/ml)**	**CHI3L1 Serum2 (ng/ml)**	**CHI3L1 Serum3 (ng/ml)**
1	212.393	122.499	134.640
2	213.084	123.270	136.510
3	214.086	123.879	134.799
4	213.106	123.171	139.058
5	215.698	120.569	137.121
6	216.774	122.562	137.633
7	214.119	124.475	136.278
8	218.647	126.309	139.374
Mean	**214.74**	**123.32**	**136.93**
STDEV	**2.14**	**1.67**	**1.75**
%CV	**1.00**	**1.35**	**1.28**

Intra-Assay Precision: CHI3L1 in Human Plasma			
Replicate Number	**CHI3L1 Plasma1 (ng/ml)**	**CHI3L1 Plasma2 (ng/ml)**	**CHI3L1 Plasma3 (ng/ml)**
1	197.460	109.827	110.664
2	199.832	113.939	114.317
3	202.813	115.476	117.664
4	208.918	118.405	119.545
5	205.706	118.154	121.546
6	208.059	115.892	120.859
7	204.323	114.935	118.793
8	209.121	116.194	118.580
Mean	**204.53**	**115.35**	**117.75**

STDEV	**4.30**	**2.70**	**3.61**
%CV	**2.10**	**2.34**	**3.06**

Intra-Assay Precision: CHI3L1 in Human CSF			
Replicate Number	**CHI3L1 CSF1 (ng/ml)**	**CHI3L1 CSF2 (ng/ml)**	**CHI3L1 CSF3 (ng/ml)**
1	812.23	951.56	759.73
2	806.15	970.98	765.73
3	823.11	967.15	769.91
4	814.11	976.18	771.80
5	825.35	972.20	783.35
6	836.63	980.77	779.34
7	804.42	986.92	767.83
8	854.41	999.67	785.95
Mean	**822.05**	**975.68**	**772.96**
STDEV	**16.88**	**14.23**	**9.11**
%CV	**2.05**	**1.46**	**1.18**

4. IMPACT

We have made novel adeno-associated viruses (AAVs) that specifically modulate chitinase gene expression in glial cells. With these new AAVs, we will demonstrate the ability to target gene therapy approaches to subsets of glial cells within the CNS. We have also completed validation of the Chit-1 immunoassay and finishing validation of the CHI3L1 immunoassay. These assays will be valuable to measure chitinases in human biofluids and monitor impact of treatments on inflammatory mechanisms relevant to ALS and other neurodegenerative diseases.

5. CHANGES / PROBLEMS

The overall project directions and aims of the study have not changed.

6. PRODUCTS

Two novel AAVs have been produced to date, and all AAVs will be made available to the scientific community upon publication of our initial results. Novel immunoassays for chitinase protein have been generated to quantify levels in human biofluids.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Robert Bowser, PhD – Principal Investigator. Oversees the overall project and reviews experimental design, results and interpretations with group members. Contributes to all reports and publications.

Fredric Manfredsson, PhD – co-Principal Investigator. Oversees all aspects of generation and use of viral vectors. Meets with group members to review results and interpretations of data. Contributes to all reports and publications.

Ivette Sandoval, PhD – co-Investigator. In charge of AAV design, construction and production. She contributes to animal based studies and image analysis. Contributes to the publication of results.

David Medina, PhD – co-Investigator. In charge of animal behavioral studies, molecular studies and tissue based immunostaining. Contributes to the publication of results.

Jiyan An – Research technician in charge of the biomarker assay development and use to measure chitinase proteins in human biofluids.

Chelsea Tran – PhD graduate student contributes to animal surgeries, chitinase immunostaining of human tissue sections and mouse tissue sections.

All work is performed at the Barrow Neurological Institute.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report.

9. APPENDICES

None