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14. ABSTRACT

shall state the purpose, scope, and major findings and be an up-to-date report of the progress in terms of results and significance. Abstracts will be submitted to the Defense Technical Information Center (DTIC) and shall not contain proprietary information. Subject terms are keywords that may have been previously assigned to the proposal abstract or are keywords that may be significant to the research.

A history of TBI increases the odds of developing AD by 2.5 times in the general population, and 4-6 times for military veterans. Although significant associations between mild TBI and risk of AD have been observed, the precise mechanism by which TBI might lead to AD and/or AD-related symptoms are not yet understood. Histologically, AD is characterized by amyloid- and neurofibrillary protein aggregates, suggesting a loss of protein processing is a key feature of AD. MicroRNAs (miRNA) are small non-coding RNAs that regulate mRNA translation, and may be a significant cause of protein dysregulation.
To date, we have established molecular biology techniques that allow us to measure miRNA in cerebrospinal fluid (CSF) from living donors. Further, we have established and validated changes in the miRNA using a biostatistical pipeline to identify biomarker candidates from our assays. We are now building a bioinformatics pipeline to associate altered miRNA signatures with predicted changes in mRNA regulation and altered protein expression that may correlate with AD-related pathologies.

15. SUBJECT TERMS
NONE LISTED

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1. Introduction

A history of traumatic brain injury (TBI) increases the odds of developing Alzheimer's disease (AD) by 2.5 times in the general population and 4-6 times for military veterans, and accelerates cognitive decline. Although significant associations have been observed, the precise mechanism by which TBI might lead to AD and/or AD-related symptoms are not yet understood. Protein biomarkers related to known AD pathologies and measured in cerebrospinal fluid (CSF) are very sensitive markers of AD, but they lack specificity, often showing up in individuals with no clinical signs of AD. It is not clear whether these protein biomarkers reflect necessary, but insufficient, processes leading to AD, or whether they reflect an early disease stage that, given enough time, will lead to AD. Histologically, AD is characterized by amyloid- and neurofibrillary protein aggregates, suggesting altered protein processing is a key feature of AD. MicroRNAs (miRNAs) are a recently discovered class of small non-coding RNA that regulate mRNA translation, and may be significant contributors to protein dysregulation. Our investigative team has shown that the miRNA distribution in CSF is altered in civilians with AD. Specifically, the signature of miRNA expression in AD is a decrease in abundance or the absence of a subset of miRNAs, which is consistent with the signature pathology of protein over expression and accumulation in AD. Further, when considering the TBI history of our subjects, we find that those with a history of TBI are over-represented in our AD group, and we find a specific group of miRNAs regulated in this population. We hypothesize that TBI induces an alteration in CSF miRNA patterns that reflect the initial molecular responses to brain injury that precede, and may drive, changes in protein expression that contribute to the development of AD.

2. Keywords

Mild traumatic brain injury (mTBI), Alzheimer's disease (AD), microRNA (miRNA), cerebrospinal fluid (CSF), biomarker, deployment, blast injury

3. Accomplishments

Specific Aim 1: CSF miRNA Measurement from mTBI and Controls

Our preliminary data from CSF obtained from living donors with Alzheimer's Disease (AD) shows that there is a 2.5 times higher incidence of self-reported TBI in our AD population, suggesting that TBI may initiate molecular changes that lead to AD. We used quantitative PCR arrays (qPCR) to measure miRNA in banked CSF from: (i) deployed veterans with TBI, (ii) deployed veterans with no lifetime history of TBI, and (iii) community controls with no lifetime history of TBI. We will identify miRNAs that are regulated by mTBI, deployment, or the interaction between mTBI and deployment.

CSF samples were acquired from our collaborator Dr. Elaine Peskind, collected as a part of the baseline assessment of her VA Merit review-funded longitudinal study. All Veterans had documented duty in Iraq and/or Afghanistan. Veterans with TBI were defined as those veterans who had at least one blast exposure with acute symptoms, vs non-TBI with no history of head trauma of any severity. Non-deployed community civilian participants were only included if they had no history of head trauma of any severity. All volunteers were in the age range of 18-60 years old, had a body mass index of 18-36 (inclusive), and were excluded if they experienced seizures, insulin dependent diabetes, had a current diagnosis of substance abuse, psychiatric

disorder, dementia, or were on any medications that might influence cognitive performance. Table 1 shows the targeted and actual enrollment numbers. We recognize the importance of including females and minorities; however, the participants in the originating study are from previously all-male Military Occupational Specialties (MOS), thus we have attempted to match variables in selecting community controls for the study.

Table 1: Enrollment Characteristics					
Group	Target Enrollment	Actual Enrollment	Age	Passed QC	
				TLDA A	TLDA B
Veterans, with mTBI (VetTBI)	50	46	33 +/- 9.9	45	45
Veterans, no mTBI (VetCTRL)	50	18	32 +/- 7.1	18	18
Civilians, no mTBI (CivCTRL)	60	53	34 +/- 9.3	52	52
Reference Standard[1]		18	*NA*	17	18
1. Uniform reference standard CSF run with every batch of 7 experimental samples					

Relative abundance of each miRNA was determined using ExpressionSuite software (Thermo Fisher Scientific), and non-changing miRNA were identified as normalizers (Vandesompele, De Preter et al. 2002). We considered 110 miRNA for further analysis that were reliably measured in at least 20% of samples and did not have a technical exclusion in more than 20% of samples.

Specific Aim 2: Identify miRNA as biomarkers of mTBI

Our data showed a distinct miRNA signature in CSF from AD subjects compared to controls. We will analyze the subset of mTBI specific miRNA identified in Aim 1 to determine their potential to serve as biomarkers for mTBI. Further analyses will compare mTBI miRNA to AD miRNA identified in our separate AD studies to test the hypothesis that mTBI may directly initiate processes leading to AD neurodegeneration.

In our AD studies, we developed a consensus method for identification of candidate biomarkers in qPCR studies that allowed us to examine a large number of candidate miRNA in an initial discovery study, and then use progressively stricter filtering and acceptance criteria as the study expanded to larger data sets. We applied several orthogonal statistical models to the data, then considered which targets were indicated in multiple tests to identify candidate targets that might be missed by one test, and filtered out spurious targets that are only identified in a single statistical model (Lusardi, Phillips et al. 2017, Wiedrick, Phillips et al. 2019). We utilized a similar method to identify candidate biomarkers in the mTBI study samples.

Using the 110 miRNA that met our inclusion criteria, biostatistical modeling focused on identifying veterans with a history of TBI (VetTBI) from community controls (CivCTRL) and veteran controls (VetCTRL). Random forest classification was performed on the data using a CHAID (chi-square automated interaction detection) model to build individual decision trees.

We visualized the data using a multidimensional scaling (MDS) plot, which calculates sample proximity based on relative expression of each of the 110 miRNA (Fig 1). In this plot, each dot represents a single sample; orange are VetTBI, purple are VetCTRL, and blue are CivCTRL. The orange dotted line is a reference representing the 50% probability of predicting a TBI; at this level, sensitivity is 41%, specificity is 81%. Moving the line up would increase the sensitivity (true positives identified), but reduce the specificity (or increasing the false positive rate). Similarly, the dotted purple line represents the 50% probability of predicting the VetCTRL status; in this case, sensitivity is poor – only 18%, but specificity is relatively high – 95%.

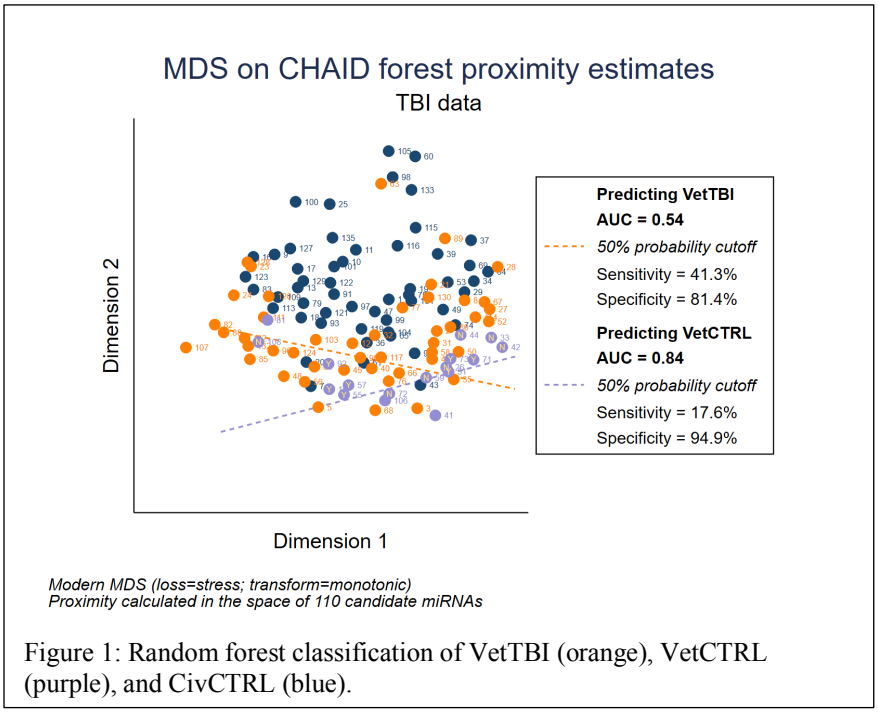

Figure 1: Random forest classification of VetTBI (orange), VetCTRL (purple), and CivCTRL (blue).

Our hypothesis in this study was that miRNA could be used to distinguish between VetTBI and CivCTRL, and we expected that the VetCTRL would be more similar to CivCTRL. While the MDS plot illustrates general stratification by group, there is significant overlap. More surprising to us was the observation that the VetCTRL group is more different from CivCTRL than VetTBI. We have discussed this with our collaborator Dr. Elaine Peskind, who suggested that we might gain some insight into the VetCTRL group by including their MOS information, inferred from a combat experiences scale questionnaire. The hypothesis was that deployed veterans in a non-combat unit may have had more toxin exposures that might explain variation in the VetCTRL samples. VetCTRLs were coded by Dr. Peskind as Yes – forward combat exposure (8), No – no forward combat exposure (7); data was unavailable for 3 samples. We overlaid this information into the MDS plot, with the expectation that it might reveal clusters by MOS (yellow letters Y/N on the purple circles, Fig. 1). Again, we found no apparent correlation in the MDS distribution and MOS that might clarify the results.

The stratification suggests that there might be an overwhelming effect of deployment on the miRNA profiles, thus we performed secondary analyses to better understand this possibility. We calculated an approximately 0.7 area under the receiver operating curve (AUC) for distinguishing CivCTRL from VetTBI. If the VetCTRL are (as they appear in the MDS plot) an extreme version of the VetTBI, we would expect adding them to the comparison would strengthen our ability to distinguish veterans by sharpening the boundary. However, CivCTRL vs. (VetTBI + VetCTRL) results in an unchanged AUC of 0.7, suggesting to us that there is not a uniform “deployment effect” acting in both the VetCTRL and VetTBI groups. Conversely, considering VetCTRL vs. CivCTRL alone or (CivCTRL + VetTBI) both lead to AUC of approximately 0.8, further indication that VetCTRL is distinct from both groups.

We also used Mahalanobis distance from the CivCTRL center to visualize the data (Fig. 2). Each circle represents an individual sample, with colors indicated as for Fig. 1. Samples are distributed along the x-axis in the order that they were processed. Y-axis values (decreasing) represent how similar the sample is to the population of CivCTRL. As with the MDS in Fig. 3, this plot shows weak stratification of the Civilian and Veteran populations, but also a large overlap in the distributions, suggesting an unidentified factor (not deployment, not TBI) may better explain the differences in the data.

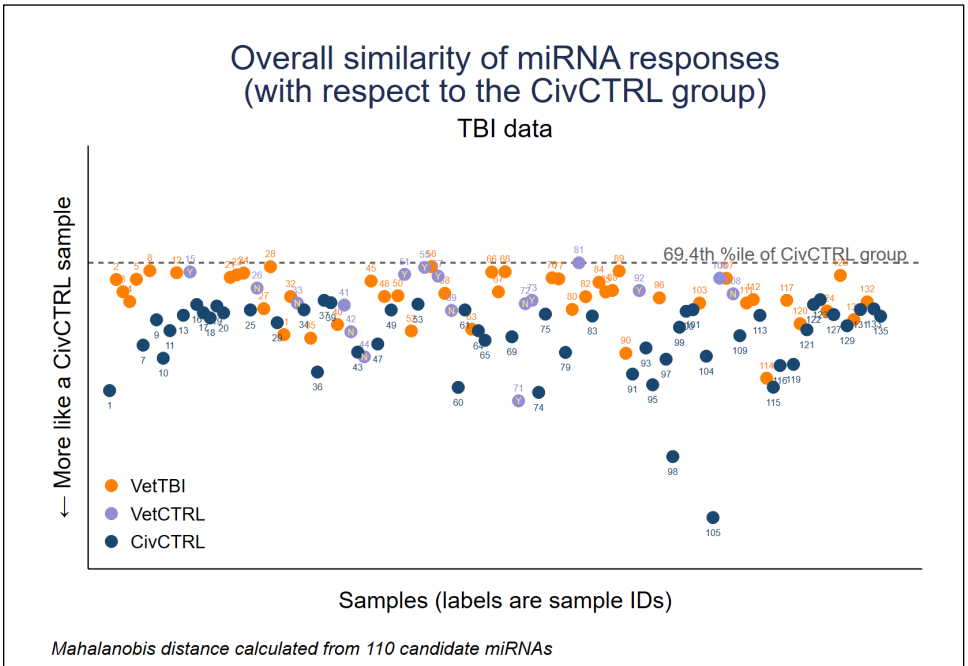

Figure 2: Mahalanobis distance from CivCTRL samples.

We conclude that in this data set, it is not possible to distinguish among our three cohorts in a clinically relevant manner using multimarker modeling. Additionally, it seems that the VetCTRL group is distinct from both the CivCTRL and the VetTBI group, rather than an intermediate state. These results may reflect the heterogeneity of the samples, which have been matched to the best of our ability, but had large variations in age and frequency of blast exposure. Additionally, the VetCTRL sample size is well below the size planned for our analysis, thus the unanticipated findings in the VetCTRL group may simply be the result of the small sample size, which we plan to address in future studies.

Specific Aim 3: AD Pathway-Directed Bioinformatics Evaluation of mTBI-regulated miRNA

In Aim 1, we identify miRNA regulated by mTBI and deployment. In this exploratory aim, we will perform miRNA target prediction studies and AD pathway directed ontology analysis to inform additional hypotheses governing the relationship between mTBI and AD. We will examine the influence of miRNA regulated in deployment on these pathways to better understand the role of deployment in increased rates of AD in veterans with an mTBI.

Despite the fact that we did not identify a set of candidate biomarkers for the VetTBI group, we did compare changes in individual miRNA expression between the VetTBI and the CivCTRL donors to determine whether they might offer insight into changes initiated by deployment-related blast TBI. We acknowledge that, for the reasons identified above, deployment status and TBI history can only explain a small portion of the changes seen in the miRNA expression, and interpretation is included solely to place the current study in the context of existing AD and TBI studies.

As our focus for this analysis was simply differential expression of individual miRNAs, we utilized a more traditional analysis of changes in miRNA expression between groups using delta Cq (dCq) analysis of the CivCTRL and VetTBI samples. We identified non-changing miRNAs in all samples with amplification curves and detection thresholds that passed ExpressionSuite criteria (amplification score > 1, Cq confidence > 0.8, and Cq quantification < 30) which ensured miRNA reliability and stability. NormqPCR (Perkins, Dawes et al. 2012) was used to assess and select the three miRNA that we used for normalization: hsa-miR-222, hsa-miR-342-3p, and hsa-miR-146a (Thermo Fisher catalog numbers 002276, 002260, and 000468). The geometric mean of these three miRNAs was used to calculate the dCq for each miRNA on a given card; higher dCq values indicate lower expression. For differential expression analysis, we excluded miRNA with heavy technical errors (failed QC), limiting analysis to miRNA that were detected in at least 80% of samples, and used a Welch Two Sample T-test (R) to identify the most likely differentially expressed miRNA (Table 2). The results show differential expression of 8 miRNAs between the CivCTRL and VetTBI groups. Fold change (FC) and confidence interval (CI) are included for reference.

Table 2: Differentially Expressed miRNA

			CivCTRL		**VetTBI**		**Change in TBI with respect to Control**	
Probe	*Accession*	*p value*	*Mean dCq*	*SD dCq*	*Mean dCq*	*SD dCq*	*FC*	*CI*
hsa-miR-197-000497	hsa-miR-197-3p	0.0008	-0.07	1.63	0.53	1.81	0.66	0.19 - 2.32
hsa-miR-20b-001014	hsa-miR-20b-5p	0.0015	3.02	1.97	4.02	2.12	0.50	0.11 - 2.19
hsa-miR-20a-000580	hsa-miR-20a-5p	0.0021	-1.19	1.75	-0.33	2.13	0.55	0.13 - 2.41
hsa-miR-191-002299	hsa-miR-191-5p	0.0039	-0.31	1.61	0.09	1.59	0.76	0.25 - 2.28
hsa-miR-17-002308	hsa-miR-17-5p	0.0071	-2.12	1.62	-1.44	1.53	0.62	0.22 - 1.81
mmu-miR-140-001187	hsa-miR-140-5p	0.0148	2.57	1.70	3.19	2.15	0.65	0.15 - 2.88
hsa-miR-30b-000602	hsa-miR-30b-5p	0.0216	-0.43	1.65	0.25	2.17	0.63	0.14 - 2.82
hsa-miR-30c-000419	hsa-miR-30c-5p	0.0233	-1.44	1.64	-0.84	2.03	0.66	0.16 - 2.69

We then performed analysis to determine whether each group had a change in the presence of a miRNA in each group as our machine-learning based biomarker evaluation revealed several miRNA of interest because of their differential rate of detection between the CivCTRL and VetTBI samples. Thus, we revisited the detection rates of miRNAs to see whether any might indicate robust changes in miRNAs found in CSF. We limited our analysis to miRNA that were broadly detected in at least one group, and set a threshold of a 15% change in the rate of censoring between the CivCTRL and VetTBI samples (Table 3). These data show that there are 6

miRNAs that are found more frequently in the TBI group, and 1 miRNA found more frequently in the CivCTRL group.

Table 3: Differentially Censored miRNA			*% Detected*		
Probe	**Accession**	**ID**	**CivCntl**	**TBI**	**Change**
hsa-miR-502-3p-002083	hsa-miR-502-3p	MIMAT0004775	15.38	40.00	24.62
hsa-miR-362-3p-002117	hsa-miR-362-3p	MIMAT0004683	3.85	26.67	22.82
hsa-miR-324-3p-002161	hsa-miR-324-3p	MIMAT0000762	35.29	57.50	22.21
hsa-miR-127-000452	hsa-miR-127-3p	MIMAT0000446	36.54	57.78	21.24
hsa-miR-452-002329	hsa-miR-452-5p	MIMAT0001635	11.76	29.55	17.78
hsa-miR-1-002222	hsa-miR-1-3p	MIMAT0000416	21.15	36.36	15.21
hsa-miR-548a-001538	hsa-miR-548a-3p	MIMAT0003251	74.51	47.73	-26.78

This is the first study to examine CSF miRNA expression exclusively in the post-acute time frame after a mild TBI. We identified multiple miRNAs that are either differentially expressed (8 miRNAs – Table 2) or are indicative to TBI status (7 miRNAs – Table 3). Out of those miRNAs, six have been previously associated with TBI in the literature. We examined published reports of TBI in humans to understand whether the signatures measured in other studies reflect the miRNA changes found in our study. It is important to note that, at the time of baseline sampling for this study, participants were cognitively normal, while the published reports are all from an acute post-injury time frame. LaRocca et al. evaluated miRNA in saliva and serum from mixed martial arts competitors before and up to 3 weeks following a match, showing increased expression of miR-20a in saliva, and miR-30b in both serum and saliva (LaRocca, Barns et al. 2019), both of which we found to be decreased in the VetTBI group (Table 2). In an emergency setting, elevated miR-20a and miR-362 were measured in serum following mild/moderate and severe TBI (Bhomia, Balakathiresan et al. 2016), while we found a decrease in miR-20a (Table 2) at our delayed time point, but an increase in the number of individuals with detectable miR-362 (Table 3). We find detectable levels of miR-502 more commonly in our VetTBI group (Table 3), and it has been suggested candidate for a serum biomarker for mild TBI, distinguishing serum from mild TBI from healthy volunteers, severe TBI, and from extracranial injuries (Di Pietro, Ragusa et al. 2017). Increased serum miR-191 has been investigated as a marker for TBI (Yang, Song et al. 2016), though we see it at depressed levels at our later time point. These results lend credibility to the present results, despite the differences in subject demographics and biofluids assayed it is promising that there is overlap in miRNAs found to be differentially expressed in TBI.

Our biostatistical approaches used in Aim 2 for biomarker discovery suggest that the VetCTRL are different from both the CivCTRL and the VetTBI group, supporting the hypothesis that there may be a deployment effect that confounds our understanding of brain injury in a conflict setting. These outcomes support further studies designed to understand the effect of deployment on miRNA expression in the brain and responses to brain injury.

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

Nothing to Report.

How were the results disseminated to communities of interest?

A manuscript is in preparation for submission to Frontiers in Neurology.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?

Our findings support that variability occurs in individual responses to brain injury, including blast injury. In addition, published studies report that several miRNAs are altered in acute responses to TBI consistent with our finding of specific miRNAs that continue to be dysregulated at the later time points that we examined. Further, we show evidence that deployment alone is sufficient to alter CSF miRNA expression. Together, these outcomes support that further studies are needed to address the underlying mechanisms that contribute to or regulate changes in CSF miRNA expression.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. Changes/Problems

Changes in approach and reasons for change

Nothing to Report.

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

Enrollment numbers for the Veterans with no lifetime history of mTBI (Vet-Cntl) were not met, limiting our ability to determine whether any of the changes that we saw are deployment related.

Changes that had a significant impact on expenditures

No problems or changes had an impact on expenditure.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

6. Products

• Publications, conference papers, and presentations

Relevant Publications

Lusardi, T. A., J. I. Phillips, J. T. Wiedrick, C. A. Harrington, B. Lind, J. A. Lapidus, J. F. Quinn and J. A. Saugstad (2017). "MicroRNAs in Human Cerebrospinal Fluid as Biomarkers for Alzheimer's Disease." J Alzheimers Dis **55**(3): 1223-1233. PMID:27814298

Saugstad, J. A., T. A. Lusardi, K. R. Van Keuren-Jensen, J. I. Phillips, B. Lind, C. A. Harrington, T. J. McFarland, A. L. Courtright, R. A. Reiman, A. S. Yeri, M. Y. S. Kalani, P. D. Adelson, J. Arango, J. P. Nolan, E. Duggan, K. Messer, J. C. Akers, D. R. Galasko, J. F. Quinn, B. S. Carter and F. H. Hochberg (2017). "Analysis of extracellular RNA in cerebrospinal fluid." J Extracell Vesicles **6**(1): 1317577. PMID:28717417

Relevant Presentations

Lusardi, T: MicroRNA in Cerebral Spinal Fluid as Biomarkers of Alzheimer's Disease Risk After Brain Injury. IPR, Feb. 17, 2017

Lusardi, T: MicroRNAs in Human Cerebrospinal Fluid as Biomarkers for Alzheimer's Disease. Extracellular RNA Communication Consortium (ERCC), Nov. 11, 2016

• Website(s) or other Internet site(s)

Nothing to Report.

• Technologies or techniques

Nothing to Report.

• Inventions, patent applications, and/or licenses

Nothing to Report.

• Other Products

Nothing to Report.

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name:	**Dr. Joseph Quinn**
Project Role:	PI
Researcher Identifier (ORCID ID):	0000-0001-7305-2256
Nearest person month worked:	0.6
Contribution to Project:	Dr. Quinn has provided clinical guidance, scientific review, and reporting assistance.
Funding Support:	*See below*

Name:	**Dr. Theresa A. Lusardi**
Project Role:	Co – PI
Researcher Identifier (ORCID ID):	0000-0003-0699-5662
Nearest person month worked:	3
Contribution to Project:	Dr. Lusardi has coordinated sample and metadata transfer with University of Washington collaborators, performed QC evaluations for pilot studies, developed the bioinformatics pipeline, prepared reports.
Funding Support:	*See below*

Name:	**Dr. Julie A. Saugstad**
Project Role:	Key Personnel
Researcher Identifier (ORCID ID):	0000-0002-2996-9611
Nearest person month worked:	1
Contribution to Project:	Dr. Saugstad is an expert molecular biologist, who has provided technical and organizational guidance for the miRNA assays.
Funding Support:	Dr. Saugstad's work for this project was funded by this award

Name:	**Dr. Ursula S. Sandau**
Project Role:	Key Personnel
Researcher Identifier (ORCID ID):	0000-0002-3646-7089
Nearest person month worked:	6
Contribution to Project:	Dr. Sandau is an experienced molecular biologist, who has performed all TLDA assays for this project. She has been active in the development of quality assessment standards and interpretation of the results.
Funding Support:	Dr. Sandau's work for this project was funded by this award

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

There have been no changes to active support for Dr. Joseph Quinn

What other organizations were involved as partners?

Organization Name	Seattle Institute for Biomedical and Clinical Research (SIBCR)
Location of Organization	1660 S. Columbian Way, MS S-151, Seattle, WA 98108-1532
Partner's Contribution	*Collaboration*: Provide banked CSF samples, corresponding clinical and biomarker data. Analytic support, including

	integration of findings resulting from this project with ongoing multimodal studies of the same participant group. Contribute to preparation of abstracts and manuscripts resulting from the research.

8. Special Reporting Requirements

None.

9. Appendices

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Yang, T., J. Song, X. Bu, C. Wang, J. Wu, J. Cai, S. Wan, C. Fan, C. Zhang and J. Wang (2016). "Elevated serum miR-93, miR-191, and miR-499 are noninvasive biomarkers for the presence and progression of traumatic brain injury." Journal of Neurochemistry **137**(1): 122-129.