Plasma extracellular vesicles as a source of biomarkers in traumatic brain injury

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OBJECTIVE The objective of this study was to isolate extracellular vesicles (EVs) from plasma in a cohort of patients with traumatic brain injury (TBI) and analyze their contents for novel biomarkers that could prove useful for rapid diagnosis and classification of brain injury during initial evaluation.

METHODS Plasma EVs were isolated by serial ultracentrifugation from patients with TBI (n = 15) and healthy controls (n = 5). Samples were obtained from the TRACK-TBI biorepository (2010–present). Size and concentration were determined by nanoparticle tracking. Glial fibrillary acidic protein (GFAP) concentration was determined in EV protein. EV RNA was isolated and deep sequencing of short noncoding RNA was performed.

RESULTS Plasma EVs are physically similar but contained approximately 10 times more GFAP in TBI patients with altered consciousness than patients and controls with normal consciousness. Eleven highly differentially expressed microRNAs (miRNAs) were identified between these groups. Genes targeted by these miRNAs are highly associated with biologically relevant cellular pathways, including organismal injury, cellular development, and organismal development. Multiple additional coding and noncoding RNA species with potential biomarker utility were identified.

CONCLUSIONS Isolating plasma EVs in patients with TBI is feasible. Increased GFAP concentration—a validated plasma TBI marker—in EVs from TBI patients with altered consciousness, along with differential expression of multiple miRNAs targeting TBI-relevant pathways, suggests that EVs may be a useful source of TBI biomarkers. Additional evaluation in larger patient cohorts is indicated.

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KEYWORDS extracellular vesicle; biomarker; microRNA; plasma; diagnostic; traumatic brain injury

EXTRACELLULAR vesicles (EVs) are membrane-bound particles released by the majority of human cells, including cells within the CNS.^{1,2} They contain DNA, messenger RNA (mRNA), noncoding RNA, and proteins that are released into the microenvironment, and play key roles in cell-to-cell communication between neurons, glia, and endothelial cells for complex functions including moderation of the blood-brain barrier.^{3–5} EVs are a heterogeneous group composed of microvesicles (> 100 nm) and smaller exosomes (< 100 nm) that are generated through distinct cellular pathways and have distinct cargo. EVs have generated significant interest in neurooncology; tumor-derived EVs shape the microenvironment by down-regulating the immune response and upregulating angiogenic factors, among other complex actions.^{3–6}

Severe traumatic brain injury (TBI) affects more than 200,000 individuals annually in the US and is a leading cause of mortality in men under 35 years of age.⁷ Mild TBI is underreported but may be as much as 10 times more common than severe TBI, and is increasingly recognized as a contributor to chronic neurological dysfunction.^{8,9} Plasma biomarkers in TBI have generated substantial excitement recently with the identification of multiple brain-specific molecules (glial fibrillary acidic protein [GFAP], ubiquitin carboxy-terminal hydrolase L1 [UCH-L1], neurofilament light, tau) in the blood of patients with TBI and recent FDA approval of a device (Brain Trauma Indicator, Banyan Biomarkers) for rapid measurement of GFAP and UCH-L1 levels in plasma.¹⁰ These biomarkers are used to help clarify TBI diagnosis and prognosis, particularly

ABBREVIATIONS EV = extracellular vesicle; GCS = Glasgow Coma Scale; GFAP = glial fibrillary acidic protein; lincRNA = long intervening noncoding RNA; miRNA = microRNA; mRNA = messenger RNA; rRNA = ribosomal RNA; snoRNA = small nucleolar RNA; snRNA = small nuclear RNA; TBI = traumatic brain injury; TRACK-TBI = Transforming Research and Clinical Knowledge in Traumatic Brain Injury; UCH-L1 = ubiquitin carboxy-terminal hydrolase L1.

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in mild TBI. EVs have not yet been extensively studied in TBI, although one small study has identified a higher number of EVs in CSF of two subjects with severe TBI (Glasgow Coma Scale [GCS] score 3–8) compared to normal controls, and further isolated both GFAP and UCH-L1 in higher quantities in the TBI subjects compared to controls.¹¹ While these findings are intriguing, obtaining similar results in plasma would be preferable to CSF as plasma is much more readily available. EVs provide a potential source of brain-specific biomarkers that can be isolated from peripheral blood.

EVs in peripheral blood provide a lipid membrane-bound container of DNA, RNA, and proteins that are protected from circulating degradation enzymes, and therefore may provide a snapshot of the microenvironment in specific organ systems shortly before the peripheral blood sample is taken. This makes plasma EVs potentially an intriguing source of brain-specific biomarkers that may provide real-time insight into the cellular microenvironment during the initial brain injury phase, as well as during both short- and long-term recovery. Beyond diagnosis and prognosis, EVs have potential therapeutic applications as well, but the exact source of therapeutic EVs in TBI and what, if any, modification would be necessary to render them therapeutic is yet unclear. In this pilot study, we sought to assess the potential of plasma EVs as a biomarker source in TBI by demonstrating the feasibility of isolating EVs, characterizing their size and frequency, and determining the presence of both established (GFAP) and novel (short noncoding RNA) TBI biomarkers within them, comparing plasma samples from TBI patients to healthy controls. A difference in RNA expression profiles contained within the EVs of TBI patients compared to healthy controls was hypothesized.

Methods

Study Population

This study was a joint analysis between the University of Pittsburgh and the Mayo Clinic. IRB approval was obtained at both institutions. Subject plasma samples (approximately 500 μ l each) were selected from the TRACK-TBI (Transforming Research and Clinical Knowledge in Traumatic Brain Injury) biorepository from the University of Pittsburgh.¹² Acquisition and sharing of these samples was approved by the University of Pittsburgh IRB. This project was reviewed by the Mayo Clinic IRB and believed to be IRB-exempt as it was secondary research on biopspecimens for which consent is not required and the identity of human subjects cannot be readily ascertained by the investigators. A total of 20 plasma samples were obtained: 15 samples were obtained after TBI, and 5 samples were obtained from healthy controls. Patients with complicated mild (GCS score 13–15 with evidence of injury on CT scan), moderate (GCS score 9–12), and severe (GCS score 3–8) TBI were included. Given the pilot nature of this study, attempts were made to select patients with a wide range of injury mechanisms accompanying all severities of TBI as well as from uninjured, healthy controls. Demographic and injury information is listed in Table 1.

Isolation of EVs, Western Blot, and GFAP Analysis

EVs within plasma were isolated using serial density gradient ultracentrifugation per our established protocol.¹³ Concentrated EVs were then analyzed via nanotracker particle analysis using NanoSight (Malvern, NanoSight NS300). NanoSight allows for characterization of EVs, including both size and concentration within a given sample. As a part of this protocol for isolating EVs, a western blot analysis was performed to identify common EV proteins, confirming the presence of EVs in the sample. For western blot analysis, proteins were separated by electrophoresis on 4%–20% sodium dodecyl sulfate–polyacrylamide gel. Following membrane transfer, the proteins were probed using the following antibodies: anti-Alix (Cell Signaling, no. 2171S, mouse monoclonal), anti-CD9 (Cell Signaling, no. 13174S, rabbit monoclonal), anti-CD63 (Santa Cruz Biotechnology, no. sc-5275, mouse monoclonal), and anti-HSP90 (Cell Signaling, no. 4874S, rabbit polyclonal). Secondary antibody was horseradish peroxidase–conjugated goat antirabbit or goat antimouse (Jackson ImmunoResearch, no. 115-035-003). Detection was conducted using enhanced chemiluminescence (ThermoFisher Scientific, no. 32106). For GFAP analysis, EVs were sonicated to break their membranes. GFAP expression was then tested in all samples via ELISA using a commercially available GFAP kit (abcam, no. ab223867) per the manufacturer's instructions.

MicroRNA Analysis

Short noncoding RNA was isolated from samples using an miRNAeasy kit and QiaCube (QIAGEN). Preliminary quality assessment was performed using NanoDrop (ThermoFisher). Samples then underwent next-generation sequencing using the Illumina MiSeq 2000 with NebNext multiplex oligos for Illumina adapters (adapter sequence: AGATCGGAAGAGCACACGTCT). Analysis of short noncoding RNA sequencing was performed in collaboration with the Bioinformatics Core of the Mayo Clinic Center for Individualized Medicine. Sequencing data were analyzed using the OASIS 2.0 platform with the reference genome HG38; this investigated 28,263 unique RNA entities covering microRNA (miRNA), Piwi-interacting RNA, small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), and ribosomal RNA (rRNA) species. Differential expression analysis was used to identify differences in miRNA expression between groups.

Statistical Analysis

Descriptive statistics with medians, interquartile ranges, and proportions were used to describe continuous and categorical variables, respectively. Biomarker levels were treated as continuous data. The Mann-Whitney U-test was used to assess for between-group differences for continuous variables. All statistical analysis was performed with JMP software.

Results

EVs were isolated from 15 TBI subjects, including 6 with CT-positive mild TBI (GCS score 13–15), 3 with CT-positive moderate TBI (GCS score 9–12), and 6 with CT-

positive severe TBI (GCS score 3–8), as well as 5 healthy control subjects with no history of TBI or neurodegeneration. Given the relatively small sample size, subjects were divided into those who presented with an altered level of consciousness (GCS score ≤ 14) and those with a normal level of consciousness (GCS score of 15 and healthy controls). Demographic and injury information is listed in Table 1.

Isolation of EVs and GFAP Analysis

EVs were successfully isolated in all samples analyzed. EV size and concentration were available for each sample (Fig. 1). EV size, concentration, GFAP concentration, and RNA yield for the entire cohort are shown in Table 2. There were no significant differences in EV size, concentration, or RNA yield between normal controls/TBI patients with normal consciousness (GCS score = 15) and TBI patients with altered consciousness (GCS score ≤ 14 ; Fig. 2). In contrast, subsequent analysis of GFAP yield by ELISA demonstrated a significantly higher GFAP concentration in subjects with an altered level of consciousness (GCS score ≤ 14) as compared to those with normal GCS scores (mean GFAP concentration [$\pm$ standard error] 2204.2 pg/ml $\pm$ 1067.2 pg/ml vs 207.8 pg/ml $\pm$ 270.8 pg/ml, $p = 0.02$; Fig. 2).

Short Noncoding RNA Sequencing

RNA was successfully isolated from all samples, and RNA yields from the isolated EVs are listed in Table 2. Short noncoding RNA sequencing for plasma EVs from TBI patients and healthy controls was analyzed on the Illumina HiSeq 2500 platform and processed via our CAP-miRSeq pipeline. This allowed unbiased identification of known and novel short noncoding RNA species beyond simply miRNAs. Differential expression analysis was performed for miRNA sequences identified with thresholds of log 2-fold change (0.7) and a p value < 0.1 , given the low number of samples with widely varying demographics, injury mechanisms, and presentation GCS scores. Using these criteria, trends for 9 highly upregulated miRNAs and 2 highly downregulated miRNAs were identified in TBI patients with an altered level of consciousness, as compared to TBI patients and healthy controls with a normal level of consciousness (Fig. 3A, Table 3). These reached statistical significance ($p \leq 0.05$) for 7 of the 11 individual miRNA sequences identified. Furthermore, Ingenuity pathway analysis of highly differentially expressed miRNAs among all TBI patients relative to controls identified top disease and disorder pathways targeted, including cancer, gastrointestinal disease, and organismal injuries/abnormalities, while the top molecular and cellular pathways identified included cell death/survival, cell-to-cell signaling, and cellular assembly/organization (Table 4).

miRNAs are well characterized in existing databases, which facilitated their analysis for the present study. However, they actually made up a minority of RNA species found within plasma EVs (Fig. 3B). The majority of the RNA found was protein-coding mRNA. Due to the specific parameters of our sequencing paradigm, which was aimed at short noncoding RNA, we were unable to mean-

TABLE 1. Cohort demographics, injury mechanisms, and presentation GCS scores

Cohort	Age (yrs), Sex	Injury Mechanism	Presentation GCS Score
TBI patients			
TR-1345	22, F	MVC	15
TR-1134	41, M	MVC	15
TR-1146	32, M	Fall	15
TR-1395	46, M	MVC	14
TR-1178	25, M	MVC	14
TR-1123	72, M	Fall	13
TR-1161	33, M	Assault	11
TR-1252	24, M	MVC	10
TR-1082	55, F	Fall	9
TR-1330	35, M	Other	6T
TR-1174	59, M	MVC	3
TR-1086	56, F	Fall	3
TR-1219	45, M	MVC	3
TR-1233	30, M	MVC	3
TR-1159	23, M	MVC	3
Healthy controls			
HC-1054	25, M	—	15
HC-1052	31, M	—	15
HC-1015	40, M	—	15
HC-1019	53, F	—	15
HC-1058	63, M	—	15

MVC = motor vehicle collision.

ingfully quantify the protein-coding transcripts found in the present data. Nevertheless, they are present in abundance. Similarly, multiple additional species of short noncoding RNA were found, including snoRNA, snRNA, rRNA, and long intervening noncoding RNA (lincRNA).

Discussion

In this pilot analysis of plasma EV isolation and sampling in TBI, we demonstrated successful EV isolation from plasma samples drawn at the time of initial hospital evaluation. Plasma EVs from TBI patients with altered consciousness (GCS score ≤ 14) are physically similar to plasma EVs from TBI patients and normal donors with normal consciousness (GCS score = 15) but are markedly enriched for GFAP, an established plasma biomarker in TBI. EVs are known to be rich sources of nucleic acids, proteins, and metabolites. The presence of GFAP in plasma EV suggests that they also likely harbor transcriptomic, proteomic, and metabolomic signatures potentially unique to TBI diagnosis that could be isolated and analyzed. As an example of this potential, our deep sequencing of short noncoding RNA identified 11 highly differentially expressed miRNA sequences between TBI patients with altered consciousness and TBI patients/healthy controls with normal consciousness. Furthermore, these miRNAs targeted genes associated with highly bio-

FIG. 1. Nanoparticle tracking analysis. Representative histograms (**A and B**) and photomicrographs (**C and D**) of EV analysis in a healthy control (**A and C**) and TBI subject (**B and D**). The histograms in both the healthy control (**A**) and TBI patient (**B**) demonstrate a substantial exosome peak (< 100 nm) and multiple smaller microvesicle peaks (> 100 nm). Original magnification $\times 20$. Western blot (**E**) shows expression of common EV-associated proteins in EVs isolated with this protocol. Figure is available in color online only.

logically relevant pathways in trauma (e.g., organismal injury and cell death/survival). These findings suggest that brain-specific EVs are released shortly after TBI, resulting in an altered level of consciousness, and that these EVs are obtainable via peripheral blood draws during initial evaluation in the emergency department. Given the highly specific and measurable contents of the EVs, their isolation and analysis provide opportunities for the identification of potentially novel biomarkers that can be correlated with injury severity, prognosis, or response to therapeutic interventions.

It is important to note that there is a constant stream of EVs circulating in peripheral blood from nearly every cell in the body, even in a healthy, resting state.¹⁴ EVs are not solely produced by the CNS after trauma, and likely make up only a small fraction of the total circulating EV burden at any given time, even after trauma. This is consistent with our findings, given that there was no significant difference between EV concentration or size in any of the trauma subjects compared to each other or healthy controls. Therefore, it is likely that multiple organ systems,

not just the CNS, change their EV release patterns after trauma. Identification of brain-specific EVs is challenging and remains under investigation. For this analysis, we chose to analyze bulk plasma EVs (not brain-derived EVs specifically) and demonstrated that GFAP, a brain-specific biomarker released after trauma, is increased in plasma EVs following TBI with altered consciousness. This suggests that, even in bulk plasma EVs, relevant CNS biomarkers are increased following TBI. It also suggests that EVs from peripheral blood are another quantifiable source of GFAP in cases of TBI, perhaps having uses in the initial evaluation and diagnosis of TBI.^{8,10,12}

Importantly, the same bulk plasma EVs enriched for the brain-specific biomarker GFAP also contained miRNA signatures that significantly differed between subjects with an altered level of consciousness as compared to normal level of consciousness at the time of peripheral blood draw. These miRNAs have hundreds of downstream targets within the transcriptome and represent novel biomarkers that may be useful for diagnosis, prognosis, and management in TBI. Our pathway analysis demonstrated that these

TABLE 2. MV/E concentration, mean size, GFAP concentration, and RNA concentration

Study Subject (GCS Score)	MV/E Concentration (particles/ml)	MV/E Size (nm)	GFAP Concentration (pg/ml)	RNA Concentration (µg/ml)
TBI				
TR-1345 (15)	36,300,000,000	85.2	2166.68	61.4
TR-1134 (15)	93,900,000,000	68.6	Undetectable	37.1
TR-1146 (15)	145,000,000,000	89.5	Undetectable	30.7
TR-1395 (14)	12,100,000,000	107.1	3489.56	42.4
TR-1178 (14)	89,400,000,000	67.7	136.4	46
TR-1123 (13)	13,400,000,000	124.9	2164.03	57.6
TR-1161 (11)	282,000,000,000	71.7	Undetectable	33
TR-1252 (10)	18,700,000,000	137.2	466.6	54.7
TR-1082 (9)	20,100,000,000	139	Undetectable	62.4
TR-1330 (6T)	8,480,000,000	92.7	1381.7	47.6
TR-1174 (3)	11,600,000,000	154.1	Undetectable	76.8
TR-1086 (3)	32,200,000,000	105	221.99	32.3
TR-1219 (3)	28,400,000,000	85.1	3834.9	42.6
TR-1233 (3)	31,700,000,000	106.6	13122.5	86.6
TR-1159 (3)	11,300,000,000	108.3	1633.1	16
Healthy control				
HC-1054 (15)	64,200,000,000	64.6	Undetectable	55.9
HC-1052 (15)	12,200,000,000	114	Undetectable	65.3
HC-1015 (15)	45,800,000,000	114.8	Undetectable	40.2
HC-1019 (15)	53,400,000,000	79.1	Undetectable	53.2
HC-1058 (15)	55,200,000,000	97.8	Undetectable	17
Analysis				
Mean altered GCS score	4.7 e+10 ± 2.2 e+10	108.3 ± 7.7	2204.2 ± 1067.2	49.8 ± 5.6
Mean normal GCS score	6.3 e+10 ± 1.4 e+10 (NS)	89.2 ± 6.7 (NS)	207.8 ± 270.8 (p = 0.02)	45.1 ± 5.9 (NS)

MV/E = microvesicles/exosomes; NS = nonsignificant.

up- and downregulated miRNAs play a role in cell maintenance, assembly, and death, as well as nervous system function. Further analysis could focus on protein-coding mRNA, snoRNA, snRNA, rRNA, and a wide range of as yet unknown protein signatures contained within these EVs. Each sample is a snapshot in time, and the signatures could change over time, in response to either further insult (persistently raised intracranial pressure, decreased cere-

bral blood flow, poor oxygen delivery, etc.) or therapeutic maneuvers (medications and/or surgical intervention). The signatures obtained and analyzed from EVs could be a technique to monitor function and recovery of the CNS at a cellular level using the body's own cell-to-cell signaling mechanisms. This area of research remains in relative infancy, and significant work is required to better understand the factors surrounding EV release after many types

FIG. 2. EV characteristics. Comparison between TBI patients and normal controls with normal consciousness (GCS score = 15) versus TBI patients with altered consciousness (GCS score $\leq$ 14) for EV concentration (A), EV size (B), EV GFAP yield (C), and EV RNA yield (D). Note that GFAP yield is significantly higher in TBI patients with altered consciousness but no significant differences are seen in EV concentration, size, or RNA yield. Values represent mean $\pm$ standard error. * $p < 0.05$.

FIG. 3. Plasma exosomal RNA expression. **A:** Differential miRNA expression. Volcano plot of fold change versus p value for miRNA sequences identified by short noncoding RNA sequencing of plasma EVs in TBI patients with altered consciousness (GCS score ≤ 14) versus TBI patients and healthy controls with normal consciousness (GCS score = 15). Sequences trending to high differential expression (log 2-fold change 0.7 and $p < 0.1$) shown in red. **B:** Relative frequency of miRNA versus other RNA species in plasma exosomal RNA.

of insult. It will also require a better understanding of how to isolate organ system–specific EVs to improve the signal-to-noise ratio for studying biomarkers from these organ systems in response to injury. Lastly, the novel biomarkers themselves will have to be rigorously correlated with clinical presentations, disease states, and outcomes to ensure that the signatures in question truly are specific to the disease process and its treatment.

Assessing the biological significance of specific differentially expressed miRNA sequences identified by deep sequencing in this pilot study must be conducted cautiously, given our small patient sample sizes. Nevertheless, several intriguing possibilities can be observed. Although not directly implicated in TBI previously, miR-1 (increased in EVs from TBI patients with altered consciousness) up-regulation has been reported in neurons undergoing apoptosis in response to hypoxia.¹⁵ miR-143 has been implicated

in neuronal injury following ischemia and in regulating blood-brain barrier integrity.¹⁶ Finally, miR-9 (6.84 log 2-fold change increase in TBI with decreased consciousness, $p < 0.01$ in our data) has, in fact, been previously identified as a plasma extracellular miRNA biomarker in TBI in mice, although not in humans.¹⁷

Limitations

There are a number of limitations to this analysis, nearly all due to the small sample size, which includes multiple mechanisms of TBI, widely varying GCS scores at presentation, different patient demographics and characteristics, as well as only 5 healthy controls, and an initial sample size of only 500 μ l of plasma. These sample size limitations precluded the ability to perform reproducibility analyses and other standard validation techniques, potentially leading us to false conclusions from our pilot data. Despite the

TABLE 3. Up- and downregulated miRNA sequences in patients with altered presentation GCS scores compared to normal presentation GCS scores

miRNA Sequence	Log 2-Fold Change	p Value
Upregulated miRNA		
hsa-miR-1-3p	1.51	<0.01
hsa-miR-143-3p	1.19	<0.01
hsa-miR-151b	3.71	0.09
hsa-miR-27a-3p	0.89	0.02
hsa-miR-29a-3p	1.37	<0.01
hsa-miR-328-3p	4.18	0.09
hsa-miR-9-3p	6.84	<0.01
hsa-miR-99a-5p	1.32	0.05
hsa-miR-99b-5p	1.15	0.06
Downregulated miRNA		
hsa-miR-155-5p	-2.99	0.03
hsa-miR-30c-5p	-1.24	0.09

limitations of the cohort, significant differences were identified in GFAP concentration. Given the heterogeneous cohort, less stringent thresholds were required when analyzing the differential expression profiles of miRNA between groups, specifically a log fold change of 0.7 and a p value < 0.1, resulting in the identification of 11 miRNA sequences that appear to differ between groups, but not all of which reach the threshold of $p \leq 0.05$. Given that this was a pilot analysis of 15 patients and 5 controls, a broad net was cast in order to identify any potential areas of interest that would warrant further analysis on a more stringent level. These specific miRNA sequences will be further analyzed in a more directed fashion with a much larger cohort and more stringent statistical analysis. The possibility of contamination of the RNA sequencing analysis must be considered, especially with such a small number of samples in this pilot study, and this furthers the need for larger studies to confirm or refute these findings.

We were also limited by the inability to completely isolate brain-specific EVs from systemic EVs in this analysis, and that likely somewhat decreased the signal-to-noise ratio for identification of TBI signatures. The significantly increased GFAP concentration in altered LOC patients would suggest that our bulk plasma EV samples were enriched for brain-derived EVs, but the actual proportion is unknown and could be quite small. We cannot state with complete certainty that GFAP is not simply a co-isolated contaminant in the ultracentrifugation process based on this analysis. Further studies will be required to identify how best to preserve the brain-specific EVs and discard the systemic EVs, after which differential expression analysis would likely reveal highly significant differences between groups. Nonetheless, this analysis provides a starting point to demonstrate that this analysis is feasible. Significant differences were quantifiable, even with a very small amount of plasma (500 μ l) from only 20 subjects with widely varying clinical and injury demographics. Further studies should attempt to isolate brain-specific EVs and analyze

TABLE 4. Top pathways for diseases and disorders or molecular and cellular functions targeted by differentially expressed plasma exosomal miRNAs, comparing TBI patients with altered consciousness (GCS scores ≤ 14) and TBI patients and healthy controls with normal consciousness (GCS scores = 15)

Variable	p Value Range	No. of Molecules
Diseases & disorders		
Cancer	4.75E-02 to 1.35E-12	10
Gastrointestinal disease	3.84E-02 to 1.35E-12	10
Organismal injury & abnormalities	4.78E-02 to 1.35E-12	10
Respiratory disease	8.49E-03 to 1.35E-12	8
Reproductive system disease	3.43E-02 to 1.00E-08	9
Molecular & cellular functions		
Cell death & survival	4.13E-02 to 8.72E-04	6
Cell-to-cell signaling & interaction	1.97E-02 to 9.05E-04	4
Cellular assembly & organization	2.82E-02 to 9.05E-04	4
Cellular development	4.22E-02 to 9.05E-04	9
Nervous system development & function	3.04E-02 to 9.05E-04	6

them in much larger, uniform injury and presentation GCS score cohorts.

Conclusions

EVs can be isolated from peripheral blood after TBI, and they may contain brain-specific biomarkers that can be analyzed and potentially used in the diagnosis, prognosis, and treatment monitoring of TBI.

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Disclosures

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions

Conception and design: Puffer, Okonkwo, Parney. Acquisition of data: Puffer, Okonkwo, Parney. Analysis and interpretation of data: Puffer, Cumba Garcia, Himes. Drafting the article: Puffer, Cumba Garcia, Himes. Critically revising the article: Puffer, Cumba Garcia, Jung, Okonkwo, Parney. Reviewed submitted version of manuscript: Puffer, Okonkwo, Parney. Approved the final version of the manuscript on behalf of all authors: Puffer. Statistical analysis: Puffer, Cumba Garcia, Himes, Jung, Parney. Administrative/technical/material support: Meyer, Okonkwo. Study supervision: Okonkwo, Parney.

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