Methods for the identification and characterization of extracellular vesicles in cardiovascular studiesfrom exosomes to microvesicles

Sean Davidson, Chantal Boulanger, Elena Aikawa, Lina Badimon, Lucio Barile, Christoph Binder, Alain Brisson, Edit Buzas, Costanza Emanueli, Felix Jansen, et al.

► To cite this version:

Sean Davidson, Chantal Boulanger, Elena Aikawa, Lina Badimon, Lucio Barile, et al.. Methods for the identification and characterization of extracellular vesicles in cardiovascular studiesfrom exosomes to microvesicles. Cardiovascular Research, In press, 1149 (1), pp.45-63. 10.1093/cvr/cvac031 . inserm-03620308

HAL Id: inserm-03620308

https://inserm.hal.science/inserm-03620308

Submitted on 25 Mar 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

3 **Authors:** Sean M Davidson[1], Chantal M. Boulanger[2#], Elena Aikawa[3], Lina Badimon[4], Lucio
4 Barile[5], Christoph J. Binder[6], Alain Brisson[7], Edit Buzas[8], Costanza Emanueli[9], Felix
5 Jansen[10], Miroslava Katsur[1], Romaric Lacroix[11,12], Sai Kiang Lim[13,14], Nigel Mackman[15],
6 Manuel Mayr[16], Philippe Menasché[17,18], Rienk Nieuwland[19,20] Susmita Sahoo[21], Kaloyan
7 Takov[16], Thomas Thum[22,23], Pieter Vader[2,24], Marca H.M. Wauben[25], Kenneth Witwer[26],
8 Joost P.G. Sluijter[18]
9
10 [#]corresponding author

11
12 Author addresses:
13 [1] The Hatter Cardiovascular Institute, University College London WC1E 6HX, United
14 Kingdom
15 [2] PARCC, INSERM, University of Paris, Paris, France
16 [3] Center for Excellence in Vascular Biology, Department of Medicine, Brigham and Women's
17 Hospital, Harvard Medical School, Boston, MA 02115, USA
18 [4] Cardiovascular Science Program-ICCC, IR-Hospital de la Santa Creu i Santa Pau-
19 IIBSantPau, CiberCV, Autonomous University of Barcelona, Barcelona
20 [5] Laboratory for Cardiovascular Theranostics, Istituto Cardiocentro Ticino, Ente Ospedaliero
21 Cantonale and Faculty of Biomedical Sciences, Università Svizzera italiana, 6900, Lugano,
22 Switzerland
23 [6] Department of Laboratory Medicine, Medical University of Vienna, Vienna, Austria
24 [7] Molecular Imaging and NanoBioTechnology, UMR-5248-CBMN, CNRS-University of
25 Bordeaux-IPB, Bat. B14, Allée Geoffroy Saint-Hilaire, 33600, Pessac, France
26 [8] Department of Genetics, Cell- and Immunobiology, Semmelweis University, HCEMM-SU
27 and ELKH-SE Immune Proteogenomics Extracellular Vesicle Research Group, Budapest,
28 Hungary
29 [9] National Heart and Lung Institute, Imperial College London, Hammersmith Campus,
30 London, W12 0NN England, United Kingdom
31 [10] Heart Center, Department of Internal Medicine II, University Hospital Bonn, Germany
32 [11] Aix Marseille University, INSERM 1263, Institut National de Recherche pour l'Agriculture,
33 l'Alimentation et l'Environnement (INRAE), Centre de Recherche en CardioVasculaire et
34 Nutrition (C2VN), Marseille, France
35 [12] Haematology and Vascular biology department, CHU La Conception, APHM, Marseille,
36 France
37 [13] Institute of Medical Biology and Institute of Molecular and Cell Biology, Agency for
38 Science, Technology and Research, Singapore, Singapore
39 [14] Department of Surgery, Yong Loo Lin School of Medicine, National University of
40 Singapore, Singapore, Singapore.
41 [15] UNC Blood Research Center, Department of Medicine, University of North Carolina at
42 Chapel Hill, Chapel Hill NC
43 [16] King's College London British Heart Foundation Centre, School of Cardiovascular
44 Medicine and Sciences, London, UK
45 [17] Department of Cardiovascular Surgery, Hôpital Européen Georges Pompidou, Paris,
46 France.
47 [18] Laboratory of Experimental Cardiology, Cardiology, UMC Utrecht Regenerative Medicine
48 Center and Circulatory Health Laboratory, Utrecht University, University Medical Center
49 Utrecht, Utrecht, The Netherlands
50 [19] Vesicle Observation Center, Amsterdam UMC, University of Amsterdam, Amsterdam, The
51 Netherlands
52 [20] Laboratory of Experimental Clinical Chemistry, Amsterdam UMC, University of
53 Amsterdam, Amsterdam, The Netherlands

54 [21] Cardiovascular Research Institute, Icahn School of Medicine at Mount Sinai, New York,
55 NY, USA
56 [22] Institute of Molecular and Translational Therapeutic Strategies, Hannover Medical School,
57 Hannover, Germany
58 [23] Fraunhofer Institute of Toxicology and Experimental Medicine, Hannover, Germany
59 [24] CDL Research, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht,
60 The Netherlands.
61 [25] Utrecht University, Faculty of Veterinary Medicine, Department of Biomolecular Health
62 Sciences, Yalelaan 2, Utrecht, The Netherlands
63 [26] Departments of Molecular and Comparative Pathobiology and Neurology, Johns Hopkins
64 University School of Medicine, Baltimore, US
65
66
67 **Corresponding author:**
68 Chantal M. Boulanger, PhD
69 Paris Cardiovascular Research Center
70 56 rue Leblanc
71 75015 Paris, France
72 Tel +331 5398 8086
73 Chantal.boulanger@inserm.fr

74
75 **Manuscript type:** Original article
76 **Short title**: Methods for studying extracellular vesicles
77 **Words**: 9,321 (18,619 with references)
78 **Tables:** 4
79 **Figures**: 3
80 **Box**: 1
81
82

83 **Abstract (179 of 200 words)**

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

97

98 **1. Pathophysiological relevance of EVs in the cardiovascular field**

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

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

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

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

139 Currently, there are more than 250 clinical trials registered to use EVs in a range of diseases
140 (ClinicalTrials.gov), as either biomarkers for response to drug treatment or as direct therapeutic
141 mediators. It is therefore crucial that appropriate methods are used to separate, validate and
142 characterize EVs, both to improve their clinical application, and to provide fundamental insights and

143 in-depth analyses of their mechanism of action. The aim of this document is to provide guidance on
144 these critical methodological issues and highlight key points for consideration in the design of
145 experiments using EVs. Some of the methods described can be applied generally to all studies using
146 EVs, but we provide CV-specific methods where relevant.

147 *1.1 Definition of extracellular vesicles and use of terminology*

148 Three main classes of EVs can be distinguished by their mechanism of production: exosomes,
149 microvesicles and apoptotic bodies (**Figure 1**). Microvesicles and apoptotic bodies are released
150 directly via outward budding of the plasma membrane in living or dying cells, respectively, and carry
151 proteins, lipids, nucleic acids and other active components that can affect target cells and modify
152 their behaviour.[4, 5, 31] Exosomes are produced by inward budding of late stage endosomes, thereby
153 forming intraluminal vesicles in multivesicular bodies (MVBs), which are released upon fusion of the
154 limiting membrane of the MVB with the cell membrane.[32] The formation of MVBs and subsequent
155 fusion with the plasma membrane is a highly orchestrated mechanism involving the Endosomal
156 Sorting Complexes Required For Transport (ESCRT) machinery, which includes the proteins
157 Hepatocyte Growth Factor-Regulated Tyrosine Kinase Substrate (HRS), Tumour Susceptibility Gene
158 101 Protein (TSG101), Signal Transducing Adapter Molecule 1 (STAM1) and Programmed Cell Death
159 6-Interacting Protein (PDCD6IP or ALIX), although ESCRT-independent mechanisms have also been
160 reported.[32] Precisely how cargo is sorted into exosomes is unclear, although some binding motifs
161 have been suggested.[32]

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

176 ## 2. Source of EVs

177 For investigations of cardiovascular EV function, primary cells, blood or explanted cardiac tissue may
178 be preferred. When the aim is to develop EVs as therapeutic agents, and large quantities are
179 required, readily expandable cells or cell lines may be preferable. Mesenchymal Stromal Cells (MSC)
180 are a popular source as they are cytoprotective, can improve cardiac contractility and calcium
181 handling and have beneficial immunomodulatory effects including in the setting of atherosclerosis
182 and pulmonary hypertension.[14, 33-35]

183 EVs from many different sources have been shown to improve cardiac function following MI,
184 including cardiac stem cells,[36] cardiovascular progenitor cells (CPC),[37], endothelial progenitor cells,[38]
185 cardiosphere-derived cells,[39] embryonic stem cells[40] and iPSC-derived cardiomyocytes[41](reviewed in
186 [5]). EVs from the epicardium can promote proliferation of cardiomyocytes.[42] EVs can also be

187 beneficial against other forms of injury such as doxorubicin/trastuzumab-induced cardiac toxicity.[26]
188 On the other hand, EVs can be detrimental, for example contributing to vascular smooth muscle cell
189 calcification.[15, 16] As yet, there is little consensus on the ideal source of EVs, however one head-to-
190 head comparison suggests CPC may be more efficacious than BM-MSC.[25]

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

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

200

201 3. Methods of separation

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

203 *3.1 Separation of EVs from cell culture medium*

204 Several techniques have been developed for the separation of EVs from cell culture medium, each
205 with their advantages and disadvantages (**Table 1**). Most procedures are based on separation by
206 size, and/or density, although many other extracellular particles may share these characteristics with
207 EVs. A protocol of differential centrifugation or ultracentrifugation published by Thery *et al*. is
208 commonly used to separate both sEVs and lEVs (**Box 1**).[54] A subsequent density-gradient separation
209 using sucrose or, preferably, iodixanol, further improves EV purity.[55] Size-exclusion chromatography
210 has become popular since it effectively removes part of the contaminating soluble protein, and
211 columns can be readily made or purchased (**Figure 2D**).[56, 57] Precipitation of sEVs is possible using
212 polyethylene glycol (PEG)-based reagents, for example in HEK293 or MSC cultures,[58] but the purity
213 obtained is generally inferior to other techniques.[55, 59] Ultrafiltration is more commonly used as an
214 initial clean-up step to remove larger (e.g.: >0.8 μM) contaminants because membranes can become
215 blocked when filtering large volumes and because of concerns that high pressures may damage the
216 membranes of larger EVs. Affinity isolation, typically using antibodies, provides highly pure isolates
217 although at the expense of yield, and only a subset of EVs might be isolated.[60] Furthermore, the
218 procedure to recover EVs from antibodies could affect their functionality and requires testing.[61]
219 Diafiltration, asymmetric flow field-flow fractionation (AF4)[62] and tangential flow filtration[63] purify
220 and concentrate sEV fractions and are scalable, but AF4 requires specialized and expensive
221 equipment.

222 Several head-to-head comparisons of EV separation procedures have been published[55, 59, 64, 65], for
223 human plasma, urine and also specific cardiac-derived progenitor cells, but ultimately, the optimal
224 method and obtained quantity depends on the source of the biofluid, the amount of available
225 biofluid and the intended use. For clinical analyses of thousands of blood samples for EV-associated
226 biomarkers, rapid precipitation might be sufficient but for mechanistic studies, purer EVs is essential.
227 The use of cell culture medium as a source of EVs allows for more rigorously controlled conditions
228 for EV production, but the cell culture environment differs from *in vivo* physiology. Given the
229 challenge of removing contaminating serum EVs, protein and lipoproteins, when highly pure EVs are
230 required for 'omics analysis or functional investigation, it is advisable to harvest EVs from cells grown

in chemically defined medium rather than EV depleted serum or serum-replacement supplements. However, control experiments must be in place to assess cell viability and contents of contaminating apoptotic bodies, when removing serum. EV-depleted serum may be used but still contains large quantities of proteins and lipoproteins which can co-isolate with EVs / are common contaminants of EVs and procedural controls are necessary to check for potential contaminant.[66]

3.2 Separation of EVs from blood

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

3.3 Separation of EVs from tissue

The isolation of EVs from tissues has considerable scientific interest for understanding their local and remote roles in cardiovascular disease development. Their presence should first be confirmed *in situ*, e.g. electron microscopy can identify the presence of vesicle structures in pathological samples such as human atherosclerotic plaques, ischaemic heart and muscles, or the brain[74, 75]. EV separation from fresh tissues represents a challenging task as the method used should ensure that isolated vesicles come from the extracellular space and do not result from tissue homogenization (cell death, membrane self-assembly; **Table 2**). Gentle mechanical disruption of tissue, optionally followed by enzymatic treatment, can be used to release EVs.[43] EVs have been released by collagenase perfusion of Langendorff-perfused rat hearts followed by differential centrifugation.[76, 77] Appropriate controls should be considered to estimate the effects of the procedure. Therefore, using tissues from genetically modified models and processing healthy tissues or tissues from sham animal models in parallel to pathological samples might help evaluate the direct effect of tissue homogenization.[49, 74, 78] Furthermore, the effect of the enzymatic cocktail on EV numbers and protein expression also requires investigation.[76]

4. General principles for EV identification and characterization

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

First, it is important to quantify the number of EVs relative to the total lipid or protein content of EV preparations obtained. The yield of EVs should be measured relative to the amount of starting

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

284 *4.1 Techniques for identifying EVs*

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

297 Since one of the defining features of exosomes is their size, this is another informative parameter to
298 report when separating small EVs, although this is not specifically recommended in the MISEV2018
299 guidelines. The size distribution of EVs can be obtained using NTA or RPS, calculated from electron
300 microscope images, or using another technique. A second defining feature of MVB-derived
301 exosomes is that they contain proteins involved in MVB formation and/or exosome release (e.g.:
302 CD9, CD63, CD81, Alix/PDCD6IP, TSG101).[60] These can be used as positive protein markers to
303 indicate the enrichment of MVB-derived exosomes within the separated EVs. The presence of at
304 least 3 markers should be demonstrated.[31, 60] Notably, acetylcholinesterase is no longer considered a
305 generic marker of exosomes.[82]

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

308 *4.2 Electron microscopy*

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

319 **2C**).[83] Other techniques, including single EV-microarray and atomic force microscopy can provide
320 images of single EVs, as well as information on their biomechanical properties and size.[84]

321 *4.3 Flow cytometry*

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

338 *4.4 Functional analysis of EVs*

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

352 *4.5 Reporting methodology*

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

357

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

359 *5.1 Total protein content*

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

366 *5.2 Antibody-based techniques to identify specific proteins*

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

374 Western blotting can identify proteins that are associated or co-isolated with EVs and provide useful
375 information about the yield and purity of an EV preparation.[64] Importantly, it can also confirm the
376 molecular weight of the target protein. Compared with cell lysates, a disadvantage of EV samples is
377 the lack of reference ("house-keeping") proteins to use for normalisation purposes in
378 immunoblotting experiments. Therefore, equal protein amount, volume from which EVs are
379 separated or particle number are commonly used. Inclusion of the original sample, the EV-depleted
380 sample and procedural control samples are required to draw firm conclusions about enrichment of
381 proteins in the EV isolate (or depletion of contaminants). Western blotting can be challenging since it
382 requires relatively large quantities of EVs for sufficient sensitivity. Alternative versions such as dot
383 blotting or capillary electrophoresis immunoassays can provide considerably higher sensitivity.[100]

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

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

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

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

403 *5.3 Mass spectrometry of the EV proteome*

404 Proteomic analysis of EV samples by mass spectrometry (MS) provides the most comprehensive
405 analysis of the EV protein cargo (**Table 3**), and does not rely on an *a priori* selection of proteins based
406 on the availability of antibodies or other affinity reagents for specific proteins.[60, 104] MS approaches,
407 however, have an inherently lower sensitivity compared with antibody-based techniques. This is
408 mainly due to the excess amounts of highly abundant proteins (*e.g.:* albumin) in the EV preparations
409 which mask the presence of low-abundant EV proteins.[105] To address this, MS can be combined with
410 better isolation techniques for EVs that result in less contamination. It is recommended to compare
411 the EV proteome to tissue or cell source of the EV sample to identify the degree of
412 enrichment/depletion of proteins. For EVs separated from cell cultures in which media are
413 supplemented with xenogenous components (*e.g.* bovine serum), it is also recommended to
414 searches against databases of other organisms. Bovine serum proteins are a common contaminant
415 in EVs isolated from cell cultures, unless cells are grown in serum free media. Finally, independent
416 validation with an antibody-based technique is advisable since MS detects peptides, which can
417 originate from both intact and fragmented proteins. Most journals require that EV proteomic data
418 are deposited in online databases.[106]

419 *5.4 Intraluminal vs membrane proteins*

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

430

431 ## 6. <u>Methods for determining the RNA content of EVs</u>

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

440 *6.1 RNA analyses by qRT-PCR and RNA-sequencing*

441 At first, RNA cargo of EVs was based solely on the use of Taqman miR-PCRs focused on individual
442 miRNAs, and it was a challenge finding ways to normalize data. Data normalization was usually
443 implemented by spiking-in an exogenous miRNA supposedly not expressed in mammalian species,
444 such as *Caenorhabditis elegans* miRNA-39 (Cel-39) before RNA extraction. More recently, several

445 quantitative PCR (qRT-PCR) and digital PCR protocols are available to detect the miRNA cargo of
446 EVs.[114]

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

451 Certain pre-analytic confounders are well known, e.g.: heparin can interfere with PCR analyses of
452 RNAs,[118] but can be overcome by heparinase treatment. The presence of certain miRNAs is
453 suggestive of haemolysis of blood samples (e.g.: miR-486-5p, miR-451, miR-92a, and miR-16), or
454 presence of contaminating calf serum (e.g.: miR-122, miR-451a and miR-1246).[119-121] Lipoprotein
455 contamination can also create difficulties in data analyses and interpretation since they can also
456 carry miRNAs[122]. To prevent contamination of EV preparation by RNAs carried by lipoproteins and
457 extra-EV Argonaute proteins, the use of proteinase K and RNase A digestion can be implemented
458 before proceeding to RNA extraction.[112] It is useful to include a negative control without enzymatic
459 treatment and positive control samples containing RNA, to confirm complete digestion of non-
460 exosomal RNAs.

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

467 *6.2 How to evaluate the functional role of EV RNA*

468 Despite the numerous examples of studies suggesting important roles of EV-mediated RNA transfer
469 on target cell behavior, e.g. the regenerative potential of epicardium-derived extracellular vesicles
470 mediated by conserved miRNA transfer, assessing the true (patho-)physiological role of such transfer
471 is a formidable challenge, not least because of the relatively low EV RNA concentrations. For
472 investigations into general mechanisms underlying EV-mediated RNA transfer, sensitive reporter
473 systems have been developed that allow the study of EV-RNA transfer at the single cell level.[124, 125]
474 However, to prove a direct effect of endogenous RNA species on EV target cells, additional
475 challenges need to be addressed and important control experiments are required. These include
476 demonstrating that the RNA of interest: 1) full length is present inside EVs; 2) shows increased levels
477 in recipient cells upon delivery (in the absence of upregulated expression); and 3) directly mediates a
478 particular response in target cells, by interfering with its presence or function without affecting the
479 content of EVs or recipient cells in any other way. Recently published reporting guidelines on EV-
480 RNA studies should help to ensure reproducibility and to critically evaluate past and future studies
481 claiming EV-RNA-induced physiological and pathological responses.[112]
482

483 **7. Methods for determining EV lipid content**

484 *7.1 Lipid content*

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

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

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

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

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

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

523

524 8. Measurement of enzymatic activities carried by EVs

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

531 *8.1 Pro-coagulant activity*

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

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

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

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

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

558 *8.2 Fibrinolytic activity*

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

565 *8.3 Enzymatic activities*

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

574

575 ## 9. Methodologies for functional characterization of EVs

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

580 i. In addition to EV purification and isolation, "EV-depleted" samples and quality and procedural
581 controls (e.g.: unconditioned cell-culture medium processed in the same way) can help to
582 determine true EV-mediated responses. GW4869, an inhibitor of neutral sphingomyelinase 2
583 (nSMase2) and sEV release, is sometimes used as a control, but care is required in its use, as it is
584 unlikely to be specific for exosome release.[31, 154]
585 ii. Co-purified and bound molecules might affect functional assays,[155] therefore it is best to avoid
586 low-specificity methods such as general precipitation (polyethylene glycol, "salting out," the
587 basis of many commercial "exosome isolation" kits), unless these methods are combined with
588 additional separation steps.
589 iii. The biological nature of EV preparations makes normalization between conditions essential but
590 there is no clear consensus on the best way forward. Some alternatives include: starting volume
591 or the number of producing cells; total number of EVs; protein content; lipid content; metabolite
592 content; or specific markers such as levels of tetraspanins or other putative house-keeping
593 proteins or RNA species.[156] It is recommended to have 2-3 different approaches, and to clearly
594 describe each, to allow potential differences in functional outcomes to be explored.
595 iv. For clinical therapeutic interventions, the identity of the EV preparations can be defined using
596 quantifiable metrics.[79]
597 ~~v.~~ In classical dose-response experiments, the relationship between the concentration of a
598 ligand/drug and a measured outcome parameter is investigated. Such experiments should be
599 considered to understand the dose-dependency of effects, and to understand the biological
600 relevance of the quantity of EVs used. In many published works, the dose relative to
601 physiological concentration is unclear.
602 vi. Profiling of the EVs proteome and RNAome also will help to characterize their origin and also
603 potential functional activities.[157]

604 ### *9.1 Uptake and biodistribution studies*

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

612 It is challenging to document the *in vivo* biodistribution of EVs. Many studies first isolate and tag EVs
613 before injecting them *in vivo*, but these exogenous EVs may not reflect the same fate as
614 endogenously released EVs. In addition, the presence of residual contaminants from the isolation
615 procedure, the route of administration, the type of label used, the animal model and the detection
616 method may all affect *in vivo* biodistribution. If fluorescent dyes are used for EV labelling they should

617 be carefully selected. Many dyes, particularly lipophilic dyes, can form dye aggregates or micelles
618 that are of similar size to EVs, or may bind to contaminants present in the isolate, such as
619 lipoproteins and certain proteins.[161] Furthermore, lipophilic dyes might dissociate from the labelled
620 EV and be incorporate into cellular membranes *in vivo*, where long dye half-life may lead to incorrect
621 assumptions about EV distribution and longevity and diffuse freely. Genetic approaches crossing
622 ROSAmTmG mice with models expressing Cre-recombinase in a cell-specific manner have opened
623 new avenues for quantifying uncommon populations of EV, such as cardiomyocyte-derived EVs in
624 the circulation.[162] On the other hand, protein-based labels added using genetic approaches (e.g. GFP)
625 can be susceptible to proteolysis and cannot be used on samples derived from human tissues and
626 fluids. Therefore, careful control experiments are required to ensure the signal is specific and to
627 monitor the influence of any free dye. Cell-cell interaction studies and paracrine activity of secreted
628 exosomes can be studied by e.g. co-culture assays of different cell types. Some examples are
629 reported where (direct) EV-cargo loading is used to detect EV-molecule transfer, but indirect effects
630 and reduced EV functionality are examples of possible limitations of these methods.[163] Possible
631 controls include comparison with the biodistribution of free-label (no EVs) or of EVs that have been
632 physically disrupted.[164]

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

640 In conclusion, all current approaches to assess EV *in vivo* biodistribution (see **Table 4** for examples)
641 have their strengths and limitations, which must be carefully considered when designing
642 experiments.
643

644 10. Methodologies for clinical use of EVs in cardiovascular diseases

645 Potential regenerative/reparative effects of EVs in the cardiovascular system have been observed in
646 both post-infarction, and non-ischaemic chemotherapy-induced cardiomyopathy models.[1, 23, 26, 37, 39,
647 42, 167] Although EV biodistribution and direct cellular uptake still needs much attention, preclinical
648 meta-analyses indicate that stem cell-derived EV administration is associated with improvements of
649 left ventricular ejection fraction, fractional shortening and a reduction of infarct size. These benefits
650 are seen largely irrespective of the type of stem cell, timing of injection, route of delivery, dosage of
651 delivery or follow-up period.[168, 169] On the other hand, not unique to EV studies, there is a potential
652 risk of positive publication bias.[168, 169] While these positive data suggest that clinical studies may be
653 warranted, there are a number of important issues to address including those related to upscaling of
654 EV preparation processes in GMP-quality facilities using non-xenogeneic culture conditions, as well
655 as ethical and regulatory approvals.[5] Even with optimization of EV separation and characterization,
656 several practical hurdles must be overcome to maximize the therapeutic potential of EVs. In addition
657 to regenerative potential, however, EVs can play detrimental roles, for example potentially by
658 causing thrombotic complications or forming microcalcifications that destabilize atherosclerotic
659 plaques.[170] The therapies preventing this deteriorating effect are under investigations.

660 *10.1 Production and storage effects on the quality of EV preparations*

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

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

675 *10.2 Delivery strategies and biodistribution of EVs*

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

693 *10.3 Loading therapeutics into EVs*

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

701 EVs have been modified to deliver small molecules, therapeutic RNA, proteins, lipids and different
702 types of imaging molecules.[183 184] Materials can be loaded into EVs via both passive loading (e.g.
703 incubation with EVs or with EV-producing cells) or active loading (e.g. sonication, membrane
704 permeabilization, electroporation, antibody binding of EVs or transfection of EV-producing cells). EVs

705 can be labelled on the surface or intraluminally.[164] However, the labelling and loading procedure
706 may alter physical, chemical and therapeutic properties of EVs or EV-mimetics. Moreover,
707 therapeutic loading might be overestimated as observed for electroporation procedures that cause
708 siRNA aggregate formation in the EV preparation.[185] Therefore, a thorough *in vitro* and *in vivo*
709 evaluation of their uptake, stability, efficacy and toxicity is necessary to develop suitable methods
710 for future clinical studies. Recent research suggests that EVs of various sizes can naturally carry
711 intact viruses used in therapeutics such as adeno-associated viruses (AAVs), (reviewed in [181],[157] and
712 may thereby be able to circumvent antibody neutralization.

713

714 **11. Conclusion**

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

731 **12. Tables**

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

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

733

734

735

736

737

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

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

740 The importance of these points will vary depending on the intended use of the EVs, and must be
741 evaluated separately for each experiment.
742 [a]As noted in the main text, these solutions can introduce problems of their own. e.g. EV removal
743 from serum also removes other components, and it is probably not possible to remove 100% of the
744 EVs. Serum-free medium may negatively affect cell health and EV quality.
745

746

747

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

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

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

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

755

756

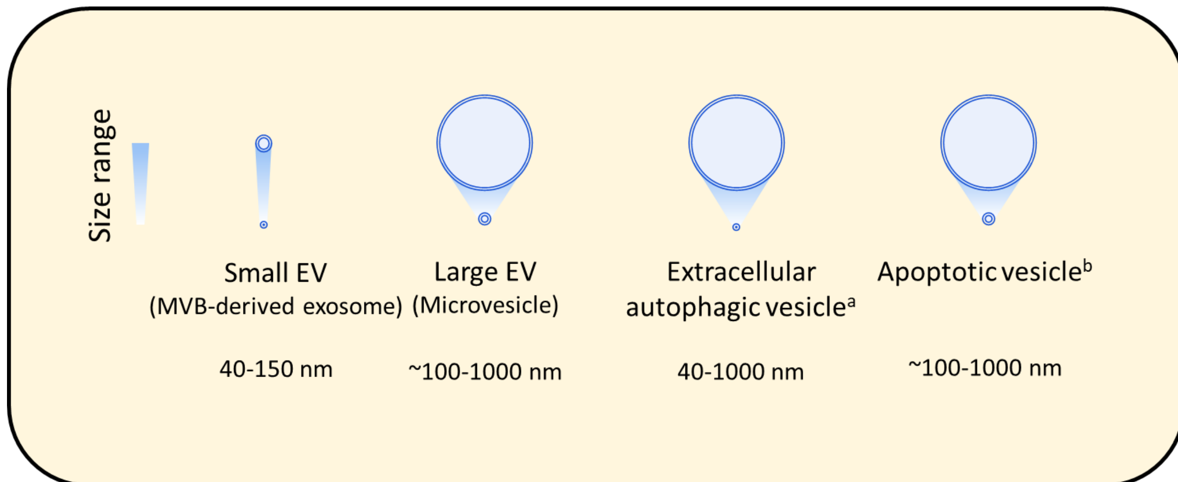
757 ***Table 4 – examples of EV labelling for direct transfer and biodistribution studies***

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

758

759 **13. <u>Figures</u>**

760
761

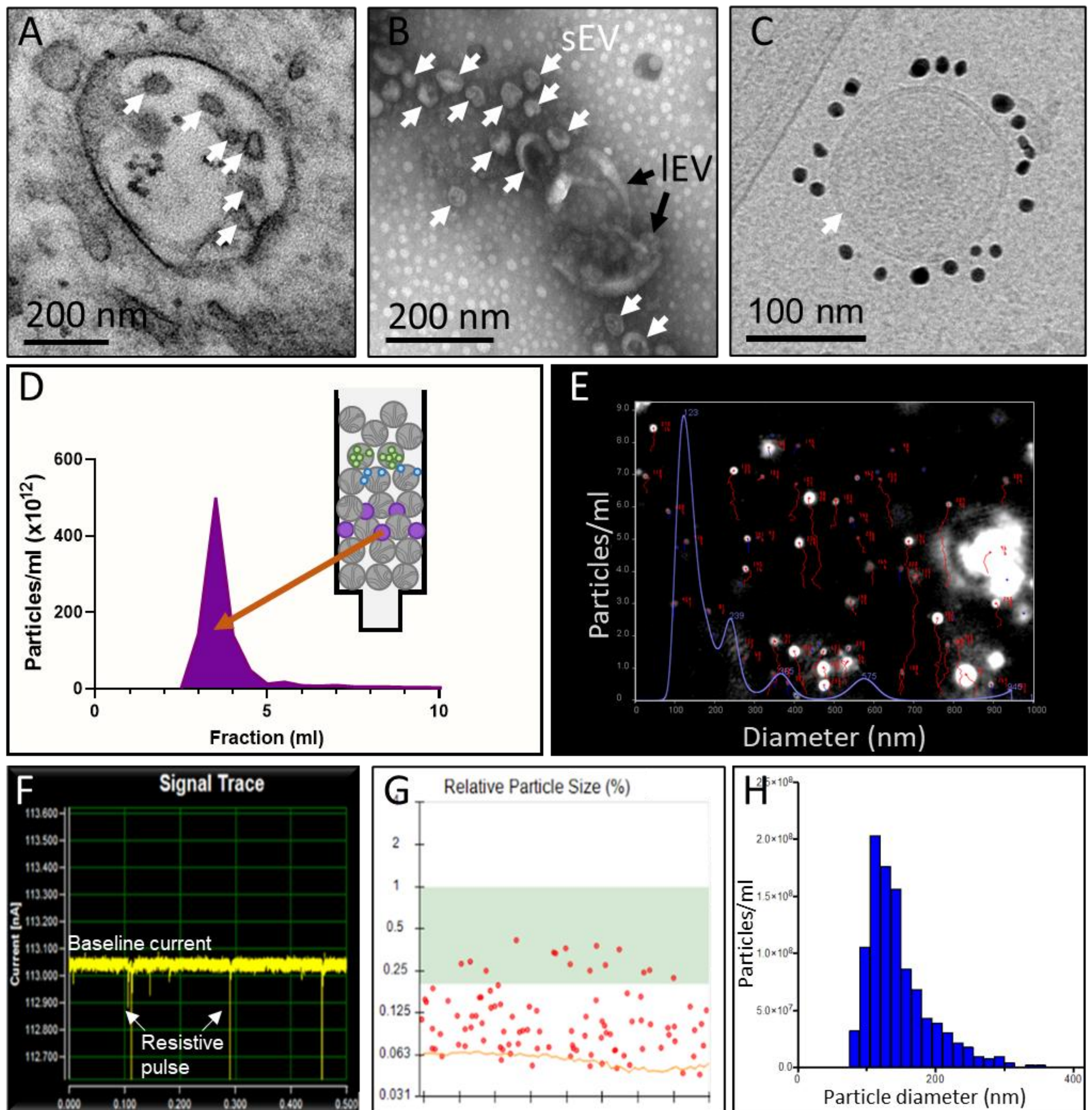


762

763 ***Figure 1.***

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

768



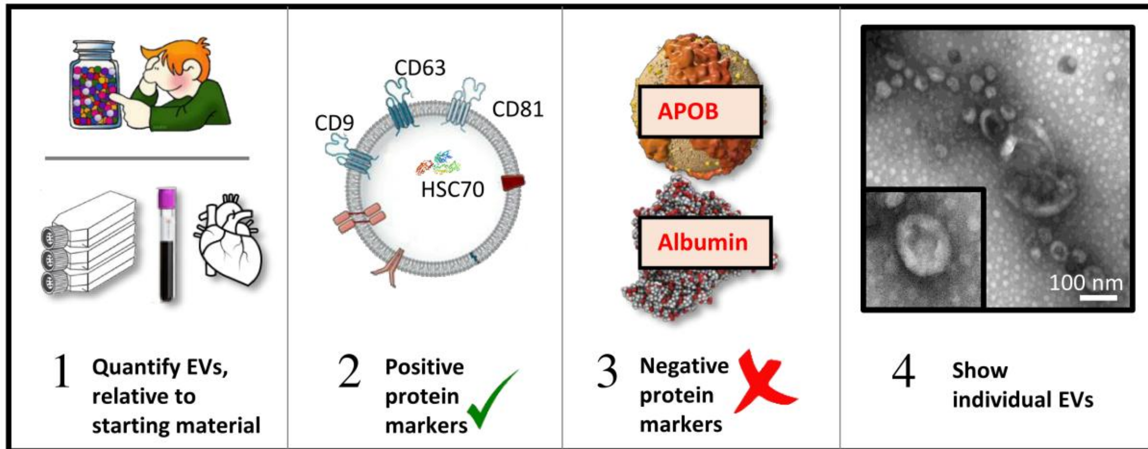
769

770 ***Figure 2***.

771 Representative images of different techniques of EV characterization.

772 A) Transmission electron micrography (TEM) of multi-vesicular body (MVB) containing
773 exosomes (arrows) in primary HUVECs.
774 B) Transmission electron micrography (TEM) of negative-stained EVs isolated from HUVECs
775 (sEV = small EVs, lEV = large EVs).
776 C) Cryo-TEM of a single CD81+ EV from iPS-derived cardiovascular progenitor cells.[37] The lipid
777 bilayer is clearly resolved (arrow).
778 D) Fractionation of sEVs (purple) from proteins (green, blue) by size-exclusion chromatography.

779 E) Single frame from nanoparticle tracking analysis (NTA) of an sEV sample under constant
780 flow, showing particle tracks (red) and particle size-distribution (blue).
781 F) Representative trace of EV sample obtained using resistive pulse sensing (RPS).
782 G) Individual particles detected by RPS, with size determined relative to calibration beads of a
783 known size.
784 H) Size distribution of EVs obtained by RPS.



785

786 ***Figure 3.***

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

796

797 **14. Box 1**

798 799	**The standard differential ultracentrifugation protocol for EV isolation, originally published by Thery et al.[54]**
800	1. Centrifuge sample at 300 g for 10 min, at 4°C. (Remove cells and cell debris)
801	2. Centrifuge supernatant at 2,000 g for 10 min, at 4°C. (Remove larger complexes)
802	3. Centrifuge supernatant at 10,000 g for 30 min, at 4°C. (Microvesicles are in the pellet).
803	4. Centrifuge supernatant at 100,000 g for 70 min, at 4°C in ultracentrifuge. (EVs are in the pellet)
804	5. Re-suspend the pellet containing EVs and contaminating proteins.
805	6. Centrifuge 100,000 g 70 min, 4°C in ultracentrifuge to wash. (sEVs/exosomes are in the pellet).

806

807
808 ## 15. Author contributions

809 All co-authors contributed to the draft of the document; SD, JS and CMB synthesized all
810 contributions and handled the revision of the paper.
811
812
813 ## 16. Funding

814 This work was supported by the Hatter Foundation [to SMD], the British Heart Foundation
815 [PG/18/44/33790 to SMD]; by the Project EVICARE (No. 725229) of the European Research Council
816 (ERC) and PPS grant (No. 2018B014) to J.P.G.S./P.V, the Dutch Ministry of Economic Affairs,
817 Agriculture and Innovation and the Netherlands CardioVascular Research Initiative (CVON): the
818 Dutch Heart Foundation to J.P.G.S.; by INSERM, the French National Agency for Research (ANR-16-
819 CE92-0032-02) and the Fondation pour la Recherche Médicale (FRM EQU202003010767) [to CMB].
820 MM is a BHF Chair Holder (CH/16/3/32406) with BHF program grant support (RG/16/14/32397), and
821 a holder of a BHF Special Project grant to participate in the ERA-CVD Transnational Grant
822 "MacroERA: Noncoding RNAs in cardiac macrophages and their role in heart failure"; by the Austrian
823 Science Fund (SFB-54 "InThro") [to CJB]; it is funded by the EU Horizon 2020 project COVIRNA (Grant
824 Agreement # 101016072), the Spanish Ministry of Economy and Competitiveness of Science
825 [PID2019-107160RB-I00], the Carlos III Institute of Health [CIBERCV CB16/11/00411 and RICORS
826 2021 – TERAV] cofounded by FEDER; and the Fundación Investigación Cardiovascular-Fundación
827 Jesus Serra [to L.B.]; by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation)
828 – JA 2351/2-1 and Project-ID 397484323 – TRR 259 and the Corona Foundation (F.J), by National
829 Institutes of Health grant numbers R01HL136431, R01HL147095, and R01HL141917 [to EA]; by the
830 EU Horizon 2020 project Cardioregenix [GA 825670 to TT] and Deutsche Forschungsgemeinschaft
831 [Transregio TRR 267 to TT]; by the US NIH National Cancer Institute [NCI to KWW] and Office of the
832 Director [UG3CA241694 to KWW]; by Higher Education Institutional Excellence Program –
833 Therapeutic development [NKFIH OTKA120237, NVKP_16-1-2016-0017 to EIB], [VEKOP-2.3.2-16-
834 2016-00002, VEKOP-2.3.3-15-2016-00016, H2020-MSCA-ITN-2017-722148 TRAIN EV to EIB]; EU's
835 Horizon 2020 research and innovation program under grant agreement [739593 to EIB].
836
837
838
839 ## 17. Conflicts of Interest

840 L.B has performed advisory board work and received speaker fees from Sanofi and Novartis, and is
841 founder and shareholder of Glycardial Diagnosis SL and Ivestatin Therapeutics, SL (all outside of this
842 work) ; CJB is a board member of Technoclone. AB is founder and CEO of Exo-Analysis. TT has filed
843 and licensed patents in the field of noncoding RNAs and targeted delivery strategies and is founder
844 and shareholder of Cardior Pharmaceuticals GmbH (outside of the topic of this review). RL discloses
845 grants from Stago and a patent on microvesicle fibrinolytic activity licensed to Stago. EIB is member
846 of the Advisory Board of Sphere Gene Therapeutics Inc. (Boston, US). MHMW discloses a
847 collaborative research agreement with BD Biosciences Europe, Erembodegem, Belgium to optimize
848 flow cytometric analysis of EVs.
849

850 **18. References**

851
852

853 1. Davidson SM, Andreadou I, Barile L, Birnbaum Y, Cabrera-Fuentes HA, Cohen MV, Downey
854 JM, Girao H, Pagliaro P, Penna C, Pernow J, Preissner KT, Ferdinandy P. Circulating blood cells
855 and extracellular vesicles in acute cardioprotection. *Cardiovasc Res* 2019;**115**:1156-1166.
856 2. Ridger VC, Boulanger CM, Angelillo-Scherrer A, Badimon L, Blanc-Brude O, Bochaton-Piallat
857 ML, Boilard E, Buzas EI, Caporali A, Dignat-George F, Evans PC, Lacroix R, Lutgens E,
858 Ketelhuth DFJ, Nieuwland R, Toti F, Tunon J, Weber C. Microvesicles in vascular homeostasis
859 and diseases. Position Paper of the European Society of Cardiology (ESC) Working Group on
860 Atherosclerosis and Vascular Biology. *Thromb Haemost* 2017;**117**.
861 3. Properzi F, Logozzi M, Fais S. Exosomes: the future of biomarkers in medicine. *Biomark Med*
862 2013;**7**:769-778.
863 4. Lv Y, Tan J, Miao Y, Zhang Q. The role of microvesicles and its active molecules in regulating
864 cellular biology. *J Cell Mol Med* 2019;**23**:7894-7904.
865 5. Sluijter JPG, Davidson SM, Boulanger CM, Buzas EI, de Kleijn DPV, Engel FB, Giricz Z,
866 Hausenloy DJ, Kishore R, Lecour S, Leor J, Madonna R, Perrino C, Prunier F, Sahoo S,
867 Schiffelers RM, Schulz R, Van Laake LW, Ytrehus K, Ferdinandy P. Extracellular vesicles in
868 diagnostics and therapy of the ischaemic heart: Position Paper from the Working Group on
869 Cellular Biology of the Heart of the European Society of Cardiology. *Cardiovasc Res*
870 2018;**114**:19-34.
871 6. Loyer X, Vion AC, Tedgui A, Boulanger CM. Microvesicles as cell-cell messengers in
872 cardiovascular diseases. *Circ Res* 2014;**114**:345-353.
873 7. Badimon L, Suades R, Fuentes E, Palomo I, Padro T. Role of Platelet-Derived Microvesicles As
874 Crosstalk Mediators in Atherothrombosis and Future Pharmacology Targets: A Link between
875 Inflammation, Atherosclerosis, and Thrombosis. *Front Pharmacol* 2016;**7**:293.
876 8. Vanhaverbeke M, Attard R, Bartekova M, Ben-Aicha S, Brandenburger T, de Gonzalo-Calvo D,
877 Emanueli C, Farrugia R, Grillari J, Hackl M, Kalocayova B, Martelli F, Scholz M, Wettinger SB,
878 Devaux Y, CA EU-CCA. Peripheral blood RNA biomarkers for cardiovascular disease from
879 bench to bedside: A Position Paper from the EU-CardioRNA COST Action CA17129.
880 *Cardiovasc Res* 2021.
881 9. Deddens JC, Vrijsen KR, Colijn JM, Oerlemans MI, Metz CH, van der Vlist EJ, Nolte-'t Hoen EN,
882 den Ouden K, Jansen Of Lorkeers SJ, van der Spoel TI, Koudstaal S, Arkesteijn GJ, Wauben
883 MH, van Laake LW, Doevendans PA, Chamuleau SA, Sluijter JP. Circulating Extracellular
884 Vesicles Contain miRNAs and are Released as Early Biomarkers for Cardiac Injury. *J*
885 *Cardiovasc Transl Res* 2016;**9**:291-301.
886 10. Emanueli C, Shearn AI, Laftah A, Fiorentino F, Reeves BC, Beltrami C, Mumford A, Clayton A,
887 Gurney M, Shantikumar S, Angelini GD. Coronary Artery-Bypass-Graft Surgery Increases the
888 Plasma Concentration of Exosomes Carrying a Cargo of Cardiac MicroRNAs: An Example of
889 Exosome Trafficking Out of the Human Heart with Potential for Cardiac Biomarker Discovery.
890 *PLoS One* 2016;**11**:e0154274.
891 11. Castellani C, Burrello J, Fedrigo M, Burrello A, Bolis S, Di Silvestre D, Tona F, Bottio T, Biemmi
892 V, Toscano G, Gerosa G, Thiene G, Basso C, Longnus SL, Vassalli G, Angelini A, Barile L.
893 Circulating extracellular vesicles as non-invasive biomarker of rejection in heart transplant. *J*
894 *Heart Lung Transplant* 2020;**39**:1136-1148.
895 12. Jansen F, Yang X, Proebsting S, Hoelscher M, Przybilla D, Baumann K, Schmitz T, Dolf A, Endl
896 E, Franklin BS, Sinning JM, Vasa-Nicotera M, Nickenig G, Werner N. MicroRNA expression in
897 circulating microvesicles predicts cardiovascular events in patients with coronary artery
898 disease. *J Am Heart Assoc* 2014;**3**:e001249.

899 13. Aliotta JM, Pereira M, Amaral A, Sorokina A, Igbinoba Z, Hasslinger A, El-Bizri R, Rounds SI,
900 Quesenberry PJ, Klinger JR. Induction of pulmonary hypertensive changes by extracellular
901 vesicles from monocrotaline-treated mice. *Cardiovasc Res* 2013;**100**:354-362.
902 14. Aliotta JM, Pereira M, Wen S, Dooner MS, Del Tatto M, Papa E, Goldberg LR, Baird GL,
903 Ventetuolo CE, Quesenberry PJ, Klinger JR. Exosomes induce and reverse monocrotaline-
904 induced pulmonary hypertension in mice. *Cardiovasc Res* 2016;**110**:319-330.
905 15. Kapustin AN, Chatrou ML, Drozdov I, Zheng Y, Davidson SM, Soong D, Furmanik M, Sanchis P,
906 De Rosales RT, Alvarez-Hernandez D, Shroff R, Yin X, Muller K, Skepper JN, Mayr M,
907 Reutelingsperger CP, Chester A, Bertazzo S, Schurgers LJ, Shanahan CM. Vascular smooth
908 muscle cell calcification is mediated by regulated exosome secretion. *Circ Res*
909 2015;**116**:1312-1323.
910 16. Durham AL, Speer MY, Scatena M, Giachelli CM, Shanahan CM. Role of smooth muscle cells
911 in vascular calcification: implications in atherosclerosis and arterial stiffness. *Cardiovasc Res*
912 2018;**114**:590-600.
913 17. Akawi N, Checa A, Antonopoulos AS, Akoumianakis I, Daskalaki E, Kotanidis CP, Kondo H, Lee
914 K, Yesilyurt D, Badi I, Polkinghorne M, Akbar N, Lundgren J, Chuaiphichai S, Choudhury R,
915 Neubauer S, Channon KM, Torekov SS, Wheelock CE, Antoniades C. Fat-Secreted Ceramides
916 Regulate Vascular Redox State and Influence Outcomes in Patients With Cardiovascular
917 Disease. *J Am Coll Cardiol* 2021;**77**:2494-2513.
918 18. Akbar N, Digby JE, Cahill TJ, Tavare AN, Corbin AL, Saluja S, Dawkins S, Edgar L, Rawlings N,
919 Ziberna K, McNeill E, Oxford Acute Myocardial Infarction S, Johnson E, Aljabali AA, Dragovic
920 RA, Rohling M, Belgard TG, Udalova IA, Greaves DR, Channon KM, Riley PR, Anthony DC,
921 Choudhury RP. Endothelium-derived extracellular vesicles promote splenic monocyte
922 mobilization in myocardial infarction. *JCI Insight* 2017;**2**.
923 19. Akbar N, Braithwaite AT, Corr EM, Koelwyn GJ, van Solingen C, Cochain C, Saliba AE, Corbin
924 A, Pezzolla D, Moller Jorgensen M, Baek R, Edgar L, De Villiers C, Gunadasa-Rohling M,
925 Banerjee A, Paget D, Lee C, Hogg E, Costin A, Dhaliwal R, Johnson E, Krausgruber T,
926 Riepsaame J, Melling GE, Shanmuganathan M, Oxford Acute Myocardial Infarction S, Bock C,
927 Carter DRF, Channon KM, Riley PR, Udalova IA, Moore KJ, Anthony D, Choudhury RP. Rapid
928 neutrophil mobilisation by VCAM-1+ endothelial extracellular vesicles. *Cardiovasc Res* 2022.
929 20. Boulanger CM, Loyer X, Rautou PE, Amabile N. Extracellular vesicles in coronary artery
930 disease. *Nat Rev Cardiol* 2017;**14**:259-272.
931 21. Martinez MC, Andriantsitohaina R. Extracellular Vesicles in Metabolic Syndrome. *Circ Res*
932 2017;**120**:1674-1686.
933 22. Jansen F, Li Q, Pfeifer A, Werner N. Endothelial- and Immune Cell-Derived Extracellular
934 Vesicles in the Regulation of Cardiovascular Health and Disease. *JACC Basic Transl Sci*
935 2017;**2**:790-807.
936 23. Timmers L, Lim SK, Arslan F, Armstrong JS, Hoefer IE, Doevendans PA, Piek JJ, El Oakley RM,
937 Choo A, Lee CN, Pasterkamp G, de Kleijn DP. Reduction of myocardial infarct size by human
938 mesenchymal stem cell conditioned medium. *Stem Cell Res* 2007;**1**:129-137.
939 24. Lai RC, Arslan F, Tan SS, Tan B, Choo A, Lee MM, Chen TS, Teh BJ, Eng JK, Sidik H, Tanavde V,
940 Hwang WS, Lee CN, El Oakley RM, Pasterkamp G, de Kleijn DP, Tan KH, Lim SK. Derivation
941 and characterization of human fetal MSCs: an alternative cell source for large-scale
942 production of cardioprotective microparticles. *J Mol Cell Cardiol* 2010;**48**:1215-1224.
943 25. Barile L, Cervio E, Lionetti V, Milano G, Ciullo A, Biemmi V, Bolis S, Altomare C, Matteucci M,
944 Di Silvestre D, Brambilla F, Fertig TE, Torre T, Demertzis S, Mauri P, Moccetti T, Vassalli G.
945 Cardioprotection by cardiac progenitor cell-secreted exosomes: role of pregnancy-associated
946 plasma protein-A. *Cardiovasc Res* 2018;**114**:992-1005.
947 26. Milano G, Biemmi V, Lazzarini E, Balbi C, Ciullo A, Bolis S, Ameri P, Di Silvestre D, Mauri P,
948 Barile L, Vassalli G. Intravenous administration of cardiac progenitor cell-derived exosomes

949 protects against doxorubicin/trastuzumab-induced cardiac toxicity. *Cardiovasc Res*
950 2020;**116**:383-392.
951 27. Maring JA, Lodder K, Mol E, Verhage V, Wiesmeijer KC, Dingenouts CKE, Moerkamp AT,
952 Deddens JC, Vader P, Smits AM, Sluijter JPG, Goumans MJ. Cardiac Progenitor Cell-Derived
953 Extracellular Vesicles Reduce Infarct Size and Associate with Increased Cardiovascular Cell
954 Proliferation. *J Cardiovasc Transl Res* 2019;**12**:5-17.
955 28. Pula G, Mayr U, Evans C, Prokopi M, Vara DS, Yin X, Astroulakis Z, Xiao Q, Hill J, Xu Q, Mayr
956 M. Proteomics identifies thymidine phosphorylase as a key regulator of the angiogenic
957 potential of colony-forming units and endothelial progenitor cell cultures. *Circ Res*
958 2009;**104**:32-40.
959 29. Prokopi M, Pula G, Mayr U, Devue C, Gallagher J, Xiao Q, Boulanger CM, Westwood N,
960 Urbich C, Willeit J, Steiner M, Breuss J, Xu Q, Kiechl S, Mayr M. Proteomic analysis reveals
961 presence of platelet microparticles in endothelial progenitor cell cultures. *Blood*
962 2009;**114**:723-732.
963 30. Hu S, Li Z, Shen D, Zhu D, Huang K, Su T, Dinh PU, Cores J, Cheng K. Exosome-eluting stents
964 for vascular healing after ischaemic injury. *Nat Biomed Eng* 2021.
965 31. Thery C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, Antoniou A,
966 Arab T, Archer F, Atkin-Smith GK, Ayre DC, Bach JM, Bachurski D, Baharvand H, Balaj L,
967 Baldacchino S, Bauer NN, Baxter AA, Bebawy M, Beckham C, Bedina Zavec A, Benmoussa A,
968 Berardi AC, Bergese P, Bielska E, Blenkiron C, Bobis-Wozowicz S, Boilard E, Boireau W,
969 Bongiovanni A, Borras FE, Bosch S, Boulanger CM, Breakefield X, Breglio AM, Brennan MA,
970 Brigstock DR, Brisson A, Broekman ML, Bromberg JF, Bryl-Gorecka P, Buch S, Buck AH, Burger
971 D, Busatto S, Buschmann D, Bussolati B, Buzas EI, Byrd JB, Camussi G, Carter DR, Caruso S,
972 Chamley LW, Chang YT, Chen C, Chen S, Cheng L, Chin AR, Clayton A, Clerici SP, Cocks A,
973 Cocucci E, Coffey RJ, Cordeiro-da-Silva A, Couch Y, Coumans FA, Coyle B, Crescitelli R, Criado
974 MF, D'Souza-Schorey C, Das S, Datta Chaudhuri A, de Candia P, De Santana EF, De Wever O,
975 Del Portillo HA, Demaret T, Deville S, Devitt A, Dhondt B, Di Vizio D, Dieterich LC, Dolo V,
976 Dominguez Rubio AP, Dominici M, Dourado MR, Driedonks TA, Duarte FV, Duncan HM,
977 Eichenberger RM, Ekstrom K, El Andaloussi S, Elie-Caille C, Erdbrugger U, Falcon-Perez JM,
978 Fatima F, Fish JE, Flores-Bellver M, Forsonits A, Frelet-Barrand A, Fricke F, Fuhrmann G,
979 Gabrielsson S, Gamez-Valero A, Gardiner C, Gartner K, Gaudin R, Gho YS, Giebel B, Gilbert C,
980 Gimona M, Giusti I, Goberdhan DC, Gorgens A, Gorski SM, Greening DW, Gross JC, Gualerzi
981 A, Gupta GN, Gustafson D, Handberg A, Haraszti RA, Harrison P, Hegyesi H, Hendrix A, Hill
982 AF, Hochberg FH, Hoffmann KF, Holder B, Holthofer H, Hosseinkhani B, Hu G, Huang Y, Huber
983 V, Hunt S, Ibrahim AG, Ikezu T, Inal JM, Isin M, Ivanova A, Jackson HK, Jacobsen S, Jay SM,
984 Jayachandran M, Jenster G, Jiang L, Johnson SM, Jones JC, Jong A, Jovanovic-Talisman T, Jung
985 S, Kalluri R, Kano SI, Kaur S, Kawamura Y, Keller ET, Khamari D, Khomyakova E, Khvorova A,
986 Kierulf P, Kim KP, Kislinger T, Klingeborn M, Klinke DJ, 2nd, Kornek M, Kosanovic MM, Kovacs
987 AF, Kramer-Albers EM, Krasemann S, Krause M, Kurochkin IV, Kusuma GD, Kuypers S,
988 Laitinen S, Langevin SM, Languino LR, Lannigan J, Lasser C, Laurent LC, Lavieu G, Lazaro-
989 Ibanez E, Le Lay S, Lee MS, Lee YXF, Lemos DS, Lenassi M, Leszczynska A, Li IT, Liao K, Libregts
990 SF, Ligeti E, Lim R, Lim SK, Line A, Linnemannstons K, Llorente A, Lombard CA, Lorenowicz
991 MJ, Lorincz AM, Lotvall J, Lovett J, Lowry MC, Loyer X, Lu Q, Lukomska B, Lunavat TR, Maas
992 SL, Malhi H, Marcilla A, Mariani J, Mariscal J, Martens-Uzunova ES, Martin-Jaular L, Martinez
993 MC, Martins VR, Mathieu M, Mathivanan S, Maugeri M, McGinnis LK, McVey MJ, Meckes
994 DG, Jr., Meehan KL, Mertens I, Minciacchi VR, Moller A, Moller Jorgensen M, Morales-
995 Kastresana A, Morhayim J, Mullier F, Muraca M, Musante L, Mussack V, Muth DC, Myburgh
996 KH, Najrana T, Nawaz M, Nazarenko I, Nejsum P, Neri C, Neri T, Nieuwland R, Nimrichter L,
997 Nolan JP, Nolte-'t Hoen EN, Noren Hooten N, O'Driscoll L, O'Grady T, O'Loghlen A, Ochiya T,
998 Olivier M, Ortiz A, Ortiz LA, Osteikoetxea X, Ostergaard O, Ostrowski M, Park J, Pegtel DM,
999 Peinado H, Perut F, Pfaffl MW, Phinney DG, Pieters BC, Pink RC, Pisetsky DS, Pogge von

1000 Strandmann E, Polakovicova I, Poon IK, Powell BH, Prada I, Pulliam L, Quesenberry P,
1001 Radeghieri A, Raffai RL, Raimondo S, Rak J, Ramirez MI, Raposo G, Rayyan MS, Regev-Rudzki
1002 N, Ricklefs FL, Robbins PD, Roberts DD, Rodrigues SC, Rohde E, Rome S, Rouschop KM,
1003 Rughetti A, Russell AE, Saa P, Sahoo S, Salas-Huenuleo E, Sanchez C, Saugstad JA, Saul MJ,
1004 Schiffelers RM, Schneider R, Schoyen TH, Scott A, Shahaj E, Sharma S, Shatnyeva O, Shekari
1005 F, Shelke GV, Shetty AK, Shiba K, Siljander PR, Silva AM, Skowronek A, Snyder OL, 2nd, Soares
1006 RP, Sodar BW, Soekmadji C, Sotillo J, Stahl PD, Stoorvogel W, Stott SL, Strasser EF, Swift S,
1007 Tahara H, Tewari M, Timms K, Tiwari S, Tixeira R, Tkach M, Toh WS, Tomasini R, Torrecilhas
1008 AC, Tosar JP, Toxavidis V, Urbanelli L, Vader P, van Balkom BW, van der Grein SG, Van Deun
1009 J, van Herwijnen MJ, Van Keuren-Jensen K, van Niel G, van Royen ME, van Wijnen AJ,
1010 Vasconcelos MH, Vechetti IJ, Jr., Veit TD, Vella LJ, Velot E, Verweij FJ, Vestad B, Vinas JL,
1011 Visnovitz T, Vukman KV, Wahlgren J, Watson DC, Wauben MH, Weaver A, Webber JP, Weber
1012 V, Wehman AM, Weiss DJ, Welsh JA, Wendt S, Wheelock AM, Wiener Z, Witte L, Wolfram J,
1013 Xagorari A, Xander P, Xu J, Yan X, Yanez-Mo M, Yin H, Yuana Y, Zappulli V, Zarubova J, Zekas
1014 V, Zhang JY, Zhao Z, Zheng L, Zheutlin AR, Zickler AM, Zimmermann P, Zivkovic AM, Zocco D,
1015 Zuba-Surma EK. Minimal information for studies of extracellular vesicles 2018 (MISEV2018):
1016 a position statement of the International Society for Extracellular Vesicles and update of the
1017 MISEV2014 guidelines. *J Extracell Vesicles* 2018;**7**:1535750.
1018 32. Mathieu M, Martin-Jaular L, Lavieu G, Thery C. Specificities of secretion and uptake of
1019 exosomes and other extracellular vesicles for cell-to-cell communication. *Nat Cell Biol*
1020 2019;**21**:9-17.
1021 33. Zhao J, Li X, Hu J, Chen F, Qiao S, Sun X, Gao L, Xie J, Xu B. Mesenchymal stromal cell-derived
1022 exosomes attenuate myocardial ischaemia-reperfusion injury through miR-182-regulated
1023 macrophage polarization. *Cardiovasc Res* 2019;**115**:1205-1216.
1024 34. Takafuji Y, Hori M, Mizuno T, Harada-Shiba M. Humoral factors secreted from adipose
1025 tissue-derived mesenchymal stem cells ameliorate atherosclerosis in Ldlr-/- mice. *Cardiovasc*
1026 *Res* 2019;**115**:1041-1051.
1027 35. Mayourian J, Ceholski DK, Gorski PA, Mathiyalagan P, Murphy JF, Salazar SI, Stillitano F, Hare
1028 JM, Sahoo S, Hajjar RJ, Costa KD. Exosomal microRNA-21-5p Mediates Mesenchymal Stem
1029 Cell Paracrine Effects on Human Cardiac Tissue Contractility. *Circ Res* 2018;**122**:933-944.
1030 36. Kompa AR, Greening DW, Kong AM, McMillan PJ, Fang H, Saxena R, Wong RCB, Lees JG,
1031 Sivakumaran P, Newcomb AE, Tannous BA, Kos C, Mariana L, Loudovaris T, Hausenloy DJ,
1032 Lim SY. Sustained subcutaneous delivery of secretome of human cardiac stem cells promotes
1033 cardiac repair following myocardial infarction. *Cardiovasc Res* 2021;**117**:918-929.
1034 37. Lima Correa B, El Harane N, Gomez I, Rachid Hocine H, Vilar J, Desgres M, Bellamy V,
1035 Keirththana K, Guillas C, Perotto M, Pidial L, Alayrac P, Tran T, Tan S, Hamada T, Charron D,
1036 Brisson A, Renault NK, Al-Daccak R, Menasche P, Silvestre JS. Extracellular vesicles from
1037 human cardiovascular progenitors trigger a reparative immune response in infarcted hearts.
1038 *Cardiovasc Res* 2021;**117**:292-307.
1039 38. Chen CW, Wang LL, Zaman S, Gordon J, Arisi MF, Venkataraman CM, Chung JJ, Hung G,
1040 Gaffey AC, Spruce LA, Fazelinia H, Gorman RC, Seeholzer SH, Burdick JA, Atluri P. Sustained
1041 release of endothelial progenitor cell-derived extracellular vesicles from shear-thinning
1042 hydrogels improves angiogenesis and promotes function after myocardial infarction.
1043 *Cardiovasc Res* 2018;**114**:1029-1040.
1044 39. Gallet R, Dawkins J, Valle J, Simsolo E, de Couto G, Middleton R, Tseliou E, Luthringer D,
1045 Kreke M, Smith RR, Marban L, Ghaleh B, Marban E. Exosomes secreted by cardiosphere-
1046 derived cells reduce scarring, attenuate adverse remodelling, and improve function in acute
1047 and chronic porcine myocardial infarction. *Eur Heart J* 2017;**38**:201-211.
1048 40. Khan M, Nickoloff E, Abramova T, Johnson J, Verma SK, Krishnamurthy P, Mackie AR,
1049 Vaughan E, Garikipati VN, Benedict C, Ramirez V, Lambers E, Ito A, Gao E, Misener S, Luongo
1050 T, Elrod J, Qin G, Houser SR, Koch WJ, Kishore R. Embryonic stem cell-derived exosomes

1051 promote endogenous repair mechanisms and enhance cardiac function following myocardial
1052 infarction. *Circ Res* 2015;**117**:52-64.
1053 41. Gao L, Wang L, Wei Y, Krishnamurthy P, Walcott GP, Menasche P, Zhang J. Exosomes
1054 secreted by hiPSC-derived cardiac cells improve recovery from myocardial infarction in
1055 swine. *Sci Transl Med* 2020;**12**.
1056 42. Villa Del Campo C, Liaw NY, Gunadasa-Rohling M, Matthaei M, Braga L, Kennedy T, Salinas G,
1057 Voigt N, Giacca M, Zimmermann WH, Riley PR. Regenerative potential of epicardium-derived
1058 extracellular vesicles mediated by conserved miRNA transfer. *Cardiovasc Res* 2021.
1059 43. Savina A, Furlan M, Vidal M, Colombo MI. Exosome release is regulated by a calcium-
1060 dependent mechanism in K562 cells. *J Biol Chem* 2003;**278**:20083-20090.
1061 44. Ribeiro-Rodrigues TM, Laundos TL, Pereira-Carvalho R, Batista-Almeida D, Pereira R, Coelho-
1062 Santos V, Silva AP, Fernandes R, Zuzarte M, Enguita FJ, Costa MC, Pinto-do OP, Pinto MT,
1063 Gouveia P, Ferreira L, Mason JC, Pereira P, Kwak BR, Nascimento DS, Girao H. Exosomes
1064 secreted by cardiomyocytes subjected to ischaemia promote cardiac angiogenesis.
1065 *Cardiovasc Res* 2017;**113**:1338-1350.
1066 45. Gollmann-Tepekoylu C, Polzl L, Graber M, Hirsch J, Nagele F, Lobenwein D, Hess MW, Blumer
1067 MJ, Kirchmair E, Zipperle J, Hromada C, Muhleder S, Hackl H, Hermann M, Al Khamisi H,
1068 Forster M, Lichtenauer M, Mittermayr R, Paulus P, Fritsch H, Bonaros N, Kirchmair R, Sluijter
1069 JPG, Davidson S, Grimm M, Holfeld J. miR-19a-3p containing exosomes improve function of
1070 ischaemic myocardium upon shock wave therapy. *Cardiovasc Res* 2020;**116**:1226-1236.
1071 46. Huang P, Wang L, Li Q, Tian X, Xu J, Xu J, Xiong Y, Chen G, Qian H, Jin C, Yu Y, Cheng K, Qian L,
1072 Yang Y. Atorvastatin enhances the therapeutic efficacy of mesenchymal stem cells-derived
1073 exosomes in acute myocardial infarction via up-regulating long non-coding RNA H19.
1074 *Cardiovasc Res* 2020;**116**:353-367.
1075 47. Hou Z, Qin X, Hu Y, Zhang X, Li G, Wu J, Li J, Sha J, Chen J, Xia J, Wang L, Gao F. Longterm
1076 Exercise-Derived Exosomal miR-342-5p: A Novel Exerkine for Cardioprotection. *Circ Res*
1077 2019;**124**:1386-1400.
1078 48. Bei Y, Xu T, Lv D, Yu P, Xu J, Che L, Das A, Tigges J, Toxavidis V, Ghiran I, Shah R, Li Y, Zhang Y,
1079 Das S, Xiao J. Exercise-induced circulating extracellular vesicles protect against cardiac
1080 ischemia-reperfusion injury. *Basic Res Cardiol* 2017;**112**:38.
1081 49. Loyer X, Zlatanova I, Devue C, Yin M, Howangyin KY, Klaihmon P, Guerin CL, Kheloufi M, Vilar
1082 J, Zannis K, Fleischmann BK, Hwang DW, Park J, Lee H, Menasche P, Silvestre JS, Boulanger
1083 CM. Intra-Cardiac Release of Extracellular Vesicles Shapes Inflammation Following
1084 Myocardial Infarction. *Circ Res* 2018;**123**:100-106.
1085 50. Zietzer A, Steffen E, Niepmann S, Dusing P, Hosen MR, Liu W, Jamme P, Al-Kassou B, Goody
1086 PR, Zimmer S, Reiners KS, Pfeifer A, Bohm M, Werner N, Nickenig G, Jansen F. MicroRNA-
1087 mediated vascular intercellular communication is altered in chronic kidney disease.
1088 *Cardiovasc Res* 2020.
1089 51. Davidson SM, Riquelme JA, Takov K, Vicencio JM, Boi-Doku C, Khoo V, Doreth C, Radenkovic
1090 D, Lavandero S, Yellon DM. Cardioprotection mediated by exosomes is impaired in the
1091 setting of type II diabetes but can be rescued by the use of non-diabetic exosomes in vitro. *J
1092 Cell Mol Med* 2018;**22**:141-151.
1093 52. Wang X, Huang W, Liu G, Cai W, Millard RW, Wang Y, Chang J, Peng T, Fan GC.
1094 Cardiomyocytes mediate anti-angiogenesis in type 2 diabetic rats through the exosomal
1095 transfer of miR-320 into endothelial cells. *J Mol Cell Cardiol* 2014;**74**:139-150.
1096 53. Palviainen M, Saari H, Karkkainen O, Pekkinen J, Auriola S, Yliperttula M, Puhka M,
1097 Hanhineva K, Siljander PR. Metabolic signature of extracellular vesicles depends on the cell
1098 culture conditions. *J Extracell Vesicles* 2019;**8**:1596669.
1099 54. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from
1100 cell culture supernatants and biological fluids. *Curr Protoc Cell Biol* 2006;**Chapter 3**:Unit 3 22.

1101 55. Paolini L, Zendrini A, Di Noto G, Busatto S, Lottini E, Radeghieri A, Dossi A, Caneschi A,
1102 Ricotta D, Bergese P. Residual matrix from different separation techniques impacts exosome
1103 biological activity. *Sci Rep* 2016;**6**:23550.
1104 56. Boing AN, van der Pol E, Grootemaat AE, Coumans FA, Sturk A, Nieuwland R. Single-step
1105 isolation of extracellular vesicles by size-exclusion chromatography. *J Extracell Vesicles*
1106 2014;**3**.
1107 57. Nordin JZ, Lee Y, Vader P, Mager I, Johansson HJ, Heusermann W, Wiklander OP, Hallbrink
1108 M, Seow Y, Bultema JJ, Gilthorpe J, Davies T, Fairchild PJ, Gabrielsson S, Meisner-Kober NC,
1109 Lehtio J, Smith CI, Wood MJ, El Andaloussi S. Ultrafiltration with size-exclusion liquid
1110 chromatography for high yield isolation of extracellular vesicles preserving intact biophysical
1111 and functional properties. *Nanomedicine* 2015;**11**:879-883.
1112 58. Ludwig AK, De Miroschedji K, Doeppner TR, Borger V, Ruesing J, Rebmann V, Durst S, Jansen
1113 S, Bremer M, Behrmann E, Singer BB, Jastrow H, Kuhlmann JD, El Magraoui F, Meyer HE,
1114 Hermann DM, Opalka B, Raunser S, Epple M, Horn PA, Giebel B. Precipitation with
1115 polyethylene glycol followed by washing and pelleting by ultracentrifugation enriches
1116 extracellular vesicles from tissue culture supernatants in small and large scales. *J Extracell*
1117 *Vesicles* 2018;**7**:1528109.
1118 59. Dong L, Zieren RC, Horie K, Kim CJ, Mallick E, Jing Y, Feng M, Kuczler MD, Green J, Amend SR,
1119 Witwer KW, de Reijke TM, Cho YK, Pienta KJ, Xue W. Comprehensive evaluation of methods
1120 for small extracellular vesicles separation from human plasma, urine and cell culture
1121 medium. *J Extracell Vesicles* 2020;**10**:e12044.
1122 60. Jeppesen DK, Fenix AM, Franklin JL, Higginbotham JN, Zhang Q, Zimmerman LJ, Liebler DC,
1123 Ping J, Liu Q, Evans R, Fissell WH, Patton JG, Rome LH, Burnette DT, Coffey RJ. Reassessment
1124 of Exosome Composition. *Cell* 2019;**177**:428-445 e418.
1125 61. Kang YT, Kim YJ, Bu J, Cho YH, Han SW, Moon BI. High-purity capture and release of
1126 circulating exosomes using an exosome-specific dual-patterned immunofiltration (ExoDIF)
1127 device. *Nanoscale* 2017;**9**:13495-13505.
1128 62. Zhang H, Freitas D, Kim HS, Fabijanic K, Li Z, Chen H, Mark MT, Molina H, Martin AB, Bojmar
1129 L, Fang J, Rampersaud S, Hoshino A, Matei I, Kenific CM, Nakajima M, Mutvei AP, Sansone P,
1130 Buehring W, Wang H, Jimenez JP, Cohen-Gould L, Paknejad N, Brendel M, Manova-Todorova
1131 K, Magalhaes A, Ferreira JA, Osorio H, Silva AM, Massey A, Cubillos-Ruiz JR, Galletti G,
1132 Giannakakou P, Cuervo AM, Blenis J, Schwartz R, Brady MS, Peinado H, Bromberg J, Matsui
1133 H, Reis CA, Lyden D. Identification of distinct nanoparticles and subsets of extracellular
1134 vesicles by asymmetric flow field-flow fractionation. *Nat Cell Biol* 2018;**20**:332-343.
1135 63. Andriolo G, Provasi E, Lo Cicero V, Brambilla A, Soncin S, Torre T, Milano G, Biemmi V,
1136 Vassalli G, Turchetto L, Barile L, Radrizzani M. Exosomes From Human Cardiac Progenitor
1137 Cells for Therapeutic Applications: Development of a GMP-Grade Manufacturing Method.
1138 *Front Physiol* 2018;**9**:1169.
1139 64. Takov K, Yellon DM, Davidson SM. Comparison of small extracellular vesicles isolated from
1140 plasma by ultracentrifugation or size-exclusion chromatography: yield, purity and functional
1141 potential. *J Extracell Vesicles* 2019;**8**:1560809.
1142 65. Mol EA, Goumans MJ, Doevendans PA, Sluijter JPG, Vader P. Higher functionality of
1143 extracellular vesicles isolated using size-exclusion chromatography compared to
1144 ultracentrifugation. *Nanomedicine* 2017;**13**:2061-2065.
1145 66. Lehrich BM, Liang Y, Fiandaca MS. Foetal bovine serum influence on in vitro extracellular
1146 vesicle analyses. *J Extracell Vesicles* 2021;**10**:e12061.
1147 67. Yuana Y, Boing AN, Grootemaat AE, van der Pol E, Hau CM, Cizmar P, Buhr E, Sturk A,
1148 Nieuwland R. Handling and storage of human body fluids for analysis of extracellular
1149 vesicles. *J Extracell Vesicles* 2015;**4**:29260.
1150 68. Lacroix R, Judicone C, Mooberry M, Boucekine M, Key NS, Dignat-George F, The ISSCW.
1151 Standardization of pre-analytical variables in plasma microparticle determination: results of

1152 the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. *J*
1153 *Thromb Haemost* 2013;**11**:1190-1193.
1154 69. Palviainen M, Saraswat M, Varga Z, Kitka D, Neuvonen M, Puhka M, Joenvaara S, Renkonen
1155 R, Nieuwland R, Takatalo M, Siljander PRM. Extracellular vesicles from human plasma and
1156 serum are carriers of extravesicular cargo-Implications for biomarker discovery. *PLoS One*
1157 2020;**15**:e0236439.
1158 70. Vicencio JM, Yellon DM, Sivaraman V, Das D, Boi-Doku C, Arjun S, Zheng Y, Riquelme JA,
1159 Kearney J, Sharma V, Multhoff G, Hall AR, Davidson SM. Plasma exosomes protect the
1160 myocardium from ischemia-reperfusion injury. *J Am Coll Cardiol* 2015;**65**:1525-1536.
1161 71. Simonsen JB. What Are We Looking At? Extracellular Vesicles, Lipoproteins, or Both? *Circ Res*
1162 2017;**121**:920-922.
1163 72. Zhang X, Borg EGF, Liaci AM, Vos HR, Stoorvogel W. A novel three step protocol to isolate
1164 extracellular vesicles from plasma or cell culture medium with both high yield and purity. *J*
1165 *Extracell Vesicles* 2020;**9**:1791450.
1166 73. Clayton A, Boilard E, Buzas EI, Cheng L, Falcon-Perez JM, Gardiner C, Gustafson D, Gualerzi A,
1167 Hendrix A, Hoffman A, Jones J, Lasser C, Lawson C, Lenassi M, Nazarenko I, O'Driscoll L, Pink
1168 R, Siljander PR, Soekmadji C, Wauben M, Welsh JA, Witwer K, Zheng L, Nieuwland R.
1169 Considerations towards a roadmap for collection, handling and storage of blood extracellular
1170 vesicles. *J Extracell Vesicles* 2019;**8**:1647027.
1171 74. Leroyer AS, Ebrahimian TG, Cochain C, Recalde A, Blanc-Brude O, Mees B, Vilar J, Tedgui A,
1172 Levy BI, Chimini G, Boulanger CM, Silvestre JS. Microparticles from ischemic muscle
1173 promotes postnatal vasculogenesis. *Circulation* 2009;**119**:2808-2817.
1174 75. Perrotta I, Aquila S. Exosomes in human atherosclerosis: An ultrastructural analysis study.
1175 *Ultrastruct Pathol* 2016;**40**:101-106.
1176 76. Crescitelli R, Lasser C, Lotvall J. Isolation and characterization of extracellular vesicle
1177 subpopulations from tissues. *Nat Protoc* 2021.
1178 77. Claridge B, Rai A, Fang H, Matsumoto A, Luo J, McMullen JR, Greening DW. Proteome
1179 characterisation of extracellular vesicles isolated from heart. *Proteomics* 2021;**21**:e2100026.
1180 78. Leroyer AS, Isobe H, Leseche G, Castier Y, Wassef M, Mallat Z, Binder BR, Tedgui A,
1181 Boulanger CM. Cellular origins and thrombogenic activity of microparticles isolated from
1182 human atherosclerotic plaques. *J Am Coll Cardiol* 2007;**49**:772-777.
1183 79. Witwer KW, Van Balkom BWM, Bruno S, Choo A, Dominici M, Gimona M, Hill AF, De Kleijn D,
1184 Koh M, Lai RC, Mitsialis SA, Ortiz LA, Rohde E, Asada T, Toh WS, Weiss DJ, Zheng L, Giebel B,
1185 Lim SK. Defining mesenchymal stromal cell (MSC)-derived small extracellular vesicles for
1186 therapeutic applications. *J Extracell Vesicles* 2019;**8**:1609206.
1187 80. van der Pol E, Coumans FA, Grootemaat AE, Gardiner C, Sargent IL, Harrison P, Sturk A, van
1188 Leeuwen TG, Nieuwland R. Particle size distribution of exosomes and microvesicles
1189 determined by transmission electron microscopy, flow cytometry, nanoparticle tracking
1190 analysis, and resistive pulse sensing. *J Thromb Haemost* 2014;**12**:1182-1192.
1191 81. Webber J, Clayton A. How pure are your vesicles? *J Extracell Vesicles* 2013;**2**.
1192 82. Liao Z, Jaular LM, Soueidi E, Jouve M, Muth DC, Schoyen TH, Seale T, Haughey NJ, Ostrowski
1193 M, Thery C, Witwer KW. Acetylcholinesterase is not a generic marker of extracellular
1194 vesicles. *J Extracell Vesicles* 2019;**8**:1628592.
1195 83. Arraud N, Linares R, Tan S, Gounou C, Pasquet JM, Mornet S, Brisson AR. Extracellular
1196 vesicles from blood plasma: determination of their morphology, size, phenotype and
1197 concentration. *J Thromb Haemost* 2014;**12**:614-627.
1198 84. Ridolfi A, Brucale M, Montis C, Caselli L, Paolini L, Borup A, Boysen AT, Loria F, van Herwijnen
1199 MJC, Kleinjan M, Nejsum P, Zarovni N, Wauben MHM, Berti D, Bergese P, Valle F. AFM-Based
1200 High-Throughput Nanomechanical Screening of Single Extracellular Vesicles. *Anal Chem*
1201 2020;**92**:10274-10282.

1202 85. Nolan JP. Flow Cytometry of Extracellular Vesicles: Potential, Pitfalls, and Prospects. *Curr*
1203 *Protoc Cytom* 2015;**73**:13 14 11-13 14 16.
1204 86. Welsh JA, Van Der Pol E, Arkesteijn GJA, Bremer M, Brisson A, Coumans F, Dignat-George F,
1205 Duggan E, Ghiran I, Giebel B, Gorgens A, Hendrix A, Lacroix R, Lannigan J, Libregts S, Lozano-
1206 Andres E, Morales-Kastresana A, Robert S, De Rond L, Tertel T, Tigges J, De Wever O, Yan X,
1207 Nieuwland R, Wauben MHM, Nolan JP, Jones JC. MIFlowCyt-EV: a framework for
1208 standardized reporting of extracellular vesicle flow cytometry experiments. *J Extracell*
1209 *Vesicles* 2020;**9**:1713526.
1210 87. Libregts S, Arkesteijn GJA, Nemeth A, Nolte-'t Hoen ENM, Wauben MHM. Flow cytometric
1211 analysis of extracellular vesicle subsets in plasma: impact of swarm by particles of non-
1212 interest. *J Thromb Haemost* 2018;**16**:1423-1436.
1213 88. Amabile N, Cheng S, Renard JM, Larson MG, Ghorbani A, McCabe E, Griffin G, Guerin C, Ho
1214 JE, Shaw SY, Cohen KS, Vasan RS, Tedgui A, Boulanger CM, Wang TJ. Association of
1215 circulating endothelial microparticles with cardiometabolic risk factors in the Framingham
1216 Heart Study. *Eur Heart J* 2014;**35**:2972-2979.
1217 89. Krankel N, Strassler E, Uhlemann M, Muller M, Briand-Schumacher S, Klingenberg R, Schulze
1218 PC, Adams V, Schuler G, Luscher TF, Mobius-Winkler S, Landmesser U. Extracellular vesicle
1219 species differentially affect endothelial cell functions and differentially respond to exercise
1220 training in patients with chronic coronary syndromes. *Eur J Prev Cardiol* 2021;**28**:1467-1474.
1221 90. Koganti S, Eleftheriou D, Gurung R, Hong Y, Brogan P, Rakhit RD. Persistent circulating
1222 platelet and endothelial derived microparticle signature may explain on-going pro-
1223 thrombogenicity after acute coronary syndrome. *Thromb Res* 2021;**206**:60-65.
1224 91. Anselmo A, Frank D, Papa L, Viviani Anselmi C, Di Pasquale E, Mazzola M, Panico C, Clemente
1225 F, Soldani C, Pagiatakis C, Hinkel R, Thalmann R, Kozlik-Feldmann R, Miragoli M, Carullo P,
1226 Vacchiano M, Chaves-Sanjuan A, Santo N, Losi MA, Ferrari MC, Puca AA, Christiansen V,
1227 Seoudy H, Freitag-Wolf S, Frey N, Dempfle A, Mercola M, Esposito G, Briguori C, Kupatt C,
1228 Condorelli G. Myocardial hypoxic stress mediates functional cardiac extracellular vesicle
1229 release. *Eur Heart J* 2021;**42**:2780-2792.
1230 92. Boyden S. The chemotactic effect of mixtures of antibody and antigen on
1231 polymorphonuclear leucocytes. *J Exp Med* 1962;**115**:453-466.
1232 93. Takov K, He Z, Johnston HE, Timms JF, Guillot PV, Yellon DM, Davidson SM. Small
1233 extracellular vesicles secreted from human amniotic fluid mesenchymal stromal cells possess
1234 cardioprotective and promigratory potential. *Basic Res Cardiol* 2020;**115**:26.
1235 94. Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for
1236 analysis of cell migration in vitro. *Nat Protoc* 2007;**2**:329-333.
1237 95. Todorova D, Simoncini S, Lacroix R, Sabatier F, Dignat-George F. Extracellular Vesicles in
1238 Angiogenesis. *Circ Res* 2017;**120**:1658-1673.
1239 96. Baker M, Robinson SD, Lechertier T, Barber PR, Tavora B, D'Amico G, Jones DT, Vojnovic B,
1240 Hodivala-Dilke K. Use of the mouse aortic ring assay to study angiogenesis. *Nat Protoc*
1241 2011;**7**:89-104.
1242 97. Gimona M, Brizzi MF, Choo ABH, M. D, M. DS, Grillari J, Hermann DM, Hill AF, de Kleijn D, Lai
1243 RC, Lai C, Lim R, M. M-T, Muraca M, Ochiya T, Ortiz LA, Toh WS, Yi YW, Witwer KW, Giebel B,
1244 Lim SK. Critical considerations for the development of potency tests for therapeutic
1245 applications of mesenchymal stromal cell (MSC)-derived small extracellular vesicles.
1246 *Cytotherapy* 2021;**In press**.
1247 98. Consortium E-T, Van Deun J, Mestdagh P, Agostinis P, Akay O, Anand S, Anckaert J, Martinez
1248 ZA, Baetens T, Beghein E, Bertier L, Berx G, Boere J, Boukouris S, Bremer M, Buschmann D,
1249 Byrd JB, Casert C, Cheng L, Cmoch A, Daveloose D, De Smedt E, Demirsoy S, Depoorter V,
1250 Dhondt B, Driedonks TA, Dudek A, Elsharawy A, Floris I, Foers AD, Gartner K, Garg AD,
1251 Geeurickx E, Gettemans J, Ghazavi F, Giebel B, Kormelink TG, Hancock G, Helsmoortel H, Hill
1252 AF, Hyenne V, Kalra H, Kim D, Kowal J, Kraemer S, Leidinger P, Leonelli C, Liang Y, Lippens L,

1253 Liu S, Lo Cicero A, Martin S, Mathivanan S, Mathiyalagan P, Matusek T, Milani G, Monguio-
1254 Tortajada M, Mus LM, Muth DC, Nemeth A, Nolte-'t Hoen EN, O'Driscoll L, Palmulli R, Pfaffl
1255 MW, Primdal-Bengtson B, Romano E, Rousseau Q, Sahoo S, Sampaio N, Samuel M, Scicluna
1256 B, Soen B, Steels A, Swinnen JV, Takatalo M, Thaminy S, Thery C, Tulkens J, Van Audenhove I,
1257 van der Grein S, Van Goethem A, van Herwijnen MJ, Van Niel G, Van Roy N, Van Vliet AR,
1258 Vandamme N, Vanhauwaert S, Vergauwen G, Verweij F, Wallaert A, Wauben M, Witwer KW,
1259 Zonneveld MI, De Wever O, Vandesompele J, Hendrix A. EV-TRACK: transparent reporting
1260 and centralizing knowledge in extracellular vesicle research. *Nat Methods* 2017;**14**:228-232.
1261 99. Ghosh R, Gilda JE, Gomes AV. The necessity of and strategies for improving confidence in the
1262 accuracy of western blots. *Expert Rev Proteomics* 2014;**11**:549-560.
1263 100. Nelson GM, Guynn JM, Chorley BN. Procedure and Key Optimization Strategies for an
1264 Automated Capillary Electrophoretic-based Immunoassay Method. *J Vis Exp* 2017.
1265 101. Welton JL, Webber JP, Botos LA, Jones M, Clayton A. Ready-made chromatography columns
1266 for extracellular vesicle isolation from plasma. *J Extracell Vesicles* 2015;**4**:27269.
1267 102. Im H, Shao H, Park YI, Peterson VM, Castro CM, Weissleder R, Lee H. Label-free detection
1268 and molecular profiling of exosomes with a nano-plasmonic sensor. *Nat Biotechnol*
1269 2014;**32**:490-495.
1270 103. Daaboul GG, Gagni P, Benussi L, Bettotti P, Ciani M, Cretich M, Freedman DS, Ghidoni R,
1271 Ozkumur AY, Piotto C, Prosperi D, Santini B, Unlu MS, Chiari M. Digital Detection of
1272 Exosomes by Interferometric Imaging. *Sci Rep* 2016;**6**:37246.
1273 104. Kowal J, Arras G, Colombo M, Jouve M, Morath JP, Primdal-Bengtson B, Dingli F, Loew D,
1274 Tkach M, Thery C. Proteomic comparison defines novel markers to characterize
1275 heterogeneous populations of extracellular vesicle subtypes. *Proc Natl Acad Sci U S A*
1276 2016;**113**:E968-977.
1277 105. Karimi N, Cvjetkovic A, Jang SC, Crescitelli R, Hosseinpour Feizi MA, Nieuwland R, Lotvall J,
1278 Lasser C. Detailed analysis of the plasma extracellular vesicle proteome after separation
1279 from lipoproteins. *Cell Mol Life Sci* 2018;**75**:2873-2886.
1280 106. Kalra H, Simpson RJ, Ji H, Aikawa E, Altevogt P, Askenase P, Bond VC, Borras FE, Breakefield
1281 X, Budnik V, Buzas E, Camussi G, Clayton A, Cocucci E, Falcon-Perez JM, Gabrielsson S, Gho
1282 YS, Gupta D, Harsha HC, Hendrix A, Hill AF, Inal JM, Jenster G, Kramer-Albers EM, Lim SK,
1283 Llorente A, Lotvall J, Marcilla A, Mincheva-Nilsson L, Nazarenko I, Nieuwland R, Nolte-'t Hoen
1284 EN, Pandey A, Patel T, Piper MG, Pluchino S, Prasad TS, Rajendran L, Raposo G, Record M,
1285 Reid GE, Sanchez-Madrid F, Schiffelers RM, Siljander P, Stensballe A, Stoorvogel W, Taylor D,
1286 Thery C, Valadi H, van Balkom BW, Vazquez J, Vidal M, Wauben MH, Yanez-Mo M, Zoeller M,
1287 Mathivanan S. Vesiclepedia: a compendium for extracellular vesicles with continuous
1288 community annotation. *PLoS Biol* 2012;**10**:e1001450.
1289 107. Osteikoetxea X, Sodar B, Nemeth A, Szabo-Taylor K, Paloczi K, Vukman KV, Tamasi V, Balogh
1290 A, Kittel A, Pallinger E, Buzas EI. Differential detergent sensitivity of extracellular vesicle
1291 subpopulations. *Org Biomol Chem* 2015;**13**:9775-9782.
1292 108. Foers AD, Chatfield S, Dagley LF, Scicluna BJ, Webb AI, Cheng L, Hill AF, Wicks IP, Pang KC.
1293 Enrichment of extracellular vesicles from human synovial fluid using size exclusion
1294 chromatography. *J Extracell Vesicles* 2018;**7**:1490145.
1295 109. Mayr M, Grainger D, Mayr U, Leroyer AS, Leseche G, Sidibe A, Herbin O, Yin X, Gomes A,
1296 Madhu B, Griffiths JR, Xu Q, Tedgui A, Boulanger CM. Proteomics, metabolomics, and
1297 immunomics on microparticles derived from human atherosclerotic plaques. *Circ Cardiovasc*
1298 *Genet* 2009;**2**:379-388.
1299 110. Lasser C, Shelke GV, Yeri A, Kim DK, Crescitelli R, Raimondo S, Sjostrand M, Gho YS, Van
1300 Keuren Jensen K, Lotvall J. Two distinct extracellular RNA signatures released by a single cell
1301 type identified by microarray and next-generation sequencing. *RNA Biol* 2017;**14**:58-72.
1302 111. Chevillet JR, Kang Q, Ruf IK, Briggs HA, Vojtech LN, Hughes SM, Cheng HH, Arroyo JD,
1303 Meredith EK, Gallichotte EN, Pogosova-Agadjanyan EL, Morrissey C, Stirewalt DL, Hladik F, Yu

1304 EY, Higano CS, Tewari M. Quantitative and stoichiometric analysis of the microRNA content
1305 of exosomes. *Proc Natl Acad Sci U S A* 2014;**111**:14888-14893.
1306 112. Mateescu B, Kowal EJ, van Balkom BW, Bartel S, Bhattacharyya SN, Buzas EI, Buck AH, de
1307 Candia P, Chow FW, Das S, Driedonks TA, Fernandez-Messina L, Haderk F, Hill AF, Jones JC,
1308 Van Keuren-Jensen KR, Lai CP, Lasser C, Liegro ID, Lunavat TR, Lorenowicz MJ, Maas SL,
1309 Mager I, Mittelbrunn M, Momma S, Mukherjee K, Nawaz M, Pegtel DM, Pfaffl MW,
1310 Schiffelers RM, Tahara H, Thery C, Tosar JP, Wauben MH, Witwer KW, Nolte-'t Hoen EN.
1311 Obstacles and opportunities in the functional analysis of extracellular vesicle RNA - an ISEV
1312 position paper. *J Extracell Vesicles* 2017;**6**:1286095.
1313 113. Zietzer A, Hosen MR, Wang H, Goody PR, Sylvester M, Latz E, Nickenig G, Werner N, Jansen
1314 F. The RNA-binding protein hnRNPU regulates the sorting of microRNA-30c-5p into large
1315 extracellular vesicles. *J Extracell Vesicles* 2020;**9**:1786967.
1316 114. Bellingham SA, Shambrook M, Hill AF. Quantitative Analysis of Exosomal miRNA via qPCR
1317 and Digital PCR. *Methods Mol Biol* 2017;**1545**:55-70.
1318 115. Godoy PM, Bhakta NR, Barczak AJ, Cakmak H, Fisher S, MacKenzie TC, Patel T, Price RW,
1319 Smith JF, Woodruff PG, Erle DJ. Large Differences in Small RNA Composition Between Human
1320 Biofluids. *Cell Rep* 2018;**25**:1346-1358.
1321 116. Veziroglu EM, Mias GI. Characterizing Extracellular Vesicles and Their Diverse RNA Contents.
1322 *Front Genet* 2020;**11**:700.
1323 117. Li S, Li Y, Chen B, Zhao J, Yu S, Tang Y, Zheng Q, Li Y, Wang P, He X, Huang S. exoRBase: a
1324 database of circRNA, lncRNA and mRNA in human blood exosomes. *Nucleic Acids Res*
1325 2018;**46**:D106-D112.
1326 118. Boeckel JN, Thome CE, Leistner D, Zeiher AM, Fichtlscherer S, Dimmeler S. Heparin
1327 selectively affects the quantification of microRNAs in human blood samples. *Clin Chem*
1328 2013;**59**:1125-1127.
1329 119. Pritchard CC, Kroh E, Wood B, Arroyo JD, Dougherty KJ, Miyaji MM, Tait JF, Tewari M. Blood
1330 cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies. *Cancer*
1331 *Prev Res (Phila)* 2012;**5**:492-497.
1332 120. Wei Z, Batagov AO, Carter DR, Krichevsky AM. Fetal Bovine Serum RNA Interferes with the
1333 Cell Culture derived Extracellular RNA. *Sci Rep* 2016;**6**:31175.
1334 121. Tosar JP, Cayota A, Eitan E, Halushka MK, Witwer KW. Ribonucleic artefacts: are some
1335 extracellular RNA discoveries driven by cell culture medium components? *J Extracell Vesicles*
1336 2017;**6**:1272832.
1337 122. Ben-Aicha S, Escate R, Casani L, Padro T, Pena E, Arderiu G, Mendieta G, Badimon L, Vilahur
1338 G. High-density lipoprotein remodelled in hypercholesterolaemic blood induce epigenetically
1339 driven down-regulation of endothelial HIF-1alpha expression in a preclinical animal model.
1340 *Cardiovasc Res* 2020;**116**:1288-1299.
1341 123. Das S, Extracellular RNACC, Ansel KM, Bitzer M, Breakefield XO, Charest A, Galas DJ, Gerstein
1342 MB, Gupta M, Milosavljevic A, McManus MT, Patel T, Raffai RL, Rozowsky J, Roth ME,
1343 Saugstad JA, Van Keuren-Jensen K, Weaver AM, Laurent LC. The Extracellular RNA
1344 Communication Consortium: Establishing Foundational Knowledge and Technologies for
1345 Extracellular RNA Research. *Cell* 2019;**177**:231-242.
1346 124. Zomer A, Maynard C, Verweij FJ, Kamermans A, Schafer R, Beerling E, Schiffelers RM, de Wit
1347 E, Berenguer J, Ellenbroek SIJ, Wurdinger T, Pegtel DM, van Rheenen J. In Vivo imaging
1348 reveals extracellular vesicle-mediated phenocopying of metastatic behavior. *Cell*
1349 2015;**161**:1046-1057.
1350 125. de Jong OG, Murphy DE, Mager I, Willms E, Garcia-Guerra A, Gitz-Francois JJ, Lefferts J,
1351 Gupta D, Steenbeek SC, van Rheenen J, El Andaloussi S, Schiffelers RM, Wood MJA, Vader P.
1352 A CRISPR-Cas9-based reporter system for single-cell detection of extracellular vesicle-
1353 mediated functional transfer of RNA. *Nat Commun* 2020;**11**:1113.

1354 126. Durcin M, Fleury A, Taillebois E, Hilairet G, Krupova Z, Henry C, Truchet S, Trotzmuller M,
1355 Kofeler H, Mabilleau G, Hue O, Andriantsitohaina R, Martin P, Le Lay S. Characterisation of
1356 adipocyte-derived extracellular vesicle subtypes identifies distinct protein and lipid
1357 signatures for large and small extracellular vesicles. *J Extracell Vesicles* 2017;**6**:1305677.
1358 127. Record M, Silvente-Poirot S, Poirot M, Wakelam MJO. Extracellular vesicles: lipids as key
1359 components of their biogenesis and functions. *J Lipid Res* 2018;**59**:1316-1324.
1360 128. Subra C, Grand D, Laulagnier K, Stella A, Lambeau G, Paillasse M, De Medina P, Monsarrat B,
1361 Perret B, Silvente-Poirot S, Poirot M, Record M. Exosomes account for vesicle-mediated
1362 transcellular transport of activatable phospholipases and prostaglandins. *J Lipid Res*
1363 2010;**51**:2105-2120.
1364 129. Rautou PE, Mackman N. Microvesicles as risk markers for venous thrombosis. *Expert Rev*
1365 *Hematol* 2013;**6**:91-101.
1366 130. Visnovitz T, Osteikoetxea X, Sodar BW, Mihaly J, Lorincz P, Vukman KV, Toth EA, Koncz A,
1367 Szekacs I, Horvath R, Varga Z, Buzas EI. An improved 96 well plate format lipid quantification
1368 assay for standardisation of experiments with extracellular vesicles. *J Extracell Vesicles*
1369 2019;**8**:1565263.
1370 131. Osteikoetxea X, Balogh A, Szabo-Taylor K, Nemeth A, Szabo TG, Paloczi K, Sodar B, Kittel A,
1371 Gyorgy B, Pallinger E, Matko J, Buzas EI. Improved characterization of EV preparations based
1372 on protein to lipid ratio and lipid properties. *PLoS One* 2015;**10**:e0121184.
1373 132. Szentirmai V, Wacha A, Nemeth C, Kitka D, Racz A, Heberger K, Mihaly J, Varga Z. Reagent-
1374 free total protein quantification of intact extracellular vesicles by attenuated total reflection
1375 Fourier transform infrared (ATR-FTIR) spectroscopy. *Anal Bioanal Chem* 2020;**412**:4619-
1376 4628.
1377 133. Smith ZJ, Lee C, Rojalin T, Carney RP, Hazari S, Knudson A, Lam K, Saari H, Ibanez EL, Viitala T,
1378 Laaksonen T, Yliperttula M, Wachsmann-Hogiu S. Single exosome study reveals
1379 subpopulations distributed among cell lines with variability related to membrane content. *J*
1380 *Extracell Vesicles* 2015;**4**:28533.
1381 134. Skotland T, Sagini K, Sandvig K, Llorente A. An emerging focus on lipids in extracellular
1382 vesicles. *Adv Drug Deliv Rev* 2020;**159**:308-321.
1383 135. Burrello J, Biemmi V, Dei Cas M, Amongero M, Bolis S, Lazzarini E, Bollini S, Vassalli G, Paroni
1384 R, Barile L. Sphingolipid composition of circulating extracellular vesicles after myocardial
1385 ischemia. *Sci Rep* 2020;**10**:16182.
1386 136. Tsiantoulas D, Perkmann T, Afonyushkin T, Mangold A, Prohaska TA, Papac-Milicevic N,
1387 Millischer V, Bartel C, Horkko S, Boulanger CM, Tsimikas S, Fischer MB, Witztum JL, Lang IM,
1388 Binder CJ. Circulating microparticles carry oxidation-specific epitopes and are recognized by
1389 natural IgM antibodies. *J Lipid Res* 2015;**56**:440-448.
1390 137. Binder CJ, Papac-Milicevic N, Witztum JL. Innate sensing of oxidation-specific epitopes in
1391 health and disease. *Nat Rev Immunol* 2016;**16**:485-497.
1392 138. Nieuwland R, Gardiner C, Dignat-George F, Mullier F, Mackman N, Woodhams B, Thaler J.
1393 Toward standardization of assays measuring extracellular vesicle-associated tissue factor
1394 activity. *J Thromb Haemost* 2019;**17**:1261-1264.
1395 139. Key NS, Mackman N. Tissue factor and its measurement in whole blood, plasma, and
1396 microparticles. *Semin Thromb Hemost* 2010;**36**:865-875.
1397 140. Lacroix R, Vallier L, Bonifay A, Simoncini S, Mege D, Aubert M, Panicot-Dubois L, Dubois C,
1398 Dignat-George F. Microvesicles and Cancer Associated Thrombosis. *Semin Thromb Hemost*
1399 2019;**45**:593-603.
1400 141. Exner T, Joseph J, Low J, Connor D, Ma D. A new activated factor X-based clotting method
1401 with improved specificity for procoagulant phospholipid. *Blood Coagul Fibrinolysis*
1402 2003;**14**:773-779.
1403 142. Hisada Y, Alexander W, Kasthuri R, Voorhees P, Mobarrez F, Taylor A, McNamara C, Wallen
1404 H, Witkowski M, Key NS, Rauch U, Mackman N. Measurement of microparticle tissue factor

1405 activity in clinical samples: A summary of two tissue factor-dependent FXa generation
1406 assays. *Thromb Res* 2016;**139**:90-97.
1407 143. Hisada Y, Mackman N. Measurement of tissue factor activity in extracellular vesicles from
1408 human plasma samples. *Res Pract Thromb Haemost* 2019;**3**:44-48.
1409 144. Berckmans RJ, Sturk A, van Tienen LM, Schaap MC, Nieuwland R. Cell-derived vesicles
1410 exposing coagulant tissue factor in saliva. *Blood* 2011;**117**:3172-3180.
1411 145. Poncelet P, Robert S, Bailly N, Garnache-Ottou F, Bouriche T, Devalet B, Segatchian JH, Saas
1412 P, Mullier F. Tips and tricks for flow cytometry-based analysis and counting of microparticles.
1413 *Transfus Apher Sci* 2015;**53**:110-126.
1414 146. Vallier L, Bouriche T, Bonifay A, Judicone C, Bez J, Franco C, Guervilly C, Hisada Y, Mackman
1415 N, Houston R, Poncelet P, Dignat-George F, Lacroix R. Increasing the sensitivity of the human
1416 microvesicle tissue factor activity assay. *Thromb Res* 2019;**182**:64-74.
1417 147. Tatsumi K, Antoniak S, Monroe DM, 3rd, Khorana AA, Mackman N, Subcommittee on H,
1418 Malignancy of the S, Standardization Committee of the International Society on T,
1419 Hemostasis. Evaluation of a new commercial assay to measure microparticle tissue factor
1420 activity in plasma: communication from the SSC of the ISTH. *J Thromb Haemost*
1421 2014;**12**:1932-1934.
1422 148. van Es N, Hisada Y, Di Nisio M, Cesarman G, Kleinjan A, Mahe I, Otten HM, Kamphuisen PW,
1423 Berckmans RJ, Buller HR, Mackman N, Nieuwland R. Extracellular vesicles exposing tissue
1424 factor for the prediction of venous thromboembolism in patients with cancer: A prospective
1425 cohort study. *Thromb Res* 2018;**166**:54-59.
1426 149. Lacroix R, Thaler J. ISTH SSC Vascular Biology Project 5: Comparison of the sensitivity and the
1427 specificity of assays to measure TF-EVs in plasma samples. 2019.
1428 150. Vallier L, Cointe S, Lacroix R, Bonifay A, Judicone C, Dignat-George F, Kwaan HC.
1429 Microparticles and Fibrinolysis. *Semin Thromb Hemost* 2017;**43**:129-134.
1430 151. Cointe S, Harti Souab K, Bouriche T, Vallier L, Bonifay A, Judicone C, Robert S, Armand R,
1431 Poncelet P, Albanese J, Dignat-George F, Lacroix R. A new assay to evaluate microvesicle
1432 plasmin generation capacity: validation in disease with fibrinolysis imbalance. *J Extracell*
1433 *Vesicles* 2018;**7**:1494482.
1434 152. Briens A, Gauberti M, Parcq J, Montaner J, Vivien D, Martinez de Lizarrondo S. Nano-
1435 zymography Using Laser-Scanning Confocal Microscopy Unmasks Proteolytic Activity of Cell-
1436 Derived Microparticles. *Theranostics* 2016;**6**:610-626.
1437 153. Shimoda M. Extracellular vesicle-associated MMPs: A modulator of the tissue
1438 microenvironment. *Adv Clin Chem* 2019;**88**:35-66.
1439 154. Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. Secretory mechanisms and
1440 intercellular transfer of microRNAs in living cells. *J Biol Chem* 2010;**285**:17442-17452.
1441 155. Sodar BW, Kittel A, Paloczi K, Vukman KV, Osteikoetxea X, Szabo-Taylor K, Nemeth A,
1442 Sperlagh B, Baranyai T, Giricz Z, Wiener Z, Turiak L, Drahos L, Pallinger E, Vekey K, Ferdinandy
1443 P, Falus A, Buzas EI. Low-density lipoprotein mimics blood plasma-derived exosomes and
1444 microvesicles during isolation and detection. *Sci Rep* 2016;**6**:24316.
1445 156. Gouin K, Peck K, Antes T, Johnson JL, Li C, Vaturi SD, Middleton R, de Couto G, Walravens AS,
1446 Rodriguez-Borlado L, Smith RR, Marban L, Marban E, Ibrahim AG. A comprehensive method
1447 for identification of suitable reference genes in extracellular vesicles. *J Extracell Vesicles*
1448 2017;**6**:1347019.
1449 157. Sahoo S, Adamiak M, Mathiyalagan P, Kenneweg F, Kafert-Kasting S, Thum T. Therapeutic
1450 and Diagnostic Translation of Extracellular Vesicles in Cardiovascular Diseases: Roadmap to
1451 the Clinic. *Circulation* 2021;**143**:1426-1449.
1452 158. Ortega FG, Roefs MT, de Miguel Perez D, Kooijmans SA, de Jong OG, Sluijter JP, Schiffelers
1453 RM, Vader P. Interfering with endolysosomal trafficking enhances release of bioactive
1454 exosomes. *Nanomedicine* 2019;**20**:102014.

1455 159. Ibrahim AG, Cheng K, Marban E. Exosomes as critical agents of cardiac regeneration
1456 triggered by cell therapy. *Stem Cell Reports* 2014;**2**:606-619.
1457 160. Mulcahy LA, Pink RC, Carter DR. Routes and mechanisms of extracellular vesicle uptake. *J*
1458 *Extracell Vesicles* 2014;**3**.
1459 161. Takov K, Yellon DM, Davidson SM. Confounding factors in vesicle uptake studies using
1460 fluorescent lipophilic membrane dyes. *J Extracell Vesicles* 2017;**6**:1388731.
1461 162. Hegyesi H, Pallinger E, Mecsei S, Hornyak B, Kovacshazi C, Brenner GB, Giricz Z, Paloczi K,
1462 Kittel A, Tovari J, Turiak L, Khamari D, Ferdinandy P, Buzas EI. Circulating cardiomyocyte-
1463 derived extracellular vesicles reflect cardiac injury during systemic inflammatory response
1464 syndrome in mice. *Cell Mol Life Sci* 2022;**79**:84.
1465 163. Han Y, Jones TW, Dutta S, Zhu Y, Wang X, Narayanan SP, Fagan SC, Zhang D. Overview and
1466 Update on Methods for Cargo Loading into Extracellular Vesicles. *Processes (Basel)* 2021;**9**.
1467 164. de Abreu RC, Fernandes H, da Costa Martins PA, Sahoo S, Emanueli C, Ferreira L. Native and
1468 bioengineered extracellular vesicles for cardiovascular therapeutics. *Nat Rev Cardiol*
1469 2020;**17**:685-697.
1470 165. Beer KB, Fazeli G, Judasova K, Irmisch L, Causemann J, Mansfeld J, Wehman AM. Degron-
1471 tagged reporters probe membrane topology and enable the specific labelling of membrane-
1472 wrapped structures. *Nat Commun* 2019;**10**:3490.
1473 166. Verweij FJ, Revenu C, Arras G, Dingli F, Loew D, Pegtel DM, Follain G, Allio G, Goetz JG,
1474 Zimmermann P, Herbomel P, Del Bene F, Raposo G, van Niel G. Live Tracking of Inter-organ
1475 Communication by Endogenous Exosomes In Vivo. *Dev Cell* 2019;**48**:573-589 e574.
1476 167. Lai RC, Arslan F, Lee MM, Sze NS, Choo A, Chen TS, Salto-Tellez M, Timmers L, Lee CN, El
1477 Oakley RM, Pasterkamp G, de Kleijn DP, Lim SK. Exosome secreted by MSC reduces
1478 myocardial ischemia/reperfusion injury. *Stem Cell Res* 2010;**4**:214-222.
1479 168. Yang L, Zhu J, Zhang C, Wang J, Yue F, Jia X, Liu H. Stem cell-derived extracellular vesicles for
1480 myocardial infarction: a meta-analysis of controlled animal studies. *Aging (Albany NY)*
1481 2019;**11**:1129-1150.
1482 169. Zwetsloot PP, Vegh AM, Jansen of Lorkeers SJ, van Hout GP, Currie GL, Sena ES, Gremmels H,
1483 Buikema JW, Goumans MJ, Macleod MR, Doevendans PA, Chamuleau SA, Sluijter JP. Cardiac
1484 Stem Cell Treatment in Myocardial Infarction: A Systematic Review and Meta-Analysis of
1485 Preclinical Studies. *Circ Res* 2016;**118**:1223-1232.
1486 170. Goettsch C, Hutcheson JD, Aikawa M, Iwata H, Pham T, Nykjaer A, Kjolby M, Rogers M,
1487 Michel T, Shibasaki M, Hagita S, Kramann R, Rader DJ, Libby P, Singh SA, Aikawa E. Sortilin
1488 mediates vascular calcification via its recruitment into extracellular vesicles. *J Clin Invest*
1489 2016;**126**:1323-1336.
1490 171. Gimona M, Brizzi MF, Choo ABH, Dominici M, Davidson SM, Grillari J, Hermann DM, Hill AF,
1491 de Kleijn D, Lai RC, Lai CP, Lim R, Monguio-Tortajada M, Muraca M, Ochiya T, Ortiz LA, Toh
1492 WS, Yi YW, Witwer KW, Giebel B, Lim SK. Critical considerations for the development of
1493 potency tests for therapeutic applications of mesenchymal stromal cell-derived small
1494 extracellular vesicles. *Cytotherapy* 2021;**23**:373-380.
1495 172. Gobin J, Muradia G, Mehic J, Westwood C, Couvrette L, Stalker A, Bigelow S, Luebbert CC,
1496 Bissonnette FS, Johnston MJW, Sauve S, Tam RY, Wang L, Rosu-Myles M, Lavoie JR. Hollow-
1497 fiber bioreactor production of extracellular vesicles from human bone marrow mesenchymal
1498 stromal cells yields nanovesicles that mirrors the immuno-modulatory antigenic signature of
1499 the producer cell. *Stem Cell Res Ther* 2021;**12**:127.
1500 173. de Almeida Fuzeta M, Bernardes N, Oliveira FD, Costa AC, Fernandes-Platzgummer A,
1501 Farinha JP, Rodrigues CAV, Jung S, Tseng RJ, Milligan W, Lee B, Castanho M, Gaspar D, Cabral
1502 JMS, da Silva CL. Scalable Production of Human Mesenchymal Stromal Cell-Derived
1503 Extracellular Vesicles Under Serum-/Xeno-Free Conditions in a Microcarrier-Based
1504 Bioreactor Culture System. *Front Cell Dev Biol* 2020;**8**:553444.

1505 174. Wu JY, Li YJ, Hu XB, Huang S, Xiang DX. Preservation of small extracellular vesicles for
1506 functional analysis and therapeutic applications: a comparative evaluation of storage
1507 conditions. *Drug Deliv* 2021;**28**:162-170.
1508 175. van der Spoel TI, Vrijsen KR, Koudstaal S, Sluijter JP, Nijsen JF, de Jong HW, Hoefer IE, Cramer
1509 MJ, Doevendans PA, van Belle E, Chamuleau SA. Transendocardial cell injection is not
1510 superior to intracoronary infusion in a porcine model of ischaemic cardiomyopathy: a study
1511 on delivery efficiency. *J Cell Mol Med* 2012;**16**:2768-2776.
1512 176. Morishita M, Takahashi Y, Nishikawa M, Takakura Y. Pharmacokinetics of Exosomes-An
1513 Important Factor for Elucidating the Biological Roles of Exosomes and for the Development
1514 of Exosome-Based Therapeutics. *J Pharm Sci* 2017;**106**:2265-2269.
1515 177. Mol EA, Lei Z, Roefs MT, Bakker MH, Goumans MJ, Doevendans PA, Dankers PYW, Vader P,
1516 Sluijter JPG. Injectable Supramolecular Ureidopyrimidinone Hydrogels Provide Sustained
1517 Release of Extracellular Vesicle Therapeutics. *Adv Healthc Mater* 2019;**8**:e1900847.
1518 178. Smyth T, Kullberg M, Malik N, Smith-Jones P, Graner MW, Anchordoquy TJ. Biodistribution
1519 and delivery efficiency of unmodified tumor-derived exosomes. *J Control Release*
1520 2015;**199**:145-155.
1521 179. Hu L, Wickline SA, Hood JL. Magnetic resonance imaging of melanoma exosomes in lymph
1522 nodes. *Magn Reson Med* 2015;**74**:266-271.
1523 180. Hernandez MJ, Gaetani R, Pieters VM, Ng NW, Chang AE, Martin TR, van Ingen E, Mol EA,
1524 Sluijter JPG, Christman KL. Decellularized Extracellular Matrix Hydrogels as a Delivery
1525 Platform for MicroRNA and Extracellular Vesicle Therapeutics. *Adv Ther (Weinh)* 2018;**1**.
1526 181. Sahoo S, Kariya T, Ishikawa K. Targeted delivery of therapeutic agents to the heart. *Nat Rev*
1527 *Cardiol* 2021.
1528 182. Ilahibaks NF, Lei Z, Mol EA, Deshantri AK, Jiang L, Schiffelers RM, Vader P, Sluijter JPG.
1529 Biofabrication of Cell-Derived Nanovesicles: A Potential Alternative to Extracellular Vesicles
1530 for Regenerative Medicine. *Cells* 2019;**8**.
1531 183. Vader P, Mol EA, Pasterkamp G, Schiffelers RM. Extracellular vesicles for drug delivery. *Adv*
1532 *Drug Deliv Rev* 2016;**106**:148-156.
1533 184. Mackie AR, Klyachko E, Thorne T, Schultz KM, Millay M, Ito A, Kamide CE, Liu T, Gupta R,
1534 Sahoo S, Misener S, Kishore R, Losordo DW. Sonic hedgehog-modified human CD34+ cells
1535 preserve cardiac function after acute myocardial infarction. *Circ Res* 2012;**111**:312-321.
1536 185. Kooijmans SAA, Stremersch S, Braeckmans K, de Smedt SC, Hendrix A, Wood MJA, Schiffelers
1537 RM, Raemdonck K, Vader P. Electroporation-induced siRNA precipitation obscures the
1538 efficiency of siRNA loading into extracellular vesicles. *J Control Release* 2013;**172**:229-238.
1539 186. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of
1540 mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell*
1541 *Biol* 2007;**9**:654-659.
1542 187. Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJ. Delivery of siRNA to the mouse
1543 brain by systemic injection of targeted exosomes. *Nat Biotechnol* 2011;**29**:341-345.
1544 188. Luo W, Dai Y, Chen Z, Yue X, Andrade-Powell KC, Chang J. Spatial and temporal tracking of
1545 cardiac exosomes in mouse using a nano-luciferase-CD63 fusion protein. *Commun Biol*
1546 2020;**3**:114.
1547 189. Neckles VN, Morton MC, Holmberg JC, Sokolov AM, Nottoli T, Liu D, Feliciano DM. A
1548 transgenic inducible GFP extracellular-vesicle reporter (TIGER) mouse illuminates neonatal
1549 cortical astrocytes as a source of immunomodulatory extracellular vesicles. *Sci Rep*
1550 2019;**9**:3094.

1551