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Methods for the identification and characterization of extracellular vesicles in cardiovascular studies – from exosomes to microvesicles

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Abstract (179 of 200 words)

Extracellular vesicles (EVs) are nanosized vesicles with a lipid bilayer that are released from cells of the cardiovascular system, and are considered important mediators of intercellular and extracellular communication. Two types of EV of particular interest are exosomes and microvesicles, which have been identified in all tissue and body fluids and carry a variety of molecules including RNAs, proteins, and lipids. EVs have potential for use in the diagnosis and prognosis of cardiovascular diseases and as new therapeutic agents, particularly in the setting of myocardial infarction and heart failure. Despite their promise, technical challenges related to their small size make it challenging to accurately identify and characterize them, and to study EV-mediated processes. Here, we aim to provide the reader with an overview of the techniques and technologies available for the separation and characterization of EVs from different sources. Methods for determining the protein, RNA and lipid content of EVs are discussed. The aim of this document is to provide guidance on critical methodological issues and highlight key points for consideration for the investigation of EVs in cardiovascular studies.

1. Pathophysiological relevance of EVs in the cardiovascular field

In recent years, extracellular vesicles (EVs) such as exosomes and microvesicles have gained significant interest as mediators of intercellular communication in both the healthy physiological state and during pathophysiological stress.[1-4] All cell types in the cardiovascular system release EVs.[5] However, most mechanistic studies use cell culture-derived EVs. EVs are also detected in plasma, where they are derived primarily from erythrocytes, platelets, endothelial and immune cells.[6] The plasma EV content responds to environmental changes and can regulate pro-inflammatory and innate immune responses, coagulation pathways and atherogenic interactions.[7] It is therefore of interest to understand the function of EVs in the cardiovascular system.

Several characteristics make EVs promising biomarkers for cardiovascular pathologies.[1] For example, EVs are secreted into body fluids such as blood, lymph and pericardial fluid, and EV molecular cargo reflects the state of the cell of origin. Therefore, by purifying EVs it is possible to enrich for diagnostic markers that may otherwise be obscured by the large quantity of proteins present in the fluid.[3] For example, acute coronary syndrome results in the rapid appearance of EVs in plasma that can be purified, aiding the identification of specific miRNAs,[8] in comparison to the detection of cardiac miRNAs in total plasma, which is inferior to high sensitivity assays for traditional markers of damaged myocardium such as troponins [9, 10]. Cardiac allograft rejection can be predicted with an accuracy of 86% based on the concentration and contents of EVs released by the transplanted heart into the blood, potentially eliminating the need for endomyocardial biopsy.[11] miRNA signatures in circulating large EVs, in contrast to freely circulating miRNAs, predicted the occurrence of cardiovascular events in patients with coronary artery disease,[12] highlighting the prognostic potential of EV-miRNA expression pattern.

In certain situations, EVs can contribute to the mechanism of cardiovascular diseases. For example, sEVs contribute to the development of pulmonary arterial hypertension,[13, 14] and to vascular calcification.[15, 16] Adipocyte-derived extracellular vesicles and their ceramide content have impact on cardiac mortality in advanced atherosclerosis.[16, 17] Endothelial EVs released during myocardial infarction can mobilize splenic neutrophils and monocytes following their transcriptional activation and could contribute to attenuated cardiac function.[18, 19] Therefore, EVs are emerging as key players in different stages of disease development of cardiovascular disease and metabolic syndrome (reviewed in[20-22]).

EVs are also promising therapeutic agents for treating cardiovascular disease. They have been shown to mediate various beneficial effects of conditioned medium from stem cells.[23, 24] EVs can be separated from tissue-culture medium "conditioned" by the growth of cells, and there is growing interest in using such EVs for treating a variety of cardiovascular pathologies.[5] For example, EVs purified from medium conditioned by cardiac progenitor cells (Exo-CPC), but not from normal dermal fibroblasts, are cardioprotective and proangiogenic in models of myocardial infarction and chemotherapy-induced cardiotoxicity,[25, 26] and stimulate cardiovascular cell proliferation following myocardial infarction.[27] Similarly, platelet-derived EVs in endothelial progenitor cell cultures contributed to their proangiogenic activity.[28, 29] In another example, EV coating of stents accelerated their re-endothelialization and reduced in-stent restenosis compared to drug-eluting and bare metal stents in mice.[30]

Currently, there are more than 250 clinical trials registered to use EVs in a range of diseases (ClinicalTrials.gov), as either biomarkers for response to drug treatment or as direct therapeutic mediators. It is therefore crucial that appropriate methods are used to separate, validate and characterize EVs, both to improve their clinical application, and to provide fundamental insights and in-depth analyses of their mechanism of action. The aim of this document is to provide guidance on these critical methodological issues and highlight key points for consideration in the design of experiments using EVs. Some of the methods described can be applied generally to all studies using EVs, but we provide CV-specific methods where relevant.

1.1 Definition of extracellular vesicles and use of terminology

Three main classes of EVs can be distinguished by their mechanism of production: exosomes, microvesicles and apoptotic bodies (**Figure 1**). Microvesicles and apoptotic bodies are released directly via outward budding of the plasma membrane in living or dying cells, respectively, and carry proteins, lipids, nucleic acids and other active components that can affect target cells and modify their behaviour.[4, 5, 31] Exosomes are produced by inward budding of late stage endosomes, thereby forming intraluminal vesicles in multivesicular bodies (MVBs), which are released upon fusion of the limiting membrane of the MVB with the cell membrane.[32] The formation of MVBs and subsequent fusion with the plasma membrane is a highly orchestrated mechanism involving the Endosomal Sorting Complexes Required For Transport (ESCRT) machinery, which includes the proteins Hepatocyte Growth Factor-Regulated Tyrosine Kinase Substrate (HRS), Tumour Susceptibility Gene 101 Protein (TSG101), Signal Transducing Adapter Molecule 1 (STAM1) and Programmed Cell Death 6-Interacting Protein (PDCD6IP or ALIX), although ESCRT-independent mechanisms have also been reported.[32] Precisely how cargo is sorted into exosomes is unclear, although some binding motifs have been suggested.[32]

The umbrella term "EVs" encompasses various types of membrane-enclosed vesicles, including exosomes, microvesicles, extracellular autophagic vesicles and apoptotic bodies, and these can have overlapping size ranges (**Figure 1**). However, there is no consensus on specific markers that can distinguish EV types. Consequently, and since it is challenging to isolate individual EV types with high purity, it is preferable to refer to the separated vesicles simply as "EVs" and report the purification methods used for their separation and characterization. The International Society of Extracellular Vesicles in their position paper, MISEV2018 strongly recommended the use of operational terms, based on: size [e.g.: small(s), medium(m) or large(l) EVs); density range (e.g.: low-, middle-, or high- density EVs]; biochemical composition (e.g.: $CD63^{+ve}$ EVs or Annexin 5^{+ve} EVs); or culture- or cell-type of origin (e.g.: hypoxic EVs, cardiomyocyte-derived EVs, etc.), unless the biogenesis of the EVs was determined.[31] However, it must be recognized that many of these terms are protocol-dependent and relative, so it is important that their use is clearly defined. Here, we use the term "sEVs" to refer to purified samples enriched in small EVs and MVB-derived exosomes, and "lEVs" to refer to preparations enriched in larger EVs and shed microvesicles.

2. Source of EVs

For investigations of cardiovascular EV function, primary cells, blood or explanted cardiac tissue may be preferred. When the aim is to develop EVs as therapeutic agents, and large quantities are required,

readily expandable cells or cell lines may be preferable. Mesenchymal Stromal Cells (MSC) are a popular source as they are cytoprotective, can improve cardiac contractility and calcium handling and have beneficial immunomodulatory effects including in the setting of atherosclerosis and pulmonary hypertension.[14, 33-35]

EVs from many different sources have been shown to improve cardiac function following MI, including cardiac stem cells,[36] cardiovascular progenitor cells (CPC),[37], endothelial progenitor cells,[38] cardiosphere-derived cells,[39] embryonic stem cells[40] and iPSC-derived cardiomyocytes[41](reviewed in [5]). EVs from the epicardium can promote proliferation of cardiomyocytes.[42] EVs can also be beneficial against other forms of injury such as doxorubicin/trastuzumab-induced cardiac toxicity.[26] On the other hand, EVs can be detrimental, for example contributing to vascular smooth muscle cell calcification.[15, 16] As yet, there is little consensus on the ideal source of EVs, however one head-to-head comparison suggests CPC may be more efficacious than BM-MSC.[25]

Certain stimuli can alter EV production and function, in a cell-type dependent manner, including calcium,[43] hypoxia/ischaemia,[44] shock wave therapy,[45] atorvastatin,[46] and exercise.[47, 48] Conversely, cardiovascular disease can alter EV production and function. For example, myocardial infarction increase EV release,[49] EV-miR-mediated vascular intercellular communication is altered in patients with CAD and CKD, promoting CKD-induced endothelial dysfunction,[50] and diabetes mellitus impairs EV function.[51, 52]

Cells can be cultured in standard tissue culture flasks, or bioreactor flasks or hollow fibre reactors may be used to maximize production. However, it is important to realise that culture conditions can affect sEV contents and activity significantly.[53]

3. Methods of separation

The optimal method for separating EVs depends on which biofluid or tissue is used as a source.

3.1 Separation of EVs from cell culture medium

Several techniques have been developed for the separation of EVs from cell culture medium, each with their advantages and disadvantages (**Table 1**). Most procedures are based on separation by size, and/or density, although many other extracellular particles may share these characteristics with EVs. A protocol of differential centrifugation or ultracentrifugation published by Thery *et al*. is commonly used to separate both sEVs and lEVs (**Box 1**).[54] A subsequent density-gradient separation using sucrose or, preferably, iodixanol, further improves EV purity.[55] Size-exclusion chromatography has become popular since it effectively removes part of the contaminating soluble protein, and columns can be readily made or purchased (**Figure 2D**).[56, 57] Precipitation of sEVs is possible using polyethylene glycol (PEG)-based reagents, for example in HEK293 or MSC cultures,[58] but the purity obtained is generally inferior to other techniques.[55, 59] Ultrafiltration is more commonly used as an initial clean-up step to remove larger (e.g.: >0.8 µM) contaminants because membranes can become blocked when filtering large volumes and because of concerns that high pressures may damage the membranes of larger EVs. Affinity isolation, typically using antibodies, provides highly pure isolates although at the expense of yield, and only a subset of EVs might be isolated.[60] Furthermore, the procedure to recover EVs from antibodies could

affect their functionality and requires testing.[61] Diafiltration, asymmetric flow field-flow fractionation (AF4)[62] and tangential flow filtration[63] purify and concentrate sEV fractions and are scalable, but AF4 requires specialized and expensive equipment.

Several head-to-head comparisons of EV separation procedures have been published[55, 59, 64, 65], for human plasma, urine and also specific cardiac-derived progenitor cells, but ultimately, the optimal method and obtained quantity depends on the source of the biofluid, the amount of available biofluid and the intended use. For clinical analyses of thousands of blood samples for EV-associated biomarkers, rapid precipitation might be sufficient but for mechanistic studies, purer EVs is essential. The use of cell culture medium as a source of EVs allows for more rigorously controlled conditions for EV production, but the cell culture environment differs from *in vivo* physiology. Given the challenge of removing contaminating serum EVs, protein and lipoproteins, when highly pure EVs are required for 'omics analysis or functional investigation, it is advisable to harvest EVs from cells grown in chemically defined medium rather than EV depleted serum or serum-replacement supplements. However, control experiments must be in place to assess cell viability and contents of contaminating apoptotic bodies, when removing serum. EV-depleted serum may be used but still contains large quantities of proteins and lipoproteins which can co-isolate with EVs / are common contaminants of EVs and procedural controls are necessary to check for potential contaminant.[66]

3.2 Separation of EVs from blood

A critical consideration when separating EVs from blood is the pre-analytical procedures (**Table 2**).[67, 68] For instance, EVs can be separated from either plasma or serum, but serum preparation causes platelet activation, which releases large numbers of platelet-derived EVs, and the thrombus formed traps some of the EVs.[69] The yield of EVs separated from plasma can be affected by the type of anticoagulant used and requires great care to prevent platelet activation and haemolysis. It is possible to use any of the methods described above to separate EVs from platelet-free plasma. Plasma contains only ~10^8-10^{10} sEVs / ml and ~10^6 lEVs / ml compared to ~10^{16} lipoprotein particles/ml and large quantities of albumin, globulins and other proteins and substances, which greatly complicates the isolation of EVs.[70, 71] However, by combining several orthogonal methods it is possible to improve both yield and purity of EVs.[72] Given the many variables that can substantially influence EV yield and purity, it is essential that all pre-analytical procedures and residual contaminants are comprehensively reported alongside the separation method.[73]

3.3 Separation of EVs from tissue

The isolation of EVs from tissues has considerable scientific interest for understanding their local and remote roles in cardiovascular disease development. Their presence should first be confirmed *in situ*, e.g. electron microscopy can identify the presence of vesicle structures in pathological samples such as human atherosclerotic plaques, ischaemic heart and muscles, or the brain[74, 75]. EV separation from fresh tissues represents a challenging task as the method used should ensure that isolated vesicles come from the extracellular space and do not result from tissue homogenization (cell death, membrane self-assembly; **Table 2**). Gentle mechanical disruption of tissue, optionally followed by enzymatic treatment, can be used to release EVs.[43] EVs have been released by collagenase perfusion of Langendorff-perfused rat hearts followed by differential centrifugation.[76, 77] Appropriate controls should be considered to estimate the effects of the procedure. Therefore, using tissues from genetically modified models and

processing healthy tissues or tissues from sham animal models in parallel to pathological samples might help evaluate the direct effect of tissue homogenization.[49, 74, 78] Furthermore, the effect of the enzymatic cocktail on EV numbers and protein expression also requires investigation.[76]

4. General principles for EV identification and characterization

A number of recommendations have been published regarding how to characterize and confirm identity, yield and purity of EVs,[2, 5] but the most authoritative are The Minimal Information for Studies of Extracellular Vesicles ("MISEV") guidelines published by the International Society for Extracellular Vesicles (ISEV).[31] A key overriding principle of the guidelines is that multiple, complementary techniques should be used to characterize EVs. Other guidelines have made quantifiable metrics to define the identity of MSC-sEV preparations, and facilitate stratification and comparison of different MSC-sEV preparations for therapeutic purposes.[79]

First, it is important to quantify the number of EVs relative to the total lipid or protein content of EV preparations obtained. The yield of EVs should be measured relative to the amount of starting material (e.g.: number of secreting cells, volume of biofluid, or mass of tissue). This calculation should be performed every time EVs are isolated since it can vary significantly. Second, the presence of at least three positive protein markers of EVs (described below) is strongly suggested. Third, it is preferable to evaluate the presence of nonvesicular co-isolated components, e.g.: apolipoproteins A1, A2 and B (APOA1, APOA2, APOB), and albumin from plasma/serum isolates. Fourth, the presence of individual EVs should be demonstrated using, for example, electron microscopy or scanning probe microscopy. If an image with a single vesicle is shown then a wide-field image should also be shown, which helps to illustrate the purity. The most appropriate technique for characterization depends on the type of EV (large or small), as discussed below.

4.1 Techniques for identifying EVs

The most widely used techniques for quantifying EVs include light scattering techniques such as dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), and resistive pulse sensing (RPS) (**Figures 2E-H**). However, the robustness and comparability of measurements is hampered by the lack of standardization, and quantification of EVs is less straightforward than it seems.[80] For example, each technology has different limitations and potential biases towards certain size ranges. An important limitation of most widely used techniques is that they measure all particles, and cannot distinguish between sEVs and lipoprotein particles, protein aggregates, EV aggregates or other contaminants. Consequently, less pure isolates can paradoxically give the false impression of containing greater numbers of EVs. For this reason, it is preferable to use additional measurements such as total protein and/or lipid content to indicate the yield and purity.[81] Alternatively, quantification of EV marker proteins by ELISA (enzyme-linked immunosorbent assay) or Western blot (semi-quantitative) can be useful for comparing yields.

Since one of the defining features of exosomes is their size, this is another informative parameter to report when separating small EVs, although this is not specifically recommended in the MISEV2018 guidelines. The size distribution of EVs can be obtained using NTA or RPS, calculated from electron

microscope images, or using another technique. A second defining feature of MVB-derived exosomes is that they contain proteins involved in MVB formation and/or exosome release (e.g.: CD9, CD63, CD81, Alix/PDCD6IP, TSG101).[60] These can be used as positive protein markers to indicate the enrichment of MVB-derived exosomes within the separated EVs. The presence of at least 3 markers should be demonstrated.[31, 60] Notably, acetylcholinesterase is no longer considered a generic marker of exosomes.[82]

Large EVs have a less well-defined size-range but can be analysed using similar techniques as for small EVs, or using flow cytometry, which is described below.[2, 80]

4.2 Electron microscopy

Transmission electron microscopy (TEM) allows imaging at the single EV level, visualizing their size and morphology, as well as detecting the presence of contaminants. Negative staining with uranyl acetate is the most common method. Of note, drying during preparation results in a typical "collapsed vesicle" or "cup-shaped" appearance (**Figure 2B**).[54] Nowadays, the gold-standard method for imaging biological objects is cryo-TEM, which preserves their native hydrated structure via rapid freezing. Cryo-TEM presents several major advantages, including better capacity to distinguish *bona fide* EVs from non-vesicular particles and to determine the actual EV size, and to characterize heterogeneous EV samples, particularly the presence of EV aggregates either contained in the original sample or induced by isolation procedures. Combining EM with immuno-gold labelling aids with phenotyping of EVs in complex media, such as pure plasma or heterogenous media (**Figure 2C**).[83] Other techniques, including single EV-microarray and atomic force microscopy can provide images of single EVs, as well as information on their biomechanical properties and size.[84]

4.3 Flow cytometry

Flow cytometry is an attractive technique for EV analysis, as flow cytometers are robust platforms, widely available and designed for high throughput quantitative analysis of single particles based on light scattering and fluorescence. However, flow cytometers are designed to analyse cells and several requirements need to be met to improve rigor and reproducibility of EV analysis.[85] Flow cytometric analysis of sEVs (<300 nm size) is particularly challenging due to their dim fluorescence and scatter signals.[85] In this respect, it is extremely important to calibrate flow cytometers, confirm detection of single EVs and be aware of the sensitivity of the platform used and potential interference by unbound fluorescent probes.[86, 87] Nevertheless, the use of single EV flow cytometric analysis has reached a level were reproducible comparisons of EV concentration measurements can be nearly performed, for example of circulating EVs in patients with CVD.[88-90] Marker proteins of interest for cardiovascular studies include those such as CD61 and CD144 for platelets and endothelium respectively, CD147 (SIRPα) for cardiomyocytes, CD235a for erythroid-derived EVs and leucocyte/lymphocyte- and monocyte-derived EVs (CD45/CD3 and CD14).[88-91] The MIFlowCyt-EV Framework, drafted by an EV flow cytometry working group of ISEV-ISAC-ISTH (www.evflowcytometry.org), provided a consensus report for EV flow cytometric studies,[86] advising the minimal experimental information that should be reported.

4.4 Functional analysis of EVs

Ideally, the functional activity of EVs would be assayed using a simple, *in vitro* potency assay as a surrogate for their *in vivo* functionality, but no single, universal method has been identified. In the cardiovascular field, EV function is commonly assessed using an assay of *in vitro* angiogenesis, cell viability, contractility, or combinations thereof. Commonly used in vitro assays of angiogenesis include the scratch assay,[91] Boyden chamber migration assay,[92, 93] endothelial tube formation[94], and vessel sprouting assays.[44, 95, 96] An accurate measure of sEV quantity and purity is important when conducting dose-response experiments of their functionality. At present there is no consensus on which measure of quantity (particle number, protein content, quantity of starting cells, etc) is preferable,[31] but whichever normalization technique is used (preferably more than one) it should be reported and justified. Furthermore, appropriate (procedural) controls should be included to proof that effects are EV-mediated. For the use of EVs as therapeutic tools, *in vitro* potency assays are required to predict the effectiveness of EV preparations for clinical use, but this depends on the ability to convincingly identify the mechanism of action and quantify the biological activity.[97]

4.5 Reporting methodology

Finally, to aid reproducibility and transparency, isolation and characterization methodology should be reported in public databases and repositories such as EV-TRACK, a crowdsourcing knowledgebase (http://evtrack.org) that centralizes EV biology and methodology with the goal of stimulating authors, reviewers, editors and funders to put experimental guidelines into practice.[98]

5. Chapter 4: Methods for determining the protein content of EVs

5.1 Total protein content

Total protein content in an EV preparation can be estimated using standard protein assays such as bicinchoninic acid (BCA) assay or Bradford assay, or variations thereof, optimised for low protein concentrations. Quantification of total protein in an EV sample and comparison with particle counts may give an indication of its purity. It has been suggested that pure sEV isolates contain concentrations of < 1 µg protein / 10^{10} EV particles,[81] although this is not necessarily universally applicable, because there are not yet methods available that can measure all EVs.

5.2 Antibody-based techniques to identify specific proteins

There may be subpopulations of EVs with different protein content that can be detected using antibodies. Some can be used as marker proteins to identify the cell type of origin within the cardiovascular system (see section 3.3). In addition to EV marker proteins, hundreds of additional proteins can be identified, which may be either genuine EV components or co-isolated proteins. The most common approaches to detect and quantify the relative levels of EV proteins are antibody-based experimental methods (**Table 3).**[31] All antibody-based techniques require the use of appropriate controls to confirm antibody specificity.[99]

Western blotting can identify proteins that are associated or co-isolated with EVs and provide useful information about the yield and purity of an EV preparation.[64] Importantly, it can also confirm the

molecular weight of the target protein. Compared with cell lysates, a disadvantage of EV samples is the lack of reference ("house-keeping") proteins to use for normalisation purposes in immunoblotting experiments. Therefore, equal protein amount, volume from which EVs are separated or particle number are commonly used. Inclusion of the original sample, the EV-depleted sample and procedural control samples are required to draw firm conclusions about enrichment of proteins in the EV isolate (or depletion of contaminants). Western blotting can be challenging since it requires relatively large quantities of EVs for sufficient sensitivity. Alternative versions such as dot blotting or capillary electrophoresis immunoassays can provide considerably higher sensitivity.[100]

The question of which proteins should be investigated as potential contaminants is debated, but the best guideline is provided by MISEV.[31] Depending on the source of EVs, it can be useful to verify the removal of lipoproteins (e.g.: APOB, APOA1, APOA2) and serum albumin (**Figure 3**), and proteins from endoplasmic reticulum or plasma membrane.

ELISA is a well-established technique that can provide sensitive antibody-based detection in multi-well formats. A sandwich ELISA format (combining separate capture and detection antibodies) is likely to be required when using enzyme-linked or fluorescent detection, but a highly sensitive immunoassay variant based on time-resolved fluorescence called DELFIA (dissociation-enhanced lanthanide fluorescence immunoassay) is able to detect EV-associated molecules using a single detection antibody.[64, 101] Similar to dot blots, immunoassays provide good sensitivity for small sample amounts, but require thoroughly validated antibodies and do provide information to validate the molecular weight.

EV flow cytometry can be used to detect surface protein markers as indicated above. Immuno-gold labelling can be performed for visualization using TEM or cryo-TEM, although it is not quantitative, and it is mostly used to label EV membrane proteins. Detection of immunogold label on non-EV particles in the sample may indicate that the target is only a contaminant in the EV isolate.

Novel antibody-based approaches such as surface plasmon resonance[102] and interferometric imaging[103] have also been utilized for EV protein characterisation, but they usually require expensive specialised equipment and consumables which limits their widespread use.

5.3 Mass spectrometry of the EV proteome

Proteomic analysis of EV samples by mass spectrometry (MS) provides the most comprehensive analysis of the EV protein cargo (**Table 3**), and does not rely on an *a priori* selection of proteins based on the availability of antibodies or other affinity reagents for specific proteins.[60, 104] MS approaches, however, have an inherently lower sensitivity compared with antibody-based techniques. This is mainly due to the excess amounts of highly abundant proteins (*e.g.:* albumin) in the EV preparations which mask the presence of low-abundant EV proteins.[105] To address this, MS can be combined with better isolation techniques for EVs that result in less contamination. It is recommended to compare the EV proteome to tissue or cell source of the EV sample to identify the degree of enrichment/depletion of proteins. For EVs separated from cell cultures in which media are supplemented with xenogenous components (*e.g.* bovine serum), it is also recommended to searches against databases of other organisms. Bovine serum proteins are a common contaminant in EVs isolated from cell cultures, unless cells are grown in serum

free media. Finally, independent validation with an antibody-based technique is advisable since MS detects peptides, which can originate from both intact and fragmented proteins. Most journals require that EV proteomic data are deposited in online databases.[106]

5.4 Intraluminal vs membrane proteins

Determining whether a protein is intraluminal, membrane or external to the EVs is of great importance for understanding the structure, origin and function.[31] Mixing a broad-range protease (*e.g.* proteinase K) with an EV-containing sample in presence or absence of detergent can help to establish whether a protein is intraluminal or present on the surface/outside of the EVs. Notably, EV subtypes have different sensitivities to detergents.[107] Detergents will also disrupt other lipid structures such as lipoproteins, another common contaminant in EV preparations. Protease treatment can also determine the topology of membrane proteins or the degree of contamination of an EV sample,[108] but proteases will digest the extracellular domains of EV membrane proteins. Alternatively, surface labelling can be performed to enrich for EV membrane proteins and distinguish them from intraluminal cargo.[109]

6. Methods for determining the RNA content of EVs

EVs carry various species of RNA, including microRNA (miRNA), circular RNA (circRNAs), vault RNA, small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), Y RNA, transfer RNA (tRNA), long non-coding RNA (lncRNA) and messenger (mRNA), as well as fragments thereof.[60] EV subtypes differ in their RNA cargo profile, according to parent cell type and environment, as well as stochastic principles, and the method of isolation used.[110] Although most attention has focused on the miRNA content of EVs, miRNAs might only represent a minor constituent of EVs relative to other RNA species.[111] The mechanism for sorting RNAs to EVs might include association with RNA-binding proteins, specific RNA motifs and RNA modifications.[112, 113]

6.1 RNA analyses by qRT-PCR and RNA-sequencing

At first, RNA cargo of EVs was based solely on the use of Taqman miR-PCRs focused on individual miRNAs, and it was a challenge finding ways to normalize data. Data normalization was usually implemented by spiking-in an exogenous miRNA supposedly not expressed in mammalian species, such as *Caenorhabditis elegans* miRNA-39 (Cel-39) before RNA extraction. More recently, several quantitative PCR (qRT-PCR) and digital PCR protocols are available to detect the miRNA cargo of EVs.[114]

Advances in RNA-sequencing technologies have enabled the identification of EV-derived RNAs in nearly all human biofluids,[115] and associated with pathophysiological phenotypes.[116] The use of RNA-sequencing approaches has provided a better understanding of the diversity of the EV-embedded RNAs.[46, 60, 117]

Certain pre-analytic confounders are well known, e.g.: heparin can interfere with PCR analyses of RNAs,[118] but can be overcome by heparinase treatment. The presence of certain miRNAs is suggestive of haemolysis of blood samples (e.g.: miR-486-5p, miR-451, miR-92a, and miR-16), or presence of contaminating calf serum (e.g.: miR-122, miR-451a and miR-1246).[119-121] Lipoprotein contamination can also create difficulties in data analyses and interpretation since they can also carry miRNAs[122]. To

prevent contamination of EV preparation by RNAs carried by lipoproteins and extra-EV Argonaute proteins, the use of proteinase K and RNase A digestion can be implemented before proceeding to RNA extraction.[112] It is useful to include a negative control without enzymatic treatment and positive control samples containing RNA, to confirm complete digestion of non-exosomal RNAs.

In order to compare data, several manually curated database were developed: Vesiclepedia (http://www.microvesicles.org/) and Exo-carta (http://www.exocarta.org/) include RNAs, lipids and proteins identified in different classes of EVs. More recently, the extracellular RNA communication (ExRNA) consortium (https://commonfund.nih.gov/exrna) was created by the NIH to establish foundational knowledge and technologies for extracellular RNA research (https://exrna-atlas.org/).[123]

6.2 How to evaluate the functional role of EV RNA

Despite the numerous examples of studies suggesting important roles of EV-mediated RNA transfer on target cell behavior, e.g. the regenerative potential of epicardium-derived extracellular vesicles mediated by conserved miRNA transfer, assessing the true (patho-)physiological role of such transfer is a formidable challenge, not least because of the relatively low EV RNA concentrations. For investigations into general mechanisms underlying EV-mediated RNA transfer, sensitive reporter systems have been developed that allow the study of EV-RNA transfer at the single cell level.[124, 125] However, to prove a direct effect of endogenous RNA species on EV target cells, additional challenges need to be addressed and important control experiments are required. These include demonstrating that the RNA of interest: 1) full length is present inside EVs; 2) shows increased levels in recipient cells upon delivery (in the absence of upregulated expression); and 3) directly mediates a particular response in target cells, by interfering with its presence or function without affecting the content of EVs or recipient cells in any other way. Recently published reporting guidelines on EV-RNA studies should help to ensure reproducibility and to critically evaluate past and future studies claiming EV-RNA-induced physiological and pathological responses.[112]

7. Methods for determining EV lipid content

7.1 Lipid content

The phospholipid bilayer membrane of EVs consists primary of phosphatidylcholine, in addition to phosphatidylethanolamine and phosphatidylserine.[62, 126] The sEV membrane is relatively rigid due to its enrichment in sphingomyelin and cholesterol, and contains domains with an ordered lipid phase ("lipid rafts"; reviewed in [127]).

Notably, EVs also carry lipids involved in signalling such as eicosanoids together with functional phospholipases and enzymes of the prostaglandin pathway.[128] The lipid composition of large EVs is closer to that of the plasma membrane, which they originate from.[126] Translocation of phosphatidylserine to the outer leaflet upon cellular activation has been suggested to be a prerequisite for large EV biogenesis[127] . EVs with externalized phosphatidylserine are highly pro-coagulant, leading to venous thrombosis, particularly in the presence of tissue factor (TF).[129]

Total lipid content can be easily measured using a sensitive assay.[130] The total protein-to-lipid ratio of an EV sample can then be used as an indication of EV concentration and purity.[130, 131] However, like protein assays, lipid assays are affected by the presence of contaminating lipoproteins.

MS is increasingly used to determine the complete lipidomic profile of EV samples[62, 126]. Furthermore, targeted lipidomic strategies can be developed based on the results of untargeted MS-based lipidomics. Newer techniques include total reflection Fourier-transform infrared spectroscopy (ATR-FTIR)[132] and Raman spectroscopy.[133] Raman spectroscopy reveals the chemical composition of single sEVs, and can identify different subpopulations of EVs based on their overall biochemical composition, including cholesterol content, phospholipids-to-cholesterol ratio, and surface protein expression.[133]

Most lipidomic studies of sEVs show an enrichment from cells to sEVs for cholesterol and sphingomyelin (representing approx. 40-50% and 10-20% of total small EV lipids, respectively).[134] Phosphatidylcholine and phosphatidylserine are in general the most abundant glycerophospholipids while phosphatidic acid, phosphatidylglycerol and phosphatidylinositol tend to be lower. Compared to cells, the content of phosphatidylcholine and phosphatidylinositol is generally lower in small EVs, while sphingolipids are increased. Certain lipids such as triacylglycerols and cholesteryl esters are found in lipoproteins and lipid droplets, and a high content of these lipids in EV preparations might be indicative for co-isolated or contaminating particles. There is evidence that sphingolipid composition of circulating EVs is altered after myocardial ischaemia.[135] Of note, ceramide content in adipocyte-derived EVs regulate vascular redox state in obese patients and is associated with cardiovascular mortality.[17] EV lipid composition is also dependent on EV type. MVB-derived small EVs have a higher cholesterol content than EV types released from the plasma membrane.[131] In line with this, sEVs show the highest resistance to detergent lysis among EVs.[107]

A subset of circulating EVs display oxidation-specific epitopes (OSE), which are immunogenic adducts derived from (phospho)lipid peroxidation.[136] Thus, OSE+ EVs may be practical markers of pathology-associated oxidative stress and may reflect pathological conditions better than EVs. Several different types of OSE can be identified using specific antibodies, including malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and phosphocholine-containing oxidized phospholipids (PC).[137]

8. Measurement of enzymatic activities carried by EVs

EVs harbour active enzymes on their membrane. Most surface enzymes are not easily detectable although the functional activity of EVs can still be measured due to the amplification of the detection signal through the enzymatic process for such enzymes, including e.g. the generation of factor Xa.[138] Moreover, in most cases, both activators and inhibitors of a biological process are present at the same EV membrane. The overall functional activity of EVs will reflect the combined effects of these molecules.

8.1 Pro-coagulant activity

Large EVs possess procoagulant activities. This is mainly determined by the exposure of anionic phospholipids, especially phosphatidylserine which allows the binding of coagulation factors to the EV surface, as well as the exposure of active TF on some subsets of EVs.[139] Assays measuring the functional capacity of EVs to generate factor Xa, thrombin, or a fibrin clot have been developed.[140]

Phosphatidylserine contributions can be evaluated measuring a phospholipid-dependent coagulation time after EV dilution in a phospholipid-depleted plasma and activation with factor Xa (FXa) and calcium.[141] Other assays combine solid-phase capture of EVs by annexin V and thrombin generation.

A second group of assays focuses on the measurement of TF-dependent procoagulant activity of EVs. Thrombin generation in platelet-free plasma or purified EVs spiked in EV-free plasma is initiated in the presence of phospholipids without TF. High concentrations of TF-EVs are necessary for detection with this assay. Other studies evaluating the value of EVs as a biomarker of thrombosis have measured procoagulant EVs with FXa generation assays, using either EVs captured on coated plate or EV isolation using ultra-centrifugation (UC).[142, 143] A more global assay also monitors fibrin generation after incubating plasma EVs isolated by UC in the presence of anti-TF or anti-FXII blocking antibodies.[144]

In clinical practice, all these assays are currently limited either by a lack of specificity, a low sensitivity, or irreproducibility when UC is used to isolate EVs. For example, measurement of TF by flow cytometry remains challenging because of the low levels of TF and some concerns about anti-TF antibody specificity.[145] To tackle such issues, a new EV-TF activity assay was recently developed using a new inhibitory anti-TF antibody and a more sensitive protocol.[146]

Comparisons of assays measuring EV-TF activity suggest that Factor Xa generation assays are more sensitive than the Zymuphen assay,[147] and a poor correlation was found between results of the factor Xa generation assay and the fibrin generation test.[148] ISTH initiated a new collaborative project to compare the analytical performance of different assays measuring EV-TF in plasma samples[149] to progress towards an optimal method to measure EV procoagulant activity in plasma samples.

8.2 Fibrinolytic activity

EVs have ambivalent functions in haemostasis since they also possess fibrinolytic activity. A subset of EVs may indeed vector plasminogen activators such as urokinase.[150] Just as for procoagulant assays, the use of UC can result in poor reproducibility of fibrinolytic assays. To overcome this limitation, a hybrid assay combining specific capture of EVs and measurement of their plasmin generation capacity has been developed.[151] High resolution laser scanning confocal microscopy could be also used to detect EV enzymatic activity using fluorescent reporters.[152] However, throughput is limited.

8.3 Enzymatic activities

Presence of acetylcholinesterase is no longer used as a reliable EV marker; neurons and red blood cells produce this activity in abundance, whereas it is almost undetectable in other cell types and often associated with non-vesicular structures.[82] Several metalloproteases, e.g. disintegrin metalloproteases and tissue inhibitor of metalloproteases have been reported in different EV preparations; these activities could confer on EVs the capacity to promote cell proliferation and remodelling of the microenvironment, which could contribute to EV therapeutic potential.[153] However, it remains crucial to demonstrate that the enzymatic activity is associated with EVs and not with soluble mediators, and does not result from co-isolation during the purification procedure.

9. Methodologies for functional characterization of EVs

Due to the variable quality of the tools and technologies used to study EVs, complete and accurate reporting of methods is essential. These include the above-mentioned isolation and characterization techniques, but to understand the functional interaction and potential of different EV preparations, other points should be taken into consideration.

i. In addition to EV purification and isolation, "EV-depleted" samples and quality and procedural controls (e.g.: unconditioned cell-culture medium processed in the same way) can help to determine true EV-mediated responses. GW4869, an inhibitor of neutral sphingomyelinase 2 (nSMase2) and sEV release, is sometimes used as a control, but care is required in its use, as it is unlikely to be specific for exosome release.[31, 154]

ii. Co-purified and bound molecules might affect functional assays,[155] therefore it is best to avoid low-specificity methods such as general precipitation (polyethylene glycol, "salting out," the basis of many commercial "exosome isolation" kits), unless these methods are combined with additional separation steps.

iii. The biological nature of EV preparations makes normalization between conditions essential but there is no clear consensus on the best way forward. Some alternatives include: starting volume or the number of producing cells; total number of EVs; protein content; lipid content; metabolite content; or specific markers such as levels of tetraspanins or other putative house-keeping proteins or RNA species.[156] It is recommended to have 2-3 different approaches, and to clearly describe each, to allow potential differences in functional outcomes to be explored.

iv. For clinical therapeutic interventions, the identity of the EV preparations can be defined using quantifiable metrics.[79]

v. In classical dose-response experiments, the relationship between the concentration of a ligand/drug and a measured outcome parameter is investigated. Such experiments should be considered to understand the dose-dependency of effects, and to understand the biological relevance of the quantity of EVs used. In many published works, the dose relative to physiological concentration is unclear.

vi. Profiling of the EVs proteome and RNAome also will help to characterize their origin and also potential functional activities.[157]

9.1 Uptake and biodistribution studies

To understand specific uptake of EV species or how different EV subpopulations are produced, several potent inhibitors are commonly used, including chloroquine, neutral sphingomyelinase inhibitors, or genetic removal of Rab-protein family members.[27, 158, 159] Inhibitors of micropinocytosis, endocytosis (clathrin, caveolin or lipid-raft dependent), phagocytosis or membrane fusion are also suggested to decipher *in vitro* the different routes and mechanisms of EV uptake by target cells.[160] Since these suggested compounds lack specificity, it is important to keep in mind that they only *suggest* potential mechanisms. No EV-specific interventions have been reported thus far.

It is challenging to document the *in vivo* biodistribution of EVs. Many studies first isolate and tag EVs before injecting them *in vivo*, but these exogenous EVs may not reflect the same fate as endogenously released EVs. In addition, the presence of residual contaminants from the isolation procedure, the route of administration, the type of label used, the animal model and the detection method may all affect *in vivo* biodistribution. If fluorescent dyes are used for EV labelling they should be carefully selected. Many dyes, particularly lipophilic dyes, can form dye aggregates or micelles that are of similar size to EVs, or may bind to contaminants present in the isolate, such as lipoproteins and certain proteins.[161] Furthermore, lipophilic dyes might dissociate from the labelled EV and be incorporate into cellular membranes *in vivo*, where long dye half-life may lead to incorrect assumptions about EV distribution and longevity and diffuse freely. Genetic approaches crossing ROSAmTmG mice with models expressing Cre-recombinase in a cell-specific manner have opened new avenues for quantifying uncommon populations of EV, such as cardiomyocyte-derived EVs in the circulation.[162] On the other hand, protein-based labels added using genetic approaches (e.g. GFP) can be susceptible to proteolysis and cannot be used on samples derived from human tissues and fluids. Therefore, careful control experiments are required to ensure the signal is specific and to monitor the influence of any free dye. Cell-cell interaction studies and paracrine activity of secreted exosomes can be studied by e.g. co-culture assays of different cell types. Some examples are reported where (direct) EV-cargo loading is used to detect EV-molecule transfer, but indirect effects and reduced EV functionality are examples of possible limitations of these methods.[163] Possible controls include comparison with the biodistribution of free-label (no EVs) or of EVs that have been physically disrupted.[164]

Investigation of endogenous EV biodistribution requires genetic labelling strategies, such as degron-tagged reporters or pH-sensitive fluorophores, which provide a stronger EV labelling than that of the parent cell.[165, 166] However, these approaches might be restricted to one specific subset of endogenous EVs. The EV-mediated transfer of Cre recombinase into floxed reporter cells appears to be an elegant method to study *in vivo* EV distribution and uptake.[166] Another technique is to detect tissue uptake of a miRNA unique to the EVs, such as a foreign miRNA that the EVs have been engineered to express.[25]

In conclusion, all current approaches to assess EV *in vivo* biodistribution (see **Table 4** for examples) have their strengths and limitations, which must be carefully considered when designing experiments.

10. Methodologies for clinical use of EVs in cardiovascular diseases

Potential regenerative/reparative effects of EVs in the cardiovascular system have been observed in both post-infarction, and non-ischaemic chemotherapy-induced cardiomyopathy models.[1, 23, 26, 37, 39, 42, 167] Although EV biodistribution and direct cellular uptake still needs much attention, preclinical meta-analyses indicate that stem cell-derived EV administration is associated with improvements of left ventricular ejection fraction, fractional shortening and a reduction of infarct size. These benefits are seen largely irrespective of the type of stem cell, timing of injection, route of delivery, dosage of delivery or follow-up period.[168, 169] On the other hand, not unique to EV studies, there is a potential risk of positive publication bias.[168, 169] While these positive data suggest that clinical studies may be warranted, there are a number of important issues to address including those related to upscaling of EV preparation processes in GMP-quality facilities using non-xenogeneic culture conditions, as well as ethical and regulatory approvals.[5] Even with optimization of EV separation and characterization, several practical

hurdles must be overcome to maximize the therapeutic potential of EVs. In addition to regenerative potential, however, EVs can play detrimental roles, for example potentially by causing thrombotic complications or forming microcalcifications that destabilize atherosclerotic plaques.[170] The therapies preventing this deteriorating effect are under investigations.

10.1 Production and storage effects on the quality of EV preparations

Prior to *in vivo* application, it is essential to assess the reproducibility of EV content, purity and functionality in batch preparations. These measures should include evaluation of ingredients and potential co-isolations of culture medium, while also keeping in mind that these might mediate part of the observed functional effects. The production of EV preparations for use in the cardiovascular system is not uniquely different from those for use in other systems. Manufacturing of MSC-sEV preparations for therapeutic applications is currently the most advanced with several preparations in clinical trials, as highlighted elsewhere.[171]

For the isolation of EVs secreted by cells in culture, several cell-culture factories are available, including multi-layered culture flasks,[63] hollow-fibre bioreactors,[172] and microcarriers.[173] Before these systems are used, however, their impact on EV production and bioactivity must be determined. Isolated EVs are believed to be stable and can be frozen, but extensive studies are warranted to confirm that EV functionality is retained following freeze-thaw cycles and long-term storage.[174] Multiple additional considerations are essential for handling blood-derived EVs,[73] including pre-analytical methods, and quality controls.

10.2 Delivery strategies and biodistribution of EVs

Efficient EV delivery to the target organ/cells may be necessary to achieve full therapeutic potential, but it should also be considered that the primary target may not be the diseased tissue if EVs function indirectly. Both systemic and intra-organ delivery is possible and close monitoring of EV biodistribution is needed since cellular uptake of EVs might not be accurately reflected by the tracking-labels used. Due to the small size of EVs, myocardial retention might be severely hampered since even stem cells, which are much larger than EVs, are immediately washed out from the myocardium after injection.[175] EVs delivered intravenously are rapidly cleared (within minutes) and mainly distribute to the liver.[176] Biodistribution studies, in which EVs are labelled with fluorescently linked lipid or amine dyes[177], radiolabels[178] or iron oxide particles,[179] are highly warranted for mechanistic understanding of their effects. To facilitate long-term exposure of EV therapeutics, slow-release systems in which EVs are loaded and slowly exposed to the targeted tissue are key. Both natural[180] and synthetic[177] delivery systems have been developed and display enhanced beneficial effects for cardiac repair[38], with the caveat that they may require a direct intramyocardial delivery whose invasiveness may hamper their clinical acceptance. An alternative approach that has been successfully used to promote cardiac repair following myocardial infarction is thus to inject the EV-producing stem cells into a semi-permeable chamber, which is then inserted subcutaneously to release EVs (and other factors) over time.[36]

10.3 Loading therapeutics into EVs

For successful intra-myocardial delivery, many limitations and barriers have to be overcome,[181] whereas bioengineered EVs with surface and/or cargo modifications might present unique advantages. Engineered therapeutic nanoparticles include: i) vesicle-mimetics produced from cells by serial extrusion or cell membrane-cloaked nanoparticles, which have substantially greater yield and an easy purification process [182]; ii) EV-liposome hybrids, produced using simple incubation or freeze-thaw cycles, for easier uptake by target cells and for enhanced delivery; and iii) synthetic EVs, which are based on liposomes with a composition similar to EVs.

EVs have been modified to deliver small molecules, therapeutic RNA, proteins, lipids and different types of imaging molecules.[183] [184] Materials can be loaded into EVs via both passive loading (e.g. incubation with EVs or with EV-producing cells) or active loading (e.g. sonication, membrane permeabilization, electroporation, antibody binding of EVs or transfection of EV-producing cells). EVs can be labelled on the surface or intraluminally.[164] However, the labelling and loading procedure may alter physical, chemical and therapeutic properties of EVs or EV-mimetics. Moreover, therapeutic loading might be overestimated as observed for electroporation procedures that cause siRNA aggregate formation in the EV preparation.[185] Therefore, a thorough *in vitro* and *in vivo* evaluation of their uptake, stability, efficacy and toxicity is necessary to develop suitable methods for future clinical studies. Recent research suggests that EVs of various sizes can naturally carry intact viruses used in therapeutics such as adeno-associated viruses (AAVs), (reviewed in [181],[157] and may thereby be able to circumvent antibody neutralization.

11. Conclusion

In conclusion, researchers are gradually developing a better understanding of the role endogenously formed EVs in cardiovascular patho-physiology, how they may be sampled as biomarkers of cardiovascular disease, and how exogenously administered EVs might be used therapeutically. Basic procedures and principles for their purification, characterization, analysis and modification are in progress, which will facilitate detailed future mechanistic investigation. However, there are critical caveats at each step, and it is essential to bypass these pitfalls in order to avoid major setbacks and succeed in clinical translation (**Tables 1,2,3**). While relatively impure EV preparations may be shown to contain a desired biological activity useful for clinical applications, mechanistic studies may be hampered by the presence of unknown contaminants. This is essential, since approval of EVs for clinical use is likely to necessitate an effective potency assay (or an array matrix consisting of several potency assays), which would ideally reflect a proven mechanism of action.[97] Apart from better separation techniques, characterization of EV preparations is needed using orthogonal and complementary methods to define the purity of the preparations and will reveal potential sources of contamination. With the wide interest in EVs from both academia and the pharmaceutical industry, there is no doubt that methods will continually evolve and improve, which will help to advance EVs studies in cardiovascular science.

12. Tables

Table 1 Potential advantages and disadvantages of the main methods used to purify sEVs

Method of purification	Disadvantages	Advantages
Affinity-based methods	• Low yield • Non-scalable • Antibodies are expensive and difficult to remove afterwards • Protein contaminants bind to the solid phase	• Highly purified sEVs
Diafiltration	• Specialized equipment required	• Membrane pores rarely block • Re-useable
Centrifugation (Pelleting)	• Labour intensive • Non-scalable • Expensive equipment required • Relatively low purity	• Widely used • Standardised protocol (though may vary with different rotors)
Density gradient centrifugation	• Labour intensive • Non-scalable • Expensive and time consuming • It may be necessary to remove the gradient material, depending on subsequent analysis	• Widely used • Standardised protocol
Field-flow fractionation	• Expensive equipment required • Extensive optimization required	• High purity and yields can be achieved • Scalable
Precipitation	• Relatively low purity	• Very rapid • "Home-made" techniques very cheap
Size-exclusion chromatography	• Labour intensive • Contaminants of a similar size of EVs may co-isolate	• Widely used • Efficient at removing small proteins • Commercial columns available • Large columns can be made relatively cheaply for isolating sub-populations by size
Tangential flow filtration	• Expensive equipment required	• Scalable • GMP-compliant
Ultrafiltration through a membrane	• Low purity • High pressures may damage the membranes of larger EVs • Membranes can become blocked when filtering large volumes	• Scalable. • High yield • Cost-effective • More commonly used as an initial clean-up step or a concentration step post isolation

Table 2. Major factors to consider when isolating EVs from sources relevant to cardiovascular studies.

Source of EVs:	Major factors to consider	Potential solutions
Cell-culture conditioned medium containing serum	• Risk of contamination from serum components including animal-derived EVs coming from serum	• Contaminating EVs can be pre-removed from serum • Consider using serum-free medium[a]
Cell-culture conditioned medium without serum	• Risk of cell phenotypic changes/death contaminating EVs with intracellular or apoptotic vesicles	• Use short-term culture • Quantify levels of cell death
Plasma	• Care must be taken not to activate platelets during collection and handling • Platelets disrupt during a freeze-thaw cycle and hamper EV isolation • Challenging to remove contaminating blood proteins and lipoproteins	• Carefully define suitable pre-analytical procedures • Isolate EVs using a combination of orthogonal techniques
Serum	• EVs are released from activated platelets • Challenging to remove contaminating blood proteins and lipoproteins • EVs lost in the fibrin clot	• Carefully define suitable pre-analytical procedures. • Isolate EVs using a combination of orthogonal techniques.
Tissue (e.g. myocardium)	• Challenging to disrupt tissue without damaging the cell membrane • Risk of shaving epitopes from EVs when using proteolytic enzymes	• Perform control experiments to ensure cells are not disrupted • Titrate enzyme quantity and use the minimum

The importance of these points will vary depending on the intended use of the EVs, and must be evaluated separately for each experiment.

[a]As noted in the main text, these solutions can introduce problems of their own. e.g. EV removal from serum also removes other components, and it is probably not possible to remove 100% of the EVs. Serum-free medium may negatively affect cell health and EV quality.

1 ***Table 3 Advantages and disadvantages of common techniques used for EV detection[a]***

Detection method	Advantages	Disadvantages
Capillary electrophoresis immunoassay[b]	• Smaller sample volume required • Ease of automation • Fast separation and data acquisition	• Expensive instrumentation • Limit of detection poorer than solid phase detection (*e.g.* immunoassay)
DELFIA[b]	• Microplate setup • Higher throughput than immunoblotting • Sufficient sensitivity with only one antibody	• Requires plate reader with time-resolved fluorescence (TRF) detector • Risk of false positive signal with low specificity antibodies
Dot blotting[b]	• Smaller sample volume required • Protocols shorter than western blotting	• Molecular weight not determined • Risk of false positive signal with low specificity antibodies
Flow cytometry	• Suitable for large EVs (>300nm) without generic fluorescent labelling • High throughput (suitable for clinical studies) • Quantitative analysis of single EVs • Can use multiple detection antibodies • Bead-based immune capturing protocols can be used to perform EV subset analysis[b] •	• Small EVs (<300nm) are below the limit of light scatter detection of many conventional flow cytometers • Generic fluorescent EV labelling may introduce biases in EV detection of heterogeneous EV preparations • EV-associated proteins may be below the limit of detection • Lengthy sample preparation with multiple control conditions required
Imaging cytometer[b]	• Can detect single small EVs • Can use multiple detection antibodies	• Specialized equipment required • Extensive protocol development required
Immunoelectron microscopy (TEM or Cryo-TEM)[b]	• Single particle detection • Can distinguish membrane and intraluminal targets	• Expensive equipment • Mostly qualitative
Mass spectrometry	• Comprehensive picture of the EV proteome • Quantitative analysis of more than one target protein • Label-based approaches powerful for quantitative purposes	• Expensive equipment • Lengthy sample preparation • Substantial quantity required • Poor limit of detection due to the presence of high-abundant contaminants
Sandwich ELISA[b]	• Microplate setup • Higher throughput than immunoblotting	• Risk of false positive signal with low specificity antibodies
Transmission electron microscopy (TEM)	• Single EV detection • Can distinguish membrane and intraluminal targets	• Expensive equipment • Sample is dried so EV morphology is altered • Mostly qualitative data
Cryo-transmission electron microscopy (Cryo-TEM)	• As per TEM • Shows native shape of EVs	• As per TEM
Western blotting[b]	• Well-established protocols • Molecular weight determined	• Large sample volume required • Time-consuming • Usually semi-quantitative

[a]An important overarching consideration is whether isolation of EVs is necessary for subsequent analysis steps. E.g.: Some analysis techniques such as flow cytometry can be optimized to work in the presence of (diluted) plasma or serum, negating the need for purification and its attendant limitations and inherent variability.

[b]All techniques using antibodies require validation of antibody specificity and optimisation of their concentrations and blocking reagents.

Table 4 – examples of EV labelling for direct transfer and biodistribution studies

Method of EV labelling	(Animal) models	Observations	Advantages	Disadvantages	References
Lipophilic dyes (e.g. PKH26, PKH67, DiD)	• Ischaemic mouse hearts • cell lines	• EV-bound labels co-labelled with cardiac-specific cell types • direct transfer *in vitro* cultures	• Well-established protocols	• Non-EV mediated dye transfer from EVs to other cells or organs. • Free label transfer	27 161
• Donor cell RNA transfer • cel-miR-39 overexpression donor cell (lipofectamine)	• *In vitro* cell model • Perfusing isolated rat hearts	• Mouse proteins present in human cell lines • Dose-dependent presence of increased cel-miR39 levels in cultured cells and ex vivo hearts	• Intact EV sorting and mechanisms • Well-established protocols	• Variation in EV content due to donor cell changes	186 25
EV siRNA loading	• Electroporation	• Knock-down of target genes in organs	•	• Disruption of EV integrity and functionality	187
Fusion proteins	• Luciferase- or • GFP-linked labels to CD9 or CD63 • CD63-pHluorin	• Cardiac-specific EV tracking via Luciferase expression • In vivo and in vitro EV release, transfer and function	• Direct EV visualizations • EV release and organ specific uptake	• EV functionality disrupted • Limited signal detection	188 189 166
Degron reporters	• In vitro cell models	• Highly sensitive EV release	• High sensitive	• Functional tools need donor/target manipulations	165

13. Figure Legends

Figure 1.

The typical size range of the major lipid-bilayer EVs up to 1000 nm diameter.
[a]As reported by Jeppesen et al.[60]
[b]The size of apoptotic vesicles/bodies can range up to 5 µm in diameter.
Please be aware that the diameter of EVs depends on the detection method used.

Figure 2.

Representative images of different techniques of EV characterization.

A) Transmission electron micrography (TEM) of multi-vesicular body (MVB) containing exosomes (arrows) in primary HUVECs.
B) Transmission electron micrography (TEM) of negative-stained EVs isolated from HUVECs (sEV = small EVs, lEV = large EVs).
C) Cryo-TEM of a single CD81+ EV from iPS-derived cardiovascular progenitor cells.[37] The lipid bilayer is clearly resolved (arrow).
D) Fractionation of sEVs (purple) from proteins (green, blue) by size-exclusion chromatography.
E) Single frame from nanoparticle tracking analysis (NTA) of an sEV sample under constant flow, showing particle tracks (red) and particle size-distribution (blue).
F) Representative trace of EV sample obtained using resistive pulse sensing (RPS).
G) Individual particles detected by RPS, with size determined relative to calibration beads of a known size.
H) Size distribution of EVs obtained by RPS.

Figure 3.

Steps towards EV characterization, adapted from MISEV2018 guidelines.[31]
1) Determine the quantity of EVs obtained, relative to the amount of starting material.
2) Verify the presence of at least three positive protein markers of small EVs, including one transmembrane or GPI-anchored protein (eg: CD9, CD63, CD81, NT5E/CD73), and one cytosolic, luminal protein (eg: ALIX/PDCD6IP, HSC70). For large EVs, a wide range of surface markers such as integrins from the cell of origin may be used.
3) Preferably, demonstrate the relative abundance of significant contamination by non-vesicular, co-isolated components such as lipoproteins (APOB, APOA1, APOA2) or albumin.
4) Characterize individual EVs, with images of single EVs (both wide-field and close-up).

14. Box 1

The standard differential ultracentrifugation protocol for EV isolation, originally published by Thery et al.[54]

1. Centrifuge sample at 300 g for 10 min, at 4°C. (Remove cells and cell debris)
2. Centrifuge supernatant at 2,000 g for 10 min, at 4°C. (Remove larger complexes)
3. Centrifuge supernatant at 10,000 g for 30 min, at 4°C. (Microvesicles are in the pellet).
4. Centrifuge supernatant at 100,000 g for 70 min, at 4°C in ultracentrifuge. (EVs are in the pellet)
5. Re-suspend the pellet containing EVs and contaminating proteins.
6. Centrifuge 100,000 g 70 min, 4°C in ultracentrifuge to wash. (sEVs/exosomes are in the pellet).

15. Author contributions

All co-authors contributed to the draft of the document; SD, JS and CMB synthesized all contributions and handled the revision of the paper.

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17. Conflicts of Interest

L.B has performed advisory board work and received speaker fees from Sanofi and Novartis, and is founder and shareholder of Glycardial Diagnosis SL and Ivestatin Therapeutics, SL (all outside of this work) ; CJB is a board member of Technoclone. AB is founder and CEO of Exo-Analysis. TT has filed and licensed patents in the field of noncoding RNAs and targeted delivery strategies and is founder and shareholder of Cardior Pharmaceuticals GmbH (outside of the topic of this review). RL discloses grants from Stago and a patent on microvesicle fibrinolytic activity licensed to Stago. EIB is member of the Advisory Board of Sphere Gene Therapeutics Inc. (Boston, US). MHMW discloses a collaborative research agreement with BD Biosciences Europe, Erembodegem, Belgium to optimize flow cytometric analysis of EVs.

18. References

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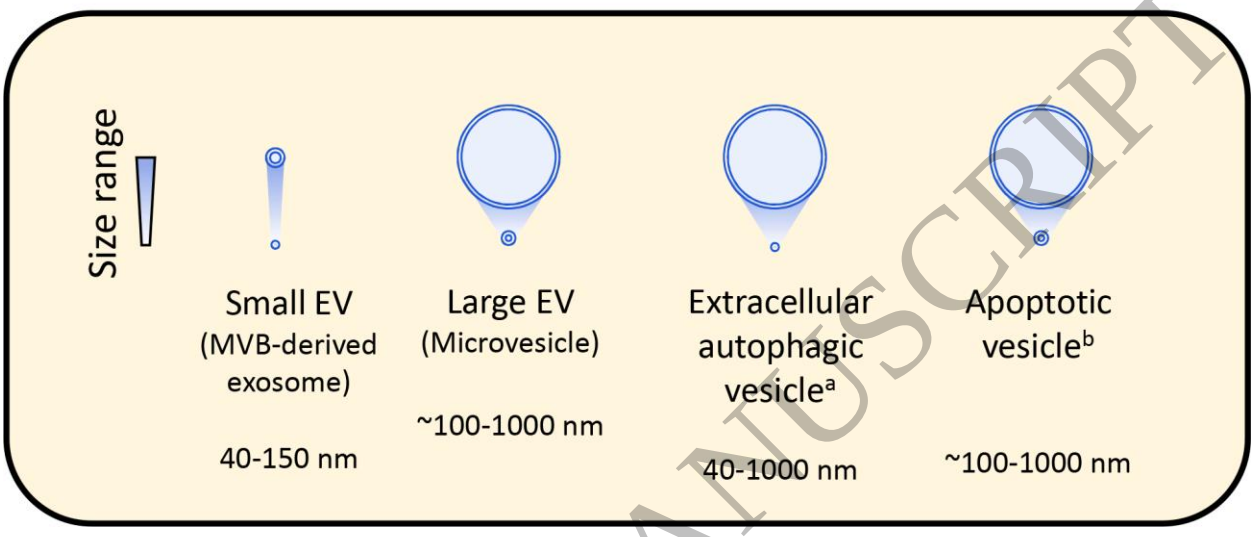
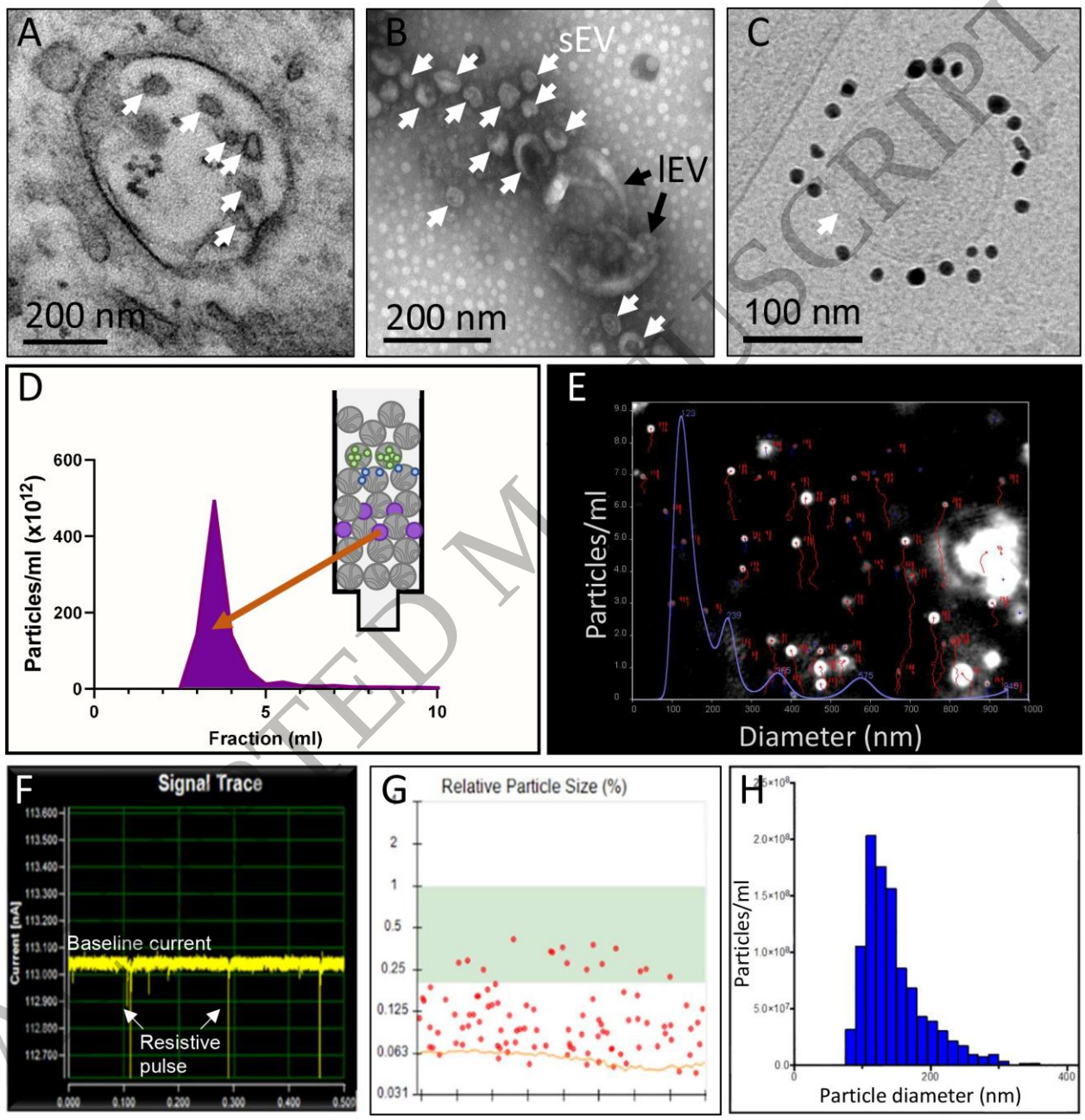
Figure 1



165x98 mm (6.2 x DPI)

Figure 2



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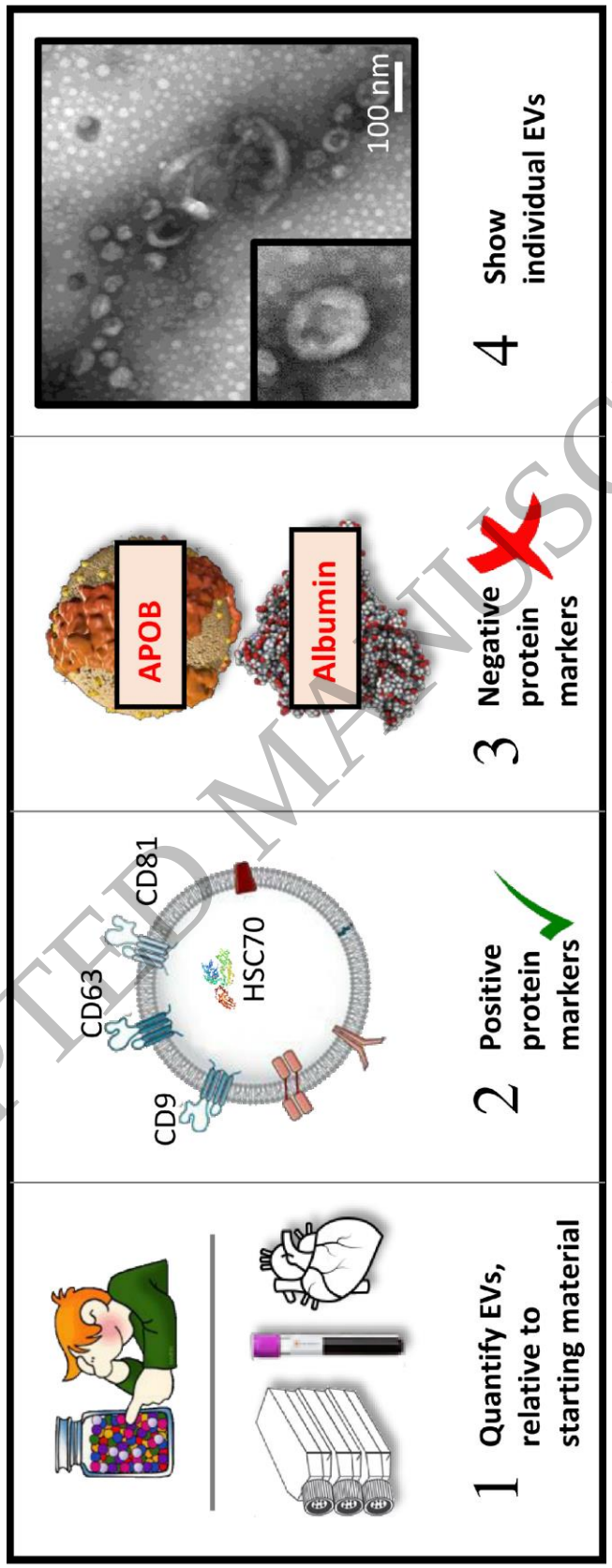

89x229 mm (6.2 x DPI)