AWARD NUMBER: W81XWH-19-2-0002

TITLE: Characterizing Concussion Using Brain Derived Exosomes

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CONTRACTING ORGANIZATION: University of Pennsylvania, Philadelphia, PA

REPORT DATE: May 2021

TYPE OF REPORT: Annual Report

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
May 2021	Annual	01May 2020-30Apr2021

4. TIT E AND SUBTIT E

Characterizing Concussion Using Brain Derived Exosomes

5a. CONTRACT NUMBER
W81XWH-19-2-000 2

5b. GRANT NUMBER
GRANT12426605

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

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8. PERFORMING ORGANIZATION REPORT NUMBER

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

U.S. Army Medical Research and Materiel 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

Traumatic brain injury (TBI) currently afflicts 357,000 enlisted military men and women in the US Armed Services. For the most common form of TBI, Mild Traumatic Brain Injury (mTBI) most patients recover within a year following the incident, but 10-20% of mild cases result in a long-term disability including seizures and emotional and behavioral issues. Although much has been learned about molecular changes in the brain following injury, access to these biomarkers following mTBI is lacking. The accurate diagnosis and precise individual clinical management of traumatic brain injury (TBI) is limited by the lack of accessible molecular biomarkers that are informative regarding the unique mixture of injury mechanisms in each TBI patient.

15. SUBJECT TERMS
Traumatic brain injury, diagnosis

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	40	**19b. TELEPHONE NUMBER** *(include area code)*

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1. INTRODUCTION:

Traumatic brain injury (TBI) currently afflicts 357,000 enlisted military men and women in the US Armed Services. For the most common form of TBI, Mild Traumatic Brain Injury (mTBI) most patients recover within a year following the incident, but 10-20% of mild cases result in a long-term disability including seizures and emotional and behavioral issues. Although much has been learned about molecular changes in the brain following injury, access to these biomarkers following mTBI is lacking. The accurate diagnosis and precise individual clinical management of traumatic brain injury (TBI) is limited by the lack of accessible molecular biomarkers that are informative regarding the unique mixture of injury mechanisms in each TBI patient.

We hypothesize that we can address this challenge by developing a microchip-based diagnostic to characterize TBI recovery and history using the RNA cargo found in brain-derived extracellular vesicles (EVs). Unlike prior work that has mainly focused on single biomarkers, our approach measures a panel of circulating EV miRNA markers processed with machine learning algorithms to more comprehensively capture the state of the injured and recovering brain. We piloted this approach and successfully classified the severity, time elapsed since initial injury, and history of multiple injuries of TBI in an animal model and with clinical samples. Our proposed chip combines two technologies, developed in my lab, to create an ultrasensitive, automated exosome diagnostic: **1.** Magnetic nanopore isolation of EV subpopulations from the injured and recovering brain, and **2**. Time-domain encoded optofluidics for rapid highly multiplexed digital droplet exosomal RNA detection. Our approach can measure the state of injury and recovery in TBI in a minimally invasive fashion, opening new opportunities to improve molecular diagnosis, prognosis, and precision medicine for TBI injury.

2. **KEYWORDS:**

Mild Traumatic Brain Injury, Diagnostics, Exosomes, Extracellular Vesicles

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

Traumatic brain injury (TBI) currently afflicts 357,000 enlisted military men and women in the US Armed Services. For the most common form of TBI, Mild Traumatic Brain Injury (mTBI) most patients recover within a year following the incident, but 10-20% of mild cases result in a long-term disability including seizures and emotional and behavioral issues. Although much has been learned about molecular changes in the brain following injury, access to these biomarkers following mTBI is lacking. The accurate diagnosis and precise individual clinical management of traumatic brain injury (TBI) is limited by the lack of accessible molecular biomarkers that are informative regarding the unique mixture of injury mechanisms in each TBI patient.

Phase 1

Major Task 1: Next Generation Technology Development

Subtask 1: Finite element design optimization of next generation TENPO.

Intended completion date 6/1/2020. Status: 100% complete.

Subtask 2: Next generation TENPO characterization.

Intended completion date 6/1/2020. Status: 100% complete.

Subtask 3: Develop, test modular magnetic nanoparticle labeling for capturing specific subsets of extracellular vesicles (EVs).

Intended completion date 6/1/2020. Status: 100% complete.

Subtask 4: Validation of EV isolation.

Intended completion date 6/1/2020. Status: 100% complete.

Subtask 5: Isolation of RNA cargo from EVs.

Intended completion date 6/1/2020. Status: 100% complete.

Subtask 6: Develop a droplet detection technology to measure at least three colors allowing ratiometric, calibration free use and expanded multiplexing.

Intended completion date 6/1/2020. Status: 100% complete.

Milestone: Next generation TENPO will be benchmarked for throughput (100 mL/hr), biomarker-selectivity, background insensitivity, and limit of detection (1000 EVs / mL in plasma).

Intended completion date 6/1/2020. Status: 100% complete.

Milestone achieved: Droplet detection platform will be benchmarked for throughput (106 droplets / sec), accuracy AUC > 0.995, and for number of colors (n > 3).

Intended completion date 6/1/2020. Status: 100% complete. Indeed, we have exceeded our original goal and have demonstrated a working 6-plex device.

Major Task 2: In Vitro Biomarker Selection

Subtask 1: To screen for surface marker candidates we will use multiple cell-culture based stretch models of injury including models using nearly pure cultures of neurons, astrocytes, and blood-brain-barrier. We will identify surface markers unique to each cell type, and RNA markers with high differential expression between injured and control (sham) state.

Intended completion date 6/1/2020. Status: 100% complete.

Milestone Achieved: A set of EV surface markers to isolate subpopulations to profile the injured and recovering brain.

Intended completion date 6/1/2020. Status: 100% complete.

Major Task 3: Pilot Clinical / Porcine Evaluation

Subtask 1: We will isolate multiple extracellular vesicle (EV) subpopulations from injured patients and healthy controls using the TENPO from *N* = 20 injured subjects and *N* = 20 controls.

Intended completion date 6/1/2020. Status: 100% complete.

Subtask 2: We will sequence EV isolated from *N* = 40 banked serum samples from a porcine injury model

Intended completion date 6/1/2020. Status: 100% complete. This work was delayed by COVID-19 shutdown. Samples have been processed and sequencing results were just recently obtained.

Milestones Achieved: We will have sequencing data of the µRNA isolated, from each EV subpopulation, for every patient.

Intended completion date 6/1/2020. Status: 100% complete.

Milestones Achieved: We will have comparisons of this sequencing data to known biological models of injury/recovery.

Intended completion date 6/1/2020. Status: 80% complete. This work was delayed by COVID-19 shutdown. Sequencing data was only recently obtained and this analysis is near completed.

Milestones Achieved: Data accumulated from each subject will be annotated with the sequencing data and analyzed.

Intended completion date 6/1/2020. Status: 100% complete.

Milestones Achieved: We will have sequencing data, from each EV sub-population, isolated from *N* = 40 banked serum samples from a porcine injury model.

Intended completion date 6/1/2020. Status: 100% complete. This work was delayed by COVID-19 shutdown. Sequencing data was only recently obtained.

Milestones Achieved: We will have compared this porcine model to our clinical data, validating it it for further use in our study.

Intended completion date 6/1/2020. Status: 80% complete. This work was delayed by COVID-19 shutdown. Sequencing data was only recently obtained and this analysis is now underway..

Milestone Achieved: HRPO/ACURO Approval

Intended completion date 6/1/2020. Status: 100% complete.

Milestone Achieved: Meeting with the FDA for guidance

Intended completion date 6/1/2020. Status: 100% complete. We have reached out to the FDA, and I was invited down to give a seminar. In light of conversations with potential commercialization partners, we have strategically chosen to obtain our next set of clinical data (later this year) before re-initiating our conversation with the FDA.

Phase 2

Major Task 1: Porcine Model Study.

Subtask 1: We have planned and have been carrying out the injury experiments on (N = 32 injured, N = 8 healthy) animals.

Intended completion date 5/1/2021. Status: 70% complete. These injury experiments will likely be complete by the next quarterly report. We have run pilot samples for Immunochemistry to validate this component of the study, and are currently working our way through the animal injuries and sample collection.

Milestone Achieved: We will use open field and T-maze tests to evaluate cognitive recovery.

Intended completion date 5/1/2021. Status: 0% complete. We have pivoted away from behavioral measurements due to feedback from our collaborators.

Milestone Achieved: We will measure the pig's sensitivity to light and sound. Auditory event-related potentials (ERPs) will be obtained from animals prior to injury, and again 1, 3 and 7 days post-TBI.

Intended completion date 5/1/2021. Status: 0% complete. We have pivoted away from behavioral measurements due to feedback from our collaborators.

Milestone Achieved: Immunochemistry will be performed on the porcine injured animals, as described in the table on the following page.

Intended completion date 5/1/2021. Status: 60% complete. We are currently working our way through the animal injuries and sample collection.

Milestone Achieved: T1-weighted and diffusion tensor imaging (DTI) sequences will be collected

Intended completion date 5/1/2021. Status: 20% complete. We are currently working our way through the animal injuries and sample collection.

Milestone Achieved: The EV RNA signatures from each of these injury types will be collected and compared to those from humans in Phase 1 and our behavioral, histology, and imaging.

Intended completion date 5/1/2021. Status: 60% complete. We are currently working our way through the animal injuries and sample collection.

Major Task 2: Next Generation Technology Development

Subtask 1: Work incorporating the TENPO EV isolation with cell-phone based droplet PCR has progressed nicely.

All individual components, including droplet production, thermal cycling, and droplet readout platforms have been developed and validated. We now have an integrated system and are working on optimization and validation of the platform.

Subtask 2: We will benchmark our chip against a commercial BioRad digital PCR system.

Intended completion date 5/1/2021. Status: 50% complete. We have just in the last month begun to benchmark our chip against a commercial BioRad digital PCR system.

Milestone achieved: We benchmark RNA detection using known quantities of RNA template, and compare results of μDFD to conventional qPCR.

Intended completion date 5/1/2021. Status: 50% complete. We have just in the last month begun to benchmark our chip against a commercial BioRad digital PCR system.

Milestone achieved: We will test samples spiked with known quantities of culture derived EVs, and compare to off-chip conventional qPCR.

Intended completion date 5/1/2021. Status: 50% complete. We have just in the last month begun to benchmark our chip against a commercial BioRad digital PCR system.

Sensitivity and specificity for RNA detection will be characterized, with a goal of sixteen parallel channels, resulting in > 10^6 droplets per minute total, with an AUC > 0.995.

Intended completion date 5/1/2021. Status: 50% complete. We have just in the last month begun to benchmark our chip against a commercial BioRad digital PCR system.

What was accomplished under these goals?

Extracellular vesicles as distinct biomarker reservoirs relative to conventional serum-based biomarkers for mild traumatic brain injury diagnosis.

Although most individuals who experience a mild traumatic brain injury (mTBI) recover within weeks after the injury, a significant number of patients suffer from persistent symptoms that include headaches, cognitive changes, and mood disturbances for months afterward.[1-3] Conventional approaches to evaluate mTBI have focused on the currently known hallmarks of moderate-to-severe brain damage, such as clinical assessment using the Glasgow Coma Scale, macroscale lesions visualized with CT imaging, and circulating neurodegenerative markers.[4] Unfortunately, these methods lack the sensitivity and specificity needed to clinically characterize milder injuries, to identify patients who are likely to have persistent symptoms in the time following mTBI, and to guide each patient to a personalized, effective therapy.[5] Because adequate biomarkers are lacking, the identification of mTBI patients in need of intervention remains mainly limited to monitoring for and treating post-concussive symptoms as they arise rather than treating the underlying pathology much earlier in the course of the disease, when therapies are more likely to be effective.

Biomarker discovery to accurately diagnose and classify an individual's TBI into categories for improving individual patient outcomes and developing new treatments has generated great interest in recent years. However, identifying sufficiently sensitive and specific biomarkers has been confounded by the particularly dynamic and heterogeneous nature of TBI. Each TBI results in a unique combination of initial tissue damage and secondary pathology including vascular dysfunction,[6,7] axonal injury,[8,9] and inflammation[10] that evolve following the injury.[11-13] These distinct aspects of an individual's TBI – each of which maps to multiple potential biomarkers in the blood – is considered key to a patient's possible recovery or progression to behavioral and cognitive deficits.[14,15] Moreover, the profiles of biomarkers in the blood are dynamic, originating from both the initial tissue damage and the multiple secondary pathologies that develop over time after the injury.[14,16-17] The complexity of biomarker expression following an injury results in diagnostics measurements that can be challenging to interpret.

To gain a more comprehensive assessment of mTBI, many researchers have shifted their attention away from measurements of single biomarkers to measurements of biomarker panels, where each constituent biomarker can be chosen to assess a different aspect of the patient's TBI.[18] For example, the combined analysis of circulating glial fibrillary acidic protein (GFAP), an astrocyte derived intermediate filament protein, and ubiquitin C-terminal hydrolase L1 (UCHL1), a neuronal cytosolic protein, accurately identifies injury severity and CT scan lesions in clinical TBI. While this assay – the Banyan Brain trauma indicator test – has demonstrated promise as a TBI diagnostic for more severe injuries, such biomarkers have not yet been identified that can reliably classify underlying TBI endophenotypes or predict patient outcomes after mTBI.[19] Other proposed TBI biomarkers have potential to directly assess specific underlying TBI pathologies. These include neurofilaments,[20,21] a major cytoskeletal component of neuronal axons, and Tau, a cytoskeletal protein whose phosphorylation and aggregation are hallmarks of neurodegenerative conditions.[22,23] Dysregulated central and peripheral immune cell function following TBI results in the release of cytokines, chemokines, and complement components that may provide an assessment of inflammation, a key driver of neurologic deficit post-TBI.[24,25]

Extracellular vesicles (EVs) have generated particular interest for multiplexed TBI diagnostics. EVs are nanoscale vesicles ranging from 100-1000nm[26] generated through a variety of mechanisms including plasma membrane budding or the fusion of multivesicular bodies (MVBs) to the cellular membrane to be released into the extracellular space.[27] EVs possess surface proteins derived from the parent cell, and cargo (proteins, mRNA, miRNAs) within the vesicle lumen that reflect the status of their cells of origin and that, when transferred to recipient cells, can act as agents of cell-cell communication.[28-30] EVs are emerging as a promising complement to plasma derived biomarkers, as they contain cargo that may play direct roles in TBI pathology, and contain surface proteins that allow brain derived EVs to be isolated from the blood. EVs and their cargo also provide a work-around to the impracticality of brain tissue biopsy by crossing the blood brain barrier[31] into CSF, peripheral circulation,[32] and other bodily fluids making them easily accessible CNS biomarkers for monitoring TBI progression.[33,34] Moreover, EVs are shed by both healthy and degenerating cells, providing a broader view into the molecular processes that occur within a tissue or organ. In the optimal form, the combination of information extracted from EVs of injured, but not necessarily degenerating, neurons with neuronal biomarkers and inflammatory mediators could lead to accurate classifications and prognoses of patients with mTBI.

On their own, the diagnostic potential of EVs has been shown in military personnel, where circulating exosome-packaged Tau and IL10 levels are elevated with mTBI and correlate with post-concussive and post-traumatic stress disorder symptoms.[35] Other studies have found variations in EV microRNA concentration after TBI.[36] In previous work we showed that by enriching brain associated EVs, which expressed the glutamate ionotropic receptor AMPA type subunit 2 (GluR2) surface marker, from plasma using a nanomagnetic chip, and analyzing RNA cargo we could identify RNA signatures that accurately classified the injury, including its presence, severity, history of previous injuries, and timing.[37-39] However, this work was limited to the RNA cargo of EVs, and did not incorporate known biomarkers of neuronal and glial cell damage or inflammation packaged in EVs.[35]

In this study, we combined conventional assessment of TBI-associated biomarkers in plasma with our approach of acquiring molecular information from brain derived EVs. Our main purpose was twofold: to determine if EVs and plasma biomarker proteins represented *independent* information for mTBI diagnostic use, and to evaluate the relative effectiveness of applying this approach on a set of mTBI patients. We used our TENPO technology to enrich for brain derived EVs and leveraged an existing ultrasensitive digital ELISA technique, single molecule array (SIMOA), to accurately determine common protein biomarker levels in these two compartments. We demonstrate that the independence of the molecular information stored in the GluR2+ EVs and in plasma allows for the development of a multianalyte approach to mTBI diagnosis. In this work we use a machine learning algorithm developed using biomarkers from both compartments as a proof-of-concept of this approach, and illustrate its ability to successfully discriminate mTBI patients from control subjects.

Due to the subtle nature of the physical injury in mTBI, we hypothesized that algorithmically combining biomarkers from both brain derived EVs and plasma could result in more sensitive and specific discrimination of mTBI patients from controls than that of any individual biomarker, or any one biomarker compartment. To test this hypothesis, we obtained human plasma samples from TBI subjects admitted to an urban, academic Level 1 trauma center (University of Pennsylvania's Penn Presbyterian Medical Center) following a head impact – representing the diversity of injury types encountered in the clinic including assault, road traffic incidents, and falls – as well as healthy control and orthopedically injured participants yielding a study size consistent with previous experiments (**Fig. 1A**).[38] Our blood-based assessment of mTBI included a panel of neuronal and glial cell damage biomarkers (UCHL1, NFL, Tau, and GFAP) and key drivers of inflammation (IL6, IL10, TNFα) quantified in both plasma and within brain derived EVs expressing GluR2. (**Fig. 1B**). We then used this data to investigate protein distribution across the two compartments and to evaluate mTBI-associated changes in biomarker concentration and signatures (**Fig. 1C**).

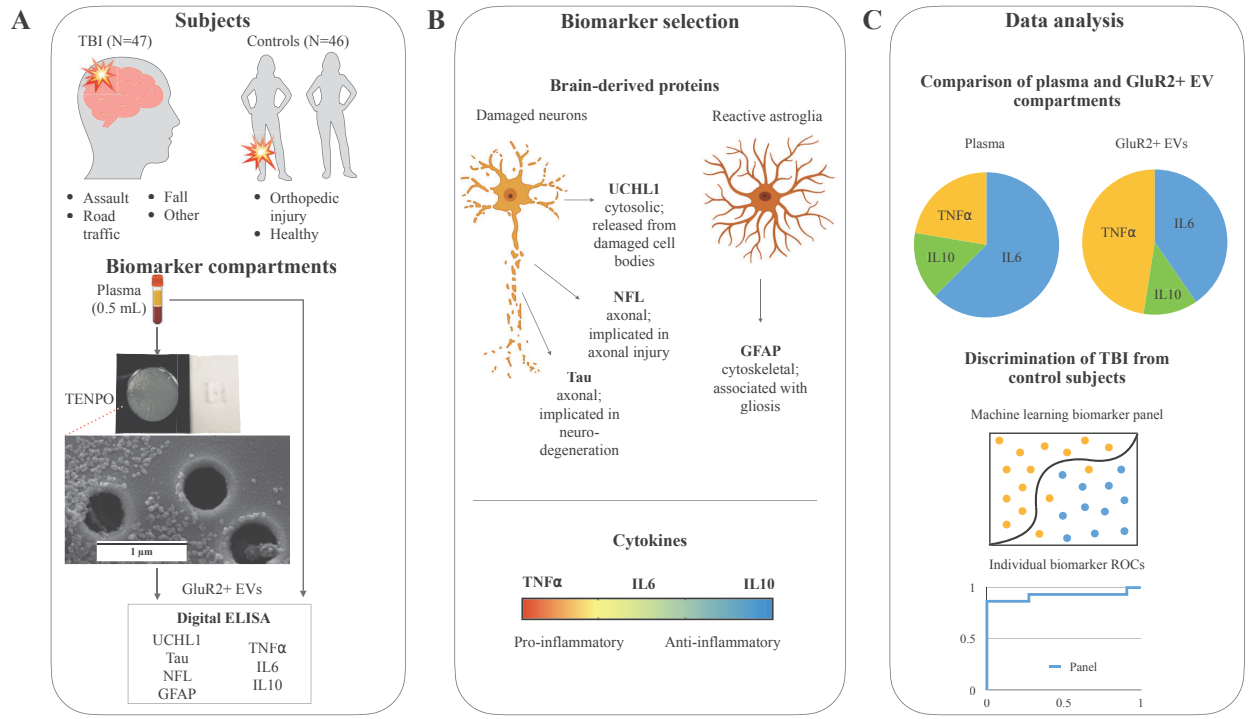

Figure 1: Project Workflow. 1) Samples were obtained from subjects sustaining TBIs through a variety of mechanisms and from a combination of orthopedic injured and healthy controls. One 500 μL aliquot of plasma from each subject was used to isolate brain derived EVs based on their expression of GluR2 using our nanofluidic platform, TENPO. Lysate from GluR2+EVs and a second 500 μL aliquot of plasma were subjected to digital ELISA assessment. **2)** Biomarkers were selected based on known or emerging role in neuronal (UCHL1, NFL, Tau) or astrocyte (GFAP) pathology, or on their roles in the spectrum of inflammatory function (TNFα, IL6, IL10). **3)** Analyses served two purposes: comparison of biomarker distribution in plasma and in GluR2+ EVs, and the discrimination of TBI and control subjects. A machine learning approach was used to combine the multiplexed data into biomarker panels for comparison with the performance of individual biomarker ROC curves and panels of biomarkers from each compartment alone.

Participant demographics
To test our hypothesis that algorithmic combination of biomarker data yields a more accurate mTBI diagnostic, we collected plasma and EV samples from GCS mild (13-15) clinical TBI patients (*n*=47; **Fig. 1**). The study design also included a control group (*n* =46) consisting of both healthy age-matched and orthopedic injured controls to assess the specificity of blood and EV-packaged biomarkers to mTBI. Although controls and mTBI subjects were of similar ages (mean = 36 years +/- 16 TBI, +/- 14 controls), there were 20% more males in the mTBI than in the control group (**Table 1**).

Plasma and brain derived GluR2+ vesicles display variable biomarker distribution across individuals To visualize the spread of the data across individuals, we first plotted log values of each biomarker across TBI and control subjects (**Fig. 2A**). For the mTBI group, coefficient of variance (CV) values for plasma biomarker levels ranged from 31% for GFAP, to 120% for TNFα. In the control subjects, CV values for plasma biomarkers ranged from 27% for IL10, and 140% for GFAP. Levels of plasma biomarkers for mTBI subjects were not significantly affected by injury type (ANOVA; $p>0.65$ across all biomarkers), and only plasma TNFα levels significantly correlated with age ($p<0.05$; $R^2=0.091$). Since this correlation was small, we collapsed all injury types into a single mTBI group for our subsequent analysis of plasma biomarker measures. In the control group, orthopedic controls exhibited significantly higher levels of plasma GFAP compared to the control mean (ANOVA; $p<0.05$; Dunett correction for multiple comparisons), and there were small correlations between age and plasma GFAP and UCHL1 levels ($p<0.05$; $R^2=0.22$ and 0.27 respectively). There were no other additional effects of control type or age on plasma biomarker levels. We thus consolidated control subjects into a single group for plasma biomarker analyses.

Our analysis and visualization of the data also revealed, similar to plasma biomarkers, that proteins packaged in GluR2+ EVs are also expressed heterogeneously across individual subjects (**Fig. 2A**). For the mTBI group, CV for GluR2+EV-packaged biomarkers ranged from 45% for IL6 to 140% for Tau. We found that neither injury/ control type nor age has an effect on the expression of EV-packaged biomarkers ($p>0.38$ (injury type, across all biomarkers); $p>0.24$ (control type, across all biomarkers)). Nor were there any significant correlations between age and GluR2+EV biomarker levels for either group ($p>0.05$ across all biomarkers for both groups) Therefore, we consolidated all injury types to a single injury group, and the two control types into a second group (supplemental tables 5-8). Furthermore, demonstrated that there is no significant difference in the number of EVs across TBI and controls ($p>0.05$), eliminating the need to normalize across EV count (**Fig. 2B**).

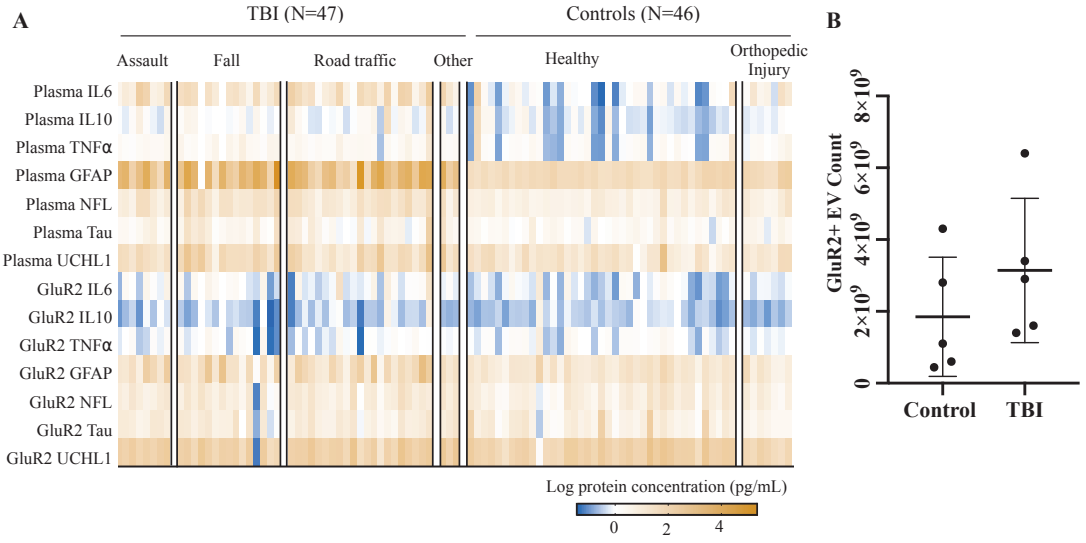

Figure 2: Expression of brain-derived proteins and cytokines is heterogeneous across TBI and controls in both plasma and GluR2+EV compartments. A) Log transformed biomarker levels plotted in heat map. Columns represent subjects, each arranged within respective TBI or control types by increasing age. **B)** Number of GluR2+ EVs isolated from 05.mL plasma from *N*=5 TBI and *N*=5 control subjects.

Once we consolidated our dataset, we hypothesized – based on other studies of mTBI biomarkers, and on the 57% rate of CT scan abnormality of our mTBI subjects (**Table 1**) – that mTBI subjects would exhibit significant elevations in conventionally-studied plasma biomarkers relative to controls.[19] To test this hypothesis, and to investigate whether mTBI was also associated with significant changes to biomarker levels in the GluR2+EV compartment, we compared mean levels of individual biomarkers in both compartments (**Fig. 3**).

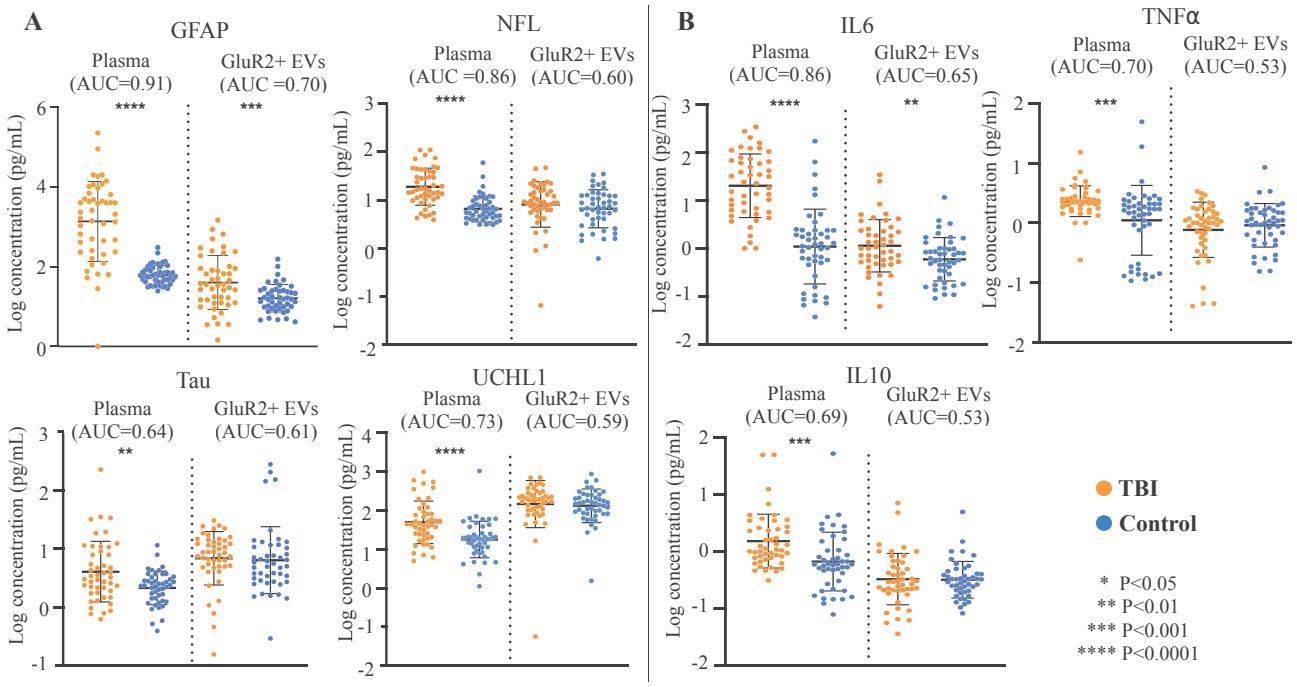

Figure 3: Mild TBI is associated with elevations in both brain-derived proteins and cytokines in plasma and GluR2+ EVs. Scatter plots of mean log biomarker values and standard deviation as error bars. Calculation of p values using student's T Test were done using log-transformed data. AUCs were generated using raw values.

Table 1. Descriptive characteristics of traumatic brain injury patient and control subjects; mean +/- SD or N (%)

Characteristics	Training set TBI patients	Training set controls	Test set TBI patients	Test set controls
N	30	31	17	15
Demographics				
Age, mean +/- SD (years)	35 +/- 14	36 +/-16	44 +/-18	28 +/- 8
Male gender- (%)	83%	55%	61%	60%
Clinical characteristic				
GCS, mean	14.4	N/A	14.5	N/A
Positive CT- n (%)	57%	N/A	72%	N/A

GCS, Glasgow Coma Scale; CT, Computed Tomography.

Brain derived EVs and plasma possess distinct protein compositions

Until this point, we analyzed the performance of single proteins, regardless of its originating in plasma or GluR2+ EVs, to best discriminate between mTBI and control samples. However, simply combining the best individually high performing biomarkers would potentially overlook combinations of biomarkers that would better predict the presence/absence of mTBI. To evaluate distinctions in biomarker information across plasma and GluR2+EV compartments, we first assessed the distribution of biomarkers in each group. In the mTBI group, plasma and GluR2+EVs displayed significantly different proportions of all measured cytokines ($p<0.001$ across all cytokines; **Fig. 4A**). Specifically, the relative abundance of IL6 was significantly elevated in plasma compared to GluR2+EVs. Conversely, GluR2+EVs contain significantly higher proportions of both IL10 and TNFα than plasma. The distribution of cytokines is also more balanced in GluR2+EVs, with IL10 and TNFα making up similar proportions, while in plasma, cytokine distribution is skewed with the relative abundance of IL6 dwarfing that of IL10 and TNFα by 11- and 5-fold respectively. In contrast to the mTBI group, the control group displayed no significant differences in proportions of IL6 or IL10 across the two compartments ($p>0.05$). In this group, only TNFα abundance differed in plasma and GluR2+EVs, showing a significant increase in the latter ($p<0.0001$). Lastly, in this group, distribution of the three cytokines exhibits more balance in both compartments, each having similar proportions of IL6 and TNFα.

Like the cytokines, our analysis also revealed differences in plasma and GluR2+EV distributions of the four brain derived proteins (**Fig 4B**). For both mTBI and control subjects, abundance of three of the four (GFAP, NFL, and UCHL1) display significant differences in plasma compared to GluR2+EVs, and Tau abundance is significantly elevated in GluR2+EVs in the mTBI group ($p<0.05$ across all brain derived proteins; **Fig. 4B**). In both groups, the distributions of these proteins are uniquely skewed in each compartment; in plasma, GFAP abounds (fold increases of 31, 136, and 15 relative to NFL, Tau, and UCHL1 respectively for mTBI group; fold increases of 10, 24, and 3 relative to NFL, Tau, and UCHL1 respectively for controls) while GluR2+EVs are dominated by UCHL1 (fold increases of 6, 15, and 17 compared to GFAP, NFL, and Tau respectively for mTBI group; fold increases of 8, 16, and 22 relative to GFAP, NFL, and Tau respectively for controls).

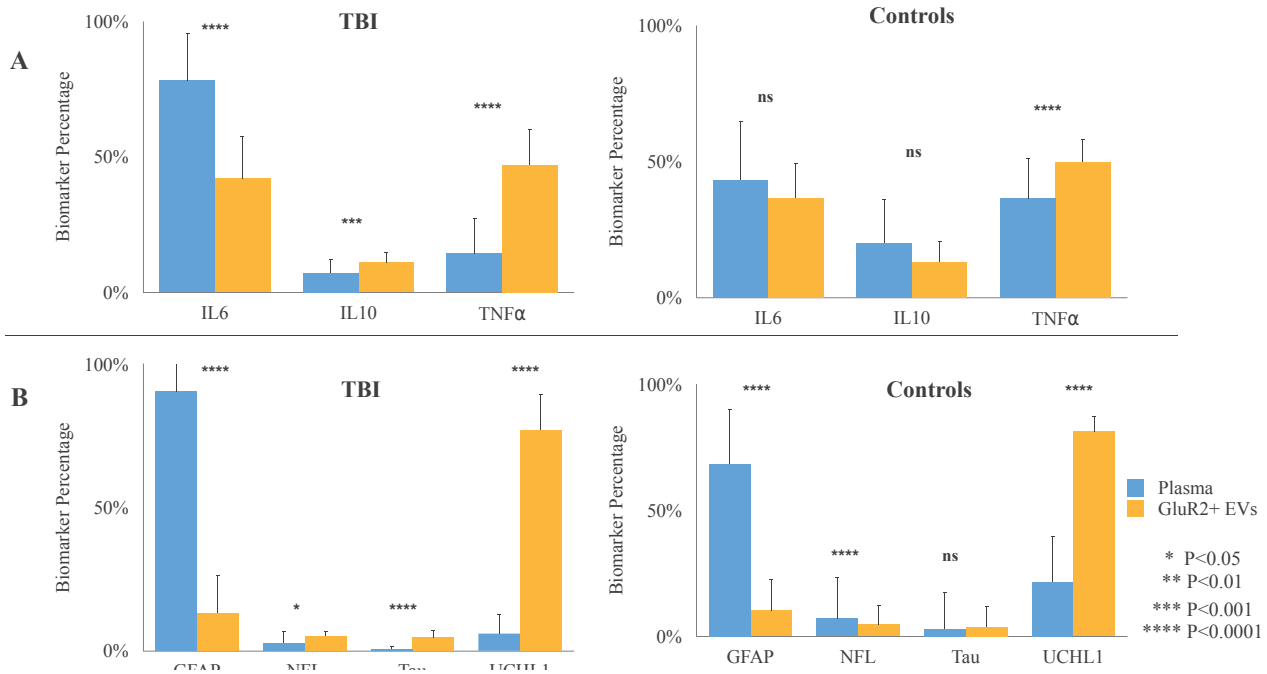

Figure 4: Plasma and brain derived EVs possess distinct protein composition that are each altered by TBI. Mean levels of each biomarker were totaled across individuals to determine the relative percentage of each **A**) cytokine and **B**) brain derived protein in plasma and GluR2+ EVs. Error bars represent SD calculated by propagation of uncertainty. T tests were performed to assess statistically significant differences in biomarker levels across compartments.

Biomarker levels correlate more within than across plasma and brain derived EV compartments

To develop a combinatorial method for discriminating TBI from control subjects, each biomarker should hold the potential to contribute unique information about each patient's TBI. To assess this, we calculated three sets of correlation values for each biomarker category (cytokines or brain derived proteins): correlations within plasma, within the GluR2+EVs, and across these two compartments (**Fig. 5A**).

We found levels of cytokines (IL6, IL10, and TNFα) and brain derived proteins (GFAP, NFL, Tau, UCHL1) correlated more with each other within the same compartment than between the plasma and EVs compartments (**Fig. 5B**). For mTBI subjects, cytokine levels are most correlated within the plasma (avg. Pearson's R= 0.61). In comparison, cytokine correlations within the EV compartment (avg Pearson's R = 0.38) and across plasma and EVs (avg Pearson's R = 0.41) were similar. Levels of brain derived biomarkers are more correlated within plasma (avg Pearson's R= 0.50) and within GluR2+ EVs (avg Pearson's R = 0.35) than across plasma and GluR2+EV compartments (avg Pearson's R= 0.0065). As with the mTBI group, cytokine levels are most correlated within plasma (avg Pearson's R = 0.80) in the control group. In contrast to the mTBI group, cytokine levels are also more correlated within EVs (avg. Pearson's R = 0.57) than across plasma and EVs (0.20). Like the mTBI group, pools of brain derived biomarkers are more distinct: levels of these proteins are most correlated in EVs and within the plasma compartment, and least correlated across compartments (avg. Pearson's R = 0.55, 0.42, 0.0065 respectively). Our results showed that different groups of biomarkers had very little correlation across compartments though levels correlated within each group, demonstrating the orthogonality of information within each.

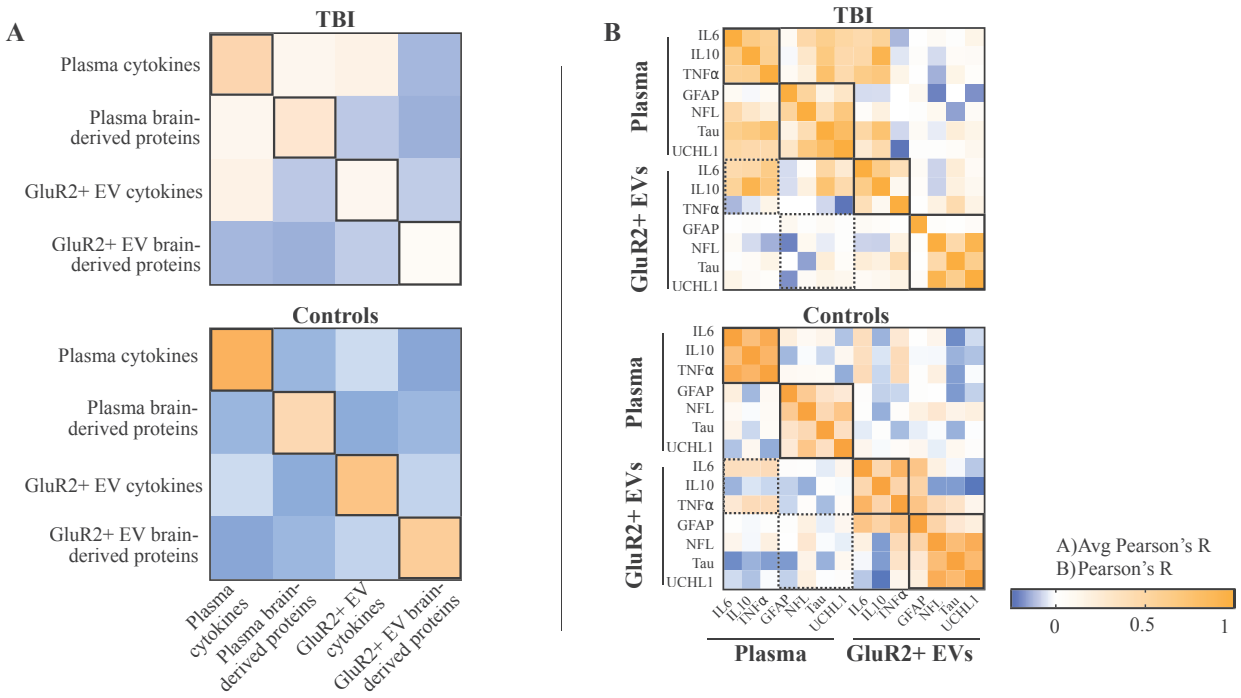

Figure 5: Biomarker levels are uncorrelated across plasma and brain derived EV compartments. Pearson's correlation coefficients calculated for all possible combination of biomarkers. **A)** Average R for each biomarker type (cytokines or brain derived markers) and for each compartment (plasma or GluR2+ EVs) were plotted in a heat map matrix for TBI patients and controls. Solid boxes indicate average R for each biomarker type-compartment combination. **B)** R for each biomarker comparison was plotted into heat map matrices for both TBI patients and controls. Solid boxes indicate R for individual biomarkers of the same type (cytokines or brain derived proteins) within each compartment. Dashed boxes indicate R values for biomarkers of the same type, but of different compartments.

Machine learning utilizes distinct biomarker information across compartments to classify mTBI.

Given the independence of information collected from plasma and plasma derived EVs, we next assessed whether we could develop a machine learning-based classifier of TBI using the complimentary biomarker information contained within each. To achieve this goal, we first applied Least Absolute Shrinkage and Selection Operator (LASSO) on our training set of data (n=61) and determined the best performing panel for discriminating TBI from controls (AUC=0.913, Accuracy=0.825, **Fig.6A**). The panel consisted of five biomarkers: plasma NFL, GFAP, IL-6 and TNFα and GluR2+ EV Tau. To further evaluate the performance of our panel, we applied it on an independent, blinded test set of 26 subjects. The panel resulted in AUC=0.92 and accuracy of 88.5% (**Fig.6B**). To demonstrate the benefit of using multi-analyte panel, we compared its performance with that of each individual biomarker (assessed using ROC analysis) and with panels comprised of biomarkers of a single compartment. Our machine learning panel led to a significant improvement in discriminating mTBI patients from controls (AUC) compared to the individual biomarkers from both plasma and GluR2+ EVs ($z > 1.96$; $p<0.05$ **Fig.6C**), except for plasma GFAP ($z = 0.71$; $p=0.47$) and plasma IL6 ($z=1.4$; $p=0.16$) when tested on the consolidated dataset (training + test). Though our panel had the highest AUC, the added benefit did not reach significance compared with the best-performing single compartment panels ($z = 1.11$; $p=0.27$ for plasma brain derived proteins; $z = 1.1$; $p=0.26$ for plasma cytokines; **Fig.6D**).

However, the panel of five biomarkers did outperform the best performing panels with fewer biomarkers, i.e. a panel of plasma NFL and GFAP ($p<0.001$) and a panel of plasma NFL, GFAP, and GluR2+ EV Tau ($p<0.01$), derived from the original panel when tested across the combined training and test set. To address the question of whether we had included enough subjects to properly train our model, we generated a learning curve on our entire subject population, including both the training and test set. We found that the model's performance plateaued beyond 58 subjects, indicating that our training set sample of 61 subjects was sufficient for the patient population in this study. Lastly, we observed a significant decline in the model's performance upon removing GluR2+ EV Tau from the panel (**Fig.6F;** $p<0.001$). Neither the plasma brain derived markers (GFAP and NFL) nor cytokines (IL6 and TNFα) affected the panel's performance when removed.

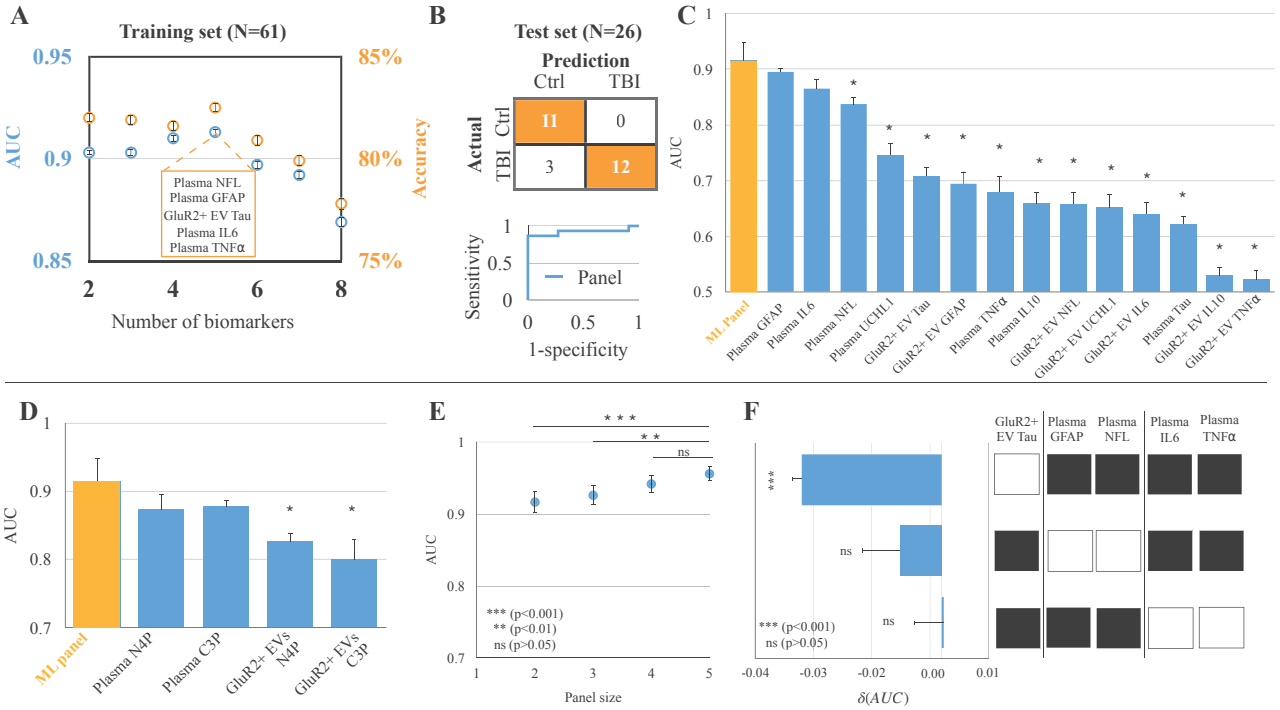

Figure 6: Machine learning combination of biomarkers from both plasma and GluR2+ EVs outperforms single biomarkers and panels consisting of a single biomarker compartment in classifying TBI. A) Performance of machine learning ensemble classifier using biomarkers from both GluR2+ EV and plasma compartments. **B)** Machine learning panel performance on user blind test subjects. **C)** Comparison of machine learning panel performance with single biomarker performance. *indicates z score >1.96; $p<0.05$ relative to machine learning (ML) panel using paired t Test while considering AUCs correlation induced by the nature of data. Error bars for single biomarker AUCs represent standard deviation of biomarker performance across 10 trials of random subsets of the dataset (90% of the total sample size) with replacement using bootstrapping. **D)** Performance of panels consisting of biomarkers from plasma (plasma Neuro4Plex, plasma Cytokine3Plex) or GluR2+ EVs (GluR2+ Neuro4Plex, GluR2+ Cytokine3Plex) alone, and panels of 2,3,4, or all 5 of the biomarkers of the machine learning panel compared to the performance of the full multi-compartment machine learning panel. * indicates z score >1.96 relative to ML panel using paired t Test while considering AUCs correlation. **E)** AUC of panels consisting of biomarkers from plasma (plasma Neuro4Plex, plasma Cytokine3Plex) or GluR2+ EVs (GluR2+ Neuro4Plex, GluR2+ Cytokine3Plex) alone, and panels of 2,3,4, or all 5 of the biomarkers of the machine learning panel compared to the performance of the full multi-compartment machine learning panel. **F)** Change in machine learning model AUC following removal of markers from the panel. White boxes indicate the marker(s) removed. Error bars indicate standard error after 50 trials.

DISCUSSION

Our study demonstrates that circulating brain derived EVs and plasma represent two distinct reservoirs of molecular information, the composition of each differentially altered by mTBI. By using both compartments to algorithmically identify a biomarker signature that persisted across individuals, we mitigated the effects of person-to-person variability in biomarker expression and accurately classified mTBI (AUC=0.92; accuracy = 88.5%). To achieve this, we combined two technologies – TENPO that can specifically enrich for GluR2+ EVs from plasma, and digital ELISA that can measure multiple protein biomarkers with 100-1000x better sensitivity than conventional ELISA – to address the challenges of accurately detecting levels of biomarkers that often circulate at levels too low to detect with conventional technologies. The combined use of biomarkers of specific TBI pathologies analyzed in the context of distinct biofluid environments from separate cellular pools is a promising approach for developing a more comprehensive assessment of the state of the injured and recovering brain.

We began our analysis with measuring circulating levels of brain derived proteins GFAP, NFL, Tau and UCHL1, which demonstrated predictive power as individual biomarkers similar to past studies of these biomarkers in mTBI.[19,47] Since a sizable proportion of mTBI subjects in this study (57%) sustained brain pathology observable through CT scan, the significant elevations in plasma levels of UCHL1 and NFL ($p<0.0001$ for each) were expected.[19] As mTBI results in few degenerating neurons,[48] it is not surprising that we did not detect significant elevations in Tau after mTBI. In contrast, reactive gliosis can be observed throughout the brain even after mild injury,[49] and as expected we found plasma GFAP as the most robust single biomarker to discriminate mTBI subjects from controls (AUC=0.89). However, we observed plasma GFAP levels were significantly elevated in orthopedic-injured controls and controls over 51 years (ANOVA; $p<0.046$ and $p<0.0001$ respectively) compared to the total control plasma GFAP mean. This finding, combined with observations that GFAP is released from other cell types of the body,[50] may complicate GFAP's specificity to brain injury, especially if it is used in isolation in polytrauma cases. We also observed significant elevations in plasma levels of IL6, IL10, and TNFα ($p<0.001$ across all cytokines), and indeed, plasma IL6 followed directly behind plasma GFAP in discriminating mTBI (AUC=0.86). But the broad role that cytokines play in mediating systemic trauma,[51] immune challenges,[52,53] and other neurological disorders[54] may limit the specificity of these biomarkers in plasma.

In our assessment of mTBI, we also included brain derived EVs expressing GluR2, an appealing alternative to co-opting the circulating neurodegenerative markers typically associated with moderate-to-severe TBI for mTBI diagnostics. On their own, the proteins in brain derived EVs performed no better than those in plasma as individual biomarkers, despite the IL6 and GFAP elevations observed in this compartment relative to controls ($p<0.01$ and $p<0.001$ respectively). However, it was intriguing to see that protein concentration of the same biomarkers across the two compartments did not correlate with each other. One potential explanation for this result is that plasma levels of some biomarkers appeared from active degeneration processes in a small population of cells, while the exosome derived measurements originate from a large population of largely intact neurons and glia responding to the mild mechanical trauma. Other studies of EV-based biomarkers of TBI have similarly observed differences in EV-contained and plasma molecular cargo. In a study measuring time-dependent changes in protein biomarkers within the total circulating EV population and plasma, investigators found no correlation between the two compartments out to 5 days after injury.[55]

Although studies on EVs and their contents is only emerging, the broader sampling of EV signatures from cells that do not later degenerate provides a new opportunity for understanding the consequences and recovery processes of mild trauma to the brain. With the broad disruption in blood-brain barrier integrity that occurs after mTBI,[56] it is possible that plasma activates pathologic cascades in neurons and glia that do not later degenerate, resulting in a cellular population that largely outnumbers actively dying or degenerating ones in mTBI that are not assessed with traditional plasma biomarkers. In experimental models of concussion and in clinical studies, degenerative changes can occur days to months following the initial mild injury, and can be further complicated by repeated, periodic opening of the blood-brain barrier.[57-60] These primed or activated neurons and glia undergo subtler forms of cellular damage or distress as they constitutively secrete exosomes, potentially as a mechanism for clearing cellular debris as they recover. We observed that GluR2+ EVs contain the same inflammatory cytokines and markers of cell damage expressed by lesioned cells.[61] Interestingly, we found UCHL1 – a deubiquitinating enzyme – dominated the EV pool of brain derived proteins (**Fig. 4**). As UCHL1 plays a neuroprotective role in brain injury by degrading reactive lipids and misfolded proteins,[62,63] the high relative abundance of this protein in GluR2+ EVs in relation to the other measured brain derived proteins suggests GluR2+EVs may serve as a protein clearing system for the damaged or distressed cells of the brain, a role for EVs that has already been demonstrated for other EV populations in other contexts and cell types.[64] Thus, our machine learning approach combines molecular information from three different categories: markers from a small number of severely damaged, degenerating cells (plasma brain derived markers), markers of broad-scale inflammation, and markers originating from a potentially less-damaged population of brain cells (brain derived EVs). By investigating what downstream molecular targets GluR2+EV-packaged UCHL1 interacts with, such as other components of the ubiquitin ligase system and potential degradation targets, we can broaden our understanding of the role the GluR2+ EV population plays in TBI pathology, and our potential pool of EV-associated biomarkers.

Our analysis of brain derived EVs was limited to those expressing GluR2+, and by expanding our approach to mTBI biomarker development – from broadening the EV subtypes that we isolate to surveil a more comprehensive set of cells affected by TBI, to advancing the technologies with which we measure and analyze this complex information – we can improve our ability to monitor mTBI outcome and identify accurate treatment strategies. Since this work we have extensively optimized the TENPO protocol, incorporating sequential wash steps following vesicle capture which greatly reduce background relative to relying on the chip's small dead volume to promote removal of unbound material.[39] Additionally, while 0.1% SDS was the lowest possible concentration allowing us to detect protein in both assays, we also aim to improve lysis buffer conditions to maximize protein yield and the efficacy of biomarker detection. We now use phosphatase/protease inhibitor cocktail and are investigating the use of non-denaturing reagents, both of which have been used in other SIMOA studies of exosome protein expression.[35,55] We are also exploring capturing circulating EVs derived from multiple brain cell types (neurons, astrocytes, microglia, endothelial cells etc), which, when combined with advancements in downstream EV cargo analysis, maximizes the molecular information at our disposal for these goals. Multianalyte approaches to disease diagnosis have already shown promise in other fields, resulting in higher accuracy in early detection and staging of cancer.[65-67] For mTBI, as the technology for isolating EVs from different populations of distressed-but-not-dying brain cells evolves, we may improve our ability to identify pathologies like gliosis and brain endothelial cell dysfunction as they occur across individual patients to better "grade" the TBI. Though we used digital ELISA in this study, a platform incompatible with point-of-care diagnostic technologies, widespread efforts to scale down these assays into portable platforms makes accessibility and clinical use of molecular diagnostic more achievable (Yelleswarapu *et al.*, 2019).[68]

Our previous work also points to the promise of miRNA cargo for a more open-ended TBI assessment than is provided by known protein biomarkers, but combining EV protein cargo information with amplifiable EV-associated miRNA results in a wealth of potential opportunities to develop more accurate and sensitive mTBI characterization. Advanced approaches to data analysis such as machine learning coupled with improved understanding of the pathologic roles brain derived EVs play in mTBI progression broadens our potential to combine this wealth of information into meaningful molecular signatures used to monitor and intervene in this insidious neurologic condition.

ExoTENPO assay development for multiple cell types

Building on this previous work, we have now expanded our ExoTENPO pulldown to isolate two distinct EV subsets, one subset enriching for EVs that originate from neurons and a second subset of EVs that originate from astrocytes. We hypothesized that i. sets of EV surface markers exist to distinctly enrich for EVs originating from neurons and EVs originating from astrocytes, ii. the RNA cargo contained in both the neuron and the astrocyte EVs would have predictive power for diagnosing and prognosing TBI, iii. the RNA cargo contained within the neuron and astrocyte derived EVs would be distinct from one another, representing independent reservoirs of information, iv. by combining the RNA cargo from the neuron and astrocyte derived EVs, more acurate classifications can be made than is possible with the neuron or astrocyte derived EVs on their own.

To this end, we use efficient two-step bio-orthogonal magnetic labeling, to enables the use of generic nanoparticles, the efficient use of affinity ligands, and amplified magnetic labeling. The minimal magnetic signature of biological material enables us to use magnetic fields and capture exosomes bound to these magnetic particles efficiently and quickly (~10 minutes), avoiding sample loss and simplifying clinical use. We apply this strategy by labeling biomarkers with affinity ligands modified with trans-cyclooctene (TCO) and then MNPs modified with 1,2,4,5-tetrazine (Tz). We use super-paramagnetic (d ~ 15 nm) cross-linked iron oxide nanoparticle (CLIO). The superparamagnetic iron oxide core is coated with dextran and cross-linked and functionalized with primary amine. We have explored targeting using CD63, CD81, and CD 9 (pan-exosome), neuronal surface markers including GluR2, L1CAM, NCAM and astrocytic markers including GLAST, ASCA-2, and GFAP. We first evaluate these surface markers using two distinct cell culture models, one including astrocytes only and the other a mix of neurons and astrocytes. Using a whole EV ELISA, we quantify the relative binding of these antibodies to the EV surfaces. (**Fig. 7A,B**)

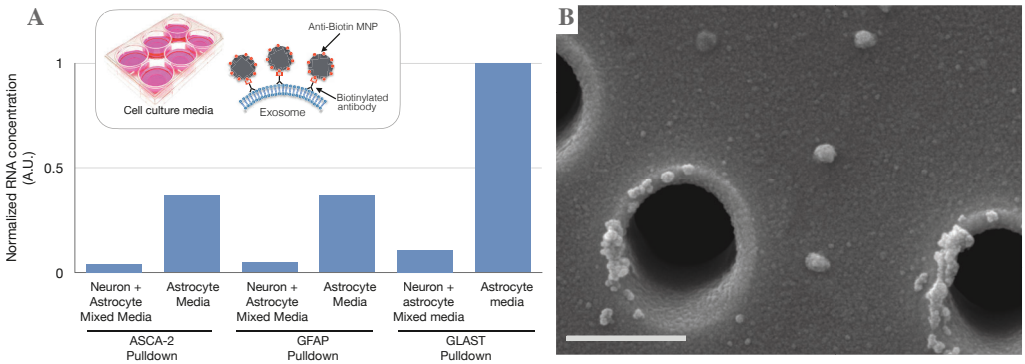

Figure 7: Characterization of GLAST pulldown. A) Enzyme Linked Immunoassay (ELISA) evaluation of GLAST pulldown surface markers for neuron and astrocyte mixed media and astrocyte media. GLAST showed the greatest specificity for astrocyte media. **B)** Scanning Electron Microscopy (SEM) micrograph showing EVs from cell culture media immunomagnetically captured using GLAST+ antibodies on our TENPO chip. Scale: 600 nm.

To discriminate TBI from control subjects, or to discriminate clinically relevant states of TBI from one another, each biomarker should hold the potential to contribute unique information about each patient's TBI. To determine if unique biomarkers could be identified in EVs isolated using ExoTENPO isolated GluR2+ EVs versus ExoTENPO isolated GLAST+ EVs, we performed a sequencing experiment on *N* =. 20 patients that experienced mild TBI (CGS mean = 13.7, Positive CT-n =10%) and *N* =. 20 control patients. The average age of the TBI patients was 42 ±18 years old and the control group was 29 ± 7 years old. The TBI patients were 50% male and 50% female, and the control patients were 71% male and 29% female. The cohorts currently being processed will address the issue of differences in age and sex between the cohorts. QIAseq miRNA Library Kit (Qiagen) was used to make the library from the isolated EV miRNA. We used a BioAnalyzer to quantify the RNA quantity and quality prior to sequencing. The library was sequenced using a HiSeq 2500 Kit (Illumina, Next-Generation Sequencing Core, University of Pennsylvania, Philadelphia, PA). A modified version of the UPenn SCAP-T RNA-Seq expression pipeline (Fisher, S A., "Safisher/ Ngs." GitHub, 2017) was used for expression quantification by aligning to the hg38 genomes. The minimum fragment length allowed past the TRIM module was adjusted to 16 bases for miRNA analysis. The number of allowed mismatches was capped at one and unannotated splices were prohibited. Expression counts were normalized by DESeq2 (24) and quantified using VERSE (25), using Gencode 25 and UCSD mm10 gene annotations, combined with MirBase v21 annotations for 3p and 5p miRNA. To generate a predictive panel of biomarkers, each biomarker needs predictive power and the constituent biomarkers should not correlate with one another, such that each biomarker carries a degree of unique information on the state of the patient. Pairwise correlation coefficients (R) between biomarkers were calculated and revealed that individual biomarkers were generally not well correlated with one another, and in particular markers in the GluR2+ EVs were less correlated with one another than with miRNA within the GLAST+ group (**Fig. 8A**). In the pilot set of data, several promising biomarkers were revealed in both the GluR2+ EVs and in the GLAST+EVs (**Fig. 8B**). An additional N ~ 50 patients and TBI samples are still being processed, and once they are, an EV miRNA biomarker panel will be selected and validated using qPCR for phase III of this work. For each of these patients we also mesure the protein biomarkers described above. The protein and miRNA biomarkers will be studied and combined together algorithmically in phase III.

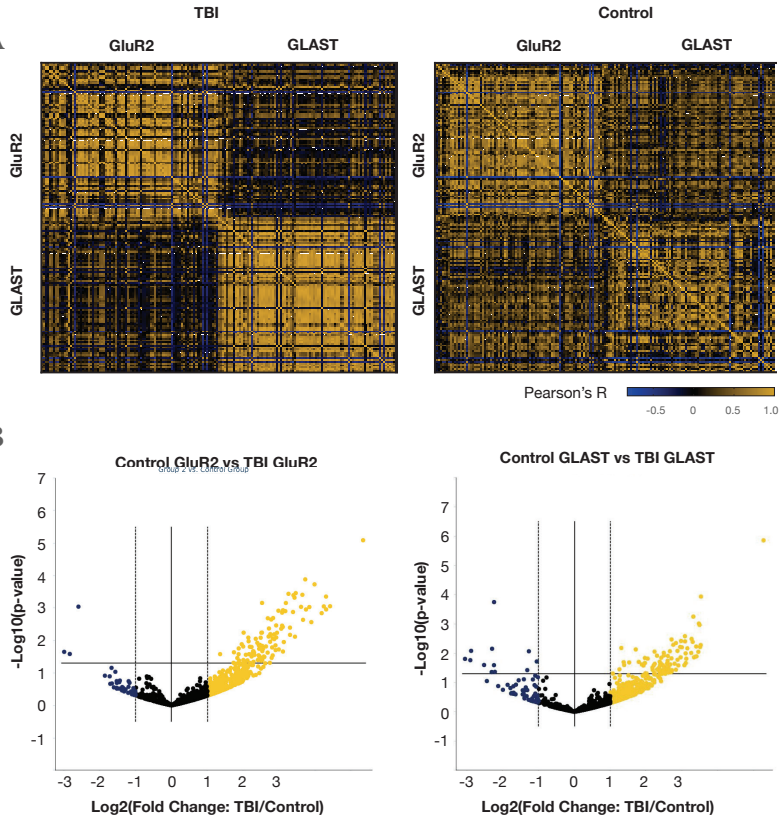

Figure 8: Sequencing analysis of EV RNA cargo using ExoTENPO multiple cell type pulldown assay. A. A cross correlogram of miRNA expression in GluR2+ and GLAST+ TENPO isolated EVs . **B.** A volcano plot for EVs isolated using GluR2+ TENPO and for EVs isolated using GLAST+ TENPO, plotting p-value for injured vs. uninjured control and fold change TBI/control for each EV miRNA. Biomarkers with a fold change > 1 are shown in yellow.

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68. Yelleswarapu V, Buser JR, Haber M, Baron J, Inapuri E, Issadore D. Mobile platform for rapid sub–picogram-per-milliliter, multiplexed, digital droplet detection of proteins. *Proc Natl Acad Sci USA.* 2019;116(10):4489-4495.

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

This funding has facilitated the training of several PhD students. Kryshawna Beard, a PhD student at University of Pennsylvania in Pharmacology, has been trained in microfluidic device design, micro/nano fabrication, assay development, cell culture, and in DNA/RNA sequencing. Zijian Yang, a PhD student at University of Pennsylvania in Mechanical Engineering, has been trained in microfluidic device design, micro/nano fabrication, optics, and exosome biology. Yasemin Atiyas, a PhD student at University of Pennsylvania in Bioengineering, has been trained in microfluidic device design, micro/nano fabrication, optics, and exosome biology. All students and post-docs in this study have encouraged to share their work at national and international meetings, including Neurotrauma, Gordon Conferences, Keystone, BMES, Pitt Con, and the International Society of Extracellular Vesicles.

All students and post-docs in this study have been encouraged to share their work at national and international meetings, and have done so at Neurotrauma, Gordon Conference on Extracellular Vesicles, Keystone on exosomes, BMES, Pitt Con, and at meetings of the International Society of Extracellular Vesicles.

How were the results disseminated to communities of interest?

Kryshawna Beard, David Issadore, and Dave Meaney annually attend Mind Your Brain, an event to share research in brain injury with survivors of traumatic brain injury, held annually at UPenn.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

In the next reporting period we will complete the final unfinished tasks in Phase 2, and begin work on Phase 3, namely transitioning to work on our pig model for brain injury.

4. **IMPACT:**

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

The accurate diagnosis and clinical management of traumatic brain injury (TBI) is currently limited by the lack of accessible molecular biomarkers that reflect the complex pathology of the brain following an injury. To address this challenge, we are developing a microchip diagnostic that can characterize TBI more comprehensively using the RNA found in brain-derived extracellular vesicles (EVs). Our approach measures a panel of EV RNA found in brain derived EVs, processed with machine learning algorithms to capture the state of the injured and recovering brain. Our diagnostic combines surface marker-specific nanomagnetic isolation of brain-derived EVs, biomarker discovery using RNA sequencing, and machine learning processing of the EV miRNA cargo to minimally invasively measure the state of TBI. This approach, which can detect signatures of injury that persist across a variety of injury types and individual responses to injury, more accurately reflects the heterogeneity of human TBI injury and recovery than conventional diagnostics, opening new opportunities to improve treatment of traumatic brain injuries.

What was the impact on other disciplines?

In addition to its intended use in traumatic brain injury, the technology and approaches that we are developing can have applications in a broad range of medical and biological applications. Because EVs are emitted by almost all cells, this approach can be applied to the early diagnosis of cancer, treatment guidance for a wide range of diseases and disorders, and for the diagnosis of infectious diseases, for example.

What was the impact on technology transfer?

The research associated with this grant has lead to the formation of a spin-out company from our lab, Chip Diagnostics. This company has secured venture capital funding and has licensed intellectual property from University of Pennsylvania.

What was the impact on society beyond science and technology?

This research is poised to fundamentally change the way that traumatic brain injuries are clinically managed. For the millions of individuals, and their loved ones, who are afflicted annually by TBI and its longterm consequences, this research has the potential to provide clarity to them and their healthcare providers on their injury, their recovery, and potential pathways towards recovery.

5. **CHANGES/PROBLEMS:**

There were ongoing delays in our research associated with the COVID related shutdown of our lab in the Spring of 2020. Our lab is now operational and we have been able to somewhat get back onto schedule, having finished the sequencing experiments of phase 1 and having made significant progress in accomplishing the aims of phase 2. However, in addition to the delays from our lab's shutdown and the delays in core facilities processing our samples, we have also had issues with hiring. Due to the pandemic, we have not been able to hire a post-doctoral fellow onto this project, as planned, and instead the work has been taken on by a team of research fellows. We are actively hiring, but because of this delay the work is almost a full year behind schedule. We believe that it is likely that we will apply for a no cost extension next year so that we can build on our promising initial results and complete this important work.

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

There were ongoing delays in our research associated with the COVID related shutdown of our lab in the Spring of 2020. In addition to the delays from our lab's shutdown and the delays in core facilities processing our samples, we have also had issues with hiring. Due to COVID, we have been delayed in hiring a post-doctoral fellow onto this project, as planned, and instead the work has been taken on by a team of research fellows. We are still attempting to hire a postdoc, but because of this delay the work is almost a full year behind schedule. We believe that it is likely that we will apply for a no cost extension next year so that we can build on our promising initial results and complete this important work.

Changes that had a significant impact on expenditures

One of the students working on this project (Yasemin Atiyas) received a Department of Defense NDSEG fellowship, which covers her stipend and her tuition. We have been continually delayed in hiring a postdoc and have had several promising candidates fall through, in some cases related to COVID. We are actively hiring to account for these changes to our budgeted spending.

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

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**

Journal publications.

J. Wu, S. Yadavali, D. Lee#, D. Issadore#, Scaling up the throughput of microfluidic droplet-based materials synthesis: A review of recent progress and outlook, In Press, 2021, federal support acknowledged.

K. Beard , Z. Yang , M. Haber, M. Flamholz , R. Diaz-Arrastia, D. Sandsmark, D. F. Meaney, D. Issadore, Extracellular vesicles as distinct biomarker reservoirs for mild traumatic brain injury diagnosis, In Press, 2021, federal support acknowledged.

N. Shah, V. Iyer, Z. Gao, Z. Zhang, V. Yelleswarapu, F. Aflatouni, A.T.C. Johnson, and D. Issadore, Graphene micro-Hall sensors for the In-flow detection of rare cells, Submitted, 2021, federal support acknowledged.

D. Issadore, V. Iyer, Z. Yang, J. Ko, R. Weissleder, Advancing Microfluidic Diagnostic Chips for Clinical Use, Submitted, 2021, federal support acknowledged.

J. Y., Kim , J. Eberwine , R. C. Anafi , S. Brem, M. Bucan, S. A. Fisher, M. S. Grady, A. E. Herr, D. Issadore, D. Lee, S. S. Rubakhin , J. Y. Sul , J. V. Sweedler, J. Wolf, K. Zaret, J. Zou, Beyond Single Cells: Subcellular 'Omics Toward a Theory of Cell Type, In Press, 2021, federal support acknowledged.

S. J. Shepherd, C. C. Warzecha, R. El-Mayta, S. Yadavali , L. Wang , J. M. Wilson, D. Issadore#, Michael J. Mitchell#, Microfluidic platform for precise throughput-invariant RNA lipid nanoparticle formulations, In Review, 2020, federal support acknowledged.

S.J. Shepherd, D. Issadore#, M. J. Mitchell#, Microfluidic formulation of nanoparticles for biomedical applications, Biomaterials, https://doi.org/10.1016/j.biomaterials.2021.120826, 2021, federal support acknowledged.

M.J. Siedlik, Z. Yang, P. S. Kadam, J. Eberwine, and D. Issadore, Micro and nano devices for studying subcellular biology, Small, doi: 10.1002/smll.202005793, 2021, federal support acknowledged.

Y. Lan, A. Wu, S. Han, S. Yadavali, D. Issadore, K.J. Stebe, D. Lee, Scalable Synthesis of Janus Particles with High Naturality, ACS Sustainable Chemistry & Engineering, https://doi.org/10.1021/acssuschemeng.0c04929, 2020, federal support acknowledged.

S. Muraoka, A. M. DeLeo, H. Tatebe, Z. Yang, Y. K. Wang, K. Yukawa-Takamatsu, Y. You , S. Ikezu , T. Tokuda , D. Issadore , R. A. Stern, T. Ikezu, Proteomic Profiling of Extracellular Vesicles Separated from Plasma of Former National Football League Players at Risk for Chronic Traumatic Encephalopathy, Aging and Disease, doi.org/10.14336/AD.2020.0908, 2020, federal support acknowledged.

K. Beard, D. F. Meaney, D. Issadore, Clinical applications of extracellular vesicles in the diagnosis and treatment of traumatic brain injury, Journal of Neurotrauma, doi: 10.1089/neu.2020.6990, 2020, federal support acknowledged.

S. Muraoka, A.M. DeLeo, M.K. Sethi, K. Yukawa-Takamatsu, Z. Yang, J. Ko, J. D. Hogan, Z. Ruan,Y. You, Y. K.Wang, M. Medalla, S. Ikezu, W. Xia1, S. Gorantla, H. E. Gendelman, D. Issadore, J. Zaia, T. Ikezu, Proteomic Profiling and Biological Characterization of Extracellular Vesicles Isolated from Human Alzheimer's Disease Brain Tissues, Alzheimer's & Dementia: The Journal of the Alzheimer's Association, doi.org/10.1002/alz.12089, 2020, federal support acknowledged.

H. Shen, T. Liu, J. Cui, P. Borole, A. Benjamin, K. Kording, D. Issadore, A Web-based Automated Machine Learning Platform to Analyze Liquid Biopsy Data, Lab on a Chip, https://doi.org/10.1039/D0LC00096E, 2020, federal support acknowledged.

Z. Yang, M. J. LaRiviere, J. Ko, J. Till, T. Christensen, S. Yee, T. Black, K. Tien, N. Bhagwat, A. Lin, A. Adallah, M. H. O'Hara, C. M. Vollmer, B. W. Katona, B. Z. Stanger, D. Issadore, and E.L. Carpenter, A multi-analyte panel consisting of extracellular vesicle miRNAs and mRNAs, cfDNA, and CA19-9 shows utility for diagnosis and staging of pancreatic adenocarcinoma, Clinical Cancer Research, DOI: 10.1158/1078-0432.CCR-19-3313, 2020, federal support acknowledged.

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers and presentations.

Nothing to report.

- **Website(s) or other Internet site(s)**

Issadore Lab Website: http://issadore.seas.upenn.edu/

- **Technologies or techniques**

The Track Etched Magnetic Nanopopre (TENPO) technology developed as part of this grant has been patented, and is now being commercialized by a venture backed spin-out company from our lab Chip Diagnostics.

- **Inventions, patent applications, and/or licenses**

D. Issadore, M. Muluneh, Magnetic Apparatus and Methods for Analyzing the Output of Microfluidic Devices, US Patent Issued - 10,473,590, 2019. Licensed to Chip Diagnostics.

D. Issadore, M. Muluneh, Magnetic Separation Filters and Microfluidic Devices, US Patent Issued - 10,335, 789, 2019. Licensed to Chip Diagnostics.

D. Issadore, M. Muluneh, Magnetic Apparatus and Method for Manufacturing a Microfluidic Device Filters and Microfluidic Devices, US Patent Issued - 10,632,462, 2020. Licensed to Chip Diagnostics.

E. Carpenter, D. Issadore, B. Stanger, Z. Yang, A Blood Based Multi-Analye Liquid Biopsy Approach for Diagnosis of Pancreatic Adenocarcinoma and Detection of Occult Meastases, Patent Filed - 62/982,254, 2020. Licensed to Chip Diagnostics.

D. Issadore, D. Lee, S. Yadavali, Silicon Chip Having Multi-Zone Through Silicon Vias and Method of Manufacturing The Same, Provisional Patent Filed - PCT/US2020/015684, 2020.

D. Issadore, D. Lee, S. Yadavali, Large Scale Microdroplet Generation Apparatus and Methods of Manufacturing Thereof, Patent Filed - 16/062,724, 2020.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

N*ame:*	*David Issadore*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-5461-8653*
Nearest person month worked:	*2.5*
Contribution to Project:	*Prof. Issadore has contributed to overseeing all aspects of the proposal, but has particularly focused on the technology development aspects..*
N*ame:*	*Dave Meaney*
Project Role:	*Co-PI*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-0954-4122*
Nearest person month worked:	*1*
Contribution to Project:	*Prof. Meaney has contributed to overseeing all aspects of the proposal, but has particularly focused on the biomarker discovery and porcine model development aspects.*
N*ame:*	*Ramon Diaz-Arrastia*
Project Role:	*Co-PI*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-6051-3594*
Nearest person month worked:	*1*
Contribution to Project:	*Prof. Diaz-Arrastia has contributed to overseeing all aspects of the proposal, but has particularly focused on n the biomarker discovery and the clinical translation aspects.*
N*ame:*	*Danielle Sandsmark*
Project Role:	*Co-I*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-1586-6961*
Nearest person month worked:	*1.8*
Contribution to Project:	*Prof. Sandsmark has contributed to overseeing all aspects of the proposal, but has particularly focused on n the biomarker discovery and the clinical translation aspects.*

N*ame:*	*Yasemin Atiyas*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	*6*
Contribution to Project:	*Yasemin has contributed mainly to the fluorescence droplet detection aspects of this work.*
Funding Support:	*Yasemin is now supported by an NDSEG fellowship*

N*ame:*	*Hanfei Shen*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	*6*
Contribution to Project:	*Hanfei has contributed mainly to the extracellular vesicle isolation aspects of this project.*

N*ame:*	*Stephanie Yang*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	*12*
Contribution to Project:	*Stephanie has contributed mainly to the fluorescence droplet detection and droplet PCR aspects of this work.*

N*ame:*	*Zijian Yang*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	*6*
Contribution to Project:	*ZJ has contributed mainly to the fluorescence droplet detection and extracellular vesicle isolation aspects of this work.*

N*ame:*	*Andrew Lin*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	*6*
Contribution to Project:	*Andrew has contributed mainly to the extracellular vesicle isolation aspects of this project.*

N*ame:*	*Sagar Yadavali*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	*2*
Contribution to Project:	*Sagar has contributed mainly to the high throughput droplet generation aspects of this project.*

N*ame:* *Kryshawna Beard*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *NA*
Nearest person month worked: *6*
Contribution to Project: *Kryshawna has contributed mainly to the extracellular vesicle isolation aspects of this project.*
Funding Support: *Kryshawna is now supported by an NIH training fellowship*

N*ame:* *Cillian Lynch*
Project Role: *Investigator*
Researcher Identifier (e.g. ORCID ID): *NA*
Nearest person month worked: *1*
Contribution to Project: *Dr. Lynch is the Laboratory Manager working with Drs. Diaz-Arrastia and Sandmark. He has managed the inventory of human biological fluids, and has carried out assays for brain-injury related biomarkers.*

N*ame:* *Leroy Wesley*
Project Role: *Research Assistant*
Researcher Identifier (e.g. ORCID ID): *NA*
Nearest person month worked: *4*
Contribution to Project: *Mr. Wesley is a research assistant working with Drs. Diaz-Arrastia and Sandsmark. He has been involved in recruiting TBI subjects and well as healthy control participants, processing and storing biological samples.*

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

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. **SPECIAL REPORTING REQUIREMENTS**

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. **APPENDICES:**