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Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines

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ABSTRACT

The last decade has seen a sharp increase in the number of scientific publications describing physiological and pathological functions of extracellular vesicles (EVs), a collective term covering various subtypes of cell-released, membranous structures, called exosomes, microvesicles, microparticles, ectosomes, oncosomes, apoptotic bodies, and many other names. However, specific issues arise when working with these entities, whose size and amount often make them difficult to obtain as relatively pure preparations, and to characterize properly. The International Society for Extracellular Vesicles (ISEV) proposed Minimal Information for Studies of Extracellular Vesicles ("MISEV") guidelines for the field in 2014. We now update these "MISEV2014" guidelines based on evolution of the collective knowledge in the last four years. An important point to consider is that ascribing a specific function to EVs in general, or to subtypes of EVs, requires reporting of specific information beyond mere description of function in a crude, potentially contaminated, and heterogeneous preparation. For example, claims that exosomes are endowed with exquisite and specific activities remain difficult to support experimentally, given our still limited knowledge of their specific molecular machineries of biogenesis and release, as compared with other biophysically similar EVs. The MISEV2018 guidelines include tables and outlines of suggested protocols and steps to follow to document specific EV-associated functional activities. Finally, a checklist is provided with summaries of key points.

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Introduction

In 2014, the ISEV board members published a Position Editorial detailing their recommendations, based on their own established expertise, on the "minimal experimental requirements for definition of extracellular vesicles and their functions" [1]. A list of minimal information for studies of extracellular vesicles (MISEV or MISEV2014) was provided, covering extracellular vesicle (EV) separation/isolation, characterization, and functional studies. The major goal of these recommendations was to sensitize researchers (especially the rapidly growing numbers of scientists newly interested in EVs), as well as journal editors and reviewers, to experimental and reporting requirements specific to the EV field. The ISEV board highlighted the need to consider these issues when making strong conclusions on the involvement of EVs, or specific populations of EVs (exosomes in particular), in any physiological or pathological situation, or when proposing EVs or their molecular cargo as biological markers. By stimulating improved reliability and reproducibility of published EV results, the MISEV2014 authors hoped to further the promise of EVs as biomarkers or for therapeutic applications even in the face of skepticism by some scientists outside the field.

As evidenced by the increasing number of EV publications in high-profile journals, proposing major roles of EVs in numerous physiological pathways from aging to cancer, infectious diseases to obesity, EV science has now clearly achieved widespread interest and enthusiasm well beyond the EV research community. However, the promotion of rigorous EV science is an ongoing process; as EV experts within the ISEV community, we are still concerned to see that major conclusions in some articles are not sufficiently supported by the experiments performed or the information reported. We therefore aim to revise and renew the MISEV recommendations and to continue to work toward their wider acceptance and implementation. In this "MISEV2018" update, a much larger group of ISEV scientists was involved through a community outreach (the MISEV2018 Survey), striving for consensus on what is absolutely necessary, what should be done if possible, and how to cautiously interpret results if all recommendations for controls cannot be followed.

We strongly believe that most of the MISEV2014 recommendations are still valid; however, discoveries and developments within the field during the past four years necessitate certain amendments. This document explains how the 2014 recommendations evolved into MISEV2018 in Tables 1, 2 and 4; provides suggestions for protein markers to validate the presence of EVs (Table 3); and, to highlight the salient points, provides outlines of examplar approaches to address some of the most important experimental issues. Importantly, a 2-page checklist summarizing the major aspects to follow in EV science is provided at the end of this article.

The authors of MISEV2014 were careful to propose feasible experiments and controls for most experimental situations, but also to suggest alternatives for particular situations in which not all guidelines could be strictly followed, such as for limiting sample quantities. However, a recent survey of members of ISEV to lay the groundwork for MISEV2018 [2] showed that, while respondents agreed almost unanimously on the need for minimal requirements, and a majority supported the MISEV2014 initiative and guidelines as published, almost a quarter of respondents found the guidelines too restrictive or too strong an imposition on the field. MISEV2018 thus provides clearer explanations of the need for each recommendation and highlights the extent of author consensus (or lack thereof) on each section. An initial draft of MISEV2018 was submitted to the entire ISEV membership as a Survey asking for agreement/disagreement and comments on each section. The survey specified that, for agree/disagree questions, > 20% "disagree" responses would prompt acknowledgment of major dissent in the final document, while > 40% "disagree" would prompt a focused survey or discussion of the ISEV board with selected survey respondents to redraft the relevant section. 329 responses were received, in which there was such broad agreement on the MISEV2018 draft that the 40% threshold was not met for any section. Nevertheless, attempts were made to address as many comments as comprehensively as possible, and thus generate a semi-final version of this text. Finally, a last round of review was conducted by all previous contributors as well as ISEV and JEV board members and numerous additional long-standing EV experts. Although not all suggestions, references, and critiques could be included in the final product, we are confident that this document represents the views of EV scientists with broad and deep expertise.

Consensus: > 99% of MISEV2018 Survey respondents agreed with the introduction. It has been modified only slightly since the survey, mostly to convey the survey mechanism and results.

Note on applicability of MISEV2018: species, cells, sample types, and experimental conditions

Does MISEV2018 apply to all EV studies, or only to some? EVs appear to be produced by almost all organisms and cell types studied. Yet EV research to date has focused on mammalian EVs, chiefly those of human or mouse origin, and not all cell types or experimental conditions have been closely investigated. In this document, as in MISEV2014, specific examples of molecular markers (such as the markers of EVs in Table 3) are based on studies of specific species, cells, and experimental conditions. Some may be broadly applicable, others less so. Nevertheless, the general principles of MISEV2018 apply to EVs produced by all organisms and all cells. The need to demonstrate presence (or enrichment) of EV markers and absence (or depletion) of putative contaminants, when contents or function of EVs are described, can be generalized to all species, cells, and conditions. We find ourselves at an exciting scientific frontier; where such markers are not yet available, we encourage their development and publication, using the principles of this document as a guide. Additional specific examples may then be incorporated into future MISEV updates.

Consensus: 93% of MISEV2018 Survey respondents agreed that the examples in this document would be based on mammalian EVs. However, applicability to non-mammalian and non-eukaryotic EVs is now addressed.

Nomenclature

ISEV endorses "extracellular vesicle" (EV) as the generic term for particles naturally released from the cell that are

delimited by a lipid bilayer and cannot replicate, i.e. do not contain a functional nucleus. Since consensus has not yet emerged on specific markers of EV subtypes, such as endosome-origin "exosomes" and plasma membrane-derived "ectosomes" (microparticles/microvesicles) [3,4] assigning an EV to a particular biogenesis pathway remains extraordinarily difficult unless, e.g. the EV is caught in the act of release by live imaging techniques. Therefore, unless authors can establish specific markers of subcellular origin that are reliable within their experimental system(s), authors are urged to consider use of operational terms for EV subtypes that refer to a) physical characteristics of EVs, such as size ("small EVs" (sEVs) and "medium/large EVs" (m/lEVs), with ranges defined, for instance, respectively, < 100nm or < 200nm [small], or > 200nm [large and/or medium]) or density (low, middle, high, with each range defined); b) biochemical composition (CD63+/CD81+-EVs, Annexin A5-stained EVs, etc.); or c) descriptions of conditions or cell of origin (podocyte EVs, hypoxic EVs, large oncosomes, apoptotic bodies) in the place of terms such as exosome and microvesicle that are historically burdened by both manifold, contradictory definitions and inaccurate expectations of unique biogenesis. If it is deemed unavoidable to use these or newly coined terms, they should be defined clearly and prominently at the beginning of each publication [5]. If confirmation of EV identity cannot be achieved according to the minimal requirements of this MISEV2018 publication, other terms such as extracellular particle (EP) might be more appropriate.

Consensus: 94% of MISEV2018 Survey respondents endorsed this nomenclature recommendation. The remainder were evenly split between dissent and a preference for no nomenclature recommendation.

Collection and pre-processing: pre-analytical variables

The first step to recover EVs is to harvest an EV-containing matrix, such as fluid from tissue culture or from an organismal compartment. During this pre-analytical phase, an extended constellation of factors, including characteristics of the source, how the source material is manipulated and stored, and experimental conditions, can affect EV recovery. Therefore, it is crucial to plan collection and experimental procedures to maximize the number of known, reportable parameters, and then to report as many pre-analytical parameters as are known.

Cell culture conditioned media

For EV isolation/characterization from conditioned media (an ISEV survey found that the majority of responding EV researchers studied conditioned medium [6]), basic characterization of the releasing cells and culture and harvesting conditions must be performed and reported. Some precautions, such as regular confirmation of cellular identity (e.g. by short tandem repeat (STR) profiling or other methods) [7,8] and identification of cell lineage and provenance including mode of immortalization [9], are advisable for all cell studies. Especially important for EV studies is that the percent of dead cells at the time of EV harvest should be indicated, since even a small percentage of cell death could release cell membranes that outnumber true released EVs. Quantifying the percentage of apoptotic and necrotic cells may also be useful. (Note, however, that when cells are treated with high concentrations of EVs, cell-adherent EVs positive for apoptotic markers may skew results [10,11]). Other relevant characteristics of the cells, including state of activation, malignancy, and senescence [12,13], should be reported where applicable.

Culture and harvesting conditions such as passage number (or days in culture for suspension cells), seeding density [14], density/confluence at harvest [14], including any relevant post-confluence characteristics such as development of polarity [15–19] (in that case, were EVs collected globally or separately, from the different parts of polarized cells?), culture volume, culture vessel or bioreactor system (if used [20,21]), surface coatings, oxygen or other gas tensions (if they differ from standard cell culture) [22,23], stimulation and other treatments [24–30], and frequency and intervals of harvest [14] should be given to allow replication [31,32]. Culture conditions prior to treatment-(s), if any, should also be given. Note that EV recovery depends not only on EV release, but also on re-uptake by cells in culture, which may vary based on culture density and other conditions. Regular checks for contamination with *Mycoplasma* (and possibly other microbes) are needed, not only because of cellular responses to contamination, but also because contaminating species can release EVs [33–36]. Exact methods of medium collection should be given, as well (e.g. decanting or pipetting from flasks, centrifugation of suspension cell cultures). The suggested parameters are of course non-inclusive, and others may be necessary to report for specific types of cells and experiments, including co-culture systems and organoid cultures [37].

All culture medium composition and preparation details should be provided in methods. This should be customary for cell culture studies, and is doubly important here since supplements like glucose [38–40], antibiotics [41], and growth factors [42] can affect EV production and/or composition. Of special note are medium components that are likely to contain EVs, such as serum. EVs are ideally obtained from culture medium conditioned by cells in the absence of fetal calf

serum (FCS or FBS), serum from other species, or other complex products such as platelet lysate, pituitary extract, bile salts, and more, to avoid co-isolation of exogenous EVs. When use of these supplements is unavoidable, experiments should include a non-conditioned medium control to assess the contribution of the medium itself. However, depending on downstream use, it may not be necessary or desirable to deplete EVs [43,44]. In the case of depletion, since nutrient or EV deprivation of cells that are normally cultured in serum- or lysate-containing medium can change cellular behavior and the nature and composition of released EVs [45,46], it is important to specify culture history (how and when the switch to serum-free medium occurred, including acclimatization steps). Alternatively, cells can be exposed during the EV release period to medium that has been pre-depleted of EVs. Here, too, effects on cells and EVs may be expected [47], and the methods and outcome of depletion vary greatly and should be reported. Several fairly efficient protocols are available, such as 100,000 x g ultracentrifugation of complete medium (or of serum following at least 1:4 dilution) for at least 18 hours [48], centrifugation at enhanced speeds (e.g. 200,000 x g [49]) for shorter periods of time, or tangential flow filtration or other forms of ultrafiltration [50]. Ultracentrifugation at around 100k x g for a few hours or without dilution will not eliminate all EVs or EV-associated RNA [51–53]. Commercial "exosome/EV-depleted" serum and other supplements are available from an increasing number of vendors. Since the method of depletion is usually not indicated, consequences on cell growth and EV release may not be predictable; the exact source, method, and reference of depleted supplements should be given, and the "exosome-free" nature of the product should be checked carefully before use [54]. Additionally, vendors are encouraged to report and benchmark the methods of depletion utilized in their products, while users should report product and lot numbers as well as any pooling of biologicals. Finally, medium preparation details, including heating (heat inactivation) or filtration steps, should be reported. For example, heat inactivation of additives such as serum leads to formation of protein aggregates that may co-precipitate with EVs and thus also change the growth-supporting properties of the serum.

Biological fluids

Since more than 30 types of biofluids exist in mammals, and lavages of numerous compartments add to this number (despite not being true biofluids), MISEV2018 does not provide an exhaustive review of the literature on pre-analytical variables related to all biofluids. Each biological fluid presents specific biophysical and chemical characteristics that makes it different from culture conditioned medium, and this must be taken into account when isolating EVs. For instance, plasma and serum are more viscous than conditioned medium. Plasma and serum contain numerous non-EV lipidic structures (low/very low/high density lipoproteins), milk is replete with fat-containing vesicles, urine with uromodulin (Tamm-Horsfall protein), bronchoalveolar lavage with surfactant, all of which will be co-isolated to various degrees with EVs. In each case, specific precautions to separate EVs from these components may be required. While detailed biofluid-specific reporting guidelines are beyond the scope of this MISEV, we encourage development of such guidelines under the MISEV umbrella.

For EV isolation/characterization from biofluids such as blood plasma, several previous ISEV position papers [55,56] and other publications (for just a few of many examples, see [57–63]) have listed reporting requirements that are important for standardization, and these are still valid today, even if many questions remain about the effects of specific pre-analytical variables on different classes of EVs. Since many of these factors have been covered in these previous publications, we do not review them exhaustively here. To give examples of considerations for blood derivatives such as plasma: donor age, biological sex, current or previous pregnancy, menopause, pre/postprandial status (fasting/non-fasting), time of day of collection (Circadian variations), exercise level and time of last exercise, diet, body mass index, specific infectious and non-infectious diseases, medications, and other factors may affect circulating EVs [64,65]. Similarly, technical factors including fluid collection volume, first-tube discard, type of container(s), time to processing, choice of anticoagulant (for blood plasma) [66–68], mixing or agitation, temperature (storage and processing), description of transport (if any), whether tube remained upright before processing, exact centrifugation or filtration procedures, degree of hemolysis, possible confirmation of platelet and lipoprotein depletion prior to storage [69–73], and other parameters should be clearly indicated. Overall, except some that are specific of plasma/serum (such as platelet removal and coagulation), the above listed technical details of collection condition apply to all biofluids and must be reported. Of course, it may be that not all variables have been recorded for archived samples, and this should be acknowledged where applicable.

Tissue

As a special case of pre-analytical issues, a rapidly increasing number of groups have reported isolation of tissue EVs. These studies may involve short-term culture of tissue explants [74] such as *ex vivo* tumors [75], or placenta [76,77], or extraction from whole tissues [78–84]. Many of the same considerations that apply for cellular and biofluids studies also apply here, including confirmation of provenance and condition. Especially for EV extraction from tissue, it is challenging to ensure that recovered vesicles are truly from the extracellular space, rather than being intracellular vesicles or artefactual particles released from cells broken during tissue harvest, processing (e.g. mechanical disruption), or storage (including freezing). This may be especially challenging in a tissue like brain, where similar procedures are used to collect synaptosomes [85]. Even apparently pure tissue-derived EVs can contain endosome components, which could correspond to components of intracellular vesicles including unreleased intraluminal vesicles of late endosomes/multivesicular bodies (MVBs) that are released artifactually during tissue processing. The recent awareness of these challenges has led researchers to perform gentle tissue disruption (i.e. with the goal of separating EVs from cells and extracellular matrix, but not disrupting cells) and several steps of further separation (including density gradients), followed by strict characterization of multiple negative markers, leading to more convincing tissue-derived EV preparations [79]. Use of genetically modified models to trace EV release from specific cells [83] is also a useful approach. More research is clearly needed and encouraged into the isolation, characterization, and function of tissue EVs, as compared with intracellular vesicles and/or non-vesicular extracellular particles (EPs).

Storage

Storage and retrieval conditions of both the matrix (e.g. biofluid, tissue, conditioned media) and isolated EVs may affect EV characteristics, including stability, number of particles, aggregation, and function [57,62,63,71,86–96]. In particular, highly purified EVs may be lost upon storage by adhering to the surfaces of the storage container. How were biofluids, tissues, or media prepared and stored (type of storage container, temperature, etc.) and for how long? Were isolated EVs analyzed or used for experiments fresh, frozen/thawed, lyophilized and reconstituted, etc.? If frozen, how was freezing and thawing performed? In what buffer(s) were EVs stored? For how long? What, if any, cryoprotectant was used? How many freeze-thaw cycles did each sample experience? If EVs were processed and stored in some other manner, details should also be provided, along with the procedure to evaluate effects of storage method and time on EV activity and other properties, where applicable.

Consensus: 96% of MISEV2018 Survey respondents endorsed the pre-analytical variables section. Since 44% of respondents also suggested at least one added literature citation or other amendment in more than 200 total comments, the section was revised to reflect as many of these suggestions as possible. However, biofluids-specific considerations and SOPs are beyond the current scope of MISEV; only illustrative examples are provided. The MISEV2018 Survey comments evidenced particular interest in urine and milk, consistent with the "Experts Meet" sessions during ISEV2016 and the results of a previous ISEV survey (which also highlighted cerebrospinal fluid) [6]. *Beginning with the blood EV roadmap announced at the ISEV2018 annual meeting* [97], *development of more specific recommendations for individual biofluids and other matrices is encouraged. The overwhelming response to this section indeed demands additional research into the effects of pre-analytical variables on EV studies.*

EV separation and concentration: how MISEV2014 evolves in 2018

Absolute purification, or complete isolation of EVs from other entities, is an unrealistic goal (as for many biological products). For this reason, and since the various combinations of EVs and media are colloids [98], here we use the terms *separation* and *concentration*. Separation (colloquially referred to as purification or isolation) of 1) EVs from other non-EV components of the matrix (conditioned medium, biofluid, tissue) and 2) the different types of EVs from each other, are achieved to various degrees by the different techniques available. Concentration is a means to increase numbers of EVs per unit volume, with or without separation. The term "enrichment" can refer to increasing concentration, i.e. EV counts relative to volume, or to increasing EV counts/markers relative to another component. The extent of separation or concentration can be assessed by characterization, which will be detailed in the next section.

How pure should an EV preparation be? The answer depends on the experimental question and EV end use, and often segregates by basic and clinical research. Highly purified EVs are needed to attribute a function or a biomarker to vesicles as compared with other particles. Less pure EVs may be required in other cases, such as when a biomarker is useful without pre-enrichment of EVs, or in certain

therapeutic situations where function is paramount, not the definitive association of function with EVs. Of note, some presumed contaminants may co-isolate with EVs and may even contribute to EV function. Therefore, the choice of separation and concentration method must be informed by factors that may vary between studies such that there is no one-size-fits-all approach. More details on this issue (function and co-isolated factors) are given in section 5c-d (p.24).

At the end of 2015, according to a worldwide ISEV survey [6], differential ultracentrifugation was the most commonly used primary EV separation and concentration technique, with various other techniques, such as density gradients, precipitation, filtration, size exclusion chromatography, and immunoisolation, used by 5–20% of respondents each. Relative success of these different methods in terms ofrecovery and specificity to EVs (as compared to non-vesicular components), or to EV subtypes, has been addressed in a previous ISEV Position Paper (see Figure 1 of [56]), and is summarized in Table 1 below. To achieve better specificity of EV or EV subtype separation, most researchers use one or more additional techniques following the primary step, such as washing in EV-free buffer, ultrafiltration, application of density gradients (velocity or flotation), or chromatography [6,99–102].

A variety of additional techniques or combinations of techniques have been or are currently being developed, some of which may become more prominent in the coming years if they achieve better recovery or specificity than legacy methods (and this must be demonstrated as in, e.g. [103]). Such methods include tangential flow filtration and variations thereon [21,104–110], field-flow fractionation (FFF) [111], asymmetric flow field-flow fractionation (AFFF, A4F, or AF4) [112–114], field-free viscoelastic flow [115], alternating current electrophoretics [116,117], acoustics [118], variations on size exclusion chromatography (SEC) [100,119–121], ion exchange chromatography [122–124], microfiltration [125], fluorescence-activated sorting [126,127] (especially for larger EVs including large apoptotic bodies [128] and large oncosomes [129]), deterministic lateral displacement (DLD) arrays [130], novel immunoisolation or other affinity isolation technologies [131–138], including lipid affinity [139], novel precipitation/combination techniques [140–142], hydrostatic filtration dialysis [143], high-throughput/high-pressure methods such as fast protein/high perfomance liquid chromatography (FPLC/HPLC) that involve some form of chromatography [144] and a wide variety of microfluidics devices that harness one or more principles, including some of those mentioned above [145–153]. Of course, combinations of methods will continue to be used and may outperform single-method approaches.

Table 1 summarizes the instructions given in MISEV2014 for EV isolation (left column), and their updates in MISEV2018 (right).

Consensus: 93% of MISEV2018 Survey respondents agreed with the original categorization of techniques by recovery and specificity in Section 3 and Table 1a; numerous amendments have been made in response to almost 90 comments. 98% agreed that reporting of all methods details should be mandated to allow reproducibility. 97% agreed with the statement of caution on proprietary kits.

EV characterization: how MISEV2014 evolves in 2018

EV characterization by multiple, complementary techniques is important to assess the results of separation methods and to establish the likelihood that biomarkers or functions are associated with EVs and not other co-isolated materials. The need for guidelines for characterization was emphasized by a consortium study led by Hendrix and colleagues [161]. These authors found that only about half of EV-related articles published within a five-year time period included positive markers of EVs, and only a small minority complemented positive with negative markers to track co-isolated non-EV components. ISEV recommends that each preparation of EVs be 1) defined by quantitative measures of the source of EVs (e.g. number of secreting cells, volume of biofluid, mass of tissue); 2) characterized to the extent possible to determine abundance of EVs (total particle number and/or protein or lipid content); 3) tested for presence of components associated with EV subtypes or EVs generically, depending on the specificity one wishes to achieve; and 4) tested for the presence of non-vesicular, co-isolated components.

Table 2 summarizes the instructions given in MISEV2014 for EV characterization, and their updates in MISEV2018. These recommendations apply to EVs from all sources, including non-mammalian and non-eukaryotic cells and organisms.

Quantification of EVs

Since quantifying EVs themselves remains difficult (see below), as minimal information, the total starting volume of biofluid, or, for conditioned medium, number of cells or mass of tissue at the time of collecting, should be indicated for each experimental use. If the

Table 1. Considerations for EV separation/enrichment.

Major recommendations of MISEV2014.	Validity and/or Update in 2018
a) There is no single optimal separation method, so choose based on the downstream applications and scientific question. Separation of non-vesicular entities from EVs is not fully achieved by common EV isolation protocols, including centrifugation protocols or commercial kits that claim EV or "'exosome'" purification.	Still valid. Any newly developed or applied technique for EV isolation must indicate to which of the 4 recovery/specificity options below it aims, and provide characterization information (see Table 2) to show the extent of success. Different methods may be positioned on a recovery vs specificity grid, ranging from low to high in each dimension. Note that the degree of specificity of a particular method might vary depending on the type of biofluid from which EVs are separated. **1) high recovery, low specificity**: methods that recover the highest amount of extracellular material, whatever its vesicular or non-vesicular nature, i.e. whole or near-whole concentrated secretome. Examples of protocols include but are not limited to: precipitation kits/polymer (PEG or others), low molecular weight cutoff centrifugal filters with no further separation step, and lengthy or very high speed ultracentrifugation without previous, lower-speed steps. **2) intermediate recovery, intermediate specificity**: methods that recover mixed EVs along with some amount of free proteins, ribonucleoproteins, and lipoproteins, depending on the matrix. Examples of protocols: size-exclusion chromatography [154,155], high molecular weight centrifugal filters [102], differential ultracentrifugation using intermediate time/speed with or without wash, tangential flow filtration, and membrane-affinity columns [155,156]. 3) **low recovery, high specificity**: methods that recover a subtype (or a few subtypes) of EVs with as few non-vesicular components as possible. Subtypes of EVs can be separated by their size (e.g. by filtration, which must be combined with another method such as SEC to eliminate non EV components), their density upon either flotation or pelleting in a density gradient, their surface protein, sugar, or lipid composition (immuno- or other affinity isolation including flow cytometry for large particles), or other biophysical properties such as surface charge. Note that the designation of "low recovery" is relative to total EVs, and that high recovery of specific subtypes may be possible using these techniques. 4) **high recovery and high specificity**, which may not be achievable as of this writing.
b) Report all details of the method(s) for reproducibility	Still valid. Methods reporting is now facilitated by the EV-TRACK knowledgebase [161] (see also checklist, p.42). ISEV strongly recommends that authors deposit experimental details with EV-TRACK. Examples for classical techniques: centrifugation (g-force, rotor, ultracentrifuge, adjusted k-factor, tube type, adaptor if relevant, time, temperature, and brake)[a]; gradients (materials, densities, volumes, and whether top-down (pelleting) or bottom-up (flotation) gradients were done, further processing); chromatography (matrix nature, pore size, volume; volume of loaded sample; volume and number of fractions, type of the elution buffer; further processing of fractions); immunoaffinity (antibody reference and amount per volume of fluid, particle number, or protein amount, incubation time and temperature, matrix, recovery). Necessary technical detail reporting may have to be established for newly developed techniques.[b-d]
c)	MISEV2018 additional recommendation: Some protocols, including those associated with many commercial kits, may result in EV populations bound to or mixed with introduced components such as antibodies, beads, polymers, and more. These materials may affect downstream profiling or functional studies and may also render the EVs unusable for therapeutic applications. Particular care in performing functional experiments must be taken (see Table 4), with controls including procedural controls and possibly with further separation of EVs.[e,f]

[a]For ultracentrifugation, the k factor can be determined from the rotor type, tube/adapter, and centrifuge speed: the k factor represents the relative pelleting efficiency of a given centrifuge rotor **at maximum rotation speed**; for runs with a rotational speed lower than the maximum rotor-speed, the k factor has to be adjusted: $k_{adj} = k \times (\text{maximum rotor speed/actual rotor speed})^2$; we recommend that all possible parameters be reported [157,201]. See also a web calculator based on a theoretical model of centrifugation and meant for conversion of protocol parameters between rotors [158].

[b]For filtration techniques, one must take care to remove cells and other large membranous structures prior to ultrafiltration; otherwise, large structures may disintegrate and re-form as small vesicles after passing through the filter [159]. Reference numbers of all filters should be specified, as filter type has been found to influence recovery profoundly [102].

[c]SEC: the pore size of the matrix should be taken into account. For example, if the pore size excludes EVs > 70 nm in diameter, a population of vesicles may be excluded.

[d]Both size exclusion chromatography (SEC) and density gradients may co-isolate EVs and certain lipoproteins. Sequential techniques may be needed to achieve separation [160,213].

[e]Note, however, continuing concerns about the specificity and effects on vesicles of certain precipitation techniques [103,278].

[f]This Table does not address several important considerations that are beyond the scope of these guidelines, such as ease of use, cost, and potential for Good Manufacturing Practice (GMP) production.

Table 2. Steps of EV characterization.

Major recommendations of MISEV2014.	Validity and/or Update in 2018
a) No recommendation on quantification	New in MISEV2018: As a rule, both the source of EVs and the EV preparation must be described quantitatively. Source: Number of cultured cells (possibly an estimate, if adherent, as well as intervals of harvest, where applicable), total starting volume of biofluid, or weight/volume/size of tissue at the time of collection must be indicated for each experimental use. The appropriate parameters will vary by source, however; e.g. for urine, volume alone may not be meaningful, and other parameters, such as creatinine level, might be reported. For the EV preparation, global quantification of EVs should be provided. There is no single perfect quantification method. The most commonly used are total protein amount and total particle number. Total lipid quantification could be also considered. None of these components are exclusively associated to EVs, though: proteins are also soluble, particles can be protein aggregates, and lipids are also present in lipoproteins. Thus, ratios of proteins:particles, lipids:particles or lipids:proteins should be reported along with global quantification estimates as a measure of purity and thus reliability of the quantity measure.
b) General characterization. Show: i. At least three positive protein markers of EVs, including at least one -transmembrane/lipid-bound protein -cytosolic protein ii. At least one negative protein marker	Still valid but has evolved with increasing knowledge of the existence of different EV types. Table 3 gives categories of proteins to consider for characterization and some examples. At least one protein of each category 1 to 3 must be evaluated in any EV preparation (at least each time pre-analytical and/or EV isolation conditions are modified). Analysis of proteins of categories 4–5 is recommended for studies that focus on one or more EV subtypes (e.g. small EVs < 200 nm, vs larger EVs: category 4), or that have identified a functional soluble factor in EVs (category 5). 1. All EVs bear proteins associated with the membrane or outer membrane (prokaryotic cells), or with plasma membrane and/or endosomes (eukaryotic cells). To demonstrate the presence of a lipid bilayer in the material analysed, at least one transmembrane or GPI-anchored extracellular protein must be shown. Examples include mammalian proteins expressed (nearly) ubiquitously in all cell types (1a), and proteins specifically expressed in some cells (1b). 2. In all EVs, the lipid bilayer encloses cytosolic material (eukaryotic cells, Gram-positive bacteria) or periplasmic material (Gram-negative bacteria) from the secreting cell. To demonstrate that the material analysed contains more than open cell fragments, at least one cytosolic/periplasmic protein with lipid or membrane protein-binding ability must be shown (2a). Other cytosolic proteins are more promiscuously associated with EVs and other structures and thus should be only optionally used as EV markers (2b). 3. Purity controls include proteins found in most common co-isolated contaminants of EV preparations: depending on the source of EVs, expected contaminants from category 3a (lipoproteins and serum-derived materials), or 3b (urine), should be evaluated. 4. Proteins present in subcellular compartments other than the plasma membrane and endosomes, which may be present in certain EV subtypes (eukaryotic cells). 5. Soluble extracellular proteins with functional activities (cytokines, growth factors, extracellular matrix) may be detected in EVs: their mode of association to EVs (via a specific or promiscuous receptor? Or internal?) should be determined. (See Table 2 part d), below.
c) Characterization of single vesicles: use two different but complementary techniques, for example: i. electron or atomic force microscopy (and show both close-up and wide-field) ii. single particle analyzers (not electron microscope-based)	Still valid, but has evolved with a rapidly increasing number of techniques used to analyze EVs. i. Techniques providing images of single EVs at high resolution, such as electron microscopy and related techniques, scanning-probe microscopy (SPM) including atomic-Force microscopy (AFM), or super-resolution microscopy: these techniques are not interchangeable in the information they provide. When reporting results, both close-up and wide-field images must be provided. ii. Single particle analysis techniques that estimate biophysical features of EVs from other techniques than high-resolution images: size measured by resistive pulse sensing (electric field displacement), or light scattering properties [nanoparticle tracking analysis (NTA), high resolution flow cytometry, multi-angle light scattering coupled to asymmetric flow field-flow fractionation (AF4)]; or fluorescence properties [fluorescence correlation spectroscopy (FCS), high-resolution flow cytometry]. Chemical composition measured by Raman spectroscopy. Other techniques are being developed that may combine these two categories but have not yet been widely used (see 4c p.20). Whatever technique is used, all experimental details for both acquisition and analysis must be reported. Note that not all techniques are equally adapted to all EVs: large EVs (> 400 nm) and very small EVs (< 50 nm) are not well quantified by all NTA; small EVs are not easy to detect by most common flow cytometers. Some large EVs (and aggregates of small EVs) can be imaged by light/fluorescence microscopy. EVs smaller than the refraction limit or resolution of a microscope can still be detected by fluorescence, but no structural information can be obtained, and a single EV cannot be distinguished from a small EV cluster purely based on structural details.
d)	MISEV2018 additional characterization. We now recommend that the topology of EV-associated components be assessed, that is, whether a component is luminal or on/at the surface of EVs, **at least for those required for a given EV-associated function**. Topology may be particularly important for certain classes of biomolecules. Protease and nuclease digestions, detergent permeabilization, and antibodies to outer epitopes (should bind) or inner epitopes (should not bind) can be used to probe topology.

latter is not possible, for instance due to culture conditions (such as periodic collection in continuous bioreactor-based cultures [162]), number of cells at initiation of culture, expected doubling time, and frequency of collection must be indicated. For some biological fluids, like urine, the volume depends strongly on pre-analytical conditions (especially intake of liquid by the donor), thus additional means of normalization should be considered, such as urinary creatinine, as routinely done in the clinic for albumin [163].

EVs have a particulate structure and contain proteins, lipids, nucleic acids, and other biomolecules. Quantification of each of these components can be used as a proxy for quantification of EVs, but none of these values is necessarily perfectly correlated with EV number.

Particle number can be measured by light scattering technologies, such as nanoparticle tracking analysis (NTA); by standard flow cytometry for larger EVs [164–167] or high resolution flow cytometry for smaller EVs [127,168–176]; by resistive pulse sensing (RPS) for a wide range of sizes, depending on pore size [177]; by cryo-EM [174]; by a platform combining surface plasmon resonance (SPR) with AFM [178]; or by other techniques with similar capabilities. Accurate quantitation may be possible only within a certain concentration and size range that varies by platform; where possible, this range (or the minimum and maximum diameter measured) should be reported along with concentration. The method of volume determination in flow cytometry should be reported and potential swarming/coincidence artefacts controlled for [179]; a more detailed guideline article on specifics of flow cytometry analysis of EVs is in preparation by members of ISEV, ISCT and ISAC. Some devices for particle quantification have the advantage of providing accurate sizing information amongst a complex mixture of particles (see Table 2-c: single vesicle analysis). This is not the case for dynamic light scattering (DLS), which is accurate only for monodisperse particle populations [180]. Particle counting by light scatter, RPS, and similar techniques typically results in overestimation of EV counts since the techniques are not specific to EVs and also register co-isolated particles including lipoproteins and protein aggregates. Possibly, ongoing development of fluorescence capabilities of NTA devices may ultimately allow EV-specific measurement [181], although assay sensitivity and the tendency of labeling antibodies and lipid dyes to form particles pose substantial hurdles to such applications [127,182]. Additionally, particle counting technologies may be biased towards certain particle size ranges (especially 50–150 nm [183,184]) because of pore sizes (RPS), size of calibrator used, sensitivity (for example, smaller particles scatter less light), and ability to cope with multidispersity (DLS versus NTA) [185]. Finally, proprietary software used for analysis of data from each device may apply unknown selection and other processing of data, resulting in differences in absolute values obtained by different software or different versions of the same software (see example in [183]).

Total protein amount can be measured by various colorimetric assays [Bradford or micro-bicinchonic acid (BCA)] or fluorimetric assays, or by global protein stain on SDS-PAGE. The EV sample concentration must be within the linear range of the reference curve. However, protein quantification can result in overestimation due to co-isolated protein contaminants (such as albumin from culture medium or plasma/serum), especially when the less specific methods of EV separation are used, or conversely can prove not sensitive enough if highly specific methods yield pure EVs. In addition, results may vary depending on the use or not of detergent to disrupt EVs and expose the entire protein content prior to performing the assay; nature and concentration of the detergent must be indicated.

Quantification of total lipids can be achieved, e.g. by sulfophosphovanilin assay [186], by measuring fluorescence of phospholipid dyes that fluoresce only when incorporated into lipid bilayers, such as DiR [187], or by total reflection Fourier-transform infrared spectroscopy [188]. However, the latter requires specialized equipment, and the former two types of assays may be insufficiently sensitive for small amount of EVs. In addition, whether these techniques equally detect all EVs independent of their specific lipid composition must still be established.

Quantification of total RNA can be performed by global RNA assays including profiles obtained by capillary electrophoresis instruments (see recommendations in Table 1 of [56]). Such measurements are difficult to recommend at this time for EV quantification or purity assays, though, since exRNAs associate in abundance with other circulating and potentially co-separating entities: chiefly ribonucleoproteins [189,190], but also a range of particles including exomeres [112] and lipoproteins [191]. RNA measurements remain, however, an important parameter to report in studies of extracellular RNA.

Quantification of specific molecules. Other methods of EV quantification, like ELISA [192] bead-based flow cytometry [193,194], aptamer- and carbon nanotube-based colorimetric assays [195], and SPR on surfaces such as antibody-coated nanorods [178,196,197], can be used to quantify the amount of one or more specific molecules in the EV preparation. These are generally

proteins (usually the tetraspanins CD9, CD63 and/or CD81, but sometimes tumor-specific proteins or other molecules such as lipids [139]) and can be used to estimate the amount of EVs containing this particular component, rather than total EVs. These methods provide additional information to the above methods and are in line with characterization recommended in part 4b (p.16).

Single and multiple measures and implications for purity. Quantification methods are the most informative for EVs recovered by separation methods with the highest expected specificity (Table 1a-category 3), and for these preparations, one quantification method may suffice; in contrast, more than one quantification should be used for EVs recovered from low-specificity methods. Importantly, ratios of the different quantification methods may provide useful measures of purity. For example, protein:particle ratio [198,199], protein: lipid ratio [186,188,200] and RNA:particle [201] have been proposed as possible purity metrics, although their applicability across protein, lipid, RNA and particle quantification methods remains to be established. Techniques that measure multiple parameters at once, such as colloidal nanoplasmonic assays or infrared (IR) spectroscopy [188,199] may be good optional methods, despite the need for specific sensors or other equipment.

Absolute EV sizing and counting methods are currently imperfect and will require further improvement, aided by appropriate EV reference standards that are now in development [202]. Nevertheless, current methods can provide a reasonable indication of particles per volume and particle size distributions that are best interpreted when combined with general (Table 2b) and single-particle (Table 2c) characterization.

Characterization of EVs by their protein composition

Selection of proteins for use as EV markers. Since MISEV2014, the growing recognition of the existence of many different types of EVs, of different sizes and cellular origins, has led to publication of several studies comparing the protein composition of at least two subtypes of EVs isolated from the same secreting cells. Some studies compared EVs recovered by medium speed centrifugation (called large oncosomes [203], ectosomes [204], microvesicles [205], cell debris [206], or large [207] or medium [208] EVs), with those recovered by 100,000 x g ultracentrifugation (called exosomes in the first four studies, small EVs in the last two), and several of these applied additional separation in density gradients. Another study used differential filtration to separate large microvesicles retained by 0.65 micron filters, and small "exosomes" passing through 0.1 micron filters [209]. Others further separated the high speed pellet to identify subpopulations of small EVs bearing different surface markers such as A33 antigen (*GPA33*) vs EPCAM [19], lipid moieties binding Cholera Toxin, Annexin-V or Shiga Toxin [139], or tetraspanins CD63, CD9, and/or CD81 [208]. EVs were also separated by floating at different densities within a sucrose gradient (defined as high density "HD-exo" vs low density "LD-exo") [210] or eluting at different time points in asymmetric flow field-flow fractionation (AF4) (small "exo-S" vs large "exo-L") [112]. These studies together provide a rich source of potential EV subtype-specific markers. However, since they were performed with different separation approaches and with different cellular sources of EVs, it is still not possible to propose specific and universal markers of one or the other type of EVs, let alone of MVB-derived "exosomes" as compared with other small EVs.

Consequently, MISEV2018 does not propose molecular markers that could characterize specifically each EV subtype. Of note, although the ISEV board tried in MISEV2014 to propose general rules applying to all EVs, some suggestions of MISEV2014 were still biased by an "exosome–oriented" view of EVs. Specifically, Table 1 of MISEV2014 listed, as primary components to analyze in EVs, 2 categories of proteins present or enriched in EVs/exosomes (membrane bound and cytosolic proteins), plus another global category of proteins « not expected in EVs/exosomes » (such as mitochondria, Golgi, or nuclear proteins), and a last category of « contaminants ». In this updated version, MISEV2018, reference to exosomes and the proteins expected or not in them (the previously called "negative controls" of "exosome" preparations) have been deleted, reflecting an evolving understanding of the subtypes of EVs and their associations with other entities. Incorporation of any given component of the cytoplasm or other cellular compartment into an EV is determined by 1) proximity to the budding membrane and size of the EV (passive loading) and 2) specific association with the membrane and any energy-dependent processes (active loading). There are of course interactions between these two domains. Leaving aside the question of active loading, the larger the EV, the more likely any randomly selected molecular or organellar entity in the cell is to be incorporated. Therefore, Golgi, endoplasmic reticulum, mitochondrial, or nuclear components may be excluded from small EVs (< 200 nm) that are presumably formed distant to these locations, or at least

strongly depleted relative to the cell (although we cannot exclude that particular components of such compartments may end up in small EVs, especially in pathologic conditions that could affect incorporation). However, such proteins may be present in larger EVs, and even more so in a very large EV, ultimately the large oncosome [211], which may be as large as some cells and contain by definition any and all components except for a whole, functional nucleus. A single negative control for such large EVs may thus be elusive.

Table 3 highlights three categories of markers that must be analysed in all bulk EV preparations to demonstrate the presence of EVs (Categories 1 and 2) and assess their purity from common contaminants (Category 3), but no universal "negative controls" relevant to a particular subtype of EVs are suggested. The three main categories are:

Category 1: Transmembrane or GPI-anchored proteins localized at the external membrane of prokaryotic cells, and plasma membrane and/or endosomes of eukaryotic cells represent hallmarks of any type of EVs: their presence demonstrates the lipid-bilayer structure specific of EVs, whether they bud directly off the plasma membrane or after transit through the endosomal pathway;

Category 2: Presence of cytosolic proteins (eukaryotic cells and Gram-positive bacteria) or periplasmic proteins (Gram-negative bacteria) demonstrates that the analysed preparation displays the structure of lipid bilayers enclosing intracellular material, as expected for any EV. Proteins presumably actively incorporated into EVs are those with ability to bind to membranes or to cytosolic sequences of transmembrane proteins. Others, like cytosolic enzymes or cytoskeletal proteins are more promiscuous EV components;

Category 3: Some proteins are major constituents of non-EV structures often co-isolated with EVs. Evaluation of their presence helps to assess the degree of purity of the EV preparation. In biofluids like blood plasma, EVs have been reported to co-isolate with other particles, including lipoproteins [212] and a variety of non-integral proteins, such as albumin or soluble acetylcholinesterase. We therefore propose apolipoproteins A1/2 and B (*APOA1/2, APOB*), and albumin (*ALB*) as the best negative markers to date [213] for plasma/serum EVs, and EVs from cells cultured in the presence of bovine serum, or liver cells that secrete lipoproteins, although it cannot be excluded that a fraction of such markers may be specifically associated with some EVs [214]. As another example, in urine, Tamm-Horsfall protein (uromodulin/*UMOD*) forms aggregates that co-precipitate with EVs unless the fluid is chemically treated [215]. Overall, however, since we cannot propose a threshold of abundance of these proteins in EV preparations below which acceptable purity is reached, we stress that it may be more appropriate to assay and report depletion than to expect a binary presence/absence of proposed negative markers.

Category 4: An additional category 4) of proteins should be evaluated if authors want to claim specificity of their study to the small EV subtype(s): Proteins localized in/on intracellular compartments of eukaryotic secreting cells other than the plasma membrane and endosomes (i.e. components of the nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, autophagosomes, peroxisomes) are found in some types of EVs, but a priori not enriched in the smaller EVs (approximately < 200 nm diameter) of plasma membrane or endosomal origin.

Category 5: Finally, category 5) covers secreted or luminal proteins that can associate with EVs by binding to specific (e.g. growth factor receptors) or to promiscuous (e.g. proteoglycan, lipid) receptors on the EV surface: their identification in EV preparations should be accompanied by exploration of the cognate EV-associated receptor(s).

Methods to assess presence of proteins in EV preparations. Several methods can be used to quantify proteins in or on EVs. Western blotting is the most commonly used, and it should be performed by loading side-by-side EV samples and source material lysates either in specified protein amount or in cell-equivalent amounts to determine if the analyzed proteins are enriched in EVs as compared with their producing cells. This comparison, however, can be easily performed only for analysis of EVs from cell culture conditioned medium; it is more difficult for biofluids (in which EVs may originate from cells in the fluid, but also from cells delimiting the fluid canals, and thus are

Table 3. Protein content-based EV characterization. At least one protein of categories 1a or 1b, 2a (optionally 2b), 3a or 3b must be analysed to demonstrate the EV nature and the degree of purity of an EV preparation. Analysis of proteins of category 4 is required when claiming specific analysis of small EVs, and of category 5 to document functional activities. Examples of proteins commonly found in mammalian cell-derived EVs are provided, but other proteins that fall into the provided categories can be used, particularly for analysis of EVs from prokaryotic (bacteria) or non-mammalian eukaryotic sources (including parasites and plants). *XX* = human gene names. *XX** or *XX*** used for families of multiple proteins, for example for integrins: *ITGA** indicates any integrin alpha chain.

Category	1- Transmembrane or GPI-anchored proteins associated to plasma membrane and/or endosomes	2- Cytosolic proteins recovered in EVs	3- Major components of non-EV co-isolated structures	4- Transmembrane, lipid-bound and soluble proteins associated to other intracellular compartments than PM/endosomes	5- Secreted proteins recovered with EVs
Use for	All EVs	All EVs	All EVs as purity control	Subtypes of EVs (e.g. large oncosomes, large EVs) and/or pathologic/atypical state	Functional component of EVs: need to determine the mode of association with EVs
	1a: non-tissue specific. Tetraspanins (*CD63, CD81, CD82*); other multi-pass membrane proteins (*CD47*; heterotrimeric G proteins *GNA**) MHC class I (*HLA-A/B/C, H2-K/D/Q*), Integrins (*ITGA*/ITGB**), transferrin receptor (*TFR2); LAMP1/2;* heparan sulfate proteoglycans including syndecans *(SDC*);* EMMPRIN *(BSG); ADAM10;* GPI-anchored 5'nucleotidase CD73 *(NT5E*), complement-binding proteins *CD55 and CD59*; sonic hedgehog *(SHH)*	2a: with lipid or membrane protein-binding ability. ESCRT-I/II/III (*TSG101, CHMP**) and accessory proteins: ALIX (*PDCD6IP), VPS4A/B; ARRDC1*; Flotillins-1 and 2 (*FLOT1/2*); caveolins (*CAV**); *EHD*; RHOA*; annexins (*ANXA**); Heat shock proteins HSC70 (*HSPA8*), and HSP84 (*HSP90AB1*) note that both are abundant also in exomeres; *ARF6;* syntenin *(SDCBP*); microtubule-associated Tau *(MAPT*, neurons)	3a: lipoproteins (produced by liver, abundant in plasma, serum). ApolipoproteinsA1/2 and B *APOA1/2, APOB; APOB100*; albumin (*ALB*)	4a: nucleus. Histones (*HIST1H***); Lamin A/C (*LMNA*)	5a: Cytokines and growth factors. e.g. *TGFB1/2; IFNG, VEGFA, FGF1/2, PDGF*, EGF*, interleukins (*IL**)…
	1b: cell/tissue specific. Some TSPANs: *TSPAN8* (epithelial cell), *CD37* and CD53 (leukocytes), *CD9* (absent from NK, B and some MSC); *PECAM1* (endothelial cells); *ERBB2* (breast cancer); *EPCAM* (epithelial); CD90 (*THY1*) (MSCs); CD45 (*PTPRC*) (immune cells), CD41 (*ITGA2B*) or CD42a (*GP9)* (platelets); Glycophorin A (*GYPA*) (RBC); *CD14* (monocytes), MHC class II (*HLA-DR /DP/DQ, H2-A**); *CD3** (T cells); Acetylcholinesterase/*AChE-S* (neurons), *AChE-E* (erythrocytes); amyloid beta A4/*APP* (neurons); multidrug resistance-associated protein (*ABCC1*)	2b: promiscuous incorporation in EVs (and possibly exomeres). Heat shock protein HSP70 (*HSPA1A*), cytoskeleton: actin (*ACT**), tubulin (*TUB**); enzymes (*GAPDH*)	3b: protein and protein/nucleic acid aggregates. Tamm-Horsfall protein (Uromodulin/*UMOD*) (urine); ribosomal proteins	4b: mitochondria *IMMT*, cytochrome C *(CYC1); TOMM20*	5b: adhesion and extracellular matrix proteins. Fibronectin (*FN1*), Collagen (*COL***), *MFGE8*; galectin3-binding protein *(LGALS3BP), CD5L;* fetuin-A *(AHSG)*
				4c: secretory pathway (endoplasmic reticulum, Golgi apparatus) calnexin (*CANX*); Grp94 (*HSP90B1*); BIP (*HSPA5*), GM130 *(GOLGA2)* 4d: others (autophagosomes, cytoskeleton…). *ATG9A*, Actinin1/4 (*ACTN1/4*), cytokeratin 18 (*KRT18*)	

Text Box 1. Example of a change from MISEV2014 as a paradigm for considering negative markers.

Argonaute proteins, chiefly AGO2, the most abundant family member in mammals, have been previously proposed as negative markers of EVs. In blood plasma and perhaps other fluids, most extracellular AGO is indeed found outside of EVs [189,190,216]. However, the canonical view of biogenesis of microRNAs (miRNAs) and their subsequent protection from degradation depends on AGO proteins. According to this reasoning, if a mature miRNA is detected, whether in cells, in EVs, or elsewhere, it is most likely protected by AGO (although other associations have been reported [56]). Limits of detection of proteins versus amplified nucleic acids may have contributed to the interpretation that miRNAs are present in EVs without AGO protein. It is in any case now established that AGO can be found in mammalian [217,218] and non-mammalian [219] EVs, not just outside EVs.

Text Box 2. Example of a possible change from MISEV2018 in the future as markers of non-EV co-isolated components.

The Lyden group recently reported that lipidic structures called "exomeres" contain lipids and a limited set of membrane-bound proteins, but do not feature a lipid bilayer and thus do not qualify as EVs. Exomeres were found to co-isolate with small EVs recovered from many cultured cell lines [112] but could be separated from EVs by AF4 (which unfortunately is not implementable in some laboratories). It may be necessary to evaluate this potential contaminant in all EV preparations. Proteomic analyses of exomeres identified various proteins in Table 3-category 2, above: heat shock proteins (*HSPA8, HSPA1A, HSP90AB1*), actin (*ACT**), tubulin (*TUB**), and *GAPDH*. Such proteins thus probably do not qualify as EV-specific components. In addition, a few proteins were identified exclusively in exomeres: hemoglobin (*HBA1/A2), IDH1, MAT1A*, transmembrane *FAT4* (protocadherin, transmembrane plasma membrane), and *EXT1/2* (exostosin, transmembrane, Golgi). Although this exhaustive study was performed with several cell lines, suggesting that the listed proteins are good candidates as specific markers of exomeres co-isolated with EVs, these results still have to be confirmed by other groups to reach validation and inclusion in the next update of the MISEV guidelines.

difficult to attribute in bulk to any given cell type). Flow cytometry of EVs decorating beads or of bulk EV populations (i.e. not designed to analyse single EVs) can be used, but with care to use appropriate negative controls (antibodies alone, isotype controls, etc) [48,220]. Numerous multiplexing approaches have been developed to analyse simultaneously the presence of a pre-designated set of surface protein markers on EVs. For instance, one platform uses flow cytometry after capture on an array of 37 beads, each bearing a specific antibody [100,194]. Other approaches use fluorescence scanning [221] or surface plasmon resonance [222,223] to quantify EV binding to a surface coated with antibodies to different antigens. These methods are population-level, not single-EV techniques, since the final result is an arbitrary unit of signal for a given protein marker in the global EV population. Finally, mass spectrometry has become increasingly economical and accessible for many laboratories, allowing fingerprint-type assessment of many proteins at once. For an exhaustive review on the currently available methods of EV analysis, see [224]. Undoubtedly, new techniques and devices will become available, including commercially, in the near term. Besides the potential of such devices for diagnostic purposes based on amount and type of secreted EVs, one of the challenges associated with the use of such devices is whether they might allow sufficient quantities of specific EVs to be purified so that cargo (RNAs, proteins) content can be analysed on a profiling level.

Non-protein components as markers of EVs. Although proteins are emphasized in the literature and here, phospholipids present in lipid bilayers are also potential positive controls for the presence of EVs [225,226], albeit non-specific as other particles (lipoproteins) may also contain these. As an example, albeit one that may not be exposed on all EVs, outer leaflet phosphatidylserine (PS) can be evidenced indirectly by binding of fluorescently-labeled PS-binding proteins, such as Annexin V [139,167] or the C1C2 domain of lactadherin/*MFGE8* [186,227]. Glycosphingolipids are similarly evidenced by binding of GM1 ganglioside [139,228]. Other lipids including cholesterol, sphingomyelin, ceramide, and phosphatidyl-choline/ethanolamine/inositol can be detected by a variety of methods [225] including Raman spectroscopy, which was recently used to analyze lipids in dried EVs [229]. However, in which ratio cholesterol, sphingomyelin, ceramide, and phosphatidyl-choline /ethanolamine/inositol are in EVs, and how this differs from ratios found in lipoproteins is not yet established: additional comparative lipidomic studies of separated EVs and liproprotein subtypes may be informative.

Alternatively, **dyes that are activated by intracellular components** can be used to label EVs. Calcein and CFSE are examples of cell permeant, non-fluorescent pro-dyes that are cleaved by intracytoplasmic enzymes, resulting in an impermeant fluorescent molecule; labelling therefore theoretically differentiates intact EVs from linearized membrane fragments, provided that the required enzymes are present in EVs [230,231]. Other tools, like a recently reported protein- and lipid-binding dye, di-8-ANEPPS [231], may be worth evaluating for sensitivity and specificity. Additionally, appropriate negative controls are necessary in all studies, such as dye only and dye plus EV-depleted matrix.

Concerning nucleic acids, both **DNA and RNA** have been detected in EVs. RNA in EV preparations can be detected by dyes [232], although some dyes may also detect non-EV-associated RNA. It is possible that some nucleic acid species could serve as negative or positive markers of some EVs. For example, strictly nuclear RNAs might be identified as negative markers in the future, while RNA associated only with cytoplasmic complexes (e.g. ribosomal RNAs or mitochondrial DNA) [233,234] may be more likely to be present in certain EVs. However, several nuclear RNAs have been found in EVs, and a variety of data have been presented on specific versus non-specific incorporation of RNAs into EVs or subtypes of EVs [56,217,235–241]. More research is thus needed before specific recommendations can be made for using nucleic acids as specific markers of EVs or EV subtypes.

Single vesicle analysis

Quantification and global protein composition apply to bulk EV preparations. However, it is important to provide some information on the individual EVs present in such bulk preparations. Two different approaches provide different types of information:

i. Techniques allowing visualization of single EVs at high resolution may provide information on both the structure and the composition of EVs, when combined with antibody-mediated detection of EV components. However, they may be difficult to exploit in a quantitative manner, with analysis of sufficient number of EVs to reach statistical power.

EVs with diameter larger than the diffraction limit of light (~ 200 nm) can potentially be visualized as single EVs by regular fluorescence microscopy and regular flow cytometry. For EVs smaller than this limit, confocal microscopy can detect fluorescent dots, but these dots can correspond either to very bright vesicles with diameter smaller than 200 nm [242], or to clusters of small dim vesicles, without the possibility to discriminate between the two [243].

All EVs can be analyzed by: electron microscopy or by other imaging techniques: SEM [244], TEM by contrasting and embedding in a mixture of uranyl compounds and methylcellulose to maintain the bilayered morphology, cryo-EM [174,245,246]; scanning-probe microscopy (SPM) including atomic force microscopy (AFM) [247]; and super-resolution microscopy [248,249]. Note that these various techniques are not necessarily interchangeable or capable of providing images of comparable quality. For example, cryo-EM clearly shows the lipid-bilayer, preserves EV size better than the dehydrating conditions used to fix samples for TEM, and may be more quantitative, as all particles in a given volume can be imaged, not just those that adhere to a surface (the grid).

ii. Single particle analysis techniques that do not provide high-resolution images but calculate biophysical parameters of single EVs can be used to quantify a large number of EVs with a higher statistical power than many single-EV techniques. For instance, size can be inferred from particle displacement pattern by nanoparticle tracking analysis [184,185,250]; light scattering and/or fluorescence detection in high resolution flow cytometry [251–255]; multi-angle light scattering combined with asymmetric flow field-flow fractionation (AF4-MALS) [256]; displacement of an electrical field in tunable resistive pulse sensing-based devices; or fluorescence correlation spectroscopy (FCS) [257–259]. Chemical composition can be evaluated by Raman tweezers microscopy [251–253].

Other recently developed technologies aim to combine the advantages of imaging with analysis of large numbers of events. They are, however, less commonly used in the EV field and require further validation in multiple laboratories. For instance, an imaging flow cytometer that captures images of single cells going through a fluidic channel can be used, with a carefully designed set of controls and settings, to image EVs of all sizes after labeling with fluorescent lipids, proteins, or antibodies [260,261]. Another recently designed device involves capture of biotinylated EVs on a streptavidin surface, followed by successive rounds of staining with fluorescent antibodies, imaging, and quenching followed by additional rounds of labeling [262]. Another example uses single-particle interferometric reflectance imaging sensing of EVs captured on antibody-coated chips [263,264], which can be accompanied by fluorescence measurements.

Whatever technique is used, all experimental details must be reported. These include the brand and version of the instrument and the software, the settings used for acquisition (diluent buffer, camera, flow rate, threshold…) and, for analysis, the precise process for EM or fluorescence microscopy and how the imaged areas were selected, as well as controls and calibration

information where relevant. For flow cytometry, an ISTH working group has recently issued recommendations [265]. Given the wide range of techniques and platforms available, many of which remain under development for EV applications, MISEV2018 cannot yet give precise protocol recommendations.

New recommendation: determine the topology of EV-associated components

Importantly, the luminal versus surface topology of various EV-associated components, including nucleic acids, proteins, glycans, etc, is not entirely strictly determined. Theoretically, components localized in the cytosol of EV-secreting cells should be inside EVs, and hence protected from mild degradation by proteases or nucleases. While this protection is usually observed, some studies have unexpectedly found proteins [266], RNAs [267], and DNA [41] on the EV surface and sensitive to digestion. It is not yet clear whether this unexpected topology is due to debris from dead or dying cells, or is instead the outcome of as-yet unknown mechanisms of transport of intracellular compartments across membranes that could occur in some physio- or pathological conditions. Certainly, even a small degree of contamination with intracellular material (with the reverse topology to EVs) would complicate interpretation.

Topology may also be important for function. A luminal active component would require membrane fusion or two membrane transport events to achieve function in a recipient cell, whereas if it is exposed at the surface of the EV, it may affect target cells without EV-cell fusion. As a result, we advise that **the actual topology of putative active components** be determined by performing mild digestions, permeabilizations, or antibody studies. To give an example, one might adopt and adapt methods developed to assess protein insertion within the endoplasmic reticulum [268]. Various protocols have also already been published in the EV community [56,217,242,266,269,270].

Example of a biochemical approach for assessing surface exposure of EV components (see for instance use in [266,270]):

for a given preparation of EVs, prepare four samples:

(1) aliquot 1 is untreated;
(2) aliquot 2 is treated with a degrading enzyme alone, which should degrade only surface-exposed components (e.g. proteins);
(3) aliquot 3 is treated with enzyme and detergent (such as Triton X-100), which will ensure degradation of both surface and internal components (to verify that enzymatic treatment worked). Note that detergents may also enhance enzymatic digestion of certain molecules independently of permeabilization of membranes; an alternative to detergent is saponin, which permeabilizes membranes.)
(4) aliquot 4 is treated with detergent alone, to make sure that detergent does not affect the downstream analysis.

For each sample, the cargo of interest is then analyzed (after careful neutralization of the enzyme) by an appropriate method (SDS-PAGE, RT-PCR, PCR…). Extinction of the signal within the enzyme-treated, detergent-untreated aliquot indicates surface exposure of the cargo.

For RNA and DNA analysis, RNAse or DNAse must be used together with proteinase to allow access of nucleases to protein-shielded nucleic acids.

Alternatively, the topology may be determined using flow cytometry and fluorescence microscopy with antibodies directed towards either external or cytoplasmic epitopes on EV membranes. Single vesicle characterization by EM or AFM coupled with immunolabeling could not only provide validation of surface-accessible targets or internal targets (with permeabilization), but also aid in differentiating differences in topology among EVs of different sizes [247].

Consensus: 97% of MISEV2018 Survey respondents endorsed the structure of the characterization section. The original version of Table 3 included more proposed markers of EV subtypes. However, although the responses ranged from 69% to 93% agreement on discreet categories of markers, some concern was communicated about the universality of subtype-specific markers based on the existing evidence. As a result, the Table was thoroughly revised to focus on classes of markers that can be applied to all EVs, not just those from certain cell types or organisms. These revisions further establish the applicability of MISEV2018 to all EVs and EV sources.

An original section on negative controls received 79% agreement and 56 comments; as a result, the approach to negative controls was substantially amended.

95% of respondents agreed that the current focus on protein markers is justified; however, lipids are now included. Although 13 respondents suggested that RNA markers could be used as generic or subtype EV markers, and several mentioned post-translational modifications, these comments were supported with limited or (usually) no references. Based on the limited endorsement of RNA markers and ongoing uncertainty about enrichment of RNAs in EVs, no specific recommendations are made by MISEV2018. Instead, further research is expected and encouraged.

92% agreed with the topology recommendation (4c).

Functional studies: how MISEV2014 evolves in 2018

Table 4 summarizes the previous and updated recommendations on functional analysis of EVs. More detailed justification for these recommendations and proposed protocols follows the Table. The goals of these recommendations are to avoid over-interpretations or classical artefacts when analyzing functions of EVs. It is important to consider several issues when attributing a functional activity to EVs in general, or an EV subtype in particular. We describe here the controls and processes that should be included in all functional studies, unless limited amounts make it impossible to perform them. For clinical applications, for instance, after a first step of pre-clinical validation following these recommendations, systematic analysis may not be possible (see previous Position Paper on clinical applications) [95].

Text Box 3.

As an aside, although we do not go into great detail on this point, many functional studies presume or investigate EV uptake. Time-courses and environmental determinants of EV uptake have been studied for some time [272-274], but challenges exist [275]. Detection within the cell of signal from an EV-labeling dye or other entity does not necessarily mean that the EV or its cargo has been internalized. Some labeling substances are very long-lived, can exist separate from the presumably labeled entity, and can form EV-mimicking particles that are difficult to separate from EVs. Another potential artifact is that labeling EVs with lipophilic or surface-coating fluorophores may modify physicochemical characteristics of EVs, thus altering detection mode and/or uptake by target cells. Although we cannot yet make firm recommendations, we urge researchers to be aware of these issues and to consider that each specific EV-donor/EV-recipient pair may behave in a different manner.

Determine the specific versus common functions of different types of EVs

An important point to keep in mind is that, when analyzing exclusively the function of a single type of EV (for instance either small EVs or large EVs that have been called ectosomes, microvesicles or microparticles in different studies), one may miss the most active EV subtype for the particular function studied. Even if a function is found in the concentrated small EV preparation, it could also be present, and even possibly more concentrated, in other EV subtypes that had been eliminated during the small EV isolation process: keeping large EVs (e.g. "microvesicles") and comparing their activity to that of small EVs should be a first step in all functional studies. In addition, when a function found in EVs may be due to soluble molecules that may or may not associate specifically with EVs, one must consider the possibility that the EV-associated function is only a minor fraction of the non-EV-bound soluble protein. Comparing quantitatively the effects of EV fraction(s), EV-depleted fraction(s), and also the unfractionated initial fluid, will identify the relative contributions of each to total activity.

Ideally, all functional studies of EVs recovered from any source (biofluid, conditioned medium…) could start by a crude separation of broad categories of EVs (e.g. large versus small EVs versus EV-depleted fraction, separated by successive centrifugation, filtration, or chromatography). Importantly, to ascribe function to different categories of EVs, each fraction should be retained for side-by-side activity analysis. However, authors who wish to analyse the function of only one subtype of EVs can justify this choice by presenting

Table 4. EV-associated and EV-excluded biological activities.

Major recommendations of MISEV2014.	Validity and/or Update in 2018
a) Dose–response studies	Still valid In addition: quantitative comparison of the activity of conditioned medium or biofluid 1) before, 2) after elimination of EVs, and 3) the EVs themselves, keeping in mind that the EV fraction may include co-isolated /contaminating materials. Additional suggested control: quantitative comparison of the activity of the targeted *versus* the "discarded" EV subtypes (see part 1 below)
b) Negative or background controls. For conditioned medium, negative control = complete medium that has not been conditioned by cells, but still processed in the same way as conditioned medium	Still valid. For biofluids, negative controls of disease-associated functions = fluids from healthy, untreated or otherwise matched donors
c) Controls to assess influence of soluble or non-EV macromolecular components c-i.) Density gradients or other rigorous separation method to show activity is intrinsic to EVs, not just associated or c-ii.) EV depletion to remove activity or c-iii.) EV/cell labelling (e.g. fluorescent labelling, with careful interpretation)	Still valid. Increasingly, it is possible to separate EVs from non-EV components by multiple methods, e.g. density gradients and size exclusion chromatography. It is recommended that functional assays be performed after rigorous separations, comparing EV and non-EV fractions to identify what proportion of activity is associated with each fraction (in case it is not EVs). If the activity is primarily associated with EVs, depleting the EVs should also deplete the activity. The refined separation must be performed at least for a set of biological replicates, but not necessarily systematically afterwards.

practical or theoretical reasons for selecting specifically this subtype for the particular experimental purpose, and further proceed with functional analyses.

Before performing functional assays, it is advisable to determine whether EVs are detected in the different fractions, in terms of total proteins, or number of particles, or ratio of total nucleic acids (RNA, DNA), or total lipids to particles (i.e. substance x-to-particle ratios): if none of these EV-associated components are detectable, the "empty" fractions may be discarded for further functional tests, once a preliminary experiment has shown lack of activity. However, any change of conditions in generation of the biofluid from which EVs are isolated (i.e. different culture conditions or treatment for the cells, different types of patients for biofluids) should be followed by re-analysis of all EV categories.

Example of an approach to compare activity of broad subcategories of EVs after separation by differential centrifugation (see example of use in [220])

From a given volume of conditioned medium:

several low speed centrifugations, transferring supernatant into a new tube each time. The pellet contains cells.

supernatant: 1x centrifugation at medium speed, to collect large EVs (if mostly live cells), large apoptotic bodies, etc., with the pellet largely devoid of cells

supernatant: 1x centrifugation at intermediate speed to collect a pellet enriched in medium size EVs and/or aggregates of small EVs (to be checked by EM)

supernatant: 1x centrifugation at high speed to collect a pellet enriched in small EVs.

Each pellet is resuspended once in buffer/medium and re-centrifuged at the same speed (= washed) before resuspension in a given volume of buffer/medium.

How to normalize amount of EVs used for comparative functional studies

The most appropriate normalization strategy to compare quantitatively the functions of different EVs will depend on the scientific question. One can choose to normalize by either characteristics of the isolated EVs, or by the source material, or by co-isolated standards. Characteristics of isolated EVs would include particle counts, total amount of a biomolecule type in the EV sample (e.g. proteins, nucleic acids, or lipids), and content or activity of specific EV-associated molecules. Source characteristics include the amount of matrix from which the EVs were obtained (initial volume of biofluid, initial mass of tissue, initial number of secreting cells, time of conditioning per cell, etc). Co-isolated standards would be traceable materials added into the matrix prior to separation [202]. Multiple normalization strategies can be pursued [120], and, as emphasized elsewhere, dose-response studies are recommended regardless of normalization method. Unfortunately, there is no clear recommendation that can be made at this point on which normalization strategy is best. Instead, the choice of normalization must be reported and justified, and relevant details of alternative strategies should be provided. For example, when studying EVs obtained from some biofluids, e.g. blood derivatives, normalization by volume may be appropriate. For other fluids, such as lavage fluids and urine, initial volume is not easily compared between donors, so another strategy might be more appropriate. As another example, for *in vitro* studies, normalizing by levels of an EV component (proteins, lipids, RNA), or by particle number may be appropriate, but the rationale should be provided, and information on the number of secreting cells should also be recorded and reported.

The ISEV survey comments on this section evidenced broad disagreement about normalization strategy, particularly on the relative merits of protein, nucleic acid, and lipid quantitation. Additional studies of normalization strategy should thus be encouraged.

Demonstrate that the activity is observed in the absence of direct cell-cell contact

Theoretically, an EV-associated function, like a soluble cytokine-dependent function, should be observed between two cells that are not in direct contact with each other. Therefore, it should be obtained when the EV-donor and an EV-recipient cell are cultured *in vitro* at a distance, through transwell co-culture systems or more sophisticated microfluidics-based culture devices, or by incubating the recipient cells with medium conditioned by the donor cells.

However, the physiological relevance of optimal conditions of these co-cultures, in terms of respective numbers of EV-donor and – recipient cells, cannot be strictly determined. If such assays yield positive results, they prove that transfer of a signal occurs in a cell contact-independent manner, in which case the next necessary step (next section p24), is to distinguish EVs from soluble components. Lack of positive results at this step would suggest that cell-cell-contact is necessary for the exchange of signal, but it could still occur by exchange of membrane–enclosed signals by transfer of plasma membrane-derived vesicles (like trogocytosis), or localized release of multivesicular body-derived vesicles at the cell-cell-contact (e.g. an immune synapse). Thus, a negative result argues against signaling at a distance but does not conclusively disprove local involvement of EVs.

Demonstrate that the activity is predominantly associated with EVs rather than with soluble mediators

Typically, an EV-associated activity is explored by 1) separation and concentration of EVs from a biofluid or cell culture media, 2) application of EVs to a recipient cell or organism, and 3) observation of a readout phenotype. However, to convincingly argue that a detected readout/function is EV-borne, it must be determined that the activity is specifically enriched in EVs (possibly with non-EV components), and not instead due to low amounts of a highly active soluble molecule remaining non-specifically in the EV preparation. This point is particularly important when the proposed or suspected active molecule on EVs is a cytokine/growth factor/metabolite usually described as secreted in a soluble form. For this step, one must compare quantitatively the activity present in/on the EVs versus in the remaining EV-depleted biofluid, using the same amounts of materials in terms of initial volume of biofluid. When evaluating the relative importance of EVs and soluble mediators, it may be worth remembering that EVs and soluble mediators may have combinatorial (e.g. synergistic) effects on cells [275,276].

Example of an approach to determine the respective contribution of EV-bound and soluble non-EV-associated factors (see example of use in [200]):

from a given volume of biofluid from which cells have been completely eliminated

split supernatant into 2 equal parts;

keep one at 4°C while processing the other with the preferred protocol allowing extensive concentration of EVs, with separation from non-EV components, but without a need to separate EV subtypes (see Table 1).

Make sure to recover the biofluid from which EVs have been separated.

Compare activity of the total biofluid, the EV-containing pellet and the EV-depleted biofluid, using material coming from the same initial volume of biofluid.

If the unprocessed supernatant must be concentrated to display activity, the supernatant after centrifugation must be concentrated in the same way, and the pellet can be resuspended in the same volume of concentrated fresh medium as concentrated supernatant, before performing the functional assay.

Demonstrate the specific association of the activity with EVs rather than with co-isolated components

Especially when dealing with concentrated preparations enriched in small EVs, one must keep in mind that such preparations potentially contain non-EV components (ribonucleoprotein aggregates, lipoproteins, exomeres, etc.). The proportion of such co-isolated components differs tremendously with the type of protocol used to separate EVs, with some (like polymer-based concentration) displaying particularly abundant contaminants, and also remnants of the precipitating agent that can affect cell function [277,278]. In the case of cells infected experimentally or unintentionally (e.g. mycoplasma) with microbes, functional microbial factors may also be co-isolated with EVs. Therefore, the functional activity of an EV preparation may be borne by EVs, or by the additional components, or by a combination of both. One must determine which of these three possibilities is the case. If small amounts of working materials do not make it possible to perform these additional investigations, the authors can explain this situation and interpret their data as activity present in EV-enriched preparations, rather than EV-specific activity.

Examples of protocols to demonstrate specific association of the activity with EVs or a given EV subtype:

From a concentrated EV preparation obtained by (ultra)centrifugation, or by centrifugal concentrator:

Option 1) separate contaminants by upward flotation into a density gradient, where only lipid-enclosed structures float upward in the tube: analyze separately activity of each fraction of the gradient in the functional assay; note that density gradient medium may have to be removed, because it can interfere with some functional assays. Alternatively, a control should be performed in which the chemical used for the density gradient is mixed with unseparated EVs.

Option 2) separate soluble contaminants from EVs by SEC column, where EVs elute in the first fractions, whereas proteins and ribonucleoproteins and some lipoproteins are eluted later: analyze separately activity of each fraction (or of pools of fractions containing EVs, intermediate, or free soluble components) in the functional assay;

Option 3) compare activity of intact EVs with that of detergent-treated samples [279]. Detergent will destroy vesicular structures, without affecting other non-lipidic particles. Of course, this technique also has limitations: 1. the effect of the same amount of detergent alone in the functional assay should be tested (it could affect target cells); 2. there could be a few contaminants that are sensitive to detergents as well (liposomes and some proteins).

Option 4) immuno-isolate from half of the biofluid all EVs bearing a surface marker of interest (for instance an integrin or a tetraspanin used to

characterize the EVs, but not expected to be required for the observed activity), and use the specific EV-depleted supernatant in the functional assay, side-by-side with the other half of unprocessed biofluid, to determine if activity has been depleted (hence was associated with the EVs of interest) or not. Using the immuno-isolated EVs in the functional assay may complicate interpretation, since the immunoprecipitate will contain antibodies and beads used for isolation that may profoundly affect interaction of EVs with the target cells. Elimination of protein-bearing EVs by immunoisolation must be demonstrated by showing the amount of EV-associated protein in biofluid before (all) and after immuno-isolation (none), and in the immuno-isolated sample (all).

Other options may arise from ongoing evolutions of the field. See section "EV separation and concentration" p11. As exemplified by AF4 [112,114], combinations of ultrafiltration and SEC [280], and tangential flow filtration combined with other filtration steps [106], novel EV separation processes are constantly being developed and published.

Determine whether a function is specific to exosomes, as compared with other small EVs

As highlighted here, it is now clear that different types of EVs can present functional activities that are as important to explore as those elicited by late endosome-derived exosomes. However, in the last decade, many studies have focused exclusively on demonstrating association of a given function with exosomes. This section explains the technical limitations of such studies, and why they are not sufficient to conclude, as is generally done, that exosomes have specific functions compared with other EVs.

In particular, numerous approaches have been taken to inhibit or stimulate exosome secretion in loss- or gain-of-function experiments. For example, in mammalian cells, exosome secretion has been *decreased* (100% inhibition is almost never achieved) by inhibiting neutral sphingomyelinases and ceramide generation (by shRNA, genetic editing, or drugs such as GW4869, spiroepoxide, cambinol and others) [281–284]; inducing ISGylation, which promotes lysosomal degradation of MVB proteins [285]; blocking Rab GTPases (by expressing dominant-negative mutants or silencing or knocking out Rab27, Rab11, Rab35, or others) [286–288], other small GTPases (RAL-1 [289]), SNARE proteins (YKT6 [290]), upstream regulators of protein sorting into MVBs (like SRC [291]), or cytoskeletal proteins (cortactin [292], microtubules [293]); or using other drugs (e.g. the sodium channel blocker amiloride [294]). Conversely, ionophores such as ionomycin (calcium signaling) or monensin (sodium transport) [295,296], or drugs inhibiting endosomal acidification and/or autophagic degradation (bafilomycin A1 [297,298]) have been used to stimulate exosome secretion. Of note, drugs inhibiting EV uptake (e.g. heparin [299]) could lead to enhanced recovery of EVs, which could be misinterpreted as an increase of exosome release.

A few studies have also proposed ways of modulating secretion of plasma membrane-derived EVs in mammals: the ARRDC1 protein uses the ESCRT machinery to induce budding of small EVs at the PM, and its blocking or depletion inhibits secretion of such EVs [300,301]. Over-activation of ARF6 has been shown to increase release of PM-derived large EVs [302]. Modulation of cytoskeletal remodelers was reported to affect PM-derived EV release [303–305], and depletion of DIAPH3 increased large oncosome release [306]. In prokaryotes, recent insights into the mechanisms of outer membrane vesicle formation [307] could suggest other molecules to test in eukaryotic cells.

These cell treatment approaches have great potential and deserve more development; however, it is important to recognize several caveats.

(1) Small EV–containing fractions potentially contain EVs originating from late endosomes ("exosomes") and others originating from the cell surface (plasma membrane), with both classes sharing common molecular players, including the ESCRT components TSG101, VPS4, and/or Alix [308–310]. Therefore consequences of decreasing or increasing global secretion of heterogeneous populations of small EVs should not be interpreted in terms of functional effects of exosomes, but rather of small EVs in general.
(2) Tools described until now to block or enhance exosome secretion have not been well evaluated for their possible effect on secretion of other EVs. For instance, ionophores, such as ionomycin, are also potent inducers of large EV and microparticle secretion [207,311]. Conversely, in one study, inhibition of neutral sphingomyelinases was shown to enhance secretion of larger plasma membrane-derived EVs while decreasing that of small EVs [312]. Another example is monensin, often used to stimulate EV secretion, being an inhibitor of apoptotic body formation [167]. Therefore, it is likely that putative exosome modulators will have different consequences in different cells and under different conditions, and it is important to carefully

quantify the toxicity of each treatment in each experimental system, to exclude artefactual effects on EV recovery due to increased cell death.

(3) Some EV release modulators affect other major intracellular pathways that might indirectly affect EV secretion and modify cell functions in general (like general intracellular trafficking, secretory, or autophagy pathways). Consequently, not only EV amount, but also EV composition may be changed, together with changes in protein expression and physiology of the secreting cells. As an example, Rab27a inhibition also decreased secretion of some non-EV-bound soluble factors [313,314]. Another caveat to consider is that disrupting the secretion of one EV type may disrupt the production of other EV types, such that the functional EV type may be masked by the over-production of an antagonistic one, leading to an erroneous conclusion that the disrupted EV type is the functional EV. Therefore, demonstrating that only late endosome-derived exosomes bear an analyzed function remains challenging. Some previous studies managed to rescue an observed effect by re-introducing purified exosomes (or rather small EV pellets) into the functional *in vitro* or *in vivo* assays [292,313,314]. This approach is indeed recommended, with careful interpretation taking into account the degree of rescue and the required amount of EVs.

Until we achieve unambiguous identification of specific, unique biogenesis machineries affecting only a given subtype of EVs, we are left with trying to isolate EV subtypes after they have left the cell. For example, if multi-tetraspanin-bearing EVs are true exosomes in a particular cell system, an EV preparation could be depleted of such EVs and the activity quantified in comparison with that of an irrelevant IgG- or mock-depleted population.

How to attribute particular effects mediated by EVs to specific EV components

Many publications include knock-out or knock-down of a certain bioactive protein or RNA in the EV donor cell, after which the effects of the modified EV on target cells are compared with the effects of non-modified EVs. If the native effect of EVs is lost, the authors conclude that EV activity was due to the specifically targeted protein or RNA. However, such a conclusion may or may not be valid in the absence of an extensive characterization of EVs released by the cells depleted for the targeted molecule. Indeed, deletion of the protein/RNA of interest may also lead to major alterations of the secreting cell, resulting in additional changes to the quantity or molecular contents of EVs, which could also explain the changes in EV-induced effects on target cells. While a complete omics analyses of the modified EV population may be beyond the scope of many studies, there should be an awareness that other EV components may have changed as well. At a minimum, a small-scale analysis of EV number or common EV-associated proteins in the modified and WT conditions must be performed. Finally, Direct EVs engineering (e.g. to deplete the particular putative active molecule) may overcome the issue of alterations in the secreting cells. However, possible loss/alteration of EV cargo due to EV manipulation may also occur.

Consider whether an EV-dependent function is specific to a given EV source

Finally, in all cases, one must be careful in claiming a specific function of EVs from a particular source: it is one thing to claim that the EV fraction from Cell X is potent (versus other fractions), another to claim that Cell X EVs are potent versus those from other cells. For example, do my mesenchymal stromal cell (MSC) EVs do something special, or do milk EVs, urine EVs, cancer cell EVs do the same? Of course, it will not be possible to compare EVs from all different sources, thus the final message must reflect this uncertainty.

Consensus: This functional section had broad support (> 94% average for each subsection) from MISEV2018 survey respondents and has been revised only minimally. The components of Table 4, on EV-associated and -excluded activities, received an average 96% endorsement. One of the least supported components of the functional section (90% agreement) was the original normalization recommendation (which are found in the function section but can be applied to characterization and other sections as well). This part was substantially revised based on the submitted comments.

General considerations

Reporting

ISEV endorses the EV-TRACK knowledgebase as a facilitating and updatable tool for comprehensive reporting of EV experimental studies [161]. EV-TRACK invites submission of details on EV isolation and characterization via a multi-step online template, then associates each with a particular study or publication and also returns an "EV-METRIC" as an aggregate

measure of the level of detail provided. ISEV strongly encourages all authors to submit their experimental protocols on EV isolation and characterization to the EV-TRACK website (evtrack.org), and to consider applying additional steps if they or reviewers/editors feel that the calculated metric is low. The important consideration is not obtaining a particular metric, which after all may vary widely between basic and clinical studies; instead, the level of detail required for approved entries in EV-TRACK ensures that the transparency and reproducibility of procedures can be assessed. Furthermore, the knowledgebase can be revised and expanded as technologies and techniques develop, with input and assistance from the community.[1] Authors are also urged to submit EV profiling data to public databases such as those curated and maintained by the European Bioinformatics Institute, the US National Center for Biotechnology Information, and the Japanese Center for Information Biology. In addition, but not as a substitute, data may be submitted to field-specific databases such as EVpedia [315,316], Vesiclepedia [317] (formerly ExoCarta [318]), and the exRNA Atlas [319].[2]

Notes

1. EV-TRACK submission and EV-METRIC calculation may assist with but do not replace appropriate peer review. Interestingly, respondents to the MISEV2018 Survey were split between advocating mandatory EV-TRACK submission and reporting and recognizing EV-TRACK as a valuable but optional tool. As a result, MISEV2018 strongly encourages but cannot mandate EV-TRACK submission, which most seem to acknowledge as highly valuable.
2. The utility of field-specific databases was questioned by some respondents, who felt that data from studies analysing EVs obtained through low-specificity methods renders these databases difficult to interpret in terms of specific association of a given molecule/sequence to EVs or an EV subtype. Submission to field-specific databases is thus important to update these resources, which are of value to identify different studies finding the same molecules, but not to demonstrate the exosomal nor EV nature of the analysed entities. It also does not substitute for deposition with publicly maintained repositories.

Exceptions to compliance with MISEV guidelines

Some situations may arise in which strict adherence to the MISEV guidelines is difficult. Not all biofluids, for example lacrimal fluid, are available in sufficient volume to separate EVs and perform multiple tests with each sample; also, only limited numbers of EVs may be harvested from small numbers of patient-derived cells, small organoids, and more. In such cases, multiple samples might be pooled to establish the reliability of the separation method(s) and characterize EVs before further characterization or functional studies are performed with individuals samples. If even this solution is impractical, authors should indicate the limit of detection of each applied EV characterization technology and demonstrate that the available material falls below this limit. However, applying this "escape clause" means that EVs cannot be rigorously demonstrated, requiring that authors mention (and reviewers insist on) the caveats of alternative interpretations, i.e. that EVs may contribute, but not necessarily exclusively, to an observed phenomenon or molecular signature.

Consensus: Section 6a (reporting requirements) was supported by 89% of MISEV2018 Survey respondents. The comments were split between those who wanted more reliance on EV-TRACK and those who found EV-TRACK submissions to be time-consuming or otherwise cumbersome. 99% of respondents agreed with mentioning exceptions to compliance in 6b.

Conclusions

Major points of MISEV2018 (see MISEV2018 quick-reference checklist, p42):

(1) "Extracellular vesicle" is the preferred generic term for the subject of our investigations, and subtypes should be defined by physical and biochemical characteristics and/or conditions/sources. When other terms are used, careful definition is required.
(2) A growing number of separation techniques and combinations thereof are available, variously balancing recovery and specificity. In all cases, the EV separation and/or isolation procedure must be reported in detail, to allow a reliable replication.
(3) As EV characterization evolves, protein and lipid markers continue to be highly useful to demonstrate presence of the generic structure of EVs. Markers chosen for characterization of EVs may differ based on cells of origin (including mammalian vs non-mammalian vs non-eukaryotic cells), and whether claims are generic to all kinds of EVs, or instead specific to subtypes of EVs (4-b p16, Table 2, Table 3). Further, newly identified EV-associated components must be validated by 1) showing their specific association with EVs or subtypes and 2) if functionally relevant: topology in/outside EVs of the functional molecule (4-d p.21).

(4) Function. Demonstration that a function is associated specifically with EVs released from the cell (Table 4) requires demonstration that the function occurs without cell-cell contact (5-b p22) and is not present (or is present to a substantially reduced extent) in the soluble, non-EV-associated secreted factors (5-c, d p23-24). Demonstration that a function is specific to exosomes (EVs of endosomal origin), as compared with other types of small EVs, is not recommended as a major point of any EV study due to the issues elaborated in section 5-e p25. However, if authors wish to make this point, the currently proposed exosome-specific tools cannot be taken for granted, and several controls should be performed to evaluate their action on other EVs, on secretion of non-EV products, and on the general physiology of secreting cells. Additional techniques for separation of these vesicles and/or genetic tools specifically affecting their secretion, may become available in the future (5-e p25).
(5) The EV-TRACK knowledgebase is endorsed by ISEV to showcase and enhance rigor and reproducibility in EV studies, consistent with the MISEV guidelines.
(6) Finally, there are exceptions to every rule. MISEV2018 is meant to guide and improve the field, not stifle it. If MISEV recommendations and requirements cannot be met, authors will then need to explain their unique situation and describe their attempts to meet the guidelines and the reason for failure. These guidelines will also continue to evolve.

Consensus: 89% of ISEV2018 Survey respondents had no major changes; 29 comments were taken into account in revisions of this section.

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Disclosure statement

No potential conflict of interest was reported by the authors. In addition, they declare that they took responsibility for avoiding any introduction of possible conflicting material into the text. For example all names of companies and trademarks were avoided in the main text to reduce the likelihood of preferential treatment to specific services or products of for-profit entities.

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Authors' contributions

CT and KWW led the manuscript drafting team, prepared the online surveys, analyzed survey results and other input, communicated with authors and prospective authors, and prepared all revisions. All other authors contributed as specified in the text and in footnotes to the author affiliations. All authors reviewed and approved the final draft of the manuscript.

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References, especially those provided to illustrate methods and approaches, are representative only, and are not meant to be a comprehensive review of the literature. Most references were derived from suggestions provided in the MISEV2018 Survey results. Each reference was checked by multiple authors. Citation implies deemed relevance of scientific content and not an endorsement by the authors or ISEV of any particular journal or editorial practice.

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MISEV2018 Checklist

Numbers refer to sections listed in the Table of contents from: C. Théry and K.W.Witwer, et al, "Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines", J Extracell Vesicles 2018;7:1535750.

+++ Mandatory ++ Mandatory if applicable + Encouraged

1-Nomenclature

Mandatory

+++ Generic term extracellular vesicle (**EV**): **With demonstration** of **extracellular** (no intact cells) and **vesicular** nature per these characterization (Section 4) and function (Section 5) guidelines **OR**

+++ Generic term, e.g., extracellular particle (**EP**): no intact cells but MISEV guidelines not satisfied

Encouraged (choose one)

+ Generic term extracellular vesicle (**EV**) + **specification** (size, density, other)

+ Specific term for subcellular origin: e.g., ectosome, microparticle, microvesicle (from plasma membrane), exosome (from endosomes), **with demonstration** of the subcellular origin

+ Other specific term: **with definition of specific criteria**

2-Collection and pre-processing

Tissue Culture Conditioned medium (CCM, Section 2-a)

+++ General cell characterization (identity, passage, mycoplasma check…)

+++ Medium used before and during collection (additives, serum, other)

++ exact protocol for depletion of EVs/EPs from additives in collection medium

+++ Nature and size of culture vessels, and volume of medium during conditioning

++ specific culture conditions (treatment, % O2, coating, polarization…) before and during collection

+++ Number of cells/ml or /surface area and % of live/dead cells at time of collection (or at time of seeding with estimation at time of collection)

+++ Frequency and interval of CM harvest

Biofluids or Tissues (Sections 2-b and -c)

++ Donor status if available (age, sex, food/water intake, collection time, disease, medication, other)

+++ Volume of biofluid or volume/mass of tissue sample collected per donor

++ Total volume/mass used for EV isolation (if pooled from several donors)

+++ All known collection conditions, including additives, at time of collection

+++ Pre-treatment to separate major fluid-specific contaminants before EV isolation

+++ Temperature and time of biofluid/tissue handling before and during pre-treatment

++ For cultured tissue explants: volume, nature of medium and time of culture before collecting conditioned medium

++ For direct tissue EV extraction: treatment of tissue to release vesicles without disrupting cells

Storage and recovery (Section 2-d)

+++ Storage and recovery (e.g., thawing) of CCM, biofluid, or tissue before EV isolation (storage temperature, vessel, time; method of thawing or other sample preparation)

+++ Storage and recovery of EVs after isolation (temperature, vessel, time, additive(s)…)

3-EV separation and concentration

Experimental details of the method

++ Centrifugation: reference number of tube(s), rotor(s), adjusted k factor(s) of each centrifugation step (= time+ speed+ rotor, volume/density of centrifugation conditions), temperature, brake settings

++ Density gradient: nature of matrix, method of generating gradient, reference (and size) of tubes, bottom-up (sample at bottom, high density) or top-bottom (sample on top, low density), centrifugation speed and time (with brake specified), method and volume of fraction recovery

++ Chromatography: matrix (nature, pore size,…), loaded sample volume, fraction volume, number

++ Precipitation: reference of polymer, ratio vol/vol or weight/vol polymer/fluid, time/temperature of incubation, time/speed/temperature of centrifugation

++ Filtration: reference of filter type (=nature of membrane, pore size…), time and speed of centrifugation, volume before/after (in case of concentration)

++ Antibody-based : reference of antibodies, mass Ab/amount of EVs, nature of Ab carrier (bead, surface) and amount of Ab/carrier surface

++ Other…: all necessary details to allow replication

++ Additional step(s) to concentrate, if any

++ Additional step(s) to wash matrix and/or sample, if any

Specify category of the chosen EV separation/concentration method (Table 1):

+ High recovery, low specificity = mixed EVs and non-EV components **OR**

+ Intermediate recovery, intermediate specificity = mixed EVs with limited non-EV components **OR**

+ Low recovery, high specificity = subtype(s) of EVs with as little non-EV as possible **OR**

+ High recovery, high specificity = subtype(s) of EVs with as little non-EV as possible

4-EV characterization

Quantification (Table 2a, Section 4-a)

+++ Volume of fluid, and/or cell number, and/or tissue mass used to isolate EVs

+++ Global quantification by at least 2 methods: protein amount, particle number, lipid amount, expressed per volume of initial fluid or number of producing cells/mass of tissue

+++ Ratio of the 2 quantification figures

Global characterization (Section 4-b, Table 3)

+++ Transmembrane or GPI-anchored protein localized in cells at plasma membrane or endosomes

+++ Cytosolic protein with membrane-binding or -association capacity

+++ Assessment of presence/absence of expected contaminants
(At least one each of the three categories above)
++ Presence of proteins associated with compartments other than plasma membrane or endosomes
++ Presence of soluble secreted proteins and their likely transmembrane ligands
+ Topology of the relevant functional components (Section 4-d)

Single EV characterization (Section 4-c)

+++ Images of single EVs **by wide-field and close-up**: e.g. electron microscopy, scanning probe microscopy, super-resolution fluorescence microscopy
+++ Non-image-based method analysing large numbers of single EVs: NTA, TRPS, FCS, high-resolution flow cytometry, multi-angle light-scattering, Raman spectroscopy, etc.

5-Functional studies

+++ Dose-response assessment
+++ Negative control = nonconditioned medium, biofluid/tissue from control donors, as applicable
+++ Quantitative comparison of functional activity of total fluid, vs EV-depleted fluid, vs EVs (after high recovery/low specificity separation)
+++ Quantitative comparison of functional activity of EVs vs other EPs/fractions after low recovery/high specificity separation
+ Quantitative comparison of activity of EV subtypes (if subtype-specific function claimed)
+ Extent of functional activity in the absence of contact between EV donor and EV recipient

6-Reporting

+ Submission of methodologic details to EV-TRACK (evtrack.org) with EV-TRACK number provided (strongly encouraged)
+++ Submission of data (proteomic, sequencing, other) to relevant public, curated databases or open-access repositories
+ Data submission to EV-specific databases (e.g., EVpedia, Vesiclepedia, exRNA atlas)
++ Temper EV-specific claims when MISEV requirements cannot be entirely satisfied (Section 6-b)