Analytical and Bioanalytical Chemistry (2023) 415:1239–1263
https://doi.org/10.1007/s00216-022-04174-5

REVIEW

A review on comparative studies addressing exosome isolation methods from body fluids

Tânia Soares Martins[1] · Margarida Vaz[1] · Ana Gabriela Henriques[1]

Received: 31 March 2022 / Revised: 17 May 2022 / Accepted: 10 June 2022 / Published online: 15 July 2022

Abstract

Exosomes emerged as valuable sources of disease biomarkers and new therapeutic tools. However, extracellular vesicles isolation with exosome-like characteristics from certain biofluids is still challenging which can limit their potential use in clinical settings. While ultracentrifugation-based procedures are the gold standard for exosome isolation from cell cultures, no unique and standardized method for exosome isolation from distinct body fluids exists. The complexity, specific composition, and physical properties of each biofluid constitute a technical barrier to obtain reproducible and pure exosome preparations, demanding a detailed characterization of both exosome isolation and characterization methods. Moreover, some isolation procedures can affect downstream proteomic or RNA profiling analysis. This review compiles and discussed a set of comparative studies addressing distinct exosome isolation methods from human biofluids, including cerebrospinal fluid, plasma, serum, saliva, and urine, also focusing on body fluid specific challenges, physical properties, and other potential variation sources. This summarized information will facilitate the choice of exosome isolation methods, based on the type of biological samples available, and hopefully encourage the use of exosomes in translational and clinical research.

Keywords Extracellular vesicles · Isolation methods · Biofluids

Overview on exosomes

Exosomes are the smallest subtype of extracellular vesicles (EVs), ranging from 30 to 150 nm in diameter. These nanovesicles of endocytic origin are formed by plasma membrane budding, resulting in early endosomes that mature into multivesicular bodies (MVBs) which bud inward, creating intraluminal vesicles. In the final step, the MVBs can either intermediate the intracellular protein degradation process by fusion with lysosomes, or can fuse with the plasma membrane leading to exosomes release [1, 2]. These nanovesicles carry a variety of molecular cargo including proteins, lipids, and RNA [2]. This ability to pack material was initially thought to be only related to their function as cellular waste disposal systems. However, exosomes emerged as key players in numerous pathways, by mediating cellular communication and signalling events, including inflammation [3] and apoptosis [4]. Indeed, these nanovesicles have been widely related to cancer development [5, 6], and more recently with the pathogenesis of neurodegenerative diseases as Alzheimer's disease (AD) and Parkinson's disease [1, 7].

Exosomes can also be released from numerous types of cells including fibroblasts, intestinal epithelial cells, and neurons, being found in many biofluids as blood [8, 9], cerebrospinal fluid (CSF) [10], saliva [11], urine [12], and breast milk [11, 13]. Hence, these nanovesicles are likewise being addressed as ideal sources for biomarker discovery representing potential tools in disease diagnosis [1, 14]. Nonetheless, exosome isolation methods still require optimization and characterization to define the best procedures to be employed in clinical practice.

Published in the topical collection *Advances in Extracellular Vesicle Analysis* with guest editors Lucile Alexandre, Jiashu Sun, Myriam Taverna, and Wenwan Zhong.

✉ Ana Gabriela Henriques
aghenriques@ua.pt

Tânia Soares Martins
martinstania@ua.pt

Margarida Vaz
margaridavaz@ua.pt

[1] Biomarker Discovery Team, Neurosciences and Signalling Group, Institute of Biomedicine (iBiMED), Department of Medical Sciences, University of Aveiro, 3810-193 Aveiro, Portugal

Therefore, many research focused on these topics and initiatives promoted by the blood EVs work group are being directed to increase standardization and reproducibility in blood-derived EVs research [15].

Exosome isolation methods

Considering the increasing interest in this field, several techniques for exosome isolation and characterization have emerged (Fig. 1) [2, 14, 16]. Regarding the methods of isolation, ultracentrifugation (UC) is one of the most employed,

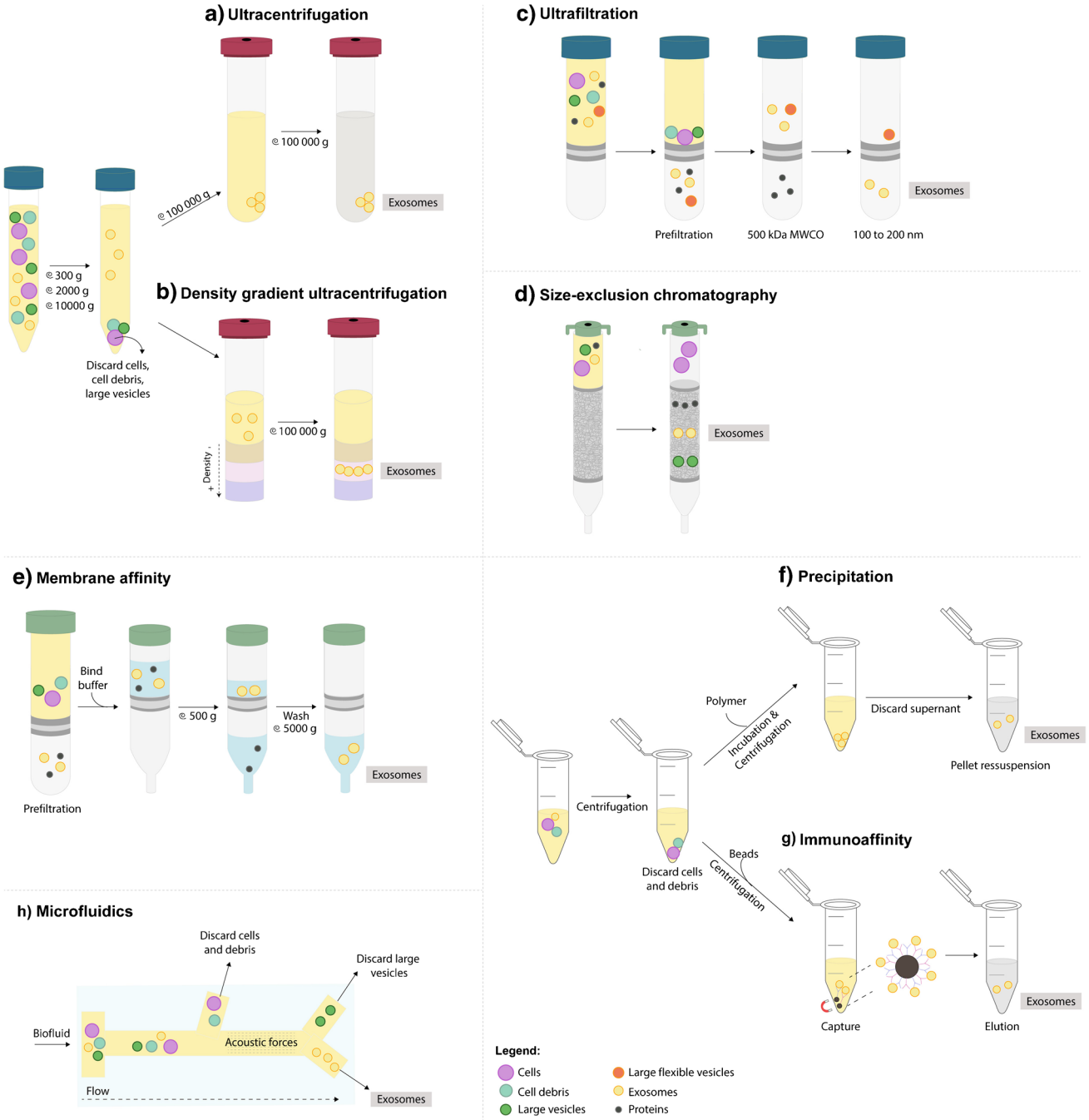

Fig. 1 Schematic representation of the most common exosome isolation methods. (**a**) Ultracentrifugation. (**b**) Density gradient ultracentrifugation step. (**c**) Ultrafiltration. (**d**) Size exclusion chromatography. (**e**) Membrane affinity. (**f**) Precipitation-based methods. (**g**) Immunoaffinity. (**h**) Microfluidics (acoustic)

consisting in the application of a high centrifugal force to a fluid to allow the deposition of particles according to their size (Fig. 1a). At first, samples need to be centrifuged at low speed to remove dead cells and cell debris and then, centrifuged at increasing speeds (~ 10,000 g) to sediment larger EVs, as microvesicles. This is followed by a high-speed ultracentrifugation step at (~ 100,000 g) to pellet small EVs such as exosomes. Usually, an additional UC step is performed to wash the exosome pellet in phosphate-buffered saline (PBS) and decrease protein contaminants [17]. Although UC is the standard method for exosome isolation, it is a time-consuming process that requires an expensive equipment (ultracentrifuge) and renders in low exosome yield. Further, biofluids with higher viscosity require longer UC steps and higher centrifugation periods which can compromise exosome integrity. UC can also be combined with a density gradient (dgUC) of sucrose, Nycodenz (iohexol), or iodixanol to separate particles according to their density, yielding exosome preparations with higher purity. In this method, samples are placed in the top of a density gradient medium (higher densities in the top) and exosomes move through density gradient medium during the UC step (Fig. 1b). The principle behind is that under centrifugation, particles with different sedimentation coefficients settle in distinct layers that can be further collected. Exosomes float until get equilibrium density, ranging from 1.10 to 1.21 g/mL on a sucrose gradient, forming a fraction zone that can be easily recovered [18–20].

Other exosome isolation methods available that separate vesicles according to size are ultrafiltration (UF), size exclusion chromatography (SEC), and field-flow fractionation (FFF). In UF, one or several filters with distinct molecular weight cut-offs or size are used to isolate exosomes, separating them from large particles (Fig. 1c). Although UF does not require specialized equipment and is easy to perform, it presents low efficiency due to the clogging of filter units. To solve this problem, tangential flow filtration (TFF) had arisen. In contrast with the typical filtration, where pressure is applied in the same direction of the flow, in TFF the pressure is applied perpendicularly. This avoids the clogging and, by manipulating hydrodynamic flow forces, it allows a more efficient separation of vesicles from small protein contaminants, starting from high fluid volumes [21]. Another advantage of this procedure is that filters retentate can be recirculated and filtered repeatedly, increasing the protocol efficiency, and TFF use provides isolation of biological active EVs [22], while in typical UF the pressure applied frequently leads to vesicle deformation or lysis. In TFF applied to sequential filtration, at first, the biofluid is prefiltered to remove cells, cell debris, and large rigid vesicles. Next, TFF can be performed to exclude free proteins, using 500-kDa molecular weight cut-off (MWCO) hollow fibers and then, the biofluid can be filtered using 100- to 200-nm filters to separate exosomes from large flexible vesicles [23]. In SEC, vesicles in biofluids are separated according to their size when passing through a porous polymeric phase with beads, multiple tunnels, and pores (Fig. 1d). Vesicles with small hydrodynamic diameters usually ranging from 30 to 200 nm are trapped into pores and lately eluted. Particles with sizes higher than 1 µm do not enter the porous phase and particles smaller than exosomes enter in the porous zone but are not retained. The eluate is collected in several fractions, and, after column void volume, the first fractions contain large vesicles whereas the following contain small EVs (e.g., exosomes), and the last fractions have smaller particles or proteins. SEC is also a time-consuming method but provides pure exosome isolations, preserving exosome integrity, and thus, SEC can be combined with other techniques to increase EVs purity and yield [18–20, 24]. Membrane affinity–based spin column is another isolation technique based on chromatography (Fig. 1e). In this method developed by Qiagen (ExoEasy), the binding of EVs to a column membrane is promoted based on a vesicle-specific biochemical feature, but this interaction does not distinguish between exosomes and apoptotic bodies, cells, or cell debris, requiring a previous centrifugation step or filtration. Other larger particles or protein aggregates are removed through column washes and then EVs are eluted intact in an aqueous buffer with inorganic salts. As an option, EVs can be lysed and eluted with QIAzol and then, total RNA extracted upon addition of chloroform and centrifugation (ExoRNeasy) [25, 26]. In the recent field-flow fractionation method, fluids are injected into a channel with a permeable membrane that works as an accumulation wall. Then, these are subjected simultaneously to a longitudinal parabolic fluid that carries EVs along the channel and to a perpendicular gradient or force field that accumulate these vesicles at the bottom wall, according to their size. The asymmetric FFF is the most common gradient applied but others can be used as electrostatic forces or centrifugal force. In asymmetric FFF, smaller vesicles became positioned further from the wall while larger vesicles are pushed to it and, thus, small vesicles are eluted first than the larger [27, 28]. Although this is a rapid and efficient procedure, it requires trained individuals.

Since exosomes present negative charged membrane components (e.g., phosphatidylserine), several charge-based isolation methodologies have arisen to explore this property, including ion-exchange techniques or electrophoresis. In ion-exchange methods, negative-charged membranes interact with anionic positive charged surfaces (e.g., cationic polymer-coated beads) and, after, EVs are eluted by the addition of buffers with high salt concentration [29, 30]. In electrophoresis, EVs are separated according to their ability to move when an electric field is applied [31].

Precipitation is a method employed to isolate exosomes from body fluids by altering EVs solubility. This involves a low-speed centrifugation step to remove cells and debris followed by mixing and incubation with water-excluding polymers, like polyethylene glycol (PEG), that link to water molecules and enhance the precipitation of less soluble components, as exosomes. Subsequently, the mixture is centrifuged at low speed, pelleting EVs with exosome-like characteristics (Fig. 1f). Polymer-based isolation renders high exosome yields, preserving EVs integrity. However, it can also co-precipitate protein aggregates and other materials as lipoproteins, and the solely polymer reagent can interfere with downstream analysis as mass spectrometry, decreasing its sensitivity [18–20]. Nonetheless, the pellet resuspension in water or PBS promotes the disassembly of the polymer network. Polymers are biochemically inert and if needed the remaining residual polymers can be easily removed using gel filtration resin columns [32].

In addition to these methods, the discovery of exosome surface proteins and receptors allowed the development of immunoaffinity-based methods. These are commonly based on interactions between antigens, as surface exosome markers, and antibody coupled to magnetic beads (Fig. 1g). Further, the beads-exosomes complexes formed are characterized by several techniques as flow cytometry and electron microscopy. The main disadvantage of exosome immunocapture methods is that only subpopulations of exosomes are isolated, depending on the target exosome marker and, to note, sometimes it is difficult to elute exosomes from beads, decreasing its yield [18–20]. An innovative technology proposes the use of noncovalent interactions to allow a better disassembly of exosomes from beads. Superparamagnetic beads coupled with anti-CD63 (an exosomal marker) antibodies, based in the interaction between β-cyclodextrin and 4-aminoazobenzene, were constructed to capture exosomes that were then eluted by the addition of the competitive host molecule α-cyclodextrin [33]. This new methodology was efficient in exosome elution and render in pure exosome isolations [33], encouraging the use of immunoaffinity-based methods.

Microfluidic techniques have recently been developed to isolate exosomes from very small amounts of fluids in a rapid, automated, and cost-effective manner, even with high-throughput capacity [18–20]. These small platforms also allow both the isolation and the analysis of exosome cargo which is a great advantage for biomarker discovery and application in clinics. Several isolation principles have been used in microfluidics as immunoaffinity, size-based separation, and contact-free microfluidics. In immunoaffinity-based microfluidics, exosomes are captured by antibodies immobilized in the surface of the platform. As in typical immunoaffinity, these antibodies recognize specific exosome markers and, then, exosomes are eluted after buffer addition. Efforts have been made to improve the antibody immobilization surface area (e.g., using nanoporous structures) and to reduce the non-specific antibody binding to other non-exosome vesicles. To note, beyond exosome isolation, affinity-based microfluidics also allow simultaneous RNA extraction [34]. Another example is the ExoChip commercial device, with surfaces fabricated in polydimethylsiloxane and containing anti-CD63 antibodies immobilized. This device allowed exosome isolation and quantification from serum, after a fluorescent labelling, electron microscopy analysis, and the isolation of intact RNA from immobilized vesicles [35]. An evolved version of this method for isolation and characterization of cancer-specific exosomes has been published, and it is based on lipid-affinity microfluidics exosome isolation instead of immunoaffinity. Annexin V was immobilized on the chip surface and specifically interacted with phosphatidylserine, externalized only in the outer surface of cancer cells and exosome membrane [36]. It is expected that other affinity-based devices will be developed to meet the needs of specific exosome subpopulation isolation. Size-based microfluidics uses several strategies to separate the exosomes from fluids as nanoporous filters and porous silicon nanowire-on-micropillar structures. ExoTIC was the first size-based microfluidics device developed and consisted on the filtration of small volumes of biological fluids through a nanoporous membrane, separating exosomes with higher yield than typical UC [37]. Nanowire-based microfluidic devices were composed of several nanowires fixed in micropillars. These devices allow the isolation of specific size vesicles subpopulations by adjusting the space between nanowires, leading to vesicles trapping and filtering out cells, large vesicles, and proteins. Finally, exosomes were simply eluted with PBS by dissolving the nanowires [38]. In addition, microfluidics deterministic lateral displacement had been used to separate vesicles according to their size in pillar arrays [39, 40]. Recently, ZnO nanowire arrays with a bifunctional peptide were developed to capture cancer exosomes [41]. Regarding contact-free microfluidics, viscoelastic flow, acoustic, and electrophoresis are the most common. In viscoelastic flow microfluidics, distinct size vesicles migrate according to the manipulation of elastic lift forces applied in a viscoelastic medium [42]. In acoustic-based microfluidics, the application of ultrasound waves exerts differential forces into particles and causes the separation according to their physical properties (Fig. 1h). These devices also require a very small sample volume, which is an important advantage in clinical research. A combination of microfluidics technology and acoustics was employed in exosome isolation from blood, providing high yield and purity. Additionally, this method allowed automation of the EVs isolation process and the resulting vesicles were intact and retained biological activity [43]. Despite the promising results, these recent microfluidics-based methodologies need additional validation and

run of large-scale tests [18–20]. Exosomes can be isolated through electrophoresis and microfluidic devices had been developed, taking advantage of this principle. Electroactive strategies can be used to drive exosomes along the devices and, for example, through membranes [44].

As discussed, several methods can be employed to isolate exosomes and novel approaches are still arising. All techniques exhibit advantages and limitations, and these have been summarized in Table 1, for the most common exosome isolation methods.

Exosome quantification and characterization techniques

According to MISEV2014 and MISEV2018 recommendations, the characterization of EV preparations must include the quantitative description of the EVs source and isolation procedure details, including the starting volumes of body fluids, cell culture media, or mass volume, when isolating EVs from tissues, and report of EVs size and concentrations.

Table 1 Advantages and disadvantages of exosome isolation methods

Methods	Advantages	Disadvantages
Ultracentrifugation and ultracentrifugation with density gradient (dgUC)	○ Reduced reagents cost ○ Increased purity of isolated exosomes (with dgUC)	• Time-consuming and labor-intensive process • Expensive equipment (ultracentrifuge) • Large sample starting volumes • Low exosome yield • Possible exosomes damage due to high-speed centrifugation (required by biofluids with higher viscosity) and contamination with particles with the same density • Additional purification steps may be required
Ultrafiltration	○ Fast and easy to perform ○ Special equipment not required	• Possible exosomes loss due to trapping in membranes • Deterioration of vesicles caused by shear stress
Size exclusion chromatography	○ Easy to perform ○ Preserves exosome integrity (structure and biological function)	• Time-consuming method • Contamination by other particles (e.g., lipoproteins) • Sample dilution
Membrane affinity	○ Easily available commercially	• High reagents cost • Not suitable for large sample volume
Field-flow fractionation	○ Fast and efficient procedure ○ Identify small vesicle subpopulations	• Trained individuals and specific equipment
Charge-based	○ Preserves exosome integrity ○ In combination with other methods, improves purity of exosome preparations	• Not always suitable for biological fluids with complex matrices, as blood, because these contain other charged molecules
Precipitation	○ Easy to perform and no need of specialized equipment ○ Suitable for large sample volumes but usually requires small sample volumes ○ High exosome yields ○ Easily available commercially	• Moderate reagents cost • Co-precipitation of protein aggregates and other materials as lipoproteins • Polymers can interfere with downstream analysis
Immunoaffinity	○ High purity ○ Isolation of exosome subpopulations of interest ○ Easily available commercially ○ Special equipment is not required ○ Small sample volumes	• High reagents cost • Non-specific binding of antibodies selected • Isolation of exosomes subpopulations • Difficult to elute exosomes from beads, impacting exosome structure
Microfluidics	○ Rapid, high-throughput, and automated capacity ○ Cost-effective ○ Allow simultaneously exosome isolation and characterization ○ Very small sample volumes	• Requires training • Standardization and validation are needed in large cohorts • High costs in device development • Scalability may be challenging

Characterization of single vesicles must be carried out through techniques that provide high-resolution images of EVs, based on electron microscopy, and using single-particle analyzers to detail EVs biophysical features. Total protein and lipid content can also be quantified and the purity ratios (proteins to particles, lipids to particles, or lipids to proteins) should be determined. In addition, at least three positive protein markers (transmembrane and cytosolic proteins) and one negative marker should be assessed in EVs preparations. Non-vesicular, co-isolated contaminants can also be addressed [45, 46].

A critical step after EVs isolation with exosome-like characteristics is its characterization in terms of particle size, morphology, concentration, and markers. Therefore, advanced methodologies have been developed to this end.

Nanoparticle tracking analysis (NTA) is used to measure particle size and concentration. To do so, a laser beam hits nanoparticles in liquid suspension and the light scattered is captured by a camera, generating several frames. According to Brownian motion, the smaller particles move faster than the larger. Using video analysis software, the movement of each particle is tracked and analyzed individually, and the diffusion coefficient determined. Then, this coefficient is used to calculate particle hydrodynamic diameter through the Stokes–Einstein equation. Particle concentrations are determined according to the number of particles in suspension, tracked individually. NTA provides a direct quantification of small particles with sizes between 40 and 1000 nm and concentrations between 10^7 and 10^9 particles per mL, requiring a minimum of 500 µL of particle suspension for the analysis. NTA results include particle diameter, concentration, size distributions, and 3D plots that combine size, particle number, and intensity of light scattered, facilitating the visualization of distinct nanoparticle subpopulations. Since NTA is based on Brownian motion, it is sensitive to the presence of aggregates or higher particles which can constitute a disadvantage of the technique. Despite that, NTA allows direct quantification of EVs in a quick and easy manner and it can also detect fluorescently labeled EVs [47, 48].

An alternative technique to measure EVs size is dynamic light scattering (DLS) and, like NTA, it is based on the Brownian motion principle. However, while NTA determines the individual particle size based on the diffusion coefficient, DLS calculates sizes according to intensity changes of scattered light, measuring a bulk of nanoparticles [49]. Although DLS is a very sensitive method, it presents limitations when distinguishing heterogeneous vesicle populations. In addition, the presence of larger particles or aggregates makes the detection of small nanoparticles difficult and influences the size determinations by DLS in a greater extent than by NTA [47, 50].

More recently, tunable resistive pulse sensing (TRPS) has been applied to measure the number and diameter of EVs by passing particles through the pores of a membrane with applied voltage, using small sample volumes, around 30 µL. As particles are forced to pass pores, the current flowing decreases. These changes in the electric current are proportional to the volume of each individual nanoparticle, and the nanoparticle flow rate allows to calculate the concentration of EVs. TRPS disadvantages include its lower sensitivity to detect small vesicles and the possibility of pore blockage by particles, resulting in measurement changes [2].

The preferred techniques employed to determine vesicle morphology are transmission electron microscopy (TEM) or cryo-electron microscopy (cryo-EM) [51, 52]. TEM is a commonly used technique; however, it must be considered that the dehydrating conditions used in the fixation of samples, the use of metal contrasting agents, and drying can affect the shape of EVs, generating the cup-shaped structure as an artifact in exosome preparations [52, 53]. In cryo-EM, samples are vitrified and, thus preserved in their native hydrated state, allowing to observe exosomes' spherical shape and, clearly, the lipid bilayer [52, 54].

For confirmation of exosomal nature of preparations, transmembrane or GPI-anchored proteins and cytosolic proteins must be analyzed. Among the most commonly assessed are tetraspanins (e.g., CD63, CD81) and endosomal sorting complex required for transport (ESCRT)-I/II/III components and its accessory proteins (e.g., TSG101, ALIX, Flotillin's 1 or 2, heat shock proteins 70 or 90). The presence of exosome-negative markers as calnexin, albumin, or apolipoproteins must be determined to assess the purity of EVs preparations [45]. The most used techniques to evaluate exosome markers include Western blotting and flow cytometry (that can also measure particle size and number) [51]. Enzyme-linked immunosorbent assay (ELISA) is another commonly used method that employs immunoaffinity technology to characterize and quantify exosomes [55–57]. Recently, ultrasensitive single-molecule array (SIMOA) assays were developed to measure the levels of tetraspanins CD9, CD63, and CD81 in CSF- and plasma-derived EVs [58].

Human biofluids as sources of exosomes: challenges and considerations

The isolation of EVs from biofluids can hold a huge potential to advance biomarker discovery, either in the diagnostic or therapeutic fields. Nevertheless, exosome isolation from biofluids is challenging since each biofluid has its specific composition and physical properties, requiring comparison of the various isolation methods to identify the best approaches to be used in each situation. In this review, some exosome isolation challenges and considerations are addressed for human plasma, serum, CSF, saliva, and urine.

In the case of blood, both plasma and serum are viscous fluids, highly concentrated in proteins (e.g., as albumin)

which turn difficult the isolation of pure exosome preparations. It was reported that plasma is more viscous than serum, and thus a trend for lower sedimentation efficiency and higher microvesicles size was observed in the former biofluid, after vesicle isolation through UC [59]. Moreover, other considerations should be taken, as suggested by the ISEV position paper [60]. Although most studies use plasma for EVs isolation, evidence supports that EVs released from platelets soon after blood collection account for a huge percentage of EVs in serum, which might be an advantage for some type of studies [60]. Hence, it would be relevant to conduct additional studies focusing on plasma and serum differences during EVs preparation.

Further, several pre-analytical factors must be taken into consideration prior EVs isolation, since these can change EVs yield and cargo or interfere with downstream applications, namely the time between blood collection and EVs isolation; the type of blood collection tubes; the transport conditions; the temperature and storage periods; the centrifugation; the fasting status; and the physical exercise.

Focusing on some of these factors, the time and type of blood collection tubes are pre-analytical variables that can influence the content in EVs. A study comparing serum collection tubes and ethylenediaminetetraacetic acid (EDTA), heparin, and citrate phosphate dextrose adenine (CPDA) reported that after collection with CPDA and EDTA tubes, the EVs concentration significantly decreased about 1 h after the blood collection, increased after 8 h and, then, returned to the initial levels after 24 h, while for serum or heparin tubes, no changes were observed [61]. Heparin and EDTA tubes were associated with a higher number of EVs while citrate tubes with lowest EVs concentration, although not statistically significant [62, 63]. Another study reported increased concentrations of serum EVs when compared with EVs from EDTA-plasma, citrate, or acid citrate dextrose [64]. No changes in EVs size or morphology had been reported in the use of either citrate, sodium citrate theophylline adenosine dipyridamole, EDTA, or heparin collection tubes [63]. Furthermore, other authors reported that heparin interacted with downstream polymerase chain reaction analysis and directly with EVs, by decreasing their binding and blocking EVs transfer to recipient cells [65].

The impact of transportation was also addressed comparing blood samples placed in an orbital shaker for 1 h, with samples without shaking. The agitation prompted an increase in several EV markers in blood collected in EDTA tubes, which leads to the speculation that the EVs adhered to platelets could be released with shaking [61]. When comparing short (days) and long (months) storage periods, and storage at room temperature and 4 °C, variations were likewise observed. For short-term, storage at low temperatures (−20 °C to −160 °C) showed relatively low signal intensities as detected by EV markers when compared to storage at room temperature or 4 °C. By contrast, for long-term storage, a tendency towards increased signal intensities with lower temperatures was observed [61]. When freezing the samples, heparin tubes provided more stable samples and EDTA tubes presented the highest variations [61]. In particular, long-term storage of plasma EVs at −80 °C or storage of urinary-derived exosomes at −80 °C for 1 week to 7 months did not affect EVs stability [25, 66]. It is mandatory to address the ideal storage conditions for exosomes since it can vary depending on the type of biofluid source [67]. Moreover, successive freeze and thaw cycles are discouraged [68].

The impact of the fasting status at the time of blood collection for EVs isolation is still unclear. Plasma concentrations in EVs, measured with NTA, did not change significantly after the ingestion of a high-fat meal but the plasma concentration of EVs strongly correlated with plasma very-low-density lipoproteins (VLDL) and serum triglyceride concentrations after meal ingestion suggest that NTA particle count may be influenced by the presence of these particles [63]. Another study that evaluated the number of particles through TRPS reported a higher number of particles in the postprandial state, when compared with fasting, and numerous lipoproteins, including low-density lipoproteins (LDL) in both fasting and after meal, although no differences were observed in particle sizes [69]. Chylomicrons, the largest lipoproteins, typically increase in circulation after meals [70] and are metabolized further in VLDL, intermediate-density lipoproteins, LDL, and high-density lipoproteins. All of these lipoproteins are highly abundant in blood and represent relevant contaminants of EVs preparations derived from this biofluid [69], being difficult to distinguish these from exosomes due to size overlap. The literature suggests that blood collection in fasting state is preferable although interpreting conclusions should be cautious, and efforts are still being made to improve EVs isolation and obtain purest preparations under these conditions. The combination of distinct isolation techniques was proven to reduce lipoprotein contamination. Lower amounts of APOE and APOB were observed in serum-derived EVs obtained through cushion UC when compared with UC. Decreased lipoprotein contamination was also obtained in EVs preparations from density gradient cushion UC or from the combination of qEV and dgUC when compared to EVs obtained only through dgUC [71]. In addition, the combination of UC, an iodixanol density cushion, and SEC decreased lipoprotein contamination in plasma-derived EVs. After UC, EVs pellet was placed on the top of a density cushion and the obtained high-density band was then collected and placed in a SEC column. EVs were mainly eluted in fractions 8 and 9 as evidenced by high levels of flotillin-1 and low levels of ApoA1 [72]. A three-step protocol was also stablished to isolate EVs from blood or cell culture media. At first, EVs were isolated using

PEG or UC, and then, floated in an iohexol density gradient during 16 h of UC. Finally, EVs were applied to SEC-based columns. ApoA1 and ApoB100 previously identified in density gradient fractions containing EVs were highly reduced after the SEC step [73]. Additional methods have also been developed to separate exosomes from lipoproteins. Some of these are the acoustofluidic-based separation or agarose gel electrophoresis. As referred above, acoustofluidics is based on the different particle's behavior under acoustic wave pressure. This allowed to distinguish between IDL, VLDL, and chylomicrons, which moved to acoustic pressure antinodes, whereas EVs and HDL particles moved to acoustic pressure nodes [74]. Since EVs, HDL, VLDL, and LDL particles are negatively charged, these can move when an electric field is applied. Hence, lipoproteins and EVs can be separated in 1% agarose gel electrophoresis with tris–acetate-EDTA, according to their size and zeta potential properties. Also, lipoproteins are dyed blue with Sudan Black B which detects triglycerides. EVs are not enriched in triglycerides and thus are not stained. In electrophoresis, the first blue leading band contained HDL and the final leading band contained a mixture of LDL and VDLD. EVs were located between these two bands. Fractions were collected and analyzed by DLS and Western blot [75]. Although the method combination can reduce contaminants, this increases the costs and complexity of the protocols and, unfortunately, leads to the loss of exosomes along the procedures. Beyond lipoproteins or protein aggregates, viruses are frequent contaminants in blood-derived EVs, which prompted the development of new isolation methods such as the nanoscale flow cytometry for EVs separation from viruses [76]. Finally, physical exercise was reported to change the EVs concentration and cargo. A general increase of EVs concentration in circulation after exercise was observed, in both humans and mice [77–80].

The use of blood-derived EVs in the neurodegeneration field rise with the appearance of a two-step neuronal-enrichment exosome isolation methodology [81–83]. It combined EVs isolation from a biofluid through ExoQuick (ExoQ), a precipitation-based method, with a subsequent immunoprecipitation step with antibodies against specific neuronal surface markers (NCAM, L1CAM) [84], astrocyte (GLAST, GFAP, GS) [85], or oligodendrocyte-specific markers (PLP, CNP) [86]. The main purpose of this workflow was the isolation of subpopulations of EVs which could be neuronally derived and, thus, hold important biomarkers for neurodegenerative disease diagnostics. The characterization of L1CAM-positive EVs revealed the enrichment in neuronal-specific proteins [81] and several studies found reproducible altered protein levels between controls and individuals with neurodegenerative diseases, as Alzheimer's or Parkinson's diseases, when testing these L1CAM-enriched blood-derived exosomes. However, concerns regarding the L1CAM enrichment neuronal specificity arise as this protein was found expressed in other tissues than brain (e.g., kidneys). It was likewise observed that L1CAM can exist in both transmembrane and soluble forms and, that the former can be cleaved by metalloproteases, generating a soluble ectodomain which is released extracellularly, plus a cytosolic domain bound to the plasma membrane [87–89]. In addition, another L1CAM soluble form was recently found, generated through alternative splicing [90]. Since most of the antibodies used in the neuronal EVs isolation, particularly in the immunoprecipitation step, were raised against the L1CAM ectodomain, the specificity of the bound, only to transmembrane L1CAM forms present on EVs, cannot be ensured. Besides it is expected that these antibodies also bind to soluble L1CAM forms. Importantly, in a recent study that used SEC and dgUC to separate EVs from soluble proteins, in both plasma and CSF, it was shown that the majority of L1CAM found in EVs fractions was not bound to EVs, but instead was mainly present as a soluble form [91]. Furthermore, it was observed that the majority of exosomal markers CD9, CD63, and CD81 measured through SIMOA assays were not eluted in the same fractions as L1CAM and albumin, for both SEC and density gradient methods. Complementary Western blot analysis of L1CAM present in CSF and plasma was also carried out, using two antibodies, one directed to the external and the other to the internal L1CAM domain. For CSF, only a 200-kDa band corresponding to the binding of the external domain antibody was observed, whereas in plasma two bands at approximately 220 kDa were detected, reflecting the bound to both internal and external domains. In addition, a mass spectrometry analysis of plasma L1CAM did not detect the transmembrane domain [91]. At this point, it is not clear which is the proportion of EVs that bound L1CAM in biofluids and the exact nature of exosomes obtained by this enrichment procedure.

Hence, further validation of analytical tools used in EVs isolation and biomarker discovery are still needed. The identification of new neuron-specific targets may improve the use of EVs in the clinical research in a wide range of brain disorders, either as a source of biomarkers or as new avenues to the use of EVs as drug delivery vehicles.

In the context of neurological disorders and biomarker discovery, exosomes isolated from distinct biofluids are indeed the focus of intensive research. Cerebrospinal fluid–derived exosomes represent important tools at this level. This biofluid is present in the brain ventricles and the subarachnoid spaces, being collected through lumbar puncture, an invasive procedure [92]. CSF-derived exosomes are expected to carry mainly neuronal-derived proteins and nucleic acids; however, EVs concentrations are lower in CSF when compared with blood-derived exosomes [24]. EVs isolation from CSF requires higher starting sample volumes, which is often difficult to obtain, mainly in the case of healthy individuals. The collection, storage, and processing

of CSF are susceptible to a wide range of variability sources which strongly affect the downstream molecular analysis, as is the case for Aβ, total-Tau, and P-Tau 181 (the biomarker triplet measured in AD neurochemical-based diagnosis), making it difficult to establish universal cut-offs. In an attempt to standardize procedures, reduce these sources of intra- and inter-laboratory variabilities, and encourage the use of CSF biomarkers, the international quality control (QC) program was implemented by the Alzheimer's Association, and headed by the Clinical Neurochemistry Laboratory at the University of Gothenburg, Sweden [93, 94]. Several sources of variability comprising pre-analytical, analytical, and post-analytical factors were identified, including biological interindividual variability and CSF collection procedures, sample storage and shipment, not excluding CSF contamination with blood during lumbar puncture procedure (pre-analytical); training and equipment, kits lot-to-lot variability in the biomarker triplet analysis (analytical), and data handling (post-analytical) [93, 94]. Sources of variabilities identified for this CSF biomarker triplet can be extended to CSF-derived EVs analysis, but studies of this nature are still lacking for these and other targets or human biofluids.

Saliva is a more easily accessible fluid, also holding potential as a source of biomarkers. Nonetheless, until now few studies used saliva to address physiological or pathological conditions, particularly in the field of neurodegenerative diseases. Sample collection needs urgent standardization since it was reported that saliva collection and composition depend on variables as the location in the oral cavity, the productive salivary gland (parotid, submandibular, sublingual, or minor salivary glands), oral cavity diseases, smoking, drinking, and food intake. Even the time of saliva collection for EVs isolation could constitute a source of variability since saliva composition can change according to the circadian cycle [95, 96]. Complementary studies addressing the impact of non- and stimulated-saliva collection procedures on EVs isolation, as well as the effects of blood contamination either derived from teeth brush or oral cavity wounds, are still needed. Other important aspects that should be considered during EVs isolation from the saliva are the presence of cells and solid contaminants that should be removed by low-speed centrifugation or filtration; samples viscosity, which require the sample dilution in PBS; and the high abundance in immunoglobulins and amylase that can mask the presence of lower abundant proteins. Some strategies used to remove amylase were affinity adsorption [97] and affinity chromatography columns combined with filter systems [98].

Likewise, urine collection involves a non-invasive procedure, it is available in large amounts and, importantly, urine-derived EVs are highly stable. One of the major challenges in exosome isolation from this biofluid is removing the Tamm-Horsfall glycoprotein (THP) or uromodulin which is the most abundant urine protein. This protein can trap or bind EVs [99], leading to EVs co-precipitation at low-speed centrifugations that are usually carried out to remove cell debris and other contaminants. A recent study characterized the first pellet of the differential centrifugation protocol (low speed ≈ 21,000 g) obtained after the remotion of THP by Tris (2-carboxyethyl) phosphine hydrochloride. This pellet, frequently discarded, presented EVs within the size of 40–250 nm with round morphology, and proteomic analysis revealed the presence of exosomal markers. Data support that this pellet obtained at lower centrifugation speed can potentially constitute a source of EVs-related biomarkers [100]. Other methods were used to extract THP, such as combination with various chaotropic reagents aiming its denaturation [101], salt precipitation using NaCl [102], addition of $ZnSO_4$ to promote THP oligomerization and easier sedimentation [103], salting-out CHAPS lysis buffer [99], sucrose gradient, or the single use of dithiothreitol (DTT) to disrupt the cysteine-cysteine interactions [104]. Nevertheless, some protocols were not completely efficient on THP remotion, and agents as DTT could promote protein remodulation with consequences for downstream analysis. Although less abundant than THP, other proteins can constitute important contaminants of urinary EVs preparations, as albumin [105], aquaporin-1 and aquaporin-2 [106] uroplakin, and prokaryotes [107].

Comparative studies on exosome isolation from biofluids

Considering all challenges in EVs isolation from biofluids, the question to be answered lies on the best method to apply in each case. An overview of the literature allowed to identify 55 comparative studies where EVs were isolated from one or more body fluids, using different methodologies (Table 2). The literature search was performed in the PubMed and included two keywords schemes: (1) "((Exosomes) OR (Extracellular vesicles)) AND ((Serum) OR (Plasma) OR (CSF) OR (Saliva) OR (Urine)) AND (Isolation methods comparison)" and (2) "((Exosomes) OR (Extracellular vesicles)) AND ((Serum AND Plasma) OR (Serum AND CSF) OR (Serum AND Saliva) OR (Serum AND Urine) OR (Plasma AND CSF) OR (Plasma AND Saliva) OR (Plasma AND Urine) OR (CSF and Saliva) OR (CSF and Urine) OR (Saliva and Urine)) AND (isolation methods). Review articles and non-comparative studies or comparative studies based on cell culture models or animals were excluded. The data collected will be subsequently discussed. For EVs isolation from CSF, four comparative studies evaluating the different EVs isolation methodologies performance were found, two of which isolated vesicles from CSF and the other two compared EVs isolation from CSF, plasma, and

Table 2 Summary table of comparative studies focusing on exosome isolation methods in human biofluids. Several exosome isolation methodologies were compared using human serum, plasma, cerebrospinal fluid, saliva, and urine. The comparison addressed particle size, yield, exosome markers tested, and purity. *Contamination or negative exosome markers

Biofluids	Exosome isolation method	Starting volume (µl)	Mode size	Particle yield	Purity	Exosome markers	Ref
CSF	- UC - UF liquid chromatography	8 mL	TEM: Similar particle size	NTA yield: UF > UC	-	CD9, Syntenin	[108]
	- SEC – EVSecond L70 - MA – ExoEasy - IA – ExoIntact - IA – MagCapture	1.1 – 1.2 mL	NTA: ExoEasy > others	NTA: ExoEasy > Others	Albumin only detected in ExoEasy	CD63, CD81, Albumin*	[109]
Plasma	- UC - UC + SEC	500 µL	-	NTA: UC + SEC > UC	UC + SEC > UC	CD9, CD63	[110]
	- UC - Col – AppiEV - PP (ExoQ) - MA – ExoEasy	500 µL	TEM: cup-shaped, within expected size range NTA: trend for ExoEasy > others	NTA: AppiEV > others	-	CD81, Hsp70, TSG101, Calnexin*, ApoB*	[111]
	- UC - UCcush - PP – agglutination by lectins - PP – SubX reagent (affinity precipitation) - IA – Lonza kit	1 mL	AFM and cryo-EM: spherical shape NTA and DLS: similar size, within expected size range	NTA: UC > UCcush > Lonza > SubX > agglutination by lectins	Trend for Lonza > SubX > Uccush > UC > agglutination by lectins	CD63, CD81, Calnexin*	[112]
	- UC - microfluidic device ATPS	UC: 5 mL Microfluidics: -	-	NTA: Microfluidics > UC	-	CD63, CD9, TSG101, ApoA1*	[113]
	- UC - PP – ExoQ - PP – TEI - PP – Wayen Exosome Isolation Kit - PP – Ribo Exosome Isolation Reagent - PP – miRCURY Exosome kits	3 mL	TEM: cup-shaped vesicles NTA: similar median size between methods, within expected size range	NTA: Precipitation > UC	UC > Precipitation	CD9, CD63, CD81, TSG101, Albumin*, Calnexin*, ApoA1*	[114]
	- UC - PP – ExoQ - MA – ExoEasy - Clustering-and-Scattering Method	1 mL	TEM: Clustering method = morphology to UC; vesicle shaped, all within expected size range	NTA: Trend for clustering method > ExoQ > ExoEasy > UC	Trend for clustering method > ExoEasy > UC > ExoQ	Alix, CD9, CD81, TSG101	[115]
	- UC - Exodisc-B (centrifugal device)	-	-	NTA: Exodisc-B > UC	Exodisc-B > UC	CD9, CD81, Albumin*	[116]

Table 2 (continued)

Biofluids	Exosome isolation method	Starting volume (µl)	Mode size	Particle yield	Purity	Exosome markers	Ref
	- UC - PP – ExoQ - PP – TEI	UC: 1 mL ExoQ & TEI: 250 µL	TEM: intact EV except for ExoQ	NTA: ExoQ > TEI > UC	UC = ExoQ = TEI	CD9, CD63	[117]
	- SEC – qEV - MA – ExoEasy	2 mL	TEM, NTA: ExoEASY > qEV	NTA: ExoEASY > qEV	qEV > ExoEasy	CD63, CD81, Syntenin-1, TSG101, Albumin*, Calnexin*	[26]
	- UC - PP – ExoQ - PP – Macherey - Filter- ExoTIC	P: ExoTIC – 10 µL to 500 µL; UC, ExoQ & Macherey – 500 µL	SEM, NTA: ExoTIC > ExoQ, Macherey	NTA: ExoTIC > Macherey and ExoQ > UC	-	-	[37]
	- UC - MA – ExoRNeasy	4 mL	NTA: UC = ExoRNeasy	NTA: ExoRNeasy = UC	-	TSG101	[25]
	- Uccush - PP – PROSPR	500 µL to 1 mL	Cryo-EM: PROSPR = UCcush	-	PROSPR > UCcush	Alix, CD9, CD63, CD81	[118]
	- UC - SEC – Seph2B, SephCL-4B and SephS-400	UC: 7 mL Seph: 500 µL to 800 µL	TEM, DLS: Within expected size range	WB: Little quantities isolated in both Seph and UC methods	SephC-4B and SephS-400 > Seph2B > UC	CD63, TSG101, Albumin*	[119]
	- SEC – ExoS - SEC – qEV - PP – ExoQ	ExoS: 250 µL ExoQ: 250 µL qEV: 1 mL	TEM, TRPS: Within expected size range	TRPS: Trend for: ExoQ > ExoS > qEV	qEV > ExoS > ExoQ	Flotillin-1, Albumin*, Calnexin*	[120]
	- UC - dgUC OptiPrep - IA – EI	UC: 5 mL dgUC OptiPrep: 5 or 25 mL EI: 5 mL	TEM: Within expected size range	WB: dgUC OptiPrep > UC > EI	dgUC Optiprep > EI and UC	Alix, CD9, CD63, Flotillin-1, Hsp70, TFRC, TSG101	[121]
Serum	- UC - SEC – qEV - PP – TEI - iSUF	500 µL	TRPS: UC > iSUF	TRPS: iSUF > TEI > UC and qEV	iSUF > TEI > qEV > UC	CD9, CD63	[122]
	- UC - SEC – ExoS	UC: 2 mL ExoS: 100 µL	TEM: UC – heterogeneous cup-shaped vesicles, ExoS – three distinct vesicle populations but less enrichment in CD9 or CD63 immunostaining NTA: Trend for UC > ExoS	NTA: ExoS > UC	-	CD9, CD63, CD81: UC > ExoS	[123]
	- Ultra-high-speed centrifugation (Ultra-Exo) - PP – Prekit-Exo	-	TEM and cryo-EM: Ultra-Exo – cup-shaped vesicles; Prekit-Exo – not obvious structure and presence of impurities NTA: Prekit-Exo > Ultra-Exo	NTA: Trend for Prekit-Exo > Ultra-Exo	-	-	[124]

Table 2 (continued)

Biofluids	Exosome isolation method	Starting volume (µl)	Mode size	Particle yield	Purity	Exosome markers	Ref
	- UC - Column – Norgen kit - PP – ExoQ - MA – ExoEasy - EV-CATCHER - IA – Miltenyi CD63 kit - IA – Mojosort streptadividin beads - IA – MagCapture Tim 4 kit - IA – Dynabeads MyOne T1 carboxylic acid beads - IA – ExoCap CD63 + kit - IA – Dynabeads Streptavidin MyOne T1 beads - IA – ExoFlow CD63 IP kit	100 µL	NTA: ExoQ and Norgen > Others	NTA: Trend for Norgen > ExoQ > EV-CATCHER > UC > ExoFlow > Mag-Capture & ExoEasy	-	CD9, CD63, CD81, Albumin*, ApoA1*, ApoB*	[125]
	- UC - PP – ExoQ	500 µL	TEM: cup-shaped morphology and protein contaminant for both NTA: 30–200 nm; trend for UC > ExoQ	NTA: Trend for ExoQ > UC	Trend for UC > ExoQ	CD9, CD63, TSG101	[126]
	- UC - UCcush - dgUC - dgUCcush - SEC (qEV1 – fractions 7 and 8, qEV2 – fractions 6 and 9) - SEC – qEV-dgUC - PP – ExoQ Plus	200 µL	TEM: cup-shaped vesicles NTA: Trend for dgUC > UCcush > UC & qEV1 > ExoQ Plus & qEV2	NTA: Trend for ExoQ Plus and qEV1 > qEV2, UC, UCcush, dgUC > dgUCcush > qEV1-dgUC	Trend for qEV1 > qEV2 > dgUC > ExoQ Plus > dgUC-cush > UC > UCcush > qEV1-dgUC	ApoE, CD63, TSG101, ApoB*	[71]
	- UC - PP – ExoQ - MA – ExoEasy	-	NTA: Only UC and ExoQ within expected size range; ExoEasy > UC > ExoQ	NTA: ExoQ > ExoEasy > UC	UC > ExoEasy > ExoQ	CD9, CD63, CD81, Albumin*	[127]

Table 2 (continued)

Biofluids	Exosome isolation method	Starting volume (µl)	Mode size	Particle yield	Purity	Exosome markers	Ref
	- SEC – ExoS - PP – ExoQ - PP + IA – ExoQ Plus - PP + IA – Exo-Flow - MA – ExoEasy - PA – ME kit	ExoS: 100 µL - 500 µL ExoQ: 250 µL ExoQ Plus: 250 µL Exo-Flow: 250 µL ExoEasy: 200 µL to 4 mL ME kit: 1 mL	NTA: Similar size	NTA: ExoQ & ExoQ Plus & ExoS > ExoEasy > ExoFlow > ME	ExoQ Plus & ExoEasy > ExoS & Exo-Flow > ME and ExoQ	CD63, CD9, TSG101, Albumin*, ApoB*	[128]
	- Centrifugation at 40000 g - PP – ExoQ - Modified protocol (40000 g + ExoQ)	Centrifugation: 1,2 or 4 mL ExoQ or modified protocol: 1 mL	TEM: expected size range for centrifugation	-	Centrifugation > ExoQ and modified protocol (based on MS)	CD63	[129]
	- multipleUC - SEC – qEV - multipleUC + qEV	2 mL	TEM: Similar size and morphology	NTA: Trend for qEV > multipleUC + qEV > multipleUC	Trend for multipleUC > multipleUC + SEC > SEC	CD63, Albumin*, Calnexin*	[130]
	- UC - SEC – qEV - SEC – ExoS Midi - PP – miRCURY - MA – ExoRNeasy	1 mL	TEM: Within size range and typical morphology NTA: Trend for ExoRNeasy > UC & qEV & ExoS midi > miRCURY	NTA: miRCURY & ExoS Midi > qEV > UC > ExoRNeasy	qEV & ExoS Midi > ExoRNeasy > miRCURY & UC	CD63, CD81, Synthenin, TSG101, Albumin*, Calnexin*	[131]
	- UC - PP – ExoQ - PP – TEI - PP – miRCURY	50 µL to 5 mL or 100 µL to 1 mL	TEM: Within size range NTA: Trend for UC > miRCURY, TEI, ExoQ	NTA: miRCURY, TEI, qEV > UC	-	CD9, CD63	[132]
	- UC - PP – ExoQ - PP – TEI	500 µL	TEM and NTA: Within expected size range	NTA: Trend for ExoQ & TEI > UC	Trend for UC > ExoQ & TEI	CD9, CD63, TSG101, Calnexin*	[133]
	- UC - SEC – ExoS - PP – ExoQ - PP – TEI - PP – PEG - UF – ExoMir	250 µL	NTA: Within expected size range	NTA: Trend for ExoQ = TEI = PEG > ExoS and ExoMir > UC	-	CD81, TSG101	[134]
	- UCcush - PP – ExoQ	62.5, 125, 250, 330 µL	TEM, NTA: Within expected size range	NTA: ExoQ > UCcush	ExoQ > UCcush	CD9, LAMP2, Albumin*, Grp94*	[135]
Saliva	- UC - PP – ExoQuick-CG	5 mL	TEM: UC has cup-shaped structure vs ExoQ-CG more prone to aggregation NTA: Trend for PEG > UC	NTA: ExoQ-CG (2x) > UC	UC > ExoQ	CD9, CD63, CD81, Flotillin 1, TSG101	[136]
	- UC - ExoQ	500 µL	TEM: Within expected size range but trend for ExoQ > UC	ELISA: Trend for ExoQ > UC	UC > ExoQ	CD9, CD63, CD81	[137]

Table 2 (continued)

Biofluids	Exosome isolation method	Starting volume (µl)	Mode size	Particle yield	Purity	Exosome markers	Ref
Urine	- UC - Col – Urine Exosome Purification and RNA Isolation Midi Kit - HFD	HFD: 50 mL UC: 30 mL Col: 10 mL	TEM, NTA: Typical morphology; HFD and UC > Col	NTA: HFD and UC > Col	UC > HFD > Col	CD9, TSG101, Albumin*, THP*	[138]
	- UC - SEC – qEV - PP – ExoQ-TC Plus	100 mL	-	NTA: Trend for qEV > UC > ExoQ	-	CD9, TSG101	[139]
	- UC - UC-SEC - Conc-SEC - PP – PEG - PEG-SEC	50 mL	TEM, NTA: Cup-shaped vesicles and similar size although PEG tend to isolate larger vesicles	NTA: Trend for UC > UC-SEC > Conc-SEC, PEG > PEG-SEC	TEM: Trend for UC-SEC, UC, Conc-SEC > PEG, PEG-SEC	CD9, CD63, CD81, TSG101	[140]
	- UC - IA – EVTRAP	10 mL	-	WB: Trend for EVTRAP > UC	EVTRAP > UC	CD9	[141]
	- UC - HFD	UC: 200 mL HFD: 200 mL	TEM: heterogeneous population, round or cup-shaped morphology NTA: within expected size range, similar size distribution	NTA: Similar	-	ALIX, CD9, CD63, TSG101	[142]
	- UC - PP – ExoQ - PP – TEI - PP – Ymir - PP – miRCURY	1 mL	TEM, NTA: Similar size for UC and Ymir	NTA: Similar yield for UC and Ymir	-	CD9, CD63, Hsp70, Rab5, Aquaporin 2*	[143]
	- UC - PP – ExoQ - UF	UC and UF: 25 mL ExoQ: 10 mL	DLS: Within expected size range and trend for: ExoQ > UF > UC	DLS: Similar yield	Trend to UF > ExoQ, UC	Aquaporin 2*	[144]
	- UC - UCcush - UC + 0.22 µm filtration - PP – ExoQ and modExoQ - UF – VS20	UC: 25 mL ExoQ: 10 mL VS20: 15 mL	-	ELISA: ExoQ mod > UC & VS20 > ExoQ & UC + 0.22 µm > UCcush	UC, UCcush, modExoQ > UC + 0.22 µm, VS20, ExoQ	Alix, TSG101	[145]
	- UC - UF – VS100 - Filtration – VVLP	14.5 mL	TEM: Within expected size range	-	-	CD10, Erzin, NHE3, Albumin*	[146]
CSF, Plasma	- UC (with and without wash) - SEC – qEV: Izon 35 nm or 70 nm - PP – ExoQ - PP – ExoQ Ultra	500 µL	-	SIMOA of Exo markers: P: Izon 35 nm > ExoQ > UC > ExoQ Ultra > Izon 70 nm > UC with wash CSF: ExoQ > Izon 70 nm > Izon 35 nm > UC > ExoQ Ultra > UC with wash	P: Izon 35 nm > Others CSF: Izon 70 nm > Others	CD9, CD63, CD81	[58]

Table 2 (continued)

Biofluids	Exosome isolation method	Starting volume (µl)	Mode size	Particle yield	Purity	Exosome markers	Ref
CSF, Plasma, Serum	- UC - SEC – ExoS - PP – ExoQ - PP – TEI	S/P: 250 µL CSF: 5 mL	TEM, NTA trend for: S: ExoQ > ExoS > TEI P: ExoQ > TEI > ExoS CSF: ExoS > ExoQ > TEI	NTA or EXOCET: S & P: ExoS > ExoQ > TEI > UC CSF: Trend for TEI & ExoS > ExoQ > UC	S: ExoS > ExoQ & TEI P: ExoS > ExoQ & TEI CSF: Similar	NCAM, RAB11, TSG101, Albumin*, Calnexin*	[24]
Plasma, Serum	- UC - SEC – qEV - qEV-UC - qEV-UF - SEC – ExoS	500 µL	NTA trend for Plasma: UC and ExoS > qEV-UC > qEV > qEV-UF or Serum: ExoS > UC > qEV-UC > qEV > qEV-UF TEM: Within expected size	NTA S & P: qEV-UF > qEV > qEV-UC > ExoS > UC	Pure small EVs	CD9, CD63, Flotillin-1, TSG101, Calnexin*	[147]
	- UC - MA – ExoEasy Maxi Kit	-	NTA: ExoEasy = UC	NTA: ExoEasy = UC	-	-	[148]
	- UC - PP – TEI	UC: 1 mL TEI: 2 mL	TEM and cryo-EM: cup-shaped particles within expected size range NTA: UC = TEI	-	Both had lipid and protein contamination; Trend for TEI > UC	CD9, CD63, CD81	[149]
	- UC - PP – Hi-efficiency	500 µL	TEM: cup-shaped morphology, vesicles within expected size range	-	Hi-efficiency and UC: High purity; P: UC > Hi-efficiency; Plasma exo > serum exo;	CD9, TSG101	[150]
	- PP – ExoQ - PP – TEI - PP – REI	150 µL	NTA: Within expected size range	NTA S & P: REI > ExoQ > TEI	Serum & Plasma: REI > ExoQ > TEI	CD63, TSG101, Albumin*	[151]
Plasma, Serum, Saliva	- UCcush - EkD	UCcush (1 mL of serum) EkD (50 µL of biofluid)	TEM: Serum exo with expected size and morphology but protein aggregates and lipoproteins were observed; NTA: Within expected size but larger saliva-derived vesicles	NTA for P, S and Sal: EkD > UCcush	-	CD81, TSG101	[152]
Plasma, Saliva, Urine	- UCcush - Chitosan	P: 250 µL Sal & U: 1 mL	NTA trend for P & U: UCcush > Chitosan NTA trend for Sal: Similar	NTA for P: Chitosan > UCcush or U and Sal: UC > Chitosan	-	CD9, CD63, Flotillin-1, Hsp70, ApoA1*, ApoB*, Calnexin*	[153]
Serum, Saliva	- UC - Charge-based-Prot - PP – PEG - P/PEG	S: 750 µL Sal: 2.5 mL	TEM, NTA: Similar size	NTA (both): P/PEG > UC, Prot, PEG	Similar	CD9, CD63, CD81, ApoA1*, ApoB100*	[154]

Table 2 (continued)

Biofluids	Exosome isolation method	Starting volume (µl)	Mode size	Particle yield	Purity	Exosome markers	Ref
Serum, Urine	- UC - dgUC - SEC – qEV - MA – ExoEasy - nanoDLD	UC: S – 5 mL, U – 20 mL dgUC: 500 µL of UC resuspended sample qEV: 500 µL ExoEasy: S – 4 mL, U – 16 mL nanoDLD: 500 µL	TEM, NTA: Similar size but larger vesicles identified for serum exo isolated through UC and ExoEasy	NTA (yield): Trends for S – nanoDLD > qEV > UC & ExoEasy & dgUC; Trends for Urine – nanoDLD > ExoEasy > UC > qEV > dgUC	-	TSG101, Calnexin*	[155]
	- S: PP – ExoQ and TEI - U: UC and NBioExo (column)	S: 250 µL U: 5 mL	NTA trend for Serum: ExoQ > TEI or Urine: UC > NBioExo	NTA: Similar	-	-	[156]

Abbreviations: *AFM*, atomic force microscopy; *ApoA*, apolipoprotein; *AppiEV*, anionic polysaccharide-modified filter papers for the isolation of EVs; *ATPS*, aqueous two-phase system; *Col*, column-based method; *Conc-SEC*, concentration of urine samples followed by SEC; *Cryo-EM*, cryo-electron microscopy; *CSF*, cerebrospinal fluid; *dg*, density gradient; *DLS*, dynamic light scattering; *EI*, EpCAM immunoaffinity pull down; *EkD*, electrokinetic device; *ELISA*, enzyme-linked immunosorbent assay; *ExoQ*, ExoQuick Exosome Precipitation Solution (System Biosciences); *ExoS*, Exo-spin blood (Cell Guidance Systems); *ExoS Midi*, Exo-spin Midi Columns (Cell Guidance Systems); *EV-CATCHER*, Extracellular Vesicle Capture by AnTibody of Choice and Enzymatic Release; *EVs*, extracellular vesicles; *ExoTIC*, Exosome Total Isolation Chip; *EVTRAP*, extracellular vesicles total recovery and purification; *HFD*, hydrostatic filtration dialysis; *IA*, Immunoaffinity method; *iSUF*, immunomagnetic sequential ultrafiltration; *MA*, membrane affinity method; *NBioExo*, Norgen Biotek Urine Exosome RNA Isolation Kit; *NTA*, nanoparticle tracking analysis; *NanoDLD*, nanoscale deterministic lateral displacement arrays; *P*, plasma; *PA*, peptide affinity; *P/PEG*, protamine/polyethylene glycol (Merck); *PEG*, polyethylene glycol; *PP*, precipitation-based method; *PROSPR*, protein organic solvent precipitation; *Prot*, protamine; *REI*, RIBO exosome isolation reagent; *S*, serum; *Sal*, saliva; *SEC*, size exclusion chromatography; *SEM*, scanning electron microscopy; *Seph2B*, Sepharose 2B (Sigma); *SephCL-4B*, Sepharose CL-4B (Sigma); *SephS-400*, Sephacryl S-400 (Sigma); *SIMOA*, single-molecule array; *TEI*, total exosome isolation kit (Invitrogen); *TEM*, transmission electron microscopy; *THP*, Tamm-Horsfall protein; *TRPS*, tunable resistive pulse sensing; *U*, urine; *UC*, ultracentrifugation; *UCcush*, cushion ultracentrifugation; *UF*, ultrafiltration; *VS*, Vivaspin concentrator (Sartorius); *VVLP*, hydrophilized polyvinylidene difluoride filter; *WB*, Western blot

serum (Table 2). In these studies, the CSF starting volumes ranged between 500 µL and 8 mL and UC was compared with SEC, precipitation, ultrafiltration liquid chromatography, membrane affinity, and immunoaffinity methods. UC had the lowest particle yield, compared with precipitation and ultrafiltration methods, as determined by NTA [24, 108] and also by SIMOA, when evaluating the enrichment in exosome markers in UC, SEC, and precipitation methods [58]. Another study compared MagCapture and ExoIntact (two immunoaffinity bead–based methods), ExoEasy (based on membrane affinity), and EVSecond L70 (based on SEC). ExoEasy outperformed the other methods in particle yield and enrichment in CD63 and CD81 but its exosome preparations were enriched in albumin [109]. None of the four studies compared the exosome isolation methodologies regarding total RNA or miRNAs yield.

Regarding plasma, information from twenty-four comparative studies was collected, of which nine also included particle isolation from other biofluids (Table 2). Plasma volumes used for exosome isolation ranged from 10 µL to 25 mL, with UC-based methodologies requiring the highest plasma volume, as described for CSF. Ultracentrifugation with OptiPrep density gradient used 25 mL of plasma [121]; however, most of UC protocols used 500 µL or 1 mL of plasma. The lower plasma volume (10 µL) was applied to exosome total isolation chip, based on filtration [37]. Typically, all studies reported vesicles with cup-shaped morphology within the exosomes expected size. Likewise, precipitation and/or SEC methodologies presented higher particle yields when compared with UC [24, 37, 114, 115, 117, 147, 153]. Nonetheless, the combination of UC with the use of discontinuous iodixanol gradient was reported to be useful to increase particle yield and purity [121]. Recent methodologies as the microfluidic device [113], the clustering and scattering method [115], the Exodisc-B centrifugal device [116], and the electrokinetic devices outperformed UC in yield [152]. The recent column-based AppiEV method, using an anionic polysaccharide-modified filter, presented similar particle sizes to UC, but it isolated more EVs than UC, ExoQ, or ExoEasy [111]. To consider, some studies did not assess the purity of EVs isolated which is an important aspect since higher particle yields are no warranty of high-quality of EVs preparations. In general, for plasma-derived EVs isolation, the highest purity was reported for SEC-based methodologies when compared with UC, membrane affinity, and/or precipitation methodologies [24, 26, 58, 119, 120]. Nonetheless, others reported that UC rendered in purest preparations when compared with precipitation-based methods [114, 115].

As mentioned above, few information is depicted in the literature regarding plasma pre-processing variables, but EDTA tubes for blood collection were the most common used tubes [24–26, 37, 112, 115–117, 120, 148, 149]. Further, RNA yields obtained from plasma-derived exosomes isolated using UC, membrane affinity, and precipitation- and column-based methods were compared in several studies and, in general, similar RNA patterns were obtained for the distinct methodologies [26, 111, 114, 147–149, 151]. However, the clustering-and-scattering method was superior in terms of RNA yield and purity than ExoEasy, UC, and ExoQ [115] and exosomes isolated using Exodisc-B had five times more RNA than EVs isolated through UC [116]. Higher amounts of DNA were found in serum-derived exosomes when compared with plasma-derived exosomes [148]. In addition, the three most used markers to characterize plasma-derived EVs preparations were TSG101, CD63, and CD9 and, as negative markers, calnexin, ApoA1, and ApoB.

Serum-derived EVs isolation performance was addressed in twenty-five comparative studies that employ distinct methodologies in serum, or including other biofluids (Table 2). Biofluid starting volumes used vary between 50 µL and 5 mL. UC and precipitation-based methods were the most used isolation methodologies and required the highest starting sample amounts. EVs isolated were compatible with exosome morphology and size but there was a trend for larger vesicles isolated through UC when compared with precipitation and/or SEC-based methods [71, 122, 123, 126, 127, 132] and larger vesicles obtained with membrane affinity–based methods when compared with UC [127, 131], although within the expected size range. Overall, precipitation and/or SEC-based methods isolated more particles than UC [24, 71, 123–127, 130–135, 147, 154, 155] and vesicle preparations employing SEC or UC exhibited superior purity than precipitation methods [24, 126, 127, 133]. Interestingly, one study showed that plasma-derived EVs isolated through UC contained more exosomal proteins, identified by mass spectrometry, than serum-derived EVs, suggesting that EVs from plasma present higher purity [150]. Another study reported that despite ExoSpin isolated more particles than UC, this cannot be understood as a higher enrichment in exosomes because in TEM preparations only a few vesicles from ExoSpin immunostained for exosome markers. The enrichment in exosome markers for UC was also confirmed by ELISA assessment of CD9, CD63, and CD81 levels [123]. Recently, a new Extracellular Vesicle Capture by AnTibody of CHoice and Enzymatic Release (EV-CATCHER) method was developed to improve the immunobased isolation of small EVs, using CD63, CD9, or CD81 antibodies coupled to beads. This selective EV purification technique was compared with other 7 EVs immuno- and 2 column-based methods, ExoQ and UC. EV-CATCHER provided high yield of small EVs and was suitable for high-throughput small-RNA sequencing [125]. Immunomagnetic sequential ultrafiltration (iSUF) is another new EVs purification methodology that combines tangential flow filtration,

a centrifuge enrichment, and an immunocapture step. The iSUF was compared with TEI, qEV, and UC and rendered in high yields and pure EVs preparations [122]. This study shows that the combination of distinct methods can be useful in small EVs isolation. Concerning RNA profiling, precipitation-based methods, immunomagnetic sequential ultrafiltration, or nanochips provided higher exosomal miRNA yields than UC [122, 131, 134, 155]; nonetheless, exosome preparations obtained through UC were more pure than ExoQ and contain less free miRNAs than ExoQ [126]. Like for plasma, the most common exosomal markers tested in these preparations were TSG101, CD63, CD81, and CD9 and the negative markers were calnexin, ApoA1, and ApoB.

In sum, for plasma- and serum-derived exosome isolation, most of the studies compared UC, precipitation-based methods, and SEC, suggesting that these are the most commonly used thus far, although novel approaches are arising in the field. In terms of particle yield, precipitation and/or SEC outperformed UC but the purest blood-derived exosome preparations were reported to be obtained through UC and/or SEC. Some studies combined UC and SEC methods in an attempt to improve exosome isolation but consensus still need to be reached in terms of yield or purity.

Five comparative studies focused on EVs isolation from saliva or saliva and other biofluids (Table 2). From these, only three studies detailed the saliva collection conditions, one of them collected unstimulated saliva [152]; other collected the saliva between 9 and 11 am to reduce circadian cycle variations and asked participants to not eat or drink 1 h before collection [136] or wait 30 s after water consumption and before saliva collection [153]. Saliva starting volumes vary between 50 µL and 5 mL and EVs preparations presented nanovesicles within the expected exosomal size range. Similar to the other biofluids, a trend for lower particle yields obtained through UC was observed when compared with precipitation methods or electrokinetic device [136, 137, 152, 154], except in comparison with chitosan, a naturally occurring polymer [153]. However, in terms of EVs purity, UC presented higher purity than the other methods [136, 137]. No differences in RNA yields were described for exosome preparations obtained with UC or PEG-based methods [154]. The characterization of saliva-derived EVs also included the assessment of CD63, CD81, and CD9.

Information was also collected from twelve comparative studies focusing on EVs isolation from urine, from which three also included nanovesicle isolation from other biofluids. The starting volumes ranged from 500 µL to 200 mL and the most used method to remove THP from urine was the addition of DTT [139, 140, 145]. EVs obtained through the distinct methods had exosomal compatible morphology but, in terms of particle yield, controversies arise. Some studies reported that UC isolated more urinary particles than SEC [140], precipitation methods [139, 140, 153], while others reported that SEC [139], but also nanoDLD (microfluidics) and membrane affinity–based methods [155] or EV-TRAP based on functionalized magnetic beads [141], isolated more nanovesicles than UC. Regarding purity, it was reported that EVs preparations obtained through UC had higher purity than precipitation-based methodologies [145] or hydrostatic filtration dialysis (HFD) [138], whereas other studies showed that ultrafiltration [144] or EV-TRAP [141] provides more pure EVs preparations than UC. Distinct results were also found regarding RNA yield. Total EVs RNA yields were similar between UC, HFD, or spin columns [138, 142] while superior miRNA yield was reported for UC when compared with SEC or ExoQ [139]. In addition, lower miRNA or mRNA was obtained for exosome preparations using UC when compared with precipitation-based methods, as Ymir [143] or ExoQ modified protocol [145]. The latter comprises incubation with a higher volume of precipitation reagent (3.3 mL) than recommended in datasheet (2 mL), and a centrifugation at 10,000 g instead of 1500 g. In the comparative studies collected, CD9, TSG101, and CD63 were the most used markers to characterize exosomal preparations nature.

Future comparative studies should focus on the comparison of new methodologies and on the identification of pre-analytical variability sources, thus contributing to more reproducible and standardized approaches.

EVs biobanking

In the era of precision medicine, biomarkers gain increased importance not only for disease diagnosis but also as potential contributors for a personalized therapy. Considering the exosomal potential as sources of biomarkers and therapeutic vehicles, it is of maximum importance to biobank exosomes. In this context, it became of extreme importance to define and standardize the best method for each biofluid and annotate pre-analytical variability sources, as the daily time of biofluid collection, sample collection tubes, time, and temperature storage conditions.

Standardization of EVs isolation workflows and implementation of QC metrics for EVs biobanking would be essential to ensure high quality of the exosomes preparations and increase data reproducibility. A new ISEV Subcommittee on scientific rigor and reproducibility was stablished and a survey carried out in 2019 revealed that only a small percentage of respondents had biobanking EVs, and that most of the biobank users did not perform any kind of sample QC [157]. It is important to implement QC measures, focusing on sample hemolysis, platelet counting, and other blood chemical parameters, and address EVs purity by monitoring the amount of the typical contaminants, as the content in albumin and lipoproteins. Biobanking

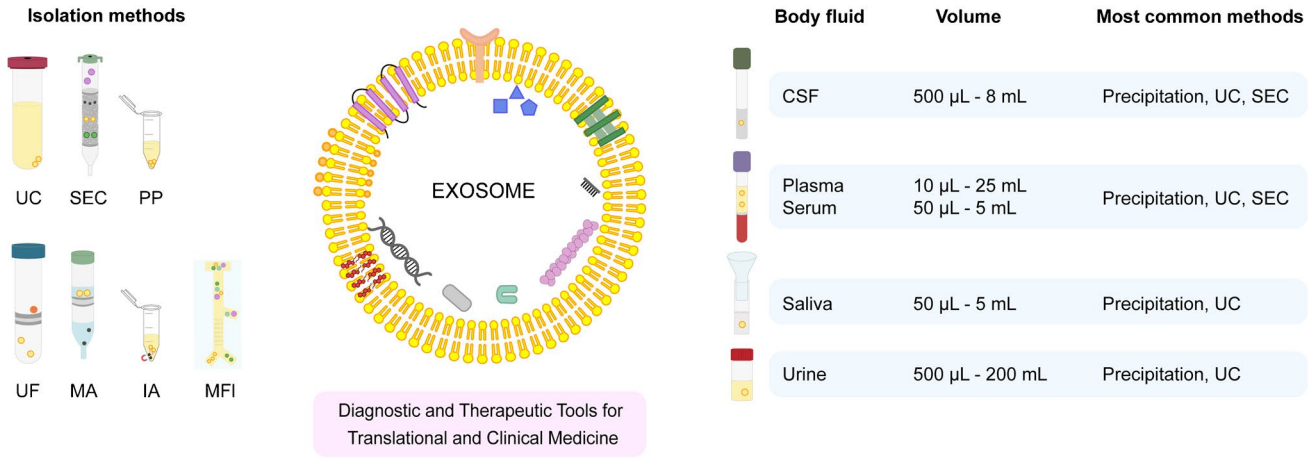

Fig. 2 Methods for exosome isolation in distinct body fluids. Several exosome isolation methodologies for human body fluids have been developed (left). These were addressed in comparative studies, as described in Table 2. For each biofluid, the volumes range used in these studies as well as the most used methods were represented on right. Exosomes obtained from human peripheral biofluids can constitute useful diagnostic and/or therapeutic tools. *Abbreviations*: CSF, cerebrospinal fluid; IA, immunoaffinity; MA, membrane affinity; MFI, microfluidics; PP, precipitation-based method; SEC, size exclusion chromatography; UC, ultracentrifugation; UF, ultrafiltration

exosomes and the implementation of good practices and QC measures would accelerate the translation of EVs discoveries to clinical practice.

Conclusion and future perspectives

The unique nature of each human body fluid represents a set of challenges when isolating EVs, among which are nanovesicle abundance, the distinct sample viscosity, and the presence of different co-contaminants. To overcome this, several exosome isolation methodologies have been developed and combined, also to ensure a better balance between EVs yield and purity. Further, diverse pre-analytical and analytical variability sources co-exist and must be properly identified and controlled. Moreover, in clinical settings, the human sample volumes are limited, as well as human and material resources, prompting the choice for methods that can provide high EVs yield, in a cost- and time-effective way. Comparative studies addressing the performance of distinct exosome methodologies, in one biofluid or more, constitute important tools to help the translation of EVs from bench to bedside, and these have been summarized in Table 2. For the body fluids included in this review, UC, precipitation-based, and SEC-based methods were the most commonly used, exhibiting a good performance in terms of balance between EVs yield and purity (Fig. 2).

A unique exosome isolation method suitable for all biofluids would be the ideal solution, but it is unexpected. Instead, simplified, high yield, relatively pure, and high-throughput new techniques will certainly arise considering the distinct biofluid biochemical properties. Also, new EVs characterization approaches will contribute to better distinguish EVs preparations with exosome-like characteristics from contaminants. This will facilitate the choice of the most suitable exosome isolation method for each biofluid. In addition, establishment of standardized procedures will be fundamental to improve exosome preparation reproducibility and quality. The ideal exosome isolation method would comprise an automatized platform allowing the extraction, characterization, and analysis of EVs content in a simple way. Hence, efficient, reproducible, and standardized EVs isolation procedures will facilitate the use of exosomes as sources of biomarkers or as therapeutic vehicles, opening avenues for its potential application in translational and clinical medicine.

Author contribution TSM—conception, literature search, data analysis, manuscript writing. MV—literature search, data analysis, manuscript writing. AGH—conception, manuscript writing and critical revision. All authors read and approved the final manuscript.

Funding This work was funded by the Alzheimer's Association under Grant 2019-AARG-644347. TSM is supported by the Fundação para a Ciência e Tecnologia (FCT) of the Ministério da Educação e Ciência under the individual PhD grant SFRH/BD/145979/2019 and MV under the individual PhD grant UI/BD/151354/2021.

Data availability Not applicable.

Declarations

Ethics approval Not applicable.

Source of biological material Not applicable.

Statement on animal welfare Not applicable.

Competing interests The authors declare no competing interests.

References

1. Soares Martins T, Trindade D, Vaz M, Campelo I, Almeida M, Trigo G, et al. Diagnostic and therapeutic potential of exosomes in Alzheimer's disease. J Neurochem. 2020;156(2):162–81. https://doi.org/10.1111/jnc.15112.
2. Kurian TK, Banik S, Gopal D, Chakrabarti S, Mazumder N. Elucidating methods for isolation and quantification of exosomes: a review. Mol Biotechnol. 2021;63:249–66. https://doi.org/10.1007/s12033-021-00300-3.
3. Borges FT, Melo SA, Özdemir BC, Kato N, Revuelta I, Miller CA, et al. TGF-β1-containing exosomes from injured epithelial cells activate fibroblasts to initiate tissue regenerative responses and fibrosis. J Am Soc Nephrol. 2013;24(3):385–92. https://doi.org/10.1681/ASN.2012101031.
4. Cantaluppi V, Biancone L, Figliolini F, Beltramo S, Medica D, Deregibus MC, et al. Microvesicles derived from endothelial progenitor cells enhance neoangiogenesis of human pancreatic islets. Cell Transplant. 2012;21(6):1305–20. https://doi.org/10.3727/096368911X627534.
5. Zhu W, Huang L, Li Y, Zhang X, Gu J, Yan Y, et al. Exosomes derived from human bone marrow mesenchymal stem cells promote tumor growth in vivo. Cancer Lett. 2012;315(1):28–37. https://doi.org/10.1016/j.canlet.2011.10.002.
6. Bunduc S, Gede N, Váncsa S, Lillik V, Kiss S, Juhász MF, et al. Exosomes as prognostic biomarkers in pancreatic ductal adenocarcinoma—a systematic review and meta-analysis. Transl Res. 2022;S1931–5244(22):00001–9. https://doi.org/10.1016/j.trsl.2022.01.001.
7. Mathews PM, Levy E. Exosome production is key to neuronal endosomal pathway integrity in neurodegenerative diseases. Front Neurosci. 2019;13:1–14. https://doi.org/10.3389/fnins.2019.01347.
8. Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L, et al. Detection of microRNA expression in human peripheral blood microvesicles. PLoS ONE. 2008;3(11): e3694. https://doi.org/10.1371/journal.pone.0003694.
9. Caby M-P, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. Exosomal-like vesicles are present in human blood plasma. Int Immunol. 2005;17(7):879–87. https://doi.org/10.1093/intimm/dxh267.
10. Yagi Y, Ohkubo T, Kawaji H, Machida A, Miyata H, Goda S, et al. Next-generation sequencing-based small RNA profiling of cerebrospinal fluid exosomes. Neurosci Lett. 2017;636:48–57. https://doi.org/10.1016/j.neulet.2016.10.042.
11. Lässer C, Seyed Alikhani V, Ekström K, Eldh M, Torregrosa Paredes P, Bossios A, et al. Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages. J Transl Med. 2011;9:9. https://doi.org/10.1186/1479-5876-9-9.
12. Elsharkawi F, Elsabah M, Shabayek M, Khaled H. Urine and serum exosomes as novel biomarkers in detection of bladder cancer. Asian Pacific J Cancer Prev. 2019;20(7):2219–24. https://doi.org/10.31557/APJCP.2019.20.7.2219.
13. Schuh CMAP, Cuenca J, Alcayaga-Miranda F, Khoury M. Exosomes on the border of species and kingdom intercommunication. Transl Res. 2019;210:80–98. https://doi.org/10.1016/j.trsl.2019.03.008.
14. Yoo YK, Lee J, Kim H, Hwang KS, Yoon DS, Lee JH. Toward exosome-based neuronal diagnostic devices. Micromachines. 2018;9(12):634. https://doi.org/10.3390/mi9120634.
15. Clayton A, Boilard E, Buzas EI, Cheng L, Falcón-Perez JM, Gardiner C, et al. Considerations towards a roadmap for collection, handling and storage of blood extracellular vesicles. J Extracell Vesicles. 2019;8:1. https://doi.org/10.1080/20013078.2019.1647027.
16. Hou R, Li Y, Sui Z, Yuan H, Yang K, Liang Z, et al. Advances in exosome isolation methods and their applications in proteomic analysis of biological samples. Anal Bioanal Chem. 2019;411(21):5351–61. https://doi.org/10.1007/S00216-019-01982-0/FIGURES/5.
17. Théry C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol. 2006;Chapter 3:Unit 3.22. https://doi.org/10.1002/0471143030.cb0322s30
18. Momen-Heravi F, Balaj L, Alian S, Mantel P-Y, Halleck AE, Trachtenberg AJ, et al. Current methods for the isolation of extracellular vesicles. Biol Chem. 2013;394(10):1253–62. https://doi.org/10.1515/hsz-2013-0141.
19. Yakimchuk K. Exosomes: isolation methods and specific markers. Mater Methods. 2015;5:1450. https://doi.org/10.13070/mm.en.5.1450.
20. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in exosome isolation techniques. Theranostics. 2017;7(3):789–804. https://doi.org/10.7150/thno.18133.
21. Busatto S, Vilanilam G, Ticer T, Lin WL, Dickson DW, Shapiro S, et al. Tangential flow filtration for highly efficient concentration of extracellular vesicles from large volumes of fluid. Cells. 2018;7(12):273. https://doi.org/10.3390/CELLS7120273.
22. Kim JY, Rhim W-K, Yoo Y-I, Kim D-S, Ko K-W, Heo Y, et al. Defined MSC exosome with high yield and purity to improve regenerative activity. J Tissue Eng. 2021;12:20417314211008624. https://doi.org/10.1177/20417314211008626.
23. Heinemann ML, Ilmer M, Silva LP, Hawke DH, Recio A, Vorontsova MA, et al. Benchtop isolation and characterization of functional exosomes by sequential filtration. J Chromatogr A. 2014;1371:125–35. https://doi.org/10.1016/J.CHROMA.2014.10.026.
24. Soares Martins T, Catita J, Martins Rosa I, A. B. da Cruz e Silva O, Henriques AG, da Cruz e Silva OAB, et al. Exosome isolation from distinct biofluids using precipitation and column-based approaches. PLoS One. 2018;13(6):e0198820. https://doi.org/10.1371/journal.pone.0198820.
25. Enderle D, Spiel A, Coticchia CM, Berghoff E, Mueller R, Schlumpberger M, et al. Characterization of RNA from exosomes and other extracellular vesicles isolated by a novel spin column-based method. PLoS ONE. 2015;10(8): e0136133. https://doi.org/10.1371/journal.pone.0136133.
26. Stranska R, Gysbrechts L, Wouters J, Vermeersch P, Bloch K, Dierickx D, et al. Comparison of membrane affinity-based method with size-exclusion chromatography for isolation of exosome-like vesicles from human plasma. J Transl Med. 2018;16(1):1. https://doi.org/10.1186/s12967-017-1374-6.
27. Zhang H, Lyden D. Asymmetric-flow field-flow fractionation technology for exomere and small extracellular vesicle separation and characterization. Nat Protoc 2019 144. 2019;14(4):1027–53. https://doi.org/10.1038/s41596-019-0126-x.
28. Wu B, Chen X, Wang J, Qing X, Wang Z, Ding X, et al. Separation and characterization of extracellular vesicles from human plasma by asymmetrical flow field-flow fractionation. Anal Chim Acta. 2020;1127:234–45. https://doi.org/10.1016/J.ACA.2020.06.071.

29. Kim H, Shin S. ExoCAS-2: rapid and pure isolation of exosomes by anionic exchange using magnetic beads. Biomed 2021;9(1):28. https://doi.org/10.3390/BIOMEDICINES9010028.
30. Kosanović M, Milutinović B, Goč S, Mitić N, Janković M. Ion-exchange chromatography purification of extracellular vesicles. Biotechniques. 2017;63(2):65–71. https://doi.org/10.2144/000114575/ASSET/IMAGES/LARGE/FIGURE4.JPEG.
31. Morani M, Mai TD, Krupova Z, Defrenaix P, Multia E, Riekkola ML, et al. Electrokinetic characterization of extracellular vesicles with capillary electrophoresis: a new tool for their identification and quantification. Anal Chim Acta. 2020;1128:42–51. https://doi.org/10.1016/J.ACA.2020.06.073.
32. Peterson MF, Otoc N, Sethi JK, Gupta A, Antes TJ. Integrated systems for exosome investigation. Methods. 2015;87:31–45. https://doi.org/10.1016/J.YMETH.2015.04.015.
33. Cai S, Luo B, Jiang P, Zhou X, Lan F, Yi Q, et al. Immuno-modified superparamagnetic nanoparticles *via* host–guest interactions for high-purity capture and mild release of exosomes. Nanoscale. 2018;10(29):14280–9. https://doi.org/10.1039/C8NR02871K.
34. Chen C, Skog J, Hsu CH, Lessard RT, Balaj L, Wurdinger T, et al. Microfluidic isolation and transcriptome analysis of serum microvesicles. Lab Chip. 2010;10(4):505–11. https://doi.org/10.1039/B916199F.
35. Kanwar SS, Dunlay CJ, Simeone DM, Nagrath S. Microfluidic device (ExoChip) for on-chip isolation, quantification and characterization of circulating exosomes. Lab Chip. 2014;14(11):1891–900. https://doi.org/10.1039/C4LC00136B.
36. Kang YT, Purcell E, Palacios-Rolston C, Lo TW, Ramnath N, Jolly S, et al. Isolation and profiling of circulating tumor-associated exosomes using extracellular vesicular lipid–protein binding affinity based microfluidic device. Small. 2019;15(47):1903600. https://doi.org/10.1002/SMLL.201903600.
37. Liu F, Vermesh O, Mani V, Ge TJ, Madsen SJ, Sabour A, et al. The exosome total isolation chip. ACS Nano. 2017;11(11):10712–23. https://doi.org/10.1021/acsnano.7b04878.
38. Wang Z, Wu HJ, Fine D, Schmulen J, Hu Y, Godin B, et al. Ciliated micropillars for the microfluidic-based isolation of nanoscale lipid vesicles. Lab Chip. 2013;13(15):2879. https://doi.org/10.1039/C3LC41343H.
39. Salafi T, Zhang Y, Zhang Y. A review on deterministic lateral displacement for particle separation and detection. Nano-Micro Lett 2019 111. 2019;11(1):1–33. https://doi.org/10.1007/S40820-019-0308-7.
40. Wunsch BH, Smith JT, Gifford SM, Wang C, Brink M, Bruce RL, et al. Nanoscale lateral displacement arrays for the separation of exosomes and colloids down to 20 nm. Nat Nanotechnol. 2016;11(11):936–40. https://doi.org/10.1038/NNANO.2016.134.
41. Suwatthanarak T, Thiodorus IA, Tanaka M, Shimada T, Takeshita D, Yasui T, et al. Microfluidic-based capture and release of cancer-derived exosomes via peptide–nanowire hybrid interface. Lab Chip. 2021;21(3):597–607. https://doi.org/10.1039/D0LC00899K.
42. Liu C, Guo J, Tian F, Yang N, Yan F, Ding Y, et al. Field-free isolation of exosomes from extracellular vesicles by microfluidic viscoelastic flows. ACS Nano. 2017;11(7):6968–76. https://doi.org/10.1021/ACSNANO.7B02277/SUPPL_FILE/NN7B02277_SI_001.PDF.
43. Wu M, Ouyang Y, Wang Z, Zhang R, Huang P-H, Chen C, et al. Isolation of exosomes from whole blood by integrating acoustics and microfluidics. Proc Natl Acad Sci U S A. 2017;114(40):10584–9. https://doi.org/10.1073/pnas.1709210114.
44. Davies RT, Kim J, Jang SC, Choi E-J, Gho YS, Park J. Microfluidic filtration system to isolate extracellular vesicles from blood. Lab Chip. 2012;12(24):5202. https://doi.org/10.1039/c2lc41006k.
45. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles. 2018;7(1):1535750. https://doi.org/10.1080/20013078.2018.1535750.
46. Lötvall J, Hill AF, Hochberg F, Buzás EI, Di Vizio D, Gardiner C, et al. Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. J Extracell vesicles. 2014;3:26913. https://doi.org/10.3402/jev.v3.26913.
47. Filipe V, Hawe A, Jiskoot W. Critical evaluation of nanoparticle tracking analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates. Pharm Res. 2010;27(5):796–810. https://doi.org/10.1007/s11095-010-0073-2.
48. Gardiner C, Ferreira YJ, Dragovic RA, Redman CWG, Sargent IL. Extracellular vesicle sizing and enumeration by nanoparticle tracking analysis. J Extracell Vesicles. 2013;2. https://doi.org/10.3402/JEV.V2I0.19671.
49. Kim A, Ng WB, Bernt W, Cho NJ. Validation of size estimation of nanoparticle tracking analysis on polydisperse macromolecule assembly. Sci Rep 2019 91. 2019;9(1):1–14. https://doi.org/10.1038/s41598-019-38915-x.
50. Pecora R. Dynamic light scattering measurement of nanometer particles in liquids. J Nanoparticle Res. 2000;2:123–31. https://doi.org/10.1023/A:1010067107182.
51. Gurunathan S, Kang M-H, Jeyaraj M, Qasim M, Kim J-H. Review of the isolation, characterization, biological function, and multifarious therapeutic approaches of exosomes. Cells. 2019;8(4):307. https://doi.org/10.3390/cells8040307.
52. Emelyanov A, Shtam T, Kamyshinsky R, Garaeva L, Verlov N, Miliukhina I, et al. Cryo-electron microscopy of extracellular vesicles from cerebrospinal fluid. Camussi G, editor. PLoS One. 2020;15(1):e0227949. https://doi.org/10.1371/journal.pone.0227949.
53. Jung MK, Mun JY. Sample preparation and imaging of exosomes by transmission electron microscopy. J Vis Exp. 2018;2018(131):56482. https://doi.org/10.3791/56482.
54. Chuo STY, Chien JCY, Lai CPK. Imaging extracellular vesicles: current and emerging methods. J Biomed Sci. 2018;25:95. https://doi.org/10.1186/s12929-018-0494-5.
55. Zarovni N, Corrado A, Guazzi P, Zocco D, Lari E, Radano G, et al. Integrated isolation and quantitative analysis of exosome shuttled proteins and nucleic acids using immunocapture approaches. Methods. 2015;87:46–58. https://doi.org/10.1016/J.YMETH.2015.05.028.
56. Logozzi M, Di Raimo R, Mizzoni D, Fais S. Immunocapture-based ELISA to characterize and quantify exosomes in both cell culture supernatants and body fluids. Methods Enzymol. 2020;645:155. https://doi.org/10.1016/BS.MIE.2020.06.011.
57. Logozzi M, De Milito A, Lugini L, Borghi M, Calabrò L, Spada M, et al. High levels of exosomes expressing CD63 and Caveolin-1 in plasma of melanoma patients. Cao Y, editor. PLoS One. 2009;4(4):e5219. https://doi.org/10.1371/journal.pone.0005219.
58. Ter-Ovanesyan D, Norman M, Lazarovits R, Trieu W, Lee J-H, Church GM, et al. Framework for rapid comparison of extracellular vesicle isolation methods. Elife. 2021;10:70725. https://doi.org/10.7554/ELIFE.70725.
59. Momen-Heravi F, Balaj L, Alian S, Trachtenberg AJ, Hochberg FH, Skog J, et al. Impact of biofluid viscosity on size and

sedimentation efficiency of the isolated microvesicles. Front Physiol. 2012;3:162. https://doi.org/10.3389/fphys.2012.00162.
60. Witwer KW, Buzás EI, Bemis LT, Bora A, Lässer C, Lötvall J, et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. J Extracell Vesicles. 2013;2(1):20360. https://doi.org/10.3402/jev.v2i0.20360.
61. Bæk R, Søndergaard EKL, Varming K, Jørgensen MM. The impact of various preanalytical treatments on the phenotype of small extracellular vesicles in blood analyzed by protein microarray. J Immunol Methods. 2016;438:11–20. https://doi.org/10.1016/j.jim.2016.08.007.
62. Lacroix R, Judicone C, Mooberry M, Boucekine M, Key NS, Dignat-George F, et al. Standardization of pre-analytical variables in plasma microparticle determination: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. J Thromb Haemost. 2013;11(6):1190–3. https://doi.org/10.1111/jth.12207.
63. Jamaly S, Ramberg C, Olsen R, Latysheva N, Webster P, Sovershaev T, et al. Impact of preanalytical conditions on plasma concentration and size distribution of extracellular vesicles using nanoparticle tracking analysis. Sci Rep. 2018;8(1):17216. https://doi.org/10.1038/s41598-018-35401-8.
64. Palviainen M, Saraswat M, Varga Z, Kitka D, Neuvonen M, Puhka M, et al. Extracellular vesicles from human plasma and serum are carriers of extravesicular cargo—implications for biomarker discovery. PLoS ONE. 2020;15(8): e0236439. https://doi.org/10.1371/journal.pone.0236439.
65. Atai NA, Balaj L, Van Veen H, Breakefield XO, Jarzyna PA, Van Noorden CJF, et al. Heparin blocks transfer of extracellular vesicles between donor and recipient cells. J Neurooncol. 2013;115(3):343–51. https://doi.org/10.1007/s11060-013-1235-y.
66. Zhou H, Yuen PST, Pisitkun T, Gonzales PA, Yasuda H, Dear JW, et al. Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. Kidney Int. 2006;69(8):1471–6. https://doi.org/10.1038/sj.ki.5000273.
67. Jeyaram A, Jay SM. Preservation and storage stability of extracellular vesicles for therapeutic applications. AAPS Journal. 2018;20(1):1. https://doi.org/10.1208/s12248-017-0160-y.
68. Glinge C, Clauss S, Boddum K, Jabbari R, Jabbari J, Risgaard B, et al. Stability of circulating blood-based microRNAs-pre-analytic methodological considerations. PLoS ONE. 2017;12(2): e0167969. https://doi.org/10.1371/journal.pone.0167969.
69. Sódar BW, Kittel Á, Pálóczi K, Vukman KV, Osteikoetxea X, Szabó-Taylor K, et al. Low-density lipoprotein mimics blood plasma-derived exosomes and microvesicles during isolation and detection. Sci Rep. 2016;6:24316. https://doi.org/10.1038/srep24316.
70. Sakai N, Uchida Y, Ohashi K, Hibuse T, Saika Y, Tomari Y, et al. Measurement of fasting serum apoB-48 levels in normolipidemic and hyperlipidemic subjects by ELISA. J Lipid Res. 2003;44(6):1256–62. https://doi.org/10.1194/jlr.M300090-JLR200.
71. Brennan K, Martin K, FitzGerald SP, O'Sullivan J, Wu Y, Blanco A, et al. A comparison of methods for the isolation and separation of extracellular vesicles from protein and lipid particles in human serum. Sci Rep. 2020;10(1):1039. https://doi.org/10.1038/s41598-020-57497-7.
72. Karimi N, Cvjetkovic A, Jang SC, Crescitelli R, Hosseinpour Feizi MA, Nieuwland R, et al. Detailed analysis of the plasma extracellular vesicle proteome after separation from lipoproteins. Cell Mol Life Sci. 2018;75(15):2873–86. https://doi.org/10.1007/s00018-018-2773-4.
73. Zhang X, Borg EGF, Liaci AM, Vos HR, Stoorvogel W. A novel three step protocol to isolate extracellular vesicles from plasma or cell culture medium with both high yield and purity. J Extracell Vesicles. 2020;9:1. https://doi.org/10.1080/20013078.2020.1791450.
74. Wu M, Chen C, Wang Z, Bachman H, Ouyang Y, Huang PH, et al. Separating extracellular vesicles and lipoproteins via acoustofluidics. Lab Chip. 2019;19(7):1174–82. https://doi.org/10.1039/C8LC01134F.
75. Zhang Y, Zhang Y, Zhang Y, Deng Z, Deng Z, Lou D, et al. High-efficiency separation of extracellular vesicles from lipoproteins in plasma by agarose gel electrophoresis. Anal Chem. 2020;92(11):7493–9. https://doi.org/10.1021/ACS.ANALCHEM.9B05675/SUPPL_FILE/AC9B05675_SI_001.PDF.
76. McNamara RP, Dittmer DP. Modern techniques for the isolation of extracellular vesicles and viruses. J Neuroimmune Pharmacol. 2020;15(3):459–72. https://doi.org/10.1007/s11481-019-09874-x.
77. Frühbeis C, Helmig S, Tug S, Simon P, Krämer-Albers EM. Physical exercise induces rapid release of small extracellular vesicles into the circulation. J Extracell Vesicles. 2015;4(1):28239. https://doi.org/10.3402/jev.v4.28239.
78. Oliveira GP, Porto WF, Palu CC, Pereira LM, Petriz B, Almeida JA, et al. Effects of acute aerobic exercise on rats serum extracellular vesicles diameter, concentration and small RNAs content. Front Physiol. 2018;9:532. https://doi.org/10.3389/fphys.2018.00532.
79. Bertoldi K, Cechinel LR, Schallenberger B, Corssac GB, Davies S, Guerreiro ICK, et al. Circulating extracellular vesicles in the aging process: impact of aerobic exercise. Mol Cell Biochem. 2018;440(1–2):115–25. https://doi.org/10.1007/s11010-017-3160-4.
80. Soares E, Reis J, Rodrigues M, Ribeiro CF, Pereira FC. Circulating extracellular vesicles: the missing link between physical exercise and depression management? Int J Mol Sci. 2021;22(2):542. https://doi.org/10.3390/IJMS22020542.
81. Mustapic M, Eitan E, Werner JK, Berkowitz ST, Lazaropoulos MP, Tran J, et al. Plasma extracellular vesicles enriched for neuronal origin: a potential window into brain pathologic processes. Front Neurosci. 2017;11:278. https://doi.org/10.3389/fnins.2017.00278.
82. Fiandaca MS, Kapogiannis D, Mapstone M, Boxer A, Eitan E, Schwartz JB, et al. Identification of preclinical Alzheimer's disease by a profile of pathogenic proteins in neurally derived blood exosomes: a case-control study. Alzheimers Dement. 2015;11(6):600-7.e1. https://doi.org/10.1016/j.jalz.2014.06.008.
83. Shi M, Liu C, Cook TJ, Bullock KM, Zhao Y, Ginghina C, et al. Plasma exosomal α-synuclein is likely CNS-derived and increased in Parkinson's disease. Acta Neuropathol. 2014;128(5):639–50. https://doi.org/10.1007/s00401-014-1314-y.
84. Goetzl EJ, Boxer A, Schwartz JB, Abner EL, Petersen RC, Miller BL, et al. Low neural exosomal levels of cellular survival factors in Alzheimer's disease. Ann Clin Transl Neurol. 2015;2(7):769–73. https://doi.org/10.1002/acn3.211.
85. Goetzl EJ, Mustapic M, Kapogiannis D, Eitan E, Lobach IV, Goetzl L, et al. Cargo proteins of plasma astrocyte-derived exosomes in Alzheimer's disease. FASEB J. 2016;30:3853–9. https://doi.org/10.1096/fj.201600756R.
86. Krämer-Albers EM, Bretz N, Tenzer S, Winterstein C, Möbius W, Berger H, et al. Oligodendrocytes secrete exosomes containing major myelin and stress-protective proteins: trophic support for axons? Proteomics Clin Appl. 2007;1(11):1446–61. https://doi.org/10.1002/prca.200700522.
87. Moos M, Tacke R, Scherer H, Teplow D, Früh K, Schachner M. Neural adhesion molecule L1 as a member of the immunoglobulin superfamily with binding domains similar to fibronectin. Nature. 1988;334(6184):701–3. https://doi.org/10.1038/334701a0.

88. Mechtersheimer S, Gutwein P, Agmon-Levin N, Stoeck A, Oleszewski M, Riedle S, et al. Ectodomain shedding of L1 adhesion molecule promotes cell migration by autocrine binding to integrins. J Cell Biol. 2001;155(4):661–73. https://doi.org/10.1083/jcb.200101099.
89. Maretzky T, Schulte M, Ludwig A, Rose-John S, Blobel C, Hartmann D, et al. L1 is sequentially processed by two differently activated metalloproteases and presenilin/γ-secretase and regulates neural cell adhesion, cell migration, and neurite outgrowth. Mol Cell Biol. 2005;25(20):9040–53. https://doi.org/10.1128/mcb.25.20.9040-9053.2005.
90. Angiolini F, Belloni E, Giordano M, Campioni M, Forneris F, Paronetto MP, et al. A novel L1CAM isoform with angiogenic activity generated by NOVA2-mediated alternative splicing. Elife. 2019;8: e44305. https://doi.org/10.7554/eLife.44305.
91. Norman M, Ter-Ovanesyan D, Trieu W, Lazarovits R, Kowal EJK, Lee JH, et al. L1CAM is not associated with extracellular vesicles in human cerebrospinal fluid or plasma. Nat Methods. 2021;18(6):631–4. https://doi.org/10.1038/s41592-021-01174-8.
92. Sakka L, Coll G, Chazal J. Anatomy and physiology of cerebrospinal fluid. Eur Ann Otorhinolaryngol Head Neck Dis. 2011;128(6):309–16. https://doi.org/10.1016/J.ANORL.2011.03.002.
93. Mattsson N, Andreasson U, Persson S, Arai H, Batish SD, Bernardini S, et al. The Alzheimer's Association external quality control program for cerebrospinal fluid biomarkers. Alzheimer's Dement. 2011;7(4):386-395.e6. https://doi.org/10.1016/j.jalz.2011.05.2243.
94. Mattsson N, Andreasson U, Persson S, Carrillo MC, Collins S, Chalbot S, et al. CSF biomarker variability in the Alzheimer's Association quality control program. Alzheimer's Dement. 2013;9(3):251–61. https://doi.org/10.1016/j.jalz.2013.01.010.
95. Choi JE, Lyons KM, Kieser JA, Waddell NJ. Diurnal variation of intraoral pH and temperature. BDJ Open. 2017;3(1):1–6. https://doi.org/10.1038/bdjopen.2017.15.
96. Dawes C. Circadian rhythms in human salivary flow rate and composition. J Physiol. 1972;220(3):529–45. https://doi.org/10.1113/jphysiol.1972.sp009721.
97. Deutsch O, Fleissig Y, Zaks B, Krief G, Aframian DJ, Palmon A. An approach to remove alpha amylase for proteomic analysis of low abundance biomarkers in human saliva. Electrophoresis. 2008;29(20):4150–7. https://doi.org/10.1002/elps.200800207.
98. Sun Y, Xia Z, Shang Z, Sun K, Niu X, Qian L, et al. Facile preparation of salivary extracellular vesicles for cancer proteomics. Sci Rep. 2016;6(1):1–11. https://doi.org/10.1038/srep24669.
99. Fernández-Llama P, Khositseth S, Gonzales PA, Star RA, Pisitkun T, Knepper MA. Tamm-Horsfall protein and urinary exosome isolation. Kidney Int. 2010;77(8):736–42. https://doi.org/10.1038/ki.2009.550.
100. Musante L, Bontha SV, La Salvia S, Fernandez-Piñeros A, Lannigan J, Le TH, et al. Rigorous characterization of urinary extracellular vesicles (uEVs) in the low centrifugation pellet - a neglected source for uEVs. Sci Rep. 2020;10(1):3701. https://doi.org/10.1038/s41598-020-60619-w.
101. Xu X, Barreiro K, Musante L, Kretz O, Lin H, Zou H, et al. Management of Tamm-Horsfall protein for reliable urinary analytics. Proteomics Clin Appl. 2019;13(6): e1900018. https://doi.org/10.1002/prca.201900018.
102. Kosanović M, Janković M. Isolation of urinary extracellular vesicles from Tamm- Horsfall protein–depleted urine and their application in the development of a lectin-exosome-binding assay. Biotechniques. 2014;57(3):143–9. https://doi.org/10.2144/000114208.
103. Liu Z, Cauvi DM, Bernardino EMA, Lara B, Lizardo RE, Hawisher D, et al. Isolation and characterization of human urine extracellular vesicles. Cell Stress Chaperones. 2018;23(5):943–53. https://doi.org/10.1007/s12192-018-0902-5.
104. Musante L, Saraswat M, Duriez E, Byrne B, Ravidà A, Domon B, et al. Biochemical and physical characterisation of urinary nanovesicles following CHAPS treatment. PLoS ONE. 2012;7(7): e37279. https://doi.org/10.1371/journal.pone.0037279.
105. Rood IM, Deegens JKJ, Merchant ML, Tamboer WPM, Wilkey DW, Wetzels JFM, et al. Comparison of three methods for isolation of urinary microvesicles to identify biomarkers of nephrotic syndrome. Kidney Int. 2010;78(8):810–6. https://doi.org/10.1038/ki.2010.262.
106. Oshikawa S, Sonoda H, Ikeda M. Aquaporins in urinary extracellular vesicles (exosomes). Int J of Mol Sci. 2016;17(6):957. https://doi.org/10.3390/ijms17060957.
107. Tataruch-Weinert D, Musante L, Kretz O, Holthofer H. Urinary extracellular vesicles for RNA extraction: optimization of a protocol devoid of prokaryote contamination. J Extracell Vesicles. 2016;5:30281. https://doi.org/10.3402/jev.v5.30281.
108. Thompson AG, Gray E, Mager I, Fischer R, Thézénas M, Charles PD, et al. UFLC-derived CSF extracellular vesicle origin and proteome. Proteomics. 2018;18(24):1800257. https://doi.org/10.1002/pmic.201800257.
109. Sjoqvist S, Otake K, Hirozane Y. Analysis of cerebrospinal fluid extracellular vesicles by proximity extension assay: a comparative study of four isolation kits. Int J Mol Sci. 2020;21(24):9425. https://doi.org/10.3390/ijms21249425.
110. Alameldin S, Costina V, Abdel-Baset HA, Nitschke K, Nuhn P, Neumaier M, et al. Coupling size exclusion chromatography to ultracentrifugation improves detection of exosomal proteins from human plasma by LC-MS. Pract Lab Med. 2021;26: e00241. https://doi.org/10.1016/J.PLABM.2021.E00241.
111. Li S, Liu Q, Geng Z, Li K, Zhao T, Liu P. Anionic polysaccharide-modified filter papers for rapid isolation of extracellular vesicles from diverse samples in a simple bind-wash-elute manner. Anal Chem. 2021;93(20):7405–12. https://doi.org/10.1021/ACS.ANALCHEM.0C02107.
112. Shtam T, Evtushenko V, Samsonov R, Zabrodskaya Y, Kamyshinsky R, Zabegina L, et al. Evaluation of immune and chemical precipitation methods for plasma exosome isolation. PLoS ONE. 2020;15(11): e0242732. https://doi.org/10.1371/journal.pone.0242732.
113. Han BH, Kim S, Seo G, Heo Y, Chung S, Kang JY. Isolation of extracellular vesicles from small volumes of plasma using a microfluidic aqueous two-phase system. Lab Chip. 2020;20(19):3552–9. https://doi.org/10.1039/d0lc00345j.
114. Peng C, Wang J, Bao Q, Wang J, Liu Z, Wen J, et al. Isolation of extracellular vesicle with different precipitation-based methods exerts a tremendous impact on the biomarker analysis for clinical plasma samples. Cancer Biomark. 2020;29(3):373–85. https://doi.org/10.3233/CBM-201651.
115. Kim J, Lee H, Park K, Shin S. Rapid and efficient isolation of exosomes by clustering and scattering. J Clin Med. 2020;9(3):650. https://doi.org/10.3390/jcm9030650.
116. Sunkara V, Kim CJ, Park J, Woo HK, Kim D, Ha HK, et al. Fully automated, label-free isolation of extracellular vesicles from whole blood for cancer diagnosis and monitoring. Theranostics. 2019;9(7):1851–63. https://doi.org/10.7150/thno.32438.
117. Serrano-Pertierra E, Oliveira-Rodríguez M, Rivas M, Oliva P, Villafani J, Navarro A, et al. Characterization of plasma-derived extracellular vesicles isolated by different methods: a comparison study. Bioengineering. 2019;6(1):8. https://doi.org/10.3390/bioengineering6010008.
118. Gallart-Palau X, Serra A, Wong ASW, Sandin S, Lai MKP, Chen CP, et al. Extracellular vesicles are rapidly purified from human

plasma by PRotein Organic Solvent PRecipitation (PROSPR). Sci Rep. 2015;5(1):14664. https://doi.org/10.1038/srep14664.
119. Baranyai T, Herczeg K, Onódi Z, Voszka I, Módos K, Marton N, et al. Isolation of exosomes from blood plasma: qualitative and quantitative comparison of ultracentrifugation and size exclusion chromatography methods. Rito-Palomares M, editor. PLoS One. 2015;10(12):e0145686. https://doi.org/10.1371/journal.pone.0145686.
120. Lobb RJ, Becker M, Wen Wen S, Wong CSF, Wiegmans AP, Leimgruber A, et al. Optimized exosome isolation protocol for cell culture supernatant and human plasma. 2015;4:27031.https://doi.org/10.3402/jev.v4.27031
121. Kalra H, Adda CG, Liem M, Ang C-S, Mechler A, Simpson RJ, et al. Comparative proteomics evaluation of plasma exosome isolation techniques and assessment of the stability of exosomes in normal human blood plasma. Proteomics. 2013;13(22):3354–64. https://doi.org/10.1002/pmic.201300282.
122. Zhang J, Nguyen LTH, Hickey R, Walters N, Wang X, Kwak KJ, et al. Immunomagnetic sequential ultrafiltration (iSUF) platform for enrichment and purification of extracellular vesicles from biofluids. Sci Rep. 2021;11(1):1–17. https://doi.org/10.1038/s41598-021-86910-y.
123. Małys MS, Aigner C, Schulz SM, Schachner H, Rees AJ, Kain R. Isolation of small extracellular vesicles from human sera. Int J Mol Sci. 2021;22(9):4653. https://doi.org/10.3390/IJMS22094653/S1.
124. Wei H, Qian X, Xie F, Cui D. Isolation of exosomes from serum of patients with lung cancer: a comparison of the ultra-high speed centrifugation and precipitation methods. Ann Transl Med. 2021;9(10):882–882. https://doi.org/10.21037/ATM-21-2075.
125. Mitchell MI, Ben-Dov IZ, Liu C, Ye K, Chow K, Kramer Y, et al. Extracellular Vesicle Capture by AnTibody of CHoice and Enzymatic Release (EV-CATCHER): a customizable purification assay designed for small-RNA biomarker identification and evaluation of circulating small-EVs. J Extracell Vesicles. 2021;10(8):e12110. https://doi.org/10.1002/JEV2.12110.
126. Cheng Y, Qu X, Dong Z, Zeng Q, Ma X, Jia Y, et al. Comparison of serum exosome isolation methods on co-precipitated free microRNAs. PeerJ. 2020;8: e9434. https://doi.org/10.7717/peerj.9434.
127. Jung HH, Kim JY, Lim JE, Im YH. Cytokine profiling in serum-derived exosomes isolated by different methods. Sci Rep. 2020;10(1):14069. https://doi.org/10.1038/s41598-020-70584-z.
128. Macías M, Rebmann V, Mateos B, Varo N, Perez-Gracia JL, Alegre E, et al. Comparison of six commercial serum exosome isolation methods suitable for clinical laboratories. Effect in cytokine analysis. Clin Chem Lab Med. 2019;57(10):1539–45. https://doi.org/10.1515/cclm-2018-1297.
129. Nguyen HQ, Lee D, Kim Y, Paek M, Kim M, Jang KS, et al. Platelet Factor 4 as a novel exosome marker in MALDI-MS analysis of exosomes from human serum. Anal Chem. 2019;91(20):13297–305. https://doi.org/10.1021/acs.analchem.9b04198.
130. An M, Wu J, Zhu J, Lubman DM. Comparison of an optimized ultracentrifugation method versus size-exclusion chromatography for isolation of exosomes from human serum. J Proteome Res. 2018;17(10):3599–605. https://doi.org/10.1021/acs.jproteome.8b00479.
131. Buschmann D, Kirchner B, Hermann S, Märte M, Wurmser C, Brandes F, et al. Evaluation of serum extracellular vesicle isolation methods for profiling miRNAs by next-generation sequencing. J Extracell Vesicles. 2018;7(1):1481321. https://doi.org/10.1080/20013078.2018.1481321.
132. Helwa I, Cai J, Drewry MD, Zimmerman A, Dinkins MB, Khaled ML, et al. A comparative study of serum exosome isolation using differential ultracentrifugation and three commercial reagents. Camussi G, editor. PLoS One. 2017;12(1):e0170628. https://doi.org/10.1371/journal.pone.0170628.
133. Tang Y-T, Huang Y-Y, Zheng L, Qin S-H, Xu X-P, An T-X, et al. Comparison of isolation methods of exosomes and exosomal RNA from cell culture medium and serum. Int J Mol Med. 2017;40(3):834–44. https://doi.org/10.3892/ijmm.2017.3080.
134. Andreu Z, Rivas E, Sanguino-Pascual A, Lamana A, Marazuela M, González-Alvaro I, et al. Comparative analysis of EV isolation procedures for miRNAs detection in serum samples. J Extracell Vesicles. 2016;5(1):31655. https://doi.org/10.3402/jev.v5.31655.
135. Caradec J, Kharmate G, Hosseini-Beheshti E, Adomat H, Gleave M, Guns E. Reproducibility and efficiency of serum-derived exosome extraction methods. Clin Biochem. 2014;47(13–14):1286–92. https://doi.org/10.1016/j.clinbiochem.2014.06.011.
136. Li M, Lou D, Chen J, Shi K, Wang Y, Zhu Q, et al. Deep dive on the proteome of salivary extracellular vesicles: comparison between ultracentrifugation and polymer-based precipitation isolation. Anal Bioanal Chem. 2020;413(2):365–75. https://doi.org/10.1007/s00216-020-03004-w.
137. Zlotogorski-Hurvitz A, Dayan D, Chaushu G, Korvala J, Salo T, Sormunen R, et al. Human saliva-derived exosomes: comparing methods of isolation. J Histochem Cytochem. 2015;63(3):181–9. https://doi.org/10.1369/0022155414564219.
138. Barreiro K, Dwivedi OP, Leparc G, Rolser M, Delic D, Forsblom C, et al. Comparison of urinary extracellular vesicle isolation methods for transcriptomic biomarker research in diabetic kidney disease. J Extracell Vesicles. 2020;10(2): e12038. https://doi.org/10.1002/jev2.12038.
139. Park S, Lee K, Park IB, Kim NH, Cho S, Rhee WJ, et al. The profiles of microRNAs from urinary extracellular vesicles (EVs) prepared by various isolation methods and their correlation with serum EV microRNAs. Diabetes Res Clin Pract. 2020;160:108010. https://doi.org/10.1016/j.diabres.2020.108010.
140. Gheinani AH, Vögeli M, Baumgartner U, Vassella E, Draeger A, Burkhard FC, et al. Improved isolation strategies to increase the yield and purity of human urinary exosomes for biomarker discovery. Sci Rep. 2018;8(1):3945. https://doi.org/10.1038/s41598-018-22142-x.
141. Wu X, Li L, Iliuk A, Tao WA. Highly efficient phosphoproteome capture and analysis from urinary extracellular vesicles. J Proteome Res. 2018;17(9):3308–16. https://doi.org/10.1021/acs.jproteome.8b00459.
142. Xu Y, Qin S, An T, Tang Y, Huang Y, Zheng L. MiR-145 detection in urinary extracellular vesicles increase diagnostic efficiency of prostate cancer based on hydrostatic filtration dialysis method. Prostate. 2017;77(10):1167–75. https://doi.org/10.1002/pros.23376.
143. Markowska A, Pendergrast RS, Pendergrast JS, Pendergrast PS. A novel method for the isolation of extracellular vesicles and RNA from urine. J Circ Biomarkers. 2017;6:184945441771266. https://doi.org/10.1177/1849454417712666.
144. Channavajjhala SK, Rossato M, Morandini F, Castagna A, Pizzolo F, Bazzoni F, et al. Optimizing the purification and analysis of miRNAs from urinary exosomes. Clin Chem Lab Med. 2014;52(3):345–54. https://doi.org/10.1515/cclm-2013-0562.
145. Alvarez ML, Khosroheidari M, Kanchi Ravi R, DiStefano JK. Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers. Kidney Int. 2012;82(9):1024–32. https://doi.org/10.1038/ki.2012.256.
146. Merchant ML, Powell DW, Wilkey DW, Cummins TD, Deegens JK, Rood IM, et al. Microfiltration isolation of human urinary

exosomes for characterization by MS. Proteomics Clin Appl. 2010;4(1):84–96. https://doi.org/10.1002/prca.200800093.
147. Tzaridis T, Bachurski D, Liu S, Surmann K, Babatz F, Gesell Salazar M, et al. Extracellular vesicle separation techniques impact results from human blood samples: considerations for diagnostic applications. Int J Mol Sci. 2021;22(17):9211. https://doi.org/10.3390/IJMS22179211/S1.
148. Wang ZY, Wang RX, Ding XQ, Zhang X, Pan XR, Tong JH. A protocol for cancer-related mutation detection on exosomal DNA in clinical application. Front Oncol. 2020;10: 558106. https://doi.org/10.3389/fonc.2020.558106.
149. Sorop A, Iacob R, Iacob S, Constantinescu D, Chitoiu L, Fertig TE, et al. Plasma small extracellular vesicles derived miR-21-5p and miR-92a-3p as potential biomarkers for hepatocellular carcinoma screening. Front Genet. 2020;11:712. https://doi.org/10.3389/fgene.2020.00712.
150. Cao F, Gao Y, Chu Q, Wu Q, Zhao L, Lan T, et al. Proteomics comparison of exosomes from serum and plasma between ultracentrifugation and polymer-based precipitation kit methods. Electrophoresis. 2019;40(23–24):3092–8. https://doi.org/10.1002/elps.201900295.
151. Ding M, Wang C, Lu X, Zhang C, Zhou Z, Chen X, et al. Comparison of commercial exosome isolation kits for circulating exosomal microRNA profiling. Anal Bioanal Chem. 2018;410(16):3805–14. https://doi.org/10.1007/s00216-018-1052-4.
152. Shi L, Kuhnell D, Borra VJ, Langevin SM, Nakamura T, Esfandiari L. Rapid and label-free isolation of small extracellular vesicles from biofluids utilizing a novel insulator based dielectrophoretic device. Lab Chip. 2019;19(21):3726–34. https://doi.org/10.1039/c9lc00902g.
153. Kumar A, Dhadi SR, Mai NN, Taylor C, Roy JW, Barnett DA, et al. The polysaccharide chitosan facilitates the isolation of small extracellular vesicles from multiple biofluids. J Extracell Vesicles. 2021;10(11): e12138. https://doi.org/10.1002/JEV2.12138.
154. Deregibus MC, Figliolini F, D'Antico S, Manzini PM, Pasquino C, De Lena M, et al. Charge-based precipitation of extracellular vesicles. Int J Mol Med. 2016;38(5):1359–66. https://doi.org/10.3892/ijmm.2016.2759.
155. Smith JT, Wunsch BH, Dogra N, Ahsen ME, Lee K, Yadav KK, et al. Integrated nanoscale deterministic lateral displacement arrays for separation of extracellular vesicles from clinically-relevant volumes of biological samples. Lab Chip. 2018;18(24):3913–25. https://doi.org/10.1039/c8lc01017j.
156. Crossland RE, Norden J, Bibby LA, Davis J, Dickinson AM. Evaluation of optimal extracellular vesicle small RNA isolation and qRT-PCR normalisation for serum and urine. J Immunol Methods. 2016;429:39–49. https://doi.org/10.1016/j.jim.2015.12.011.
157. Royo F, Théry C, Falcón-Pérez JM, Nieuwland R, Witwer KW. Methods for separation and characterization of extracellular vesicles: results of a worldwide survey performed by the ISEV Rigor and Standardization Subcommittee. Cells. 2020;9(9):1955. https://doi.org/10.3390/cells9091955.