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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.

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Traumatic brain injury, diagnosis

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

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:** *Provide a brief list of keywords (limit to 20 words).*

Mild Traumatic Brain Injury, Diagnostics, Exosomes, Extracellular Vesicles

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

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 25-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: 100% complete. 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: 100% complete.

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: 50% 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: 50% 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: 50% 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: 50% 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: 50% 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.

Status: 100% complete. 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: 100 % completed for protein analysis, in process for RNA. 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. 100 % completed for protein analysis, in process for RNA. 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. 100 % completed for protein analysis, in process for RNA. 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. 100 % completed for protein analysis, in process for RNA. We have just in the last month begun to benchmark our chip against a commercial BioRad digital PCR system.

Phase 3

Major Task 1: EV Biomarker Characterization of Endophenotypes in Clinical Samples:

Subtask 1: Clinical Statement of work.

This work is 80% completed, and we expect to complete it this summer.

Subtask 2: We will attempt to classify specific axonal, inflammatory, and vasculature endophenotypes in a variety of injury types, severities, and time-points after injury, as well as healthy controls, and compare our platform's results against

This work is 50% completed, and we expect to complete it this summer.

Milestone Achieved: For each patient we will perform MR imaging and measure a panel of blood borne markers. Comparisons will be made between our approach and these emerging gold standard techniques.

This work is 50% completed, and we expect to complete it this coming year.

Milestone Achieved: We will predict each patients' endophenotypic state using various combinations of the time-point measurements, including acute and subacute together and acute, subacute, and chronic. Results will be compared to the known truth, based on imaging and patient outcome.

This work is 50% completed, and we expect to complete it this coming year.

Major Task 2: Next Generation Technology Evaluation

Subtask 1: Our mobile EV diagnostic will be used in clinical settings and compared directly to a variety of emerging gold standard technologies, including Quanterix.

This work is 25% completed, and we expect to complete it this coming year.

Subtask 2: We will have our mobile technology be evaluated by clinicians not previously involved in this study to evaluate its capability for robust use.

This work is 25% completed, and we expect to complete it this coming year.

Milestone Achieved: Validation that a mobile platform can classify TBI endophenotypes.

This work is 25% completed, and we expect to complete it this coming year.

What was accomplished under these goals?

Developing Biomarkers of Cell-Specific Pathology via Extracellular Vesicles

Our work and that of others demonstrates the promise of brain derived extracellular vesicles (EVs), an additional source of circulating molecular information, for the development of cell-specific biomarkers of therapeutically targetable aspects of TBI. EVs are produced through diverse mechanisms; those formed through the direct budding of the plasma membrane are known as microparticles, while those released through the endocytic pathway (Del Conde et al. 2005) are called exosomes, though there is similarity in their molecular compositions (Colombo, Raposo, and Théry 2014). These nanoscale (30-1000nm), membranous particles housing luminal RNA, protein, and metabolite cargo can cross the blood-brain barrier (BBB) (Chen et al. 2016), and directly elicit systemic responses to brain damage in peripheral tissues (Dickens et al. 2017; Hazelton et al. 2018; Zhang et al. 2021; Ali et al. 2021; Yates et al. 2019; Couch et al. 2017; Xia et al. 2021). Initially thought to be merely vehicles for the clearance of unwanted materials from the cell, EVs in the CNS have since been implicated in a plethora of processes. For example, they have been demonstrated to promote the propagation of neurotoxic protein isoforms (Connors and Gutnick 1990; Asai et al. 2015; Ruan et al. 2020), protect neurons from *in vitro* stretch injury through the delivery of anti-inflammatory microRNAs (miRNAs) (Yang et al. 2019; Huang et al. 2018; Li et al. 2019), and support oligodendrocyte maturation and survival thereby potentially affecting myelination (Kurachi, Mikuni, and Ishizaki 2016; Willis et al. 2020; Osawa et al. 2017). Since each of these processes is mediated by the release and uptake of EVs from specific cell types, expanding the search from protein to EV-based biomarkers may be the key to the precise evaluation of and intervention for TBI.

Over the last several years we have evaluated the diagnostic potential of the transcriptomic and proteomic data in brain derived EVs isolated based on the presence of the Glutamate Ionotropic AMPA Receptor type 2 subunit (GluR2) on their surface — a receptor subunit primarily expressed in neurons (Van Damme et al. 2007). The GluR2 subunit renders AMPA receptors impermeable to divalent cations, protecting neurons from excitotoxic injury caused by influx of Ca^{2+} and Zn^{2+}(Greger and Esteban 2007; Liu et al. 2013)As neuronal expression of GluR2 has been shown to decrease following injury through endocytosis (Bell et al. 2007), EVs expressing GluR2 released from injured cells can be detected in the plasma of injured patients and animals, and their cargo can be leveraged for TBI diagnosis. To isolate GluR2+ EVs, we used Track Etching NanoPOre (TENPO), an emerging technology, which achieves higher specificity and throughput with smaller sample volumes and faster isolation times than traditional EV isolation methods (Jina Ko et al. 2016).

In earlier studies using TENPO, GluR2+ EV miRNA cargo was found to accurately predict features of experimental TBI such as time elapsed since injury, injury severity, and history of single or repeated injury (J. Ko et al. 2018; Jina Ko et al. 2020). Subsequent pathway analysis revealed upregulation of GluR2+ EV miRNAs involved in axon guidance, hippocampal signaling, and glutamatergic synapse maintenance (Jina Ko et al. 2020). More recently, we found that the relative abundance of Tau, NFL, GFAP, and UCHL1 in GluR2+ EVs isolated using TENPO within 24 hours of clinically mild (GCS 13 – 15) TBI (mTBI) in humans was significantly different from that of plasma, and levels of these proteins were uncorrelated across GluR2+ EVs and plasma indicating the diagnostic independence of these two biomarker pools (Beard et al. 2021). Additionally, as proof-of-concept for using EV-based information for TBI diagnosis in the clinic, we showed that an algorithmic combination of these proteins in GluR2+ EVs and plasma produces a biomarker panel that accurately distinguishes mTBI patients from controls (Beard et al. 2021). In this study, we aimed to expand our previous work to develop a method for evaluating the acute (<24 hour), neuron- and astrocyte-specific responses to mTBI by investigating molecular information in EVs released from astrocytes.

The classical view of astrocytes as merely agents of structural support is increasingly being replaced by greater appreciation of the direct role astrocytes play in neuronal function. In addition to shuttling glutamate from the synaptic cleft (Nedergaard, Ransom, and Goldman 2003), astrocytes release neuromodulators and inflammatory mediators in response to neuronal and immune cell activity (Newman 2001; Parpura et al. 1994; Henneberger and Rusakov 2012; Lau and Yu 2001). TBI and other neurologic diseases activate astrocytes, which alters their gene expression profile, morphology, proliferative ability, and function (Burda, Bernstein, and Sofroniew 2016; Choo et al. 2013; Ding et al. 2007; Orre et al. 2014; Keene et al. 2009; Serrano-Pozo et al. 2011). Similar changes occur in the resident microglia and collectively these processes define reactive gliosis, a potentially therapeutically targetable process contributing to TBI progression that is difficult to fully assess in the clinic. Reactive astrogliosis is triggered by many stimuli including ATP, tumor necrosis factor alpha (TNFα), and interleukin-1 (IL-1) which activate diverse downstream pathways. Although reactive astrogliosis plays a crucial role in TBI etiology, the current biomarkers used to assess it, such as GFAP, fail to illuminate its contribution to an individual's etiology. We therefore sought to isolate EVs released from astrocytes and evaluate their potential in assessing astrocyte responses to mTBI with the hypothesis that mTBI shifts the expression of astrocyte EV cargo. If successful, when used clinically this information could be combined with protein biomarkers of injury severity and neurodegeneration during mTBI evaluation.

Study Design

To develop a strategy for enriching for astrocyte-specific EVs from blood using TENPO, we first quantified and compared the amount of RNA recovered from EVs derived from astrocyte and neuron—astrocyte mixed cell cultures based on their expression of three astrocyte proteins, anti-astrocyte cell surface antigen-2 (ACSA-2), GFAP, and GLutamate ASpartate Transporter (GLAST). Based on these results, we selected GLAST, a membrane-expressed protein responsible for the removal of glutamate from the extracellular space. To evaluate the clinical potential of a GLAST+ EV-based assessment of astrocyte injury processes and to compare GluR2+ and GLAST+EV responses to TBI, we collected plasma from *n*=18 mTBI (<24hr of injury; GCS 13–15) and *n*=19 healthy control (HC) participants (**Figure 1A**). We isolated GluR2+ and GLAST+ EVs using TENPO and performed next-generation sequencing of their miRNA cargo to discover potential biomarkers of acute cell-specific injury processes housed within each EV population (**Figure 1B**). To evaluate the expression of emerging biomarkers of injury severity and neurodegeneration (GFAP, UCHL1, Tau, and NFL), we used the ultrasensitive dELISA technique Single Molecule Array (SIMOA) with the hypothesis that mTBI results in acute elevations of each based on our and others' previous work (Diaz-Arrastia et al. 2014; Beard et al. 2021).

The overall purpose of our analysis was to demonstrate that GLAST+ and GluR2+EV biomarker pools exhibit differing responses to acute mTBI reflecting the differing pathologic processes activated in astrocytes and neurons by the injury. To achieve this goal, we quantified and compared the TBI-induced alterations in EV miRNA expression in GluR2+ and GLAST+ EVs, applied pathway analysis to determine the signaling mechanisms activated by TBI reflected by differentially expressed miRNAs in each EV population, and assessed the correlation of miRNA levels both across the two EV populations and across EVs and plasma proteins (**Figure 1C**). Our results demonstrate the promise of GluR2+ and GLAST+ EV-based evaluation of cell-specific CNS injury mechanisms in the clinic that can be broadened for use in the diagnostic and therapeutic discovery efforts of other neurologic diseases.

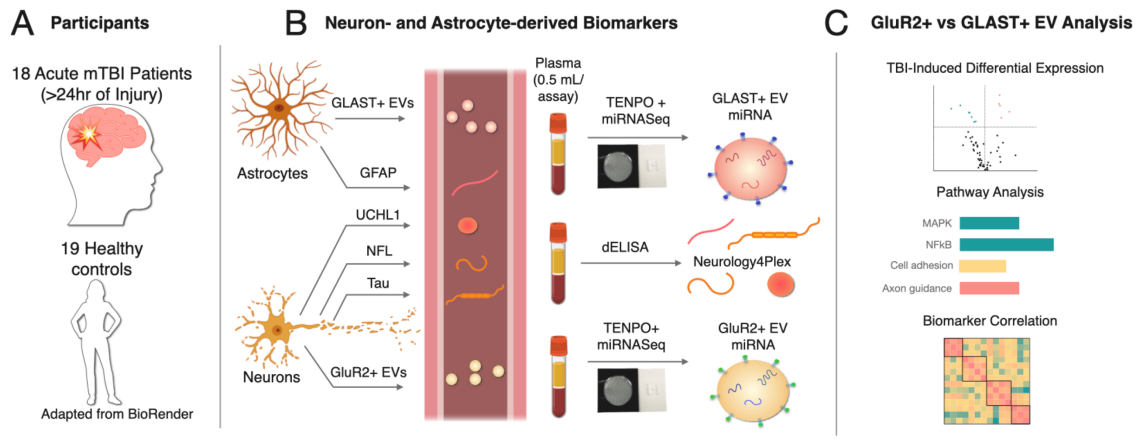


Figure 1 I Study Design. A. Samples were obtained from subjects sustaining mild TBIs (Glasgow Coma Scale Scores 13 – 15) through road traffic incidents, falls, or other causes and from healthy controls. **B.** From each participant, GluR2+ and GLAST+ EVs presumably originating from neurons and astrocytes respectively were each isolated using a 500 μL aliquot of plasma on our nanofluidic platform, TENPO, and the RNA lysate was subjected to miRNA sequencing. Using a third 500 μL aliquot of plasma, emerging protein biomarkers of injury severity released from neurons (UCHL1, NFL, Tau) and astrocytes (GFAP) were quantified with

digital ELISA. **C.** Analyses served to compare TBI-induced alterations to the miRNA content in GluR2+ and GLAST+ EVs in acute mTBI. This image was adapted from BioRender.com.

METHODS

Cell Culture

To identify a method for isolating astrocyte derived EVs from plasma using TENPO, we first utilized astrocyte and mixed culture supernatants to test candidate astrocyte proteins in their relative EV RNA yield. Cells were provided by the Neurons R Us core at the University of Pennsylvania. All procedures and protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Briefly, neocortical tissue was dissected from day 18 embryos of pregnant Sprague-Dawley rats. To prepare mixed cultures, nitex mesh-filtered (Crosswire Cloth, Bellmawr, NJ) cells were resuspended in Minimum Essential Medium (MEM) with Earl's salts, GlutaMAX (Gibco Invitrogen, Carlsbad, CA), 0.6% D-glucose (Sigma-Aldrich, St. Louis, MO), 1% Pen-Strep (Gibco Invitrogen), and 10% Horse Serum (Gibco Invitrogen). To prepare astrocyte cultures, nitex-filtered cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM, Gibco Invitrogen) with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1% Pen-Strep. Both mixed and astrocyte cultures were maintained in 37°C incubators at 5% CO_2.

Cells were cultured on either glass-bottomed dishes (MatTek, Ashland, MA) for immunofluorescence imaging, or T75 flasks (Corning, Corning, NY) for supernatant collection, each coated with poly-D-lysine (0.08 mg/ml, Sigma-Aldrich) and laminin (0.001 mg/ml, BD Biosciences, San Jose, CA) for mixed cultures and poly-D-lysine alone for astrocyte cultures. After overnight adhesion, the mixed culture plating medium was removed and replaced with Neurobasal medium with B-27 and 0.4 mM GlutaMAX until 21 days *in vitro* (DIV). At DIV 21, cultures were either fixed for imaging, or their supernatants were collected for EV analysis. Astrocyte cultures were continuously fed with DMEM supplemented with FBS and Pen-Strep and were passaged at DIV 21. Passaged astrocytes were then cultured for an additional week before being fixed for imaging or harvested for supernatant.

TENPO EV Isolation and Vesicle Lysis

To assess the EV RNA recovery of various astrocyte-specific markers in cell culture, we applied the TENPO EV isolation strategy reported in (Beard et al. 2021), utilizing ACSA-2-biotin (130-126-952, Miltenyi Biotec, Germany), GFAP (ab68428, Abcam, biotinylated using one-step biotinylation kit (130-093-385, Miltenyi Biotec)), GLAST-biotin (130-119-161, Miltenyi Biotec), or IgG (ab79558, Abcam, biotinylated using one-step biotinylation kit) in place of GluR2 antibody and performing lysis with 700 μL of QIAzol lysis reagent (Qiagen, Germantown, MD). The lysate was then extracted at a flow rate of 50mL/h for RNA isolation, and the second part of the RNeasy kit (Qiagen) was then used to extract RNA. RNA was quantified using a Qubit RNA high sensitivity assay for these studies (Agilent, Agilent, Wilmington, DE).

For subsequent human studies, EV isolation was done using a commercialized version of TENPO provided by ChipDiagnostics Inc (Philadelphia, PA). For each participant, 500 μL of plasma was incubated with GluR2-biotin (bs-10042R-biotin, Bioss, Woburn, MA) and a separate 500 μL aliquot of plasma was incubated with GLAST-biotin (Miltenyi Biotec) for 1 hour at room temperature with shaking. Samples were then incubated with anti-biotin ultrapure magnetic nanoparticles (Miltenyi Biotec) for an additional 20 minutes at room temperature. During sample incubation, TENPO devices were blocked with 2 mL of 1% F-127 in 1X PBS which was run through the TENPO chip using a negative pressure supply (Programmable Syringe Pump; Braintree Scientific, Braintree, MA) at an initial flow rate of 50 mL/h for 2 minutes followed by a slow flow at 0.5 mL/h for one hour. Chips were then rinsed with 1mL 1X PBS at 50 mL/h for 2 minutes. Next, the 500 μL samples were run through prepared chips at a flow rate of 15 mL/h. Unbound material was then cleared by three sequential washes of 1XPBS (200 μL, 800 μL, and 1000 μL each at 15 mL/h)..

After washing, 700 μL of QIAzol lysis reagent (Qiagen) was run through the device at 50 mL/h, followed by a slow flow of 0.5 mL/h for 2 minutes to allow captured EVs sufficient time to lyse. Lysate was then extracted at a flow rate of 50 mL/h for RNA isolation. The second part of the RNeasy kit (Qiagen) was then used to extract RNA from the lysate. RNA was eluted in 15 μL of RNAse-free water and stored at -80°C until quantification and library preparation. Plasma samples used for EV western blot analysis were incubated with antibody and magnetic beads and run through the device as described above but were lysed in 1X RIPA (Abcam, Waltham, MA) + HALT (ThermoFisher, Philadelphia, PA) + EDTA (ThermoFisher) + PMSF (Cell Signaling Technologies, Danvers, MA). EV protein lysate was also stored at -80°C until analysis.

Immunofluorescence Imaging

Having identified GLAST as a promising EV isolation strategy, we next aimed to confirm the specificity of its expression in astrocytes, and the specificity of GluR2 expression to neurons through immunofluorescence imaging. Cells cultured on glass-bottomed dishes were fixed with 4% paraformaldehyde for 5 minutes then rinsed with 1x PBS. Cells were permeabilized with 0.2% Triton X-100 (ThermoFisher) in PBS for 5 minutes then rinsed with PBS. 1% BSA (Invitrogen) and 2.5% normal goat serum (NGS; Invitrogen) in PBS was used to block for 45 minutes. Mouse anti-GluR2 (32-0300, ThermoFisher), mouse anti-MAP2 (MAB3418, MilliporeSigma, Burlington, MA), rabbit anti-GFAP (G9269 Sigma-Aldrich), and rabbit anti-GLAST (PA5-19709, ThermoFisher) primary antibodies were diluted at 1:500, 1:700, 1:700, and 1:500 respectively in accordance with manufacturer's recommendations and applied to cells for overnight incubation at 4°C. Cells were co-labelled with either GLAST and MAP2, or GFAP and GluR2 primary antibodies listed above. Cells were then rinsed four times with 0.2% BSA and 0.5% NGS in PBS for 5 minutes each, then incubated with Alexa Fluor donkey anti-mouse 488 (A21202, Invitrogen) and donkey anti-rabbit 594 (A21207, Invitrogen) secondary antibodies each diluted at 1:500 in 0.2% BSA and 0.5% NGS in PBS. Dishes were then rinsed three times in PBS for 10 minutes each, with 10 μg/mL Hoescht (Invitrogen) applied during the second rinse. Cells were imaged on a Leica SP5 confocal microscope at 1024x magnification.

Participant Plasma Sample Preparation

To test the hypothesis that GluR2+ and GLAST+ EVs are independent EV pools with unique clinical potential, we collected plasma from mTBI subjects admitted to the University of Pennsylvania's Penn Presbyterian Medical Center and from healthy controls using criteria approved by the University of Pennsylvania Institutional Review Board and described in Beard et al 2021. 16 TBI subjects sustained mild TBIs, defined by GCS scores of 13—15 and 2 participants were missing GCS scores but had no CT scan-visible pathology**.** We analyzed plasma samples collected within 24 hours of injury prepared from whole blood by centrifugation (1000 rpm for 10 minutes). Plasma was processed within 1 hour of collection, aliquoted at 500 μL, and frozen at –80°C until further analyses.

MiRNA Sequencing, Differential Expression Analysis, and Kyoto Encyclopedia of Genes (KEGG) Pathway Analysis

RNA quality was assessed using Pico bioanalyzer (Agilent) for nucleotide size distribution and concentration before library preparation. The QIAseq miRNA library kit (Qiagen) was used to prepare barcoded cDNA libraries of EV RNA samples. cDNA libraries were then assessed using High Sensitivity DNA bioanalyzer (Agilent) for nucleotide size and library concentration, and equivalent amounts of cDNA from each library were pooled. The pooled library was then sequenced using a NextSeq 500/550 Hi Output Kit v2.5 (FC-404-2005, Illumina, San Diego, CA). Raw sequencing data for each sample was demultiplexed using the bcl2fastq script, v2.20 from Illumina. FASTQ files were then uploaded to the Qiagen GeneGlobe site, and their legacy primary analysis pipeline was used to obtain expression counts. Expression counts for each miRNA were normalized by the sum of each miRNA count for each sample. Fisher's exact test was used to measure the p value of miRNA expression differences between mTBI and control subjects. For pathway analysis, TargetScan was used to identify the mRNAs targeted by significantly upregulated EV miRNAs. The mRNA targets for each miRNA were then subjected to Kyoto Encyclopedia of Genes (KEGG) 2021 pathway analysis.

Single Molecule Array for Plasma Biomarkers

To assess patient and control levels of biomarkers associated with injury severity and neurodegeneration, plasma samples were analyzed using the Neurology 4Plex (A) single molecule array kits (SIMOA; Quanterix, Billerica, MA) for Tau, NFL, UCHL1 and GFAP, as described in Beard et al. 2021 with 110-120 μL of plasma and quality control samples run at 4x dilution.

Statistical Analysis of Candidate Biomarkers

We calculated the effects of age, self-reported gender, and injury type in the mTBI group on the expression of biomarker candidates using Pearson's coefficients, Student's T tests, and one-way ANOVA (with 95% confidence intervals) respectively, using log-transformed data. To visualize the spread of the data across individuals, we used the log-transformed values of candidate biomarker measurements to plot scatter plots (**Figure 3**) and heat maps (**Figure 4**) using GraphPad Prism 8.1.2. For scatter plots, p values were calculated using Student's T Tests with log-transformed data for Neurology4Plex data, and Fisher's exact test for EV miRNA data. Correlations between EV miRNAs and plasma proteins were calculated using Pearson correlation. To assess each candidate biomarker's ability to discriminate mTBI from control groups, we calculated receiver operating characteristic (ROC) curves and reported the Area Under the Curve (AUC) for each. Algorithmic combination of GluR2+ and GLAST+ EV differentially expressed miRNAs was achieved using linear discriminate analysis with 5-fold cross validation.

Western Blot Analysis

The protein concentration of EV lysates was measured with the Pierce BCA Protein Assay Kit (ThermoFisher). Proteins (5-10 µg) were separated on 10% acrylamide gels by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blocked with 5% bovine serum albumin (BSA) in TBST (20 mM Tris pH 7.5; 150 mM NaCl; 0.1% Tween-20) for 1 hour. Membranes were incubated with anti-Alix (1:1000, Cell Signaling Technology, cat. # 2171S), anti-Tsg101 (1:1000, Santa Cruz Biotechnology, cat. #sc7964), and anti-Glur2/3 (1:1000, Millipore, cat. #07-598) antibodies in 5% BSA in TBST overnight at 4°C. Membranes were washed with TBST and incubated with appropriate HRP-conjugated secondary antibodies (Rockland Immunochemicals, Inc.). Chemiluminescence signals were detected with Immobilon Western Chemiluminescent HRP Substrate (Millipore) and the LI-COR Odyssey Fc Imaging system (LI-COR Biosciences).

Scanning Electron Microscopy (SEM)

Captured GLAST+ EVs were imaged by fixing them to the TENPO membrane using 2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.1 sodium cacodylate buffer, pH 7.4. Images were taken at the Cell and Developmental Biology Microscopy Core at University of Pennsylvania at 50,000x magnification.

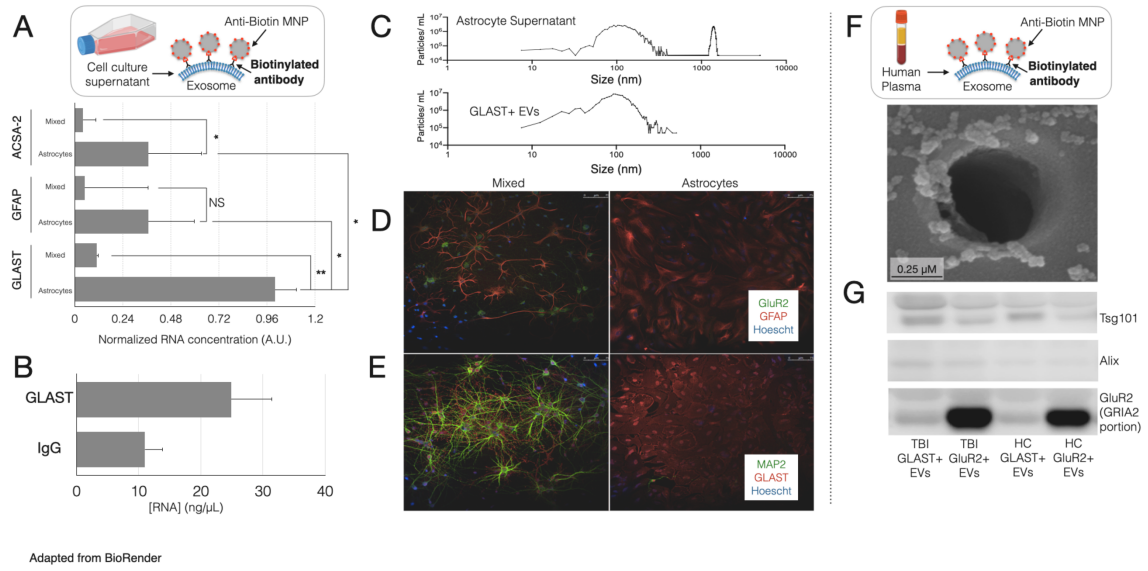
Nano Tracking Analysis (NTA)

Astrocyte-pure culture media was used to isolate GLAST+ vesicles with TENPO, which were eluted in 500 μL of PBS. NTA was performed at the Extracellular Vesicle Core facility at University of Pennsylvania.

RESULTS

GLAST is predominantly expressed in astrocytes and can be used to isolate EVs with TENPO

To develop a strategy for isolating astrocyte-specific EVs with TENPO, we first assessed the amount of TENPO-isolated EV RNA recovered from the use of three antibody candidates – ACSA-2, GFAP, and GLAST – using supernatants from rat cortical mixed and astrocyte dissociated cultures. Of the three antibodies tested, both ACSA-2+ and GLAST+ TENPO resulted in significantly more RNA recovered from astrocyte than mixed cultures when adjusting for relative astrocyte abundance (ACSA-2 p=0.032 GLAST; p=0.0016; paired T test, *n*=2 devices per condition), supporting ACSA-2 and GLAST specificity for astrocyte derived EVs. But GLAST+ TENPO resulted in significantly higher EV RNA recovered than either GFAP or ACSA-2 from astrocyte supernatant (**Figure 2A**; GLAST vs GFAP p=0.043; GLAST vs ACSA-2 p=0.043; paired T test, *n*=2 devices). Having identified GLAST as a promising candidate for use with TENPO, we next compared GLAST EV RNA yield with that of an isotype control antibody, and found that GLAST+ particle isolation from astrocyte supernatant yielded 2x more EV RNA (**Figure 2B**). The size of GLAST+ particles isolated from astrocyte supernatant was consistent with EVs (~100 nm), and the concentration of 100nm particles in the GLAST+ TENPO output was 10-fold that of the astrocyte supernatant input (**Figure 2C**).



Adapted from BioRender

Figure 2 I TENPO isolation of GLAST+ EVs. A. Average normalized RNA concentration resulting from ACSA-2, GFAP, and GLAST isolation of particles from cell culture supernatant. *p<0.05 **p<0.01. **B.** Average RNA concentration resulting from GLAST and IgG isotype control antibody isolation of particles from astrocyte supernatants. **C.** NTA assessment of particle count in astrocyte supernatant TENPO input and GLAST+ particle eluate. **D.** Immunofluorescence images of GluR2, GFAP, and Hoescht staining of mixed and astrocyte cultures at 1024x magnification. **E.** Immunofluorescence images of MAP2, GLAST, and Hoescht staining of mixed and astrocyte cultures at 1024x magnification. **F.** SEM imaging of human plasma derived EVs captured using GLAST+ TENPO at 50,000x magnification. **G.** Western blot analysis of TBI and healthy control (HC) plasma derived GluR2+ and GLAST+ EV lysate expression of EV-associated markers Tsg101 and Alix and GluR2/3. Experiment schematic images were adapted from BioRender.com.

To confirm the specificity of GLAST and GluR2 for astrocytes and neurons respectively, we next visualized the localization of each protein in rat primary cortical mixed and astrocyte cultures using immunofluorescence. These two markers stained different cell populations *in vitro*: GluR2 immunoreactivity was observed only in the neuronal cell bodies of mixed cultures, and was completely absent in GFAP-labeled astrocyte cultures (**Figure 2D**), but only astrocytes stained positive for GLAST, in both MAP2-labelled mixed and astrocyte cultures (**Figure 2E**).

To characterize GLAST+ TENPO EV isolation in human plasma, we next performed a series of experiments in accordance with standards developed by the International Society of Extracellular Vesicles to demonstrate the EV nature of the isolated particles (Lötvall et al. 2014). Under SEM imaging GLAST+EVs exhibited morphology consistent with EVs (**Figure 2F**), and GluR2+ and GLAST+ EVs from both TBI subjects and controls expressed Tsg101 and Alix (**Figure 2G**) consistent with exosomes. Western blot analysis also revealed expression of GluR2 was more prominent in GluR2+ than GLAST+ EVs, supporting the cell-specificity of these pulldowns (**Figure 2G**).

TBI alters plasma protein and EV miRNA expression

Having successfully isolated astrocyte derived EVs based on their expression of GLAST, we then obtained plasma samples from a cohort of healthy control participants (n=19) and mTBI patients (*n*=18) to test our hypotheses that acute mTBI 1) differently affects GluR2+ and GLAST+EV cargo expression and 2) results in significant elevations in plasma protein biomarkers of injury severity and neurodegeneration. To achieve this goal, we first determined the miRNA content of GLAST+ and GluR2+ EVs enriched from plasma using TENPO, and quantified protein biomarker levels using the SIMOA Neurology4Plex assay for Tau, NFL, GFAP, and UCHL1. The mTBI cohort was on average 13 years older than the HC cohort, and the HC cohort contained 18% more self-reported males (**Figure 3A**).

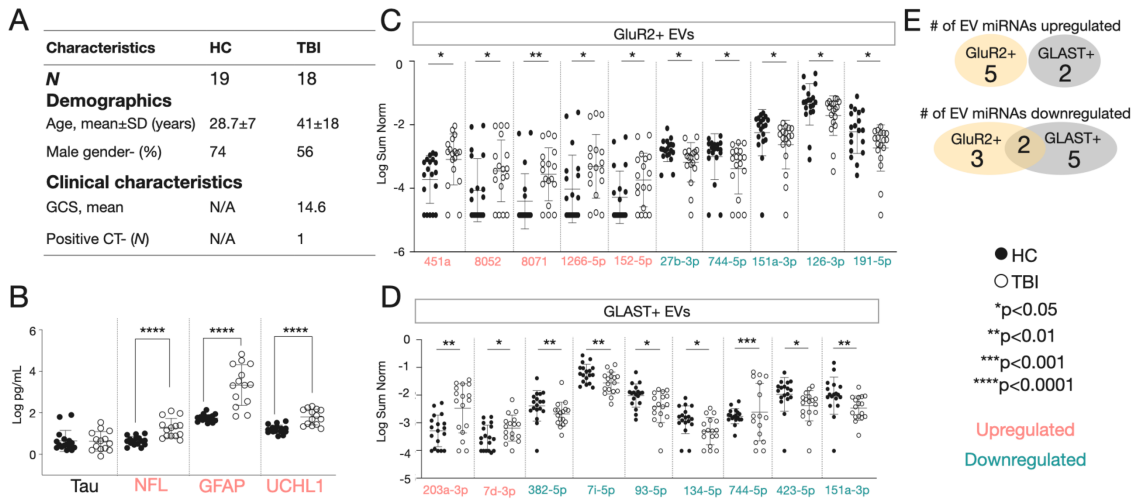


Figure 3 | Plasma Neurology4Plex and GluR2+ and GLAST+EV miRNA expression in clinical mTBI. A. Descriptive characteristics of traumatic brain injury patient and control subjects; N**,** mean ± SD, or N (%). **B.** Healthy control (HC) and traumatic brain injury patient (TBI) log transformed Neurology4Plex levels. Significance was assessed using student's T tests with log-transformed data. **C.** HC and TBI log transformed levels of GluR2+ EV miRNAs significantly up- or downregulated with TBI. **D.** HC and TBI log transformed levels of GLAST+ EV miRNAs significantly up- or downregulated with TBI. Significance of miRNA expression in GluR2+ and GLAST+ EVs was assessed using Fisher's exact test. **E.** Summary of overlap in differentially expressed miRNAs in GluR2+ and GLAST+ EVs

Consistent with our previous work and that of others, we observed significant elevation of three of the four Neurology4Plex markers in the mTBI group compared to controls (**Figure 3B;** fold change (FC) = 2.0, 1.9, 1.5; p<0.0001 for NFL, GFAP, and UCHL1). miRNA expression in both GluR2+ and GLAST+ EV populations was also significantly shifted in mTBI patients. In GluR2+ EVs, the expression of miRNAs hsa-miR-451a, hsa-miR-8052, hsa-miR-8071, hsa-miR-1266-5p, and hsa-miR-152-5p were elevated in the mTBI group compared to controls (**Fig. 3C;** FC = 2.7, 2.2, 2.1, 2.0, 1.9; p= 0.046, 0.033, 0.0074, 0.020 and 0.013 respectively), while levels of hsa-miR-27b-3p, hsa-miR-744-5p, hsa-miR-151a-3p, hsa-miR-126-3p, and hsa-miR-191-5p significantly decreased (**Figure 3C;** FC = -1.8, -1.7, -2.0, -2.9, -3.4; p= 0.017, 0.047, 0.023, 0.019 and 0.036 respectively). GLAST+ EVs, however, contained only two miRNAs – hsa-miR-203a-3p and hsa-let-7d-3p – that were significantly elevated in mTBI (**Figure 3D**; FC= 9.0, 2.7; p= 0.0049, 0.024 respectively), but seven that were significantly downregulated including hsa-miR-382-5p, hsa-let-7i-5p, hsa-miR-93-5p, hsa-miR-134-5p, hsa-miR-744-5p, hsa-miR-423-5p, and hsa-miR-151a-3p (**Figure 3D**; FC= -1.9, -2.0, -2.1, -2.1, -2.7, -2.0, -4.3; p= 0.012, 0.0094, 0.032, 0.030, 0.00032, 0.025, 0.0037 respectively). Of the differentially expressed miRNAs in the two EV pools, there was no overlap in the identify of miRNAs upregulated in each, but two miRNAs were downregulated in both GluR2+ and GLAST+ EVs (**Figure 3E;** hsa-miR-744-5p and hsa-miR-151a-3p).

Given these results, we next evaluated the diagnostic potential of these differentially expressed miRNAs by calculating their respective AUCs for discriminating mTBI patients from healthy controls. Of the plasma neurology4Plex biomarkers, GFAP resulted in the highest AUC (0.96), followed by UCHL1 (0.92), NFL (0.91) and Tau (0.55). When evaluated individually, GluR2+ and GLAST+ EV differentially expressed miRNAs performed similarly, with average AUCs of 0.72 and 0.70 respectively. The algorithmic combination of differentially expressed miRNAs in GLAST+ EVs resulted in an AUC of 0.83, while that of GluR2+ EVs was 0.64. After least absolute shrinkage and selection operator (LASSO) feature selection, these AUCs increased to 0.89 for GLAST+ EVs (using miRNAs hsa-miR-203a-3p, hsa-let-7d-3p, hsa-let-7i-5p, and hsa-miR-134-5p) and 0.74 for GluR2+ EVs (using miRNAs hsa-miR-451a, hsa-miR-8052, hsa-miR-8071, and hsa-miR-191-5p). The higher diagnostic performance of GLAST+ EV compared to GluR2+ EV differentially expressed miRNAs and the high performance of plasma GFAP relative to other neurology 4Plex markers suggest acute astrocyte injury, rather than neuronal damage, may be a major contributor to mTBI pathology in this cohort of patients.

Despite clear shifts in plasma protein and EV miRNA expression induced by mTBI, visualization of the distribution of the data across individuals reveals extensive person-to-person heterogeneity in the expression of Neurology4Plex biomarkers and that of differentially expressed EV miRNAs which may have contributed to their respective AUCs. In the control subjects, coefficient of variance (CV) values for plasma biomarkers ranged from 44% for GFAP, and 197% for Tau (**Figure 4A**). For the mTBI group, CV values for plasma biomarker levels ranged from 75% for UCHL1, to 164% for GFAP. The levels of plasma protein biomarkers for both the control and mTBI group were not significantly affected by age (see table 1A in Appendix) or self-reported gender ($p>0.081$ across all biomarkers; see table 2A in Appendix), and injury type (road traffic, fall, other) did not significantly affect mTBI patient plasma biomarker expression (ANOVA $p>0.20$ across all biomarkers; see table 3A in Appendix).

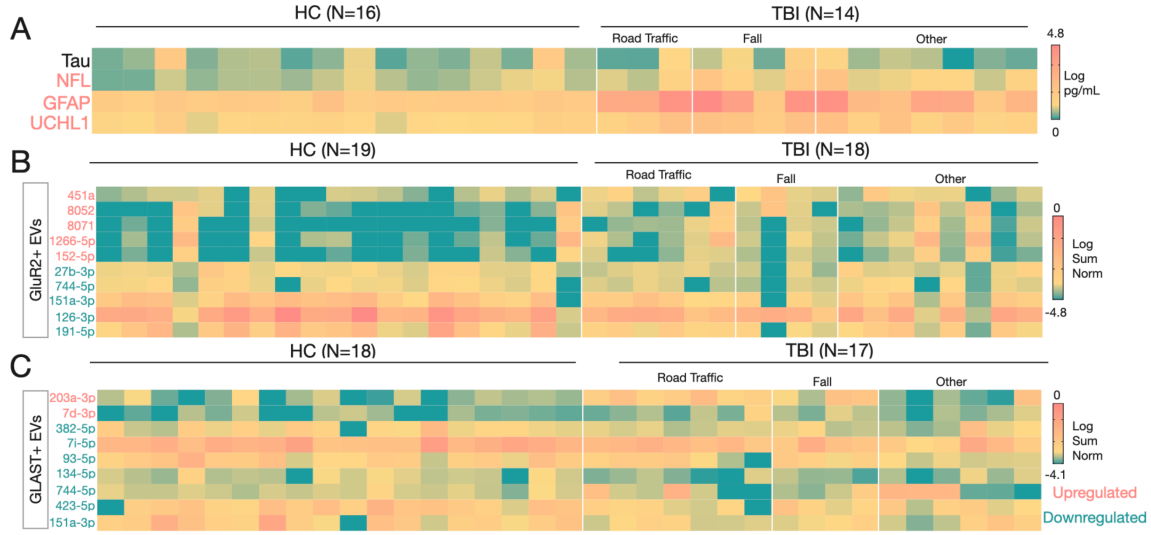


Figure 4 | Plasma Neurology4Plex, and GluR2+ and GLAST+ EV miRNA expression is heterogeneous across individuals. A. Log transformed neurology4plex levels plotted in heat map. Columns represent subjects, each arranged within the control group and respective TBI types by increasing age. **B.** Log transformed values of differentially expressed GluR2+EV miRNA levels plotted in heat map. Columns represent subjects, each arranged within the control group and respective TBI types by increasing age. **C.** Log transformed values of differentially expressed GLAST+EV miRNA levels plotted in heat map. Columns represent subjects, each arranged within the control group and respective TBI types by increasing age.

Differentially expressed GluR2+ EV miRNA levels also exhibited extensive person-to-person heterogeneity with CV values ranging from 66% for hsa-miR-744-5p to 278% for hsa-miR-8071 in controls and from 67% for hsa-miR-126-3p to 199% for hsa-miR-1266-5p in mTBI patients. Like the plasma protein biomarkers, levels of differentially expressed GluR2+ miRNAs were largely unaffected by age (see table 1B in Appendix) or self-reported gender ($p>0.13$ across all biomarkers; see table 2B in Appendix) in either group, or by injury type in mTBI patients ($p>0.50$ across all biomarkers; see table 3B in Appendix). The exception to this was hsa-miR-451a, which displayed a significant effect of self-reported gender in the control group ($p=0.016$).

For differentially expressed GLAST+ EV miRNAs, CV values ranged from 65% for hsa-miR-744-5p to 161% for hsa-miR-151a-3p in the control group and from 73% for hsa-miR-744-5p to 192% for hsa-miR-151a-3p in mTBI patients. For GLAST+ EVs, age (see table 1C in Appendix) self-reported gender ($p>0.072$ across all biomarkers; see table 2C in Appendix), and injury type in mTBI patients (ANOVA; $p>0.19$ across all biomarkers; see table 3C in Appendix) largely had nonsignificant effects on levels of differentially expressed miRNAs. However, in this EV population, injury type significantly affected hsa-93-5p levels ($p=0.040$).

GluR2+ and GLAST+ EV differentially expressed miRNAs reflect distinct signaling pathways

The promising advantage of EV-based diagnostics for TBI lies in their ability to indicate the activation of specific pathologic pathways in damaged neurons and astrocytes in response to injury. To determine signaling pathways implicated by GluR2+ and GLAST+ EV differentially expressed miRNAs, we subjected their respective mRNA targets to KEGG analysis. GluR2+ EV differentially expressed miRNAs collectively possessed 18955 mRNA targets, and the top 10 pathways implicated by these mRNAs were diverse, including processes relating to neurologic function, cell signaling, hormonal signaling, and cancer-specific pathways (**Table 1**). By contrast, GLAST+ EV differentially expressed miRNAs collectively targeted just 4825 mRNAs, but the top 10 pathways implicated by these mRNA targets were similarly diverse in function (**Table 1**). MAPK signaling, axon guidance, and signaling pathways regulating the pluripotency of stem cells were the only three pathways represented in the top 10 of both GluR2+ and GLAST+ EVs, with the remaining seven being unique to each.

GluR2	GLAST	
Axon guidance	MAPK signaling pathway	
MAPK signaling pathway	TGF-beta signaling pathway	Neurologic function
Endocytosis	FoxO signaling pathway	
Hippo signaling pathway	Signaling pathways regulating pluripotency of stem cells	Cell signaling
Sphingolipid signaling pathway	Axon guidance	Cancer
Pathways in cancer	Focal adhesion	Systemic function
Ras signaling pathway	Chronic myeloid leukemia	
Signaling pathways regulating pluripotency of stem cells	Proteoglycans in cancer	Hormonal Signaling
Cushing Syndrome	Thyroid hormone signaling pathway	
Adrenergic signaling in cardiomyocytes	Autophagy	

Table 1 | Kyoto Encyclopedia of Genes (KEGG) 2021 analysis of top 10 pathways of differentially expressed GluR2+ and GLAST+ EV miRNA targets. Ranked by p value.

GluR2+ EVs, GLAST+ EVs, and plasma biomarkers are uncorrelated

To overcome the extensive heterogeneity afflicting mTBI biomarker discovery efforts, it is important to leverage diverse sources of diagnostic information rather than relying on one dimensional analysis. Having evaluated the distinctions in the signaling pathways reflected by miRNAs significantly affected by mTBI in GluR2+ and GLAST+ EVs, we next aimed to further investigate the independence of these potential biomarkers by assessing their correlation with each other and with plasma Neurology4Plex proteins (**Fig 5A**).

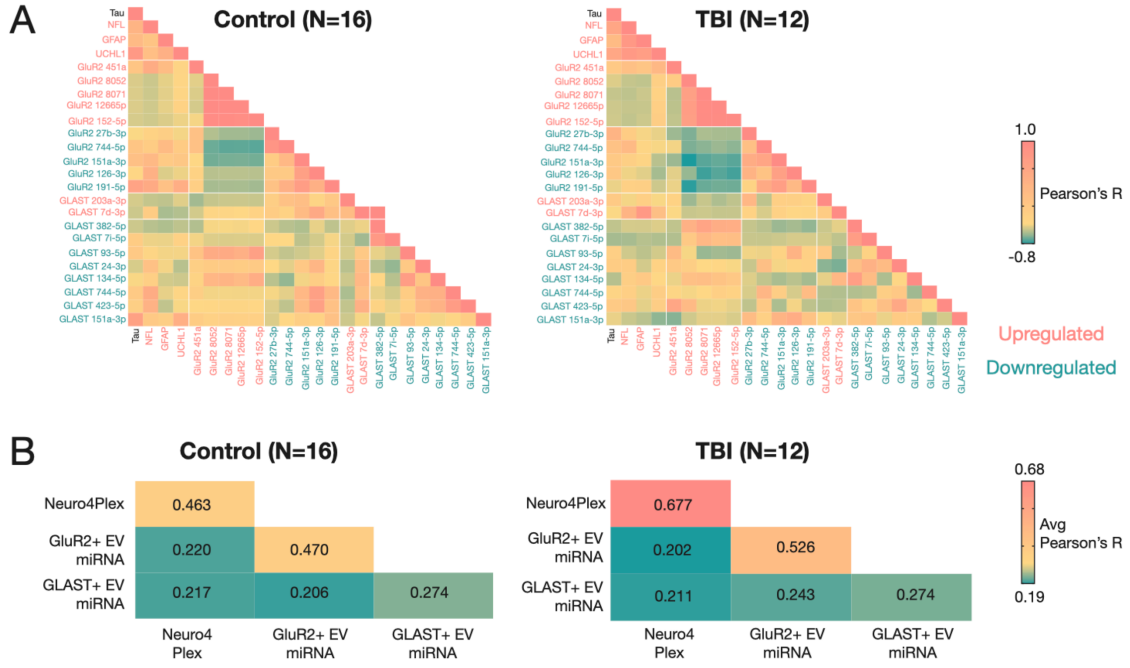


Figure 5 | Biomarker levels are less correlated across plasma, GluR2+EVs, and GLAST+EVs. A. Pearson's correlation coefficients calculated for all biomarker combinations across EV populations and plasma were plotted in a heat map matrix for mTBI patients and controls. **B.** Absolute value of Pearson's R values were averaged and plotted in a heat map matrix for mTBI patients and controls. Boxes delineate GluR2+ EVs, GLAST+ EVs, and plasma proteins.

This analysis revealed that protein and miRNA levels were more correlated within than across the two EV pools and plasma. For controls, Neurology4Plex levels were most correlated within the plasma (**Figure 5B;** avg Pearson's R = 0.463). By contrast, Neurology4Plex levels were less correlated with differentially expressed GluR2+ (avg Pearson's R= 0.220) and GLAST+ (avg Pearson's R= 0.217) EV miRNAs. Differentially expressed GluR2+ EV miRNA levels were also more correlated with each other (**Figure 5B**; avg Pearson's R= 0.470) than with Neurology4Plex (avg Pearson's R= 0.220) or GLAST+ EV differentially expressed miRNAs (avg Pearson's R= 0.206). Differentially expressed GLAST+ EV miRNAs were similarly more correlated with each other (**Figure 5B**; avg Pearson's R=0.274) than with Neurology4Plex levels (avg Pearson's R= 0.217) or GluR2+ EV differentially expressed miRNAs (avg Pearson's R= 0.206), but miRNAs in GLAST+ EVs were less correlated with each other on average than miRNAs in GluR2+ EVs and Neurology4Plex proteins in plasma

The correlation trends observed in the control group were echoed in the mTBI group. Neurology4Plex levels were most correlated with each other (**Figure 5B;** avg Pearson's R = 0.677) and less correlated with differentially expressed GluR2+ (avg Pearson's R= 0.202) and GLAST+ (avg Pearson's R= 0.211) EV miRNAs. Differentially expressed GluR2+ EV miRNA levels were also more correlated with each other (**Figure 5B**; avg Pearson's R= 0.526) than with Neurology4Plex (avg Pearson's R= 0.202) or GLAST+ EV differentially expressed miRNAs (avg Pearson's R= 0.243). Differentially expressed GLAST+ EV miRNAs were correspondingly more correlated with each other (**Figure 5B**; avg Pearson's R=0.274) than with Neurology4Plex protein levels (avg Pearson's R= 0.211) or GluR2+ EV differentially expressed miRNAs (avg Pearson's R= 0.243), but as observed in the control group, GLAST+ EV differentially expressed miRNAs were less correlated with each other on average than Neurology4Plex proteins or GluR2+ EV differentially expressed miRNAs. Interestingly, while the extent of biomarker correlation within plasma and GluR2+ EVs increased in the mTBI group (**Figure 5B**; avg Pearson's R= 0.677 and 0.526 respectively) relative to controls (**Figure 5B**; avg Pearson's R= 0.463 and 0.477 respectively), the extent of biomarker correlation within GLAST+ EVs remained unchanged across the two groups (**Figure 5B**; avg Pearson's R= 0.274 for controls and mTBI). These data collectively imply the independence of biomarker information across both EV populations and across each EV population and plasma, and suggest differing effects of mTBI on the extent of correlation of GluR2+ versus GLAST+ EV differentially expressed miRNAs.

This work illustrates that GluR2 and GLAST expression is highly specific to neurons and astrocytes respectively, and that circulating EVs can be isolated based on their expression of these receptor subunits. Acute mTBI was associated with distinct shifts in miRNA expression in each EV population, and with elevation of three plasma protein biomarkers of injury severity, GFAP, NFL, and UCHL1. Somewhat unsurprisingly, Tau, which is associated with chronic neurodegeneration, was not significantly elevated with acute mTBI, probably due to the low number of actively degenerating neurons and to the timing of the biomarker measurement. The levels of miRNAs significantly up- and downregulated with acute mTBI in each EV population were uncorrelated with these traditional protein-based TBI biomarkers, and since each EV population was associated with the activation of seven distinct pathways, the combined clinical assessment of all three biomarker types holds promise for a comprehensive evaluation of not only the severity of the injury, but also of the specific pathologic processes underlying an individual TBI patient's etiology. The differentially expressed miRNAs in each EV pool demonstrated diagnostic potential, though the panel of GLAST+ EV miRNAs outperformed that of GluR2, suggesting the potential role of astrocyte-specific injury to mTBI phenotype in this cohort of patients.

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What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

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.

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

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?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

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 3, namely transitioning to work on our clinical brain injury samples.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

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?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an 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?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*

- *adoption of new practices.*

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?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:
- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

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:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

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 had 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 remains persistently almost a full year behind schedule.

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

Describe problems or delays encountered during the reporting period 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 had 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 remains persistently almost a full year behind schedule.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

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 continue to actively hiring to account for these changes to our budgeted spending. Juhwan Park, PhD from KAIST, has recently joined our group and will transition partially onto this project.

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/ Institutional Animal Care and Use Committee approval dates.

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:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

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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.

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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. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report.

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

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

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

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

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**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Z. Yang, D. Issadore, Ultrasensitive Single Extracellular Vesicle Detection Using High Throughput Droplet Digital Enzyme-Linked Immunosorbent Assay, 2022, Submitted.

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.

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*

Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

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?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*

- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report.

8. **SPECIAL REPORTING REQUIREMENTS**

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*