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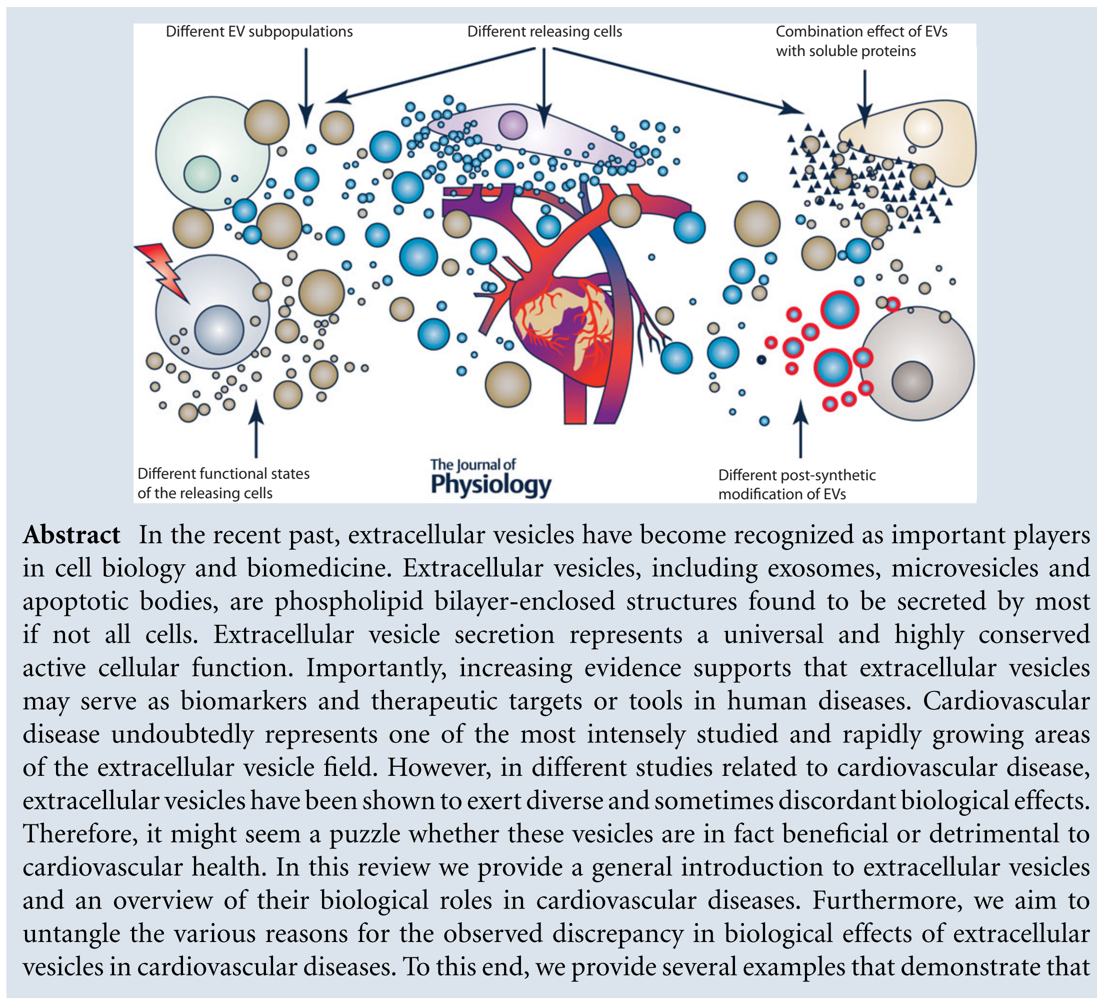
SYMPOSIUM REVIEW

Extracellular vesicles in cardiovascular disease: are they Jedi or Sith?

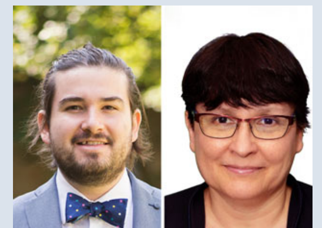
Xabier Osteikoetxea, Andrea Németh, Barbara W Sódar, Krisztina V Vukman and Edit Irén Buzás

Department of Genetics, Cell- and Immunobiology, Semmelweis University, Budapest, Hungary



Abstract In the recent past, extracellular vesicles have become recognized as important players in cell biology and biomedicine. Extracellular vesicles, including exosomes, microvesicles and apoptotic bodies, are phospholipid bilayer-enclosed structures found to be secreted by most if not all cells. Extracellular vesicle secretion represents a universal and highly conserved active cellular function. Importantly, increasing evidence supports that extracellular vesicles may serve as biomarkers and therapeutic targets or tools in human diseases. Cardiovascular disease undoubtedly represents one of the most intensely studied and rapidly growing areas of the extracellular vesicle field. However, in different studies related to cardiovascular disease, extracellular vesicles have been shown to exert diverse and sometimes discordant biological effects. Therefore, it might seem a puzzle whether these vesicles are in fact beneficial or detrimental to cardiovascular health. In this review we provide a general introduction to extracellular vesicles and an overview of their biological roles in cardiovascular diseases. Furthermore, we aim to untangle the various reasons for the observed discrepancy in biological effects of extracellular vesicles in cardiovascular diseases. To this end, we provide several examples that demonstrate that

Xabier Osteikoetxea received his BSc degree in 2010 at the University of Florida majoring in Microbiology and Cell Sciences with a minor in Chemistry. He received his MSc at the University of Pompeu Fabra in Barcelona, Spain in Biomedical Research. In 2012 he joined the Extracellular Vesicle Research Group of Semmelweis University, Budapest, Hungary as a FP7 Marie Curie early stage researcher (ITN DYNANO). **Andrea Németh** MSc, **Barbara W. Sódar** MD and **Krisztina V. Vukman** PhD are members of the same research group and share expertise in the field of extracellular vesicles. **Edit I. Buzás** MD, PhD and DSc is a Professor and Chairman in the Department of Genetics, Cell- and Immunobiology at Semmelweis University, Budapest, Hungary. She is the Head of the Extracellular Vesicle Research Group at Semmelweis University. For over a decade her research group has focused on isolation and characterization of extracellular vesicles.



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the observed functional diversity is in fact due to inherent differences among various types of extracellular vesicles.

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Corresponding author E. I. Buzás: Nagyvárad tér 4., Budapest 1089, Hungary. Email: edit.buzas@gmail.com

Abstract figure legend Underlying reasons for the observed diversity in biological effects of extracellular vesicles in cardiovascular diseases.

Abbreviations APO, apoptotic body; EV, extracellular vesicle; EXO, exosome; MV, microvesicle.

Introduction to extracellular vesicles

Different subtypes of extracellular vesicles. An increasing body of evidence supports that extracellular vesicles (EVs) are highly heterogeneous structures (Théry *et al.* 2009; György *et al.* 2011; Buzás *et al.* 2014; Yáñez-Mó *et al.* 2015). Current EV classification based on cellular biogenesis distinguishes exosomes (EXOs), originating from multivesicular bodies, from larger vesicles directly shed from the plasma membrane, such as microvesicles (MVs, which have also been termed microparticles), and apoptotic bodies (APOs) (Théry *et al.* 2009; György *et al.* 2011; Théry, 2011; Buzás *et al.* 2014; Yáñez-Mó *et al.* 2015). Although at present there are no molecular markers that clearly distinguish these subpopulations, size-based fractionation has been shown to yield distinct EV subpopulations (Crescitelli *et al.* 2013; Osteikoetxea *et al.* 2015*a*). These subpopulations include vesicles around 100 nm (considered to correspond to EXOs), which are currently attracting the most research interest. According to the size-based fractionation, vesicles of 100–1000 nm are considered to be MVs, and those ≥ 1 μm in diameter correspond to APOs. However, due to technical limitations in EV size determination, these size range cut-off values should not be considered absolute. In this review article we only indicate the EV subtype in those cases where the isolated EV population was clearly identified. In the rest of the cited references we used the collective term EV. In addition to their differences in size, there are several other parameters that differ among the EV subtypes and can have profound implications for their biological roles, and they are summarized in Table 1.

Isolation of EVs. EVs can be isolated by several different methods. Currently used EV isolation techniques are summarized with their strengths and best suited applications in Table 2. Traditionally, EVs have been isolated using differential centrifugation, which also includes high speed ultracentrifugation (Théry *et al.* 2001*a*; Valadi *et al.* 2007; Crescitelli *et al.* 2013; Osteikoetxea *et al.* 2015*a*). This common technique, encountered in many studies, allows the separation of size-based EV subpopulations with different centrifugation speeds and the processing of high volumes of sample. Several other techniques for EV isolation have also emerged to complement or replace differential centrifugation. Some techniques such as density gradient isolation (sucrose or iodixanol) are well suited to obtain EV preparations of higher purity than differential centrifugation alone (Raposo *et al.* 1996; Théry *et al.* 2001*a*,*b*; Marzesco *et al.* 2005; Van Deun *et al.* 2014; Zonneveld *et al.* 2014; Keerthikumar *et al.* 2015). Other isolation techniques that may be either faster or less dependent on instrumentation include precipitation techniques (Lee *et al.* 2012; Musante *et al.* 2012; Brownlee *et al.* 2014), microfluidic devices (Chen *et al.* 2010; Davies *et al.* 2012; Wang *et al.* 2013; He *et al.* 2014; Kanwar *et al.* 2014; Vaidyanathan *et al.* 2014), affinity capture (Wubbolts *et al.* 2003; Caby, 2005; Kim *et al.* 2012; Balaj *et al.* 2015), size-exclusion chromatography (Ogawa *et al.* 2008; Sokolova *et al.* 2011; Böing *et al.* 2014; Hong *et al.* 2014), and field-flow fractionation (Oh *et al.* 2007; Sitar *et al.* 2015). The type of isolation procedure best suited for a given experiment is dependent on the origin of the sample (e.g. biological fluid or cell conditioned medium), its volume, equipment availability, and the subsequent type of analysis. These different techniques for isolation of EVs are currently used due to their relative strengths. However, it is conceivable that future technical advances in isolation methods may bring about protocols and techniques that can be applicable to most type of experiments and samples with equal efficiency.

Detection of EVs. Similarly to EV isolation techniques, there is also a wide diversity of detection and characterization techniques currently available for EV studies. Two common techniques used for molecular characterization of EVs are Western blotting (Théry *et al.* 2001*a*) and flow cytometry (Théry *et al.* 2001*a*; Hoen *et al.* 2012; van der Vlist *et al.* 2012; van der Pol *et al.* 2014). Western blotting is routinely used for bulk molecular characterization of all EVs present in an isolate. After initial set-up and bead- or liposome-based gating for EV detection, fluorescence flow cytometry allows for the molecular characterization of larger sized EVs such as MVs (100–1000 nm) and APOs (≥ 1 μm). Furthermore, flow cytometry enables bulk molecular characterization of bead-bound EXOs (50–120 nm). The coupling of EXOs to beads for flow cytometry is necessitated since the sizes of EXOs fall below the limit of detection of most instruments. EVs may be bound to beads with antibodies against specific markers or by unspecific adsorption of

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Table 1. Characteristic parameters of different size-based EV subtypes

Parameter	Exosomes	Microvesicles	Apoptotic bodies	References
Size	50–120 nm	100–1000 nm	$\geq$1 μm	Théry *et al.* 2009; György *et al.* 2011; Théry, 2011; Buzás *et al.* 2014; Yáñez-Mó *et al.* 2015
Homogeneity	Relatively uniform	Heterogeneous	$\geq$ 2 subpopulations	Bilyy *et al.* 2012; Crescitelli *et al.* 2013; Osteikoetxea *et al.* 2015*a*
Nucleic acid content	miRNA, mRNA, DNA	Heterogeneous RNAs	rRNAs, DNA	Valadi *et al.* 2007; Skog *et al.* 2008; Hong *et al.* 2009; Waldenström *et al.* 2012; Crescitelli *et al.* 2013; Thakur *et al.* 2014
Lipid packing density	High	Intermediate	Low–intermediate	Osteikoetxea *et al.* 2015*a*
Lipid content	High cholesterol, high glycosphingolipids (ceramide, externalized phosphatidylserine, gangliosides GM1 and GM3)	Externalized phosphatidylserine	Externalized phosphatidylserine	Wubbolts *et al.* 2003; Trajkovic *et al.* 2008; Carayon *et al.* 2011; Llorente *et al.* 2013; Osteikoetxea *et al.* 2015*a*
Protein/lipid ratio	Low	Intermediate	Large	Osteikoetxea *et al.* 2015*a*
Detergent sensitivity	Low	Intermediate	Intermediate	György *et al.* 2010; Osteikoetxea *et al.* 2015*b*
Approximate density	1.13–1.19 g ml^{-1}	1.03–1.20 g ml^{-1}	1.16–1.28 g ml^{-1}	Raposo *et al.* 1996; Théry *et al.* 2001*b*; Marzesco *et al.* 2005; Théry *et al.* 2009; Keerthikumar *et al.* 2015
EM morphology	Cup shaped	Heterogeneous	Heterogeneous	Raposo *et al.* 1996; Théry *et al.* 2009; György *et al.* 2011; Crescitelli *et al.* 2013; Buzás *et al.* 2014; Osteikoetxea *et al.* 2015*a*

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Table 2. Currently used EV isolation techniques

Technique	Examples and types	Advantages	Disadvantages	Suggested choice for:	References
Differential centrifugation	Sequential centrifugation with or without gravity size filtration	Most widely used; separates EV size-based subpopulations; processes large sample volume (~20–200 ml)	>3 h; requires ultracentrifuge	Large sample volume (e.g. conditioned cell media); no prior knowledge of EV markers; large scale EV isolation	Théry *et al.* 2001*a*
Density gradient separation	Sucrose density gradient; iodixanol (Optiprep^{TM}) density gradient; PureExo exosome isolation kit^{TM}	High EV purity	>10 h; requires ultracentrifuge; low yields; small sample volumes (0.5–3ml); doesn't necessarily fractionate EV subpopulations as they share density	Purification of EV isolates; small sample volume	Raposo *et al.* 1996; Théry *et al.* 2001*a*; Théry *et al.* 2001*b*; Marzesco *et al.* 2005; Van Deun *et al.* 2014; Zonneveld *et al.* 2014; Keerthikumar *et al.* 2015
Precipitation	Polyethylene glycol; acetate; ammonium sulphate; Total Exosome Isolation^{TM}; Exoquick^{TM}; Exo-spin^{TM}	Commercial kits available; fast; no specialized equipment; simple operation	Low purity (co-precipitation of non-vesicular material); possible interference of precipitation reagents with downstream applications	Small volume (e.g. limited biological samples); fast processing time	Lee *et al.* 2012; Musante *et al.* 2012; Brownlee *et al.* 2014
Microfluidic devices	Sieving; trapping; immunological separation; nanoshearing	Simple operation; single-step isolation; on-chip EV characterization; easier standardization and higher reproducibility	Currently not readily available; small sample volume ($< 500\ \mu$l); to date has only been applied to EXOs and MVs	Small volume (e.g. limited biological samples)	Chen *et al.* 2010; Davies *et al.* 2012; Wang *et al.* 2013; He *et al.* 2014; Kanwar *et al.* 2014; Vaidyanathan *et al.* 2014
Affinity capture	Antibody coated magnetic beads; antibody coated latex beads; heparin affinity	Relatively high EV purity; some commercial kits available; simple operation	Biased by the choice of affinity reagent (only EVs with the corresponding ligand will be purified); 0.5–3 ml sample volume; > 2 h; difficulty in recovering EVs after capture for other applications	Small volume (e.g. limited biological samples) with known EV markers	Wubbolts *et al.* 2003; Caby, 2005; Kim *et al.* 2012; Balaj *et al.* 2015
Size-exclusion chromatography	Sepharose gel; Sephadex gel; Bio-gel A^{TM}; Izon qEV^{TM} column	~ 15 min; inexpensive; no specialized equipment required; simple operation	0.5–1.5 ml sample volume; doesn't fractionate EV subpopulations as they are all excluded from the gels; further dilutes EVs from the sample	Purification of EV isolates; small volume (e.g. limited biological samples); no need for prior assumption of EV markers; fast processing time	Ogawa *et al.* 2008; Sokolova *et al.* 2011; Böing *et al.* 2014; Hong *et al.* 2014
Field-flow fractionation	Flow field-flow; asymmetrical flow field-flow	Separates EVs with lower applied forces than used with centrifugation; achieves good size based separation of small EVs	Not readily available; mostly custom made; 1–2 ml sample volume; to date has only been applied to EXOs and MVs	Small volume (e.g. limited biological samples); size-based separation of EV subpopulations	Oh *et al.* 2007; Sitar *et al.* 2015

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vesicular molecules to chemically modified surfaces such as sulfate aldehyde. Antibody based bead capture can result in highly specific binding to EVs with limited adsorption of non-vesicular proteins, but this approach may also bias the measurement by excluding any EVs that lack the selected marker. On the other hand, while unspecific adsorption may also bind to non-vesicular proteins, it does not bias the measurements towards vesicles bearing a chosen marker. High resolution flow cytometry proves capable of circumventing the need for bead coupling, allowing for direct molecular characterization of individual EXOs by employing optimized set-ups with improved fluidics, lasers and detectors.

To assess EV particle size and concentration, tunable resistive pulse sensing (Maas *et al.* 2014; van der Pol *et al.* 2014; Osteikoetxea *et al.* 2015*a*,*b*), nanoparticle tracking analysis (Sokolova *et al.* 2011; Gardiner *et al.* 2013; van der Pol *et al.* 2014) and dynamic light scattering (György *et al.* 2010; Sahoo *et al.* 2011; Sokolova *et al.* 2011; Sitar *et al.* 2015) may be used. For fast quantification of EVs, both protein (Théry *et al.* 2001*a*) and lipid (Osteikoetxea *et al.* 2015*a*) content can be measured. Recently our group has shown that these two methods together can yield information about the quality, purity and subtype of EV preparations by calculating their total protein to lipid ratio (Osteikoetxea *et al.* 2015*a*). Additionally, several microscopy methods are routinely used for EV size and morphology determination including transmission electron microscopy, scanning electron microscopy and cryo-electron microscopy (Théry *et al.* 2001*a*; Turiák *et al.* 2011; Crescitelli *et al.* 2013; Buzás *et al.* 2014; Osteikoetxea *et al.* 2015*a*) as well as atomic force microscopy (György *et al.* 2010; Yuana *et al.* 2010). Many new studies are also showing the applicability of label-free techniques such as grating coupled interferometry, surface plasmon resonance, as well as Raman, infrared and electrochemical impedance spectroscopy for characterization of EVs (Lvovich *et al.* 2010; Tatischeff *et al.* 2012; Patko *et al.* 2013; Im *et al.* 2014; Rupert *et al.* 2014). Lastly, different 'omics' techniques have been applied to determine the precise molecular composition of EVs (Wubbolts *et al.* 2003; Valadi *et al.* 2007; Skog *et al.* 2008; Trajkovic *et al.* 2008; Hong *et al.* 2009; Carayon *et al.* 2011; Waldenström *et al.* 2012; Choi *et al.* 2013; Escrevente *et al.* 2013; Llorente *et al.* 2013; Thakur *et al.* 2014). All these techniques are summarized in Table 3. The International Society for Extracellular Vesicles has outlined minimal experimental requirement guidelines for the characterization of EV preparations that may be used for reference when deciding which characterization techniques to use (Lötvall *et al.* 2014). As long as these guidelines are met, the best characterization technique for EV analysis is dependent on the particular experimental aims and instrument availability. Furthermore, as long as appropriate controls such as EV-depleted negative controls or detergent lysis (György *et al.* 2010; Osteikoetxea *et al.* 2015*b*) are used to exclude the interference of non-vesicular components of the sample, the type of method used for characterization should not have a major impact on EV characterization and the biological functions observed.

Possible reasons for discordant functions of EVs

Inherent differences of EV subtypes. Given the important inherent differences between the subpopulations of EVs (Table 1), certain subpopulations might be responsible for specific biological effects. One example is a recent study showing that EVs released during remote ischaemic preconditioning (RIPC) are responsible for reduced infarct sizes in rat hearts (Giricz *et al.* 2014). Likewise another study found that following RIPC, EXOs containing miR-29a and IGF-1R are released and mediate the cardioprotective effect (Yamaguchi *et al.* 2015). In contrast, another study had found previously that MVs (a single subtype of EVs) alone could not decrease infarct sizes (Jeanneteau *et al.* 2012). Therefore, these studies show that EXOs but not MVs are the mediators of the cardioprotective effects observed following RIPC. In a different setting, apoptotic EVs have been found to limit atherosclerosis and mediate vascular protection by transfer of miR-126 (Zernecke *et al.* 2009). Consequently, these studies suggest that a given subpopulation of EVs and not the others might be responsible for a particular biological effect.

Differences in the EV-releasing cell types. Another important reason for the diversity in biological roles of EVs can be attributed to the diversity in their releasing cells. Several studies demonstrate that different cell type-derived EVs can have either protective or pathogenic effects on the cardiovascular system.

Numerous reports on endothelial cell-derived EVs show that these vesicles have vasculoprotective roles. In the study mentioned above, apoptotic EVs obtained from endothelial cells were found to be able to deliver miR-126 to other cells and to induce the expression of CXCL12 resulting in recruitment of Sca-1^{+} progenitor cells for endothelial repair and inhibition of atherosclerosis (Zernecke *et al.* 2009). Interestingly, another study has shown that miR-126-containing MVs are also released from endothelial cells and enhance endothelial recovery following electric endothelial denudation of the common carotid artery in wild-type mice. These MVs incorporated into recipient endothelial cells and promoted *in vitro* migration and proliferation in a miR-126-dependent manner (Jansen *et al.* 2013). Endothelial cell shear stress is known to induce Krüppel-like factor 2 (KLF2), a key transcription factor regulating atheroprotective gene expression changes (Boon & Horrevoets, 2009). Interestingly, it has been shown that EVs secreted by

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Table 3. Currently used EV detection techniques

Technique	Examples and types	Advantages	Disadvantages	Suggested choice for:	Reference
Flow cytometry	Scattering/fluorescence flow cytometry; impedance flow cytometry; high resolution flow cytometry	Fast phenotyping of large number of EVs; may be used for determination of EV concentration in combination with counting beads; compatible with differential detergent lysis	Except for high resolution flow cytometry, it cannot detect EXOs without binding to larger beads; substantial initial setup and gating required to analyse EVs; swarm detection may hinder measurements	Individual characterization of EV molecular markers (also for EXOs with high resolution flow cytometry)	Théry *et al.* 2001*a*; György *et al.* 2010; Hoen *et al.* 2012; van der Vlist *et al.* 2012; van der Pol *et al.* 2014; Osteikoetxea *et al.* 2015*b*
Western blotting		Well established for EV marker determination	Time consuming; relatively high amounts of EV sample needed	Bulk characterization of EV molecular markers	Théry *et al.* 2001*a*
Tunable resistive pulse sensing	Izon qNano	~50 μl sample volume; resolves multimodal particle distributions; compatible with differential detergent lysis	Requires substantial user training and experience	Determination of EV size and concentration	Maas *et al.* 2014; van der Pol *et al.* 2014; Osteikoetxea *et al.* 2015*b*
Nanoparticle tracking analysis	Malvern Nanosight; Zetaview®	Fast (~5–15 min); simple operation	Requires operational skills for the adjustment all software settings	Determination of EV size and concentration	Sokolova *et al.* 2011; Gardiner *et al.* 2013; van der Pol *et al.* 2014
Dynamic light scattering		~50 μl sample volume	Not optimal for polydisperse samples	Determination of EV size and relative concentrations	György *et al.* 2010; Sahoo *et al.* 2011; Sokolova *et al.* 2011; Sitar *et al.* 2015
Protein quantification	Bicinchoninic acid (BCA) assay; Bradford assay; Lowry	Simple bench top assays; commercial reagent kits available	Prone to EV overestimation in case of protein contamination	Fast quantification of EV proteins; EV quality control when combined with lipid quantification	Théry *et al.* 2001*a*
Lipid quantification	Sulfophosphovanilin (SPV) assay	Simple bench top assay	Requires fume hood for handling of hot sulphuric acid	Fast quantification of EV lipids; EV quality control when combined with protein quantification	Osteikoetxea *et al.* 2015*a*

(Continued)

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Table 3. Continued

Technique	Examples and types	Advantages	Disadvantages	Suggested choice for:	Reference
Electron microscopy	Transmission EM; scanning EM; cryo-EM	Visualization of EV morphology and size; identification of markers with immunolabelling techniques	Sample preparation can generate artifacts (e.g. cup shape morphology); needs substantial user training and experience	Visualization of EV morphology and size; direct visualization of EXOs	Raposo *et al.* 1996; Théry *et al.* 2001*a*; Turiák *et al.* 2011; Crescitelli *et al.* 2013; Buzás *et al.* 2014; van der Pol *et al.* 2014; Osteikoetxea *et al.* 2015*a*
Atomic force microscopy		Visualization of EV morphology and size	Needs substantial user training and experience	Visualization of EV morphology and size including EXOs	György *et al.* 2010; Yuana *et al.* 2010
Label free techniques	Grating coupled interferometry; surface plasmon resonance; Raman spectroscopy; infrared spectroscopy; electrochemical impedance spectroscopy	Determination of EV markers; determination of EV concentration; detection of EV binding; label free study of EV surface interactions	Methodology still in development for EV applications; needs substantial user training and experience	Dynamic study of molecular interaction with EVs	Lvovich *et al.* 2010; Tatischeff *et al.* 2012; Patko *et al.* 2013; Im *et al.* 2014; Rupert *et al.* 2014
'Omics' techniques	Proteomics; lipidomics; genomics; transcriptomics; glycomics	Complete molecular characterization of EVs	Relatively high amounts of EV sample needed	Determination of EV molecular composition	Wubbolts *et al.* 2003; Valadi *et al.* 2007; Skog *et al.* 2008; Trajkovic *et al.* 2008; Hong *et al.* 2009; Carayon *et al.* 2011; Waldenström *et al.* 2012; Choi *et al.* 2013; Escrevente *et al.* 2013; Llorente *et al.* 2013; Thakur *et al.* 2014

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KLF2-transfected human umbilical vein endothelial cells (HUVECs; a model for endothelial cell shear stress) could transfer the atheroprotective phenotype to smooth muscle cells and reduce atherosclerotic lesions in ApoE$^{-/-}$ mice in a miR-143- and miR-145-dependent manner (Hergenreider *et al.* 2012). Additionally, one report has shown that cardiac endothelial cell-derived EXOs can be cardioprotective in the case of heart transplantation by inducing regulatory B cells that can promote immune tolerance to alloantigens (Song *et al.* 2014).

Another cellular source of EVs, dendritic cells (DCs) have been shown to release EVs that induce immune tolerance in allograft models. A study with a rat cardiac allograft model has found that intravenous administration of donor bone marrow DC-derived EXOs induced delayed acute allograft rejection (Pêche *et al.* 2003). Work from the same group showed that allograft rejection was further delayed when EXOs were administered in combination with a novel immunosuppressant *in vivo* (Pêche *et al.* 2006). Similarly, another study with a mouse cardiac allograft model showed that EVs derived from immature DCs extended cardiac allograft survival and that this effect was prolonged when combined with the immunosuppressant drug rapamycin (Li *et al.* 2012).

EVs secreted by yet another cell type, stem cells (SCs), have been shown to be cardioprotective. A study in a mouse model of myocardial ischemia–reperfusion injury has shown that EXOs derived from mesenchymal SCs mediated their cardioprotective effect and diminished the sizes of infarcts (Lai *et al.* 2010). Furthermore, a following study from the same group found that EXOs mediated this cardioprotective effect by decreasing oxidative stress, increasing ATP levels and activating the PI3K/Akt pathway (Arslan *et al.* 2013). Another study has also shown that *in vivo* administration of EXOs derived from mouse cardiac progenitor cells protect cardiomyocytes from oxidative stress and inhibit apoptosis in an acute ischaemia–reperfusion model (Chen *et al.* 2013). Furthermore, a separate study with cardiac progenitor cells, expanded *in vitro* into cardiospheres, has found that their EXOs can induce the same beneficial therapeutic effects on infarcted mouse hearts as the cardiospheres themselves (Ibrahim *et al.* 2014). Autologous CD34^{+} SC transplantation has been shown to hold promise for refractory angina patients following ischaemic injury (Kawamoto *et al.* 2006; Losordo *et al.* 2011). Interestingly, CD34^{+} SC cell-derived EXOs were found to mediate the therapeutic effects of SCs by inducing angiogenesis in both *in vivo* and *in vitro* models (Sahoo *et al.* 2011).

While all the above-mentioned studies have observed cardioprotective roles for EVs, cardiomyocyte-derived EVs have been shown to be involved in pathogenic roles. A study has found that EVs derived from hypoxic cardiomyocytes contained functional TNF-α and were capable of inducing apoptosis when administered to normal cardiomyocytes (Yu *et al.* 2012). In another study, EXOs derived from cardiomyocytes of a type 2 diabetes rat model were shown to contain and transfer functional miR-320 to cardiac endothelial cells inhibiting their proliferation and decreasing angiogenesis (Wang *et al.* 2014). These studies show that cardiomyocyte-derived EVs transfer functional proteins and nucleic acids that may have an adverse effect on other cardiomyocytes or endothelial cells.

In addition to the previously discussed cells, other cell types may also release EVs that can have a biological effect on the heart. For example, it has been shown that EXOs secreted by cardiac fibroblasts induce a hypertrophic phenotype in cardiomyocytes by transferring miR-21 (Bang *et al.* 2014). EXOs derived from platelets of septic patients have been implicated in cardiomyopathy by inducing apoptosis in endothelial cells and cardiomyocytes leading to vascular and cardiac dysfunctions as well as coagulation (Janiszewski *et al.* 2004; Azevedo *et al.* 2007; Gambim *et al.* 2007).

Taken together, all the currently available literature suggests that endothelial cell, SC and dendritic cell-derived EVs are protective. On the other hand, cardiomyocyte-, cardiac fibroblast-, and platelet-derived EVs have so far been shown to be pathogenic. In addition to the above studies, many reports with plasma-derived EVs are also available but given the heterogeneity of cell types releasing EVs into the circulation, these particles may have disparate effects. Furthermore, preanalytical variables (such as the choice of anticoagulant and sample processing and handling) can influence the quantity and downstream effects of plasma-derived EVs (Witwer *et al.* 2013). Platelets have been found to be one of the most important sources of EVs in circulation (Arraud *et al.* 2014). One study with blood from cardiac surgery patients found higher tissue factor exposure in MVs, the majority of which were of platelet origin, compared with those of healthy individuals. Moreover, MVs from these patients were highly thrombogenic in a venous stasis thrombosis model in rats, whereas MVs from healthy individuals were not (Biró *et al.* 2003). In contrast, another study has found that higher plasma levels of MV-associated, but not the freely circulating, miR-126 and miR-199a were predictive of a lower risk of major adverse cardiovascular events in patients with stable coronary artery disease (Jansen *et al.* 2014).

Differences in the functional states of EV-releasing cells. As discussed in the previous section, most reports on EVs derived from endothelial cells have found that these structures were protective. However, it has also been shown that 16 kDa N-terminal prolactin fragment (16K PRL) induces endothelial cells to secrete miR-146a-enriched EXOs. 16K PRL is an important pro-apoptotic and pro-inflammatory factor that can initiate and drive peripartum cardiomyopathy (Hilfiker-Kleiner *et al.* 2007).

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Thus, in contrast to EXOs secreted by normal endothelial cells, these 16K PRL-induced EXOs can transfer miR-146a to cardiomyocytes and inhibit their metabolic activity and contractile function (Halkein *et al.* 2013). In another study with endothelial cells, EVs released during apoptosis or autophagy were found to differ significantly from those released during normal conditions. EVs from serum-starved cells were enriched in autophagosomes and mitochondria and also carried different danger signals such as extracellular ATP (Pallet *et al.* 2013). Interestingly, in yet another study with endothelial cells, TNF-α was shown to be able to simultaneously induce the release of MVs that exerted contrasting effects. Upon TNF-α treatment, miRNA-rich MVs were released in a RhoA/Rho kinase pathway-dependent manner and had antiapoptotic effects. However, this treatment also induced the release of miRNA-poor MVs in a caspase pathway-dependent manner with proapoptotic effects (Alexy *et al.* 2014).

Similarly to the findings with endothelial cells in different functional states, another report has shown that EXOs from ischaemic-preconditioned, but not from non-preconditioned, mesenchymal SCs imparted significant reduction of cardiac fibrosis and apoptosis following direct injection into infarcted hearts of mice (Feng *et al.* 2014).

Together, these studies show that while there are specific biological roles for EVs derived from different types of cells, the functional state of the cells from which the EVs are released should also be taken into account as it can have an important impact on the resulting biological effect.

Differences in post-synthetic modifications to molecules of EVs. Besides being released from cells in different functional states, EVs and their cargos can also undergo various post-synthetic modifications that can alter their biological effects on recipient cells. There has been growing evidence that many cardiovascular diseases are associated with an increased oxidative stress (Dhalla *et al.* 2000; Tsutsui *et al.* 2011). Correspondingly, EVs released under these circumstances may undergo oxidative post-synthetic modifications. Indeed, it has been found that MVs from ST-segment elevation myocardial infarction patients carried oxidation-specific epitopes (OSE) and are increased at the site of coronary occlusion. Additionally, these MVs were able to induce pro-inflammatory IL-8 secretion by monocytes in an OSE-dependent manner (Tsiantoulas *et al.* 2014).

EVs may have combinatorial effects on target cells. Several subtypes of EVs released by a broad range of cells in diverse functional states are simultaneously present *in vivo*. In addition to the diversity of EVs, other soluble mediators are also found in the same biological fluids. Therefore, a specific subpopulation of EVs secreted by cells in a given functional state might still have a combinatorial effect when interacting with other types of EVs or soluble mediators. In earlier work from our group, human monocytes showed a synergistically increased up-regulation of IL-8, when exposed to a combination of cell line-derived EVs and human recombinant TNF. Thus, the synergistic interaction of EVs with TNF was found to increase the biological effect of EVs alone (Szabó *et al.* 2014). In a similar manner, another study has shown a synergistic effect of EVs and IL-2 on T-cell proliferation (Wahlgren *et al.* 2012).

In summary, these studies show that the biological effects observed with isolated EVs may vary between different experimental settings because of the specific milieu in which they may act upon cells. Possible synergistic or antagonistic interactions with soluble mediators/cytokines and other EVs may thus explain functional variations observed in various experimental conditions.

EV to target cell ratio. An important parameter to consider in functional tests is the stoichiometry of EVs to recipient cells. When possible, it is recommended to show that the biological effects observed for different EVs are dose dependent and occur in physiologically relevant EV concentrations. Instead, if there is a failure to adhere to the relevant EV-to-cell stoichiometry, treatment with too few EVs might obscure the biological effect. Inversely, treatment with high excesses of EVs might produce effects that would never be encountered *in vivo*. Different studies have so far shown that endothelial cell-derived EVs have protective roles. However, one study has shown that treatment with endothelial cell-derived EVs at high concentrations, such as those observed in different cardiovascular diseases, can impair angiogenesis via oxidative stress in human umbilical vein endothelial cells, while lower physiological concentrations could not (Mezentsev *et al.* 2005). Moreover, in three other studies described earlier in this review, EVs released during RIPC were observed to either reduce or have no effect on infarct sizes (Jeanneteau *et al.* 2012; Giricz *et al.* 2014; Yamaguchi *et al.* 2015). Although the probable cause for these discordant findings is that one of the studies (Jeanneteau *et al.* 2012) only considered MVs while the other one looked at both EXOs and MVs (Giricz *et al.* 2014) or EXOs alone (Yamaguchi *et al.* 2015), one cannot rule out the possibility that too few MVs were administered in the study failing to observe a reduction in infarct sizes.

Concluding remarks

Taken together, all the studies outlined in this review support the diversity of EVs and their biological roles. However, this diversity of roles should not be confused with experimental discrepancy since ample evidence

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shows that EVs may have inherently different biological functions depending on factors such as the type of releasing cell and its functional state, the subpopulation of EVs, their post-synthetic modifications, and possible combinatorial effects with soluble factors or other EVs. Furthermore, the reasons outlined in this review may also explain possible discrepancies in biological effects found for EVs in other fields beside cardiovascular diseases.

In the continuous search for future biomarkers and tools of regenerative medicine, EVs have already shown great promise. In order to truly fulfil their envisioned potential in the clinic, the right EV candidates with beneficial rather than detrimental effects need to be identified and utilized.

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Additional information

Competing interests

All authors declare that they have no competing interests.

Author contributions

X.O. was responsible for writing the majority of the text. Tables 2 and 3 were prepared by A.N., B.W.S. and K.V.V. Table 1 was prepared by E.I.B., who also contributed with the concept of this review article. All authors approved the final version of the manuscript. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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