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REVIEW

Positive and negative selection of extracellular vesicle-based biomarkers: insights from L1CAM immunocapture and emerging immunodepletion strategies

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Abstract

Extracellular vesicles (EVs) reflect their spatiotemporal cellular origins and, when released into the peripheral bloodstream, represent sources of biomarkers for processes occurring in inaccessible tissues, such as the brain. Positive selection by immunoaffinity isolation targeting a specific EV surface marker can be used to preferentially enrich for EVs from a particular cell type over other EVs in circulation. However, the case of L1CAM immunocapture for enrichment of brain-derived neuronal EVs has exemplified the biological and technical influences affecting the outcomes of positive selection, which are discussed here. These include findings from the wider (non-EV) literature showing that soluble L1CAM is a binding partner of the common EV marker CD9. Additional studies show that CD9 is not expressed by the vast majority of neurons in the brain, but it is highly expressed by neurons of the peripheral nervous system, which could affect the interpretation of studies demonstrating the co-localisation of L1CAM and CD9 on EVs to assert isolation of EVs derived from neurons in the brain. Next, emerging negative selection strategies are discussed which, when combined with positive selection, could overcome some of the challenges associated with enriching for EV-based biomarkers. These strategies include depleting EVs on a cell-by-cell type basis, as well as targeting common EV markers to simultaneously deplete diverse, undesired EV populations whilst selecting for the EVs of interest. An example is given of how CD9 depletion could enrich and select for brain-derived neuronal EVs. Additionally, studies that have used negative selection alone to select for EVs, and the advantages of this approach for biomarker detection, are highlighted. Finally, based on these insights, considerations for

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the choice of future positive and negative selection targets are discussed, including how understanding the nuances of EV heterogeneity could facilitate the process of EV-based biomarker discovery and their translation to the clinic.

Plain Language summary

Extracellular vesicles are miniscule parcels released by cells with functions that include communication. These parcels contain molecular messages in the form of proteins and RNA which can be read by other cells that receive the parcels. Since the messages reflect the status of the sending cell, they can also warn other cells about a disease affecting the sending cell.

The parcels are transported between cells by the movement of fluids in the body, including the bloodstream. Because of this, researchers in the lab can take a blood sample then use techniques to separate the parcels from the blood and read the molecular messages contained within. This is a useful approach for understanding diseases in inaccessible tissues such as the brain because the disease processes can be indirectly studied via the parcels that were released into the blood by brain cells.

A barrier to this approach is that most of the parcels found in the blood are derived from blood cells and these mask disease-specific signals coming from the brain. To overcome this, researchers can use an approach called positive selection to target a brain cell marker on the parcels' surface and enrich brain-specific signals. However, in a large, complex structure such as the human body, many cells release parcels with overlapping markers which can again obscure brain-specific messages. This article looks at how issues such as these have been raised and addressed when targeting a protein called L1CAM to enrich parcels coming from neuronal cells in the brain. Negative selection is also discussed as an alternative approach, where the unwanted parcels are targeted for removal to enrich the desired parcels in the sample. Together, these approaches could clarify messages coming from the brain so that we can identify early warning signals (biomarkers) in disease diagnostics and design more effective treatments.

Keywords

Extracellular vesicle, biomarker, neuron, positive selection, negative selection, immunodepletion, L1CAM, CD9.



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Introduction

Neuropathological conditions cause major disease burden and mortality, but there are few effective therapies available. Moreover, differential diagnosis remains challenging due to overlapping clinical symptoms and a lack of molecular markers which can be used to distinguish different neurological disorders in the clinic (Dutta et al., 2023). The massive financial and health costs for individuals and society caused by these conditions necessitates a greater understanding of the underlying neuropathophysiological mechanisms to develop drugs to prevent their onset and identify biomarkers for risk stratification, diagnosis, and monitoring disease progression and response to therapy. However, the discovery of such biomarkers is hindered by the inaccessibility of the brain, and central nervous system (CNS) as a whole, and direct access to brain tissue is too invasive to be used in routine clinical tests. Therefore, much of our understanding of neuropathological disorders is based on animal studies (which may not translate to humans) or brain imaging techniques, which yield little information about molecular and cellular neuropathophysiological events (Trivedi and Bhat, 2023).

Liquid biopsies of peripheral biofluids (e.g. blood plasma) offer a less invasive way to monitor neuropathophysiological molecular processes in humans in real-time through analysis of brain-derived molecules in the bloodstream, such as proteins, RNA, and DNA (Trivedi and Bhat, 2023). However, currently there is a lack of biomarkers for neurological disorders that can be accessed in peripheral biofluids. Biomarker identification by analysing soluble molecules in the peripheral circulation has been hampered by the low concentration of molecules derived from the central nervous system (CNS) in the periphery, difficulties in tracing individual molecules back to their cell type of origin, and degradation of potential biomarkers by circulating enzymes (Ciferri et al., 2021; Zhou et al., 2021).

There are some cases where biomarker assays for single soluble molecules have been developed into clinical tests, such as for neurofilament light chain (NFL), which is a neuron-enriched cytoskeletal protein. The concentration of NFL in peripheral biofluids has been developed into a biomarker assay because NFL is released following axonal damage or neuronal degeneration and levels of NFL are therefore elevated in traumatic brain injury, multiple sclerosis, and other neurodegenerative diseases (Ashrafzadeh-Kian et al., 2024). However, whilst elevated NFL levels do serve as a general biomarker of neuronal damage, they are unable to distinguish neurological conditions such as Alzheimer's disease and frontotemporal dementia nor indicate the molecular events that led to the release of NFL (Truffi et al., 2023). Moreover, NFL levels can be a result of co-morbidities and lifestyle habits unrelated to the neurological disorder of interest, limiting its biomarker potential (Ladang et al., 2022). Therefore, there is increasing interest in multicomponent extracellular vesicles (EVs) in liquid biopsies as a source of biomarkers for neuropathological conditions (Zanirati et al., 2024).

Extracellular vesicles as sources of biomarkers

EVs are heterogenous spherical packages of bioactive molecules (proteins, lipids, nucleic acids, glycans, and metabolites) protected by a lipid bilayer which are released by viable cells (Welsh et al., 2024). Because EVs are stable in biofluids, they can carry molecular messages to distant cells and act in intercellular communication. The composition of EVs is determined by their mode of biogenesis and cellular origins; thus, EVs represent a molecular signature of their parent cell at that moment in time and their presence in biofluids makes them attractive for use as biomarkers (van Niel et al., 2018). The biomarker component of EVs could be represented by a single molecular biomarker in the EV cargo, or a signature of several different biomolecules within the EV unit (Foroni et al., 2020; Tutrone et al., 2023).

EVs from blood have already been characterised to identify proteomic patterns associated with (and distinguishing) different cancers in large patient cohorts (Hoshino et al., 2020) and EV-based biomarkers have been shown to outperform circulating nucleic acids in terms of sensitivity and selectivity for tumour detection (Foroni et al., 2020). Furthermore, EV-based biomarkers have already entered the clinic in the form of a gene expression array for three EV-encapsulated RNA biomarkers in urine (the commercial ExoDx^{TM} Prostate test) that provides risk stratification of prostate cancer and guides the clinical need for biopsy (Tutrone et al., 2023). With this precedent, Box 1 highlights some of the areas in which EV-based biomarkers could lead to improved clinical outcomes for patients.

The advantages EV-based biomarkers over single soluble molecules relate to 1) the multicomponent nature of EVs and 2) the protection offered by the EV lipid bilayer (Ciferri et al., 2021; Zhou et al., 2021). Firstly, being multicomponent enables EVs to concentrate multiple copies of the biomarker in a single EV unit, enhancing the signal-to-noise ratio of the biomarker (Tutrone et al., 2023). Moreover, analysis of a combination of different biomarkers (e.g. proteins and nucleic acids) within one EV can be performed to increase the specificity of the biomarker signature, which further increases the signal-to-noise ratio compared to potential biomarkers in freely circulating single molecules (Foroni et al., 2020). EVs can also be enriched from biofluids using various techniques (e.g. size-exclusion chromatography, ultracentrifugation, etc.), which further enhances the biomarker signal-to-noise ratio by removing non-EV molecules to reduce background noise. Additionally, the composition of EVs reflects their cell type of origin, so cell type-specific EVs can be isolated by targeting a cell type-specific protein exposed on the EV surface (for example, by antibody-based immunoaffinity

Box 1. Areas in which isolating EV-based biomarkers from inaccessible tissues in peripheral biofluids could lead to improved clinical outcomes for patients.

- Drug development – providing insight into the mechanisms driving disease progression, and thus targets for new drugs to prevent the development of disease states
- Risk stratification – determining the likelihood of an individual developing a disease and whether the individual would benefit from preventative treatment
- Diagnosis – especially in cases where differential diagnosis is required due to overlapping clinical symptoms, or in cases where early diagnosis can improve clinical outcomes
- Monitoring disease progression – guiding clinical decisions regarding treatment options
- Monitoring response to treatment – informing clinical decisions on whether a therapy is having the desired outcome, and especially in the development of new drugs to treat diseases to determine if they are effective

isolation; Mondal and Whiteside, 2021). This yields a population of EVs which can be analysed to provide insight into cell type-specific processes during disease, which also amplifies the biomarker signal-to-noise ratio by removing the background noise caused by EVs from other cells (Amin et al., 2024).

Secondly, the EV lipid bilayer protects the biomarker cargo from degradation by circulating enzymes (e.g. proteases and RNases). This can prolong the amount of time an EV-enclosed biomarker remains in circulation, as well as enabling molecular biomarkers to transfer between distal tissues via the bloodstream whilst contained within an EV (Zhao et al., 2019). The protection conferred by EVs extends to the ex vivo setting (e.g. after a blood sample has been taken in the clinic) and, so long as the EV membrane remains intact, cargo molecules are protected during sample processing (e.g. centrifuging blood to acquire plasma) and storage. Indeed, EV biomarker cargo can remain stable within biofluids stored for multiple years (Jeyaram and Jay, 2017). Therefore, the stability of EVs and their biomarkers during sample processing and storage makes them clinically amenable because clinical assays do not need to be performed immediately.

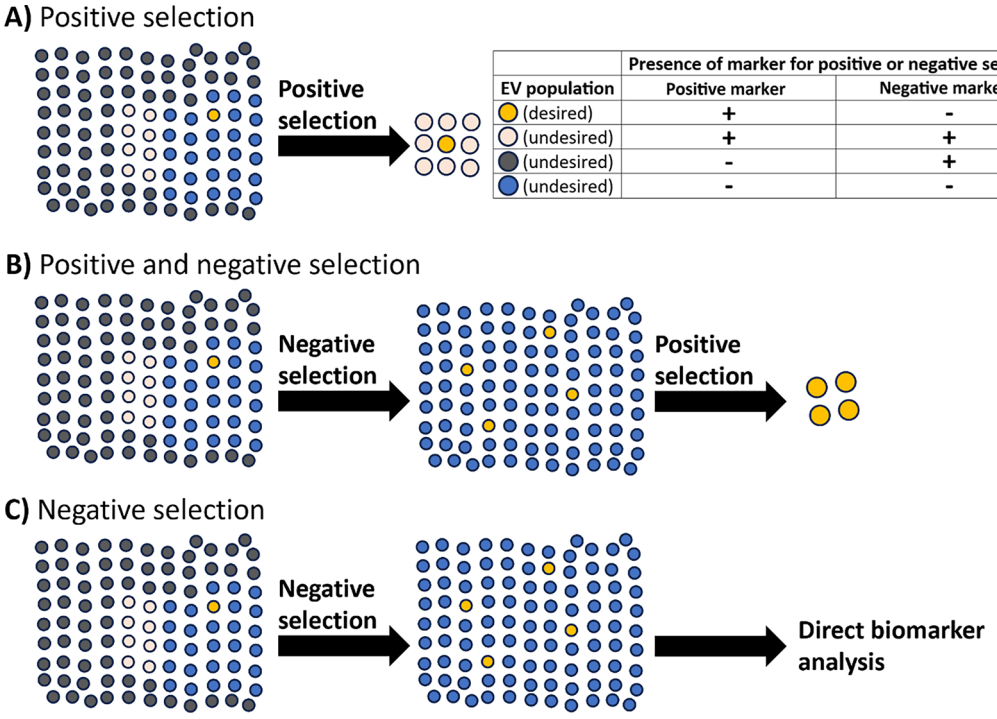
Extracellular vesicle-based biomarkers of neuropathological conditions

Because brain-derived (BD) EVs enter the peripheral circulation (Banks et al., 2020; Rufino-Ramos et al., 2022; Rufino-Ramos et al., 2023), the isolation and analysis of BDEVs from patient blood samples could offer an unprecedented source of biomarkers for cell type-specific neuropathological molecular processes. However, although EVs are relatively abundant in blood plasma (approximately 10^{10} EVs/ml; Johnsen et al., 2019), most of these originate from blood cells and platelets, whilst tissue-derived EVs make up a relatively small proportion of the total EVs in circulation (Li et al., 2020). For example, EVs derived from brain tissue have been estimated to constitute <1% of the total blood plasma EV population (Li et al., 2020), or $<10^8$ EVs/ml. Therefore, because EVs released by peripheral cells make up the majority of EVs in circulation, molecular patterns that reflect cell type-specific changes in the brain are masked by peripheral EVs, obscuring potential biomarkers of neurological disorders.

To enable analysis of brain-derived EV-based biomarkers in peripheral biofluids, immunoaffinity isolation can be performed to positively select for EVs on a cell-by-cell type basis (Figure 1A) and unmask biomarkers of neurological disorders by reducing the background noise of peripheral EVs (Ganesh et al., 2023). However, this approach relies on the identification of specific cell markers, while the use of a single protein to definitively trace EVs back to their cellular source is complicated in large multicellular organism where most proteins show expression in multiple cell types and in multiple tissues (Uhlén et al., 2015). Moreover, the isolation of CNS neuronal EVs is further complicated by EVs derived from neurons of the peripheral nervous system (PNS; e.g. enteric neurons that innervate the gastrointestinal tract), which share the expression of many neuronal markers (Uhlén et al., 2015; Zeisel et al., 2018). This hinders the identification of EV proteins unique to CNS neurons that can be detected in EVs in the periphery and used as targets for selective immunoisolation.

The case of L1CAM for the immunocapture of brain-derived neuronal extracellular vesicles

The most widely targeted protein for immunocapture of CNS neuronal EVs from peripheral biofluids has been L1 cell adhesion molecule (L1CAM) (Gomes and Witwer, 2022), which is a single-pass type 1 membrane protein (https://www.uniprot.org/uniprotkb/P32004/entry; UniProt Consortium, 2025). Whilst L1CAM-associated biomarker signatures have been identified that correlate with neurological disease (Dunlop et al., 2023; Dutta et al., 2023) and treatment response (Athauda et al., 2019; Mustapic et al., 2019), the validity of L1CAM as a target to capture CNS neuronal EVs from the peripheral circulation is contested (Gomes and Witwer, 2022; Dutta et al., 2023). Firstly, L1CAM is not exclusively expressed by CNS neurons and transcriptomic and proteomic datasets show that L1CAM is expressed at higher levels in enteric neurons and in the kidneys and skin, as well as at lower levels in other tissues (http://mousebrain.org/genes/L1cam.html, Zeisel et al., 2018; v25.0 proteinatlas.org/ENSG00000198910-L1CAM, Uhlén et al., 2015). This indicates



	Presence of marker for positive or negative selection	
EV population	Positive marker	Negative marker
(desired)	+	-
(undesired)	+	+
(undesired)	-	+
(undesired)	-	-

Figure 1. A simplified illustration of positive and negative selection strategies to select for rare extracellular vesicle (EV) populations for biomarker discovery and detection. (A) Single-step positive selection enriches for the targeted EV population but co-isolates non-target EVs displaying the marker for positive selection. (B) A combination of positive and negative selection, where non-target EVs (including those displaying the positive selection marker) are removed by negative selection, enabling the isolation of a relatively homogeneous EV population. Negative selection also enriches the proportion of the targeted rare EV population (and associated biomarkers) by removing the majority of contaminating EVs. (C) Negative selection alone to enrich for a rare EV population for biomarker detection or discovery.

that other tissues may also release L1CAM-positive EVs into circulation, which could be enriched following L1CAM immunocapture. This is supported by estimations that 5-10% of EVs in plasma are L1CAM-positive (Mustapic et al., 2017; Kumar et al., 2022), whilst less than 1% of EVs in plasma originate from the brain (Li et al., 2020).

Secondly, Norman et al. (2021) reported that L1CAM in circulation is present as a soluble isoform and not integral or exclusive to CNS neuronal EVs. Through fractionation of blood plasma by density gradient centrifugation, as well as size-exclusion chromatography, L1CAM eluted with albumin (a proxy for soluble protein), but not in the fractions containing the EV markers CD9, CD63, and CD81 when detected using a digital ELISA (Simoa®) (Norman et al., 2021), which can detect fg/ml levels of proteins (Rissin et al., 2010). At the same time, the authors revealed the existence of a soluble, alternatively spliced isoform of L1CAM in plasma, containing both the internal and external domains of the protein, but lacking its transmembrane region. Since this isoform was indistinguishable from the integral membrane protein by Western blotting, the authors indicated that the L1CAM detected after L1CAM immunocapture from plasma could be this isoform, and that other proteins detected in the immunoprecipitate were co-isolated through direct interactions with the L1CAM antibody/beads. This was demonstrated with soluble α-synuclein, which preferentially bound the UJ127 L1CAM antibody clone compared to control IgG (Norman et al., 2021).

Subsequent studies have corroborated that the majority of L1CAM in circulation is not an integral EV component using size-exclusion chromatography, but these studies have also indicated that there is a small amount of L1CAM that elutes with the EV fraction (Dutta et al., 2021; Yan et al., 2023). Additionally, Blommer et al. (2023) and Kumar et al. (2022) reported that L1CAM could be detected on single EVs through co-localisation with canonical EV markers, as demonstrated by immunoelectron microscopy with CD63 (Kumar et al., 2022); high-resolution fluorescent confocal microscopy with ALIX (Blommer et al. 2023); and single particle interferometric imaging sensing (SP-IRIS) with CD9, CD63, and CD81 (Blommer et al. 2023). For this, both studies used a used a commercial precipitation reagent (ExoQuick®) to precipitate particles from plasma before performing L1CAM immunocapture (Blommer et al. 2023; Kumar et al., 2022).

However, the reliability and reproducibility of biomarker studies using precipitation agents to concentrate EVs before L1CAM capture has been questioned due to the associated aggregation of soluble proteins with EVs, and subsequent non-specific capture during immunoisolation, leading to high variability in inter-study results (Taha, 2023; Yan et al., 2023). For example, in the case of α-synuclein derived from the blood of healthy volunteers, Yan et al. (2023) highlighted that the 3099.9 (±2986) pg/ml concentration of α-synuclein obtained by Blommer et al. (2023) using precipitation followed by L1CAM immunocapture was approximately 233-fold higher than the 13.3 (±4.5) pg/ml concentration obtained when size-exclusion chromatography was used to separate EVs and soluble proteins (Yan et al., 2023). This difference was attributed to the tendency of α-synuclein, which is present in blood plasma at a concentration of 42 ng/ml (v25.0 proteinatlas.org/ENSG00000145335-SNCA/blood#blood_conc_ms, Uhlén et al., 2015), to precipitate and aggregate in the presence of the precipitation reagent, as well as bind to surfaces including immunocapture beads, polymers in the precipitation reagent, and EVs (Yan et al., 2023).

To try to definitively show the association of L1CAM with EVs in plasma, Nogueras-Ortiz et al. (2024) employed multiple EV isolation techniques and single-EV analysis methods to localise the ectodomain of L1CAM (as the intended target for L1CAM immunocapture) to the outer surface of EVs. This included differential ultracentrifugation followed by high-resolution confocal fluorescent microscopy to show that the L1CAM ectodomain co-localised with EV markers CD63, CD81, and ALIX on EVs derived from neuronal cells in vitro (specifically, primary rat hippocampal neurons and human induced pluripotent stem cell-derived neurons). Additionally, nanoscale multiplex flow cytometry analysis (FCA) showed that 97% of L1CAM-positive events were double positive for 'pan-tetraspanins' (CD9, CD63, and CD81) and that a higher number of L1CAM/pan-tetraspanin(++) events occurred in larger EVs that pelleted at 10,000 g compared with smaller EVs that pelleted 120,000 g (Nogueras-Ortiz et al., 2024).

Using FCA to analyse total EVs from plasma (pelleted by a single ultracentrifugation step at 120,000 g), Nogueras-Ortiz et al. (2024) also reported that 3.8% were L1CAM-positive, and 67.4% of L1CAM-positive events were co-labelled with at least one of CD9, CD63, or CD81. To show the neuronal origin of plasma-derived EVs by FCA, it was demonstrated that 33.6% of L1CAM-positive events were co-labelled with VAMP2 and 54% with β-III-tubulin (two proteins enriched in neurons), whilst only 5.0% (VAMP2) or 4.1% (β-III-tubulin) of EVs from the total plasma pool were labelled with these proteins (Nogueras-Ortiz et al., 2024). Moreover, only 2.4% L1CAM-negative events were immunopositive for VAMP2, and 1.3% for β-III-tubulin, indicating the specificity of L1CAM co-labelling with neuron-enriched proteins. Out of the total EVs in plasma, 1.3% were double-positive for L1CAM/VAMP2, 2.1% for L1CAM/β-III-tubulin, and 2.5% L1CAM/pan-tetraspanin (CD9, CD63, and CD81). Furthermore, following L1CAM immunocapture, an EV multiplexing assay was used to show that the isolated pan-tetraspanin(+) population carried GAP43, another neuron-enriched protein (Nogueras-Ortiz et al., 2024). Based on these quantitative findings, it was estimated that two-thirds of L1CAM-positive EVs were from neuronal cells, whilst one-third had a non-neuronal origin (Nogueras-Ortiz et al., 2024).

Additionally, in contrast to the results of Norman et al. (2021), Nogueras-Ortiz et al. (2024) did detect the L1CAM ectodomain using a Simoa® digital ELISA in the fractions of plasma containing CD9, CD63, or CD81 following size-exclusion chromatography (although notably both studies employed different chromatography columns and resins). The latter study also sought to address whether free, soluble L1CAM in blood could bind non-specifically to the surface of EVs (Nogueras-Ortiz et al., 2024). For this, the authors incubated EVs from HEK-293 cells (an embryonic kidney cell line that do not express L1CAM) with particle-depleted plasma for 3.5 h at 37°C before assessing the adsorption of L1CAM or APOA1 to the surface of EVs (APOA1 is a recognised component of the non-integral, surface corona coating EVs in plasma; Tóth et al., 2021). After the incubation, FCA showed that APOA1, but not L1CAM, was detected on the surface of HEK-293 EVs, demonstrating that free, soluble L1CAM in particle-depleted plasma does not bind HEK-293 EVs (Nogueras-Ortiz et al., 2024).

Unanswered questions regarding the use of L1CAM for the immunocapture of brain-derived neuronal extracellular vesicles

Together, the abovementioned studies make substantial contributions to understanding the complexities of targeting EVs from a single cellular source (CNS neurons) using immunoaffinity isolation. However, they also highlight some of the limitations of this approach and there remains unresolved questions regarding the use of L1CAM immunocapture to obtain brain-derived neuronal biomarkers. It is recognised that the methodology of L1CAM immunocapture is unable to distinguish between EVs derived from neurons of the CNS and PNS, thereby limiting the recovery of biomarkers specific for brain disorders (Nogueras-Ortiz et al., 2024). The relative contribution of EVs to the peripheral bloodstream from these two sources remains unknown (Nogueras-Ortiz et al., 2024); however, in comparison with the blood-brain barrier, the blood-nerve barrier (between peripheral neurons and the bloodstream) represents a relatively 'leaky' interface (Malong et al., 2023) by which peripheral neuron-derived EVs may enter circulation.

Notably, transcriptomic data shows that L1CAM, β-III-tubulin, and GAP43 are expressed at higher levels in peripheral neurons than the CNS, whilst VAMP2 is expressed at similar levels in CNS and PNS neurons (Table 1). Additionally, L1CAM, VAMP2, β-III-tubulin, and GAP43 have widely different protein concentrations in plasma (proteinatlas.org; Uhlén et al., 2015), ranging from 0.029 ng/ml for GAP43 to 71 ng/ml for β-III-tubulin (Table 1). The concentration of β-III-tubulin in plasma is also 17-fold the concentration of CD9 (Table 1), with CD9 representing the most abundant tetraspanin in plasma, found on the majority (approximately 62-72%) of plasma EVs (Hoshino et al., 2020; Karimi et al., 2022; Salmond et al., 2021; Tordoff et al., 2024). This indicates that some of these neuron-enriched proteins are either particularly abundant components of EVs, or they have non-neuronal sources, or they are secreted in a soluble form. Therefore, this highlights that further markers are required to delineate the origins of CNS neuronal EVs from peripheral sources.

Furthermore, it remains unknown how biomarker studies using immunocapture antibodies targeting the internal (intravesicular) domain of L1CAM apparently isolate L1CAM-positive EVs (Chen et al., 2024; Dagur et al., 2019; Kluge et al., 2022; Schaeffer et al., 2024). Since the L1CAM ectodomain is on the EV surface (Nogueras-Ortiz et al., 2024) and L1CAM is a single-pass type 1 membrane protein (https://www.uniprot.org/uniprotkb/P32004/entry; UniProt Consortium, 2025), it would be anticipated that the internal domain and C-terminus of the protein are located within the EV lumen (as illustrated in Figure 2A). Therefore, with this transmembrane model, the internal domain of L1CAM should be inaccessible to antibody binding (Figure 2A). Notably, antibodies targeting the L1CAM internal domain, which have been experimentally mapped to binding the protein's C-terminus (Chen et al., 2010; Girbes Minguez et al. 2020), would also not bind the soluble L1CAM ectodomain (released as a result of ectodomain shedding; Maretzky et al., 2005) adsorbed onto the EV surface because this cleavage product lacks the internal domain of L1CAM.

However, studies using internal domain antibodies for L1CAM immunocapture (Chen et al., 2024; Dagur et al., 2019; Kluge et al., 2022; Schaeffer et al., 2024) reported the isolation of particles with the size profile of EVs by transmission electron microscopy (Kluge et al., 2022) and nanoparticle tracking analysis (Dagur et al., 2019). Similarly, L1CAM internal domain immunocapture isolated full-length L1CAM, EV-enriched proteins (CD9, CD63, CD81, TSG101) and

Table 1. Expression of L1CAM, VAMP2, β-III-tubulin (TUBB3), GAP43, and CD9 in neurons of the central and peripheral nervous system (of mice), as well as their concentration in human plasma. Comparative expression levels (with arbitrary units) for neurons of the central nervous system (CNS) and peripheral nervous system (PNS) were obtained from mousebrain.org (Zeisel et al., 2018) because quantitative transcriptomic data for PNS neurons is not available in the Human Protein Atlas. N.B. CD9 expression in CNS neurons was only detected in one neuronal subtype. Protein concentrations in plasma were obtained from the Human Protein Atlas version 25.0 (Uhlén et al., 2015) at proteinatlas.org/ENSG00000198910-L1CAM, /ENSG00000220205-VAMP2, /ENSG00000258947-TUBB3, /ENSG00000172020-GAP43, and/ENSG00000010278-CD9.

Protein	Highest expression level in CNS neurons	CNS neuron subtype showing highest expression	Highest expression level in PNS neurons	PNS neuron subtype showing highest expression	Concentration in human plasma
L1CAM	2.3	Cholinergic neurons, hindbrain (rhombencephalon)	7.4	Cholinergic enteric neurons (neural crest)	13 ng/ml
VAMP2	15.7	Excitatory neurons, hindbrain (rhombencephalon)	13.0	Noradrenergic erector muscle neurons (neural crest)	5.2 ng/ml
β-III-tubulin	18.3	Cholinergic neurons, septal nucleus, Meissnert and diagonal band (diencephalon)	115.1	Noradrenergic neurons, sympathetic (neural crest)	71 ng/ml
GAP43	42.7	Cholinergic neurons, septal nucleus, Meissnert and diagonal band (diencephalon)	79.7	Noradrenergic erector muscle neurons (neural crest)	0.029 ng/ml
CD9	0.9	Central canal neurons, spinal cord (spinal cord)	79.7	Noradrenergic erector muscle neurons (neural crest)	4.2 ng/ml

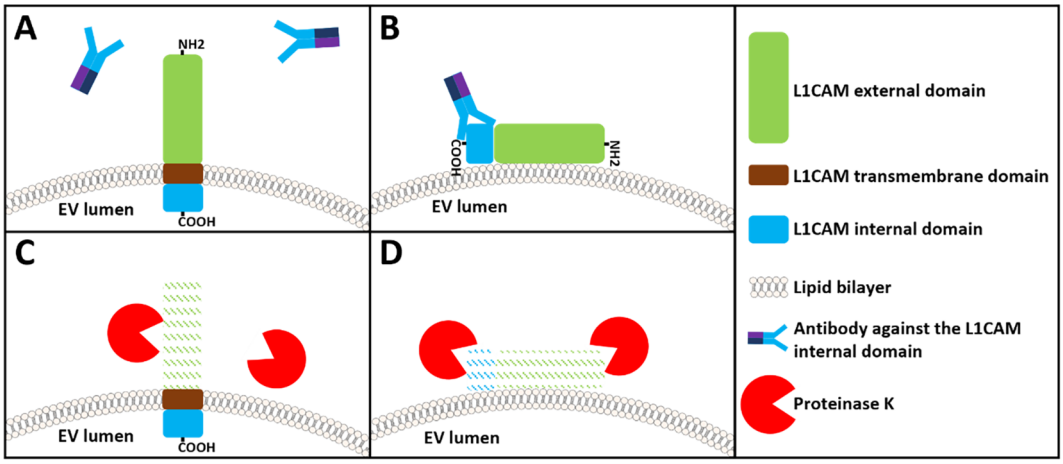


Figure 2. Simplified illustrations of L1CAM at the extracellular vesicle (EV) surface and the effects of proteinase K treatment. (A) The anticipated single-pass transmembrane form of L1CAM in the extracellular vesicle (EV) membrane, where the intraluminal location of the internal domain does not permit binding by antibodies targeting the L1CAM internal domain. (B) An alternative model where the soluble isoform of L1CAM (lacking the transmembrane domain) is adhered to the EV surface, enabling binding by the L1CAM internal domain antibody. (C) With the transmembrane form of L1CAM seen in (A), proteinase K digests the exposed external domain of L1CAM, but the internal domain remains protected by the EV lipid bilayer, enabling the subsequent detection of the internal domain in downstream assays. (D) Proteinase K digests both the internal and external domains of L1CAM when the soluble isoform of L1CAM is adhered to the EV surface (or free in solution), as shown in (B). Consequently, the internal domain of L1CAM would not be detected in downstream assays. Of note, both forms of L1CAM seen in (A) and (B) may exist on the same EV or within the same EV preparation.

neuron-enriched proteins (β-III-tubulin, synaptophysin, enolase 2) (Dagur et al., 2019; Kluge et al., 2022). Since these studies detected L1CAM with a molecular weight >32 kDa (the size of the internal domain and transmembrane region alone; Maretzky et al., 2005), it indicates that both the internal domain and ectodomain of the protein were captured by the internal domain antibody (Dagur et al., 2019; Kluge et al., 2022). To explain this, Gomes and Witwer (2022) postulated that the L1CAM internal domain could be exposed to antibodies on damaged EVs or particles with an inverted membrane topology formed from cell membrane fragments, enabling immunocapture of full-length L1CAM and other proteins.

An alternative explanation is that the L1CAM internal domain antibodies capture the soluble, alternatively spliced isoform of L1CAM (described in plasma by Norman et al., 2021) bound to the surface of EVs (illustrated in Figure 2B). Although it was shown that soluble L1CAM did not bind exogenous EVs in particle-depleted plasma when compared with APOA1 (Nogueras-Ortiz et al., 2024), the binding of soluble L1CAM to EVs may depend on context and specific protein-protein interactions since the binding of EVs and soluble plasma proteins is specific and not universal (Su et al., 2024). Notably, the concentration of APOA1 in plasma is ~160,000 ng/ml (v25.0 proteinatlas.org/ENSG00000118137-APOA1/blood#blood_conc_ms, Uhlén et al., 2015), approximately 12,308-fold higher than L1CAM (13 ng/ml; v25.0 proteinatlas.org/ENSG00000198910-L1CAM/blood#blood_conc_ms). Whilst interactions between EVs and free L1CAM molecules in plasma would be comparatively rare, L1CAM is one of the most abundant soluble proteins secreted by neurons (Tüshaus et al., 2020). Therefore, it remains to be explored if full-length soluble L1CAM could be adsorbed onto the surface of EVs in the neuronal microenvironment, which could explain how studies performing L1CAM immunocapture using the internal domain antibody show the presence of neuron-enriched proteins in the immunoprecipitate (Dagur et al., 2019; Kluge et al., 2022). This could be tested experimentally by incubating L1CAM-negative EVs with conditioned media from in vitro cultures of neurons. Further controls are also required to show that the observed EV isolation is not the result of off-target binding of the immunocapture antibody, as observed with α-synuclein for the UJ127 clone against the L1CAM ectodomain (Norman et al., 2021).

It is also noteworthy that soluble L1CAM is a CD9 binding protein via its ectodomain (Schmidt et al., 1996; Jouannet et al., 2016). This was demonstrated in immunoprecipitation experiments with soluble cell lysates derived from N2A neuroblastoma (Schmidt et al., 1996) or U2 osteosarcoma cells (Jouannet et al., 2016), with L1CAM and CD9 found to form a stable and specific molecular complex that was concentration-dependent and saturable (Schmidt et al., 1996). Thus, this interaction could provide a mechanism by which soluble full-length L1CAM binds the EV surface, leaving its internal domain exposed for immunocapture. Relevant to this interaction is that CD9 inhibits ADAM10 and ADAM17 (Arduise et al., 2008; Gutiérrez-López et al., 2011), two metalloproteases that cleave L1CAM to release the ectodomain

(Maretzky et al., 2005), and it is unknown how this negative regulation could impact the presence of full-length L1CAM on CD9-positive EVs.

Notably, the vast majority of neurons in the adult CNS do not express CD9 (Figure 3), as demonstrated by proteomic and transcriptomic datasets (proteinatlas.org, Uhlén et al., 2015; mousebrain.org, Zeisel et al., 2018), as well as in studies investigating the expression of CD9 in the nervous system (e.g. Doh-Ura et al., 2000; Kagawa et al., 1997; Table 2). Instead, these same datasets show that CD9 is highly expressed by PNS neurons (Figure 3, Table 1, Table 2), providing a physiological context for which soluble L1CAM could bind CD9-positive EVs secreted by PNS neurons. Together, context-dependent CD9 expression and CD9-L1CAM interactions need to be taken into account when assessing the presence of L1CAM on EVs. This is especially important because 1) several EV analysis platforms using CD9-L1CAM binding have been used to demonstrate the presence of EV-integral L1CAM (when soluble L1CAM is a CD9 binding protein) and 2) these platforms have also been used to assert the presence of brain-derived neuronal EVs through the

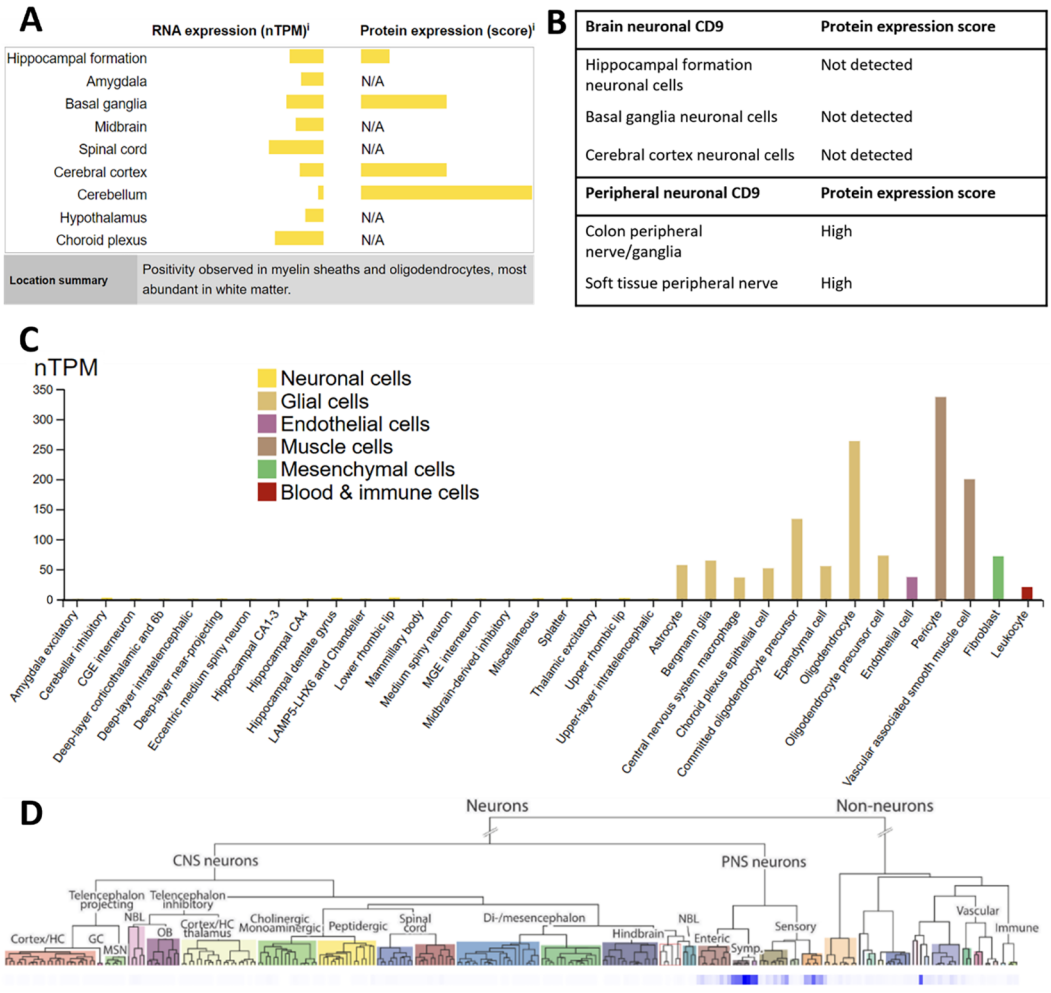


Figure 3. Summary of CD9 expression in the nervous system. (A) The distribution of CD9 expression in different brain regions and the location summary for CD9 expression in the brain displayed in the Human Protein Atlas. (B) Summary of neuronal CD9 protein detection in the brain from the Human Protein Atlas (where neuronal CD9 detection data is available). (C) Single-cell RNA expression profile for CD9 in the brain, adapted from the Human Protein Atlas. (D) Heatmap of single-cell RNA sequencing data from the Mouse Brain Atlas showing the distribution of CD9 expression in neuronal and non-neuronal cells in the adolescent mouse nervous system; darker blue indicates higher expression. Panel A-C image credit: Human Protein Atlas (Uhlén et al., 2015) with images reproduced from v24.0 proteinatlas.org/ENSG00000010278-CD9/tissue and proteinatlas.org/ENSG00000010278-CD9/brain (A and B) and adapted from v24.0 proteinatlas.org/ENSG00000010278-CD9/single+cell (C) under the Creative Commons Attribution-ShareAlike 4.0 International License. Panel D image credit: Mouse Brain Atlas reproduced with permission (http://mousebrain.org/genes/Cd9.html; Zeisel et al., 2018).

Table 2. Studies investigating the expression of CD9 in the nervous system and a summary of the cells in which CD9 was detected. Briefly, CD9 expression was detected in some mouse and rat embryonic and neonatal neurons in the central nervous system (CNS), but CNS neuronal CD9 expression disappeared as the animals developed. In non-neuronal cells, CD9 was detected in neural stem cells (and neural stem cell extracellular vesicles), myelinating oligodendrocytes, astrocytes, ependymal cells, and microglia. In the peripheral nervous system (PNS), CD9 was detected in peripheral neurons, satellite cells, and myelinating Schwann cells.

Publication	Organism	CD9 expression summary and detection method
Banerjee and Patterson, 1995	Rat (neonatal and embryonic)	• CD9 is expressed in myelinating Schwann cells in primary culture, shown by in situ hybridisation • CD9 is expressed in sympathetic and sensory neurons in primary culture, shown by in situ hybridisation
Doh-Ura et al., 2000	Mouse and human (adult)	• Mouse astrocytes and oligodendrocytes in the brain were positive for CD9 by immunohistochemistry • Mouse neuronal cells in the brain were negative for CD9 by immunohistochemistry • In human brains, the same pattern was observed by immunohistochemistry (astrocytes and oligodendrocytes CD9-positive, neurons CD9-negative)
Ishibashi et al., 2004	Mouse (8 weeks old)	• CD9 is detected in oligodendrocytes and Schwann cells, shown by immunohistochemistry • CD9 is enriched in the PNS compared with the CNS, shown by immunohistochemistry and Western blotting
Kagawa et al., 1997	Mouse (postnatal day 10 to adults)	• CD9 was detected in oligodendrocyte myelin in brain white matter but not in axons by immunohistochemistry • Primary oligodendrocytes, astrocytes, and microglia were CD9-positive by immunohistochemistry • No neurons or neuronal precursors in the brain were stained with the CD9 antibody in this study
Kaprielian et al., 1995	Rat (adult)	• Superior cervical ganglion neurons, dorsal root ganglion neurons, and Schwann cells expressed CD9, as shown by in situ hybridisation
Llorens-Bobadilla et al., 2015	Mouse (8-12 weeks old)	• Adult subventricular zone quiescent neural stem cells expressed CD9, shown by single-cell RNA sequencing, flow-cytometry, and immunohistochemistry • Ependymal cells were CD9-positive by immunohistochemistry • Cortical and striatal astrocytes were CD9-positive by flow cytometry • Some axons were CD9-positive by immunohistochemistry
Morton et al., 2018	Mouse (neonatal)	• Neonatal subventricular zone neural stem cells were CD9-positive by immunohistochemistry • Extracellular vesicles from neonatal subventricular zone neural stem cells were CD9-positive by Western blot
Nakamura et al., 1996	Human (adult)	• CD9 was not detected in neurons or astroglia in human cerebral white matter, but was detected in the myelin of oligodendrocytes by immunohistochemistry • CD9 was detected in myelin sheaths in the PNS by immunohistochemistry
Polanco et al., 2018	Mouse (embryonic)	• Embryonic primary hippocampal neurons transfected with either mCherry-CD9–10 or Dendra2-CD9–10 were positive for CD9 by immunofluorescent staining • Notably, here CD9 expression was under the influence of the promoter of the genetic construct

Table 2. *Continued*

Publication	Organism	CD9 expression summary and detection method
Sanchez-Arias et al., 2020	Mouse (neonatal)	• Neonatal primary neurons transfected with mCherry-CD9-10 then studied after 10 days in vitro culture were positive for CD9 by immunofluorescent staining • Notably, here CD9 expression was under the influence of the promoter of the genetic construct
Schmidt et al., 1996	Rats (11-day old embryos, postnatal day 4, and adult) and mouse (6 day old)	• CD9 positivity was observed in the cerebellum and PNS tissue of rats by immunohistochemistry • Small cerebellar neurons, astrocytes, and oligodendrocytes in primary culture derived from 6-day old mice were positive for CD9 by immunohistochemistry • Motor neurons in primary culture derived from 11-day old rat embryos were positive for CD9 by immunohistochemistry
Tole and Patterson, 1993	Rat (embryonic and adult)	• CD9 positivity was observed in embryonic sympathetic neurons, dorsal root ganglion neurons, and embryonic spinal motor neurons by immunohistochemistry • Staining of CD9 in motor neurons disappeared as the embryos developed • Immunohistochemistry in adult tissues showed CD9-positivity in oligodendrocytes, astrocytes, satellite cells, and Schwann cells, whilst CD9 labelling of neurons in the spinal cord was inconclusive and the majority of cerebellar neurons were negative for CD9

presence of CD9 and L1CAM (Bravo-Miana et al., 2024; Hotta et al., 2022; Nogueras-Ortiz et al., 2024), even though the vast majority of neurons in the brain do not express CD9 (Figure 3, Table 2).

Clarification over the association of L1CAM with EVs could come through proteinase K assays, which have already been used to demonstrate that the L1CAM ectodomain exists on the EV surface, or free in solution, in brain-derived EV preparations (You et al., 2023). Such assays involve proteinase K digesting the exposed proteins in the EV preparation, whilst the EV lipid bilayer protects proteins within the EV lumen (Théry et al., 2018). Subsequent analysis (e.g. Western blotting) then reveals which proteins had been digested (i.e. those proteins on the EV surface or free in solution) (Théry et al., 2018). Thus, this approach could also be used to resolve the association of the L1CAM internal domain with EVs, where protection of the internal domain following proteinase K treatment would confirm that L1CAM exists on EVs as a transmembrane protein with the internal domain located within the EV lumen (Figure 2C). Notably, in such a case, following digestion L1CAM would be detected at ~32 kDa by Western blotting using an internal domain antibody because the ectodomain would have been digested by proteinase K (Maretzky et al., 2005). Conversely, if both the internal domain and ectodomain were exposed to proteinase K digestion (either because L1CAM exists as the isoform free in solution or bound to the EV surface), the internal domain would not be detected following proteinase K treatment (Figure 2D). Of note, related to this, You et al. (2023) targeted ATP1A3 to enrich for neuronal EVs from plasma and used the XVIF9-G10 clone antibody that has also been shown to bind the cytoplasmic (and anticipated intravesicular) domain of the ATP1A3 protein at the protein's N-terminus (Arystarkhova and Sweadner, 1996). However, when You et al. (2023) performed a proteinase K assay with EVs derived from human brain tissue (enriched using a protocol of enzymatic digestion and density gradient centrifugation) followed by Western blotting for ATP1A3 with the XVIF9-G10 clone antibody, proteinase K was shown to completely digest the epitope, indicating that the internal domain of ATP1A3 was exposed to proteinase K, not protected by the EV membrane, and thus external to the EV (You et al., 2023). Further clarity is therefore required on how these proteins are located in relation to EVs and the EV lipid bilayer.

Negative selection by immunodepletion: a key step for enrichment and specificity of rare extracellular vesicle-based biomarkers?

Because of concerns over the specificity of L1CAM to brain-derived neuronal EVs, other targets have been investigated for CNS neuronal EV isolation from the periphery. These include ATP1A3, GluR2, GAP43, NLGN3, and NCAM (Eitan et al., 2023; Fiandaca et al., 2015; Ko et al., 2016; You et al., 2023). However, these all show expression in peripheral neurons, and some are expressed at high levels in other non-neuronal cell types in the body (proteinatlas.org, Uhlén et al., 2015; mousebrain.org, Zeisel et al., 2018), indicating that they may be unable to distinguish between EVs from neurons of

the CNS and PNS, or EVs released by other cell types that express these proteins. In this regard, it is notable that studies targeting L1CAM (for example, Dunlop et al., 2023; Hotta et al., 2022; Kumar et al., 2022), GAP43 and NLGN3 (Eitan et al., 2023), and ATP1A3 (You et al., 2023) from plasma show that the immunocaptured EVs are enriched in CD9, which is highly expressed by PNS neurons but not expressed by the vast majority of CNS neurons (Figure 3, Table 1, Table 2). This suggests that a sizeable proportion of these EVs are actually originating from PNS neurons/other non-neuronal sources, rather than from neurons in the brain.

Together, this indicates that the single-step immunocapture approach of targeting enriched neuronal proteins for CNS neuronal EV isolation is unable to distinguish CNS-derived neuronal EVs from PNS-derived EVs in circulation; thereby, obscuring potentially critical insights into mechanisms underlying neuropathophysiological processes and masking brain-derived biomarkers. Moreover, the molecular overlap of PNS and CNS neurons remains an obstacle to identifying new targets for positive selection of CNS neuronal EVs that are 1) only expressed in CNS neurons; 2) released in CNS neuronal EVs; and 3) released into peripheral biofluids in sufficient number for biomarker discovery and detection. The need for more selective and specific isolation of CNS neuronal EVs (and associated biomarkers) has directed attention towards the strategy of combining positive selection of CNS neuronal EVs with negative selection by depleting contaminating EV populations (Nogueras-Ortiz et al., 2024). Figure 1B shows an illustration of this approach, where positive and negative selection are combined to select for a rare EV population, thereby amplifying the signal-to-noise ratio of cell type-specific biomarkers.

Such an approach has not been widely applied in EV research but, in cases where negative selection has been used, this has mostly been to remove EVs on a cell-by-cell type basis by targeting cell type-enriched markers to deplete abundant EV populations. In blood plasma, the majority of circulating EVs are blood cell-derived (Li et al., 2020) and the most abundant EVs by cell type originate from platelets (constituting between 30-51% of all plasma EVs; Auber and Svenningsen, 2022; Li et al., 2020). Therefore, depletion of platelet EVs, as well as EVs derived from other blood cell types, has been used to successfully enrich for rare EV populations in plasma. Notarangelo et al. (2019) combined CD41 depletion with CD235 depletion to deplete platelet and erythrocyte EVs, which together have been estimated to constitute <55% of the total EVs in plasma (Auber and Svenningsen, 2022; Li et al., 2020). This achieved a 4-5 fold enrichment of rare EVs (and associated biomarkers) derived from breast cancer cells in plasma EV samples, thereby demonstrating that the inclusion of a negative selection step increased the amount of material for biomarker detection in rare EV populations (Notarangelo et al., 2019).

Ko et al. (2016) also used negative selection by depleting CD45 (a leukocyte EV marker) and CD61 (a platelet EV marker) to enrich for and detect neuronal EVs in serum using a microfluidic device. For this, serum loaded into the microfluidics chamber was incubated with CD45/CD61 negative selection beads for 30 minutes, as well as positive selection beads targeting CD81. Using negative pressure to draw the serum through the device, the larger (7 μm) negative selection beads were then separated from the sample on a 5 μm porous membrane before the smaller (2.2 μm) positive selection beads were trapped on a 1 μm porous membrane. Following washes, a custom-made ELISA was then performed to detect the neuron-enriched protein, GluR2, in the positively selected EV population (Ko et al., 2016).

Using this device, Ko et al. (2016) reported that the inclusion CD45/CD61 depletion removed background EVs to enable the detection of GluR2 in mouse serum (that had been spiked with cortical neuron cultured media), when GluR2 could not be detected without the negative selection step. Similarly, the immunodepletion step enhanced the detection of changes in the level of GluR2-positive EVs in mouse serum following mild traumatic brain injury, showing that depletion of abundant blood EVs can be used to amplify the biomarker signal originating from neuronal EV populations bearing GluR2 (Ko et al., 2016). In another example combining positive and negative selection, Ganesh et al. (2023) found that preceding neuronal EV capture from plasma with either depletion of CD235-positive EVs derived from erythrocytes (constituting 4-5% of total plasma EVs; Auber and Svenningsen, 2022; Li et al., 2020), abundant free plasma proteins (albumin and immunoglobulin), or combined CD235/albumin/immunoglobulin depletion, increased the number of proteins that were identified in neuronal EVs isolated by positive selection using a proprietary platform and analysed by mass spectrometry-based proteomics, thereby increasing the number of potential biomarker proteins that were detected.

Collectively, these examples demonstrate that immunodepletion is an effective strategy to enrich for rare EV populations, and their associated biomarkers, when used in combination with positive selection. However, strategies targeting cellular markers to deplete abundant EVs in plasma have the limitation that they only deplete EVs released by specific cell types. Similarly, solely focussing on depletion of abundant EVs released by particular cells may be unable to separate two EV populations from closely related cell types (e.g. CNS and PNS neurons) which both release EVs bearing the desired target for positive selection. Concerns have also been raised that depleting abundant plasma proteins could compromise EV recovery due to interactions between plasma proteins and specific EV populations (Su et al., 2024).

Taking advantage of the heterogeneity of common extracellular vesicle markers for immunodepletion

An alternative negative selection approach is targeting a common EV marker not found on the EV population of interest to simultaneously deplete multiple contaminating EV populations from different cell types. Almousa et al. (2025) developed a method of negative selection through depletion of the common EV marker CD63 to enable the enrichment of bacterial EVs from mouse faecal samples. Since no universal surface marker existed to isolate EVs from diverse bacteria by positive selection, performing CD63 depletion instead maximised the removal of heterogenous murine EVs from the sample (faecal EVs isolated by ultracentrifugation) by targeting a single protein common to eukaryotic EVs but absent from the bacterial EVs (Almousa et al., 2025).

To examine the selectivity of CD63 depletion in enriching for bacterial EVs, unbiased metaproteomic analysis was then performed. Of the proteins identified in total faecal EVs from control mice, 64.2% (4133 proteins) were of bacterial origin, whilst 35.8% (2308 proteins) were derived from the mouse. However, following CD63 depletion, the percentage of proteins identified as bacterial rose to 90.3% (6692 proteins), with 9.7% (723) derived from the murine host. Correspondingly, the CD63-positive fraction contained 98.4% (4707) proteins of eukaryotic origin, and 1.6% (79) bacterial origin (Almousa et al., 2025). Immunogold electron microscopy and Western blotting for the eukaryotic EV markers CD63 and syntenin, as well as the bacterial markers LPS and outer membrane protein C, were also used to confirm the separation of bacterial EVs from those derived from the murine host. Thus, the role of bacterial EVs in pain sensitivity in a mouse model of diet induced obesity could be analysed by limiting the confounding presence of murine EVs, thereby enabling the observed biological effects to be attributed to the bacterial-enriched faecal EV population (Almousa et al., 2025).

This approach could also be used in human biomarker studies due to the heterogeneity of markers on EVs derived from different cellular and subcellular origins (Kugeratski et al., 2021; Spitzberg et al., 2023; Su et al., 2024; Zarovni et al., 2025). Thus, rather than depleting contaminating EVs on a cell-by-cell type basis, this approach would target a single, common marker for negative selection. This could enable the separation of the desired rare EV population from a multitude of EVs released by diverse cell types in a variety of biofluids (Almousa et al., 2025).

For example, the common EV marker CD9 is not expressed by the vast majority of neurons in the brain (Figure 3, Table 1, Table 2) and brain-derived neuronal EVs are a desired source of biomarkers for neurological disorders (Nogueras-Ortiz et al., 2024). Conversely, CD9 is the most abundant tetraspanin in blood plasma (Tordoff et al., 2024), found on 62-72% of plasma EVs (Hoshino et al., 2020; Tordoff et al., 2024). CD9 is also relatively common in EVs derived from different cell types and biofluids (Hoshino et al., 2020; Ko et al., 2021; Spitzberg et al., 2023). Therefore, CD9 depletion could be used to enrich for CNS-derived neuronal EVs in a variety of biological samples, for example by removing 62-72% of contaminating plasma EVs. In contrast, a depletion strategy targeting a cell marker for platelet EVs (constituting ~30-51% of EVs in plasma; Auber and Svenningsen, 2022; Li et al., 2020) would be of limited use and could only be employed in certain biofluids. While there may be scope for combining the depletion of common EV markers with the depletion of markers of abundant cell type-specific EVs to further enrich for rare EV populations, the case for each additional depletion target needs to be considered. For example, combining CD9 depletion with CD41 depletion to remove platelet EVs (as the most abundant EVs by cell type in plasma) would have a negligible effect since 98% of CD41-positive EVs are CD9-positive (Khanna et al., 2023).

Moreover, depleting common EV markers could enable the isolation of relatively pure cell type-specific EV populations compared to using positive selection alone by removing contaminating EVs that also display the positive selection target (and so are co-isolated during immunoisolation). For example, CD9 is highly expressed by PNS neurons but is not expressed by neurons in the brain (Figure 2, Table 1, Table 2), indicating that negative selection to deplete CD9 will deplete CD9-positive PNS neuronal EVs and select for CNS neuronal EVs for biomarker analysis. Thus, CD9 depletion could be used to overcome the molecular overlap of positive selection targets shared between neurons of the CNS and PNS (Table 1). Accordingly, CD9 has been used as a marker to distinguish cells of the CNS and PNS by immunofluorescence microscopy and single-cell RNA sequencing (Ishibashi et al., 2004; Zeisel et al., 2018). This suggests that a strategy of CD9 depletion in combination with positive selection of neuron-enriched proteins could be used to separate brain-derived neuronal EVs (and associated biomarkers) from EVs released by PNS neurons and other cell types, which could not be achieved using a negative selection approach targeting e.g. platelet EVs for depletion.

Untouched isolation to select for tissue-derived extracellular vesicles

Whilst a combination of positive and negative selection is required for isolation of specific EV populations from a single cellular source, negative selection by itself has also been employed to enrich for rare EVs derived from tissue (as depicted in Figure 1C). Yu et al. (2022) developed an ‘untouched’ isolation strategy by using immunodepletion to separate tumour

cell-derived EVs from heterogenous tissue-derived EVs without the need for a positive isolation step. After enriching for EVs from tissue interstitial fluid using an enzymatic digestion and ultracentrifugation protocol, non-tumour cell EVs were depleted by targeting CD45, CD144, CD41, and CD235 (removing leukocyte, endothelial, platelet, and erythrocyte EVs, respectively). By simultaneously depleting EVs from these four different cell types, which had previously constituted 62.4% of the total EV pool (52.4% leukocyte EVs, 5.1% endothelial EVs, 3.4% platelet EVs, 1.5% erythrocyte EVs), the proportion of tumour-derived EVs in the EV preparation increased from 37.6% (pre-negative selection) to 92.5% (post-negative selection) (Yu et al., 2022).

There were multiple reasons for omitting positive immunoaffinity isolation of the desired EV population. Firstly, it eliminated the need to identify a cell type-specific EV marker for positive selection of the tumour EV population and enabled enrichment of the entire heterogenous assortment of tumour cell-derived EVs. This overcame a limitation of positive selection strategies that only select for EVs bearing the positive selection target, whilst disregarding EV subpopulations released by the cell of interest that do not carry that marker, potentially missing EVs with biomarker cargo that may inform about the disease state (Ramirez et al., 2018).

Secondly, since the tumour-derived EVs did not go through the immunocapture procedure, there was no need to separate the EVs from an antibody-coated surface, which can require non-physiological conditions (e.g. acid treatment/high-salt concentrations), as well as being time-consuming and inefficient in eluting intact EVs (Karimi et al., 2022). Moreover, following EV elution, antibodies remaining bound to the positive selection target on the EV surface can prevent subsequent antibody binding in detection assays, alter the protein's structure and distribution on the membrane, and block EV internalisation by recipient cells (Chen et al., 2018; de la Torre-Escudero et al., 2019, Hoshino et al., 2015). Therefore, the untouched negative selection strategy enabled the natural properties of the EVs of interest to be preserved (Yu et al., 2022).

Thirdly, negative selection can be relatively time and labour-efficient since it does not require the washing steps performed in positive selection to separate off-target molecules adsorbed on the immunocapture surface, or an elution step (Kabe et al., 2019). For example, Sharafeldin et al. (2023) demonstrated that the majority of CD9-positive EVs could be depleted within 30 minutes from neat serum, where they constitute 75-86% of total EVs (Hoshino et al., 2020; Sharafeldin et al., 2023). This was achieved by using a magnetic field to promote interactions between EVs and the magnetic immunocapture beads within a microfluidics device (Sharafeldin et al., 2023). Therefore, the single-step negative selection approach could shorten the duration and hands-on processing time of clinical assays for EV-based biomarkers, whilst amplifying the biomarker signal-to-noise ratio by removing abundant EV populations unrelated to the disease state.

Based on these advantages of omitting the positive selection step, the 'untouched' tumour cell-derived EVs enriched by Yu et al. (2022) were maintained in a biocompatible form (termed 'natural EVs' by the authors) during the isolation procedure, which enabled direct experimentation with biological systems. It was found that these natural tumour cell-derived EVs remained detectable in circulation almost four times longer (120 minutes) than the tissue-derived EVs (pre-negative selection), which only remained in circulation for 40 minutes after being injected into the tail veins of mice (Yu et al., 2022). Additionally, the tumour cell-derived EVs displayed a specific tropism for the lymph nodes, whereas the heterogenous tissue-derived EVs did not display the same tissue distribution specificity. Moreover, EVs derived from an in vitro cell line corresponding to the tumour type (oral squamous cell carcinoma) did not interact with CD8 T cells and B cells to the same extent as the ex vivo tumour cell-derived EVs. This demonstrates the limitations of EVs isolated from in vitro cultures in replicating the biological effects of EVs produced in a physiological environment (Yu et al., 2022).

The purity of EVs derived from a single cellular source obtained by Yu et al. (2022) from tissue using negative selection alone might be difficult to achieve in more complex biofluids such as plasma due to the diversity of cells that contribute to the total EV pool. However, single-step negative selection may be sufficient to amplify the signal-to-noise ratio of pre-determined EV-based biomarkers in plasma that had previously been identified through positive selection or combined positive and negative selection strategies. For the abovementioned reasons, this would facilitate the enrichment and direct detection of EV-based biomarkers derived from a single cellular origin without the need for further sample processing (e.g. positive selection), especially if the negative selection antibodies could be integrated into a microfluidics device (Yu et al., 2022).

Alternatively, the 'untouched' single-step negative selection approach could be used for biomarker discovery by unveiling correlative changes in peripheral EVs that mirror disease states in tissue. Profiling total EVs from blood (without positive or negative selection) does not yield cell type-specific disease information (Fordjour et al., 2023). However, it has been used to identify biomarker signatures in blood samples through changes in peripheral EV miRNA

content in Alzheimer's disease and amyotrophic lateral sclerosis (Cheng et al., 2020; Vassileff et al., 2024). This method has the advantage that it does not require isolation of cell type-specific EVs, so the identified biomarkers could feasibly be used in clinical tests with minimal processing of the sample. Incorporating single-step negative selection could benefit this total-profiling approach by removing abundant EVs to unmask biomarker signatures that had otherwise been obscured, whilst only adding a limited amount of hands-on labour.

The use of negative EV markers to screen for contaminating EV populations in rare EV isolates has also been proposed to determine the origin of detected biomarker signals before more comprehensive analysis and clarify whether the biological signal truly comes from the EVs of interest (Newman et al., 2023). The introduction of negative markers as a screening tool could be particularly useful where positive markers alone are unable to distinguish two EV populations released by closely related cell types (for example, EVs from neurons of the CNS and PNS).

Selection of future targets to isolate biomarkers in rare extracellular vesicle populations

There are now many datasets from which candidate targets for positive and negative selection of rare EV populations can be identified. These include the Human Protein Atlas (proteinatlas.org, Uhlén et al., 2015) and the Mouse Brain Atlas (mousebrain.org; Zeisel et al., 2018), as well as EV proteomics atlases of cells, tissues, biofluids, and disease states (Hoshino et al., 2020; Huang et al., 2023; Kugeratski et al., 2021; Liu et al., 2022). However, after selecting a candidate EV target, the case of L1CAM exemplifies the biological and technological factors that could affect cell type-specific EV isolation and may influence the interpretation of biological signals in biomarker studies.

These factors include alternative (non-EV) mechanisms of secretion of the candidate immunoisolation target, such as those involving alternatively spliced forms or ectodomain shedding (Norman et al., 2021). For ectodomain shedding, there are databases (SheddomeDB; Huang and Wu, 2023), predictive tools (DEEPSMP; Cao et al., 2020), and tissue-specific studies (e.g. for the mouse brain; Lobete et al., 2024) that can indicate whether a membrane protein is likely to be shed through proteolytic processing. Relatedly, 71% of identified glycoproteins in the murine neuronal secretome have been shown to be shed ectodomains (Tüshaus et al., 2020) and as many as 8% of annotated human transmembrane proteins are predicted to undergo ectodomain shedding (Tien et al., 2017). Whilst alternative secretory mechanisms do not preclude the utility of a candidate target for EV immunoisolation (Nogueras-Ortiz et al., 2024), they may need to be considered as potential confounding factors during the experimental process. In that regard, proteinase K assays have been used to show that the targeted protein domains exist outside of the EV lumen (You et al., 2023) and could be used to clarify the association of transmembrane immunocapture targets with EVs (Figure 2C), as well as differentiate intravesicular biomarker cargo from free, soluble forms of the same protein (Gilboa et al., 2024).

Negative selection approaches have emerged as alternative enrichment strategies to positive selection alone, with advantages that include enhancing the biomarker signal and preserving natural properties of EVs (Ganesh et al., 2023; Ko et al., 2016; Notarangelo et al., 2019; Yu et al., 2022). Whilst ideal targets for positive selection are specific for EVs from the cell type of interest, negative selection targets benefit from being general markers of EVs to deplete diverse, non-target EV populations (Almousa et al., 2025). Therefore, shifting away from using CD9, CD63, and CD81 to define EV populations irrespective of EV cellular and subcellular origins, and taking advantage of EV marker heterogeneity (Kugeratski et al., 2021; Spitzberg et al., 2023; Su et al., 2024), could facilitate discovery of common EV markers as negative selection targets.

Incorporating negative selection into EV biomarker detection protocols could also promote future clinical translation by reducing pre-analytical variability caused by the release of EVs into blood samples ex vivo. Platelet EVs released during blood processing and storage are a major source of contamination with EVs enriched in CD41, CD42, CD62, and CD9 that are not related to the disease state (Małys et al., 2023) and the degree of contamination varies depending on methodological differences (Karimi et al., 2022). Therefore, depletion of platelet-derived EVs by targeting platelet-enriched proteins will facilitate standardisation in the detection of biomarkers in EVs by removing contaminating platelet EVs which could mask the biomarker signal to differing extents depending on ex vivo treatment of the blood sample.

Additionally, context-dependent biological factors need to be accounted for in EV immunoisolation studies since the presence of surface markers on EVs can change depending on cell activation states (Yang et al., 2018), pathological conditions, or the age and sex of the sample donor (Huang et al., 2025). Similarly, technical factors during the EV isolation procedure can influence the presence of surface markers on EVs. For example, enzymatic digestion of tissue to access EVs within the intercellular space was shown to also digest exposed domains of EV proteins, affecting the recovery of certain markers on EVs derived from brain tissue (Matamoros-Angles et al., 2024). Moreover, EV isolation methods used to separate total EVs from biofluids, and freezing of the sample, both differentially impact recovery of

certain EV populations and, thus, the presence of the targeted EV population in the sample (Droste et al., 2021; Gelibter et al., 2022; Paget et al., 2022).

Finally, the utility of a candidate for immunoisolation may depend on the availability of a commercial antibody for the targeted molecule since generating and validating new antibodies can be time consuming, costly, and technically challenging. Advances in affinity agents, like the development of oligonucleotide aptamers targeting CD9, CD63, and PTK7 (An et al., 2023; Xue et al., 2021; Zhang et al., 2019), may reduce the costs associated with scalable antibody production for clinical assays of novel EV-based biomarkers (Roy et al., 2021) and enable capture and release of intact EVs under physiological conditions (Brambilla et al., 2021). Additionally, the use of emerging biosensing technologies, which enable real-time analysis of binding kinetics to EVs (Yildizhan et al., 2021), could streamline the process of optimising binding agents for immunoisolation. With the correct affinity agent, the capture efficiency for the targeted EV population can be as high as 100% (Dias et al., 2024; Fortunato et al., 2022), facilitating the recovery of low abundance EV populations for detection of their biomarker cargo in clinical assays.

Ethics and consent

Ethical approval and consent were not required.

Data statement

No data are associated with this article.

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✗ **Suman Dutta**
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This is a timely and interesting review on an important problem in extracellular vesicle (EV) biomarker research: how to enrich rare brain-related EV populations from peripheral blood. The manuscript is relevant and overall well written. The discussion of the L1CAM literature is extensive, and I agree that the field needs to think more seriously about selection strategies and may not need to rely solely on single-step positive immunocapture from crude samples. However, after reading it carefully, I think the central conclusions currently extend beyond the strength of the supporting evidence.

Major comments

The CD9-depletion idea is interesting, but at present it is still largely conceptual. The manuscript presents this more strongly than the available evidence supports, especially given that the paper itself acknowledges that such combined positive/negative selection strategies have not yet been widely established in EV research. At the moment, this reads more like a promising hypothesis than a validated framework.

The manuscript moves too quickly from cell-type expression data to EV identity. A central argument is that mature CNS neurons largely lack CD9, whereas PNS neurons express it, and therefore CD9-positive 'neuronal' EVs in plasma may substantially derive from peripheral neurons or other non-neuronal sources in circulation. This is plausible, but it is still an inference. Cellular transcriptomic/proteomic expression should not be treated as equivalent to EV-surface composition without direct validation.

The conflict between Norman et al. and Nogueras-Ortiz et al. is summarized, but not decisively resolved. The review appropriately discusses both the soluble-L1CAM model and the later single-EV studies arguing for L1CAM-positive EVs. However, it does not define clearly what

evidence should now be required to resolve this discrepancy. The manuscript should propose a more explicit validation roadmap for future studies.

The discussion of internal-domain L1CAM immunocapture reveals a major mechanistic problem that needs a firmer conclusion. The manuscript correctly notes that, if L1CAM is present as a type I transmembrane protein on intact EVs, its internal domain should be intraluminal and inaccessible to antibody binding. Yet several cited studies using antibodies against the internal domain reportedly isolate EV-like particles. At present, the manuscript lists possible explanations, but it should state more directly that this is a major unresolved issue with important implications for interpretation of prior biomarker studies.

The article should place stronger emphasis on orthogonal validation of EV topology and target localization. I agree with the idea that proteinase K-type approaches could help distinguish EV-integral from soluble or surface-associated forms of L1CAM. However, this point currently appears too late and too softly. In a review centered on L1CAM, I would expect a more explicit statement that claims of successful neuronal EV capture require orthogonal validation of membrane topology and exclusion of soluble-protein carryover and antibody-driven artifacts.

Methodological heterogeneity across cited studies is not analyzed rigorously enough. The manuscript does note that precipitation-based workflows can produce aggregation of soluble proteins and nonspecific capture, and it cites the marked discrepancy in α-synuclein measurements between precipitation-based and SEC-based workflows. This is an important point. However, the manuscript needs a more systematic comparison of how different EV isolation methods influence the apparent evidence for L1CAM-positive EVs, because this is central to why the literature remains so inconsistent.

The proposed CD9-depletion strategy does not sufficiently address loss of relevant non-neuronal CNS EV populations. The manuscript itself summarizes evidence that CD9 is present in several non-neuronal CNS cell types, including oligodendrocytes, astrocytes, microglia, ependymal cells, and neural stem-cell-related populations. This means CD9 depletion may not simply remove peripheral contaminants; it may also deplete biologically relevant CNS-derived EVs, depending on the disease context. This trade-off should be discussed more explicitly.

The later sections on negative selection and untouched isolation are less critical than the L1CAM/CD9 section. These sections are interesting, but they are more descriptive and rely on examples that are not fully analogous to rare CNS-neuronal EV isolation from human plasma. The manuscript becomes less analytical in these sections and would benefit from a more balanced discussion of what can and cannot yet be translated from those model systems to plasma-based neurological biomarker studies.

Minor comments

- There is some repetition, especially around CD9 expression and the limitations of single-step positive selection.
- The sentence on page 6 contains a duplicated phrase: ‘used a used a commercial precipitation reagent’.
- A summary table comparing positive selection alone, combined positive/negative selection, and negative-selection-only approaches would improve the review and make the argument easier to follow.

Recommendation: Major revision

Overall, this manuscript addresses a relevant and unsettled topic, and it contains several worthwhile ideas. However, in its current form, it presents a conceptual model more strongly than the current evidence allows. Therefore, the manuscript requires major revision prior to further consideration, including clearer separation of hypothesis from demonstration, more rigorous treatment of methodological limitations, and a more explicit validation framework for future work in this area.

Is the topic of the review discussed comprehensively in the context of the current literature?
Partly

Are all factual statements correct and adequately supported by citations?
Partly

Is the review written in accessible language?
Yes

Are the conclusions drawn appropriate in the context of the current research literature?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Neurodegenerative disease, Biomarker, Extracellular vesicles, Assay development

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 20 March 2026

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This manuscript reviews strategies for enriching extracellular vesicle (EV) biomarkers, focusing on L1CAM-based immunocapture and proposing negative selection approaches to improve specificity. The topic is timely and relevant given ongoing debates regarding the validity of L1CAM as a marker for neuronal EVs and the broader challenges associated with isolating tissue-specific

EV populations.

Major Comments

1. The manuscript places substantial emphasis on the limitations associated with the positive immunoaffinity isolation of brain-derived neuronal extracellular vesicles (BDN-EVs) using L1CAM-targeting antibodies. In addition, it provides a comprehensive and well-contextualized review of the literature on CD9 expression across diverse cell types within the nervous system, highlighting evidence for the minimal or absent expression of CD9 in mature neurons and, by extension, on the surface of neuron-derived EVs. By the midpoint of the review, the synthesis of these observations implicitly supports a potential dual-step isolation strategy involving initial negative selection using CD9 antibodies, followed by positive selection with L1CAM antibodies, to enrich for a more specific BDN-EV fraction. However, this concept is not explicitly articulated or developed into a clear methodological framework. As presented, the CD9-negative/L1CAM-positive selection approach remains largely conceptual and insufficiently operationalized. The manuscript would be significantly strengthened by a more explicit and technically detailed proposal of this strategy, including experimental design considerations, validation approaches (e.g., orthogonal markers, functional assays, or omics-based characterization), and a critical discussion of potential limitations such as antibody specificity, EV heterogeneity, and yield trade-offs.
2. The remaining sections of the review (e.g., the section entitled “Untouched isolation to select for tissue-derived extracellular vesicles”) are comparatively underdeveloped relative to the L1CAM/CD9-focused discussion. These sections are largely descriptive, primarily summarizing published studies without sufficient critical synthesis. In addition, they lack structured presentation – such as summary tables, schematic diagrams, or comparative figures – which would facilitate clearer interpretation and enhance the analytical depth of the review. Notably, there is also a lack of systematic evaluation of the advantages and limitations of the described approaches, as well as an absence of clearly articulated future research directions, both of which are essential for providing a balanced and forward-looking perspective.
3. Given that the studies cited in this review employ heterogeneous methodologies for the isolation of extracellular vesicles (EVs) from biological specimens, a systematic comparative evaluation of these approaches is warranted. Differential ultracentrifugation, while widely used and cost-effective, typically yields a limited recovery (approximately 5–7% of total EVs) and is associated with substantial co-isolation of soluble proteins, as well as potential disruption of vesicle integrity due to high centrifugal forces. Polymer-based precipitation methods, in contrast, enable substantially higher recovery rates (up to ~95–98%) and generally preserve vesicle structure; however, they suffer from poor specificity, resulting in significant co-precipitation of contaminating biomolecules, including soluble proteins, as well as extracellular DNA and RNA. Size-exclusion chromatography (SEC) represents an alternative approach that mitigates many of these limitations, allowing for the isolation of a high proportion of EVs (>90%) with improved purity and preserved structural integrity. Nevertheless, SEC typically results in sample dilution, which may necessitate additional concentration steps depending on downstream applications.

Minor Comments

- Ensure consistent use of terminology (preferably **“extracellular vesicles (EVs)”** rather than alternating with “exosomes”).
- The review is affected by substantial redundancy, with several key concepts reiterated multiple times throughout the text. This is particularly evident in the repeated description of methodologies for isolating blood cell-derived extracellular vesicles (EVs) via negative

selection using markers such as CD45, CD144, and CD41. Similarly, the assertion that CD9 – unlike in the peripheral nervous system – is not expressed in central nervous system neurons is redundantly restated. Such repetition detracts from the overall coherence and conciseness of the manuscript. Careful editing to eliminate redundancy, along with minor language refinement, would significantly enhance clarity, readability, and overall scientific rigor.

- Page 6, Last sentence: “For this, both studies used a used a commercial precipitation reagent (ExoQuick®) to precipitate particles from plasma before performing L1CAM immunocapture.” Remove duplicated phrase.
- Page 14, paragraph 4: “Therefore, CD9 depletion could be used to enrich for CNS-derived neuronal EVs in a variety of biological samples, for example by removing 62-72% of contaminating plasma EVs.” Instead of “contaminating" it would be more appropriate to use the term "non-brain derived" or similar one.

Strengths

- Addresses a timely and important methodological issue in EV biomarker research.
- Provides critical discussion of L1CAM-based EV enrichment strategies.
- Proposes alternative conceptual approaches for improving EV biomarker specificity.

Recommendation:
Major revision
The manuscript raises important points but would benefit from a more balanced discussion of the literature and further development of the proposed negative selection strategies.

Is the topic of the review discussed comprehensively in the context of the current literature?
Yes

Are all factual statements correct and adequately supported by citations?
Yes

Is the review written in accessible language?
Yes

Are the conclusions drawn appropriate in the context of the current research literature?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Extraction, purification, and validation of extracellular vesicles (EVs) from different biological samples, including neuron-derived and hepatocyte-derived EVs. Extraction and analysis of EVs RNA and proteins.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.