

Review

The Link Among Neurological Diseases: Extracellular Vesicles as a Possible Brain Injury Footprint

Fausta Ciccocioppo^{a,b} Paola Lanuti^{a,b} Diego Centonze^{c,d} Sebastiano Miscia^{a,b}
Marco Marchisio^{a,b}

^aDepartment of Medicine and Aging Sciences, University "G. D'Annunzio" Chieti-Pescara, Chieti, Italy,

^bCenter on Aging Science and Translational Medicine (Ce.S.I.-Me.T.), University "G. D'Annunzio" Chieti-Pescara, Chieti, Italy, ^cLaboratory of Synaptic Immunopathology, Department of Systems Medicine, Tor Vergata University, Rome, Italy, ^dUnit of Neurology, IRCCS Neuromed, Pozzilli, Italy

Key Words

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Abstract

Extracellular vesicles (EVs), referred as membranous vesicles released into body fluids from all cell types, represent a novel model to explain some aspects of the inter-cellular cross talk. It has been demonstrated that the EVs modify the phenotype of target cells, acting through a large spectrum of mechanisms. In the central nervous system, the EVs are responsible of the wide range of physiological processes required for normal brain function and neuronal support, such as immune signaling, cellular proliferation, differentiation, and senescence. Growing evidences link the EV functions to the pathogenic machinery of the neurological diseases, contributing to the disease progression and spreading. Extracellular vesicles are involved in the brain injury by multimodal ways; they propagate inflammation across the blood brain barrier (BBB), mediate neuroprotection and modulate regenerative processes. For these reasons, extracellular vesicles represent a promising biomarker in neurological disorders as well as an interesting starting point for the development of novel therapeutic strategies. Herein, we review the role of the EVs in the pathogenesis of neurological disease, discussing their potential clinical applications.

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Introduction

The term “extracellular vesicles” (EVs) refers to membrane-surrounded vesicles, that, together with metabolite solutions, ions, proteins and polysaccharides makes up the extracellular milieu. Growing evidences have proposed the EVs as novel mediators of the inter-cellular cross talk. Extracellular vesicles determine the modification of the phenotype of target cells, acting through a large spectrum of mechanisms [1]. They represent specific ‘packages’ containing different bioactive materials, such as cytosolic and membrane proteins, mRNAs, non-coding RNAs, and even DNA fragments [2]. Extracellular vesicles are released virtually from all cell types and represent multimodal signaling vehicles able to travel wide range of distances in many body fluids. As a matter of fact, the EVs have been found in the peripheral blood, in the milk, in the saliva, in the cerebrospinal fluid (CSF), in the tears and in the urine, where they carry specific biological messages [3–5]. Extracellular vesicles, can be categorized as exosomes, activation- or apoptosis-induced microvesicles (MVs)/ microparticles and apoptotic bodies, based on their biogenesis and their size [6]. However, they also include other vesicular structures originating from plasma membranes, such as exosome-like vesicles that lack lipid raft micro-domains and membrane particles [7, 8].

Exosomes are small vesicles (approximately 50 – 100 nm in diameter) surrounded by a phospholipid bilayer, released by exocytosis of multivesicular bodies (MVBs) [9]. They expose phosphatidylserine on their surfaces, and CD63, CD81, CD9, LAMP1 and TSG101 are considered common exosome markers [6]. Exosomes exert their biological functions by different ways, including direct surface contact between the EVs and the target cells, the endocytosis, the EV-cell membrane fusion and the horizontal transfer of the mRNA/miRNA, the oncogenic receptors and the HIV particles [10–13]. Exosomes have been largely described both as mediators of the immune cell functions (involving dendritic, T and B cells, as well as macrophages), as well as regulators of the tumor mechanisms, where their key role is linked to presentation of the antigen and to immunomodulatory activity [10, 14].

Microvesicles have been predominantly described as platelets, endothelial and red blood cells products. Their diameters measure 100 – 1, 000 nm [10, 15], and are surrounded by a phospholipid bilayer that may or not expose phosphatidylserine on the membrane surface [16]. The regulated release of the MVs, by budding/ablation of the plasma membrane, is induced upon the activation of cell surface receptors. Microvesicles have pro-coagulant functions and represent a form of secretion for the IL1b. The role of the MVs has been also described in the pathogenesis of rheumatoid arthritis, in the mechanisms associated to tumor pro-invasive characteristics, and in the induction of oncogenic cellular transformation and fetomaternal communication [6].

Apoptotic Bodies are approximately 1 – 5 µm in diameter; they are released as blebs from cells undergoing apoptosis and are characterized by phosphatidylserine externalization [17, 18]. Apoptotic bodies horizontally transfer oncogenes and/or DNA, are involved in the presentation of the T cell epitopes upon their uptake by phagocytic cells and in the representation of the B cell autoantigens [6].

Regardless of differences mentioned above, the terms of the “EVs”, “microvesicles” and “exosomes” have been interchangeably used in the literature, therefore, confounding the evaluation of obtained results. However, given that EVs are characterized by small size, the EV detection require several pre-analytical enrichment steps (i.e. the centrifugation/ultracentrifugation, the ultrafiltration, the size exclusion chromatography, the immunocapture, the hydrostatic dialysis or the hydrostatic filtration dialysis (HFD). For these reasons, their final characterization uses highly manipulated material [19, 20]. In this context, the final measurement may not reflect the initial characteristics of the sample [21]. For this reasons, several working-groups composed by experts in the field, are studying standardization methods for the EV clear identification and analysis [22, 23].

Physiological role of EVs in the Central Nervous System

In the Central Nervous System (CNS), the EVs have been involved in the rich network of intercellular connections responsible for the maintenance of the physiological homeostasis as well as for the development of the pathogenic machinery leading to neurological diseases (neurodegenerative disorders, as well as brain tumors and stroke).

It has been demonstrated that the EVs released by neurons and glial cells are able to pass across the brain blood barrier (BBB), through a mechanism known as trans-cytosis [24, 37, 40]. This allows the systemic propagation of physio-pathological information; the EVs have been proposed, therefore, as peripheral biomarker candidates for neurological diseases [25-27] (Fig. 1). Extracellular vesicle biogenesis give rise to their specific cargo packaging, which is strictly related both to the characteristics of their relative parental cells and to the stimulus which has determined their release [27]. It has been shown, that microglial-derived EVs expose CD13 and monocarboxylate transporter 1 [28], the neural-derived EVs move the cell adhesion molecule L1, the GPI-anchored prion protein and the subunits of glutamate receptors [29]; while the astrocyte-derived exosomes carry functional glutamate transporters and mitochondrial DNA [30, 31]. In addition, the oligodendrocytic-derived exosomes transport myelin and associated lipids [32]. As already underlined, the content of the EV depends on the stimulus received. It is known that several mechanisms, such as the synaptic activity, the depolarization, the function of sphingolipid-metabolizing enzymes and the PARK9 influence the release of exosomes from neurons [29, 33-35]. On the other hand, the serotonin-Wnt3a and the neurotransmitter glutamate regulate the EV production from microglia and oligodendrocytes, respectively [36-38].

Extracellular vesicles are also responsible of several physiological processes required for normal brain functions and neuronal support, including immune signaling, cellular

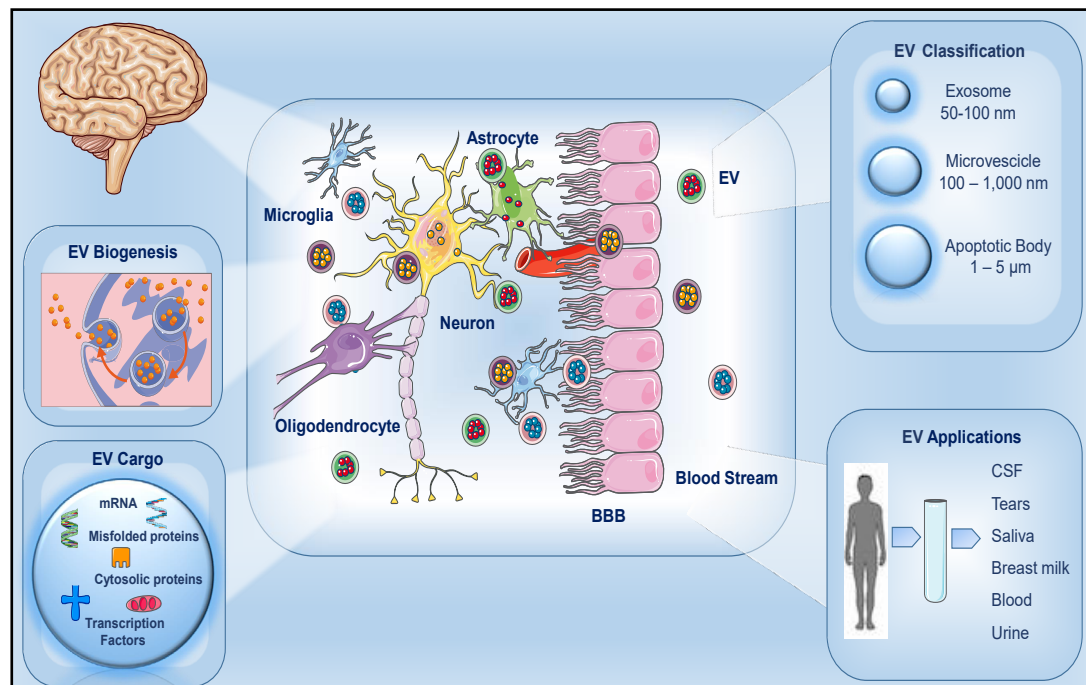


Fig. 1. Extracellular Vesicle' Origin, Cargo and Clinical Applications. Extracellular Vesicles secreted by neurons and glial cells are able to cross the brain blood barrier (BBB) through a mechanism known as trans-cytosis and their impact on target cells depends by the cargo that they shuttle. Thus, the EVs could represent a diagnostic and functional biomarker as well as suitable therapeutic agents in neurological diseases. Source: Servier Medical Art by Servier and modified under the following terms: Creative Commons Attribution 3.0 Unported license (CC BY 3.0).

proliferation, differentiation, and senescence [39-41]. The EVs transfer synaptic proteins, mRNAs and miRNAs, therefore allowing the cell-to-cell communication, modulating functions and phenotypes of target cells [42, 43]. Extracellular vesicles are also involved in the clearance of the unwanted materials and cellular waste [22]. Moreover, they show a key role in the synaptic activity [29, 38, 44], as well as in promoting neuroprotection and regeneration in brain diseases [45-48].

The neuron-glia cross-talk EV-mediated appears linked to synaptic functions, to neurovascular integrity and to myelination in the CNS. It has been demonstrated that the EVs carry several proteins linked to synaptic plasticity mechanisms, such as the α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor components and the trafficking protein Evi/Wntless, involved in the formation of synaptic buttons [29, 35, 49-52]. Extracellular vesicles are also involved in the brain vascular integrity maintenance through the transfer of the miR-132 into endothelial cells, followed by the upregulation of the adherent junction protein Cdh5 expression [53]. In addition, acting through a Rho-associated coiled-coil protein kinase (ROCK) activation and regulation of actomyosin contractility, the EVs are involved in myelination and re-myelination processes [54]. Extracellular vesicles convey miR-219 into oligodendrocyte precursor cells (OPC) increasing the OPC numbers and their myelin production, thereby repressing the expression of negative regulators of myelination [55-57]. The glial-originated EVs appear to offer neuron support, providing a regulatory feedback on presynaptic activity, both in the excitatory and the inhibitory neurotransmission [50]. The neuronal internalization of the oligodendrocyte-derived EVs [58], leads to functional cargo recovery and to genetic modulations of the specific plasticity-related targets, such as the VGF nerve growth factor inducible (VGF) and the brain-derived neurotrophic factor (BDNF) [38, 59]. On the other hand, the microglial-secreted EVs lead to increased presynaptic release of neurotransmitters, through a stimulation of the neuronal sphingolipid metabolism the amplifies the excitatory neurotransmission [44, 60]. The glial EVs have been shown to also carry several enzymes, supporting the neuronal energy metabolism [28, 32, 61].

Results

Emerging concepts propose the EVs as key mediators in the information network linked to the pathogenic machinery of the neurological diseases. Extracellular vesicles are involved in the brain injury through multimodal ways; they propagate inflammation across the BBB, but also mediate neuroprotection and modulate regenerative processes. The EV-mediated signaling appears to support neuronal survival during ischemic stress [62], it is also linked to brain cancer progression [63] and contributes to protein aggregation processes and clearance in neurodegenerative diseases [50]. In Table 1 we have resumed the EV roles in neurological disorders.

Neurodegenerative Diseases

Neurodegenerative disorders such as Parkinson's disease (PD), Alzheimer's disease (AD) and Amyotrophic Lateral Sclerosis (ALS) represent relevant issues for public health. In the aforementioned pathologies the lack of preclinical biomarkers for the identification of the early stages of the toxic protein aggregation processes makes impossible the administration of specific treatments to control the iceberg pathogenic machinery [64-66]. According to these reasons, the research focuses its interest on the EVs as potential source of information in early pathological disease stages Extracellular vesicles could represent diagnostic and functional biomarkers as well as suitable therapeutic agents in neurodegenerative diseases, allowing the monitoring of the pathogenic status in real time [67]. Recent literature describes the EV shuttle role in the spreading of misfolded proteins through a prion like mechanism in the cerebral "proteinopathies", such as the β -amyloid (Ab) and the tau protein in AD, the α -synuclein protein in PD and the TDP-43 in ALS [68-70]. The gene alteration, the

Table 1. The Extracellular Vesicles' Roles in Neurological Disorders

Neurological Diseases	EV Involvement	References
	Mediation of protein aggregation processes and neurotoxic aggregates clearance;	[50,79,80]
Proteinopathies	Contribution to mitochondrial and lysosomal dysfunction;	
	Induction of specific apoptotic pathways.	[72,74,87]
	Positive modulation of excitatory transmission;	[44,63,111]
Multiple Sclerosis	Increasing of the BBB permeability;	[113–115]
	Promotion of trans-endothelial recruitment of inflammatory cells;	[116]
	Reduction of T-cell activation, during pregnancy.	[27,119]
Stroke	Support of neuronal survival;	[62]
	Modulation of cognitive dysfunctions.	[27]
	Releasing of soluble factors and mediation of the signaling machineries related to dysregulated cell growth and hypoxic environment development;	[127]
	Mediation of the immune system inhibition and development of the responsive environment for metastasis;	[133]
Brain Tumors	Induction of the tumor promoting effects in nearby cells with a prion-like model through to shuttle the onco-proteins, ephrins and chemokine receptors, but also DNA, mRNAs, miRNAs and other small noncoding RNAs;	[12,70,128–132]
	Alteration in gene expression and angiogenesis, through the modulation of endothelial cells;	[70,127,129,136]
	Spreading of virus pathologies linked to carcinogenesis.	[70,134]

protein translation, the lysosomal dysfunction and the RNA transfer promote the misfolded protein shuttle from a “diseased cell” to a “healthy cell target” producing aggregation and accumulation of the misfolded protein in the target recipient cells [71, 72]. In the Parkinson’s disease, the cellular overexpression and the aggregation of α -synuclein in Lewy bodies and Lewy neurites result linked to increased transport of α -synuclein via EV [73]. In this contest, the lysosomal dysfunction is involved in the cell-to-cell transmission of α -synuclein oligomers packaged in the EVs, representing a second attempt to prevent toxic protein accumulation [74]. Recent studies on the SH-SY5Y cells have described that the α -synuclein is conveyed via exosomes [75], providing the catalytic conditions for nucleation and toxic misfolded protein accumulation [76]. It has been shown that the CSF level of α -synuclein protein packaged in EVs is straightly related to the cognitive impairment in the PD patients [77]. Furthermore, additional data reinforced the hypothesis that the EVs could referee the neurodegenerative machinery by increasing the induction of specific apoptotic pathways [63, 78]. Similarly, the Ab protein is a proteolytic product of the amyloid precursor protein (APP), which is sequentially cleaved by secretase (BACE1) and the gamma-secretase complex; its aggregation and the related toxic accumulation has been implicated in the Alzheimer’s disease neuropathology [67]. According to the current view, it has been suggested that the EVs represent a multimodal way for the spreading of the Ab and Tau neuropathology among neurons [79–81]. The evidence that the Ab peptides (i.e. APP, APPC terminal fragments, APP intra-cellular domain, Ab) are exosomes-associated, together with the evidence that some typical exosome proteins (e.g., flotillins, Alix) have been found in the amyloid plaques, could explain the plaque formation in the AD brain [67, 82–84]. However, the role of the EVs in AD is controversial. In such a context, several data have described that the EVs mediate Ab neurotoxicity by neutralizing the expression of the surface proteins in the EVs [85]. It has been also described that the EVs play a key role as scavengers of neurotoxic Ab. In the mouse model of the Alzheimer’s disease, after the intracerebral inoculation of the neuronal-derived

EV [86] that contain in glycosphingolipids bound the neurotoxic Ab, the microglial-derived EVs are involved in the Ab clearance and, lastly, the Ab pathology resulted decreased [34, 68]. Furthermore, data show that the EVs could contribute to mitochondrial dysfunction, spreading neuronal injury in the Alzheimer's disease brains [87].

Extracellular vesicles involvement has been demonstrated also in the Amyotrophic Lateral Sclerosis, a neurodegenerative disease associated to SOD1 gene mutations and characterized by motor neurons degeneration. It has been shown that, in the ALS cellular models, the spread of the misfolded SOD1 protein could be associated to prion-like transmission mechanisms modulated by EVs [88, 89]. An additional misfolded protein in the ALS is the TAR DNA-binding protein of 43 kDa (TDP-43), which represents the major neuropathological hallmark in the Amyotrophic Lateral Sclerosis brain inclusions [90]. Aggregates of the TDP43 are packaged in the EVs and they have detected in the body fluids [91]. Extracellular vesicles also enclose the RNA transcripts, such as piRNA, miRNA and tRNA, conveyed to the microenvironment and/or to long distances [92-95]. Thus, the EVs mediate both inter-cellular communication between cells and trans-cellular communication between brain and distant organs [10, 96]. Therefore, the small RNA transcripts, released by EVs into biological fluids, exert specific biological effects on target cells, modulating gene expressions [97-99]. Deregulation of the miRNAs has been described in the neurodegenerative disorders; it has been demonstrated that the miR-132 and the miR-212 are down-regulated in Alzheimer's disease and in Fronto-Temporal Dementia brain tissues [100-104]. Thus, specific dysregulated microRNAs conveyed by the EVs in the CSF could be able to distinguish different neurodegenerative disorders [105, 106]. The whole of these surprising evidences remark, *in vivo*, the multimodal way through which the EVs modulate the spread of neuropathological features in different neurodegenerative disorders.

Multiple Sclerosis

Multiple sclerosis (MS) is the most common immune-mediated inflammatory demyelinating disease, in the central nervous system, associated to autoreactive lymphocyte action leading to inflammation, demyelination and axonal degeneration [107, 108].

In reason of the ascertained role of the extracellular vesicles in immunomodulation, their involvement in MS results highly intriguing, representing one of the first neurological disorders in which the EVs have been detected. In particular, the involvement of the oligodendrocyte-derived EVs and the endothelium-derived EVs in the activation of the CD4+ and the CD8+ lymphocytes in the CSF of the MS patients has been described [109, 110]. The additional data underlined increased numbers of the myeloid-derived EVs in the CSF from the MS patients and proposed their positive modulator role on the excitatory transmission [44, 63, 111].

In the plasma samples of the MS patients, higher levels of the endothelium-derived EVs have been found and significant increase of the CD31-expressing EVs was evidenced during the acute phase in the MS patients; while higher levels of the CD51-expressing EVs were found both in remission and exacerbation phases, possibly reflecting the related acute vs chronic endothelium dysfunction status [112].

The endothelial-derived-EVs and the platelet-derived EVs result also increased in the Multiple sclerosis along with the elevation of CD62p expression, which is described as a platelets activation marker. In this contest, it has been described that the extracellular vesicles participate to the disruption of the BBB, increasing the permeability of endothelial layers *in vitro* [113-115] and promote the monocyte activation in the plasma, mediating the trans-endothelial recruitment of inflammatory cells [116].

Recent data have described the phenotypes of the EVs stemming from different cellular lineages (i.e. from leukocytes, monocytes and platelets), both in Multiple Sclerosis patients and healthy subjects. The level of the all EV subsets resulted higher in relapsing-remitting patients than in the secondary progressive patients and controls, suggesting that the spreading of the extracellular vesicles could reflect the inflammatory vs the chronic degeneration status, respectively [117]. It has been described a linear correlation between the higher CSF level of

the EVs in the MS patients and the gadolinium enhancing MRI lesions, index of acute phase in the natural history of disease [111]. In addition, recent data have described the RNA profile of the serum EVs in the MS subjects, characterizing four different peripheral EVs subsets, respect to their miRNA contents (i.e. hsa-miR-122-5p, hsa-miR-196b-5p, hsa-miR-301a-3p, hsa-miR-532-5p). Those miRNAs identified the MS patients respect to control subjects and the upregulation of the EVs conveying in the serum the miRNAs profile mentioned above resulted linked to the relapse phase of the disease as well as to a gadolinium enhancement on brain magnetic resonance imaging [67, 118]. According to the immunomodulation role of the EVs in the MS, several studies have been detected, in the serum of the pregnant MS woman, the EVs able to decrease T-cell activation, probably leading the well-known immune privileged status in the MS during pregnancy, and suggesting that EVs could modulate the diseases status [27, 119–121]. All in all, these findings recall in the mind the possible role of the EVs as biomarker of the immune status in the Multiple Sclerosis patients.

Stroke

Stroke is a focal cerebral insult leading to death or severe neurological disability. Discovery of the biomarkers for cerebral vascular risk identification and stratification of the stroke patient represents a strong focus of interest. In the stroke pathology, the characterization of the EV profiles *in vivo*, could represent a powerful diagnostic and prognostic tool as well as an index of therapeutic response. Limited data are available on the use of the EVs as biomarkers or as neuroprotective treatment in stroke [27, 122]. A recent study has described the faster cognitive decline of stroke patients respect to healthy subjects, beyond than the subacute phase, and also to 6 years after the stroke incident was happened [123]. In this case, EVs could act as mediators and/or shuttles of functional biomarkers, providing novel potential diagnostic approaches for the improvement of the cognitive dysfunction management after stroke event [27]. Literature have proposed the mRNA profiles as a potential diagnostic biomarkers of the stroke. Nevertheless, the mRNA profiles showed a good sensibility but reduced specificity to discriminate other disorders, such as cardiovascular risk factors, hypoglycemia, myocardial infarction or hemorrhagic stroke from ischemic stroke [27, 122].

Of note, some differentially regulated miRNAs have been associated to stroke severity and outcome in the plasma of patients and in the animal models of stroke [124]. The latter showed the involvement of the miR-133b, conveyed by the stromal-derived EVs, in neural structure modification [105, 125]. It has also been demonstrated that along with miRNAs, also the monitoring of different proteins, such as the MMP-9, the S100 β , the ICAM1 and the GFAP represent potentially useful diagnostic biomarkers in stroke [122, 126]. The investigations of the miRNA, the mRNA or the protein cargoes in the EVs profile could open novel diagnostic, prognostic and therapeutic perspectives in stroke [27].

Brain Tumors

Common processes linked to disease initiation and spread (i.e. genetic and epigenetic features, hypoxic environment exposure, mutagens and senescence factors) have been described for neurodegenerative diseases and brain cancers. Growing studies describe a network of the EVs-mediated cellular interactions, which are strictly linked to cancer advancement [63]. As matter of fact, the tumor-derived EVs release soluble factors and mediate signaling machineries related to dysregulated cell growth and hypoxic environment development [127]. Furthermore, proteins as onco-proteins, ephrins and chemokine receptors, but also DNA, mRNAs, miRNAs and other small noncoding RNAs are packaged into the cancer-derived EVs [12, 128–132]. In line with their immunomodulatory role, the extracellular vesicles stemming from primary tumor cells result involved in the immune system inhibition as well as in development of the responsive environment for metastasis in the cancer machinery [133]. In addition, just as in neurodegenerative diseases, also in cancer progression, has been described a prion-like model, in which cancer cells-derived EVs induce tumor promoting effects in nearby cells [70]. The viruses-derived EVs, known to be linked to certain cancers, such as human papillomavirus (HPV), human immuno- deficiency

virus (HIV), and human T cell lymphotropic (T cell leukemia/lymphoma) virus (HTLV)-1, could spread the pathology through an EVs-dependent mechanism [70, 134]. In this context, glioblastoma-derived EVs promoted the proliferation of cultured cells from which they were originated [130, 135] and when they are put into co-cultured with endothelial cells induce the alteration in gene expression and angiogenesis, through the modulation of endothelial cells [70, 127, 129, 136]. All these evidences, underline the involvement of the EVs in cancer physiopathology and their potential use in the prognostic and therapeutic monitoring.

Conclusion

All in all, these data underline that circulating EVs could be proposed as reliable biomarkers, representing an intriguing starting point for the development of novel therapeutic strategies, based on EV modulation. However, in this scenario, the limit of the translation of the EV analysis into the clinical practice come from different highly discussed questions, yet not solved, in this field. First of all, the heterogeneous EV nomenclature available in current literature determines a real problem when data have to be compared and reproduced [137]. Also, it must be taken into account that the EV detection presents enormous technological issues and also their biological roles are nowadays not fully characterized [1, 19]. In particular, the ideal method should detect EV larger than 50 nm and larger directly from fresh body fluids. It has to rely on a technique able to determine the concentration, as well as the phenotype of EVs being able to identify also the smallest EV compartment [19]. Therefore, further efforts need to be planned to improve those lacking points, in order to measure the real power of the extracellular vesicles as a novel tool in neurological diseases.

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Disclosure Statement

The authors have no ethical and/or conflicts of interest to declare. The datasets generated during and/or analyzed during the current study are available in the reference list. All authors read and approved the final manuscript.

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