doi: 10.21860/medflum2025_330052

Clinical Potential of Extracellular Vesicles in Central Nervous System Pathologies

Klinički potencijal ekstracelularnih vezikula u patologiji središnjeg živčanog sustava

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Abstract. The number of neurological disorders has been increasing for the last 30 years, and they are now among top 10 causes of mortality. As for neurodegenerative diseases in which Alzheimer's and Parkinson's disease predominate, there is a very small number of available and mostly symptomatic drugs. It is therefore a priority to find appropriate therapies, as well as biomarkers for early detection and monitoring of such disorders. Extracellular vesicles are formed in almost all cells, they transfer macromolecules like DNA, RNA and proteins to give an image of the tissue from which they originate. This makes them an excellent diagnostic as well as therapeutic tools. Therefore, there is a substantial goal in the need to find an adequate procedure for isolation of vesicles, in order to find a good biomarker from body fluids that does not require invasive methods.

Keywords: biomarkers; central nervous system diseases; extracellular vesicles

Sažetak. Broj neuroloških poremećaja raste zadnjih 30 godina te su oni sada unutar deset vodećih uzroka smrtnosti u svijetu. Što se tiče neurodegenerativnih bolesti u kojima prevladavaju Alzheimerova i Parkinsonova bolest, postoji vrlo mali broj dostupnih, i to većinom simptomatskih lijekova. Prioritetno je stoga pronaći odgovarajuće terapije, ali i biomarkere za rano otkrivanje i praćenje takvih poremećaja. Izvanstanične vezikule stvaraju se u gotovo svim stanicama, prenose makromolekule, DNA i RNA te daju sliku tkiva od kojeg potiču. To ih čini potencijalno dobrim dijagnostičkim, ali i terapeutskim alatom. Stoga se javlja potreba za pronalaženjem adekvatnog postupka izolacije vezikula kako bi se iz tjelesnih tekućina pronašao dobar biomarker bez korištenja invazivnih metoda.

Ključne riječi: biomarkeri; bolesti središnjeg živčanog sustava; izvanstanične vezikule

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INTRODUCTION

Not so long ago, extracellular vesicles (EVs) were considered as biological waste or even "cell dust" but today's situation is completely different. They are now used as diagnostic and genetic tools but also and equally important as a therapeutic agent. Stem cells have some unique biological traits, and possible healing uses and EVs from stem cells are getting noticed for treating neurological diseases. These EVs can effectively improve disease conditions by speeding up the recovery of damaged tissues[1]. Corynoxine-B, a naturally occurring autophagy inducer, has been used for both Alzheimer's (AD) and Parkinson's disease (PD). Fe65-engineered HT22 hippocampal neuron cell-derived exosomes loaded with Corynoxine-B, caused autophagy in neuronal cells that expressed amyloid-β precursor protein which improved cognitive loss in AD mice[2].

EVs are differently sized membranous particles secreted from all types of brain cells. They come in a variety of sizes; 1) exosomes (30-150 nm), 2) ectosomes (100-1000 nm) and 3) apoptotic bodies (50-5000 nm) and are used for intercellular communication[3]. Inside the Central Nervous System (CNS), exosomes are used for communication between neurons, microglia and other neural cells[4]. More importantly, they transfer functional cargo to maintain standard physiological function but also spread disease signals (via prions, peptides and proteins) in onset of neurodegenerative diseases[5, 6]. Misfolded proteins, impaired protein homeostasis and neuron degeneration are some of the common features of neurodegenerative diseases and when different cell types in the CNS release EVs in circulation, they become logical and appropriate biomarkers for corresponding diseases[7]. Certain proteins, such as CD81, C63, TSG101 and HSP70 are often utilized as exosome markers since they are exclusively used in the synthesis of EVs, regardless of their origin[8] (Figure 1).

Virtually all cell types secrete extracellular vesicles (EVs). Inside the CNS they play a role in tissue repair and neuronal survival but also in promotion of CNS pathologies by carrying pathogenic proteins such as amyloid peptides and alpha synuclein.

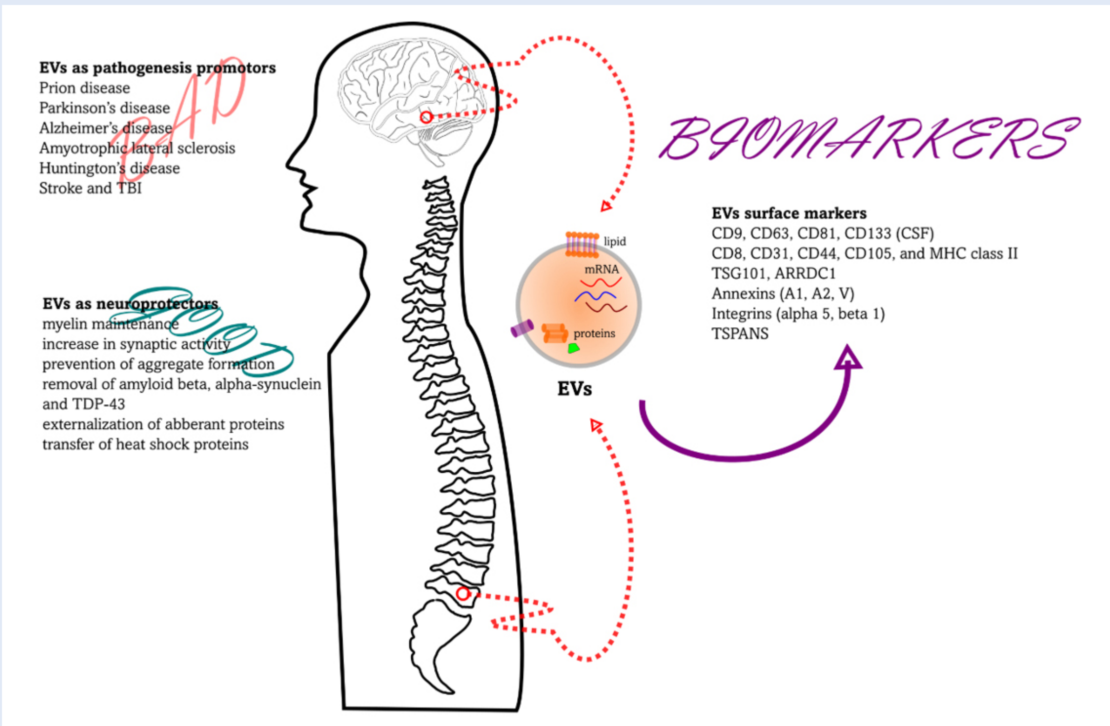

Figure 1. Role of EVs in CNS pathologies

Additionally, EVs mediate communication between the tumour cells and their surroundings in aggressive brain tumours, especially glioblastoma multiforme (GBM). Angiogenesis, tumour invasion, treatment resistance, and immunomodulation are all known to be facilitated by EVs produced from GBM[9]. Furthermore, a variety of GBM exosomal cargoes control these activities[8]. Epidermal growth factor receptor (EGFR) is often associated and amplified in GBM and EGFRvIII is present in high concentrations within GBM EVs, making it a potential biomarker. A study involving clinical samples from GBM patients after tumour resection indicated that EVs from cerebrospinal fluid (CSF) could serve as a diagnostic tool to accurately identify patients with EGFRvIII-positive GBM[10, 11].

During the onset of neurological diseases, the Blood-Brain barrier (BBB) can disappear completely (in some extreme cases) or become more permeable, probably because of inflammation. In terms of CNS, it is necessary to further investigate EVs ability to traverse the BBB in both directions to serve as therapeutics or to elucidate their neuroprotective properties. Interaction of EVs with CNS barrier is done in following manner: 1) Barrier cells (brain microvascular endothelial cells) can release EVs, 2) Brain-derived or circulating EVs can signal to the CNS barrier and 3) EVs can pass the barrier during disease[12]. For instance, EVs originated from erythrocytes are loaded with alpha-synuclein, they cross the BBB and facilitate the onset of Parkinson's pathology[13]. Viruses and immune cells employed in neuroimmunity also traverse the BBB regularly[14, 15].

LIQUID BIOPSY EMPLOYING EVS

Liquid biopsy uses the analysis of non-solid tissues such as urine, serum and cerebrospinal fluid (CSF) without any invasive procedure. Furthermore, it is new and advanced approach for determining biomarkers to make a more accurate disease prognosis and to regulate treatment response. Beside EVs, microRNA (miRNA), transfer-RNA (tRNA), cell-freeDNA (cfDNA) and circulating tumor cells all serve as major components of liquid biopsy in CNS pathologies[16]. Because EVs are found in nearly all body fluids, they are very useful diagnostic instrument. Their composition includes variety of proteins, carbohydrates, lipids, DNA, and RNA, reflecting the cells from which they originated. Hence, EVs provide a tissue-free biopsy that enables disease monitoring and diagnosis[5, 6], however most of these studies are con-

Table 1. Selected tissue-free markers in CNS pathologies.

Type of disorder	Source	Key
Traumatic brain injury		
tau, A*β*42 and IL10[19]	neuronal derived exosomes	significantly higher levels of tau, A*β*42 and IL-10 in mild TBI patients
ccfDNA[20]	blood EVs	significant increase of ccf-DNA in traumatic brain injury patients
miR-21[21]	brain EVs	increased in the injured hemisphere relative to sham surgery control mice
Alzheimer's disease		
A*β*42[22]	NDEVs	increased A*β*42 linked to improved memory and cognitive function in AD patients
miR-92a, miR-181c, miR-210[23]	plasma	significant increase in AD patients
miR-let-7e[24]	NDEVs	significant increase in AD patients
Parkinson's disease		
miR-19b-3p, miR-136-3p, miR-331-5p[25]	CSF-exosomes	upregulation
***α*-Synuclein**[26]	CSF, plasma	a small percentage of newly synthesized
***α*-Synuclein**[27]	L1CAM-positive EVs	*α*-synuclein is present in the lumen of vesicles when classifying individuals who are very susceptible to PD, L1EV α-synuclein in conjunction with prodromal indicators should be taken into account.

IL-10 = interleukin−10; Aβ42 = amyloid-*β* 42; TBI = traumatic brain injury; ccfDNA = circulating cell-free DNA; AD = Alzheimer disease; PD = Parkinson disease; ND = neuron-derived; EVs = extracellular vesicles; miR = microRNA; CSF = cerebrospinal fluid ducted *in vitro*, just as many cancer research. Moreover, there is a question of limited availability of clinical samples and sometimes this is suitable for techniques like size-exclusion chromatography and centrifugation[5]. Nevertheless, there are many obstacles before EVs can be successful used as biomarkers in clinical context. One of the biggest obstacles is large range of isolation techniques with varying EVs enrichment capabilities. Namely, there are over 100 specific isolation methods and more than 1000 documented protocols[17]. Size, shape, density on one side and EVs antigen expression on the other side govern the usage of correct enrichment and detection technique. Furthermore, separation of EVs from biological samples with particles/contaminants of similar size is also difficult and arises a question of better tools and methods such as super resolution microscopy for instance[18]. Some of the selected biomarkers of CNS pathologies are presented in Table 1[19–27].

EVS AS BIOMARKERS IN ALZHEIMER'S AND PARKINSON'S DISEASE

The CNS pathology consists of acute injuries such as trauma or stroke, and of chronic neurodegenerative processes such as Alzheimer's or Parkinson's disease. Neurodegenerative disorders suffer from frequent misdiagnosis due to symptom overlap with virtually nontherapeutic options.

AD is the most common type of dementia marked by an extensive preclinical phase and increasingly irreversible damage. Therefore, early detection in asymptomatic preclinical phase can help with lowering the speed of the disease advancement since there are no viable treatment options. Moreover, existing biomarkers are far from perfect since their classification accuracy is lower than what is considered appropriate for clinical diagnosis and their dynamic range does not cover the full course of the disease[28]. Fortunately, EVs with their specific cargo offers a noninvasive route to complement expensive PET/MRI (positron emission tomography, PET/ Magnetic resonance imaging, MRI) biomarkers and biomarkers from CSF which are obtained by an invasive procedure[29]. Therefore, there is an evident need for a development of an inexpensive, non-invasive and reliable blood biomarker; however, measurements in the liquid phase of plasma have not demonstrated sufficient sensitivity and specificity for clinical use. A new platform for neurological disorders involves EVs enriched for neuronal origin and those contain increased concentrations of signalling molecules necessary for cellular metabolism or to be used as biomarkers and to follow response to therapeutic interventions.

The AD itself involves intracellular inclusions of hyper-phosphorylated tau protein and extracellular deposition of beta-amyloid (Aβ) polymerized protein[30] with EVs from CSF loaded with neurotoxic Aβ and tau aggregates[31].

AD can be detected up to 10 years before clinical onset by checking autolysosomal proteins in neurally derived blood exosomes[32]. Other proteins such as alpha- or beta-globin are increased in neuron-originated EVs from AD patients[33]. Moreover, EVs have been suggested as a vessels for the propagation of misfolded proteins and as a contributors for cognitive part of certain neurological diseases[22]. Besides the protein content, brain derived EVs contain different miRNA and tRNA from the originating tissue[34, 35]. Recent work provides evidence for cell type-specific EV proteins in different brain cell types leading to novel markers development. These newly identified cell type-specific proteins including NCAM1, ATP1A3, LCP1, LRP1, ITGA6, LAMP2, and FTH1 showed higher enrichment in EVs over brain tissue[35].

Early PD diagnosis is also important as well as its differentiation from other neurodegenerative disorders[36]. One of the most prominent proteins in the onset of PD is α-synuclein (aSyn). The physiological role of aSyn is still unknown, however PD and other synucleinpathies are characterized by its aggregation and misfolding. Transition of aSyn between the cells is governed by exosomes[30]. Several other proteins were identified inside the neuron-derived EVs; amyloid-P as an acute phase reactant, increased clusterin in PD patients and upregulated gelsolin[37].

In terms of CSF, a total aSyn is an excellent biomarker candidate for PD which is decreased in CSF possibly due to intracellular accumulation[38] and more specific phosphorylated aSyn. Furthermore, level of aSyn with clusterin in L1CAM-immunocaptured exosomes enables the classification of synucleinopathies through a validated blood test[39].

Additionally, axonal degeneration can be assessed due to elevation in neurofilament light chain (NfL) in CSF opposed to decrease of CSF ubiquitin C-terminal hydrolase L1 (UCHL-1). Two astrocyte markers, glial fibrillary acidic protein (GFAP) and S100B are characteristic for PD[40].

Many human disorders are characterised with dysregulation in miRNA expression including PD. These miRNAs modulate the essential PD genes and play crucial role in the PD pathogenesis[41, 42]. Cao *et al.* found differentially expressed miR-19b, miR-24 and miR-195[43] while Starhof *et al.* found differentially expressed miR-7-5p, miR-331-5p and miR-145-5p[44]. However, the entirety of miRNA methodology needs further validation and standardization since the field of circulating miRNA is still very young and lacks consistency. Studies need to understand all the technical and non-technical factors affecting the miRNA profiles but the development of the informatics tools to map, analyse and read small RNA is a step in the right direction[45].

EVS AS BIOMARKERS IN TRAUMATIC BRAIN INJURY

Several million people suffer from traumatic brain injury (TBI) every year and a process has been considered biphasic. First phase is a mechanical insult and mostly irreversible. In second phase neurological damage continues and neuroinflammation steps in disrupting the blood brain barrier (BBB) with EVs playing the important role in exacerbation of the process[46].

For successful detection and classification of TBI type and evaluation of clinical response, there is a need of a well-founded blend of ongoing imaging techniques, clinical work and possible biomarkers. Currently available biomarkers are very accurate for assessing TBI but are limited since they require very sensitive analyses. Secondly, a reliable fluid biomarker should be released to accessible body fluids, should correlate with the TBI severity and their level should be higher in TBI patients versus healthy controls[47]. Since EVs are loaded with different signalling molecules involved in promotion of trauma, it is of most importance to characterize their content to use them as potential biomarkers. The usage of neurological and neuroimaging techniques such as computed tomography scans and magnetic resonance imaging, does not clearly assess some physiological parameters of TBI[48].

At a present time, there are several biomarker types for TBI corresponding to pathophysiology of TBI: biomarkers for neuronal, axonal, dendritic, astroglia and synaptic injury. Some other candidates include miRNAs, circulating DNA and proteins such as dendritic microtubule-associated protein-2 (MAP2) for instance[47].

> Extracellular vesicles (EVs) carry and protect different molecules such as proteins, lipids, DNAs and RNAs. They can be isolated from blood, urine or CSF and offer a great diagnostic potential. Furthermore, their biocompatibility makes them also a potential therapeutic vessels.

MicroRNA molecules are emerging as diagnostic biomarkers but also as possible therapeutics for TBI injuries[49]. Multiple rodent models of TBI showed different miRNAs elevated in biofluids such as plasma, serum and CSF. In a study of different miRNA from CSF after severe TBI the most abundant miRNAs were miR-451a, miR-16-5p, miR-144-3p, miR-20a-5p, let-7b-5p, miR15a-5p, and miR-21-5p. They were detected throughout 12 days after TBI, and some of the miRNAs were enriched in EVs but EVs were present in all analysed CSF pools[50]. MiR-711 alleviated neurological dysfunction in a mouse model of TBI[51]. Likewise, miR-144 alleviated brain edema and improved cognition in rat TBI model[52].

Furthermore, EVs play a pivotal role in cell-to-cell communication as regulators of immune response after TBI[48] including the activation of peripheral immune cells and moving the glia to the site of injury[53]. Specific component of microglial EVs, namely miR-124-3p, is upregulated after TBI and exerts anti-inflammatory properties[54].

In case of circulating DNA, they are much more labile than miRNAs and total plasma DNA does not reflect brain only, it is doubtful that DNA test will serve as TBI diagnostic test. Nevertheless, significant increase of ccf-DNA in TBI and adenocarcinoma was found by using qPCR and quantitative PicoGreen™ fluorescence assay leading to rapid diagnostic effect[20]. According to Regner *et al.* there is a correlation between ccf-DNA and TBI severity and mortality[55].

One of the biomarkers essential for a long-term prediction of TBI severity is a protein Ubiquitin C-terminal hydrolase-L1 (UCH-L1) and was one of the first found in CSF[56]. Together with Glial fibrillary acidic protein (GFAP), they represent a biomarker panel for two predominant brain cell types and GFAP was elevated in several rat models of TBI[57]. In humans, plasma EVs from patients with consciousness problems had 10x more GFAP than control for instance[58].

Though the precise mechanism is still being determined, there is also discussion about TBI being a risk factor for age-related disorders like AD or PD. In the continuation of TBI incapacitating acute effect there is also an acceleration of (A*β*) synthesis/accumulation with hyperphosphorylation of tau leading to damage of the BBB[59].

CONCLUSIONS

Different cell types of the body produce extracellular vesicles with their cargo reflecting their cellular origin. That cargo consists of peptides, proteins, DNA and RNA molecules used in normal physiological functions but also in the onset of various pathological processes and diseases. There is an enormous diagnostic potential of EVs given their biocompatibility, furthermore, EVs can be isolated from blood, urine, CSF as well as other biological fluids with goal to provide a non or minimally invasive type of biopsy with identification of robust biomarkers. Isolation of EVs from blood or CSF is ongoing challenge with focus on higher yielding extraction methods contrary to costly alternative of MRI and other large instruments. Additionally, current and future nanotechnology research will focus on EVs therapeutic possibilities widening their role in diagnosing, monitoring and treating different CNS pathologies.

Conflicts of Interest: Authors declare no conflicts of interest.

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