Extracellular vesicle heterogeneity and its impact for regenerative medicine applications

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This work is financially supported by a Van Herk Foundation grant to SvdW, ERC Consolidator Grant EVICARE (#725229) of the European Research Council (ERC) to JS, and a PPS grant (No. 2018B014) to JS and PV.
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Running title: Functional EV heterogeneity in regenerative medicine

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The number of text pages: 20 (with big spaces)
Number of tables: 3
Number of figures: 5
Number of references: 201
Number of words in the Abstract: 205

Abbreviations:
ADP-ribosylation factor 6 (ARF6)
Arrestin-domain-containing protein 1 (ARRDC1)-mediated microvesicles (ARMMs),
Asymmetrical-flow field-flow fractionation (AF4)
Density gradient centrifugation (DGC)
Differential ultracentrifugation (UC)
Endosomal sorting complexes required for transport (ESCRT)
Epidermal growth factor receptor (EGFR)
Extracellular particles (EPs)
Extracellular vesicles (EVs)
High density EVs (HD-EVs)
Highly negatively charged EVs (HNC-EVs)
Intraluminal vesicles (ILVs)
Large EVs (IEVs)
Low density EVs (LD-EVs)
Lower negative charged EVs (LNC-EVs)
Medium density EVs (MD-EVs)
Multivesicular bodies (MVBs)
Phosphatidylserine (PS)
Size exclusion chromatography (SEC)
Small EVs (sEVs)
Total EV (tEV)
Vacuolar sorting proteins (VSPs)
Abstract

Extracellular vesicles (EVs) are cell-derived membrane enclosed particles that are involved in physiological and pathological processes. EVs are increasingly being studied for therapeutic applications in the field of regenerative medicine. Therapeutic application of stem cell-derived EVs has shown great potential to stimulate tissue repair. However, the exact mechanisms through which they induce this effect has not been fully clarified. This may to a large extent be attributed to a lack of knowledge on EV heterogeneity. Recent studies suggest that EVs represent a heterogeneous population of vesicles with distinct functions. The heterogeneity of EVs can be attributed to differences in their biogenesis, and as such, they can be classified into distinct populations that can then be further subcategorized into various subpopulations. A better understanding of EV heterogeneity is crucial for elucidating their mechanisms of action in tissue regeneration. This review provides an overview of the latest insights on EV heterogeneity related to tissue repair, including the different characteristics that contribute to such heterogeneity, as well as the functional differences among EV subtypes. It also sheds light on the challenges that hinder clinical translation of EVs. Additionally, innovative EV isolation techniques for studying EV heterogeneity are discussed. Improved knowledge of active EV subtypes would promote the development of tailored EV therapies and aid researchers in the translation of EV-based therapeutics to the clinic.

Keywords: Extracellular vesicles, EV, Heterogeneity, Regeneration, Subpopulations, Exosomes, Ectosomes, Biogenesis, Isolation
Significance Statement

Within this review we discuss the differences in regenerative properties of EV subpopulations, and implications of EV heterogeneity for development of EV-based therapeutics. We aim to provide new insights into which aspects are leading to heterogeneity in EV preparations, and stress the importance of EV heterogeneity studies for clinical applications.
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Introduction

Intercellular communication has classically been thought to be accomplished via soluble mediators, such as cytokines and hormones, and direct contact of neighboring cells. More recently, cells have also been shown to communicate through the exchange of extracellular vesicles (EVs). EVs are a heterogeneous population of phospholipid bilayer-enclosed particles that are secreted by all cell types and can be internalized by recipient cells (Wikander et al., 2019; Witwer et al., 2013). To date, it remains unclear if cells produce EVs primarily to excrete waste or obsolete products (Pan & Johnstone, 1983), for intercellular signaling, for functional cargo delivery, or a combination of all. However, there is convincing evidence that EVs are able to physiologically affect recipient cells through ligand-receptor interactions, or through cargo delivery by fusion with the plasma membrane or after endocytosis in the target cell (Bonafede & Mariotti, 2017; Dykes, 2017; Raposo et al., 1996; Valadi et al., 2007; Zitvogel et al., 1998).

Recent studies have demonstrated the existence of EV populations and subpopulations with very diverse morphologies (Lässer et al., 2018). Due to their heterogeneous nature, EVs are able to play versatile roles in physiological and pathological processes (Bellingham et al., 2012; Bonafede & Mariotti, 2017; Inamdar et al., 2017; Selmaj et al., 2017; Whiteside, 2017; Willms et al., 2018). EVs from different stem- and progenitor cells have been suggested to mediate processes related to coagulation, cell proliferation, apoptosis, angiogenesis, and inflammation, which allow them to accelerate tissue repair and regeneration (HRashed et al., 2017; Kirkham et al., 2022). For this reason, tissue regeneration using stem cell-derived EVs has been studied intensively and is showing great potential for therapeutic applications (Lai et al., 2010; Liang et al., 2014; Vizoso et al., 2017). For example, mesenchymal stromal cell (MSC)-derived EVs are as effective as transplanting their producing cells to protect against hyperoxic lung injuries (Ahn et al., 2018). Furthermore, EVs are the active component of the paracrine secretion of progenitor cells to stimulate cardiac repair (Barile et al., 2014; Gnecchi et al., 2005; Ibrahim et al., 2014; Lai et al., 2010; Maring et al., 2019; Yang et al., 2019). An early clinical trial demonstrated that EVs are able to improve renal function and ameliorate the immune reaction in patients with kidney diseases (Nassar et al., 2016). Importantly,
administration of EVs is safe and has multiple advantages over cell therapy. Compared to cells, EVs are better equipped to handle storage and shipping conditions. Furthermore, when comparing EVs to their secreting induced pluripotent stem cells, EVs have less off-target effects, such as adverse immune cell reactions and risk of tumor development (Qiao et al., 2020; Yu et al., 2014). These findings highlight the potential of using EVs as reparative or regenerative treatment. However, the exact mechanism by which they induce their therapeutic responses has not been elucidated.

Distinct functionality among different EV subtypes has been reported, but the underlying mechanisms are still poorly understood (Collino et al., 2017; Cosenza et al., 2018; Lopez-Verrilli et al., 2016; H. Zhang et al., 2018). This lack of knowledge about functional EV heterogeneity complicates the understanding of their therapeutic role. To exploit and improve the functional effects of EVs in tissue regeneration, it is important to understand if different EV subtypes act synergistically or, rather, have a redundant effect. Furthermore, it is essential to recognize and control the different drivers of EV heterogeneity in order to improve reproducibility of enhance their therapeutic effects (Lener et al., 2015; Sluijter et al., 2018).

When describing EV heterogeneity, it is important to define EV nomenclature (Théry et al., 2018). Different EV populations and subpopulations can be classified based on biogenesis or on physical properties. Based on biogenesis, multivesicular endosome-derived vesicles are often called “exosomes”, while plasma membrane-derived vesicles are called “ectosomes” or “microvesicles”. However, it is almost impossible to assign isolated EVs to a particular biogenesis pathway after their release. Therefore, EVs are in practice preferably classified based on physical properties. In this review, isolated EV subtypes separated on size will be called “small EVs (sEVs)” and “large EVs (lEVs)”. Non-membranous protein aggregates/particles will be called extracellular particles (EPs). In general remarks, the comprehensive term of “EVs” will be used.

This review aims to evaluate the reparative and regenerative potential of different EV subtypes and emphasizes the importance of EV heterogeneity studies for therapeutic applications. First, we will describe different aspects which lead to EV heterogeneity, including biogenesis, biological environment, and stochasticity. Next, the different EV separation techniques that allow separation of
EV subtypes will be discussed, highlighting challenges for possible clinical implementation. Further sections will critically discuss studies that have made a comparison between functional effects of different EV subtypes, followed by our perspectives on critical issues that need to be addressed in future research. We believe that a better understanding of active EV subpopulations would benefit the development of more tailored EV therapies and would guide researchers to select the most optimal EV isolation procedure and/or subpopulation for their applications.

**Origins of EV heterogeneity**

**Biogenesis of different EV populations and subpopulations**

EVs are a heterogeneous population of vesicles varying in size, cargo, surface characteristics and intracellular origin (Lässer et al., 2018). Based on their biogenesis, EVs are classified into two main populations: *exosomes* and *ectosomes*, both of which may be subcategorized into different subpopulations (Figure 1). Exosomes originate from multivesicular bodies (MVBs) and are released when MVBs fuse with the cell membrane. Ectosomes (also referred to as microvesicles or shedding vesicles) are released by direct outward budding of the cell membrane (Van Niel et al., 2018a; Willms et al., 2016). A better understanding of the biogenesis, the loading of their content, and secretion of exosomes and ectosomes will help to understand what drives EV heterogeneity.

**Exosome** biogenesis starts when early endosomes mature into late endosomes and then turn into MVBs by inward budding forming intraluminal vesicles (ILVs) (Klumperman & Raposo, 2014). Although ILVs are precursors of exosomes, not all ILVs end up to be exosomes. MVBs can fuse with lysosomes for degradation or with the cell membrane for the secretion of exosomes (Klumperman & Raposo, 2014). The coexistence of different MVB populations suggest the existence of ILV subpopulations with some being fated for excretion and others for degradation, but the regulation of this balance remains unclear (Buschow et al., 2009; Klumperman & Raposo, 2014; Van Niel et al., 2018b). When degradation by lysosomes is prevented, exosome secretion is stimulated which indicates that MVB populations destined to be degraded by lysosomes also have capabilities to release
exosomes (Ortega et al., 2019; Villarroya-Beltri et al., 2016). Furthermore, it has been shown that exosomes are released together with histones from amphisomes, a hybrid organelle between an autophagosome and a MVB (Jeppesen et al., 2019).

Because highly abundant cytosolic and cytoskeletal proteins are absent from classically described exosomes, inclusion of cytosolic components during exosome biogenesis is probably a highly regulated rather than a random process (Jeppesen et al., 2019). Multiple ILV formation- and loading mechanisms have been described, suggesting the existence of different exosome subpopulations. It is however unclear if these machineries are specific for particular MVB populations. Yet it is shown that single MVBs contain morphologically distinct ILVs, indicating that different exosome subpopulations can be formed in a single MVB population (Zabeo et al., 2017). The most widely described loading system depends on the endosomal sorting complexes required for transport (ESCRT) machinery which is responsible for the sorting of ubiquitinated proteins and the budding process of ILVs (Hromada et al., 2017; Rezaie et al., 2018). Alix and TSG101, often used as marker proteins to demonstrate presence of exosomes, as well as various vacuolar sorting proteins (VSPs), are important regulators of the ESCRT complex (Friand et al., 2015; Hanson & Cashikar, 2012; Hurley, 2010).

MVB formation may also occur in an ESCRT-independent pathway, involving two lipid metabolism enzymes: neutral sphingomyelinase that catalyzes the formation of ceramide, and phospholipase D2 (PLD2) which catalyzes bisphosphatidic acid formation. Proteins and lipids involved in and recognized as markers of these pathways include ceramide, flotillin and the small G protein ADP-ribosylation factor 6 (ARF6) (Ghossoub et al., 2014; Kowal et al., 2014; Trajkovic et al., 2008).

For protein sorting into ILVs, the syntenin-syndecan system is important. Alix connects cargo sorted by syntenin-syndecan to the ESCRT machinery to be specifically loaded in exosomes (Fares et al., 2017). This cargo loading pathway requires activation of PLD2 by ARF6 (Ghossoub et al., 2014). Additionally, tetraspanins, including CD9, CD63, CD81 and CD82 are involved in cargo selection in an ESCRT- and ceramide-independent manner (Chairoungdua et al., 2010; Perez-Hernandez et al., 2013; van Niel et al., 2011). The existence of alternative pathways of ILV formation indicates biological heterogeneity of exosomes.
Ectosomes are generated by blebbing of the plasma membrane. Therefore, ectosomes reflect the composition of the plasma membrane more closely than exosomes (El Andaloussi et al., 2013). Ectosomes can be generated via multiple mechanisms, some overlapping with exosome biogenesis systems. Calcium is one of the key factors during ectosome generation, triggering several mechanisms of cytoskeletal remodeling. Increased calcium levels activate cytosolic proteases, including calpain and caspase, that can disrupt the cytoskeleton to induce ectosome production (Coleman et al., 2001; B. Li et al., 2012; Morel et al., 2011; Piccin et al., 2007). Additionally, high calcium concentrations affect enzymes involved in the regulation of cell membrane phospholipid asymmetry. Loss of phospholipid asymmetry leads to disruption of cytoskeletal anchorage allowing membrane budding (Piccin et al., 2007). In addition, cholesterol-rich microdomains, so-called lipid rafts, contribute to ectosome biogenesis and sorting of specific lipid- and protein cargo via anchors on the inner leaflet of the cell membrane (Del Conde et al., 2005; Shen et al., 2011). Similar to exosomes, ARF6, ceramide and the PLD1 isoform, which is more abundantly expressed on the cell membrane, are involved in ectosome biogenesis (Ghossoub et al., 2014; Hozumi et al., 2022; Muralidharan-Chari et al., 2009). ARF6 is one of the proteins involved in selective loading of proteins such as integrin β1 and MHC-1, but can also cause actin-myosin based contraction leading to ectosome shedding (D’Souza-Schorey & Clancy, 2012; Muralidharan-Chari et al., 2009).

A variety of different ectosome subpopulations, released via different biogenesis pathways, have been described during different physiological cell stages or by different cell types (Table 1). The most well-studied subpopulation is apoptotic bodies. Apoptosis induces phospholipid reorganization leading to membrane blebbing and fragmentation of apoptotic bodies from the cell membrane (Bevers et al., 1982; Martin et al., 1995). They differ from other ectosomes as they are only formed during programmed cell death and are characterized by the presence of organelles (Hristov et al., 2004; Van Niel et al., 2018b). Next to membrane blebbing, it is also described that apoptotic bodies are excreted in a ‘beads-on-a-string’ structure, facilitating a sorting process where nuclear content is selectively excluded from these apoptotic bodies (Atkin-Smith et al., 2015; Coleman et al., 2001; Halicka et al.,
Apoptotic bodies contain phosphatidylserine (PS) on the outside of their membrane and seem to be cleared by macrophages (Fadok et al., 1992; Martínez & Freyssinet, 2001; Taylor et al., 2008). Another type of ectosome, distinct in terms of biogenesis from the mentioned ectosomes, are midbody remnants. During the cell division stage, cells remain connected via a small midbody which is rich in microtubules. After completion of cell division this midbody remnant is secreted into the extracellular space and can be taken up by surrounding cells. Midbody remnants from colon cancer cells were able to promote cellular transformation and an invasive phenotype of fibroblasts (Rai et al., 2021).

During cell migration, another type of ectosomes is formed called migrasomes which are formed at the end of long retraction fibers. Migrasomes contain smaller vesicles which are released when the fibers break with the cell body during migration, a process called migracytosis (Ma et al., 2015). During migration, cytosolic components can actively be transported into migrasomes. TSPAN4 has been suggested to be the most specific marker for migrasomes, but their formation is furthermore dependent on TSPAN7, cholesterol and integrins (Huang et al., 2019; Jiang et al., 2019). Migrasome-derived vesicles can be taken up by neighboring cells and play a role in vascular homeostasis and correct positioning in embryos (Jiang et al., 2019; Y. Zhang et al., 2020). In addition to migrasomes, a different type of ectosomes derived from cellular extensions are protrusion-derived ectosomes. Mechanisms involved in the scission of these protrusion-derived ectosomes may depend on the ESCRT machinery, which is also important for exosome biogenesis, actomyosin contractility, and external forces (D’Angelo et al., 2023). Protrusion-derived ectosomes were first discovered in macrophages, which release cholesterol-rich ectosomes during movement of filopodia and lamellipodia. In contrast to migrasomes, these particles are directly released from the membrane and may play a role in cholesterol transport and contribute to cholesterol deposition in atherosclerotic plaques (Hu et al., 2019). More recently, other protrusion-derived ectosomes have been described, including filopodium-derived ectosomes (Nishimura et al., 2021) and microvilli-derived ectosomes (Hurbain et al., 2022).

Exopheres are a subpopulation of large ectosomes only observed in specific cell types and are formed to maintain homeostasis. They are released by stressed cardiomyocytes to eject dysfunctional
mitochondria and unwanted cargo (Nicolás-Ávila et al., 2020). These exophores express PS on their surface which marks them for uptake by macrophages. Exosphere formation is driven by autophagy machinery to maintain metabolic stability and has also been described for homeostasis in the central nervous system, where exophores are shed from neurons to be degraded by glial cells (Davis et al., 2014; Melentijevic et al., 2017; Nicolás-Ávila et al., 2020). Large oncosomes are very large ectosomes specifically derived from tumor cells. They are highly abundant in proteins associated with cancer progression and are able to transfer these oncoproteins such as epidermal growth factor receptor (EGFR) to other tumor cells to alter the tumor microenvironment (Al-Nedawi et al., 2008; Di Vizio et al., 2009, 2012; Minciacchi et al., 2015).

In glial cells, a rapid release of a specific type of ectosomes has been described. These intracellular membrane-derived ectosomes have a cytoplasmic origin, presumably from the endoplasmic reticulum and can squeeze through pores in the plasma membrane to be released. This process seems to be driven by caveolae, and these PS-bearing intracellular vesicles seem to be mainly involved in cytokine release (A. M. Andrews & Rizzo, 2016; S. Li et al., 1996; Sun et al., 2021). Arrestin-domain-containing protein 1 (ARRDC1)-mediated microvesicles (ARMMs), are a type of small ectosomes which require the ESCRT proteins TSG101 and VSP4 for release. Distinct from exosomes, ARMMs lack endosomal marker proteins such as CD63 and LAMP1 (Nabhan et al., 2012). In summary, based on biogenesis there are multiple subpopulations of ectosomes described of which some are unique for specific cell types or cell stages.

Despite distribution of protein markers among EV populations being rather cell-type dependent, some overlap may be recognized (Table 1). In general, syntenin, LAMP1/2 and various proteins that are part of the ESCRT complex are often considered exosome markers (Andreu & Yáñez-Mó, 2014; Greening et al., 2017; Mathieu et al., 2021). Although the tetraspanins CD9, CD63 and CD81 are frequently referred to as exosome marker proteins, especially CD9 and CD81 can also be found in ectosomes (Crescitelli et al., 2013; Mathieu et al., 2021). Ectosomes are enriched in plasma membrane markers, however only very few markers have been identified for ectosomes, of which annexin A1/2,
basigin and SLC3A2 have been proposed to be the most specific (Jeppesen et al., 2019; Mathieu et al., 2021).

Next to these classical EV populations, EPs are defined as a separate class. Exomeres and supermeres are subpopulations of EPs and have a distinct protein, nucleic acid and lipid composition, with lower abundance of exosome and ectosome markers compared to EV populations. To date, the biogenesis of these EPs has not been unraveled (Lee et al., 2019; H. Zhang et al., 2018; Q. Zhang et al., 2019, 2021).

**Biological environment**

Next to different biogenesis pathways, multiple other factors influence variation in the secretion of EV subtypes. Cellular source, physiological state and biological environment are additional important drivers of EV heterogeneity and affect EV composition and function. EVs isolated from MSCs that are derived from different tissues differ in composition, functional effects and immunogenic characteristics (Almeria et al., 2022). In addition, paracrine factors of cells change as a response to external stimuli such as inflammatory signals (de Jong et al., 2012; Kilpinen et al., 2013), ATP (J. Wang et al., 2017), heat stress (T. Chen et al., 2009, 2011), increased intracellular calcium levels (Montecalvo et al., 2012; Savina et al., 2003), hypoxia (Noman et al., 2011; Park et al., 2010) and many other factors (Jaiswal & Sedger, 2019; H. Y. Kim et al., 2022). Influencing cell culturing conditions is therefore an interesting approach to boost EV production and functional activity. Especially hypoxic-preconditioning seems to have beneficial effects on the immunoregulatory and regenerative aspects of MSC-derived EVs (Almeria et al., 2019; S. Andrews et al., 2021; Bian et al., 2014; Cheng et al., 2020; Collino et al., 2019; Ge et al., 2021; Zhu et al., 2018). In endothelial cells, EV biogenesis pathways were altered after stimulation with TNF-α, which changed the effect of EV subpopulations on apoptosis regulation (Alexy et al., 2014). Furthermore, protein and RNA composition of endothelial cell-derived EVs is modified after hypoxia, hyperglycemia, and inflammatory stimuli such as TNF-α (de Jong et al., 2012). Hypoxia, acidosis, and pro-inflammatory...
cytokines influence MSC-EV yield, composition and immunomodulatory effect on T-cells (S. Andrews et al., 2021).

Aging of EV producing cells is another factor which can significantly impact EV function. EVs derived from MSCs with a higher cell passage stimulate endothelial migration to a lesser extent than EVs derived from younger MSCs (Patel et al., 2017). Even the polarity of epithelial cells affects the release of different EV subpopulations. Basolateral secreted EVs are enriched in TSG101, CD9 and CD81, whereas apically secreted EVs express CD63, HSP70, annexin A1 and flotillin-1. As these markers differ, EV cargos are likely to be selectively sorted as well into different EVs depending on epithelial polarity. While basolateral EV release depends on the ceramide pathway, release from the apical side relies on the alix-syntenin-syndecan machinery (Q. Chen et al., 2016; Matsui et al., 2021).

Different culture platforms have also been shown to influence EV composition (J. Y. Kim et al., 2021; Kucharzewska & Belting, 2013). Whereas 2D culture is the most common cell culture platform for EV production, bioreactors are more widely being used for upscale purposes. When MSCs are grown in a hollow fiber bioreactor, MSC-EV production is boosted and yields EVs more effective in reducing acute kidney injury as compared to normal 2D culture of MSCs (Cao et al., 2020). Similarly, a vertical-wheel bioreactor to produce MSC-derived EVs renders higher EV yields with a similar purity compared to 2D culture. However, such culture systems seem to induce release of different EV subtypes as the EVs harvested from these cultures substantially differ in protein marker expression (de Almeida Fuzeta et al., 2020). Similarly, it has been reported that MSC-derived EVs carry a higher amount of immunomodulatory cytokines when MSCs are grown in a 3D environment (H. Kim et al., 2020).

**Stochasticity**

Finally, stochasticity also plays a major role in EV heterogeneity. Protein and RNA levels and their incorporation in EVs varies in a stochastic manner, as a result of diffusion, steric hindrance, chemical processes and other mechanistic drivers. As a consequence, stochasticity can lead to EV batch-to-batch variation. Additionally, it is possible that every single EV includes a partly random portion of
cytoplasm and membrane cargo, which makes it plausible that each EV is unique (Pegtel & Gould, 2019).

Techniques to separate EVs for functional studies

Great progress has been made in EV separation techniques to study their functions. It is possible to discriminate distinct subtypes of EVs based on specific biophysical properties. There is however no consensus on the optimal method for EV isolation and separation, and choice of isolation technique will impact the selection and enrichment of specific EV subpopulations (Veerman et al., 2021; X. Zhang et al., 2020). An overview of the described EV separation techniques is given in Figure 2.

Differential ultracentrifugation (UC) is the most widely used method to enrich EVs and to study differences between lEVs and sEVs (Brennan et al., 2020). Very large EVs can be pelleted at centrifugal forces of around 2,000 x g, lEV fractions are pelleted at 10,000-20,000 x g, and sEVs at forces around 100,000 x g (Crescitelli et al., 2013). When UC was applied with forces in the range of 167,000-367,000 x g, two EP subpopulations could also be purified, referred to as exomeres and supermeres. (Lee et al., 2019; Q. Zhang et al., 2019, 2021). Despite often being suggested, there is no evidence that differential UC is capable to fully separate microvesicles/ectosomes (in the lEV pellet) and exosomes (in the sEV pellet) (Bobrie et al., 2012; Pegtel & Gould, 2019; S. E. Wang, 2019). Although UC is a relatively straightforward method, standardization is important as centrifugation time, rotor type, and number of washing steps, among others, can greatly influence EV yield and purity (Cvjetkovic et al., 2014; Nigro et al., 2021). Moreover, the sedimentation profile can vary between different cell lines (Jeppesen et al., 2014). In addition, applied high forces in UC may lead to aggregation of EVs, while protein complexes and other small particles can be co-pelleted or even generated (Coumans et al., 2017; Linares et al., 2015; Pegtel & Gould, 2019), and potentially negatively influence EV functionality (Mol et al., 2017). Despite these disadvantages, numerous papers show that UC is an useful method to separate lEVs from sEVs that differ in protein, nucleotide and lipid composition (Crescitelli et al., 2013; Haraszi et al., 2016). Among different studies, a large
variety in EV proteins have been used to characterize sEVs versus lEVs, with some marker proteins seemingly quite selectively detected in either sEVs or lEVs. sEVs are more enriched in proteins of the ESCRT machinery such as TSG101, alix, several VSPs, as well as syntenin and extracellular matrix proteins. lEVs are more enriched in endoplasmic reticulum proteins, mitochondrial proteins, cytoplasmic proteins, α-actinin-4, ceramides, PS and annexin A1 and A2. CD9, CD63, CD81 and flotillin 1/2 are highly expressed in sEVs but are also found in lEVs (Choi et al., 2019; Crescitelli et al., 2020; Durcin et al., 2017; Haraszti et al., 2016; Jeppesen et al., 2019; Keerthikumar et al., 2015; Kowal et al., 2016; Menck et al., 2013).

**Density gradient centrifugation (DGC),** using a sucrose or iodixanol gradient, is mainly used to separate EVs from non-EV contaminants (Jeppesen et al., 2019). However, this method can also be applied to separate EVs in low- (LD-EVs), medium- (MD-EVs) or high density fractions (HD-EVs) (Brennan et al., 2020). Separation is based on the principle that particles with different physical characteristics (such as size) are migrating to their equilibrium density at a different pace (Aalberts et al., 2012; Willms et al., 2016). DGC is however time consuming and generally results in low recovery rates (Seo et al., 2022; Willis et al., 2017). Some consensuses can be found by comparing studies that have employed DGC to separate LD-EVs from HD-EVs. As expected, LD-EVs are generally larger in size compared to HD-EVs. LD-EVs generally seem to be more enriched for α-actinin-4 and annexin A2 while HD-EVs are more enriched for extracellular matrix proteins, HSP90, as well as mitochondrial and endoplasmic reticulum proteins. General EV markers TSG101, alix, CD9, CD63, CD81 and flotillin 1/2 seem to be relatively more abundant in LD-EVs, but are found also found in HD-EVs (Q. Chen et al., 2016; Crescitelli et al., 2020; Jeppesen et al., 2019; Kowal et al., 2016; Lässer et al., 2017; X. Wang et al., 2021; Willms et al., 2016). On top of differences in protein expression, EVs separated using DGC also differ in their lipid and RNA composition (Brouwers et al., 2013; Lässer et al., 2017; Palma et al., 2012; Willms et al., 2016). In addition, using DGC, a novel double-membraned EV subpopulation enriched for mitochondrial proteins was identified. These so-called mitovesicles were distinct from classical EVs in morphology and were altered in cargo during
pathological processes where mitochondrial dysfunction occurs. Due to the lack of classical exosome (aliX, TSG101) and ectosome (annexin A1/2) marker protein expression, mitovesicles have been acclaimed distinct from classical ectosomes and exosomes (D’Acunzo et al., 2021).

**Size exclusion chromatography (SEC)** is a method where particles are separated based on size according to the higher permeability of small particles in the stationary phase compared to larger particles (Gámez-Valero et al., 2016). Depending on the type of resin, SEC is capable of separating EVs with distinct sizes (Ortega et al., 2019). SEC is easy to use and the structure and biological function of the EVs is better preserved with less aggregation as compared to UC (Baranyai et al., 2015; Gámez-Valero et al., 2016; P. Li et al., 2017; Mol et al., 2017; Nordin et al., 2015). Nevertheless, the recovery rate is low because particles can get trapped in the stationary phase (X. Zhang et al., 2020). SEC has been used to separate EVs based on size. Using Sepharose CL-2B resin, two distinct subtypes of MSC-derived sEVs were distinguished. CD63 and flotillin-1 were enriched in larger sEVs (>80 nm), while aliX and TSG101 were abundant in smaller sEVs (<80 nm) (Willis et al., 2017). Another study separated two subtypes of sEVs, differing in size, using Sephacryl S-1000 resin, which has a better separation capacity in the size range of the sEVs (Willms et al., 2016). In this study, a larger sEV type enriched for α-actinin-4 could be separated from a smaller sEV type with a higher expression of ephrin type-A receptor 2, while aliX and TSG101 were present in both subtypes.

**Asymmetrical-flow field-flow fractionation (AF4)** uses the combination of a laminar parabolic flow and a variable crossflow in a channel to separate particles on size, based on their diffusion properties (H. Zhang & Lyden, 2019). AF4 is a gentle method preserving EV integrity and has high recovery rates due to the lack of a stationary phase or exposure to high forces (Kang et al., 2008; H. Zhang et al., 2018). AF4 is highly reproducible and fast with the ability to separate particles in a very wide size range with adjustable separation profiles. At the same time, the large variety of possible settings requires a significant amount of method optimization. Multiple groups have employed AF4 to separate EVs based on size, or to separate EVs from lipoproteins in serum samples (H. Zhang &
Lyden, 2019; H. Zhang et al., 2018; Sitar et al., 2015; Petersen et al., 2014; Y. B. Kim et al., 2020; Wu et al., 2020; Multia et al., 2020). Using UC to pellet sEVs and EPs, followed by separation of sEV and EP subtypes with AF4, exomeres with a size of around 35 nm were discovered alongside two sEV classes of 60-80 nm (Exo-S) and 90-120 nm (Exo-L) (H. Zhang et al., 2018). The two sEV classes were enriched for proteins involved in endosomal pathways, while exomeres which lacked an external membrane were enriched for HSP90. The two distinguished sEV subtypes were also distinct in protein content. ‘Exo-L’ was enriched for annexin A1 and VSP4, while ‘Exo-S’ was more abundant in flotillin-1/2. There was no difference in the expression between CD9, CD63, CD81, TSG101 and alix. Comparing AF4-separated exomeres to exomeres pelleted using very high forces in UC, as discussed in abovementioned studies, some overlap in appearance and protein content can be observed.

**Ultrafiltration** is a method using ultrafine membranes with a specific pore size to filter particles based on size. Ultrafiltration is a simple and gentle method without a need for high forces, thereby being rather quick and unexpensive, however, particles could be lost due to adherence to or in the membrane. Furthermore, there are only limited membrane pore sizes available, and it is difficult to separate EVs from larger volumes, especially when small pore sizes are used. As a result of the increasing interest in ultrafiltration, several chips based on ultrafiltration are developed to separate EV classes based on size (Z. Chen et al., 2020; D. Kim et al., 2020; F. Liu et al., 2017; Mireles et al., 2020). These ultrafiltration-based chips seem to have higher recovery rates, however additional quality and purity validation studies on EVs purified using these chips should show their true potential.

**Microfluidic techniques** are a relatively novel way to separate EV classes based on size. Separation with this technique is relatively quick with minimal external force applied. One example is nanoscale deterministic lateral displacement that separates EVs based on their trajectories in a pillar array. With this method it is possible to separate particles between 20 and 110 nm with good resolution (Wunsch et al., 2016). This technique and other microfluidic techniques are gentle and suitable for good
separation of sEV with distinct sizes (Shin et al., 2017). Nevertheless, microfluidic systems can suffer from poor scalability and clogging which makes them less suitable for large functional EV heterogeneity studies (Asghari et al., 2020; Hattori et al., 2019; C. Liu et al., 2017).

**Affinity-based methods** separate EVs based on expression of specific markers. Usually, affinity-based methods do not need any pre-purification, are easy to use, and yield highly pure EV preparations. Capturing EVs with magnetic beads is a regularly used method to characterize EVs expressing a specific marker, often combined with antibodies against tetraspanins or tissue specific markers (Brett et al., 2017; Karimi et al., 2022; Lai et al., 2016; Sharma et al., 2018; Tauro et al., 2013). With this method, highly specific subsets of EVs can be rapidly collected for further characterization. Although it is possible to remove EVs from the magnetic beads, removing connected antibodies without hampering EV integrity or yield remains very challenging. Therefore, this technique is currently less suitable for functional studies. Flow cytometry techniques in combination with affinity-based capture or labeling are also used to separate distinct EV subtypes based on surface composition (Groot Kormelink et al., 2016; Koliha et al., 2016). Flow cytometry allows for high throughput and single EV screenings of surface markers, but is rarely able to sort sufficient quantities for functional studies (Groot Kormelink et al., 2016). Other affinity based methods, such as immobilized metal affinity chromatography or specific capture using aptamers, are also being used to purify specific EV subtypes (Bellotti et al., 2021; Ellington & Szostak, 1990; Nakai et al., 2016; Tran et al., 2020; Tuerk & Gold, 1990). An advantages of capturing EVs with aptamers compared to antibodies is that aptamers allow straightforward elution of EVs. Despite affinity-based separation techniques being promising to study EV heterogeneity and ideal to capture or deplete specific EV subtypes, they are relatively expensive. Furthermore, one needs pre-defined markers for separation, for which biological information may often be lacking.

**Charge-based separation** can be performed using anion exchange chromatography and uses a positive charged resin to separate EVs based on charge (Heath et al., 2018). Negatively charged EVs
are captured in the column, after which different fractions can be released during the elution step with good recovery rates (Kosanović et al., 2017; Seo et al., 2022). However, salt conditions vary strongly during a run and could impact the stability of EVs. When anion exchange chromatography was used for EV separation, two EV fractions were distinguished. EVs with a lower negative charge (LNC-EVs) were smaller in size compared to highly negatively charged EVs (HNC-EVs, respectively 148 nm and 191 nm). LNC-EVs expressed higher levels of HSP70, alix, HSP90, TSG101, flotillin-2, CD9 and CD81, while HNC-EVs were enriched in cytoskeleton proteins, ribosomal proteins, annexin A2, MHC molecules and ARF6. Lipid analysis revealed that the ratio of sphingolipids to phospholipids was slightly higher in LNC-EVs compared to HNC-EVs (Seo et al., 2022). Capillary electrophoresis is another charge-based separation method suggested for EV heterogeneity studies. While it has been shown that different subtypes of EVs can be separated with this technique, the scalability and resolution is currently insufficient for functional EV heterogeneity studies (Morani et al., 2020; Piotrowska et al., 2020).

Challenges regarding EV subtype separation techniques and other technical aspects for functional studies

Many techniques that are able to separate EV subtypes are available, with each technique having advantages and disadvantages with regards to separation resolution, recovery rates, scalability and purity (Table 2). Despite biological differences, separating exosomes from ectosomes or subpopulations thereof with current separation techniques remains extremely challenging, due to the overlap in biophysical properties such as size, density, morphology and protein marker expression (Brennan et al., 2020; Van Niel et al., 2018a). The majority of separation techniques separates EV subtypes based on size, while the correlation between EV size and biogenesis pathways is largely absent (Figure 3A). Alternatively, there are some techniques that separate EV subtypes based on charge differences, however also here a correlation with biogenesis pathway is lacking. In addition, separation resolution remains an issue for proper functional characterization. Purifying EV subtypes based on differences in surface marker
expression seems to be the most biologically relevant approach. However, to date, a lack of knowledge about subpopulation specific markers makes it difficult to select appropriate targets (Figure 3B). In addition, difficulties with regards to elution and scalability hampers use of these methods for functional studies (Ingato et al., 2016). Capturing EVs with aptamers allowing straightforward elution will have some benefit over more traditional methods, but scalability issues remain.

Besides challenges regarding the separation and functional recovery of EV subtypes, there are technical challenges in relation to variations in biophysical properties that need to be considered when comparing EV subtypes in functional studies. One of these challenges includes quantification (Figure 3C). Larger EVs may carry a different relative protein, lipid or nucleotide content than smaller EVs due to their lower surface area-to-volume ratios. This makes quantitative comparisons between EV subtypes based on absolute protein, lipid or nucleotide levels not straightforward. In addition, smaller EVs suffer relatively more from detection limitations with the existing particle quantification techniques, including those based on light scattering or fluorescence, than larger EVs (Collino et al., 2017; Cosenza et al., 2018).

Next to quantification, a main technical challenge for performing functional studies using EV subtypes is their low recovery rate (Figure 3D). Additional separation and concentration steps for purifying EV subtypes have a negative impact on recovery, for example due to adsorption to filters or plastics (Görgens et al., 2022; van de Wakker et al., 2022). As a result, there is an urgent demand for techniques that combine high separation resolution with high EV subtype recovery.

Next to the heterogeneity of EVs themselves, the presence of non-vesicular co-isolates such as protein complexes, aggregates and lipoproteins should also be considered (Figure 3E). It is not unlikely that these co-isolates are partly responsible for some of the reported beneficial functions of the isolated EVs (Whittaker et al., 2020). When EVs are not properly separated from contaminants, functions of these co-isolates could be mistakenly coupled to EVs. This is especially relevant when comparing functions of EV subtypes, where after improper purification different EV subtype preparations may
contain these contaminants to different degrees. Indeed, co-isolated EPs such as exomeres and supermeres are reported to be functionally active, which underlines the importance of taking non-vesicular co-isolates into account (Q. Zhang et al., 2019, 2021). Furthermore, co-isolated or EV-associated proteasomes derived from MSCs were shown to be involved in cardiac repair (Lai et al., 2012) and carry other functions (Abou Karam et al., 2022; Bochmann et al., 2014; Dekel et al., 2021).

Optimization of EV subtype purification is a prerequisite for defining therapeutically active EV subtypes, and would be beneficial for EV treatment reproducibility and effectivity. Although major progress has been made in the last decade, important drawbacks in current separation methods still exist. Novel or improved separation methods regarding EV recovery and separation resolution are needed to increase our knowledge about the involvement of different EV subtypes in tissue regeneration for further development of EV therapeutics.

Differences in function among EV subtypes in regenerative medicine

In the subsequent section, functional differences of EV subtypes are discussed. A general overview of functional studies that have taken EV heterogeneity into account is provided, followed by an in-depth analysis of functional EV heterogeneity reported in regenerative medicine studies.

Differences in function among EV subtypes

Studying functional EV heterogeneity remains to be a major challenge. However, the improvement in separation and detection techniques over the last decade has enabled initial attempts to study functional differences between EV subtypes.

Multiple studies have revealed the diverse functionality for different EV subtypes. The majority of these studies performed UC, thereby separating sEVs from lEVs. For cancer cells, differences in uptake properties and function were observed for sEVs and lEVs. sEVs, but not lEVs, were able to induce cell proliferation and migration of neuroblastoma cells (Keerthikumar et al., 2015). In addition, sEVs showed higher uptake rates in epidermoid carcinoma cells as compared to lEVs (Choi et al., 2019). Moreover, there is evidence that some subtypes of lEVs have distinct functional properties.
related to cancer progression (Al-Nedawi et al., 2008; Di Vizio et al., 2009; Meehan et al., 2016; Minciacchi et al., 2015).

Also lymphocytes react differently upon exposure to different EV subtypes. EVs derived from dendritic cells carry MHC complexes allowing them to activate T-cells. sEVs and lEVs were equally efficient to induce CD4+ T-cell activation in vitro, but different T-helper cell responses were promoted. sEVs mainly induced the T-helper cell 1 responses, while lEVs stimulated the secretion of T-helper cell 2 cytokines (Tkach et al., 2017).

As an alternative to size-based separation, anion exchange chromatography for EV separation based on charge differences has been applied to study functional differences between EV subtypes. LNC-EVs, with a relatively low negative charge, derived from cytotoxic T-cells showed prevention of tumor metastasis by depleting mesenchymal cells in a mouse model of lung metastasis, compared to HNC-EVs with a relatively high negative charge which had no effect. Furthermore, differences in cell uptake were seen, with LNC-EVs more likely to be taken up by MSCs while phagocytosis by Kupffer cells was observed more for HNC-EVs (Seo et al., 2022).

Comparing functional effects between sEVs and EPs, exomeres have been shown to be especially involved in metabolism, glycolysis and mTOR signaling. However, a large overlap in function was shown for exomeres and sEVs which could be the result of incomplete separation (H. Zhang et al., 2018; Q. Zhang et al., 2019). Another study revealed that exomeres, but not sEVs, derived from HEK cells induce cell proliferation of recipient HEK cells (Lee et al., 2019). In terms of their biodistribution, sEVs were shown to be taken up in the lymph nodes in higher quantities whereas exomeres were taken up more in the liver (H. Zhang et al., 2018). Next to exomeres, supermeres were also shown to be highly enriched for proteins involved in metabolism. It was shown that supermeres increased lactate secretion, transferred drug resistance and altered liver metabolism in vivo, but all these functions were also observed for exomeres and/or sEVs. In contrast, supermeres showed much higher in vivo uptake in multiple organs compared to exomeres and sEVs (Q. Zhang et al., 2021). The observed functional activities of exomeres and supermeres also suggest that EPs may partially be responsible for the described functional properties previously attributed to EV populations.
Altogether, these studies have revealed that functional differences between different EV subtypes exist.

**Differences in function among EV subtypes in regenerative medicine**

A wide variety of studies has demonstrated the potential of stem cell-derived EVs for tissue regeneration. However, only few studies directly compared different EV subtypes for their functional regenerative capacity (an overview is shown in Table 3). Most of these studies have used UC to compare the regenerative effects of sEVs versus lEVs. In the context of kidney regeneration, sEVs seem to display the most promising efficacy. sEVs derived from MSCs and endothelial colony-forming cells induced proliferation and protection against hypoxic apoptosis in endothelial cells in vitro. Furthermore, in an acute kidney injury mouse model, sEVs were able to improve renal function and reduce morphological injuries. lEVs did not induce any of the abovementioned effects, neither in vitro nor in vivo (Bruno et al., 2017; Burger et al., 2015). The regenerative character of sEVs might partly be explained by their immunoregulatory role, since sEVs, but not lEVs, have been shown to be able to prevent upregulation of pro-inflammatory proteins such as intercellular adhesion molecule-1 (ICAM-1) in endothelial cells. Interestingly, lEVs in fact showed an opposite effect by stimulation of ICAM-1 expression and induction of apoptotic responses in vitro (Burger et al., 2015). However, such immunoregulatory roles of EVs may be highly context dependent. Stimulating EV-producing cells with TNF-α led to increased ICAM-1 expression on both sEVs and lEVs and abolished the immunoregulatory effect of sEVs. In this context, ICAM-1-positive sEVs were able to stimulate monocyte recruitment, whereas lEVs were capable of stimulating ICAM-1 expression in recipient endothelial cells (Hosseinkhani et al., 2018, 2020).

Next to separation via UC, DGC has also been used to separate different EV subtypes to discriminate their effects in renal repair. Three classes of EVs derived from MSCs were recovered from low-, middle- and high-density gradient fractions, and termed LD-EVs, MD-EVs and HD-EVs. LD-EVs, MD-EVs and HD-EVs were internalized by renal cells, but only LD-EV and MD-EVs induced tubular epithelial cell proliferation. Furthermore, MD-EVs could protect hypoxic tubular epithelial cells from...
apoptosis after ischemia-reperfusion injury. In contrast, HD-EVs did not affect proliferation or apoptosis rates. Interestingly, this MD-EV fraction that appeared most effective in renal regeneration have some overlap in protein expression (e.g. CD63 and CD81) with functionally active sEVs described in other studies (Table 3) (Collino et al., 2017).

Similar to the findings for kidney regeneration, sEVs seem to have more favorable neurotrophic parameters compared to lEVs when separated using UC. While MSCs-derived lEVs have been shown to exert an inhibitory effect on cortical neural growth in vitro, sEVs result in a significant improvement of neurite outgrowth. Interestingly, the total population of EVs showed similar improvement as sEVs, which indicates that the inhibitory effect of lEVs was neutralized by the positive effect of the sEVs (Lopez-Verrilli et al., 2016). The neuro-protective potential of sEVs was further confirmed in a study using EVs derived from stem cells originating from the dental pulp of human exfoliated deciduous teeth (SHEDs) cultured in a bead-based bioreactor. After inducing oxidative stress, sEVs protected neurons from apoptosis, while lEVs did not show anti-apoptosis properties. Remarkably, only sEVs derived from SHEDs growing in the bioreactor but not sEVs from standard 2D culturing conditions were able to protect these cells. More in-depth characterization of the sEVs produced in both these culturing systems could give more insights in the involved drivers of the observed functional differences (Jarmalavičiūtė et al., 2015).

In osteoarthritis and rheumatoid arthritis models, both sEVs and lEVs derived from MSCs show beneficial effects, but sEVs to a higher degree (Cosenza et al., 2017, 2018). In osteoarthritis-like chondrocyte cultures, sEVs and lEVs showed similar chondroprotective functions whereas sEVs had a higher anti-apoptotic effect after staurosporine-induced apoptosis (Cosenza et al., 2017). In a collagenase-induced arthritis mouse model, both sEVs and lEVs showed significant prevention of cartilage damage to a similar extent. However, only sEV treatment significantly reduced osteophyte formation. (Cosenza et al., 2017). In inflammatory arthritis, sEVs and lEVs were both shown to have a dose-dependent anti-inflammatory effect on both T- and B-cells, and decreased the production of pro-inflammatory cytokines by macrophages. In murine models, however, sEVs, but not lEVs, suppressed development of inflammatory arthritis, even when up to 2.4 fold higher doses of lEVs
were applied. A potential explanation between these apparent differences in \textit{in vitro} and \textit{in vivo} properties is that the biodistribution of sEVs and lEVs may differ (Cosenza et al., 2018).

The above discussed studies consistently suggest higher regenerative properties for sEVs as compared to lEVs, but there is also evidence for specific functional properties of lEVs. When pathfinder cell-derived sEVs and lEVs were compared in a mouse model of diabetes, lEVs showed better reparative properties in the pancreas in comparison with sEVs that had no effect (McGuinness et al., 2016). Furthermore, only lEVs isolated from the plasma of healthy subjects were found to stimulate endothelial migration, although only to a very minor extent (Kränkel et al., 2020). However, the potential contribution of co-purified contaminants such as lipoprotein particles, which may largely outnumber EVs in plasma (Van Deun et al., 2020), and their relative distribution across the different EV fractions was not considered here.

Altogether, EV subtypes separated on size show distinct functional properties in various disease-specific experimental models. In most studies, the regenerative effects of sEVs were more pronounced as compared to lEVs, including in models of injuries of kidney, neurons and cartilage (Bruno et al., 2017; Burger et al., 2015; Cosenza et al., 2017, 2018; Jarmalavičiute et al., 2015; Lopez-Verrilli et al., 2016). In contrast, two studies suggest that lEVs fractions are more effective for regenerative applications in damaged endothelium and pancreas (Kränkel et al., 2020; McGuinness et al., 2016). Naturally, there were variations in EV isolation protocols among the described studies, which could have contributed to these apparent differences in observed effects (\textbf{Table 3}). To better define the functional involvement of specific EV subtypes, more studies describing in-depth characterization of cargo and its relation to EVs’ regenerative properties are needed.

\textbf{Challenges and scientific roadblocks for translation to clinical implementation regarding EV heterogeneity}
Despite the potential of EV therapeutics, there are several challenges regarding their clinical implementation (Sluijter et al., 2018). While it is known that EVs can transfer proteins, lipids, and nucleic acids to target cells, the specific mechanisms by which these molecules are transferred and how they elicit a regenerative response in the target cells are not well understood (Roefs et al., 2020) (Figure 4A). Part of the challenge to unravel the functional involvement of EVs is their inherent heterogeneity. Determining the functional contribution of different EV subtypes is complex as illustrated by the relatively limited number of studies that have attempted to address this. The usage of different EV sources, separation techniques, quantification strategies and characterization techniques furthermore inhibits extrapolation of the results from these studies.

For regenerative applications, it is currently unknown if the therapeutic activity of EVs is a collective effect or an effect which is driven by specific EV subtypes (Figure 4B). Potentially, regenerative functions of active EV subtypes could be diminished by the presence of other non-functional, or even counteracting, EV subtypes. Enrichment of functionally active EV subtypes will likely increase EVs’ therapeutic effects and would be beneficial for reproducibility. Reduced heterogeneity will also make EV quantification more straightforward. In contrast, when the desired effect is driven by synergistic functions of different EV subtypes, or co-isolates, there is no need for complex separation steps and basic isolation methods for yield optimization would be beneficial. Thus, increased knowledge about functional EV heterogeneity is of crucial importance to determine if and how EV treatments can be improved (Bauer et al., 2022).

Batch-to-batch variability forms another important hurdle for the translation of EV therapeutics (Figure 4C). It has been observed that independent MSC-EV batches produced in a standardized manner differ in EV activity (Bauer et al., 2022; Madel et al., 2020). Clonal drift, which is even seen in individual MSC batches (Ratajczak et al., 2006), and environmental factors may impact the production of different EV subtypes and their function on target tissues. An increased understanding which environmental factors impact the release of beneficial EV subtypes will raise the possibility to control these parameters for improved batch-to-batch reproducibility. Additionally, it will aid the development of potency assays to identify differences among active and non-active EV batches.
Finally, due to the abovementioned variabilities in cellular environmental conditions, suboptimal EV separation methods, and stochasticity, it remains highly challenging to trace the biogenetic origin of EV subtypes after their secretion (Figure 4D). Unraveling the biogenesis of functionally active EV subtypes would enable novel ways to increase the production of functionally active EV subpopulations to enhance their therapeutic effectivity.

Overall, the current lack of knowledge about the heterogeneity of EVs represents a major challenge to their clinical implementation. Increased understanding of EV heterogeneity is needed to overcome abovementioned challenges and to advance the translation of EVs to clinical applications for tissue regeneration.

**Future perspectives**

In the search for novel therapeutics, stem cell-derived EVs have demonstrated enormous potential as mediators of tissue repair and regeneration (Kirkham et al., 2022; Tsiapalis & O’Driscoll, 2020; Yang et al., 2019). Currently, the importance of EV heterogeneity is gaining more awareness, however there is still little known about differences in functions among EV subpopulations. Based on the existence of distinct biogenesis pathways and sorting mechanisms, it is evident that several subpopulations of both exosomes as ectosomes exist. Despite current knowledge about EV biogenesis, extensive research is still needed to understand the underlying pathways which drive EV heterogeneity. Due to the suggested specific signaling and communication of different EV subtypes, it is likely that only specific EV subtypes mediate regenerative therapeutic effects, and that other EV subtypes may even have unwanted or opposing effects. Understanding which EV subpopulations are functionally active or bear specific therapeutic cargo is important to manipulate EV preparations, in order to enhance their potency or select active EV subtypes and thereby improve reproducibility (Figure 5A).

Clearly, choice of isolation procedure is important for selection and collection of EV subtypes and can influence their functional properties. The majority of functional EV heterogeneity studies to date have been performed using size-based separation techniques, mainly UC. The use of more advanced
techniques, such as AF4, may allow for size separation of EVs with a higher resolution. However, all size-based techniques rely on the assumption of a correlation between EV size and functionality. Use of affinity separation techniques to select well-defined EV subtypes based on their surface marker expression seems to be a more biologically relevant approach, but is still hampered by issues related to scalability and recovery. Since all separation techniques have advantages and disadvantages (summarized in Table 2), a combination of different techniques may be necessary, but could lead to low EV recovery rates impairing the possibility to perform functional studies. Therefore, future advances in EV separating techniques are needed to allow more in-depth functional studies.

In the light of guidelines for EV studies (Théry et al., 2018), adequate EV characterization and accurate EV quantification is especially important to be able to draw reliable conclusions regarding EV heterogeneity. Quantification of the relative presence of different EV subtypes is highly challenging due to variations in their physicochemical characteristics and content (Collino et al., 2017; Cosenza et al., 2018). Therefore, we recommend that quantification of EV subtypes should be performed preferably with multiple, complementary methods which also allow determination of indicators of purity (e.g. protein/particle ratio). This will also allow better comparisons among different studies. In-depth cargo characterization will furthermore help to better define what content of functionally active EV subtypes is linked to what beneficial activity. To this end, novel developments in quantification of small EVs will help in identifying and analyzing specific EV subtypes (Bordanaba-Florit et al., 2021). These methods include techniques such as single-vesicle analysis and multidimensional flow cytometry, which can provide more detailed and accurate information on the size, shape, and composition of individual EVs.

As a consequence of different physiological cell stages and external stimuli, biogenesis pathways of cells are continuously altered leading to different paracrine functions. It is therefore likely that modification of biogenesis pathways to boost functionally active EV subtypes is an innovative approach to enhance therapeutic effects (Figure 5B). However, EV biogenesis is only partly understood and knowledge about the relationship between EV biogenesis and activity is lacking.
Despite description of multiple pathways and their molecular mediators involved in EV production and release (Baietti et al., 2012; Colombo et al., 2013), we are still unable to block or stimulate production of specific EV subpopulations. Intervening with specific environmental factors or culture conditions and thereby indirectly modifying EV biogenesis pathways is an interesting strategy to boost the production of regenerative EV subtypes. Alteration of the culturing conditions to boost regenerative effects of EVs has already been shown in various studies (Jaiswal & Sedger, 2019; H. Y. Kim et al., 2022). However, how these external factors influence functional EV heterogeneity should be addressed in future research.

Finally, loading pro-regenerative EVs with active cargo for therapeutic delivery is a potential approach to stimulate additional regenerative therapeutic signaling (Ramasubramanian et al., 2020) (Figure 5C). In many studies, to induce EV loading, therapeutic cargo is either engineered to associate to known high abundance general EV marker proteins (Somiya & Kuroda, 2022; Sutaria et al., 2017), or overexpressed in EV donor cells to be loaded into EVs in a stochastic matter (Mathiyalagan & Sahoo, 2017; Pascucci et al., 2014). A more tailored approach would be to induce targeted drug loading into EV subtypes that display intrinsic pro-regenerative activity or that show affinity for the target cell type (Lässer et al., 2018). However, this requires discovery of specific marker proteins that define such EV subtypes.

Altogether, advances are being made in the understanding of functional properties of EV subtypes. Effective EV fractions should be studied in more detail to discover the specific components involved in their therapeutic functions. We foresee that EV heterogeneity studies will provide leads in two directions: 1) to select the most active EV subtypes and 2) to exclude non-functional EVs subtypes, with both directions aiming to improve efficiency and reproducibility of EV treatments. In turn, improved knowledge about functionally active EV subtypes will help with the development of engineered EVs or EV mimetic treatments approaches.

Acknowledgements
This work is financially supported by a Van Herk Foundation grant to SvdW, ERC Consolidator Grant EVICARE (#725229) of the European Research Council (ERC) to JS, and a PPS grant (No. 2018B014) to JS and PV.

Data Availability Statement

There are no datasets in our paper.

Disclosure statement

PV. serves on the scientific advisory board of Evox Therapeutics Ltd.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: van de Wakker, Vader, Sluijter, Meijers
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## Table 1: Characteristics of EV populations and subpopulations

<table>
<thead>
<tr>
<th>Population</th>
<th>Subpopulation</th>
<th>Size</th>
<th>Suggested marker or cargo</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exosomes</td>
<td></td>
<td>30-150 nm</td>
<td>Syntenin, LAMP1/2, alix, TSG101, CD63</td>
<td>(Andreu &amp; Yáñez-Mó, 2014; Greening et al., 2017; Mathieu et al., 2021)</td>
</tr>
<tr>
<td>Ectosomes</td>
<td></td>
<td>50-10,000 nm</td>
<td>Annexin A1/2, basigin, SLC3A2</td>
<td>(Jeppesen et al., 2019; Mathieu et al., 2021)</td>
</tr>
<tr>
<td>Apoptotic bodies</td>
<td></td>
<td>50-2000 nm</td>
<td>ICAM-3, phosphatidyserine, histones, mitochondrial content</td>
<td>(Atkin-Smith et al., 2015; Fadok et al., 1992; Martínez &amp; Freyssinet, 2001; Taylor et al., 2008; Torr et al., 2012)</td>
</tr>
<tr>
<td>Exopheres</td>
<td></td>
<td>~3.5 µm</td>
<td>Phosphatidyserine, LC3, mitochondrial content</td>
<td>(Melentijevic et al., 2017; Nicolás-Avila et al., 2020)</td>
</tr>
<tr>
<td>Large oncosomes</td>
<td></td>
<td>1-10 µm</td>
<td>Cytokeratin 18, caveolin-1, ARF6, GAPDH, HSPA5</td>
<td>(Di Vizio et al., 2012; Minciacchi et al., 2015)</td>
</tr>
<tr>
<td>Intracellular membrane-derived ectosomes</td>
<td></td>
<td>50–120 nm</td>
<td>Phosphatidyserine, cytokines</td>
<td>(Sun et al., 2021)</td>
</tr>
<tr>
<td>ARMMs</td>
<td></td>
<td>~50 nm</td>
<td>TSG101, VSP4</td>
<td>(Nabhan et al., 2012)</td>
</tr>
<tr>
<td>Midbody remnants</td>
<td></td>
<td>200-600 nm</td>
<td>Microtubules</td>
<td>(Rai et al., 2021)</td>
</tr>
<tr>
<td>Migrasomes</td>
<td></td>
<td>50-100 nm</td>
<td>TSPAN4, TSPAN7, cholesterol, integrins</td>
<td>(Huang et al., 2019; Jiang et al., 2019)</td>
</tr>
<tr>
<td>Protrusion-derived ectosomes</td>
<td></td>
<td>~30 nm</td>
<td>Cholesterol, HSP90, cytoskeletal proteins, prominin-1</td>
<td>(D’Angelo et al., 2023; Hu et al., 2019; Hurbain et al., 2022; Nishimura et al., 2021)</td>
</tr>
<tr>
<td>Extracellular particles (biogenesis unknown)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exomeres</td>
<td></td>
<td></td>
<td>HSP90, argonaute, amyloid precursor proteins</td>
<td>(H. Zhang et al., 2018; Q. Zhang et al., 2019)</td>
</tr>
<tr>
<td>Supermeres</td>
<td></td>
<td></td>
<td>TGF-β1, HSPA13, enolase 2</td>
<td>(Q. Zhang et al., 2021)</td>
</tr>
</tbody>
</table>
Table 2: Overview of advantages and disadvantages of EV separation techniques

<table>
<thead>
<tr>
<th>Separation type</th>
<th>Time</th>
<th>Purity</th>
<th>Scalability</th>
<th>Recovery</th>
<th>Suitability for functional heterogeneity studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultracentrifugation</td>
<td>-</td>
<td>++</td>
<td>+/-/+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Density gradient centrifugation</td>
<td>-</td>
<td>++</td>
<td>--</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Size exclusion chromatography</td>
<td>+/-</td>
<td>+</td>
<td>+/- +/</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>Asymmetrical-flow field-flow fractionation</td>
<td>+/-</td>
<td>+</td>
<td>+/- +/</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Microfluidic techniques</td>
<td>+</td>
<td>+</td>
<td>--</td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>Affinity based methods</td>
<td>+/-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>--</td>
</tr>
<tr>
<td>Anion exchange chromatography</td>
<td>+/-</td>
<td>+</td>
<td>+/- +/</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Therapeutic target</td>
<td>EV source</td>
<td>Non-EV removal</td>
<td>Fractions ¹</td>
<td>EV marker enrichment</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------------------</td>
<td>----------------------------------</td>
<td>----------------</td>
<td>-------------</td>
<td>--------------------------------------------------------------------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Kidney</td>
<td>In vitro: murine tubular epithelial cells</td>
<td>Bone marrow MSCs</td>
<td>3,000 x g</td>
<td>tEV-100K sEV-10K sEV-100K</td>
<td>IEV: CD29, CD44, CD73, CD105 sEV: CD63, CD9, CD29, CD44, CD47, CD105 (Bruno et al., 2017)</td>
</tr>
<tr>
<td></td>
<td>In vivo: acute kidney injury mouse model</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>In vitro: human umbilical vein endothelial cells</td>
<td>Endothelial colony-forming cells</td>
<td>2,500 x g</td>
<td>IEV-20K sEV-100K</td>
<td>IEV: Caveolin-1 sEV: TSG101, CD63 (Burger et al., 2015)</td>
</tr>
<tr>
<td></td>
<td>In vivo: acute kidney injury mouse model</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>In vitro: murine tubular epithelial cells</td>
<td>Bone marrow MSCs</td>
<td>1,500 x g</td>
<td>HD-EV tEV-100K sEV-10K sEV-100K</td>
<td>IEV: CD29, annexein A2</td>
</tr>
<tr>
<td></td>
<td>In vivo: acute kidney injury mouse model</td>
<td></td>
<td></td>
<td></td>
<td>MD-EV: CD63, CD81, CD29, integrin α5, annexin A2, HLA-l</td>
</tr>
<tr>
<td>Immune cells</td>
<td>In vitro: T- and B- cells</td>
<td>Bone marrow MSCs</td>
<td>300 + 2,500 x g</td>
<td>tEV-100K sEV-18K sEV-100K</td>
<td>IEV: CD29, integrin β1 sEV: CD9 and CD81, HSP70, TSG101, alix</td>
</tr>
<tr>
<td></td>
<td>In vivo: Collagen-induced arthritis mouse model</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cartilage</td>
<td>In vitro: murine chondrocytes</td>
<td>Murine bone marrow MSCs</td>
<td>300 + 2,500 x g</td>
<td>tEV-18K sEV-100K sEV-10K sEV-100K</td>
<td>IEV: CD29, integrin β1 sEV: CD9, CD81, HSP70, TSG101, alix</td>
</tr>
<tr>
<td></td>
<td>In vivo: Collagen-induced arthritis mouse model</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neural tissue</td>
<td>In vitro: cortical neuron culture and dorsal root ganglia cultures</td>
<td>Menstrual MSCs</td>
<td>200 + 2,000 x g</td>
<td>tEV-100K sEV-10K sEV-100K sEV-100K</td>
<td>IEV: CD73, CD90, CD105, integrin α1, CD63, HLA-l sEV: CD63, TSG101, HSP70, HSP90</td>
</tr>
<tr>
<td></td>
<td>In vivo: Collagen-induced arthritis mouse model</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neural tissue</td>
<td>In vitro: dopaminergic neuron (suspension-like) culture</td>
<td>SHEDs cultured in a bioreactor</td>
<td>300 + 2,000 x g</td>
<td>IEV-20K sEV-100K sEV-100K sEV-100K</td>
<td>IEV: CD73, CD90, CD105, integrin α1, CD63, HLA-L sEV: CD63, TSG101, HSP70, HSP90</td>
</tr>
<tr>
<td></td>
<td>In vivo: Collagen-induced arthritis mouse model</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>In vitro: diabetes mouse model</td>
<td>Pathfinder cells</td>
<td>1,000 x g</td>
<td>IEV-16K sEV-120K sEV-100K</td>
<td>IEV: Integrin-β1, CD40 sEV: CD9, CD63, CD81, TSG101, Rab5B, integrin β1</td>
</tr>
<tr>
<td></td>
<td>In vivo: diabetes mouse model</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelium</td>
<td>In vitro: human aortic endothelial cells</td>
<td>Plasma samples of chronic coronary syndrome patients and healthy controls</td>
<td>Filter 1 µm Filter 0.2 µm</td>
<td>IEV-16K sEV-100K sEV-100K sEV-100K</td>
<td>IEV: TSG101 sEV: TSG101, CD63, CD81</td>
</tr>
<tr>
<td></td>
<td>In vivo: human aortic endothelial cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ EV fractions showing significant regenerative effects are marked in **bold**, while damaging effects are marked in italic and underlined total EV (tEV).

*Table 3: Overview of regenerative function comparison studies between lEVs and sEVs*
Figure 1: Schematic representation of subpopulations of extracellular vesicles and their biogenesis. Exosomes are derived from different multivesicular bodies populations and from amphisomes (Jeppesen et al., 2019; Klumperman & Raposo, 2014). Ectosomes are generated by blebbing of the plasma membrane (El Andaloussi et al., 2013). Apoptotic bodies are a subpopulation of ectosomes that are formed by blebbing and fragmentation during apoptosis (Martin et al., 1995). Exopheres are large ectosomes that contain dysfunctional mitochondria (Nicolás-Ávila et al., 2020). Large oncosomes are large ectosomes purposely released by tumor cells (Minciacchi et al., 2015). Intracellular membrane-derived ectosomes are derived from the endoplasmic reticulum and squeeze through pores in the plasma membrane (Sun et al., 2021). ARMMs (arrestin-domain-containing protein 1 (ARRDC1)-mediated microvesicles), are a distinct type of small ectosomes (Nabhan et al., 2012). Midbody remnants are vesicles formed between cells during cell division (Rai et al., 2021). Migrasomes are formed at the end of long retraction fibers and contain smaller vesicles. When the fibers break during migration the smaller vesicles are released (Ma et al., 2015). Protrusion-derived ectosomes are released during movement of filopodia and lamellipodia (Hu et al., 2019).
Figure 3: Technical challenges for isolation and functional comparison of EV subtypes. There are several technical challenges regarding functional EV heterogeneity studies. 

A. While most EV subtype separation methods are based on differences in size, the correlation between EV size and biogenesis is largely absent. 

B. Specific markers to capture unique EV subpopulations with affinity-based methods are unknown. 

C. As EV subtypes may differ in size and content, it is challenging to perform comparative quantification studies. 

D. Additional separation steps necessary to purify EV subpopulations have a negative impact on EV recovery. 

E. Non-vesicular co-isolates, including lipoproteins, histones, proteasomes and other large protein complexes, may contaminate specific EV subtypes to different degrees.
Figure 4: Translational challenges regarding EV heterogeneity. A. The mechanisms through which EVs exert their regenerative functions are not well understood. B. It is currently unknown if EV subtypes function in an additive, synergistic or counteractive manner. C. Batch-to-batch variability in the production of different EV subtypes due to clonal drift or environmental factors impacts EV efficacy. D. It is highly challenging to trace the biogenetic origin of EVs after their secretion.
Figure 5: Future perspectives for the use of EV heterogeneity studies to assist the development of EV therapeutics. 

A. Selection of functional active EV subtypes will most likely lead to increased potency of EV treatments. 

B. Stimulation of the production of functionally active EV subpopulations with external stimuli will increase the potency of EV preparations. 

C. Selection of the most suitable EV subpopulation for cargo loading will lead to a more tailored, synergistic approach.
Separation based on size

Pelleting IEVs ~10,000-20,000 x g
Pelleting sEVs ~100,000 x g
Pelleting EPs >167,000 x g

Loading sample
Centrifugation
Low density
High density

Laminar flow in
Slot flow
Detector flow
Parabolic flow
Sample-free
Sample-rich
Cross flow

Separation based on affinity

Separation based on charge

EV binding to resin
Elution with low salt
Elution with high salt
A. Absence of a correlation between EV size and biogenesis

B. Lack of knowledge about subpopulation specific markers

C. Quantification challenges

D. Recovery challenges

E. Non-vesicular co-isolates
A. Mechanism of action of EVs not well understood

B. Additive, synergistic or counteractive function?

C. Functional diversity among EV batches

D. Challenging to trace the biogenetic origin of EV subtypes
A. Selection of functionally active EV subpopulations

B. Stimulation of the production of functionally active EV subpopulations

C. Selection of beneficial EV subpopulations for cargo loading