



King's Research Portal

DOI:

[10.1093/cvr/cvac031](https://doi.org/10.1093/cvr/cvac031)

Document Version

Peer reviewed version

[Link to publication record in King's Research Portal](#)

Citation for published version (APA):

Davidson, S. M., Boulanger, C. M., Aikawa, E., Badimon, L., Barile, L., Binder, C. J., Brisson, A., Buzas, E., Emanuelli, C., Jansen, F., Katsur, M., Lacroix, R., Lim, S. K., Mackman, N., Mayr, M., Menasché, P., Nieuwland, R., Sahoo, S., Takov, K., ... Sluijter, J. P. G. (2022). Methods for the identification and characterization of extracellular vesicles in cardiovascular studies - from exosomes to microvesicles. *Cardiovascular Research*, Article cvac031. Advance online publication. <https://doi.org/10.1093/cvr/cvac031>

Citing this paper

Please note that where the full-text provided on King's Research Portal is the Author Accepted Manuscript or Post-Print version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version for pagination, volume/issue, and date of publication details. And where the final published version is provided on the Research Portal, if citing you are again advised to check the publisher's website for any subsequent corrections.

General rights

Copyright and moral rights for the publications made accessible in the Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the Research Portal

Take down policy

If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

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

3 Sean M Davidson¹, Chantal M. Boulanger^{2#}, Elena Aikawa³, Lina Badimon⁴, Lucio Barile⁵,
4 Christoph J. Binder⁶, Alain Brisson⁷, Edit Buzas⁸, Costanza Emanuelli⁹, Felix Jansen¹⁰,
5 Miroslava Katsur¹, Romaric Lacroix^{11,12}, Sai Kiang Lim^{13,14}, Nigel Mackman¹⁵, Manuel Mayr¹⁶,
6 Philippe Menasché^{17,18}, Rienk Nieuwland^{19,20} Susmita Sahoo²¹, Kaloyan Takov¹⁶, Thomas
7 Thum^{22,23}, Pieter Vader^{2,24}, Marca H.M. Wauben²⁵, Kenneth Witwer²⁶, Joost P.G. Sluijter¹⁸
8 #corresponding author

9 ¹ The Hatter Cardiovascular Institute, University College London WC1E 6HX, United Kingdom

10 ² PARCC, INSERM, University of Paris, Paris, France

11 ³ Center for Excellence in Vascular Biology, Department of Medicine, Brigham and Women's
12 Hospital, Harvard Medical School, Boston, MA 02115, USA

13 ⁴ Cardiovascular Science Program-ICCC, IR-Hospital de la Santa Creu i Santa Pau-IIBSantPau,
14 CiberCV, Autonomous University of Barcelona, Barcelona

15 ⁵ Laboratory for Cardiovascular Theranostics, Istituto Cardiocentro Ticino, Ente Ospedaliero
16 Cantonale and Faculty of Biomedical Sciences, Università Svizzera italiana, 6900, Lugano,
17 Switzerland

18 ⁶ Department of Laboratory Medicine, Medical University of Vienna, Vienna, Austria

19 ⁷ Molecular Imaging and NanoBioTechnology, UMR-5248-CBMN, CNRS-University of
20 Bordeaux-IPB, Bat. B14, Allée Geoffroy Saint-Hilaire, 33600, Pessac, France

21 ⁸ Department of Genetics, Cell- and Immunobiology, Semmelweis University, HCEMM-SU and
22 ELKH-SE Immune Proteogenomics Extracellular Vesicle Research Group, Budapest, Hungary

23 ⁹ National Heart and Lung Institute, Imperial College London, Hammersmith Campus, London,
24 W12 0NN England, United Kingdom

25 ¹⁰ Heart Center, Department of Internal Medicine II, University Hospital Bonn, Germany

26 ¹¹ Aix Marseille University, INSERM 1263, Institut National de Recherche pour l'Agriculture,
27 l'Alimentation et l'Environnement (INRAE), Centre de Recherche en CardioVasculaire et
28 Nutrition (C2VN), Marseille, France

29 ¹² Haematology and Vascular biology department, CHU La Conception, APHM, Marseille,
30 France

1 ¹³ Institute of Medical Biology and Institute of Molecular and Cell Biology, Agency for Science,
2 Technology and Research, Singapore, Singapore

3 ¹⁴ Department of Surgery, Yong Loo Lin School of Medicine, National University of Singapore,
4 Singapore, Singapore.

5 ¹⁵ UNC Blood Research Center, Department of Medicine, University of North Carolina at Chapel
6 Hill, Chapel Hill NC

7 ¹⁶ King's College London British Heart Foundation Centre, School of Cardiovascular Medicine
8 and Sciences, London, UK

9 ¹⁷ Department of Cardiovascular Surgery, Hôpital Européen Georges Pompidou, Paris, France.

10 ¹⁸ Laboratory of Experimental Cardiology, Cardiology, UMC Utrecht Regenerative Medicine
11 Center and Circulatory Health Laboratory, Utrecht University, University Medical Center Utrecht,
12 Utrecht, The Netherlands

13 ¹⁹ Vesicle Observation Center, Amsterdam UMC, University of Amsterdam, Amsterdam, The
14 Netherlands

15 ²⁰ Laboratory of Experimental Clinical Chemistry, Amsterdam UMC, University of Amsterdam,
16 Amsterdam, The Netherlands

17 ²¹ Cardiovascular Research Institute, Icahn School of Medicine at Mount Sinai, New York, NY,
18 USA

19 ²² Institute of Molecular and Translational Therapeutic Strategies, Hannover Medical School,
20 Hannover, Germany

21 ²³ Fraunhofer Institute of Toxicology and Experimental Medicine, Hannover, Germany

22 ²⁴ CDL Research, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The
23 Netherlands.

24 ²⁵ Utrecht University, Faculty of Veterinary Medicine, Department of Biomolecular Health
25 Sciences, Yalelaan 2, Utrecht, The Netherlands

26 ²⁶ Departments of Molecular and Comparative Pathobiology and Neurology, Johns Hopkins
27 University School of Medicine, Baltimore, US

28 **Corresponding author:**

29 Chantal M. Boulanger, PhD
30 Paris Cardiovascular Research Center
31 56 rue Leblanc
32 75015 Paris, France
33 Tel +331 5398 8086
34 Chantal.boulanger@inserm.fr

35 **Manuscript type:** Original article

36 **Short title:** Methods for studying extracellular vesicles

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Abstract (179 of 200 words)

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

ACCEPTED MANUSCRIPT

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

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

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

22 In certain situations, EVs can contribute to the mechanism of cardiovascular diseases. For example, sEVs
23 contribute to the development of pulmonary arterial hypertension,^{13,14} and to vascular calcification.^{15,16}
24 Adipocyte-derived extracellular vesicles and their ceramide content have impact on cardiac mortality in
25 advanced atherosclerosis.^{16,17} Endothelial EVs released during myocardial infarction can mobilize splenic
26 neutrophils and monocytes following their transcriptional activation and could contribute to attenuated
27 cardiac function.^{18,19} Therefore, EVs are emerging as key players in different stages of disease
28 development of cardiovascular disease and metabolic syndrome (reviewed in²⁰⁻²²).

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

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

9 *1.1 Definition of extracellular vesicles and use of terminology*

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

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

37 **2. Source of EVs**

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

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

5 EVs from many different sources have been shown to improve cardiac function following MI, including
 6 cardiac stem cells,³⁶ cardiovascular progenitor cells (CPC),³⁷ endothelial progenitor cells,³⁸ cardiosphere-
 7 derived cells,³⁹ embryonic stem cells⁴⁰ and iPSC-derived cardiomyocytes⁴¹(reviewed in ⁵). EVs from the
 8 epicardium can promote proliferation of cardiomyocytes.⁴² EVs can also be beneficial against other
 9 forms of injury such as doxorubicin/trastuzumab-induced cardiac toxicity.²⁶ On the other hand, EVs can
 10 be detrimental, for example contributing to vascular smooth muscle cell calcification.^{15, 16} As yet, there is
 11 little consensus on the ideal source of EVs, however one head-to-head comparison suggests CPC may be
 12 more efficacious than BM-MSC.²⁵

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

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

21 **3. Methods of separation**

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

23 *3.1 Separation of EVs from cell culture medium*

24 Several techniques have been developed for the separation of EVs from cell culture medium, each with
 25 their advantages and disadvantages (**Table 1**). Most procedures are based on separation by size, and/or
 26 density, although many other extracellular particles may share these characteristics with EVs. A protocol
 27 of differential centrifugation or ultracentrifugation published by They *et al.* is commonly used to
 28 separate both sEVs and IEVs (**Box 1**).⁵⁴ A subsequent density-gradient separation using sucrose or,
 29 preferably, iodixanol, further improves EV purity.⁵⁵ Size-exclusion chromatography has become popular
 30 since it effectively removes part of the contaminating soluble protein, and columns can be readily made
 31 or purchased (**Figure 2D**).^{56, 57} Precipitation of sEVs is possible using polyethylene glycol (PEG)-based
 32 reagents, for example in HEK293 or MSC cultures,⁵⁸ but the purity obtained is generally inferior to other
 33 techniques.^{55, 59} Ultrafiltration is more commonly used as an initial clean-up step to remove larger (e.g.:
 34 >0.8 µM) contaminants because membranes can become blocked when filtering large volumes and
 35 because of concerns that high pressures may damage the membranes of larger EVs. Affinity isolation,
 36 typically using antibodies, provides highly pure isolates although at the expense of yield, and only a
 37 subset of EVs might be isolated.⁶⁰ Furthermore, the procedure to recover EVs from antibodies could

1 affect their functionality and requires testing.⁶¹ Diafiltration, asymmetric flow field-flow fractionation
2 (AF4)⁶² and tangential flow filtration⁶³ purify and concentrate sEV fractions and are scalable, but AF4
3 requires specialized and expensive equipment.

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

18 3.2 Separation of EVs from blood

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

31 3.3 Separation of EVs from tissue

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

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

4 **4. General principles for EV identification and characterization**

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

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

23 *4.1 Techniques for identifying EVs*

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

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

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

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

9 4.2 Electron microscopy

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

22 4.3 Flow cytometry

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

1 4.4 Functional analysis of EVs

2 Ideally, the functional activity of EVs would be assayed using a simple, *in vitro* potency assay as a
 3 surrogate for their *in vivo* functionality, but no single, universal method has been identified. In the
 4 cardiovascular field, EV function is commonly assessed using an assay of *in vitro* angiogenesis, cell
 5 viability, contractility, or combinations thereof. Commonly used *in vitro* assays of angiogenesis include
 6 the scratch assay,⁹¹ Boyden chamber migration assay,^{92,93} endothelial tube formation⁹⁴, and vessel
 7 sprouting assays.^{44,95,96} An accurate measure of sEV quantity and purity is important when conducting
 8 dose-response experiments of their functionality. At present there is no consensus on which measure of
 9 quantity (particle number, protein content, quantity of starting cells, etc) is preferable,³¹ but whichever
 10 normalization technique is used (preferably more than one) it should be reported and justified.
 11 Furthermore, appropriate (procedural) controls should be included to proof that effects are EV-
 12 mediated. For the use of EVs as therapeutic tools, *in vitro* potency assays are required to predict the
 13 effectiveness of EV preparations for clinical use, but this depends on the ability to convincingly identify
 14 the mechanism of action and quantify the biological activity.⁹⁷

15 4.5 Reporting methodology

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

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

21 5.1 Total protein content

22 Total protein content in an EV preparation can be estimated using standard protein assays such as
 23 bicinchoninic acid (BCA) assay or Bradford assay, or variations thereof, optimised for low protein
 24 concentrations. Quantification of total protein in an EV sample and comparison with particle counts may
 25 give an indication of its purity. It has been suggested that pure sEV isolates contain concentrations of < 1
 26 µg protein / 10¹⁰ EV particles,⁸¹ although this is not necessarily universally applicable, because there are
 27 not yet methods available that can measure all EVs.

28 5.2 Antibody-based techniques to identify specific proteins

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

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

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

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

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

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

24 Novel antibody-based approaches such as surface plasmon resonance¹⁰² and interferometric imaging¹⁰³
25 have also been utilized for EV protein characterisation, but they usually require expensive specialised
26 equipment and consumables which limits their widespread use.

27 *5.3 Mass spectrometry of the EV proteome*

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

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

4 *5.4 Intraluminal vs membrane proteins*

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

14 **6. Methods for determining the RNA content of EVs**

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

23 *6.1 RNA analyses by qRT-PCR and RNA-sequencing*

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

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

33 Certain pre-analytic confounders are well known, *e.g.*: heparin can interfere with PCR analyses of
 34 RNAs,¹¹⁸ but can be overcome by heparinase treatment. The presence of certain miRNAs is suggestive of
 35 haemolysis of blood samples (*e.g.*: miR-486-5p, miR-451, miR-92a, and miR-16), or presence of
 36 contaminating calf serum (*e.g.*: miR-122, miR-451a and miR-1246).¹¹⁹⁻¹²¹ Lipoprotein contamination can
 37 also create difficulties in data analyses and interpretation since they can also carry miRNAs¹²². To

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

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

10 *6.2 How to evaluate the functional role of EV RNA*

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

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

26 *7.1 Lipid content*

27 The phospholipid bilayer membrane of EVs consists primary of phosphatidylcholine, in addition to
 28 phosphatidylethanolamine and phosphatidylserine.^{62, 126} The sEV membrane is relatively rigid due to its
 29 enrichment in sphingomyelin and cholesterol, and contains domains with an ordered lipid phase (“lipid
 30 rafts”; reviewed in¹²⁷).

31 Notably, EVs also carry lipids involved in signalling such as eicosanoids together with functional
 32 phospholipases and enzymes of the prostaglandin pathway.¹²⁸ The lipid composition of large EVs is
 33 closer to that of the plasma membrane, which they originate from.¹²⁶ Translocation of
 34 phosphatidylserine to the outer leaflet upon cellular activation has been suggested to be a prerequisite
 35 for large EV biogenesis¹²⁷. EVs with externalized phosphatidylserine are highly pro-coagulant, leading to
 36 venous thrombosis, particularly in the presence of tissue factor (TF).¹²⁹

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

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

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

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

25 **8. Measurement of enzymatic activities carried by EVs**

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

31 **8.1 Pro-coagulant activity**

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

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

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

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

16 Comparisons of assays measuring EV-TF activity suggest that Factor Xa generation assays are more
17 sensitive than the Zymuphen assay,¹⁴⁷ and a poor correlation was found between results of the factor Xa
18 generation assay and the fibrin generation test.¹⁴⁸ ISTH initiated a new collaborative project to compare
19 the analytical performance of different assays measuring EV-TF in plasma samples¹⁴⁹ to progress
20 towards an optimal method to measure EV procoagulant activity in plasma samples.

21 *8.2 Fibrinolytic activity*

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

28 *8.3 Enzymatic activities*

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

37

1 **9. Methodologies for functional characterization of EVs**

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

- 6 i. In addition to EV purification and isolation, “EV-depleted” samples and quality and procedural
7 controls (e.g.: unconditioned cell-culture medium processed in the same way) can help to determine
8 true EV-mediated responses. GW4869, an inhibitor of neutral sphingomyelinase 2 (nSMase2) and
9 sEV release, is sometimes used as a control, but care is required in its use, as it is unlikely to be
10 specific for exosome release.^{31, 154}
- 11 ii. Co-purified and bound molecules might affect functional assays,¹⁵⁵ therefore it is best to avoid low-
12 specificity methods such as general precipitation (polyethylene glycol, “salting out,” the basis of
13 many commercial “exosome isolation” kits), unless these methods are combined with additional
14 separation steps.
- 15 iii. The biological nature of EV preparations makes normalization between conditions essential but
16 there is no clear consensus on the best way forward. Some alternatives include: starting volume or
17 the number of producing cells; total number of EVs; protein content; lipid content; metabolite
18 content; or specific markers such as levels of tetraspanins or other putative house-keeping proteins
19 or RNA species.¹⁵⁶ It is recommended to have 2-3 different approaches, and to clearly describe each,
20 to allow potential differences in functional outcomes to be explored.
- 21 iv. For clinical therapeutic interventions, the identity of the EV preparations can be defined using
22 quantifiable metrics.⁷⁹
- 23 v. In classical dose-response experiments, the relationship between the concentration of a ligand/drug
24 and a measured outcome parameter is investigated. Such experiments should be considered to
25 understand the dose-dependency of effects, and to understand the biological relevance of the
26 quantity of EVs used. In many published works, the dose relative to physiological concentration is
27 unclear.
- 28 vi. Profiling of the EVs proteome and RNAome also will help to characterize their origin and also
29 potential functional activities.¹⁵⁷

30 *9.1 Uptake and biodistribution studies*

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

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

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

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

29 **10. Methodologies for clinical use of EVs in cardiovascular diseases**

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

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

5 *10.1 Production and storage effects on the quality of EV preparations*

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

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

20 *10.2 Delivery strategies and biodistribution of EVs*

21 Efficient EV delivery to the target organ/cells may be necessary to achieve full therapeutic potential, but
22 it should also be considered that the primary target may not be the diseased tissue if EVs function
23 indirectly. Both systemic and intra-organ delivery is possible and close monitoring of EV biodistribution
24 is needed since cellular uptake of EVs might not be accurately reflected by the tracking-labels used. Due
25 to the small size of EVs, myocardial retention might be severely hampered since even stem cells, which
26 are much larger than EVs, are immediately washed out from the myocardium after injection.¹⁷⁵ EVs
27 delivered intravenously are rapidly cleared (within minutes) and mainly distribute to the liver.¹⁷⁶
28 Biodistribution studies, in which EVs are labelled with fluorescently linked lipid or amine dyes¹⁷⁷,
29 radiolabels¹⁷⁸ or iron oxide particles,¹⁷⁹ are highly warranted for mechanistic understanding of their
30 effects. To facilitate long-term exposure of EV therapeutics, slow-release systems in which EVs are
31 loaded and slowly exposed to the targeted tissue are key. Both natural¹⁸⁰ and synthetic¹⁷⁷ delivery
32 systems have been developed and display enhanced beneficial effects for cardiac repair³⁸, with the
33 caveat that they may require a direct intramyocardial delivery whose invasiveness may hamper their
34 clinical acceptance. An alternative approach that has been successfully used to promote cardiac repair
35 following myocardial infarction is thus to inject the EV-producing stem cells into a semi-permeable
36 chamber, which is then inserted subcutaneously to release EVs (and other factors) over time.³⁶

37

1 10.3 Loading therapeutics into EVs

2 For successful intra-myocardial delivery, many limitations and barriers have to be overcome,¹⁸¹ whereas
3 bioengineered EVs with surface and/or cargo modifications might present unique advantages.
4 Engineered therapeutic nanoparticles include: i) vesicle-mimetics produced from cells by serial extrusion
5 or cell membrane-cloaked nanoparticles, which have substantially greater yield and an easy purification
6 process¹⁸²; ii) EV-liposome hybrids, produced using simple incubation or freeze-thaw cycles, for easier
7 uptake by target cells and for enhanced delivery; and iii) synthetic EVs, which are based on liposomes
8 with a composition similar to EVs.

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

21 **11. Conclusion**

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

1 **12. Tables**2 **Table 1 Potential advantages and disadvantages of the main methods used to purify sEVs**

Method of purification	Disadvantages	Advantages
Affinity-based methods	<ul style="list-style-type: none"> • Low yield • Non-scalable • Antibodies are expensive and difficult to remove afterwards • Protein contaminants bind to the solid phase 	<ul style="list-style-type: none"> • Highly purified sEVs
Diafiltration	<ul style="list-style-type: none"> • Specialized equipment required 	<ul style="list-style-type: none"> • Membrane pores rarely block • Re-useable
Centrifugation (Pelleting)	<ul style="list-style-type: none"> • Labour intensive • Non-scalable • Expensive equipment required • Relatively low purity 	<ul style="list-style-type: none"> • Widely used • Standardised protocol (though may vary with different rotors)
Density gradient centrifugation	<ul style="list-style-type: none"> • Labour intensive • Non-scalable • Expensive and time consuming • It may be necessary to remove the gradient material, depending on subsequent analysis 	<ul style="list-style-type: none"> • Widely used • Standardised protocol
Field-flow fractionation	<ul style="list-style-type: none"> • Expensive equipment required • Extensive optimization required 	<ul style="list-style-type: none"> • High purity and yields can be achieved • Scalable
Precipitation	<ul style="list-style-type: none"> • Relatively low purity 	<ul style="list-style-type: none"> • Very rapid • “Home-made” techniques very cheap
Size-exclusion chromatography	<ul style="list-style-type: none"> • Labour intensive • Contaminants of a similar size of EVs may co-isolate 	<ul style="list-style-type: none"> • 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	<ul style="list-style-type: none"> • Expensive equipment required 	<ul style="list-style-type: none"> • Scalable • GMP-compliant
Ultrafiltration through a membrane	<ul style="list-style-type: none"> • Low purity • High pressures may damage the membranes of larger EVs • Membranes can become blocked when filtering large volumes 	<ul style="list-style-type: none"> • Scalable. • High yield • Cost-effective • More commonly used as an initial clean-up step or a concentration step post isolation

3

4

5

6

1

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

Source of EVs:	Major factors to consider	Potential solutions
Cell-culture conditioned medium containing serum	<ul style="list-style-type: none"> • Risk of contamination from serum components including animal-derived EVs coming from serum 	<ul style="list-style-type: none"> • Contaminating EVs can be pre-removed from serum • Consider using serum-free medium^a
Cell-culture conditioned medium without serum	<ul style="list-style-type: none"> • Risk of cell phenotypic changes/death contaminating EVs with intracellular or apoptotic vesicles 	<ul style="list-style-type: none"> • Use short-term culture • Quantify levels of cell death
Plasma	<ul style="list-style-type: none"> • 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 	<ul style="list-style-type: none"> • Carefully define suitable pre-analytical procedures • Isolate EVs using a combination of orthogonal techniques
Serum	<ul style="list-style-type: none"> • EVs are released from activated platelets • Challenging to remove contaminating blood proteins and lipoproteins • EVs lost in the fibrin clot 	<ul style="list-style-type: none"> • Carefully define suitable pre-analytical procedures. • Isolate EVs using a combination of orthogonal techniques.
Tissue (e.g. myocardium)	<ul style="list-style-type: none"> • Challenging to disrupt tissue without damaging the cell membrane • Risk of shaving epitopes from EVs when using proteolytic enzymes 	<ul style="list-style-type: none"> • Perform control experiments to ensure cells are not disrupted • Titrate enzyme quantity and use the minimum

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

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

8

9

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

Detection method	Advantages	Disadvantages
Capillary electrophoresis immunoassay ^b	<ul style="list-style-type: none"> Smaller sample volume required Ease of automation Fast separation and data acquisition 	<ul style="list-style-type: none"> Expensive instrumentation Limit of detection poorer than solid phase detection (e.g. immunoassay)
DELFA ^b	<ul style="list-style-type: none"> Microplate setup Higher throughput than immunoblotting Sufficient sensitivity with only one antibody 	<ul style="list-style-type: none"> Requires plate reader with time-resolved fluorescence (TRF) detector Risk of false positive signal with low specificity antibodies
Dot blotting ^b	<ul style="list-style-type: none"> Smaller sample volume required Protocols shorter than western blotting 	<ul style="list-style-type: none"> Molecular weight not determined Risk of false positive signal with low specificity antibodies
Flow cytometry	<ul style="list-style-type: none"> 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 	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> Can detect single small EVs Can use multiple detection antibodies 	<ul style="list-style-type: none"> Specialized equipment required Extensive protocol development required
Immunolectron microscopy (TEM or Cryo-TEM) ^b	<ul style="list-style-type: none"> Single particle detection Can distinguish membrane and intraluminal targets 	<ul style="list-style-type: none"> Expensive equipment Mostly qualitative
Mass spectrometry	<ul style="list-style-type: none"> Comprehensive picture of the EV proteome Quantitative analysis of more than one target protein Label-based approaches powerful for quantitative purposes 	<ul style="list-style-type: none"> Expensive equipment Lengthy sample preparation Substantial quantity required Poor limit of detection due to the presence of high-abundant contaminants
Sandwich ELISA ^b	<ul style="list-style-type: none"> Microplate setup Higher throughput than immunoblotting 	<ul style="list-style-type: none"> Risk of false positive signal with low specificity antibodies
Transmission electron microscopy (TEM)	<ul style="list-style-type: none"> Single EV detection Can distinguish membrane and intraluminal targets 	<ul style="list-style-type: none"> Expensive equipment Sample is dried so EV morphology is altered Mostly qualitative data
Cryo-transmission electron microscopy (Cryo-TEM)	<ul style="list-style-type: none"> As per TEM Shows native shape of EVs 	<ul style="list-style-type: none"> As per TEM
Western blotting ^b	<ul style="list-style-type: none"> Well-established protocols Molecular weight determined 	<ul style="list-style-type: none"> Large sample volume required Time-consuming Usually semi-quantitative

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

5 ^bAll techniques using antibodies require validation of antibody specificity and optimisation of their
6 concentrations and blocking reagents.

7

8

ACCEPTED MANUSCRIPT

1 **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)	<ul style="list-style-type: none"> Ischaemic mouse hearts cell lines 	<ul style="list-style-type: none"> EV-bound labels co-labelled with cardiac-specific cell types direct transfer <i>in vitro</i> cultures 	<ul style="list-style-type: none"> Well-established protocols 	<ul style="list-style-type: none"> Non-EV mediated dye transfer from EVs to other cells or organs. Free label transfer 	27 161
<ul style="list-style-type: none"> Donor cell RNA transfer cel-miR-39 overexpression donor cell (lipofectamine) 	<ul style="list-style-type: none"> <i>In vitro</i> cell model Perfusing isolated rat hearts 	<ul style="list-style-type: none"> Mouse proteins present in human cell lines Dose-dependent presence of increased cel-miR39 levels in cultured cells and ex vivo hearts 	<ul style="list-style-type: none"> Intact EV sorting and mechanisms Well-established protocols 	<ul style="list-style-type: none"> Variation in EV content due to donor cell changes 	186 25
EV siRNA loading	<ul style="list-style-type: none"> Electroporation 	<ul style="list-style-type: none"> Knock-down of target genes in organs 	<ul style="list-style-type: none"> 	<ul style="list-style-type: none"> Disruption of EV integrity and functionality 	187
Fusion proteins	<ul style="list-style-type: none"> Luciferase- or GFP-linked labels to CD9 or CD63 CD63-pHluorin 	<ul style="list-style-type: none"> Cardiac-specific EV tracking via Luciferase expression In vivo and in vitro EV release, transfer and function 	<ul style="list-style-type: none"> Direct EV visualizations EV release and organ specific uptake 	<ul style="list-style-type: none"> EV functionality disrupted Limited signal detection 	188 189 166
Degron reporters	<ul style="list-style-type: none"> In vitro cell models 	<ul style="list-style-type: none"> Highly sensitive EV release 	<ul style="list-style-type: none"> High sensitive 	<ul style="list-style-type: none"> Functional tools need donor/target manipulations 	165

2

1 **13. Figure Legends**

2

3 **Figure 1.**

4 The typical size range of the major lipid-bilayer EVs up to 1000 nm diameter.

5 ^aAs reported by Jeppesen et al.⁶⁰

6 ^bThe size of apoptotic vesicles/bodies can range up to 5 μm in diameter.

7 Please be aware that the diameter of EVs depends on the detection method used.

8 **Figure 2.**

9 Representative images of different techniques of EV characterization.

- 10 A) Transmission electron micrography (TEM) of multi-vesicular body (MVB) containing exosomes
11 (arrows) in primary HUVECs.
- 12 B) Transmission electron micrography (TEM) of negative-stained EVs isolated from HUVECs (sEV =
13 small EVs, lEV = large EVs).
- 14 C) Cryo-TEM of a single CD81+ EV from iPS-derived cardiovascular progenitor cells.³⁷ The lipid
15 bilayer is clearly resolved (arrow).
- 16 D) Fractionation of sEVs (purple) from proteins (green, blue) by size-exclusion chromatography.
- 17 E) Single frame from nanoparticle tracking analysis (NTA) of an sEV sample under constant flow,
18 showing particle tracks (red) and particle size-distribution (blue).
- 19 F) Representative trace of EV sample obtained using resistive pulse sensing (RPS).
- 20 G) Individual particles detected by RPS, with size determined relative to calibration beads of a
21 known size.
- 22 H) Size distribution of EVs obtained by RPS.
- 23

24 **Figure 3.**

25 Steps towards EV characterization, adapted from MISEV2018 guidelines.³¹

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

1

2 **14. Box 1**3 **The standard differential ultracentrifugation protocol for EV isolation, originally published by They et**
4 **al.⁵⁴**

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

11

ACCEPTED MANUSCRIPT

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40

15. Author contributions

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

16. Funding

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

17. Conflicts of Interest

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

18. References

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

- 1 circulating microvesicles predicts cardiovascular events in patients with coronary artery disease.
2 *J Am Heart Assoc* 2014;**3**:e001249.
- 3 13. Aliotta JM, Pereira M, Amaral A, Sorokina A, Igbinoba Z, Hasslinger A, El-Bizri R, Rounds SI,
4 Quesenberry PJ, Klinger JR. Induction of pulmonary hypertensive changes by extracellular
5 vesicles from monocrotaline-treated mice. *Cardiovasc Res* 2013;**100**:354-362.
- 6 14. Aliotta JM, Pereira M, Wen S, Dooner MS, Del Tatto M, Papa E, Goldberg LR, Baird GL,
7 Ventetuolo CE, Quesenberry PJ, Klinger JR. Exosomes induce and reverse monocrotaline-
8 induced pulmonary hypertension in mice. *Cardiovasc Res* 2016;**110**:319-330.
- 9 15. Kapustin AN, Chatrou ML, Drozdov I, Zheng Y, Davidson SM, Soong D, Furmanik M, Sanchis P, De
10 Rosales RT, Alvarez-Hernandez D, Shroff R, Yin X, Muller K, Skepper JN, Mayr M,
11 Reutelingsperger CP, Chester A, Bertazzo S, Schurgers LJ, Shanahan CM. Vascular smooth muscle
12 cell calcification is mediated by regulated exosome secretion. *Circ Res* 2015;**116**:1312-1323.
- 13 16. Durham AL, Speer MY, Scatena M, Giachelli CM, Shanahan CM. Role of smooth muscle cells in
14 vascular calcification: implications in atherosclerosis and arterial stiffness. *Cardiovasc Res*
15 2018;**114**:590-600.
- 16 17. Akawi N, Checa A, Antonopoulos AS, Akoumianakis I, Daskalaki E, Kotanidis CP, Kondo H, Lee K,
17 Yesilyurt D, Badi I, Polkinghorne M, Akbar N, Lundgren J, Chuaiphichai S, Choudhury R, Neubauer
18 S, Channon KM, Torekov SS, Wheelock CE, Antoniades C. Fat-Secreted Ceramides Regulate
19 Vascular Redox State and Influence Outcomes in Patients With Cardiovascular Disease. *J Am Coll*
20 *Cardiol* 2021;**77**:2494-2513.
- 21 18. Akbar N, Digby JE, Cahill TJ, Tavaré AN, Corbin AL, Saluja S, Dawkins S, Edgar L, Rawlings N,
22 Ziberna K, McNeill E, Oxford Acute Myocardial Infarction S, Johnson E, Aljabali AA, Dragovic RA,
23 Rohling M, Belgard TG, Udalova IA, Greaves DR, Channon KM, Riley PR, Anthony DC, Choudhury
24 RP. Endothelium-derived extracellular vesicles promote splenic monocyte mobilization in
25 myocardial infarction. *JCI Insight* 2017;**2**.
- 26 19. Akbar N, Braithwaite AT, Corr EM, Koelwyn GJ, van Solingen C, Cochain C, Saliba AE, Corbin A,
27 Pezzolla D, Moller Jorgensen M, Baek R, Edgar L, De Villiers C, Gunadasa-Rohling M, Banerjee A,
28 Paget D, Lee C, Hogg E, Costin A, Dhaliwal R, Johnson E, Krausgruber T, Riepsaame J, Melling GE,
29 Shanmuganathan M, Oxford Acute Myocardial Infarction S, Bock C, Carter DRF, Channon KM,
30 Riley PR, Udalova IA, Moore KJ, Anthony D, Choudhury RP. Rapid neutrophil mobilisation by
31 VCAM-1+ endothelial extracellular vesicles. *Cardiovasc Res* 2022.
- 32 20. Boulanger CM, Loyer X, Rautou PE, Amabile N. Extracellular vesicles in coronary artery disease.
33 *Nat Rev Cardiol* 2017;**14**:259-272.
- 34 21. Martínez MC, Andriantsitohaina R. Extracellular Vesicles in Metabolic Syndrome. *Circ Res*
35 2017;**120**:1674-1686.
- 36 22. Jansen F, Li Q, Pfeifer A, Werner N. Endothelial- and Immune Cell-Derived Extracellular Vesicles
37 in the Regulation of Cardiovascular Health and Disease. *JACC Basic Transl Sci* 2017;**2**:790-807.
- 38 23. Timmers L, Lim SK, Arslan F, Armstrong JS, Hofer IE, Doevendans PA, Piek JJ, El Oakley RM,
39 Choo A, Lee CN, Pasterkamp G, de Kleijn DP. Reduction of myocardial infarct size by human
40 mesenchymal stem cell conditioned medium. *Stem Cell Res* 2007;**1**:129-137.
- 41 24. Lai RC, Arslan F, Tan SS, Tan B, Choo A, Lee MM, Chen TS, Teh BJ, Eng JK, Sidik H, Tanavde V,
42 Hwang WS, Lee CN, El Oakley RM, Pasterkamp G, de Kleijn DP, Tan KH, Lim SK. Derivation and
43 characterization of human fetal MSCs: an alternative cell source for large-scale production of
44 cardioprotective microparticles. *J Mol Cell Cardiol* 2010;**48**:1215-1224.
- 45 25. Barile L, Cervio E, Lionetti V, Milano G, Ciullo A, Biemmi V, Bolis S, Altomare C, Matteucci M, Di
46 Silvestre D, Brambilla F, Fertig TE, Torre T, Demertzis S, Mauri P, Moccetti T, Vassalli G.
47 Cardioprotection by cardiac progenitor cell-secreted exosomes: role of pregnancy-associated
48 plasma protein-A. *Cardiovasc Res* 2018;**114**:992-1005.

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

- 1 Phinney DG, Pieters BC, Pink RC, Pisetsky DS, Pogge von Strandmann E, Polakovicova I, Poon IK,
 2 Powell BH, Prada I, Pulliam L, Quesenberry P, Radeghieri A, Raffai RL, Raimondo S, Rak J, Ramirez
 3 MI, Raposo G, Rayyan MS, Regev-Rudzki N, Ricklefs FL, Robbins PD, Roberts DD, Rodrigues SC,
 4 Rohde E, Rome S, Rouschop KM, Rughetti A, Russell AE, Saa P, Sahoo S, Salas-Huenuleo E,
 5 Sanchez C, Saugstad JA, Saul MJ, Schiffelers RM, Schneider R, Schoyen TH, Scott A, Shahaj E,
 6 Sharma S, Shatnyeva O, Shekari F, Shelke GV, Shetty AK, Shiba K, Siljander PR, Silva AM,
 7 Skowronek A, Snyder OL, 2nd, Soares RP, Sodar BW, Soekmadji C, Sotillo J, Stahl PD, Stoorvogel
 8 W, Stott SL, Strasser EF, Swift S, Tahara H, Tewari M, Timms K, Tiwari S, Tixeira R, Tkach M, Toh
 9 WS, Tomasini R, Torrecilhas AC, Tosar JP, Toxavidis V, Urbanelli L, Vader P, van Balkom BW, van
 10 der Grein SG, Van Deun J, van Herwijnen MJ, Van Keuren-Jensen K, van Niel G, van Royen ME,
 11 van Wijnen AJ, Vasconcelos MH, Vechetti IJ, Jr., Veit TD, Vella LJ, Velot E, Verweij FJ, Vestad B,
 12 Vinas JL, Visnovitz T, Vukman KV, Wahlgren J, Watson DC, Wauben MH, Weaver A, Webber JP,
 13 Weber V, Wehman AM, Weiss DJ, Welsh JA, Wendt S, Wheelock AM, Wiener Z, Witte L,
 14 Wolfram J, Xagorari A, Xander P, Xu J, Yan X, Yanez-Mo M, Yin H, Yuana Y, Zappulli V, Zarubova J,
 15 Zekas V, Zhang JY, Zhao Z, Zheng L, Zheutlin AR, Zickler AM, Zimmermann P, Zivkovic AM, Zocco
 16 D, Zuba-Surma EK. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a
 17 position statement of the International Society for Extracellular Vesicles and update of the
 18 MISEV2014 guidelines. *J Extracell Vesicles* 2018;**7**:1535750.
- 19 32. Mathieu M, Martin-Jaular L, Lavieu G, Thery C. Specificities of secretion and uptake of exosomes
 20 and other extracellular vesicles for cell-to-cell communication. *Nat Cell Biol* 2019;**21**:9-17.
- 21 33. Zhao J, Li X, Hu J, Chen F, Qiao S, Sun X, Gao L, Xie J, Xu B. Mesenchymal stromal cell-derived
 22 exosomes attenuate myocardial ischaemia-reperfusion injury through miR-182-regulated
 23 macrophage polarization. *Cardiovasc Res* 2019;**115**:1205-1216.
- 24 34. Takafuji Y, Hori M, Mizuno T, Harada-Shiba M. Humoral factors secreted from adipose tissue-
 25 derived mesenchymal stem cells ameliorate atherosclerosis in Ldlr-/- mice. *Cardiovasc Res*
 26 2019;**115**:1041-1051.
- 27 35. Mayourian J, Ceholski DK, Gorski PA, Mathiyalagan P, Murphy JF, Salazar SI, Stillitano F, Hare JM,
 28 Sahoo S, Hajjar RJ, Costa KD. Exosomal microRNA-21-5p Mediates Mesenchymal Stem Cell
 29 Paracrine Effects on Human Cardiac Tissue Contractility. *Circ Res* 2018;**122**:933-944.
- 30 36. Kompa AR, Greening DW, Kong AM, McMillan PJ, Fang H, Saxena R, Wong RCB, Lees JG,
 31 Sivakumaran P, Newcomb AE, Tannous BA, Kos C, Mariana L, Loudovaris T, Hausenloy DJ, Lim SY.
 32 Sustained subcutaneous delivery of secretome of human cardiac stem cells promotes cardiac
 33 repair following myocardial infarction. *Cardiovasc Res* 2021;**117**:918-929.
- 34 37. Lima Correa B, El Harane N, Gomez I, Rachid Hocine H, Vilar J, Desgres M, Bellamy V, Keirththana
 35 K, Guillas C, Perotto M, Pidial L, Alayrac P, Tran T, Tan S, Hamada T, Charron D, Brisson A,
 36 Renault NK, Al-Daccak R, Menasche P, Silvestre JS. Extracellular vesicles from human
 37 cardiovascular progenitors trigger a reparative immune response in infarcted hearts. *Cardiovasc*
 38 *Res* 2021;**117**:292-307.
- 39 38. Chen CW, Wang LL, Zaman S, Gordon J, Arisi MF, Venkataraman CM, Chung JJ, Hung G, Gaffey
 40 AC, Spruce LA, Fazelinia H, Gorman RC, Seeholzer SH, Burdick JA, Atluri P. Sustained release of
 41 endothelial progenitor cell-derived extracellular vesicles from shear-thinning hydrogels
 42 improves angiogenesis and promotes function after myocardial infarction. *Cardiovasc Res*
 43 2018;**114**:1029-1040.
- 44 39. Gallet R, Dawkins J, Valle J, Simsolo E, de Couto G, Middleton R, Tseliou E, Luthringer D, Kreke M,
 45 Smith RR, Marban L, Ghaleh B, Marban E. Exosomes secreted by cardiosphere-derived cells
 46 reduce scarring, attenuate adverse remodelling, and improve function in acute and chronic
 47 porcine myocardial infarction. *Eur Heart J* 2017;**38**:201-211.

- 1 40. Khan M, Nickoloff E, Abramova T, Johnson J, Verma SK, Krishnamurthy P, Mackie AR, Vaughan E,
2 Garikipati VN, Benedict C, Ramirez V, Lambers E, Ito A, Gao E, Misener S, Luongo T, Elrod J, Qin
3 G, Houser SR, Koch WJ, Kishore R. Embryonic stem cell-derived exosomes promote endogenous
4 repair mechanisms and enhance cardiac function following myocardial infarction. *Circ Res*
5 2015;**117**:52-64.
- 6 41. Gao L, Wang L, Wei Y, Krishnamurthy P, Walcott GP, Menasche P, Zhang J. Exosomes secreted by
7 hiPSC-derived cardiac cells improve recovery from myocardial infarction in swine. *Sci Transl Med*
8 2020;**12**.
- 9 42. Villa Del Campo C, Liaw NY, Gunadasa-Rohling M, Matthaei M, Braga L, Kennedy T, Salinas G,
10 Voigt N, Giacca M, Zimmermann WH, Riley PR. Regenerative potential of epicardium-derived
11 extracellular vesicles mediated by conserved miRNA transfer. *Cardiovasc Res* 2021.
- 12 43. Savina A, Furlan M, Vidal M, Colombo MI. Exosome release is regulated by a calcium-dependent
13 mechanism in K562 cells. *J Biol Chem* 2003;**278**:20083-20090.
- 14 44. Ribeiro-Rodrigues TM, Laundos TL, Pereira-Carvalho R, Batista-Almeida D, Pereira R, Coelho-
15 Santos V, Silva AP, Fernandes R, Zuzarte M, Enguita FJ, Costa MC, Pinto-do OP, Pinto MT,
16 Gouveia P, Ferreira L, Mason JC, Pereira P, Kwak BR, Nascimento DS, Girao H. Exosomes
17 secreted by cardiomyocytes subjected to ischaemia promote cardiac angiogenesis. *Cardiovasc*
18 *Res* 2017;**113**:1338-1350.
- 19 45. Gollmann-Tepekoylu C, Polzl L, Graber M, Hirsch J, Nagele F, Lobenwein D, Hess MW, Blumer
20 MJ, Kirchmair E, Zipperle J, Hromada C, Muhleder S, Hackl H, Hermann M, Al Khamisi H, Forster
21 M, Lichtenauer M, Mittermayr R, Paulus P, Fritsch H, Bonaros N, Kirchmair R, Sluijter JPG,
22 Davidson S, Grimm M, Holfeld J. miR-19a-3p containing exosomes improve function of ischaemic
23 myocardium upon shock wave therapy. *Cardiovasc Res* 2020;**116**:1226-1236.
- 24 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,
25 Yang Y. Atorvastatin enhances the therapeutic efficacy of mesenchymal stem cells-derived
26 exosomes in acute myocardial infarction via up-regulating long non-coding RNA H19. *Cardiovasc*
27 *Res* 2020;**116**:353-367.
- 28 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
29 Exercise-Derived Exosomal miR-342-5p: A Novel Exerkine for Cardioprotection. *Circ Res*
30 2019;**124**:1386-1400.
- 31 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, Das
32 S, Xiao J. Exercise-induced circulating extracellular vesicles protect against cardiac ischemia-
33 reperfusion injury. *Basic Res Cardiol* 2017;**112**:38.
- 34 49. Loyer X, Zlatanova I, Devue C, Yin M, Howangyin KY, Klaihmon P, Guerin CL, Kheloufi M, Vilar J,
35 Zannis K, Fleischmann BK, Hwang DW, Park J, Lee H, Menasche P, Silvestre JS, Boulanger CM.
36 Intra-Cardiac Release of Extracellular Vesicles Shapes Inflammation Following Myocardial
37 Infarction. *Circ Res* 2018;**123**:100-106.
- 38 50. Zietzer A, Steffen E, Niepmann S, Dusing P, Hosen MR, Liu W, Jamme P, Al-Kassou B, Goody PR,
39 Zimmer S, Reiners KS, Pfeifer A, Bohm M, Werner N, Nickenig G, Jansen F. MicroRNA-mediated
40 vascular intercellular communication is altered in chronic kidney disease. *Cardiovasc Res* 2020.
- 41 51. Davidson SM, Riquelme JA, Takov K, Vicencio JM, Boi-Doku C, Khoo V, Doreth C, Radenkovic D,
42 Lavandero S, Yellon DM. Cardioprotection mediated by exosomes is impaired in the setting of
43 type II diabetes but can be rescued by the use of non-diabetic exosomes in vitro. *J Cell Mol Med*
44 2018;**22**:141-151.
- 45 52. Wang X, Huang W, Liu G, Cai W, Millard RW, Wang Y, Chang J, Peng T, Fan GC. Cardiomyocytes
46 mediate anti-angiogenesis in type 2 diabetic rats through the exosomal transfer of miR-320 into
47 endothelial cells. *J Mol Cell Cardiol* 2014;**74**:139-150.

- 1 53. Palviainen M, Saari H, Karkkainen O, Pekkinen J, Auriola S, Yliperttula M, Puhka M, Hanhineva K,
2 Siljander PR. Metabolic signature of extracellular vesicles depends on the cell culture conditions.
3 *J Extracell Vesicles* 2019;**8**:1596669.
- 4 54. They C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell
5 culture supernatants and biological fluids. *Curr Protoc Cell Biol* 2006;**Chapter 3**:Unit 3 22.
- 6 55. Paolini L, Zendrini A, Di Noto G, Busatto S, Lottini E, Radeghieri A, Dossi A, Caneschi A, Ricotta D,
7 Bergese P. Residual matrix from different separation techniques impacts exosome biological
8 activity. *Sci Rep* 2016;**6**:23550.
- 9 56. Boing AN, van der Pol E, Grootemaat AE, Coumans FA, Sturk A, Nieuwland R. Single-step
10 isolation of extracellular vesicles by size-exclusion chromatography. *J Extracell Vesicles* 2014;**3**.
- 11 57. Nordin JZ, Lee Y, Vader P, Mager I, Johansson HJ, Heusermann W, Wiklander OP, Hallbrink M,
12 Seow Y, Bultema JJ, Gilthorpe J, Davies T, Fairchild PJ, Gabrielsson S, Meisner-Kober NC, Lehtio J,
13 Smith CI, Wood MJ, El Andaloussi S. Ultrafiltration with size-exclusion liquid chromatography for
14 high yield isolation of extracellular vesicles preserving intact biophysical and functional
15 properties. *Nanomedicine* 2015;**11**:879-883.
- 16 58. Ludwig AK, De Miroschedji K, Doepfner TR, Borger V, Ruesing J, Rebmann V, Durst S, Jansen S,
17 Bremer M, Behrmann E, Singer BB, Jastrow H, Kuhlmann JD, El Magraoui F, Meyer HE, Hermann
18 DM, Opalka B, Raunser S, Epple M, Horn PA, Giebel B. Precipitation with polyethylene glycol
19 followed by washing and pelleting by ultracentrifugation enriches extracellular vesicles from
20 tissue culture supernatants in small and large scales. *J Extracell Vesicles* 2018;**7**:1528109.
- 21 59. Dong L, Zieren RC, Horie K, Kim CJ, Mallick E, Jing Y, Feng M, Kuczler MD, Green J, Amend SR,
22 Witwer KW, de Reijke TM, Cho YK, Pienta KJ, Xue W. Comprehensive evaluation of methods for
23 small extracellular vesicles separation from human plasma, urine and cell culture medium. *J*
24 *Extracell Vesicles* 2020;**10**:e12044.
- 25 60. Jeppesen DK, Fenix AM, Franklin JL, Higginbotham JN, Zhang Q, Zimmerman LJ, Liebler DC, Ping
26 J, Liu Q, Evans R, Fissell WH, Patton JG, Rome LH, Burnette DT, Coffey RJ. Reassessment of
27 Exosome Composition. *Cell* 2019;**177**:428-445 e418.
- 28 61. Kang YT, Kim YJ, Bu J, Cho YH, Han SW, Moon BI. High-purity capture and release of circulating
29 exosomes using an exosome-specific dual-patterned immunofiltration (ExoDIF) device.
30 *Nanoscale* 2017;**9**:13495-13505.
- 31 62. Zhang H, Freitas D, Kim HS, Fabijanic K, Li Z, Chen H, Mark MT, Molina H, Martin AB, Bojmar L,
32 Fang J, Rampersaud S, Hoshino A, Matei I, Kenific CM, Nakajima M, Mutvei AP, Sansone P,
33 Buehring W, Wang H, Jimenez JP, Cohen-Gould L, Paknejad N, Brendel M, Manova-Todorova K,
34 Magalhaes A, Ferreira JA, Osorio H, Silva AM, Massey A, Cubillos-Ruiz JR, Galletti G, Giannakakou
35 P, Cuervo AM, Blenis J, Schwartz R, Brady MS, Peinado H, Bromberg J, Matsui H, Reis CA, Lyden
36 D. Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric
37 flow field-flow fractionation. *Nat Cell Biol* 2018;**20**:332-343.
- 38 63. Andriolo G, Provasi E, Lo Cicero V, Brambilla A, Soncin S, Torre T, Milano G, Biemmi V, Vassalli G,
39 Turchetto L, Barile L, Radrizzani M. Exosomes From Human Cardiac Progenitor Cells for
40 Therapeutic Applications: Development of a GMP-Grade Manufacturing Method. *Front Physiol*
41 2018;**9**:1169.
- 42 64. Takov K, Yellon DM, Davidson SM. Comparison of small extracellular vesicles isolated from
43 plasma by ultracentrifugation or size-exclusion chromatography: yield, purity and functional
44 potential. *J Extracell Vesicles* 2019;**8**:1560809.
- 45 65. Mol EA, Goumans MJ, Doevendans PA, Sluijter JPG, Vader P. Higher functionality of extracellular
46 vesicles isolated using size-exclusion chromatography compared to ultracentrifugation.
47 *Nanomedicine* 2017;**13**:2061-2065.

- 1 66. Lehrich BM, Liang Y, Fiandaca MS. Foetal bovine serum influence on in vitro extracellular vesicle
2 analyses. *J Extracell Vesicles* 2021;**10**:e12061.
- 3 67. Yuana Y, Boing AN, Grootemaat AE, van der Pol E, Hau CM, Cizmar P, Buhr E, Sturk A, Nieuwland
4 R. Handling and storage of human body fluids for analysis of extracellular vesicles. *J Extracell
5 Vesicles* 2015;**4**:29260.
- 6 68. Lacroix R, Judicone C, Mooberry M, Boucekine M, Key NS, Dignat-George F, The ISSCW.
7 Standardization of pre-analytical variables in plasma microparticle determination: results of the
8 International Society on Thrombosis and Haemostasis SSC Collaborative workshop. *J Thromb
9 Haemost* 2013;**11**:1190-1193.
- 10 69. Palviainen M, Saraswat M, Varga Z, Kitka D, Neuvonen M, Puhka M, Joenvaara S, Renkonen R,
11 Nieuwland R, Takatalo M, Siljander PRM. Extracellular vesicles from human plasma and serum
12 are carriers of extravesicular cargo-Implications for biomarker discovery. *PLoS One*
13 2020;**15**:e0236439.
- 14 70. Vicencio JM, Yellon DM, Sivaraman V, Das D, Boi-Doku C, Arjun S, Zheng Y, Riquelme JA, Kearney
15 J, Sharma V, Multhoff G, Hall AR, Davidson SM. Plasma exosomes protect the myocardium from
16 ischemia-reperfusion injury. *J Am Coll Cardiol* 2015;**65**:1525-1536.
- 17 71. Simonsen JB. What Are We Looking At? Extracellular Vesicles, Lipoproteins, or Both? *Circ Res*
18 2017;**121**:920-922.
- 19 72. Zhang X, Borg EGF, Liaci AM, Vos HR, Stoorvogel W. A novel three step protocol to isolate
20 extracellular vesicles from plasma or cell culture medium with both high yield and purity. *J
21 Extracell Vesicles* 2020;**9**:1791450.
- 22 73. Clayton A, Boilard E, Buzas EI, Cheng L, Falcon-Perez JM, Gardiner C, Gustafson D, Gualerzi A,
23 Hendrix A, Hoffman A, Jones J, Lasser C, Lawson C, Lenassi M, Nazarenko I, O'Driscoll L, Pink R,
24 Siljander PR, Soekmadji C, Wauben M, Welsh JA, Witwer K, Zheng L, Nieuwland R.
25 Considerations towards a roadmap for collection, handling and storage of blood extracellular
26 vesicles. *J Extracell Vesicles* 2019;**8**:1647027.
- 27 74. Leroyer AS, Ebrahimian TG, Cochain C, Recalde A, Blanc-Brude O, Mees B, Vilar J, Tedgui A, Levy
28 BI, Chimini G, Boulanger CM, Silvestre JS. Microparticles from ischemic muscle promotes
29 postnatal vasculogenesis. *Circulation* 2009;**119**:2808-2817.
- 30 75. Perrotta I, Aquila S. Exosomes in human atherosclerosis: An ultrastructural analysis study.
31 *Ultrastruct Pathol* 2016;**40**:101-106.
- 32 76. Crescitelli R, Lasser C, Lotvall J. Isolation and characterization of extracellular vesicle
33 subpopulations from tissues. *Nat Protoc* 2021.
- 34 77. Claridge B, Rai A, Fang H, Matsumoto A, Luo J, McMullen JR, Greening DW. Proteome
35 characterisation of extracellular vesicles isolated from heart. *Proteomics* 2021;**21**:e2100026.
- 36 78. Leroyer AS, Isobe H, Leseche G, Castier Y, Wassef M, Mallat Z, Binder BR, Tedgui A, Boulanger
37 CM. Cellular origins and thrombogenic activity of microparticles isolated from human
38 atherosclerotic plaques. *J Am Coll Cardiol* 2007;**49**:772-777.
- 39 79. Witwer KW, Van Balkom BWM, Bruno S, Choo A, Dominici M, Gimona M, Hill AF, De Kleijn D,
40 Koh M, Lai RC, Mitsialis SA, Ortiz LA, Rohde E, Asada T, Toh WS, Weiss DJ, Zheng L, Giebel B, Lim
41 SK. Defining mesenchymal stromal cell (MSC)-derived small extracellular vesicles for therapeutic
42 applications. *J Extracell Vesicles* 2019;**8**:1609206.
- 43 80. van der Pol E, Coumans FA, Grootemaat AE, Gardiner C, Sargent IL, Harrison P, Sturk A, van
44 Leeuwen TG, Nieuwland R. Particle size distribution of exosomes and microvesicles determined
45 by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and
46 resistive pulse sensing. *J Thromb Haemost* 2014;**12**:1182-1192.
- 47 81. Webber J, Clayton A. How pure are your vesicles? *J Extracell Vesicles* 2013;**2**.

- 1 82. Liao Z, Jaular LM, Soueidi E, Jouve M, Muth DC, Schoyen TH, Seale T, Haughey NJ, Ostrowski M,
2 Thery C, Witwer KW. Acetylcholinesterase is not a generic marker of extracellular vesicles. *J*
3 *Extracell Vesicles* 2019;**8**:1628592.
- 4 83. Arraud N, Linares R, Tan S, Gounou C, Pasquet JM, Mornet S, Brisson AR. Extracellular vesicles
5 from blood plasma: determination of their morphology, size, phenotype and concentration. *J*
6 *Thromb Haemost* 2014;**12**:614-627.
- 7 84. Ridolfi A, Brucale M, Montis C, Caselli L, Paolini L, Borup A, Boysen AT, Loria F, van Herwijnen
8 MJC, Kleinjan M, Nejsum P, Zarovni N, Wauben MHM, Berti D, Bergese P, Valle F. AFM-Based
9 High-Throughput Nanomechanical Screening of Single Extracellular Vesicles. *Anal Chem*
10 2020;**92**:10274-10282.
- 11 85. Nolan JP. Flow Cytometry of Extracellular Vesicles: Potential, Pitfalls, and Prospects. *Curr Protoc*
12 *Cytom* 2015;**73**:13 14 11-13 14 16.
- 13 86. Welsh JA, Van Der Pol E, Arkesteijn GJA, Bremer M, Brisson A, Coumans F, Dignat-George F,
14 Duggan E, Ghiran I, Giebel B, Gorgens A, Hendrix A, Lacroix R, Lannigan J, Libregts S, Lozano-
15 Andres E, Morales-Kastresana A, Robert S, De Rond L, Tertel T, Tigges J, De Wever O, Yan X,
16 Nieuwland R, Wauben MHM, Nolan JP, Jones JC. MIFlowCyt-EV: a framework for standardized
17 reporting of extracellular vesicle flow cytometry experiments. *J Extracell Vesicles*
18 2020;**9**:1713526.
- 19 87. Libregts S, Arkesteijn GJA, Nemeth A, Nolte-'t Hoen ENM, Wauben MHM. Flow cytometric
20 analysis of extracellular vesicle subsets in plasma: impact of swarm by particles of non-interest. *J*
21 *Thromb Haemost* 2018;**16**:1423-1436.
- 22 88. Amabile N, Cheng S, Renard JM, Larson MG, Ghorbani A, McCabe E, Griffin G, Guerin C, Ho JE,
23 Shaw SY, Cohen KS, Vasani RS, Tedgui A, Boulanger CM, Wang TJ. Association of circulating
24 endothelial microparticles with cardiometabolic risk factors in the Framingham Heart Study. *Eur*
25 *Heart J* 2014;**35**:2972-2979.
- 26 89. Krankel N, Strassler E, Uhlemann M, Muller M, Briand-Schumacher S, Klingenberg R, Schulze PC,
27 Adams V, Schuler G, Luscher TF, Mobius-Winkler S, Landmesser U. Extracellular vesicle species
28 differentially affect endothelial cell functions and differentially respond to exercise training in
29 patients with chronic coronary syndromes. *Eur J Prev Cardiol* 2021;**28**:1467-1474.
- 30 90. Koganti S, Eleftheriou D, Gurung R, Hong Y, Brogan P, Rakhit RD. Persistent circulating platelet
31 and endothelial derived microparticle signature may explain on-going pro-thrombogenicity after
32 acute coronary syndrome. *Thromb Res* 2021;**206**:60-65.
- 33 91. Anselmo A, Frank D, Papa L, Viviani Anselmi C, Di Pasquale E, Mazzola M, Panico C, Clemente F,
34 Soldani C, Pagiatakis C, Hinkel R, Thalmann R, Kozlik-Feldmann R, Miragoli M, Carullo P,
35 Vacchiano M, Chaves-Sanjuan A, Santo N, Losi MA, Ferrari MC, Puca AA, Christiansen V, Seoudy
36 H, Freitag-Wolf S, Frey N, Dempfle A, Mercola M, Esposito G, Briguori C, Kupatt C, Condorelli G.
37 Myocardial hypoxic stress mediates functional cardiac extracellular vesicle release. *Eur Heart J*
38 2021;**42**:2780-2792.
- 39 92. Boyden S. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear
40 leucocytes. *J Exp Med* 1962;**115**:453-466.
- 41 93. Takov K, He Z, Johnston HE, Timms JF, Guillot PV, Yellon DM, Davidson SM. Small extracellular
42 vesicles secreted from human amniotic fluid mesenchymal stromal cells possess
43 cardioprotective and promigratory potential. *Basic Res Cardiol* 2020;**115**:26.
- 44 94. Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for
45 analysis of cell migration in vitro. *Nat Protoc* 2007;**2**:329-333.
- 46 95. Todorova D, Simoncini S, Lacroix R, Sabatier F, Dignat-George F. Extracellular Vesicles in
47 Angiogenesis. *Circ Res* 2017;**120**:1658-1673.

- 1 96. Baker M, Robinson SD, Lechertier T, Barber PR, Tavora B, D'Amico G, Jones DT, Vojnovic B,
2 Hodivala-Dilke K. Use of the mouse aortic ring assay to study angiogenesis. *Nat Protoc*
3 2011;**7**:89-104.
- 4 97. Gimona M, Brizzi MF, Choo ABH, M. D, M. DS, Grillari J, Hermann DM, Hill AF, de Kleijn D, Lai RC,
5 Lai C, Lim R, M. M-T, Muraca M, Ochiya T, Ortiz LA, Toh WS, Yi YW, Witwer KW, Giebel B, Lim SK.
6 Critical considerations for the development of potency tests for therapeutic applications of
7 mesenchymal stromal cell (MSC)-derived small extracellular vesicles. *Cytotherapy* 2021;**In press**.
- 8 98. Consortium E-T, Van Deun J, Mestdagh P, Agostinis P, Akay O, Anand S, Anckaert J, Martinez ZA,
9 Baetens T, Beghein E, Bertier L, Berx G, Boere J, Boukouris S, Bremer M, Buschmann D, Byrd JB,
10 Casert C, Cheng L, Cmoch A, Daveloose D, De Smedt E, Demirsoy S, Depoorter V, Dhondt B,
11 Driedonks TA, Dudek A, Elsharawy A, Floris I, Foers AD, Gartner K, Garg AD, Geeurickx E,
12 Gettemans J, Ghazavi F, Giebel B, Kormelink TG, Hancock G, Helmoortel H, Hill AF, Hyenne V,
13 Kalra H, Kim D, Kowal J, Kraemer S, Leidinger P, Leonelli C, Liang Y, Lippens L, Liu S, Lo Cicero A,
14 Martin S, Mathivanan S, Mathiyalagan P, Matusek T, Milani G, Monguio-Tortajada M, Mus LM,
15 Muth DC, Nemeth A, Nolte-'t Hoen EN, O'Driscoll L, Palmulli R, Pfaffl MW, Primdal-Bengtson B,
16 Romano E, Rousseau Q, Sahoo S, Sampaio N, Samuel M, Scicluna B, Soen B, Steels A, Swinnen JV,
17 Takatalo M, Thaminy S, Thery C, Tulkens J, Van Audenhove I, van der Grein S, Van Goethem A,
18 van Herwijnen MJ, Van Niel G, Van Roy N, Van Vliet AR, Vandamme N, Vanhauwaert S,
19 Vergauwen G, Verweij F, Wallaert A, Wauben M, Witwer KW, Zonneveld MI, De Wever O,
20 Vandesompele J, Hendrix A. EV-TRACK: transparent reporting and centralizing knowledge in
21 extracellular vesicle research. *Nat Methods* 2017;**14**:228-232.
- 22 99. Ghosh R, Gilda JE, Gomes AV. The necessity of and strategies for improving confidence in the
23 accuracy of western blots. *Expert Rev Proteomics* 2014;**11**:549-560.
- 24 100. Nelson GM, Guynn JM, Chorley BN. Procedure and Key Optimization Strategies for an
25 Automated Capillary Electrophoretic-based Immunoassay Method. *J Vis Exp* 2017.
- 26 101. Welton JL, Webber JP, Botos LA, Jones M, Clayton A. Ready-made chromatography columns for
27 extracellular vesicle isolation from plasma. *J Extracell Vesicles* 2015;**4**:27269.
- 28 102. Im H, Shao H, Park YI, Peterson VM, Castro CM, Weissleder R, Lee H. Label-free detection and
29 molecular profiling of exosomes with a nano-plasmonic sensor. *Nat Biotechnol* 2014;**32**:490-495.
- 30 103. Daaboul GG, Gagni P, Benussi L, Bettotti P, Ciani M, Cretich M, Freedman DS, Ghidoni R,
31 Ozkumur AY, Piotto C, Prospero D, Santini B, Unlu MS, Chiari M. Digital Detection of Exosomes by
32 Interferometric Imaging. *Sci Rep* 2016;**6**:37246.
- 33 104. Kowal J, Arras G, Colombo M, Jouve M, Morath JP, Primdal-Bengtson B, Dingli F, Loew D, Tkach
34 M, Thery C. Proteomic comparison defines novel markers to characterize heterogeneous
35 populations of extracellular vesicle subtypes. *Proc Natl Acad Sci U S A* 2016;**113**:E968-977.
- 36 105. Karimi N, Cvjetkovic A, Jang SC, Crescitelli R, Hosseinpour Feizi MA, Nieuwland R, Lotvall J, Lasser
37 C. Detailed analysis of the plasma extracellular vesicle proteome after separation from
38 lipoproteins. *Cell Mol Life Sci* 2018;**75**:2873-2886.
- 39 106. Kalra H, Simpson RJ, Ji H, Aikawa E, Altevogt P, Askenase P, Bond VC, Borrás FE, Breakefield X,
40 Budnik V, Buzas E, Camussi G, Clayton A, Cocucci E, Falcon-Perez JM, Gabrielsson S, Gho YS,
41 Gupta D, Harsha HC, Hendrix A, Hill AF, Inal JM, Jenster G, Kramer-Albers EM, Lim SK, Llorente A,
42 Lotvall J, Marcilla A, Mincheva-Nilsson L, Nazarenko I, Nieuwland R, Nolte-'t Hoen EN, Pandey A,
43 Patel T, Piper MG, Pluchino S, Prasad TS, Rajendran L, Raposo G, Record M, Reid GE, Sanchez-
44 Madrid F, Schiffelers RM, Siljander P, Stensballe A, Stoorvogel W, Taylor D, Thery C, Valadi H,
45 van Balkom BW, Vazquez J, Vidal M, Wauben MH, Yanez-Mo M, Zoeller M, Mathivanan S.
46 Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation.
47 *PLoS Biol* 2012;**10**:e1001450.

- 1 107. Osteikoetxea X, Sodar B, Nemeth A, Szabo-Taylor K, Paloczi K, Vukman KV, Tamasi V, Balogh A,
2 Kittel A, Pallinger E, Buzas EI. Differential detergent sensitivity of extracellular vesicle
3 subpopulations. *Org Biomol Chem* 2015;**13**:9775-9782.
- 4 108. Foers AD, Chatfield S, Dagley LF, Scicluna BJ, Webb AI, Cheng L, Hill AF, Wicks IP, Pang KC.
5 Enrichment of extracellular vesicles from human synovial fluid using size exclusion
6 chromatography. *J Extracell Vesicles* 2018;**7**:1490145.
- 7 109. Mayr M, Grainger D, Mayr U, Leroyer AS, Leseche G, Sidibe A, Herbin O, Yin X, Gomes A, Madhu
8 B, Griffiths JR, Xu Q, Tedgui A, Boulanger CM. Proteomics, metabolomics, and immunomics on
9 microparticles derived from human atherosclerotic plaques. *Circ Cardiovasc Genet* 2009;**2**:379-
10 388.
- 11 110. Lasser C, Shelke GV, Yeri A, Kim DK, Crescitelli R, Raimondo S, Sjostrand M, Gho YS, Van Keuren
12 Jensen K, Lotvall J. Two distinct extracellular RNA signatures released by a single cell type
13 identified by microarray and next-generation sequencing. *RNA Biol* 2017;**14**:58-72.
- 14 111. Chevillet JR, Kang Q, Ruf IK, Briggs HA, Vojtech LN, Hughes SM, Cheng HH, Arroyo JD, Meredith
15 EK, Gallichotte EN, Pogosova-Agadjanian EL, Morrissey C, Stirewalt DL, Hladik F, Yu EY, Higano
16 CS, Tewari M. Quantitative and stoichiometric analysis of the microRNA content of exosomes.
17 *Proc Natl Acad Sci U S A* 2014;**111**:14888-14893.
- 18 112. Mateescu B, Kowal EJ, van Balkom BW, Bartel S, Bhattacharyya SN, Buzas EI, Buck AH, de Candia
19 P, Chow FW, Das S, Driedonks TA, Fernandez-Messina L, Haderk F, Hill AF, Jones JC, Van Keuren-
20 Jensen KR, Lai CP, Lasser C, Liegro ID, Lunavat TR, Lorenowicz MJ, Maas SL, Mager I, Mittelbrunn
21 M, Momma S, Mukherjee K, Nawaz M, Pegtel DM, Pfaffl MW, Schiffelers RM, Tahara H, Thery C,
22 Tosar JP, Wauben MH, Witwer KW, Nolte-'t Hoen EN. Obstacles and opportunities in the
23 functional analysis of extracellular vesicle RNA - an ISEV position paper. *J Extracell Vesicles*
24 2017;**6**:1286095.
- 25 113. Zietzer A, Hosen MR, Wang H, Goody PR, Sylvester M, Latz E, Nickenig G, Werner N, Jansen F.
26 The RNA-binding protein hnRNPU regulates the sorting of microRNA-30c-5p into large
27 extracellular vesicles. *J Extracell Vesicles* 2020;**9**:1786967.
- 28 114. Bellingham SA, Shambrook M, Hill AF. Quantitative Analysis of Exosomal miRNA via qPCR and
29 Digital PCR. *Methods Mol Biol* 2017;**1545**:55-70.
- 30 115. Godoy PM, Bhakta NR, Barczak AJ, Cakmak H, Fisher S, MacKenzie TC, Patel T, Price RW, Smith
31 JF, Woodruff PG, Erle DJ. Large Differences in Small RNA Composition Between Human Biofluids.
32 *Cell Rep* 2018;**25**:1346-1358.
- 33 116. Veziroglu EM, Mias GI. Characterizing Extracellular Vesicles and Their Diverse RNA Contents.
34 *Front Genet* 2020;**11**:700.
- 35 117. Li S, Li Y, Chen B, Zhao J, Yu S, Tang Y, Zheng Q, Li Y, Wang P, He X, Huang S. exoRBase: a
36 database of circRNA, lncRNA and mRNA in human blood exosomes. *Nucleic Acids Res*
37 2018;**46**:D106-D112.
- 38 118. Boeckel JN, Thome CE, Leistner D, Zeiher AM, Fichtlscherer S, Dimmeler S. Heparin selectively
39 affects the quantification of microRNAs in human blood samples. *Clin Chem* 2013;**59**:1125-1127.
- 40 119. Pritchard CC, Kroh E, Wood B, Arroyo JD, Dougherty KJ, Miyaji MM, Tait JF, Tewari M. Blood cell
41 origin of circulating microRNAs: a cautionary note for cancer biomarker studies. *Cancer Prev Res*
42 (*Phila*) 2012;**5**:492-497.
- 43 120. Wei Z, Batagov AO, Carter DR, Krichevsky AM. Fetal Bovine Serum RNA Interferes with the Cell
44 Culture derived Extracellular RNA. *Sci Rep* 2016;**6**:31175.
- 45 121. Tosar JP, Cayota A, Eitan E, Halushka MK, Witwer KW. Ribonucleic artefacts: are some
46 extracellular RNA discoveries driven by cell culture medium components? *J Extracell Vesicles*
47 2017;**6**:1272832.

- 1 122. Ben-Aicha S, Escate R, Casani L, Padro T, Pena E, Arderiu G, Mendieta G, Badimon L, Vilahur G.
2 High-density lipoprotein remodelled in hypercholesterolaemic blood induce epigenetically
3 driven down-regulation of endothelial HIF-1alpha expression in a preclinical animal model.
4 *Cardiovasc Res* 2020;**116**:1288-1299.
- 5 123. Das S, Extracellular RNACC, Ansel KM, Bitzer M, Breakefield XO, Charest A, Galas DJ, Gerstein
6 MB, Gupta M, Milosavljevic A, McManus MT, Patel T, Raffai RL, Rozowsky J, Roth ME, Saugstad
7 JA, Van Keuren-Jensen K, Weaver AM, Laurent LC. The Extracellular RNA Communication
8 Consortium: Establishing Foundational Knowledge and Technologies for Extracellular RNA
9 Research. *Cell* 2019;**177**:231-242.
- 10 124. Zomer A, Maynard C, Verweij FJ, Kamermans A, Schafer R, Beerling E, Schiffelers RM, de Wit E,
11 Berenguer J, Ellenbroek SIJ, Wurdinger T, Pegtel DM, van Rheenen J. In Vivo imaging reveals
12 extracellular vesicle-mediated phenocopying of metastatic behavior. *Cell* 2015;**161**:1046-1057.
- 13 125. de Jong OG, Murphy DE, Mager I, Willms E, Garcia-Guerra A, Gitz-Francois JJ, Lefferts J, Gupta D,
14 Steenbeek SC, van Rheenen J, El Andaloussi S, Schiffelers RM, Wood MJA, Vader P. A CRISPR-
15 Cas9-based reporter system for single-cell detection of extracellular vesicle-mediated functional
16 transfer of RNA. *Nat Commun* 2020;**11**:1113.
- 17 126. Durcin M, Fleury A, Taillebois E, Hilairret G, Krupova Z, Henry C, Truchet S, Trotsmuller M, Kofeler
18 H, Mabileau G, Hue O, Andriantsitohaina R, Martin P, Le Lay S. Characterisation of adipocyte-
19 derived extracellular vesicle subtypes identifies distinct protein and lipid signatures for large and
20 small extracellular vesicles. *J Extracell Vesicles* 2017;**6**:1305677.
- 21 127. Record M, Silvente-Poirot S, Poirot M, Wakelam MJO. Extracellular vesicles: lipids as key
22 components of their biogenesis and functions. *J Lipid Res* 2018;**59**:1316-1324.
- 23 128. Subra C, Grand D, Laulagnier K, Stella A, Lambeau G, Paillasse M, De Medina P, Monsarrat B,
24 Perret B, Silvente-Poirot S, Poirot M, Record M. Exosomes account for vesicle-mediated
25 transcellular transport of activatable phospholipases and prostaglandins. *J Lipid Res*
26 2010;**51**:2105-2120.
- 27 129. Rautou PE, Mackman N. Microvesicles as risk markers for venous thrombosis. *Expert Rev*
28 *Hematol* 2013;**6**:91-101.
- 29 130. Visnovitz T, Osteikoetxea X, Sodar BW, Mihaly J, Lorincz P, Vukman KV, Toth EA, Koncz A,
30 Szekacs I, Horvath R, Varga Z, Buzas EI. An improved 96 well plate format lipid quantification
31 assay for standardisation of experiments with extracellular vesicles. *J Extracell Vesicles*
32 2019;**8**:1565263.
- 33 131. Osteikoetxea X, Balogh A, Szabo-Taylor K, Nemeth A, Szabo TG, Paloczi K, Sodar B, Kittel A,
34 Gyorgy B, Pallinger E, Matko J, Buzas EI. Improved characterization of EV preparations based on
35 protein to lipid ratio and lipid properties. *PLoS One* 2015;**10**:e0121184.
- 36 132. Szentirmai V, Wacha A, Nemeth C, Kitka D, Racz A, Heberger K, Mihaly J, Varga Z. Reagent-free
37 total protein quantification of intact extracellular vesicles by attenuated total reflection Fourier
38 transform infrared (ATR-FTIR) spectroscopy. *Anal Bioanal Chem* 2020;**412**:4619-4628.
- 39 133. Smith ZJ, Lee C, Rojalin T, Carney RP, Hazari S, Knudson A, Lam K, Saari H, Ibanez EL, Viitala T,
40 Laaksonen T, Yliperttula M, Wachsmann-Hogiu S. Single exosome study reveals subpopulations
41 distributed among cell lines with variability related to membrane content. *J Extracell Vesicles*
42 2015;**4**:28533.
- 43 134. Skotland T, Sagini K, Sandvig K, Llorente A. An emerging focus on lipids in extracellular vesicles.
44 *Adv Drug Deliv Rev* 2020;**159**:308-321.
- 45 135. Burrello J, Biemmi V, Dei Cas M, Amongero M, Bolis S, Lazzarini E, Bollini S, Vassalli G, Paroni R,
46 Barile L. Sphingolipid composition of circulating extracellular vesicles after myocardial ischemia.
47 *Sci Rep* 2020;**10**:16182.

- 1 136. Tsiantoulas D, Perkmann T, Afonyushkin T, Mangold A, Prohaska TA, Papac-Milicevic N,
2 Millischer V, Bartel C, Horkko S, Boulanger CM, Tsimikas S, Fischer MB, Witztum JL, Lang IM,
3 Binder CJ. Circulating microparticles carry oxidation-specific epitopes and are recognized by
4 natural IgM antibodies. *J Lipid Res* 2015;**56**:440-448.
- 5 137. Binder CJ, Papac-Milicevic N, Witztum JL. Innate sensing of oxidation-specific epitopes in health
6 and disease. *Nat Rev Immunol* 2016;**16**:485-497.
- 7 138. Nieuwland R, Gardiner C, Dignat-George F, Mullier F, Mackman N, Woodhams B, Thaler J.
8 Toward standardization of assays measuring extracellular vesicle-associated tissue factor
9 activity. *J Thromb Haemost* 2019;**17**:1261-1264.
- 10 139. Key NS, Mackman N. Tissue factor and its measurement in whole blood, plasma, and
11 microparticles. *Semin Thromb Hemost* 2010;**36**:865-875.
- 12 140. Lacroix R, Vallier L, Bonifay A, Simoncini S, Mege D, Aubert M, Panicot-Dubois L, Dubois C,
13 Dignat-George F. Microvesicles and Cancer Associated Thrombosis. *Semin Thromb Hemost*
14 2019;**45**:593-603.
- 15 141. Exner T, Joseph J, Low J, Connor D, Ma D. A new activated factor X-based clotting method with
16 improved specificity for procoagulant phospholipid. *Blood Coagul Fibrinolysis* 2003;**14**:773-779.
- 17 142. Hisada Y, Alexander W, Kasthuri R, Voorhees P, Mobarrez F, Taylor A, McNamara C, Wallen H,
18 Witkowski M, Key NS, Rauch U, Mackman N. Measurement of microparticle tissue factor activity
19 in clinical samples: A summary of two tissue factor-dependent FXa generation assays. *Thromb*
20 *Res* 2016;**139**:90-97.
- 21 143. Hisada Y, Mackman N. Measurement of tissue factor activity in extracellular vesicles from
22 human plasma samples. *Res Pract Thromb Haemost* 2019;**3**:44-48.
- 23 144. Berckmans RJ, Sturk A, van Tienen LM, Schaap MC, Nieuwland R. Cell-derived vesicles exposing
24 coagulant tissue factor in saliva. *Blood* 2011;**117**:3172-3180.
- 25 145. Poncelet P, Robert S, Bailly N, Garnache-Ottou F, Bouriche T, Devalet B, Segatchian JH, Saas P,
26 Mullier F. Tips and tricks for flow cytometry-based analysis and counting of microparticles.
27 *Transfus Apher Sci* 2015;**53**:110-126.
- 28 146. Vallier L, Bouriche T, Bonifay A, Judicone C, Bez J, Franco C, Guervilly C, Hisada Y, Mackman N,
29 Houston R, Poncelet P, Dignat-George F, Lacroix R. Increasing the sensitivity of the human
30 microvesicle tissue factor activity assay. *Thromb Res* 2019;**182**:64-74.
- 31 147. Tatsumi K, Antoniak S, Monroe DM, 3rd, Khorana AA, Mackman N, Subcommittee on H,
32 Malignancy of the S, Standardization Committee of the International Society on T, Hemostasis.
33 Evaluation of a new commercial assay to measure microparticle tissue factor activity in plasma:
34 communication from the SSC of the ISTH. *J Thromb Haemost* 2014;**12**:1932-1934.
- 35 148. van Es N, Hisada Y, Di Nisio M, Cesarman G, Kleinjan A, Mahe I, Otten HM, Kamphuisen PW,
36 Berckmans RJ, Buller HR, Mackman N, Nieuwland R. Extracellular vesicles exposing tissue factor
37 for the prediction of venous thromboembolism in patients with cancer: A prospective cohort
38 study. *Thromb Res* 2018;**166**:54-59.
- 39 149. Lacroix R, Thaler J. ISTH SSC Vascular Biology Project 5: Comparison of the sensitivity and the
40 specificity of assays to measure TF-EVs in plasma samples. 2019.
- 41 150. Vallier L, Cointe S, Lacroix R, Bonifay A, Judicone C, Dignat-George F, Kwaan HC. Microparticles
42 and Fibrinolysis. *Semin Thromb Hemost* 2017;**43**:129-134.
- 43 151. Cointe S, Harti Souab K, Bouriche T, Vallier L, Bonifay A, Judicone C, Robert S, Armand R,
44 Poncelet P, Albanese J, Dignat-George F, Lacroix R. A new assay to evaluate microvesicle plasmin
45 generation capacity: validation in disease with fibrinolysis imbalance. *J Extracell Vesicles*
46 2018;**7**:1494482.

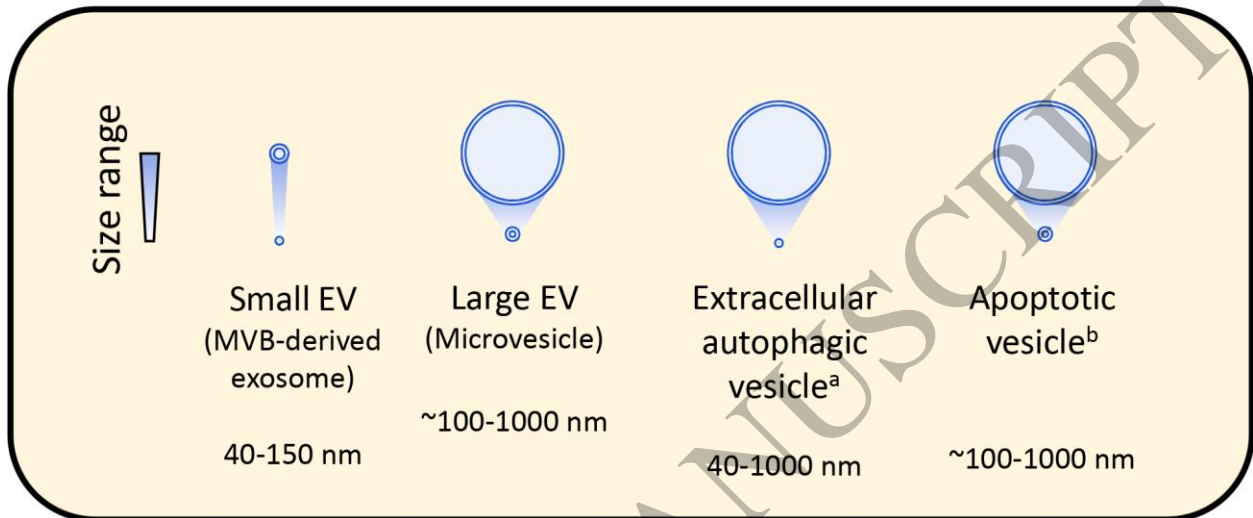
- 1 152. Briens A, Gauberti M, Parcq J, Montaner J, Vivien D, Martinez de Lizarrondo S. Nano-
2 zymography Using Laser-Scanning Confocal Microscopy Unmasks Proteolytic Activity of Cell-
3 Derived Microparticles. *Theranostics* 2016;**6**:610-626.
- 4 153. Shimoda M. Extracellular vesicle-associated MMPs: A modulator of the tissue
5 microenvironment. *Adv Clin Chem* 2019;**88**:35-66.
- 6 154. Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. Secretory mechanisms and
7 intercellular transfer of microRNAs in living cells. *J Biol Chem* 2010;**285**:17442-17452.
- 8 155. Sodar BW, Kittel A, Paloczi K, Vukman KV, Osteikoetxea X, Szabo-Taylor K, Nemeth A, Sperlagh B,
9 Baranyai T, Giricz Z, Wiener Z, Turiak L, Drahos L, Pallinger E, Vekey K, Ferdinandy P, Falus A,
10 Buzas EI. Low-density lipoprotein mimics blood plasma-derived exosomes and microvesicles
11 during isolation and detection. *Sci Rep* 2016;**6**:24316.
- 12 156. Gouin K, Peck K, Antes T, Johnson JL, Li C, Vaturi SD, Middleton R, de Couto G, Walravens AS,
13 Rodriguez-Borlado L, Smith RR, Marban L, Marban E, Ibrahim AG. A comprehensive method for
14 identification of suitable reference genes in extracellular vesicles. *J Extracell Vesicles*
15 2017;**6**:1347019.
- 16 157. Sahoo S, Adamiak M, Mathiyalagan P, Kenneweg F, Kafert-Kasting S, Thum T. Therapeutic and
17 Diagnostic Translation of Extracellular Vesicles in Cardiovascular Diseases: Roadmap to the
18 Clinic. *Circulation* 2021;**143**:1426-1449.
- 19 158. Ortega FG, Roefs MT, de Miguel Perez D, Kooijmans SA, de Jong OG, Sluijter JP, Schiffelers RM,
20 Vader P. Interfering with endolysosomal trafficking enhances release of bioactive exosomes.
21 *Nanomedicine* 2019;**20**:102014.
- 22 159. Ibrahim AG, Cheng K, Marban E. Exosomes as critical agents of cardiac regeneration triggered by
23 cell therapy. *Stem Cell Reports* 2014;**2**:606-619.
- 24 160. Mulcahy LA, Pink RC, Carter DR. Routes and mechanisms of extracellular vesicle uptake. *J*
25 *Extracell Vesicles* 2014;**3**.
- 26 161. Takov K, Yellon DM, Davidson SM. Confounding factors in vesicle uptake studies using
27 fluorescent lipophilic membrane dyes. *J Extracell Vesicles* 2017;**6**:1388731.
- 28 162. Hegyesi H, Pallinger E, Mecsei S, Hornyak B, Kovacshazi C, Brenner GB, Giricz Z, Paloczi K, Kittel
29 A, Tovari J, Turiak L, Khamari D, Ferdinandy P, Buzas EI. Circulating cardiomyocyte-derived
30 extracellular vesicles reflect cardiac injury during systemic inflammatory response syndrome in
31 mice. *Cell Mol Life Sci* 2022;**79**:84.
- 32 163. Han Y, Jones TW, Dutta S, Zhu Y, Wang X, Narayanan SP, Fagan SC, Zhang D. Overview and
33 Update on Methods for Cargo Loading into Extracellular Vesicles. *Processes (Basel)* 2021;**9**.
- 34 164. de Abreu RC, Fernandes H, da Costa Martins PA, Sahoo S, Emanuelli C, Ferreira L. Native and
35 bioengineered extracellular vesicles for cardiovascular therapeutics. *Nat Rev Cardiol*
36 2020;**17**:685-697.
- 37 165. Beer KB, Fazeli G, Judasova K, Irmisch L, Causemann J, Mansfeld J, Wehman AM. Degron-tagged
38 reporters probe membrane topology and enable the specific labelling of membrane-wrapped
39 structures. *Nat Commun* 2019;**10**:3490.
- 40 166. Verweij FJ, Revenu C, Arras G, Dingli F, Loew D, Pegtel DM, Follain G, Allio G, Goetz JG,
41 Zimmermann P, Herbomel P, Del Bene F, Raposo G, van Niel G. Live Tracking of Inter-organ
42 Communication by Endogenous Exosomes In Vivo. *Dev Cell* 2019;**48**:573-589 e574.
- 43 167. Lai RC, Arslan F, Lee MM, Sze NS, Choo A, Chen TS, Salto-Tellez M, Timmers L, Lee CN, El Oakley
44 RM, Pasterkamp G, de Kleijn DP, Lim SK. Exosome secreted by MSC reduces myocardial
45 ischemia/reperfusion injury. *Stem Cell Res* 2010;**4**:214-222.
- 46 168. Yang L, Zhu J, Zhang C, Wang J, Yue F, Jia X, Liu H. Stem cell-derived extracellular vesicles for
47 myocardial infarction: a meta-analysis of controlled animal studies. *Aging (Albany NY)*
48 2019;**11**:1129-1150.

- 1 169. Zwetsloot PP, Vegh AM, Jansen of Lorkeers SJ, van Hout GP, Currie GL, Sena ES, Gremmels H,
2 Buikema JW, Goumans MJ, Macleod MR, Doevendans PA, Chamuleau SA, Sluijter JP. Cardiac
3 Stem Cell Treatment in Myocardial Infarction: A Systematic Review and Meta-Analysis of
4 Preclinical Studies. *Circ Res* 2016;**118**:1223-1232.
- 5 170. Goettsch C, Hutcheson JD, Aikawa M, Iwata H, Pham T, Nykjaer A, Kjolby M, Rogers M, Michel T,
6 Shibasaki M, Hagita S, Kramann R, Rader DJ, Libby P, Singh SA, Aikawa E. Sortilin mediates
7 vascular calcification via its recruitment into extracellular vesicles. *J Clin Invest* 2016;**126**:1323-
8 1336.
- 9 171. Gimona M, Brizzi MF, Choo ABH, Dominici M, Davidson SM, Grillari J, Hermann DM, Hill AF, de
10 Kleijn D, Lai RC, Lai CP, Lim R, Monguio-Tortajada M, Muraca M, Ochiya T, Ortiz LA, Toh WS, Yi
11 YW, Witwer KW, Giebel B, Lim SK. Critical considerations for the development of potency tests
12 for therapeutic applications of mesenchymal stromal cell-derived small extracellular vesicles.
13 *Cytotherapy* 2021;**23**:373-380.
- 14 172. Gobin J, Muradia G, Mehic J, Westwood C, Couvrette L, Stalker A, Bigelow S, Luebbert CC,
15 Bissonnette FS, Johnston MJW, Sauve S, Tam RY, Wang L, Rosu-Myles M, Lavoie JR. Hollow-fiber
16 bioreactor production of extracellular vesicles from human bone marrow mesenchymal stromal
17 cells yields nanovesicles that mirrors the immuno-modulatory antigenic signature of the
18 producer cell. *Stem Cell Res Ther* 2021;**12**:127.
- 19 173. de Almeida Fuzeta M, Bernardes N, Oliveira FD, Costa AC, Fernandes-Platzgummer A, Farinha JP,
20 Rodrigues CAV, Jung S, Tseng RJ, Milligan W, Lee B, Castanho M, Gaspar D, Cabral JMS, da Silva
21 CL. Scalable Production of Human Mesenchymal Stromal Cell-Derived Extracellular Vesicles
22 Under Serum-/Xeno-Free Conditions in a Microcarrier-Based Bioreactor Culture System. *Front*
23 *Cell Dev Biol* 2020;**8**:553444.
- 24 174. Wu JY, Li YJ, Hu XB, Huang S, Xiang DX. Preservation of small extracellular vesicles for functional
25 analysis and therapeutic applications: a comparative evaluation of storage conditions. *Drug*
26 *Deliv* 2021;**28**:162-170.
- 27 175. van der Spoel TI, Vrijssen KR, Koudstaal S, Sluijter JP, Nijsen JF, de Jong HW, Hoefler IE, Cramer
28 MJ, Doevendans PA, van Belle E, Chamuleau SA. Transendocardial cell injection is not superior to
29 intracoronary infusion in a porcine model of ischaemic cardiomyopathy: a study on delivery
30 efficiency. *J Cell Mol Med* 2012;**16**:2768-2776.
- 31 176. Morishita M, Takahashi Y, Nishikawa M, Takakura Y. Pharmacokinetics of Exosomes-An
32 Important Factor for Elucidating the Biological Roles of Exosomes and for the Development of
33 Exosome-Based Therapeutics. *J Pharm Sci* 2017;**106**:2265-2269.
- 34 177. Mol EA, Lei Z, Roefs MT, Bakker MH, Goumans MJ, Doevendans PA, Dankers PYW, Vader P,
35 Sluijter JPG. Injectable Supramolecular Ureidopyrimidinone Hydrogels Provide Sustained Release
36 of Extracellular Vesicle Therapeutics. *Adv Healthc Mater* 2019;**8**:e1900847.
- 37 178. Smyth T, Kullberg M, Malik N, Smith-Jones P, Graner MW, Anchordoquy TJ. Biodistribution and
38 delivery efficiency of unmodified tumor-derived exosomes. *J Control Release* 2015;**199**:145-155.
- 39 179. Hu L, Wickline SA, Hood JL. Magnetic resonance imaging of melanoma exosomes in lymph
40 nodes. *Magn Reson Med* 2015;**74**:266-271.
- 41 180. Hernandez MJ, Gaetani R, Pieters VM, Ng NW, Chang AE, Martin TR, van Ingen E, Mol EA, Sluijter
42 JPG, Christman KL. Decellularized Extracellular Matrix Hydrogels as a Delivery Platform for
43 MicroRNA and Extracellular Vesicle Therapeutics. *Adv Ther (Weinh)* 2018;**1**.
- 44 181. Sahoo S, Kariya T, Ishikawa K. Targeted delivery of therapeutic agents to the heart. *Nat Rev*
45 *Cardiol* 2021.
- 46 182. Ilahibaks NF, Lei Z, Mol EA, Deshantri AK, Jiang L, Schiffelers RM, Vader P, Sluijter JPG.
47 Biofabrication of Cell-Derived Nanovesicles: A Potential Alternative to Extracellular Vesicles for
48 Regenerative Medicine. *Cells* 2019;**8**.

- 1 183. Vader P, Mol EA, Pasterkamp G, Schiffelers RM. Extracellular vesicles for drug delivery. *Adv Drug*
2 *Deliv Rev* 2016;**106**:148-156.
- 3 184. Mackie AR, Klyachko E, Thorne T, Schultz KM, Millay M, Ito A, Kamide CE, Liu T, Gupta R, Sahoo
4 S, Misener S, Kishore R, Losordo DW. Sonic hedgehog-modified human CD34+ cells preserve
5 cardiac function after acute myocardial infarction. *Circ Res* 2012;**111**:312-321.
- 6 185. Kooijmans SAA, Stremersch S, Braeckmans K, de Smedt SC, Hendrix A, Wood MJA, Schiffelers
7 RM, Raemdonck K, Vader P. Electroporation-induced siRNA precipitation obscures the efficiency
8 of siRNA loading into extracellular vesicles. *J Control Release* 2013;**172**:229-238.
- 9 186. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of
10 mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol*
11 2007;**9**:654-659.
- 12 187. Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhai S, Wood MJ. Delivery of siRNA to the mouse brain
13 by systemic injection of targeted exosomes. *Nat Biotechnol* 2011;**29**:341-345.
- 14 188. Luo W, Dai Y, Chen Z, Yue X, Andrade-Powell KC, Chang J. Spatial and temporal tracking of
15 cardiac exosomes in mouse using a nano-luciferase-CD63 fusion protein. *Commun Biol*
16 2020;**3**:114.
- 17 189. Neckles VN, Morton MC, Holmberg JC, Sokolov AM, Nottoli T, Liu D, Feliciano DM. A transgenic
18 inducible GFP extracellular-vesicle reporter (TIGER) mouse illuminates neonatal cortical
19 astrocytes as a source of immunomodulatory extracellular vesicles. *Sci Rep* 2019;**9**:3094.

20
21

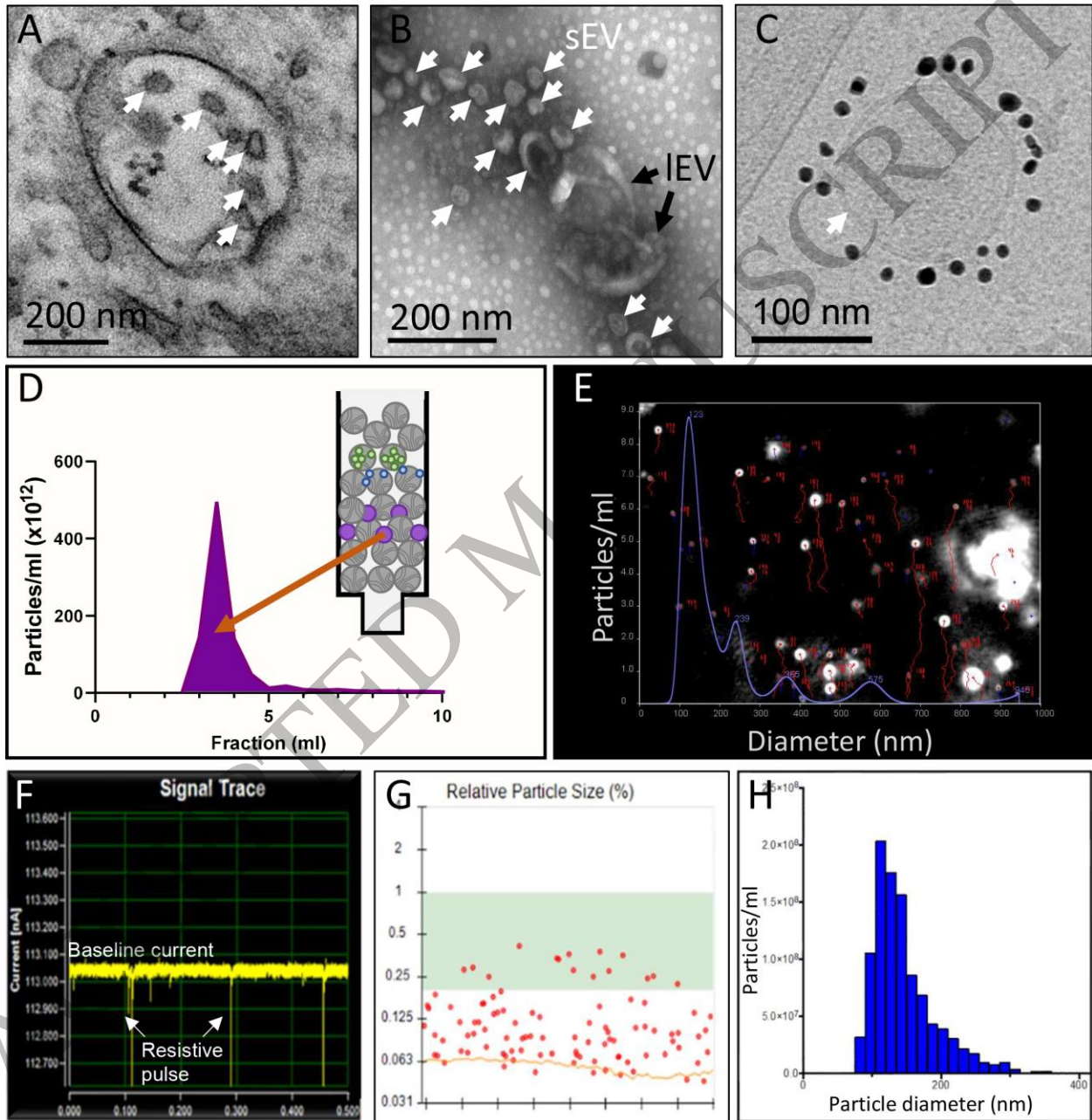
Figure 1



165x98 mm (6.2 x DPI)

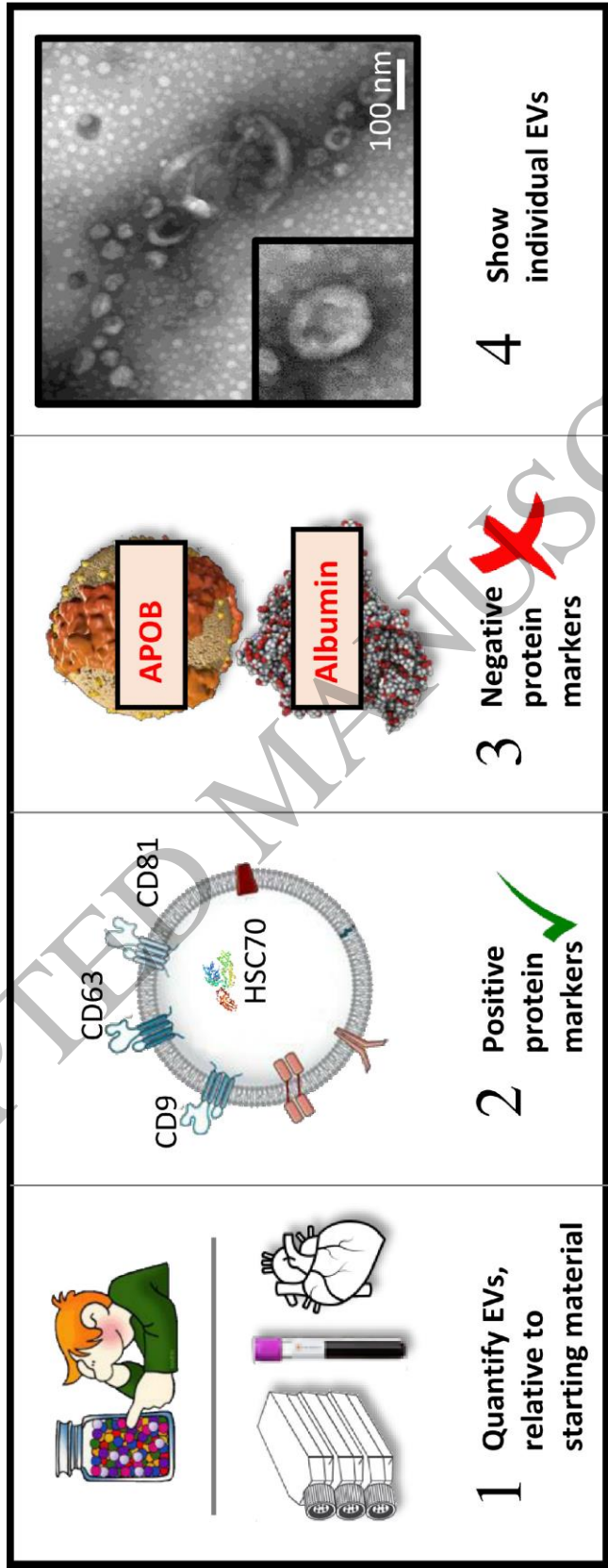
1
2
3

Figure 2



165x199 mm (6.2 x DPI)

1
2
3



1
2

89x229 mm (6.2 x DPI)