Expert Review of Proteomics

ISSN: 1478-9450 (Print) 1744-8387 (Online) Journal homepage: http://www.tandfonline.com/loi/ieru20

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To cite this article: Stefania Mondello, Eric P. Thelin, Gerry Shaw, Michelle Salzet, Carmela Visalli, Dasa Cizkova, Firas kobeissy & Andras Buki (2018): Extracellular vesicles: pathogenetic, diagnostic and therapeutic value in traumatic brain injury, Expert Review of Proteomics, DOI: 10.1080/14789450.2018.1464914

To link to this article: https://doi.org/10.1080/14789450.2018.1464914

Accepted author version posted online: 19 Apr 2018.

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Publisher: Taylor & Francis

Journal: *Expert Review of Proteomics*

DOI: 10.1080/14789450.2018.1464914

Review

Extracellular vesicles: pathogenetic, diagnostic and therapeutic value in traumatic brain injury

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Abstract

Introduction: Traumatic brain injury (TBI) is a leading cause of death and disability worldwide. Accurate classification according to injury-specific and patient-specific characteristics is critical to help informed clinical decision-making and to the pursuit of precision medicine in TBI. Reliable biomarker signatures for improved TBI diagnostics are required but still an unmet need.

Areas covered: Extracellular vesicles (EVs) represent a new class of biomarker candidates in TBI. These nano-sized vesicles have key roles in cell signaling profoundly impacting pathogenic pathways, progression and long-term sequelae of TBI. As such EVs might provide novel neurobiological insights, enhance our understanding of the molecular mechanisms underlying TBI pathophysiology and recovery, and serve as biomarker signatures and therapeutic targets and delivery systems.

Expert commentary: EVs are fast gaining momentum in TBI research, paving the way for new transformative diagnostic and treatment approaches. Their potential to sort out TBI variability and active involvement in the mechanisms underpinning different clinical phenotypes point out unique opportunities for improved classification, risk-stratification ad intervention, harboring promise of predictive, personalized, and even preemptive therapeutic strategies. Although a great deal of progress has been made, substantial efforts are still required to ensure the needed rigorous validation and reproducibility for clinical implementation of EVs.

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1. Introduction

Traumatic brain injury (TBI) is a leading cause of death and disability, especially among children and young adults, causing long-term devastating effects on the survivors and their families and posing huge costs to society. Each year more than 50 million TBIs occur worldwide with an estimated overall cost of approximately US$400 billion – 0.5% of the entire annual global output [1]. Despite these already staggering figures, according to the World Health Organization, TBI is projected to become the third leading contributor to the disease burden by 2020.

TBI is a highly heterogeneous disease, and the types of injury and recovery patterns are significantly influenced by the cause of the injury. The trauma produces the initial injury, disrupting the structural integrity of the brain, destroying tissue, and causing vascular and parenchymal damage, intracerebral hemorrhage, and axonal shearing. However, the damage to the brain surpass the initial insult, a complex cascade of biochemical and cellular events associated with secondary injury, that include disruption of the blood–brain barrier (BBB), inflammation, excessive neural excitability, and oxidative stress, is initiated [2,3]. These processes persist for months to years post-injury, perhaps lifelong, eliciting both regenerative and degenerative tissue response [3].

Because of the heterogeneous and chronic/evolving nature of TBI, the serious consequences and adverse outcome, and the significant challenges to identify definitive effective interventions, objective indicators of the different pathogenic processes that can be used for diagnosis, prognostication, and better stratification and characterization of patients, and to help optimize management and therapeutic pathways, are a priority research area [1,4].

Despite the tremendous efforts has been devoted and a number of promising acute and subacute/chronic markers have been identified [5-8], currently, while implemented regionally [9], no blood-based markers are routinely adopted worldwide in clinical practice [6,10] and the quest for novel more accurate and refined TBI biomarker is still ongoing.

This review focuses on a novel promising family of biomarkers, namely extracellular vesicles. We discuss emerging approaches and currently available evidence from experimental and human studies, highlighting their potentials to sort out patient variability and develop and guide targeted personalized, even preemptive, therapies towards precision medicine practice. Importantly, gaps and challenges which may hamper and hold back successful translation to bedside are outlined.

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2. Extracellular vesicles

Extracellular vesicles (EVs) represent a heterogeneous group of membrane vesicles of less than 1μm in diameter released into the extracellular environment from virtually all cell types (e.g., platelets, lymphocytes, astrocytes, fibroblasts, endothelium and neuronal cells etc.). They serve as carriers of many bioactive molecules including cytosolic proteins, nucleic acids (mRNA, miRNA), and lipids, and have been demonstrated to play a critical role in intercellular communication, [11,12] permitting, among others, the acquisition of new functional properties by recipient cells [13].

Despite EVs can be physiologically found in healthy individuals, data from many studies clearly show that their production increase during cell activation, oxidative stress, tissue hypoxia, and in other various pathological conditions [14].

Based upon the mechanism formation and the intracellular site origin (endosomal or cell membrane), size, markers, and contents EV can be divided into exosomes, microparticles (MPs), also called microvesicles (MVs), and apoptotic bodies.

Exosomes are small vesicles with a diameter of 30–100 nm originated from specialized compartment of the endosomal-lysosomal pathway and stored as intraluminal vesicles inside multivesicular bodies. Exosomes are constitutively or selectively released depending on the cell type [13]. Johnstone in 1987 first described exosomes as extracellularly intraendosomal vesicles released during the maturation process of reticulocytes. Importantly, the signature biomarkers expressed/contained on exosome membrane permit to track their original cell and determine the cells they will interact with [15,16]. In addition, they have a lipid bilayer structure that allows them to get through the BBB easily and confers relative stability and protection from degradation in the blood and CSF [17]. These features make them particularly suitable as candidate biomarkers of brain damages and dynamic pathophysiological processes associated with TBI, and as vehicles for delivering therapeutic molecules.

MPs/MVs are, on the other hand, irregularly shaped phospholipid vesicles with a diameter of 100–1000nm, formed by cell-membrane budding/blebbing triggered by various activation stimuli that lead to increased intracellular calcium levels [14,18]. Like exosomes, MPs can be derived from different cell types and their protein and lipid composition is indicative of their cellular origin [19]. Furthermore, as their production in brain cells is enhanced as a result of tissue damage

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and ischemic and inflammatory insults, they can provide crucial information on cell injury and/or underlying pathophysiological mechanisms.

Apoptotic bodies are another form of membrane vesicles which bud directly from the plasma membrane upon programmed cell death. They are heterogeneous in size (800–5000 nm) and morphology and may contain nuclear fragments, including histones [20,21].

A major ongoing challenge is to establish methods that allow to accurately separate the populations of membrane vesicles. Thus, despite attempts that have been made to characterize EV subtypes, in many studies investigators did not make clear-cut distinctions, and the terms 'MPs/MVs' or 'exosomes' indicate a heterogeneous mixture of different types and populations of cell-derived vesicles. In this review, we retained the definition used by the original authors.

3. Extracellular vesicles and TBI

There is now substantial evidence that EVs can originate from cells of the nervous system, [22-24], play major role in regulating and modulating neuroinflammation, and in promoting neurogenesis and neurite outgrowth, [17,25] and are possible mediators of neuronal plasticity [15]. In addition, accumulating studies demonstrated the potential systemic pathological effects of MP following brain injury.

In TBI, assessment of levels and temporal profiles of EVs in biofluids as well as the characterization of their different phenotypes, which may reflect distinct pathogenic pathways, can provide novel neurobiological insights and enhance our understanding of the molecular mechanisms underlying TBI pathophysiology (Table 1).

Elevated concentrations, altered pattern and distinct transcranial gradients of endothelial-, platelet-, and leukocyte-derived MPs in patients with severe TBI were reported by Nekludov and colleagues [26]. Highly increased concentrations of endothelial-derived MPs were interpreted as indicative of cerebral vascular damage and microvascular thrombosis and therefore capable to influence coagulation in the microvasculature of the brain.
The role of MPs to detect and track endothelial injury and vascular remodeling in the brain following TBI is also suggested by the work of Andrews and colleagues [27]. *In vitro* analysis of human adult brain microvascular endothelial cells (BMVECs) complemented with *in vivo* study indicated that mechanical injury results a time-dependent release of eMVs containing tight

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junction proteins (TJPs). Importantly, the cerebral microvascular endothelium is an integral part of the BBB, which regulates diffusion and transport of solutes into the brain, and whose impairment or altered permeability contribute to edema formation, neuroinflammation, excitotoxicity, cerebral blood flow irregularities and metabolic imbalance, ultimately, leading to delayed neuronal dysfunction and degeneration [28,29]. Thus, brain endothelial-derived MVs harbor promise as biosignatures of BBB status, compromise and dysfunction, while shedding light on potential mechanisms linking vascular pathology to neuronal dysfunction and degeneration, and providing exciting lines of investigation for novel therapeutic approaches. There is now substantial evidence that protein tau released by injured neurons is carried by exosomes [30]. Recently, this exosome-mediated secretion of phosphorylated tau has been suggested as the mechanism for tauopathy to spread in brains after TBI, possibly leading to chronic traumatic encephalopathy (CTE) and neurodegenerative disorders [31,32]. These interesting data provide exciting lines of investigation for the future.

New recent studies also suggest that MPs carrying brain-specific antigens can be used as biomarkers of TBI. Nekludov and co-workers [33] demonstrated higher circulating levels of MPs expressing glial (glial fibrillary acidic protein [GFAP] and aquaporin-4 [AQP4]) and neuronal (neuron-specific enolase [NSE]) proteins in patients with severe isolated TBI as compared to healthy controls. Interestingly, the highest concentrations of MPs expressing AQP4, protein involved in the generation of brain edema, were associated with the subsequent development of severe intracranial hypertension and overt coagulopathy.

Moreover, several lines of evidence support the idea that RNAs in exosomes are key regulators in development, apoptosis, stem cell self-renewal, differentiation and cell integrity maintenance. Recently, using different approaches, including microarrays, quantitative real-time PCR and next-generation sequencing, particular attention has been directed to the RNA signatures of exosomes derived from injured brain.
In a study by Patz and colleagues [34], 81 differentially expressed vesicle-associated RNAs were identified in CSF from brain injured patients. Several RNAs, such as microRNA (miR)-9 and miR-451, were previously reported to be linked to TBI or play a role in the regulation of neuronal processes and neurological function [35]. Furthermore, when brain injury associated MPs were added to cell lines (NTERAs), down-regulation of putative miR-451 target genes was observed, confirming the transfer of genetic information via MPs with functional consequences to target recipient cells, and the role of vesicle-associated RNAs as damage-associated regulators of gene expression in brain injured patients [34].

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Differential expression of EV-associated miRNA was found in a rodent CCI model compared with uninjured controls. Among the 5 TBI-induced miRNA, there was increased expression of miR-21 that is known to be involved in neuroprotective and regenerative pathways, reducing neurotoxicity, glial scar formation and microglia-mediated neuronal death [36].

4. Evidence for a link between microparticles and the development of coagulation and inflammation in TBI

4.1. Coagulation

Many patients with TBI have also coagulopathy, which occurs more frequently in patients with severe TBI (~60%). The coexistence of TBI and coagulopathy is associated with detrimental outcomes resulting in a nine-time higher risk of mortality and a 30-times higher risk of unfavorable outcome [37].

A number of interesting studies, using cell lines, animal models of TBI, and/or clinical samples, have started to unravel and emphasize the role of MVs in the pathogenesis of local and systemic TBI-associated coagulopathy [38-41] (Table 1).

MVs have been found to be elevated or altered in blood following isolated TBI and to likely contribute to altered coagulation after brain injury [26,33,40]. These observations have now been confirmed in different studies hypothesizing that is presumably the presence of the phosphatidylserine (PS) and tissue factor (TF) on the external membrane leaflet of MVs which promote the coagulation cascade leading to thrombin generation [40,42,43].

In a pilot study, Morel and colleagues [41] show enhanced elevated levels of procoagulant platelet- and endothelial-derived MPs in CSF as well as in blood of patients with severe TBI up to 10 days post-injury. Besides reporting on the contribution of endothelial damage and cell activation to generation of MVs after brain injury, this work provided initial evidence of an association between MVs and development of disseminated intravascular coagulopathy and poor clinical outcome after TBI.

A revealing 2015 study, in a mouse fluid percussion injury (FPI) model combined with complementary *in vitro* experiments showed a causal role for brain-derived MPs (BDMPs) - neuron- and glial cell-derived MPs - in the systemic coagulation associated with TBI by

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promoting intravascular coagulation and platelet activation. Three hours following FPI in conjunction with peak blood BDMPs concentration mice developed a hypercoagulable state, which was reversed with removal of BDMPs [38]. The procoagulant activity of BDMPs released from the injured brain was dependent on the expression of procoagulant PS and TF highly enriched on the surface of the MPs [44,45] as well as their concentration and kinetics of release [38].

Supporting these observations, administration of lactadherin, a molecule enhancing the clearance of circulating MVs through PS-mediated phagocytosis, was reported to reduce the severity of BDMP-induced platelet activation and TBI-associated coagulopathy. Lactadherin also reduced cerebral edema, improving survival and neurological outcome [39].

These findings have major implications for human studies. First, they provided insight into the pathobiology of TBI-induced coagulopathy and identified/defined potential therapeutic target to prevent or attenuate this condition. Furthermore, they indicate that BDMVs released from injured brain could be used as early predictive markers - treatment-response modifiers - and to monitor the biochemical effect (downstream effects on pathogenic mechanisms) of drugs, thus, suggesting that they might be adopted as valuable theragnostic markers in clinical trials.

As yet, the factors leading to the generation of procoagulant MPs after TBI are unclear. Whereas it is well-know that the brain is enriched in procoagulant anionic phospholipids, no correlation has been established between the extent or type of brain trauma and the release of procoagulant MPs [41]. In the future, it will be crucial to understand how specific injury and patient characteristics contribute to the MPs phenotype which, in turn, can initiate, modulate or exacerbate molecular mechanisms underlying TBI pathophysiology and recovery. Understanding such pathways might be used to make decisions about prognosis and timely treatment planning, stopping the pathological processes even before clinical symptoms occur.

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4.2 Inflammation

EVs have also been implicated in inflammation. Recent experimental and human studies have shown that EVs have immune-activating properties and inflammation-promoting activities carrying and releasing a number of proinflammatory mediators such as IL-6, IL-1β, and CC-chemokine ligand (CCL)-2 (MCP-1) [45,46], and support their role for neuroinflammation in

several neurological disease [47-49]. By contrast, anti-inflammatory effects of secreted vesicles have also been reported [13,50].

TBI initiates a complex local and systemic inflammatory response which can last from several days to weeks, or even years after the acute event. While the initial inflammatory response has been shown to be associated with protective and beneficial effects - clearing tissue debris and protecting from pathogens-, successively an enduring neuroinflammation is harmful as it may lead to the pathological progression of TBI with exacerbation of the primary injury, progressive neurodegeneration and delayed cell death [51]. These persistent inflammatory responses are considered major drivers of the development of neurodegenerative diseases or CTE. Herein, understanding how to alleviate and modulate the neuroinflammation following TBI and potential applications to treatment are a critical research priority area.

A recent seminal study by Kumar and colleagues [52] has examined the contributing role of microglial MP to promote brain inflammation in a controlled cortical impact (CCI) mouse model. The study shows that microglia-derived MPs containing elevated concentrations of pro-inflammatory molecules (e.g IL-1β and miR-155) are released into the circulation following TBI, inducing systemic response and enhancing robust neuroinflammatory responses in the injured brain through microglia activation and increased expression of pro-inflammatory molecules. In addition, the data indicate that microglial-derived MPs can seed, propagate and rapidly disseminate brain inflammation transferring the posttraumatic neuroinflammatory phenotype to non-activated cells and independently initiating inflammatory responses in injured brain areas. Using immunoblot analysis, NLRP1 inflammasome proteins have also been identified in exosomes derived from CSF of severe TBI patients [53].

As discussed, EVs could also be involved in inhibiting immune responses. A recent *in vitro – in vivo* study showed exosomal miR-124-3p to improve the neurologic outcome and reduce the inflammatory response after TBI, through inhibiting overactivation of microglia and suppressing the activity of mTOR signaling [17]. In addition, promotion of neurite outgrowth via miR-124-3p transfer by exosomes into neurons was observed. Therefore, novel neuroprotective strategies can entail the use of miRNA-manipulated microglial exosomes.

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5. Possible role of exosomes in the treatment of traumatic brain injury

The long history of failed TBI trials [1,54,55] implies a shift in thinking, and has prompted exploration of novel therapeutic strategies/approaches and targets and compounds.
Evidence is emerging that MVs/exosomes modulate inflammation, neuronal function and plasticity, regulate BBB permeability and cellular responses to brain injury [15], and mediate brain reparative processes via the provision of trophic provision [56], and synaptogenesis [32,57], supporting their potential application as therapeutic target or biomarkers and to monitor therapeutic efficacy/response. In addition, the low immunogenicity, stability and long half-life in circulation, and ability to cross the BBB [58-60] make EVs highly suitable as a tool to deliver therapeutic agents [50] (Table 1).

Over the past decade, experimental and clinical data have provided support for mesenchymal stem cells (MSCs) as a potentially promising therapy for TBI. However, the use of MSC-derived exosomes (i.e. cell-free exosome-based therapy) may offer several advantages over the more traditional cell-based therapy, including a superior safety profile (e.g. they do not induce microvascular embolism), higher stability allowing for easier storage, less invasiveness, easy delivery, low or no immunogenicity, along with no ethical issue of embryonic and fetal cells, and low or no tumorigenicity.
In a proof-of-principle study by Zhang and colleagues [61], intravenous delivery of MSC-derived exosomes improved cognitive and sensorimotor functional outcome in a rat CCI TBI model. MSC-generated exosomes have also been shown to increase vascular density, angiogenesis and neurogenesis and to reduce brain inflammation, while no effect on cortical lesion volume was observed [61].
Similar data showing that infusion of EVs isolated from human MSCs (hMSCs) suppress neuroinflammation and improve functional recovery 1 month after injury were reported by Kim and colleagues [62]. In this thorough work carried out in a mouse model for TBI extensive characterization and optimization of the producing cells was obtained, also using a marker of potent anti-neuroinflammatory effects. Moreover, the protocol for isolation and the culture conditions under which MSCs produced EVs were accurately planned and examined [62].
More recently, MSCs cultured in the three dimensional (3D) scaffolds have been shown to produce higher amount of exosomes compared to the MSCs seeded in the 2D conventional condition [63]. Exosomes generated from hMSCs cultured in 3D condition showed an enhanced therapeutic effect likely due to a different profile of proteins and genetic materials compared to

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2D exosomes, providing clear evidence that the exosome contents critically depend on the features of the MSCs as well as the surrounding environment and conditions.

Taken together, these studies demonstrate that cell-free exosome-based therapy appears a promising approach and may represent a major leap forward in our understanding of the mechanisms of pathophysiology and neuroprotection in TBI.
However, investigators still need to optimize exosome production and quality control, including definition of the producer cells and their physiological status, and refinement of methods to purify and characterize exosomes. Furthermore, dosing, timing, toxicity and mechanisms of MSC exosome treatment have to be thoroughly examined and addressed. These fundamental issues must be overcome before moving into clinical trials.

6. Extracellular vesicles - biofluids analytical challenges

The presence of EVs was detected in a number of body fluids of healthy individuals, including peripheral blood, cerebrospinal fluid, urine, saliva, making them readily accessible [64,65].

However, to date a key issue hindering progress in understanding and potential clinical adoption of EVs is the technical challenge to definitively separate exosomes from MVs. This, obviously, introduces biases when characterizing extracellular vesicle properties, for molecular analysis and interpretation of the results. Thus, a research priority in the field is to improve and standardize methods for EV isolation and analysis [66].

Among the currently available methods for isolation and purification, differential centrifugation which involves several centrifugation and ultracentrifugation steps is the most commonly used (see Table 1). Nonetheless, protocols can vary across laboratories and this lack of standardization is a source of variability leading to inconsistencies in recovery of EVs and, therefore, findings [67,68]. Other alternative methods that have been introduced include high-performance liquid chromatography (HPLC), precipitation technologies (ExoQuick™), antibody-coated magnetic beads, ultrafiltration technologies, and more recently the use microfluidic devices. The latter, in particular, can offer significant advantages, such as reducing material costs, increasing throughput and permitting multiplexing. Moreover, microfluidic technologies can used in lab-on-a-chip devices allow for small volumes of sample, shorter processing times, improved sensitivity and eventually reduced clinical care costs [67].

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Common methods of identification and quantification include Flowcytometry Electron, microscopy for the morphology identification, and Western blotting for the membrane marker identification (e.g. CD9, CD63, and CD81 have been suggested as reliable markers of exosomes [69]).
Still, there remains an urgent need for standardization of pre-analytical and analytical procedures, as well as for establishment of more efficient, reliable and reproducible extraction, identification and characterization methods of EVs.

7. Expert commentary

This review brings together information and provides a comprehensive overview on the role of extracellular vesicles in TBI. The novel insights highlight the mounting interest and enhanced understanding of EVs suggesting and supporting that this novel class/family of markers could yield transformative approaches to TBI diagnosis, characterization and treatment. The particularly exciting prospect is that besides the potential to sort out patient and injury variability, EVs can unveil pathophysiological processes initiated by TBI that can lead to secondary and long term complications, and therefore, allowing for predictive, personalized, and even preemptive therapeutic strategy and bringing personalized medicine to patients.

However, a series of critical issues still needs to be addressed to permit the deployment of EVs as brain injury biomarkers in the clinic. With respect to “analytical performance”, important technical considerations will concern the development of analytically validated methods capable of accurately and efficiently isolating, characterizing and assessing the different types and subtypes of EVs. Reliable results, reproducibility across multiple laboratories, platforms and clinical settings, and adequate sensitivity and specificity are mandatory prerequisites of any subsequent steps towards clinical application. In addition, future rigorous and independent validation studies should be designed to confirm and evaluate strength of evidence of clinical validity of the EVs, convincingly demonstrating their correlation with clinical outcomes and potential value as suitable diagnostic, prognostic or predictive signatures for TBI. In this regards, it would be essential to explore whether specific selected populations of exosomes and MVs/MPs enable the molecular discrimination of TBI according to phenotype, rejuvenating the current classification and, possibly, leading toward a new risk stratification system based on the gained biological and pathophysiological insights. It is conceivable that depending on the EVs characteristics (i.e. cellular and subcellular origin, size, cargo, and patient status) and patterns, classification algorithms can be developed which, in addition to the characterization of the acute

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injury, provide information on the subacute and chronic phases of TBI as well as on the long term clinical outcome.

As shown, a number of studies have already started cataloging the different exosome and MV profiles and identifying their links to mechanisms and pathways involved in secondary injures such as systemic coagulopathy and inflammation, thus creating opportunities for advancing our knowledge of TBI pathophysiology, monitoring the progression of the brain pathology or recovery, and programming management and medical intervention.

Nonetheless, conducting large-scale studies and addressing the effect of potential confounding factors (e.g. gender, age, race, etc.) will be instrumental to confidently interpret levels and changes in the circulating EVs and enable the successful implementation in clinical practice and diagnosis of disease. Germane to this, normative reference ranges in large cohorts representative of different healthy populations must be generated.

Finally, to achieve qualification and regulatory acceptance and, thus, be broadly used by the medical community, evidentiary standards will be needed to evaluate and define clinical utility of specific EV populations within specific context of use (COU) and to assess the added beyond current clinical practice while providing new methodological approaches for their integration and combination with other types of clinical and molecular data.

8. Five-year view

Building on these interesting and exciting data, the EV research field has now become very active and, undoubtedly, will attract a broader interest and support over the next five years. Crucial next steps of this future work will imply establishing standardized rigorous protocols, building more reliable analytical platforms, as well as conducting well-designed and properly powered studies for validation purposes and to explore potential clinical use. Sharing emerging data and samples will be also imperative for accelerating progress toward the adoption of EVs in clinical practice.

However, these complex tasks and challenges cannot be addressed by a single entity. Specific programs fostering strategic public–private collaborations and multi-party consortia, leading to dialog and cross-fertilization, and allowing exchange and pooling of expertise, knowledge and resources, will be pivotal in achieving the substantial leaps forward in EVs development and truly making an impact on widespread medical practice.

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Key issues

- EVs are an emerging class of biomarkers consisting of a heterogeneous group of membrane vesicles, including exosomes and microvesicles, which have key roles in cell signaling and intercellular communication. The analysis of their content permits to track their parental cells and determine their function as well as the cells they will interact with.

- EVs are released by brain cells and their production is enhanced following TBI. Mounting evidence suggests that they play a casual role in the pathogenesis and recovery of acute and chronic TBI, promoting TBI-associated coagulopathy and neuroinflammation and potentially propagating neurodegeneration on the one hand, while participating in immune modulation and neural repair on the other.

- The characterization of brain-derived EVs leaking into the circulation may provide crucial information and shed light on the injury and recovery patterns cell injury, leading to the identification of specific TBI phenotypes/signatures and helping sorting out patient and injury variability.

- Because of their subcellular size, ability to cross the blood-brain barrier, stability and the link with brain damage and pathophysiological mechanisms of TBI, specific selected populations of exosomes and MVs/MPs could also serve as predictive biomarkers, as well as therapeutic targets and delivery systems of neuroprotective compounds, opening a new frontier in the field of neuroprotection.

- Rigorous standardization, validation process and reproducibility, as well as more refined and accurate analytical techniques are essential to ensure the successful translation of EVs as TBI biomarkers to bedside.

- EVs yields a transformative potential in the context of TBI diagnosis and treatment, offering novel approaches to improve characterization and classification of TBI, and to develop more targeted personalized, and even preemptive therapeutic strategies towards precision medicine practice.

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Funding

This work was supported by Italian MoH GR-2013-02354960 (SM).

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. Peer reviewers on this manuscript have no relevant financial or other relationships to disclose

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Table 1. Extracellular Vesicles as biomarkers of TBI: studies and findings

Study	Type of Study	EV	Content/ Cargoes	Mechanisms	Origin	Findings	Isolation method	Identification/ Characterization method
TBI								
Nekludov et al. (2014) [26]	*In vivo*- Humans (Severe TBI patients)	MPs	Tissue factor (TF) and P-selectin	Changes in MP pattern likely reflect damage of ECs of brain vessels	Endothelial-, platelet-, and leukocyte-derived MPs	MPs were increased after TBI with and altered pattern	Serial centrifugation - 2000× g for 20 min at RT and 13,000 × g for 2min at RT	Flow cytometry
Andrew et al. (2016) [27]	*In vitro* - Human adult brain microvascular endothelial cells (BMVEC) – *In vivo* - Animals (Mice)	MPs	Tight junction proteins (TJPs)	MPs reflect damage of ECs of brain vessels and cerebral vascular remodeling following TBI	Brain endothelial-derived MPs	Brain endothelium responds to mechanical injury by producing extracellular MPs	Exoquick system	Flow cytometry, Electron microscopy, Western blotting
Nekludov et al. (2017) [33]	*In vivo*- Humans (Severe TBI patients)	MPs	Glial fibrillary acidic protein (GFAP), neuron-specific enolase (NSE) and aquaporin-4 (AQP4)	MPs may reflect brain damage or be involved in TBI pathophysiology (e.g. controlling the generation of brain edema)	Neuronal-, glial-derived MPs	Patients with severe isolated TBI have increased concentrations of circulating brain-derived MPs	Serial centrifugation - 2000 × g for 20 min at RT and 13,000 × g for 2 min at RT	Flow cytometry

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Patz et al. (2013) [34]	*In vivo* - Humans (Severe TBI patients) and - I*n vitro*	MPs	81 mature miRNA (e.g. miR-9 and miR-451)	CNS damage - Transfer of genetic material from CSF MPs to recipient cells	NR	MPs were increased in CSF after TBI and contained distinct genetic information which trigger regulation of neuronal processes and the adaptive response to injury	Serial centrifugation and filtration - 400g for 5min Ultracentrifugation 170,000 × g for 40 min at 4°C	Flow cytometry Electron microscopy, PCR, Western blotting
Harrison et al. (2016) [36]	*In vivo*- Animals (Mice)	EVs	miRNA (miR-21, miR-146, miR-7a, and miR-7b)	Mediate neuron-glia signaling	Brain – Possibly neuronal origin	Increased expression of miR-21, miR-146, miR-7a, and miR-7b in EVs and decreased expression of miR-212 in the inured mice brain	Serial centrifugation - 100,000 × g for 60 min at 4° C, 100,000 × g for 60 min at 4°C	Electron microscopy, miRNA sequencing
Coagulopathy								
Morel et al. (2008) [41]	*In vivo* – Humans (Severe TBI patients)	MPs	Procoagulant MPs - enriched in procoagulant aminophospholipids like phosphatidylserine (PS) and TF	Link with vascular damage and platelet and endothelial activation	Platelet Endothelial derived MPs	Elevated levels of procoagulant MPs in CSF and blood up to 10 days post-severe TBI - Association between MPs and development of DIC and poor clinical outcome after TBI	Serial centrifugation	ELISA
Midura et al. (2015) [40]	*In vivo*- Animals (Mice)	MPs	NR	Triggered by the injury	Platelet-derived MPs	Altered MP populations at 24h after injury, with s a decline in circulating total MP numbers and a significantly increased proportion of PMPs, likely contributing to the altered platelet role in coagulation and to the development of a post-TBI hypercoagulable state	Centrifugation 25,000 × g for 30 min	Nanoparticle Tracking Analysis

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Tian et al. (2015) [38]	*In vivo*- Animals (Mice) – *In vitro*	MPs	Procoagulant MPs - enriched in procoagulant aminophospholipids like phosphatidylserine (PS) and TF	Produced from activated or inured cells to promote coagulation	Brain-derived MPs (BDMPs) - neurons and glial	Release of procoagulant BDMPs into the circulation following TBI promotes intravascular coagulation and platelet activation. BDMPs may serve as a therapeutic target and predictive marker of TBI-associated coagulopathy	NR	Modified flow cytometry-based method
Zhou et al. (2017) [39]	*In vivo* - Animals (Mice) – *In vitro*	MVs	Phosphatidylserine (PS) on membrane vesicles and cardiolipin (CL) on mtMVs	Produced from activated or inured cells to promote coagulation	Brain-derived MVs (BDMVs) - Membrane-embedded mitochondria (mtMVs)	Lactadherin by enhancing clearance of circulating procoagulant MVs prevents TBI-induced coagulopathy, reduces cerebral edema, improves neurological function, and increases survival	NR	Flow cytometry – mtMVs were detected by the mitochondrial dye MitoTracker® Green
Inflammation								
Kumar et al. (2017) [52]	*In vivo*- Animals (Mice)	MPs	Pro-inflammatory mediators, interleukin-1β and microRNA-155	Neuroinflammation	Microglial	Microglial-derived MPs contribute to the development and dissemination of neuroinflammation after TBI by activating microglia and stimulating systemic immune responses	Ultracentrifugation 100,000 × g for 60 min at 4 °C	Flow cytometry
Huang et al. (2018) [17]	*In vitro* – *In vivo* - Animals (Mice)	Exosomes	microRNA-155	Inhibition of neuronal inflammation and promotion of neurite outgrowth	Microglial	Levels of miR-124-3p in microglial exosomes increase from acute to chronic phase of TBI / miR-124-3p transfer in microglia by exosomes inhibits neuronal inflammation and promote neurite outgrowth *in vitro*, while improves neurologic outcome and inhibits neuroinflammation *in vivo*	Serial centrifugation - 100,000 × g for 60 min at 4° C, 100,000 × g for 60 min at 4° C	Electron microscopy Nano Particle Tracking, Zeta Potential Distribution Analyzer, miRNA microarray analysis

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De Rivero Vaccari et al. (2015) [53]	*In vivo* - Humans (Severe TBI patients) *In vitro* –	Exosomes	NLRP1 inflammasome proteins	Pro-inflammatory	NR	Increased inflammasome protein expression in exosomes derived from CSF of TBI subjects - Exosomes derived from neurons deliver short-interfering RNA (siRNA) into the CNS to decrease inflammasome activation	ExoQuick	Western blot
Treatment								
Zhang et al. (2015) [61]	*In vivo*- Animals (Rats)	Exosomes	NR	Promoting functional recovery and neurovascular remodeling	Rat multipotent mesenchymal stromal cells (MSCs)	Cell-free exosomes generated from MSCs promotes cognitive and sensorimotor functional recovery by stimulating angiogenesis and neurogenesis and by reducing neuroinflammation	ExoQuick	qNano nanoporebased exosome detection system, Transmission electron microscopy, Western blot, Confocal fluorescence microscopy
Kim et al. (2016) [62]	*In vivo*- Animals (Mice)	EVs	TSG-6 (However, anti-inflammation effects are also likely due to other components)	Reducing inflammation	Human mesenchymal stem/stromal cells (hMSCs)	Administration of isolated EVs from preselected MSCs decreases levels of IL-1β in a dose-dependent manner, and rescues pattern separation and spatial learning impairments in TBI mice	Chromatography	ELISA, Nanoparticle tracking analysis
Zhang et al. (2017) [63]	*In vivo*- Animals (Rats)	Exosomes	NR	Promoting endogenous angiogenesis and neurogenesis while reducing neuroinflammation	Multipotent human bone marrow derived mesenchymal stem cells	Exosomes from hMSCs in 2D or 3D cultures improves functional recovery in rats after TBI - MSCs cultured in 3D scaffolds produce higher amount of exosomes with an enhanced therapeutic effect compared to 2D exosomes, difference likely due to a	ExoQuick	Proteomic analysis

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					(hMSCs)	different profile of proteins and genetic materials.		

Abbreviations: AQP4, aquaporin-4; BDMPs, brain-derived microparticles; BDMVPs, brain-derived microvescicles; BMVEC, microvascular endothelial cells; CL, cardiolipin; CNS, central nervous system; CSF, cerebrospinal fluid; DIC, disseminated intravascular coagulation; ECs, endothelial cells; EVs, extracellular vesicles; GFAP, glial fibrillary acidic protein; hMSCs, human mesenchymal stem cells; MPs, microparticles; mtMvs, mitochondrial microvescicles; MSCs, mesenchymal stem cells; NR, not reported; NSE, neuron-specific enolase; PMPs, platelet-derived microparticles; PCR, polymerase chain reaction; PS, phosphatidylserine; RT, room temperature; siRNA, short-interfering RNA; TBI, traumatic brain injury; TF, tissue factor; TJPs, tight junction proteins; TSG-6, TNF-alpha stimulated gene/protein 6.

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