

16 **Abstract**

17 Extracellular vesicles (EVs) are a heterogeneous group of natural particles with relevance for the
18 treatment of cardiovascular diseases. The endogenous properties of these vesicles allow them to
19 survive in the extracellular space, bypass biological barriers and deliver their biologically active
20 molecular cargo to recipient cells. Moreover, EVs can be engineered to enhance their stability,
21 bioactivity, presentation and capacity for on target binding at both cell type and tissue levels. The
22 therapeutic potential of native (i.e., EVs that were not modified via donor cell or direct modulation)
23 and engineered (i.e. EVs that were modified either pre- or post-isolation or whose
24 pharmacokinetics/presentation was altered using engineering methodologies EVs is still limitedly
25 explored in the context of cardiovascular diseases. Efforts to tap into the therapeutic potential of EVs
26 will require innovative approaches and a comprehensive integration of knowledge gathered from
27 decades of molecular compound delivery. In this review, we outline the endogenous properties of
28 EVs that make them natural delivery agents as well as those features that can be improved using
29 bioengineering approaches. We also discuss the therapeutic applications of native and engineered
30 EVs for cardiovascular applications and examine the opportunities and challenges that need to be
31 addressed to advance this research area with an emphasis on clinical translation.

32 **Key points**

- 33 • EVs secreted from stem/progenitor cells as well as differentiated somatic cells have
34 regenerative properties in the context of myocardial infarction, ischemic limb, chronic
35 wounds and stroke.

- 36 • Despite the advantage of native EVs as delivery agents, their applicability in the
37 cardiovascular context is hindered by intrinsic limitations, such as their undefined and
38 heterogenous nature and limited tropism.

- 39 • Targeting, bioactivity, kinetics and biodistribution of EVs may be improved by engineering
40 approaches using both pre- and post-isolation methodologies to functionalize and/or otherwise
41 enrich EVs.

- 42 • Enhancing EVs is key to unlock their clinical potential for cardiovascular applications.

43 **1-Introduction**

44 Cardiovascular diseases have been the most prevalent cause of death and morbidity in the
45 world for decades despite numerous breakthroughs and the discovery of novel therapies¹. In the last
46 20 years, several therapeutic interventions have been initiated, including cell-based therapies;
47 however, poor survival and/or engraftment of transplanted cells in the ischemic milieu of the cardiac
48 tissue limited their clinical efficacy². Mechanistically, the functional improvements observed with
49 cell therapies are poorly understood; however, several pieces of experimental data indicate that they
50 may act by paracrine action, mediated by the release of extracellular vesicles (EVs) and/or other
51 factors^{3,4}. Therefore, more recently, interest has been placed in cell-free therapies, in particular, those
52 based in EVs, obviating the need of transplanting large number of cells whilst having a better-defined
53 and less expensive product.

54 EVs are lipid bilayer-enclosed extracellular structures⁵ secreted by virtually all cell types
55 known, and include two major classes, namely exosomes and microvesicles⁶. Exosomes (30-150 nm),
56 intraluminal vesicles formed via invagination of the membrane of multivesicular endosomes (MVEs),
57 are released into the extracellular space upon fusion of MVEs with the cell membrane. Microvesicles
58 (MVs) (50-1000 nm), a very heterogeneous class of EVs, are characterized by their origin and
59 secretion via outward budding of the plasma membrane. Given the complexity involved in identifying
60 their biogenesis, the size of the vesicles is the most widely used parameter to distinguish both types
61 and, on that basis, we either have small EVs (sEVs) or medium/large EVs (m/lEVs)^{7,8}. In this review,
62 EVs represent sEV-enriched samples (many studies are not conclusive relatively to the biogenic
63 origin of EVs), being the exception MV examples which are clearly defined in the text.

64 EVs carry proteins, RNAs and/or microRNAs (miRNAs), among other molecules, and they
65 act as vehicles in cell-to-cell communication⁹. A large body of evidence suggests that EVs are
66 involved in many physiological and pathological cardiovascular processes, including the regulation
67 of angiogenesis^{10,11}, blood pressure^{12,13}, cardiomyocyte hypertrophy¹⁴ and apoptosis/survival¹⁵⁻¹⁷ and

68 cardiac fibrosis¹⁸. Given their ubiquitous presence in body fluids, EVs have been used as potential
69 biomarkers of cardiovascular diseases¹⁹. Moreover, because EVs are an important component of the
70 paracrine effect of stem cell-based therapies²⁰, they are candidates as a standalone therapy in the
71 context of cardiovascular diseases. Pioneering work from the group of Lim suggested the therapeutic
72 potential of EVs in protecting the heart from ischemic injury²⁰. Shortly after, the group of Sahoo
73 unravelled the pro-angiogenic potential of EVs in the setting of limb ischemia¹⁰. Ever since, a number
74 of pre-clinical studies have reported the advantages of EVs for cardiovascular regeneration and
75 protection²¹⁻²⁴. Yet, several challenges need to be addressed before clinical translation of these
76 therapies including (i) the development of platforms to monitor EVs (both the membrane and the
77 cargo) *in vivo* to determine and optimize the EV dosage regimen, the route of administration, the
78 biodistribution, potential toxicity, immunogenicity as well tumorigenesis, (ii) the characterization of
79 EV cargo in order to use well-defined EV formulations and (iii) the development of strategies to
80 modify the membrane of EVs in order to improve their accumulation in specific organs and tissues.
81 To overcome these limitations, researchers developed pre- and/or post-isolation techniques capable
82 of modulating the intrinsic properties of native EVs and modified their surface to enhance their
83 targeting efficiency and track them *in vivo*. The hypothesis of this review is that the modulation of
84 EVs by engineering approaches may unlock their clinical potential for cardiovascular applications.

85 In this review, we cover initially the EV biophysical properties (e.g., size, charge, membrane
86 composition and cargo content). This will be followed by the presentation of the therapeutic potential
87 of native EVs for cardiovascular applications. Then, we describe engineering strategies to modulate
88 the content of EVs in order to favourably alter their bioactivity, biodistribution, delivery, targeting
89 and intracellular trafficking. Finally, we present the studies performed using engineered EVs as
90 cardiac therapies and discuss how this area can move forward.

91

92

93 **2-EV properties**

94 The biophysical properties of EVs are briefly described below. These properties may be
95 modified using engineering tools, a topic that is covered in section 4. EV biogenesis is not covered
96 in this review and readers are referred to recent reviews related to this topic^{25,26}.

97

98 **2.1-EV size**

99 EV size is one of the parameters adopted to categorize EVs^{7,8}. Typically, exosomes are smaller
100 (30-150 nm in diameter) than microvesicles and their size distribution more uniform. EV size is
101 critical for their study and application due to most of the protocols used for isolation and
102 characterization relying on EV density and/or diameter²⁷. Moreover, EV size, precluded by their
103 biogenic pathway, correlates with EV composition, as does the cell line of origin²⁸. Finally, the
104 efficiency in tissue biodistribution, cell internalization and intracellular trafficking of EVs are size-
105 dependent²⁹⁻³². Indeed, it has been shown that upon systemic administration, large and/or aggregated
106 vesicles (>200 nm) may be trapped in the lung, liver and spleen, taken up by macrophages or unable
107 to extravasate and interact with non-vascular cells and tissues^{29,30}. Additionally, aggregation renders
108 EVs less spherical, which may decrease their margination and extravasation from circulation³¹. For
109 example, it has been shown that the vasculature has enhanced permeability after ischemic injury;
110 however, only small-size particles (<200 nm) cross the endothelial barrier³³.

111 At a cellular level, particles with different sizes may elicit different uptake mechanisms. For
112 example, particles with a diameter smaller than 100 nm may be taken up via clathrin- or caveolae-
113 mediated endocytosis, while larger complexes may require macropinocytosis^{34,35}. Therefore, larger
114 aggregates are more likely to be directed towards lysosomal degradation or membrane recycling³²,
115 while smaller vesicles may exhibit higher rates of effective intracellular delivery. In the cardiac
116 setting, particularly for EVs administered systemically, this is of utmost importance as EVs are
117 required to extravasate successfully to the cardiac tissue and then be efficiently taken up by the
118 relevant cell types.

119 **2.2-EV charge**

120 Another important property of EVs is their surface charge. As part of EV membrane
121 composition comes from the plasma membrane (being the remaining from other organelles including
122 Golgi complex)^{25,36}, which is rich in phosphate groups, a global negative charge is the norm for EVs,
123 just as in cells. However, the charge is also highly dependent on the sugar composition of the plasma
124 membrane which is highly dependent in the expression level of sialyltransferase in the endoplasmic
125 reticulum and Golgi apparatus³⁷. Changes in surface charge can be used to infer stability of EVs in
126 suspension, as low absolute values are thought to be typical of EVs more prone to aggregation due to
127 lessened repulsion. This, however, must be balanced with the fact that closer to neutral nanoparticles
128 are more stable in circulation, compared with highly charged ones³⁸. Both EV size and surface charge
129 are crucial in specifying the mechanism of interaction between the EV and a host of potential ligands,
130 as well as their uptake by target cells³⁹. Finally, the presence of contaminants (e.g. protein or lipid
131 aggregates) in an EV sample may affect multiple functions and parameters. Given the heterogeneity
132 in surface charge of these contaminants, aggregates may be formed between them and EVs.
133 Therefore, proper purification protocols must be ensured. This, however, is aside from the scope of
134 this review, and we direct the reader to other excellent papers on the matter^{40,41}.

135

136 **2.3-EV membrane composition**

137 Exosome membranes have higher content of cholesterol than donor cell membranes⁴², making
138 them less susceptible to the permeation of small solutes. In addition, exosome membranes contain
139 higher levels of phosphatidylserine, glycosphingolipids and sphingomyelin and lower levels of
140 phosphatidylcholine than the corresponding donor cells⁴². Moreover, exosomes have low protein to
141 lipid ratios compared with, for example, microvesicles⁴³. Indeed, high cholesterol and sphingolipid
142 content makes exosomes more resistant to detergents and high temperatures than microvesicles⁴³⁻⁴⁵.
143 Aside from their lipidic composition, exosomes are decorated with proteins and sugars which, on one

144 hand, contribute to exosome charge and maintenance of membrane structure and, on the other hand,
145 mediate the interactions of exosomes with the target cells²⁵. For example, tetraspanins are a class of
146 membrane proteins abundantly present, in clusters, in exosomes and some of them, namely CD63,
147 CD9 and CD81, are considered general exosome markers. Functionally, tetraspanins are involved in
148 membrane fusion and cellular adhesion, and, as such, play a key role in exosome internalization.
149 Other classes of proteins, such as chemokine receptors (e.g. CXC chemokine receptor type 4)⁴⁶,
150 adhesion molecules⁴⁷⁻⁴⁹ and proteoglycans (e.g. heparan sulfate)⁵⁰, have been shown to play a role in
151 mediating EV interactions with the cell surface. When these protein and sugar-based components
152 were deleted or masked, EV internalization⁵¹ as well as EV biodistribution was affected^{52,53}. EVs
153 have their transmembrane proteins in an identical topology to that of the secreting cell, conferring
154 them a degree of cellular identity and possible tropism^{10,54}.

155

156 **2.4-EV lumen**

157 From late 1990s, it has been proposed that EVs act as important players in intercellular
158 communication, particularly in the context of immune responses and cancer^{55,56}. This concept was
159 further confirmed in 2007, after the discovery that exosomes contain microRNAs (miRNAs), as well
160 as other types of RNAs, and can transfer their content to target cells ultimately affecting their
161 activity⁹. Recent studies using high-resolution density gradient fractionation and direct
162 immunoaffinity analyses further dissected EV composition⁵⁷. EVs contain different proteins and
163 RNAs in the lumen, including long and small non-coding RNAs, transfer RNA and ribosomal
164 RNA^{28,57-59}. Exosomes do not contain DNA, although it may be present in larger EVs or present in
165 exosome-enriched samples due to the co-precipitation with histones⁵⁷. Proteins, on the other hand,
166 may be sorted into EVs via post-translational modifications, such as ubiquitination⁶⁰ and
167 glycosylation⁶¹ and these pathways can be hijacked in order to specifically target proteins onto EVs.
168 Upon studying the interaction networks that can be functionally established between EV proteins, the

169 intravesicular protein content has been proposed as an organized “nanocosmos” and not cellular
170 scraps⁶².

171

172 **3-Native EVs for cardiovascular applications**

173 The first study describing the use of EVs as a potential therapeutic intervention for
174 cardiovascular diseases was published in 2010²⁰. In early 2000s, several groups have shown that upon
175 MI, transplantation of different cell types, including mesenchymal stem cells (MSCs)⁶³ and
176 hematopoietic progenitor/stem cells (CD34⁺ cells)⁶⁴, improved heart repair. A few years later, it was
177 shown that the positive effects of those stem/progenitor cells were mediated not by direct contribution
178 of the engrafted cells but by paracrine factors^{65,66}, in particular EVs^{10,20} secreted by the surviving cells
179 (**Box 1**). Since these pioneer studies, several groups have demonstrated the regenerative properties of
180 EVs secreted from stem/progenitor cells⁶⁷⁻⁷⁰ as well as differentiated somatic cells^{71,72} in the context
181 of MI^{69,70,72,73}, ischemic limb⁶⁷, chronic wounds⁷¹, among others. While there are several studies on
182 cardiovascular diseases such as atherosclerosis⁷⁴ and stroke^{75,76}, most research with EVs has pertained
183 to ischemic heart disease and MI.

184 EVs with cardiovascular efficacy have been isolated from different cell sources such as
185 MSCs⁷⁷⁻⁸³, putative cardiac progenitor cells^{15,21,68-70,84-88}, cells differentiated from pluripotent stem
186 cells^{20,72,89-93} and differentiated somatic cells^{71,94-96}. The impact of allogenic EVs has been evaluated
187 in mice^{69,79,81,87}, rats^{70,78,85,86,88} and pigs^{21,84}, through intravenous^{78,82,97} and intramyocardial<sup>21,68-
188 70,77,80,81,83,84,86,88</sup> administration. The EV dosage regimen was highly variable as between 30 to 1300
189 μg ^{78,85} and 4 to 4000 μg ^{89,92} of EVs per kg of animal have been tested for rats (300 g was assumed as
190 the weight of a rat) and mice (25 g was assumed for the weight of a mouse), respectively. Given the
191 fact that EVs are less immunogenic than their cellular counterparts (with the possible exception of
192 exosomes released from dendritic cells⁹⁸), it is not surprising to see several studies where EVs isolated

193 from human cells have been tested into non-immunosuppressed animals such as mice^{89,99}, rats^{80,85}
194 and pigs²¹.

195 EV tracking studies indicate that the intramyocardial delivery of EVs yields higher EV
196 retention in the heart than intracoronary or intravenous administration routes²¹. The pre-clinical data
197 collected so far indicate that EVs, regardless of their origin, may improve the ejection fraction (EF)
198 (up to 1.3-fold increase^{15,77,86,88} relatively to non-treated group) and reduce infarct size (up to 3-
199 fold^{79,81,84,85} decrease relatively to non-treated group). The therapeutic effect of EVs and donor cells
200 has been evaluated/discussed and the results collected until now indicate that EVs are as effective as
201 the donor cells in the context of MI^{21,80}.

202 The therapeutic role of EVs in recipient cells has been mostly ascribed to the delivery of
203 proteins and/or non-coding RNAs, in particular miRNAs. For example, the cardiovascular protective
204 role of exosomes has been attributed to miRNA-19a-3p¹⁰⁰, miRNA-21^{79,90}, miRNA-24¹⁶, miRNA-
205 22⁸¹, miRNA-29a¹⁸, miRNA-143⁹⁶, miRNA-146⁶⁹, miRNA-181b⁸⁴, miRNA-210⁹⁰, miRNA-222⁹⁶,
206 miRNA-294-3p⁹³, mir-126⁶⁷, among others. Some of the miRNAs have been identified in previous
207 studies of cell-based therapies (e.g. miRNA-19, miRNA-21, miRNA-24, miRNA-210)¹⁰¹ to be
208 relevant for cardiovascular repair while others are novel. Another important component of EVs, also
209 associated with their bioactivity, are proteins such as platelet-derived growth factor D⁷⁸ and
210 pregnancy-associated plasma protein-A¹⁵. Some of these proteins are at the EV surface and therefore,
211 do not need to be delivered to the cytoplasm of the acceptor cell. For example, pregnancy-associated
212 plasma protein A (also known as pappalysin-1) is a protein highly expressed in exosomes of cardiac-
213 resident progenitor cells¹⁵. It has been shown that this protein mediates the cardioprotection and
214 angiogenesis of cardiac-resident progenitor cell-derived EVs by cleaving the insulin-like growth
215 factor binding protein-4 in insulin-like growth factor-1 which, in turn, activates the insulin growth
216 factor receptor, ultimately leading to the phosphorylation of Akt and ERK1/2 and subsequently to
217 decreased caspase activation and reduced cardiomyocyte apoptosis. In other cases, the proteins are in

218 the lumen of EVs and need to reach the acceptor's cell cytoplasm to elicit a biological affect. For
219 example, EVs secreted by stem/progenitor cells in mice under systemic inflammation conditions
220 contain integrin-linked kinase that activate the NF-kB pathway¹⁰². The knockdown of integrin-linked
221 kinase in inflamed exosomes attenuated their inflammatory response and enhanced the endothelial
222 progenitor cell-derived exosome therapeutic activity in ischemic heart.

223 The mechanisms triggered by EVs depend on the source of EVs (as well as their content) and
224 include: (i) an improvement in cardiomyocyte and endothelial cell survival^{21,89,90,95,103} by the
225 regulation of autophagy¹⁰⁴, activation of pro-survival signalling pathways (e.g. Akt, ERK, toll-like
226 receptors)^{95,105} and a decrease in oxidative stress⁹⁰, (ii) modulation of the inflammatory response^{85,106}
227 by influencing immune cell polarization (i.e. inducing a more reparative state rather than an
228 inflammatory state)⁸⁴ and cytokine secretion⁸⁵ as well as increasing the activation of CD4-positive T
229 cells⁹⁴ (iii) a decrease in scar content²¹ and (iv) enhancement of angiogenesis^{107,108}. For example,
230 cardiosphere-derived EVs improved heart function in a mouse model of MI via miR-146, decreasing
231 apoptosis and inflammatory response and increasing cardiomyocyte proliferation and angiogenesis⁶⁹.
232 It has also been shown that extracellular matrix-derived EVs carry miR-199a-3p, which, by regulating
233 GATA-binding 4 acetylation, were able to rescue electric function in engineered and *in vivo* atria¹⁰⁹.

234 The above-mentioned studies underscore the potential of native EVs for cardiovascular
235 therapy. However, their clinical potential has not been met yet and important limitations must be
236 overcome before their establishment as an effective therapeutic tool (**Fig. 1**). These limitations may
237 be surpassed by enhancing native EV using bioengineering approaches, as detailed in the following
238 sections.

239

240 **4-Engineering the EVs**

241 Despite the therapeutic efficacy of native EVs to treat cardiovascular diseases, in recent years,
242 technologies have been developed to modulate EV and thus to enhance their bioactivity, stability,
243 targeting and presentation (by the development of EV-delivery systems) to the cardiovascular system

244 **(Fig. 2)**. In the subsequent sections, we explore how EVs may be enhanced or modified and used for
245 the treatment of cardiovascular diseases.

246

247 **4.1-Tracking the EVs**

248 Methods to track EVs *in vivo* and follow their biodistribution are very important to fully
249 evaluate their cardiovascular therapeutic potential. Fluorescence^{47,54,71,110,111}, luminescence¹¹²,
250 positron-emission tomography (PET)/magnetic resonance imaging (MRI)¹¹³ and single-photon
251 emission computed tomography (SPECT)^{114,115} imaging techniques have been used to monitor *in vivo*
252 EVs. In most of the cases, the EVs were isolated and modified with chemical ligands^{113,114}. In few
253 cases, the EV-secreting cells were genetically modified to express a reporter. For example, human
254 embryonic kidney 293T cells were engineered to express in their membrane a Gaussian luciferase
255 fused to a biotin receptor domain¹¹². The EVs could be monitored either *in vitro* or *in vivo* by
256 luminescence or fluorescence (by the interaction with fluorescent streptavidin). Although
257 fluorescence and luminescence imaging techniques are easy to operate and available in most
258 laboratories, they do not offer high sensitivity and absolute quantification. In contrast, the methods
259 that rely in PET/MRI or SPECT/computed tomography offer higher sensitivity and absolute
260 quantification while allowing the acquisition of images with anatomical details. In general, the
261 intravenous administration of labelled EVs (without any further modification besides the labelling)
262 isolated from different cell sources indicate that less than 10% of the injected EVs accumulate into
263 the non-injured heart^{54,112-115}. Yet, the accumulation of EVs in the heart is influenced by the delivery
264 route, concentration of EVs and the identity of the EV-secreting cell^{54,112-114}.

265

266 **4.2-Modulation of EVs bioactivity**

267 **4.2.1-EV-secreting cell modulation**

268 EV-secreting cells may be modulated by two different procedures: (i) by culturing them in
269 stress-induced conditions (e.g. hypoxia^{80,88,96}, serum starvation^{71,96}, inflammation¹¹⁶) and (ii) by

270 transfecting them with exogenous compounds, such as nucleic acids, especially miRNAs^{23,117,118},
271 miRNA antagonists¹¹⁹, Y-RNA⁸⁵, plasmid DNA^{77,78,120,121} and small molecules¹²¹ to enhance their
272 bioactivity (**Table 1**). For example, EVs collected from cardiac progenitor cells cultured under
273 hypoxia conditions increased the capacity of cardiac endothelial cells to form tube-like structures *in*
274 *vitro*, reduced the expression of pro-fibrotic genes in cardiac fibroblasts cultured *in vitro* and
275 improved function of the infarcted heart (in the acute phase, increased the fractional shortening from
276 30.6% to 36.4% and in the chronic phase increased from 27.6% to 34.2%) as compared to EVs
277 collected from cells cultured under normoxia conditions⁸⁸. The effect was mediated by several
278 miRNAs including miRNA-292, miRNA-210, miRNA-103, miRNA-17, miRNA-199a, miRNA-20a
279 and miRNA-15b. The modulation of EV-secreting cells might also be achieved by changing their
280 culture medium. For example, EVs collected from adipose-derived stem cells (ADSC) cultured in
281 endothelial differentiation medium showed an increase in miRNA-31 and the resulting EVs enhanced
282 endothelial cell migration, tube formation and aortic ring outgrowth compared to EVs collected from
283 ADSC grown in normal medium and thus not-enriched for miRNA-31¹²². Finally, as an example of
284 EVs collected from cells that were modulated by external agents, EVs collected from mesenchymal
285 stem cells transfected with miRNA-181a increased the pro-reparative state of peripheral blood
286 mononuclear cells and, upon administration in infarcted mice hearts, the miR-181a enriched EVs
287 increased EF (12% relatively to the baseline)²³.

288 Several cellular platforms have been developed for customizable enrichment of EVs with
289 specific proteins¹²³ and RNAs^{124,125} of interest; however, these platforms were not yet evaluated for
290 cardiovascular applications. In addition, very few studies took advantage of the cellular machinery to
291 engineer EVs with specific epitopes able to target the heart¹²⁶.

292

293 **4.2.2-Post-isolation methods**

294 Hijacking the cellular machinery to produce modulated EVs has the advantage of preserving
295 the biophysical properties of EVs relatively intact, but also has drawbacks, namely overexpressing a
296 given molecule in a cell may have unforeseen consequences for its biology, ultimately interfering
297 with EV biogenesis. Modulation strategies based on post-isolation modification of EVs may be an
298 alternative for effective control of EV loading, targeting, and delivery, regardless of their cell of
299 origin. However, post-isolation methods may mask or impair endogenous EV properties and
300 ultimately compromise EV bioactivity.

301 Membrane-permeabilizing strategies, such as electroporation (for both nucleic acids¹²⁷⁻¹²⁹ and
302 drugs¹³⁰), heat-shock or freeze-thaw procedures^{131,132}, detergent treatment¹³⁰ and sonication¹³³,
303 developed over the past decades to load cells with exogenous material, have been readily applied to
304 the EV field with a varied degree of success. Additional strategies exploited the hydrophobicity of
305 EV membranes in order to passively load compounds of interest into them^{134,135} and the modification
306 of the molecule of interest with cholesterol^{136,137}. Recent work focused on the use of well-defined
307 chemical formulations, some of which commercially available, designed with the specific purpose of
308 transfecting EVs directly¹³⁸⁻¹⁴⁰. Thus far, these studies reported loading efficiencies up to 70% but
309 the impact on the biophysical properties of EVs and the exact mechanism of action remains to be
310 elucidated.

311 Several EV formulations have been enriched using transfection agents or membrane-
312 permeabilizing strategies and evaluated in the context of cardiovascular applications to decrease
313 cardiac fibrosis, modulate the inflammatory response and to increase angiogenesis (Table 1). For
314 example, miRNA-21-5p has a critical role in the development of fibrosis after MI, regulating several
315 gene targets including SMAD family member 7 (Smad7), sprout RTK signalling antagonist 1 and
316 phosphatase and tensin homolog (PTEN)¹⁴¹. Human peripheral blood-derived EVs enriched for miR-
317 21 inhibitors reduced fibrosis in a mouse model of MI as compared to non-modified EVs. In a separate
318 example, EVs collected from cardiosphere-derived cells and enriched for miRNA-322 using

319 electroporation, reduced the infarcted area and fibrosis, and increased angiogenesis in a mouse model
320 of MI as compared to non-modified EVs¹⁴². Together, these studies demonstrate the possibility to
321 enrich EVs after their isolation and thus increase their bioactivity as compared to the non-modified
322 EVs.

323

324 **4.3-Modulation of EV biodistribution and targeting**

325 Upon systemic administration of EVs in animal models, they are quickly cleared or trapped
326 in the liver, spleen and lungs¹³⁴, with the EV half-life (minutes range), inherently dependent on the
327 identity of the EV-secreting cell¹⁴³⁻¹⁴⁵. EV biodistribution is influenced by multiple factors, including
328 the delivery route and dosage⁵⁴. It is possible that EVs retained in several organs induce a systemic
329 anti-inflammatory effect improving the regenerative capacity of the cardiovascular system, as already
330 observed with cell-based therapies⁴. However, studies clearly indicate that increased EV efficacy is
331 related with EV retention at the lesion area²¹. Therefore, in the last years, several strategies have been
332 developed to control EV biodistribution and targeting to specific organs and tissues. One strategy to
333 maximize the uptake is to increase EV stability in circulation and therefore, improve the likelihood
334 of an interaction between the EVs and the cells/tissues to be targeted. Modifying EVs with
335 polyethylene glycol (PEG), a strategy previously used for liposomes¹⁴⁶, enhanced their circulation
336 time⁴⁵ and reduced their uptake by nonspecific cells. Another strategy relies on the modification of
337 EV membrane with specific proteins¹²⁶ or peptides^{22,110,111,127,147-151} that are able to interact with
338 specific cellular receptors or extracellular matrix components expressed in the cardiovascular system
339 (Table 2). Although it is known that tetraspanins present in EV membranes have preferential binding
340 to specific cell lineages (e.g. Tspn8 binds to $\alpha 4$ and $\beta 4$ integrin chains that are expressed by
341 endothelial cells¹⁵²) these might not be enough for selective and effective organ or tissue targeting.

342 In the case of heart diseases, the intramyocardial administration of EVs has been reported in
343 pre-clinical studies; however, this route of administration is not always clinically desirable because

344 it involves a catheterization procedure, unless delivery is planned during cardiac surgeries¹⁵³. The
345 intravenous administration of EVs is a much simpler procedure and allows repeated applications;
346 however, it is more prone to off-targeted binding, increasing the potential for unwanted effects.
347 Moreover, the poor accumulation of EVs into the cardiac tissue (due to poor extravasation and lack
348 of efficient and specific epitopes for recognition) remains an important barrier. Engineered EVs can
349 eventually overcome these obstacles and deliver their therapeutic cargo into the injured heart. Two
350 approaches have been used to modify the surface of EVs for targeting the heart. In one, the EV-
351 secreting cell is genetically modified to express a peptide which is then incorporated in the membrane
352 of the secreted EVs¹⁴⁸⁻¹⁵⁰. For example, EV-secreting cells were genetically modified with a lentivirus
353 construct expressing a membrane protein (Lamp2b) fused with ischemic myocardium-targeting
354 peptide CSTSMLKAC¹⁴⁸. Although no absolute quantification was provided for the accumulation of
355 EVs in the heart, the fluorescence imaging results indicate a higher accumulation of the peptide-
356 modified EVs relatively to EVs without surface modification. As an alternative, many laboratories
357 adopted the surface modification of EVs by chemical approaches^{22,110}, mainly by following two
358 strategies: (i) physical incorporation of lipids modified with proteins (e.g. streptavidin)¹²⁶ or
359 peptides¹⁴⁷ into the membrane of the EVs and (ii) chemical incorporation of linkers to functional
360 groups (carboxylic¹¹¹ or amine^{22,110} groups) present at the surface of EVs to which peptides are
361 subsequently reacted by a copper-free click chemistry. These reactions can be performed in aqueous
362 solutions, are rapid, selective and very efficient as compared to conventional bioconjugation schemes.
363 In both strategies, the epitopes selected for targeting ischemic regions or a given cell type of interest
364 were: (i) a cyclic RGD peptide with high affinity to integrin $\alpha\beta3$ highly expressed in brain
365 endothelial cells after an ischemic event¹¹⁰, (ii) an ischemia-targeting peptide^{22,126,147,148} and (iii) a
366 cardiomyocyte-specific peptide¹⁴⁹. According to one study, introduction of approximately 263 copies
367 of the targeting peptide per exosome has been achieved¹¹⁰. Although in many reports, no absolute
368 quantification of EV accumulation was performed, the results showed that the peptide-modifications

369 can increase the EVs tropism to ischemic regions, including the brain¹¹⁰ or the heart^{22,147-150}, and
370 induce a higher therapeutic effect^{22,147}. For example, the EF of mice hearts after MI, treated with EVs
371 modified with a myocardium-targeting peptide was approximately 46% while in mice treated with
372 scramble peptide-modified EVs was approximately 38%²². Similar improvements were reported by
373 others^{147,148}. Both genetic modification of the EV-secreting cell and surface modification of EVs by
374 chemical approaches have pros and cons. The genetic approach may allow for a more standardized
375 product which is desirable to address regulatory expectations. However, this strategy has several
376 limitations including (i) changes in the biological activity of the EV as a consequence of the genetic
377 manipulation and (ii) difficulty to control the density of the targeting epitope in the surface of EVs as
378 well as to control their glycosylation state. The chemical approach may offer an effective control of
379 EV surface modification both in content (e.g. to include non-natural aminoacids to prevent peptide
380 degradation) and density (number of epitopes per surface area of EV) of the targeting epitope,
381 regardless of their cell of origin. The chemical approach may be performed during the purification
382 steps of EVs and thus amenable for clinical translation.

383

384 **4.4-Modulation of EV uptake and intracellular trafficking**

385 The internalization and intracellular trafficking of EVs can be studied using fluorescence
386 imaging techniques and labelled EVs^{154,155}. The cellular internalization of EVs seems to be influenced
387 by both the interaction between EVs and the cell membrane^{26,155} and the endocytic capacity of the
388 acceptor cell^{71,156}. Internalization of EVs may be mediated either by non-specific interactions,
389 particularly endocytic processes such as macropinocytosis and micropinocytosis⁵¹, or by specific
390 interactions, such as receptor-dependent pathways (in the case of peptide-modified EVs, see section
391 above). Little is known about the differences in the endocytic capacity of the cells, the impact of those
392 EV surface modifications in the intracellular trafficking and which EV surface modifications may
393 improve endolysosomal escape. Yet, these issues are critical because a large proportion of

394 internalized EVs are processed in the endolysosomal pathway and ultimately degraded in the
395 lysosome^{51,157}. Indeed, approximately 60% of internalized EVs colocalize with lysosomes after 48 h
396 of contact¹⁵⁵.

397 Strategies have been proposed to enhance the endolysosomal escape of EVs. In one case, EVs
398 have been coated with a combination of cationic lipids and pH-sensitive fusogenic peptides which
399 enhanced the disruption of the endolysosomal membrane leading to the efficient cytosolic release of
400 the EV cargo¹⁵⁸. In another case, EVs have been coated with arginine-rich cell-penetrating peptides
401 to induce active micropinocytosis and a more efficient release of EVs to the cell cytoplasm^{159,160}.

402

403 **4.5-Modulation of EV delivery**

404 Local administration of EVs at the injured site has been shown to increase the chance of cell
405 targeting and uptake by the cells of interest. In some cases, EVs are washed out or taken up by non-
406 relevant neighbouring cells. Interestingly, differential uptake of EVs by different cell types in an
407 ischemic hind limb has been reported⁶⁷. Local accumulation of EVs after few hours following
408 intramyocardial, intramuscular or topical administration in an infarcted pig heart²¹, an ischemic limb
409 tissue⁶⁷ or in a mouse wound healing model⁷¹, respectively, has also been demonstrated. Several
410 biomaterial-based strategies have been developed to engineer EV presentation by sustained the
411 release of EVs in the injured site including hydrogels based in hyaluronic acid^{71,161,162}, alginate^{163,164},
412 chitosan^{165,166}, collagen⁷² and amphiphilic peptides¹⁶⁷ (**Table 3**). Selection of hydrogel composition
413 took in consideration its biomedical history, degradation, in situ jellification profile, mechanical and
414 release properties. The EVs were incorporated in the hydrogels by several means. In the first case,
415 EVs were mixed with a polymer solution without involving the reaction of both
416 entities^{72,161,162,164,166,167}. The solution was then injected in the tissue of interest and physically or
417 chemically crosslinked in a few minutes retaining the EVs within the polymeric structure. In the
418 second case, the EVs were mixed with a polymer solution for formation of a polymer-EV conjugate

419 and initiate the chemical crosslinking process⁷¹. Then, the solution was administered in the tissue of
420 interest for further crosslinking and formation of a hydrogel. In the third case, the EVs were physically
421 incorporated in the hydrogel after its polymerization¹⁶⁸. In this case the hydrogel was already formed
422 and the pores were large enough to allow the diffusion of EVs within the polymeric structure. It has
423 been demonstrated that a 7 mm hydrogel patch could retain up to 3×10^{10} EVs and released the EVs
424 for more than 7 days after implantation in the heart⁷². The hydrogel-releasing EVs significantly
425 improved the activity of injured tissues relatively to EVs administered without a sustained release
426 system. For example, in two similar studies, the EF of infarcted rat hearts treated with a hydrogel
427 patch containing EVs was 40%⁷² and 25% higher than control¹⁶¹. Importantly, the kinetics of EV
428 release from the hydrogel seems to play an important role in their therapeutic effect⁷¹. For example,
429 the slow release of EVs from hydrogels implanted in skin mouse wounds was not as effective as the
430 coordinated release of EVs during skin regenerative process using remotely triggerable hydrogels⁷¹.

431

432 **5-Conclusions and perspectives**

433 In the last decade, significant progress was made in understanding the biology of EVs as well
434 as their application in the cardiovascular arena. We now have a better understanding of the
435 composition of EVs^{28,57}, the role of EVs in the communication between cells of the same or different
436 tissues in the body^{14,17,47,96} and how the content of EVs secreted by stem/progenitor cells or other
437 cardiac populations is affected by disease^{17,102}. Regarding EV applicability, substantial advances have
438 been made in the: (i) therapeutic effect of EVs in pre-clinical models of several cardiovascular
439 diseases such as MI^{21,84}, skin wound healing^{71,165}, hindlimb ischemia⁶⁷ and stroke¹¹⁰, (ii) how the
440 therapeutic effect of EVs compares to the one obtained from the transplantation of stem/progenitor
441 cells^{80,161} and (iii) how to enhance the therapeutic effect of EVs by increasing the stability and
442 targeting to a specific location, by enriching their therapeutic content, by improving their
443 internalization and intracellular trafficking and controlling their spatial and temporal release from

444 within biomaterials (**Fig. 3**). As consequence of these progresses, there are 2 observational and 2
445 interventional clinical trials actively running. In the observational clinical studies, changes in
446 epicardial fat-contained EVs in patients with atrial fibrillation (NCT03478410) and the miRNA
447 expression profile in peripheral blood exosomes of patients with MI (NCT04127591) will be
448 investigated. In the interventional clinical studies, the therapeutic effect of exosomes on cutaneous
449 wound healing (NCT02565264) and in patients with acute ischemic stroke (NCT03384433) will be
450 evaluated.

451 Further translation of EVs as potential therapies will require further advances in the
452 implementation of good practices related to EV separation and characterization, which implies the
453 use of guidelines to track and organize data on EV separation and characterization, reference materials
454 for normalization or calibration and inter-laboratory validation and reproducibility studies¹⁶⁹. Some
455 of these guidelines have been introduced in the last years by the scientific community^{7,170}. In addition,
456 further technological progresses will be needed to overcome the challenges related to the purification
457 and characterization of EVs^{28,57}. For further clinical translation of EVs in the cardiovascular area,
458 standard EV sources are needed to obtain EVs for therapeutic efficacy. EVs may be harvested from
459 autologous (i.e. donor biologic fluids or harvested cells) or exogenous sources (i.e. allogeneic
460 biological fluids or cell lines). Autologous EVs have the advantage of immuno-compatibility;
461 however, these EVs cannot be harvested on demand, may have reduced/unpredictable bioactivity
462 based on, for example, existing comorbidities and/or age of the donor and are much harder to
463 standardize as a clinical product. EVs produced from an exogenous source have the advantage of
464 being easier to standardize and store in larger quantities. Additionally, they may be standardized for
465 any given application and modified either by pre- or post-isolation modifications. However, for
466 clinical translation, further progresses are needed to optimize the administration route and the dosage
467 regimen. A clinical study in a human has shown that 10^{10} - 10^{11} EVs was an effective therapeutic
468 dosage to treat one patient with graft-versus-host disease, with multiple administrations of increasing

469 amounts¹⁷¹. This dose was estimated based on therapeutic dosages of transplanted MSCs for similar
470 purposes ($\sim 10^7$ - 10^8 MSCs); however, other studies indicate that clinical application of EVs may
471 require $> 10^{14}$ particles per dose¹⁷². High concentrations of EVs will require the use of bioreactors for
472 cell culture. For example, the culture of 10^8 MSCs for 2 weeks can generate approximately 10^{12} EV
473 particles¹⁷³.

474 Overall, targeting technologies that increase EV accumulation in the cardiovascular system
475 and thus decreasing the required dosage, and strategies to enrich EV content for specific biomolecules
476 may be the key to unlocking their use in the clinical setting. This may be ensured by coupling tracking
477 technologies, in order to mechanistically understand the biodistribution of EVs. Moreover, loading
478 of EVs with exogenous molecules and controlling their *in vivo* delivery kinetics opens a number of
479 opportunities to enhance EV bioactivity. Ultimately, engineered EVs represent a promising
480 translational cell-free, robust and customizable platform to improve the outcomes in cardiovascular
481 diseases.

482

483

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496

497 **7-Author contributions**

498 All authors contributed equally to all aspects of the article (researching data for article, substantial
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500

501 **8-Conflicts of Interest**

502 The authors declare no competing interests.

503

504 **9-References**

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879

880 **Glossary: Post isolation EV loading methods**

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882 **Electroporation** is a transfection method in which biological membranes are permeabilized upon
883 exposure to an electrical pulse.

884

885 **Heat-shock** is a transformation technique reliant on heat to induce membrane permeabilization.

886

887 **Sonication** is the use of ultrasound technology to physically disrupt biological membranes and
888 facilitate exogenous compound entry.

889

890 **Passive loading** is a strategy which relies on passive diffusion or complexation of a molecule with a
891 cell or organelle. It may rely on a number of factors such as pH, osmotic pressure, electric charge or
892 hydrophobicity.

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894 **Liposome fusion** is the conjugation of a lipidic material with a liposome formulation.

895 **Display Items**

896 **Box 1. Advantages and limitations of EVs in cardiovascular therapeutics**

897

898 The use of EVs in cardiac regeneration was preceded by extensive research in cell therapies. As non-
899 living entities, EVs overcame a major limitation of cell therapies, namely the poor survival after
900 implantation. Of note, methods that were used for cell engineering can now be efficiently used to
901 modulate EVs. Additionally, given the hostile environment of the injured myocardium, poor
902 engraftment hindered clinical success. While EVs must contend with the low infiltration and
903 endocytic rates of the heart, they are capable of overcoming the poor engraftment by being
904 internalized directly by the recipient cells. Furthermore, adverse immune responses are associated
905 with cell therapies, in particular when using allogeneic cells. This effect may be ameliorated, absent
906 or even beneficially modulated by EVs, depending on their source and/or modulation strategy. The
907 faster EV clearance, compared with their cellular counterparts, can be seen as a safeguard mechanism
908 whilst an effective targeting of EVs to the tissue of interest pave the way to their therapeutic success.
909 The limitations of EVs in cardiovascular therapeutics are related to the lack of tools for efficiently
910 target the injured myocardium which limits their clinical use. Production and standardization of
911 analytical parameters (i.e. storage, isolation and purification procedures) are some of the challenges
912 that the field needs to overcome in order to translate EV therapies from the bench to the bedside.
913 Since the physic-chemical properties of EVs are related with their cell/tissue/fluid of origin, the
914 heterogeneity present in the source will be reflected in EV composition. Thus, methods capable of
915 reducing the heterogeneity pre- as well as post-isolation are needed.

916 **Figure 1. Limitations and challenges in the use of native EVs as cardiovascular therapies.** While
917 EVs have been shown to elicit functional responses on target cells, their potential is limited in several
918 regards, including bioactivity, tracking, internalization and targeting. Therefore, several challenges
919 should be addressed to harness the full potential of EVs. **(a) Bioactivity.** EVs must elicit a bioactive
920 response in their target tissue/organ, which requires that EV cocktail of factors is potent enough to
921 have therapeutic value. Yet, native EVs are limited by the nature of their content being intrinsically
922 dependent on their donor cell features. **(b) Tracking.** The tracking of EVs is necessary to monitor
923 their *in vivo* biodistribution and targeting. Yet, without modifications, EVs are only detectable after
924 a biopsy is removed, limiting their monitorization. **(c) Targeting.** EVs should interact with specific
925 cells and deliver their content. Although native EVs have some tropism, depending on the epitopes
926 expressed by their donor cell, the process is limited. **(d) Internalization.** EVs should be taken up by
927 target cells and deliver their cargo in the cell cytoplasm. Yet, native EVs have reduced endolysosomal
928 escape and thus the ability to release their content in the cell cytoplasm.

929
930 **Figure 2. Modulation of EVs for cardiovascular therapies.** Several strategies have been used to
931 overcome limitations inherent to native EVs. These modifications can be categorized, from a
932 technical standpoint, depending on whether they were performed on the donor cell prior to EV
933 secretion, or after the purification of EVs from the medium or fluid of origin. From a biological
934 standpoint, these modifications may pertain to the membrane or lumen of the EVs. In the
935 cardiovascular context, several approaches have been used to modulate EV bioactivity, cell
936 internalization and targeting as well as to allow EV tracking. **(a) Bioactivity.** To enhance the
937 therapeutic potential of EVs, functionalization of the membrane and the lumen have been used. **(b)**
938 **Tracking.** To track *in vivo* EVs, these formulations have been labelled with fluorophores,
939 luminescence reporters or radiotracers. **(c) Targeting.** To enhance the targeting efficacy of EVs, they
940 have been modified with exogenous peptides, proteins or lipids. **(d) Internalization.** To enhance EV
941 internalization and endolysosomal escape, the vesicles have been modified with cationic lipids, pH-
942 sensitive peptides and cell-penetrating peptides.

943
944 **Figure 3. Examples of EV modulation strategies for cardiac therapies.** Both native or modulated
945 EVs have been used as cardiac therapies. The modulated EVs were enriched in therapeutically
946 relevant compounds such as miRNAs, proteins and small molecules, but also were decorated with
947 surface epitopes that improved their cardiac targeting and pharmacokinetics. EVs have been
948 administered in animal models either locally or systemically. In the local administration, biomaterial-
949 based delivery systems have been used to control the release of EVs, as well as to act as a supporting
950 scaffold for tissue regeneration. Therapies based in native or modulated EVs have translated into
951 improved cardiac function mediated by a decrease in inflammation, cardiomyocyte death, fibrosis
952 and infarct size, and increased angiogenesis.

953

Table 1. Modulation of EV bioactivity for cardiovascular applications.

Engineering	Source	EV type	Methodology	Animal model	<i>In vivo</i> outcome	Ref.
Modulation of EVs bioactivity: EV-secreting cell modulation	hAFSs	sEVs	Hypoxic preconditioning	Mouse MI	Improved cardiac repair	174
	hMSCs	sEVs + MVs	Hypoxic preconditioning	Rat MI	Improved angiogenesis and cardiac function and reduced infarct size	80
	rCPCs	sEVs	Hypoxic preconditioning	Rat MI	Improved cardiac function and reduced infarct size	88
	rCM	sEVs	Hypoxic preconditioning	Mouse MI	Improved angiogenesis	96
	hMNCs	sEVs	Hypoxic preconditioning	Mouse diabetic wounds	Improved angiogenesis and healing	71
	hASCs	MVs	Endothelial medium conditioning	Mouse subcutaneous Matrigel plug	Improved angiogenesis	122
	rMSCs	sEVs	GATA-4 overexpression	Rat MI	Restored cardiac contractile function and reduced infarct size	77
	hMSCs	sEVs	Akt overexpression	Chick allantoic membrane	Improved angiogenesis	78
	MsCs	sEVs	Fluorescent miR-181a overexpression	Mouse MI	Reduced infarct size, improved function, reduced inflammation	23
	hHEK293T cells	EVs	miRNA-146a overexpression	NA	NA	117
	hCDCs	sEVs	Y RNA overexpression	Rat MI	Reduced infarct size and CM apoptosis, reduced inflammation	85
Modulation of EVs bioactivity: post-isolation methods	hBlood	sEVs	EV loading with microRNA: ExoFect	Mouse MI	Reduced fibrosis	141
	mCPCs	sEVs	EV loading with microRNA: Electroporation	Mouse MI	Improved angiogenesis	142
	hMSCs	sEVs	EV loading with microRNA: Electroporation	Rat MI	Reduced fibrosis and improved cardiac function	118

955 AFCs (human amniotic fluid stem cells), ASCs (Adipose-derived stem cells), CSCs (Cardiac stromal cells), UMSCs
956 (umbilical mesenchymal stem cells), MSCs (Mesenchymal stem cells), MsCs (mesenchymal stromal cells), CDCs
957 (Cardiosphere-derived cells), CPCs (Cardiac progenitor cells), hMNCs (human mononuclear cells) and CM
958 (cardiomyocytes). “h” stands for human, “r” for rat and “m” for mouse.

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Table 2. Modulation of EV biodistribution and targeting.

Engineering	Source	EV type	Methodology	Model	Outcome	Ref.
Chemical modification of EV membrane with peptides/proteins	mMSCs	sEVs	Membrane modification with peptide (chemical conjugation)	Middle cerebral artery occlusion mice model	Suppression of the inflammatory response and cellular apoptosis in the lesion region	110
	mMSCs	sEVs	Membrane modification with peptide (chemical conjugation)	Mouse MI	Reduction of fibrosis and CM apoptosis and improvement of cardiac function	22
	hCDCs	sEVs	Membrane modification with peptide (chemical conjugation)	Rat MI	Reduction of fibrosis and CM apoptosis and improvement of cardiac function	147
	hCDCs	sEVs	Membrane modification with modified lipid	Rat MI	Improved targeting to the heart	126
EV-secreting cell is genetically or chemically modified to express a peptide/protein	mMSCs	sEVs	Membrane modification with peptide (genetic approach)	Mouse MI	Suppression of the inflammatory response, improved angiogenesis, reduction of myocardial apoptosis, improvement of cardiac function	148
	hHEK-293T	sEVs	Membrane modification with peptide (genetic approach)	Mouse MI	Improved targeting to the heart	150
	hCDCs	sEVs	Membrane modification with peptide (genetic approach)	Mouse MI	Improved targeting to the heart	149
	h562 cells	sEVs	Membrane modification with modified lipid	Vascular targeting in zebrafish	Improved targeting to the vasculature	151

969 MSCs (Mesenchymal stem cells), CDCs (Cardiosphere-derived cells), CPCs (Cardiac progenitor cells), hMNCs (human
970 mononuclear cells) and CM (cardiomyocytes). “h” stands for human, “r” for rat and “m” for mouse.
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981 **Table 3. Modulation of EV delivery.**

Source	EV type	Methodology	Model	Outcome	Ref.
rEPCs	sEVs	Injectable hyaluronic acid-based hydrogel	Rat MI	Improvement in angiogenesis and cardiac function, decrease in infarct size	110
rEPCs	sEVs	Injectable hyaluronic acid-based hydrogel	Rat MI	Improvement in hemodynamics, cardiac function and decrease in infarct size	162
hiPSC-derived CMs	sEVs	Encapsulation of EVs in collagen gel followed by its transplantation	Rat MI	Improvement in cardiac function, decrease in CM apoptosis, reduction in infarct size and cell hypertrophy	72
hMSCs	sEVs	Injectable peptide amphiphile hydrogel	Rat MI	Improvement in cardiac function, reducing inflammation, fibrosis and apoptosis, enhancement of angiogenesis	167
rMSCs	sEVs	Injectable alginate gel	Rat MI	Decrease in cardiac cell apoptosis, improvement in angiogenesis, improved cardiac function	164
hPRP	sEVs	Encapsulation of EVs in alginate gel followed by its transplantation	Skin wound healing diabetic rat model	Improved angiogenesis and re-epithelization	163
hMSC	sEVs	Encapsulation of EVs in chitosan gel followed by its transplantation	Skin wound healing diabetic rat model	Improved angiogenesis and re-epithelization	165
hMNCs	sEVs	Injectable hyaluronic acid-based hydrogel	Skin wound healing diabetic mouse model	Improved angiogenesis and re-epithelization	71
hMSCs	sEVs	Injectable chitosan-based gel	Mouse hindlimb ischemia model	Increased angiogenesis in ischemic hindlimbs and high limb salvage	166

982 MSCs (Mesenchymal stem cells), hMNs (human mononuclear cells), EPC (endothelial progenitor cell) and PRP
 983 (human platelet-rich plasma). “h” stands for human, “r” for rat and “m” for mouse.

984