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Comparative analysis of the minimal information for studies of extracellular vesicles guidelines: Advancements and implications for extracellular vesicle research

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ABSTRACT

In 2014, the International Society for Extracellular Vesicles (ISEV) introduced the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines to establish standards for extracellular vesicle (EV) research. These guidelines aimed to enhance reliability and reproducibility, addressing the expanding field of EV science. EVs, membrane-bound particles released by cells, play crucial roles in intercellular communication and are potential biomarkers for various conditions. Over the years, the EV landscape witnessed a surge in publications, emphasizing their roles in cancer and immune modulation. In response, the MISEV guidelines underwent evolution, leading to the MISEV2018 update. This version, generated through community outreach, provided a comprehensive framework for EV research methodologies, emphasizing separation, characterization, reporting standards, and community engagement. The MISEV2018 guidelines reflected responsiveness to feedback, acknowledging the evolving EV research landscape. The guidelines served as a testament to the commitment of the scientific community to rigorous standards and the collective discernment of experts. The present article compares previous MISEV guidelines with its 2023 counterpart, highlighting advancements, changes, and impacts on EV research standardization. The 2023 guidelines build upon the 2018 principles, offering new recommendations for emerging areas. This comparative exploration contributes to understanding the transformative journey in EV research, emphasizing MISEV's pivotal role and the scientific community's adaptability to challenges.

1. Introduction: navigating the history of the MISEV guidelines

In 2014, the International Society for Extracellular Vesicles (ISEV) board members unveiled the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines, providing a foundational framework for defining extracellular vesicles (EVs) and elucidating their functions [1]. The MISEV guidelines are designed to set standards for EV research, addressing the expanding landscape of this field. Extracellular vesicles, which are membrane-bound particles released by cells, play important roles in intercellular communication, and potentially serve as biomarkers for various physiological and pathological conditions [2]. The MISEV guidelines outlined in the MISEV2014 publication aimed to sensitize researchers, journal editors, and reviewers to the unique experimental and reporting requirements within the rapidly expanding field of EV research.

Over the ensuing years, the landscape of EV research witnessed an exponential surge in publications, bringing EVs to the forefront due to their implicated roles in cancer progression and immune network modulation [2–4]. Recognizing the growing interest surrounding EVs beyond the research community, the ISEV community initiated a concerted effort to update and refine the MISEV recommendations. The resultant MISEV2018 guidelines, born out of a community outreach effort, marked a significant evolution in response to the advancements and discoveries in the field [5]. MISEV2018, generated through a

Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; EVs, extracellular vesicles; HDL, high-density lipoprotein; ISEV, International Society for Extracellular Vesicles; LC, liquid chromatography; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MISEV, Minimal Information for Studies of Extracellular Vesicles; MS, mass spectrometry; NVEPs, non-vesicular extracellular particles; SEC, size exclusion chromatography; SF, synovial fluid; SIL, stable isotope labelled; UC, ultracentrifugation; UEV, urine extracellular vesicles.

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detailed process involving a community-wide survey, aimed to establish a consensus on essential requirements, feasible practices, and cautious result interpretation. While retaining the core validity of the MISEV2014 recommendations, MISEV2018 recognized the imperative for certain amendments. To guide researchers, the 2018 document meticulously delineated the evolution of recommendations in tables and provided practical suggestions, including protein markers and exemplar approaches. MISEV2018 responded to feedback from the ISEV community, recognizing concerns that MISEV2014 was perceived as either too restrictive or a strong imposition on the field [6]. The inclusive approach involved an extensive survey and multiple rounds of internal review, incorporating the perspectives of ISEV members, board members, and additional EV experts [5]. The guidelines adapt to field advancements, incorporating technological changes, expanding knowledge, and reflecting consensus within the EV research community over time. MISEV seeks consensus on essential requirements, feasible practices, and the interpretation of results. Researchers are encouraged to transparently report their methods and results, enabling other researchers to replicate or reproduce findings, thereby contributing to the overall reliability of EV research. Expanding upon the foundation established by the two prior iterations of the guidelines, which largely remain relevant, the 2023 guidelines enhance and elaborate on the principles introduced in MISEV2018. Additionally, they provide new recommendations and guidance to address emerging areas of development [7]. This literature review aims to identify and evaluate the advancements in methodologies, changes in recommendations, and the overall impact on the standardization and progress of EV research.

2. Guideline organization

Several sections distinguish MISEV2023 from MISEV2018, reflecting an evolution in the organizational structure. For instance, in MISEV2023, a section clarifying the scope of MISEV is introduced, along with novel sections focusing on expanded EV sources, including considerations for bacteria, biofluids, and tissues in both collection and preprocessing stages, and practical assistance on how researchers should use the guidelines. MISEV2023 prioritizes nomenclature considerations: although “exosomes” and “ectosomes” are distinct based on biogenesis, practical challenges often hinder their separate identification, favouring the use of broader terms. The guidelines encompass the nomenclature of non-vesicular particles and include supplementary sections on EV secretion, uptake, and manipulations used to block EV secretion, emphasizing specificity and the necessity for more precise approaches. Concerning EV uptake, a focused discussion delves into analyzing the process and its functional significance. Particularly, the exploration of the significance of EVs directed to the lysosome of the recipient cell becomes a pivotal consideration. In addition, the guidelines cover EV therapeutics, partly based on strong interest expressed in the November 2020 MISEV update survey [6]. A critical issue addressed is the characterization of preparations often described as exosomes in the context of therapeutic development and the extent to which these preparations should undergo further characterization. Aligning with the November 2020 survey, MISEV2023 includes a section on EV analysis *in vivo*. Rather than providing a comprehensive listing of experiments in different models, the focus is on the general methods by which EVs are marked, characterized, and functionally analyzed in these systems. This section aims to highlight complementary strategies and caveats associated with *in vivo* EV studies. A "Summary" and/or "Recommendations" section at the end of every MISEV2023 subsection presents the key insights and guidelines discussed in that subsection. It streamlines the assimilation of information, offering a convenient roadmap for researchers to grasp the essential recommendations and considerations pertinent to their specific area of interest within the broader field of EV studies. This approach enhances the guidelines’ user-friendliness, facilitating efficient navigation and aiding researchers in the application of MISEV recommendations to their work. Furthermore, the "Consensus" portion of the guidelines at the end of each section in both the MISEV2018 and 2023 guidelines plays a pivotal role in refining collective expert agreement within the EV research community. These sections serve as concise summaries, encapsulating widely accepted recommendations, key insights, and best practices outlined in each subsection. The inclusion of these consensus sections has been carried over into the MISEV2023 guidelines, emphasizing their enduring significance in guiding researchers toward a shared understanding of fundamental principles in the dynamic field of EV studies.

3. Scope and definition of EVs

The definitions of EVs have evolved slightly from MISEV2018 to MISEV2023.

3.1. *MISEV2018 definition*

In the MISEV2018 guidelines, the term "EV” is endorsed as the generic term for particles naturally released from the cell, delimited by a lipid bilayer, and incapable of replication due to the absence of a functional nucleus [5]. The guidelines acknowledge the lack of consensus on specific markers for EV subtypes, making the assignment of EVs to specific biogenesis pathways extraordinarily difficult without live imaging techniques capturing EV release. Authors are urged to use operational terms for EV subtypes based on physical characteristics, biochemical composition, or descriptions of conditions or cell of origin. Furthermore, caution is advised against using terms such as "exosome" and "microvesicle" due to historical inconsistencies and contradictory definitions associated with these terms, further emphasizing the need for clarity and standardization in EV research.

3.2. *MISEV2023 definition*

The MISEV2023 guidelines retain the definition of EV from MISEV2018 but remove "naturally released" to avoid unintended exclusion of engineered EVs or those produced under various cell culture conditions [7]. Exosomes, smaller vesicles associated with the endosomal pathway, are released when multivesicular bodies fuse with the plasma membrane, posing challenges in establishing their biogenesis pathways [8]. Ectosomes, generally larger vesicles that may have a size overlap with exosomes, are associated with direct budding from the cell surface, contributing to the challenge of establishing clear biogenesis pathways [8] (Fig. 1). Although the use of the generic term "EV," is still recommended, the use of operational terms for EV subtypes based on characteristics like size, density, molecular composition, or cellular origin, is encouraged, albeit with caution. Additionally, the guidelines address the limitations associated with size terminology and biogenesis-based terms, emphasizing the need for clear definitions. Table 1 provides a reference on EV terms discussed in the updated guidelines [7].

3.3. *Comparative analysis*

While both sets of guidelines endorse the use of “EV” as the generic term, MISEV2023 introduces refinements to address the evolving landscape of EV research. The removal of "naturally released," sustained the focus on generic terminology, and the acknowledgment of challenges associated with specific terms and operational definitions underscores the guidelines’ commitment to precision and consistency. Given the challenges posed by dependence on biogenesis-based subtyping, marked by the absence of universal markers, heterogeneity in EV populations, overlapping size profiles, limited understanding of biogenesis pathways, dynamic cellular processes, potential misleading terminology, and technological limitations, the guidelines stress the importance of adopting a nuanced and comprehensive approach in EV research. This approach encourages the consideration of various characteristics

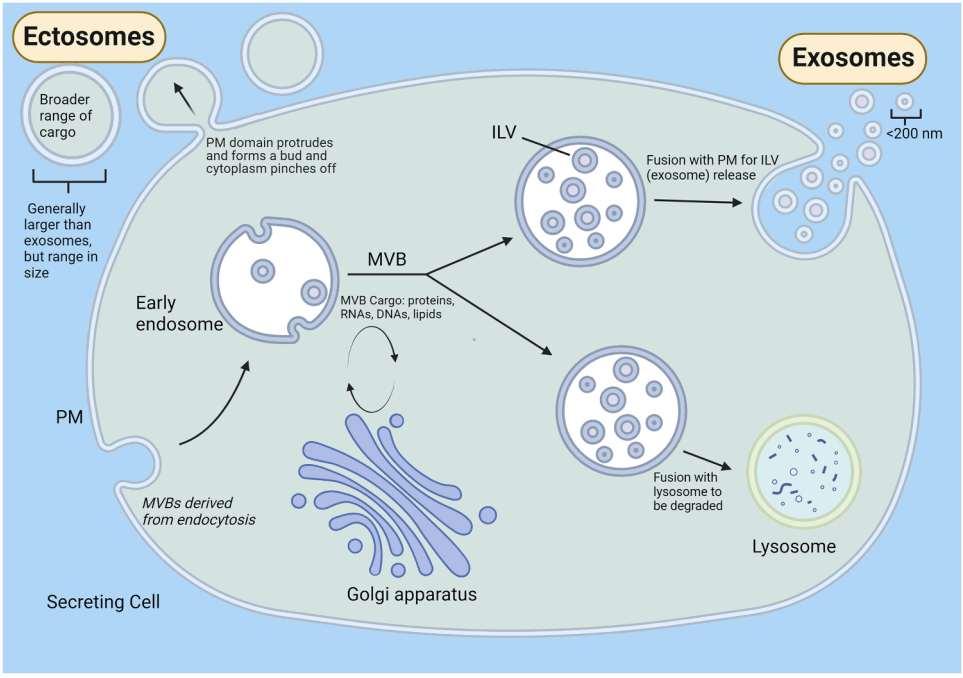

Fig. 1. Biogenesis Pathways of Exosomes and Ectosomes. Extracellular particles (EPs), Extracellular vesicles (EVs), Multivesicular bodies (MVBs), Intraluminal vesicles (ILVs), Non-vesicular extracellular particles (NVEPs), Plasma membrane (PM).

beyond biogenesis pathways for accurate categorization and interpretation in the ever-evolving field of EV research (Fig. 2).

4. The applications of MISEV

A close examination of what MISEV is and is not reveals the evolving nature of these guidelines over the years. MISEV2018 set the stage as an introduction to EV research, offering a comprehensive set of recommendations to enhance rigor, reproducibility, and transparency. It served as a tool for reviewers and editors, assisting in evaluating EV-related proposals, manuscripts, and funding applications. The guidelines were crafted to be a non-exhaustive set of examples and a framework supporting innovation across a spectrum of EV research.

MISEV2023 recognizes the dynamic nature of EV studies and, unlike a rigid checklist, positions itself as a flexible framework encouraging expert judgment. It recognizes the multitude of techniques and platforms available to researchers, providing adaptable recommendations that can accommodate the diversity inherent to EV research. This departure from a prescriptive approach allows researchers the freedom to tailor methods to their study's specific demands. MISEV2023 prominently commits to fostering innovation in EV research, positioning itself as a facilitator rather than an obstacle to innovation or publication. Researchers are encouraged to present their findings, even if certain aspects do not perfectly align with the guidelines. The focus on openness cultivates a supportive environment for diverse EV studies, recognizing the continuous exploration and discovery in the field. As a facilitator, not a restrictive force, MISEV2023 strives to nurture a collaborative and inclusive community, encouraging a broad spectrum of EV research to thrive. The evolution from MISEV2018 to MISEV2023 represents a significant shift in focus. MISEV2023 is more than guidelines; it acts as a flexible framework promoting inclusivity, innovation, and supports researchers in navigating EV studies. Serving as a guiding beacon, MISEV2023 offers adaptable principles for the diverse and dynamic nature of EV investigations.

5. Sample collection and processing

Both sets of guidelines emphasize the pivotal role of the pre-analytical phase in influencing the quantitative and qualitative aspects of EVs. Common recommendations include detailing the source of EV-containing materials, reporting donor characteristics, and providing methodological details of sample collection. However, MISEV2023 introduces sections on specific EV sources, some of which are highlighted below.

5.1. Cell culture-conditioned medium

This section covers EV-releasing cells, culture conditions, and harvesting approaches. This signifies a refinement in addressing the evolving field of EV research. This is exemplified by the detailed considerations for cell culture-conditioned media and the incorporation of new sections focusing solely on EV sources. Additionally, both guidelines highlight the necessity of reporting medium composition and preparation, especially when using complex additives like serum, ensuring the reliability of EV-related measurements. MISEV2023 also refers to the ISEV Rigor and Standardization Subcommittee, which oversees the appointment and activities of thematic task forces and special interest groups dedicated to specific sources of EVs and other EV-related topics.

5.2. Biofluid complexity

Both MISEV2018 and MISEV2023 acknowledge the complexity of handling biofluids in EV research, recognizing over 30 types of biofluids in mammals. While MISEV2018 avoids an exhaustive review of pre-analytical variables across all biofluids, both sets of guidelines stress the need for meticulous consideration due to the diverse biophysical and chemical characteristics inherent in each biological fluid. Structurally, both guidelines emphasize the unique challenges posed by specific biofluids, such as the viscosity of plasma and serum or the lipidic structures present in different biofluids. MISEV2023 explicitly calls for the development of biofluid-specific reporting guidelines, a departure

Table 1
Comparison of EV Nomenclature Guidelines (MISEV2018 vs. MISEV2023).

Term	Old (2018) Guidelines	New (2023) Guidelines	Usage
Extracellular vesicles (EVs)	Particles naturally released from cells, enclosed by a lipid bilayer, incapable of self-replication. Lack of consensus on specific markers for EV subtypes; suggests using operational terms.	Particles released from cells, enclosed by a lipid bilayer, incapable of self-replication. The term "naturally" has been omitted to include engineered EVs. Advocates for the use of the generic term "EV" over "exosomes" and "ectosomes." Encourages the use of operational extension.	Recommended
Non-vesicular extracellular particles	Previously unspecified.	Multimolecular assemblies released from cells without a lipid bilayer. Recommends using operational terms for EV subtypes.	Recommended
Extracellular particles (EPs)	To be used if EV identity cannot be confirmed according to the guidelines.	General term encompassing all particles outside the cell, including EVs and NVEPs. Recommends operational terms for EV subtypes.	Recommended
EV mimetic	Previously unspecified.	EV-like particles produced through artificial manipulation. Favored over terms implying specific biogenesis. Advises caution with operational terms.	Recommended
Artificial cell-derived vesicles (ACDVs)	Previously unspecified.	EV mimetics produced in the laboratory under induced cell disruption. Recommends operational terms for clarity.	Recommended
Synthetic vesicles (SVs)	Previously unspecified.	EV mimetics synthesized de novo or as hybrid entities. Recommends operational terms for clarity.	Recommended
Small EVs (operational term)	Defined based on size, often <200 nm. Caution necessary due to variations in measurement methods.	Diameter-based term, often <200 nm. Caution required with specific measurement methods.	Recommended, but caution required
Medium/Large EVs (operational term)	Defined based on size, often >200 nm. Caution necessary due to variations in measurement methods.	Diameter-based term, often >200 nm. Caution required with specific measurement methods.	Recommended, but caution required
Other 'operational terms'	Physical characteristics (e.g., diameter), biochemical composition, cellular origin, and conditions. Caution necessary due to method influence.	Physical attributes, including size (e.g., small extracellular vesicles or sEVs, large EVs or lEVs) and density (categorized as low, medium, or high within defined ranges), constitute one set of characteristics. Biochemical composition involves the presence of particular (macro) molecules, such as proteins. Terms referring to cellular origin and the conditions of EV generation emphasize specific aspects of biogenesis, encompassing molecular mechanisms, energy dependence (or its absence), and the functional state of the parent cell in relation to stress or cell death. Use caution with specific measurement methods.	Recommended, but caution required
Exosome	Origin from endosomal system. Likely studying a broad EV population unless subcellular origin is demonstrated. Diameter of intraluminal vesicles generally <200 nm.	Origin from endosomal system. Likely studying a broad EV population unless subcellular origin is demonstrated. Diameter of intraluminal vesicles generally <200 nm. Discourages use unless subcellular origin is demonstrated.	Discouraged
Ectosome	Origin from plasma membrane. Likely studying a broad EV population unless subcellular origin is demonstrated. Wide size range.	Origin from plasma membrane. Likely studying a broad EV population unless subcellular origin is demonstrated. Wide size range which may include exosomes. Discourages use unless subcellular origin is demonstrated.	Discouraged
Microvesicle	Often used synonymously with "ectosomes".	Origin from plasma membrane. Historically used for large EVs or all EVs. Can lead to confusion. Discourages use.	Discouraged
Exosome-like vesicles	Discouraged due to the biogenesis-related term (unless subcellular origin can be demonstrated).	Discouraged for synthesized EV mimetics, as the term 'exosome' indicates endosomal system origin.	Discouraged

EV (Extracellular Vesicle), NVEP (Non-vesicular Extracellular Particle), EP (Extracellular Particle), ACDV (Artificial Cell-Derived Vesicle), SV (Synthetic Vesicle), sEV (Small Extracellular Vesicle), lEV (Large Extracellular Vesicle).

from MISEV2018's broader scope. This targeted approach aligns with the evolving understanding of unique considerations required for different biofluids in EV isolation. Importantly, the guidelines only cover EV sources with an ISEV Task Force. Encouraging the creation of new task forces aims to enhance collaboration and refine guidelines for diverse biofluids in EV research. MISEV2023 specifically advocates for the development of biofluid-specific reporting guidelines, reflecting the evolving insights into distinct considerations in EV isolation.

5.2.1. *Bacteria*

In addressing bacterial EVs, both MISEV2018 and MISEV2023 acknowledge the challenges posed by the diverse nature of bacteria, bacterial EVs, and source material characteristics. The guidelines recognize the complexity of providing universal recommendations for sample type, pre-processing, separation, collection, and characterization due to the multitude of factors influencing bacterial EV heterogeneity.

MISEV2018 lays the groundwork by acknowledging the diverse pathways for bacterial EV formation, including blebbing and lytic biogenesis. The guidelines highlight the impact of different species, strains, and growth conditions on EV heterogeneity, echoing the importance of their role in comprehensive bacterial EV studies. While acknowledging the infancy of research on the impact of culture conditions on bacterial EV yield and composition, the guidelines offer initial recommendations for reporting culture conditions.

Building on MISEV2018, MISEV2023 delves deeper into the complexities of bacterial EV studies. Despite diversity, the guidelines stress the feasibility of certain recommendations, underscoring the importance of reporting culture details. Factors like media composition, oxygenation/aeration, culture format, and growth phase significantly impact bacterial EV characteristics. MISEV2023 introduces additional recommendations for the post-sample collection phase, urging detailed reporting of all separation/concentration methods. The guidelines caution against non-specific methods like precipitation and ultracentrifugation, which may co-isolate unwanted non-EV materials. Gentler alternatives, such as filtration and chromatography methods, are recommended. MISEV2023 also highlights the challenges in characterizing bacterial EV preparations, noting availability of validated affinity reagents for bacterial markers. MISEV2023 stresses the significance of reporting detailed characterization beyond core measurements, acknowledging the limitations imposed by the lack of validated markers for various bacterial species. Specific markers such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA) are recommended as universal markers for broad classes of bacterial EVs, with a reminder to include appropriate controls due to potential presence in non-vesicular extracellular particles (NVEPs). MISEV2023 introduces several key recommendations, such as reporting bacterial growth phase at harvest, limiting storage prior to EV separation/concentration, considering the presence of host EVs or EVs from non-target species *in vivo* and environmental sources, and highlighting the challenges posed by non-vesicular co-isolates of bacterial EVs.

5.2.2. *Blood*

Blood stands as the paramount biofluid in the realm of EV research. Its dominance arises not only from the advantages of a minimally invasive liquid biopsy, but also the wealth of clinically relevant information encapsulated within its contents [9]. The intricate characteristics of blood make it imperative for a comprehensive exploration of its pivotal role and profound significance in the field of EV research [10].

For EV isolation from blood plasma, both guidelines reference previous ISEV position papers and publications outlining essential reporting requirements [11]. While MISEV2018 emphasizes factors like donor age, sex, and technical details such as fluid collection volume,

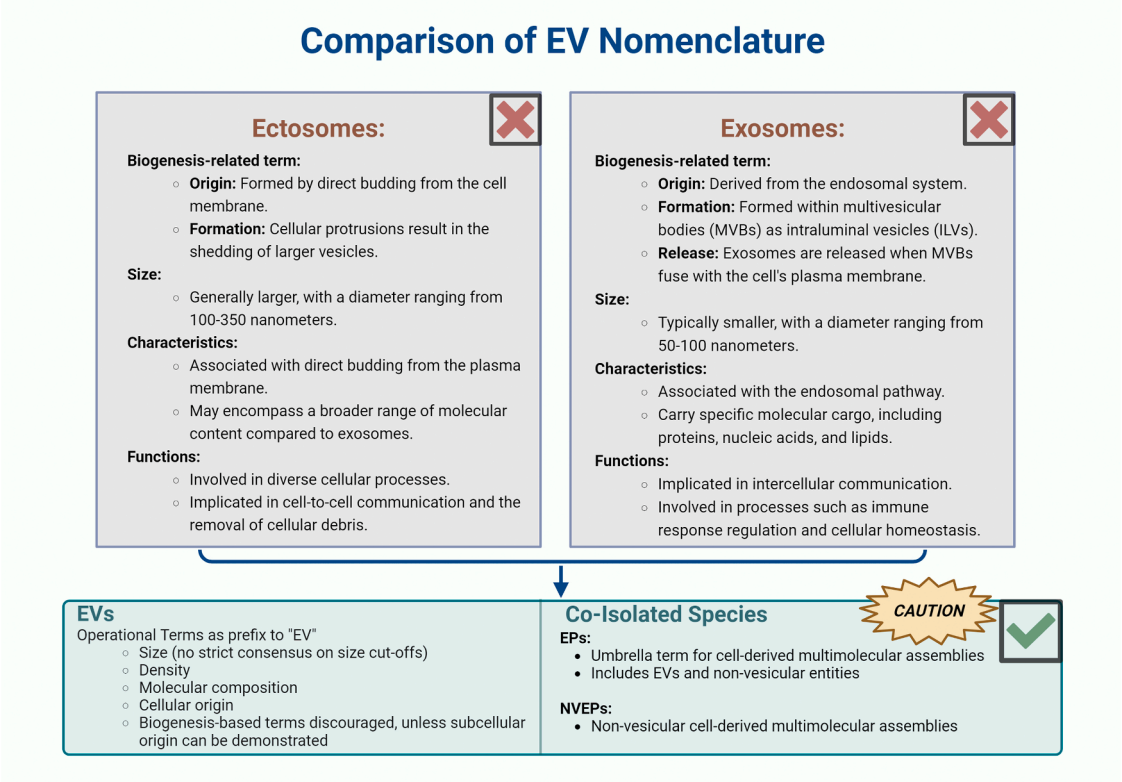

Fig. 2. EV Nomenclature Compared. Extracellular particles (EPs), Extracellular vesicles (EVs), Multivesicular bodies (MVBs), Intraluminal vesicles (ILVs), Non-vesicular extracellular particles (NVEPs).

MISEV2023 extends this by encouraging a more comprehensive exploration of pre-analytical variables' effects on various EV classes. The MISEV2023 guidelines explicitly acknowledge the ongoing questions surrounding the impact of specific pre-analytical variables, accentuating the need for continued research in this area. While certain technical details, including those specific to plasma/serum, remain consistent, MISEV2023 advocates for a more inclusive and nuanced reporting approach. Both guidelines acknowledge the challenges associated with archived samples and stress transparency regarding unrecorded variables.

In support of researchers exploring relevant factors in EV blood research, the ISEV's "Blood EVs" workshop in Helsinki in 2022 on all things related to EVs and blood, underscored the need for dependable information, particularly for novice researchers [12]. Following this workshop, a beginner's guide to studying EVs in human blood plasma and serum was published in 2024 [12]. This guide explores the composition and terminology of blood, offering comprehensive guidelines for blood collection and the preparation of plasma and serum. This complementary article to MISEV2023 emphasizes the significance of blood and its derivatives, plasma, and serum, as the most widely studied body fluids for EV research. The guide introduces basic principles for isolating and detecting blood EVs while considering relevant factors. This resource aims to support novice researchers by providing a concise, evidence-based introduction to current knowledge and available resources for studying blood EVs. Notably, the authors acknowledge the challenges in detecting rare EV types in blood due to their low concentration and highlight the ongoing exploration of cellular origins using advanced techniques. The guide discusses blood EVs' physiological and pathological functions, diseases, and potential roles in processes like coagulation. Challenges related to blood composition variations and the overlap of lipoproteins with EVs in post-prandial blood are recognized, supporting the need to address these complexities in studies, especially in the context of healthy human blood.

Isolation methods for EVs play a pivotal role in downstream analytical assays from blood plasma or serum and hinge on four key principles—size, density, charge, and molecular composition [11]. Commonly employed techniques include ultracentrifugation, density gradient centrifugation, size-exclusion chromatography, and emerging affinity-based chromatography. However, none of these methods can exclusively isolate all EVs due to the inherent overlap in size and density with platelets and lipoproteins. Commercial precipitation kits offer high yields but compromise purity, contrasting with methods like size-exclusion chromatography or density gradient centrifugation that provide higher purity at the expense of lower recovery [13]. The choice of isolation method should align with the study's objectives, identifying that different methods may impact downstream results, making direct comparisons challenging [14,15].

Major confounders in EV isolation and detection involve lipoproteins and plasma proteins, both abundant in blood [16]. Co-isolation of lipoproteins with EVs poses challenges due to overlapping size ranges and significantly higher number concentrations of lipoproteins. Despite efficient size-based separation, a 100-fold enrichment of EVs compared to high-density lipoprotein (HDL) may still result in a 10,000-fold excess of HDL particles. Plasma and serum, rich in soluble proteins, exhibit dynamic protein composition influenced by genetic and environmental factors. Analyzing the tiny fraction of EVs in plasma and serum is challenging due to extremely low concentrations. EVs from human plasma and serum carry a plasma protein corona, underlining the relevance of optimizing EV purity aligned with the study's objective.

Isolating platelets from platelet derived EVs is challenging due to the high viscosity and density of fluids hindering separation methods. The ISEV Blood EV task force recommends measuring platelet concentration before freezing samples to account for platelet fragmentation during freeze-thaw cycles. Co-isolation of confounding factors introduces biases in EV analyses, emphasizing the need to optimize blood collection and plasma/serum preparation conditions. Guidelines for the preparation of plasma and serum emphasize standardized steps, measuring confounding factors during protocol development. Quality control parameters like particle/protein or protein/lipid ratios offer insights into the efficacy of applied isolation protocols, ensuring reliable blood EV research.

The beginner's guidelines stress the importance of standardized procedures, clear reporting, and continuous education on the latest practices provided by ISEV. This parallels the MISEV2023 guidelines which were updated to include specific recommendations on blood collection and pre-processing. MISEV2023 also acknowledges the various complex components of blood (e.g., cells, lipoproteins, other factors) which can confound subsequent analyses. A common theme between both the beginner's guidelines and the MISEV2023 guidelines is the encouragement of transparent reporting. Specifically, the beginner's guidelines advocate for avoiding ambiguous terminology, assessing lipoprotein residuals, providing detailed information, and staying informed (Table 2) [12]. In summary, the tandem guidance from MISEV2023 and the beginner's guide forms a strong foundation for researchers, encouraging comprehensive exploration, standardized methodologies, and transparent reporting to navigate the complexities of blood EV research.

5.2.3. Urine

Urine is the second most-analyzed biofluid after blood in EV research for its non-invasive, serial, and large-quantity collection potential [17]. Acknowledging challenges in urine extracellular vesicle (uEV) studies, difficulties arise from the diverse cellular origin of uEVs and the dynamic urine composition influenced by factors like fluid intake, collection time, diet, exercise, age, sex, medication, and health status. Addressing these challenges, the 2023 guidelines recommend specific considerations for urine collection and storage, noting that many biobanked urine samples have not been processed to remove cells before storage, potentially necessitating uEV-specific biobanks, or new collections. The guidelines highlight the presence of urinary proteins, such as Tamm-Horsfall protein (THP), as common co-isolates/contaminants of uEV preparations and offer strategies, such as depolymerization, for mitigating their impact on uEV purity and yield.

In the territory of normalization approaches for uEV studies, the 2023 guidelines underscore the magnitude of inter- and intra-individual variation in urine concentrations, requiring careful considerations. Unlike the 2018 guidelines, the 2023 version acknowledges the absence of a consensus method or marker for robust normalization. MISEV2023 suggests the use of absolute or relative measures, including total protein, uEV number, uEV biomarkers, or normalization to urinary creatinine, osmolality, and other non-EV urine parameters. Furthermore, MISEV2023 provides specific recommendations for researchers, urging adherence to previously published ISEV recommendations and conducting uEV research using cell-free urine and cell-free urine biobanks. The guidelines also emphasize reporting the methodology and outcomes of uEV co-isolate/contaminant depletion, particularly for proteins like THP and albumin.

A study conducted by Garcìa-Flores *et al.* explored uEVs as potential prostate cancer biomarkers, highlighting their relevance in reflecting tumor biology non-invasively [18]. However, the translation of these biomarkers to clinical settings faces challenges, including the irreproducibility of results. In their research, the investigators evaluated the impact of three EV isolation methods, namely, ultracentrifugation (UC), size exclusion chromatography (SEC), and Exolute®, applying various techniques to analyze their effects on EV characteristics.

The findings suggest SEC as the most efficient method for isolating specific EVs, emphasizing the need for methodological standardization of EV isolation and characterization, particularly in clinical contexts, where large sample sizes are common. Therefore, consistently updating the guidelines is crucial for significantly enhancing the utility of EVs in clinical settings.

Table 2
Reported Recommendations for Blood EV Research.

Beginner's guidelines	MISEV2023	MISEV2018
1. **Avoid Ambiguous Terminology:** Refrain from using terms like "platelet-free plasma," "platelet-depleted plasma," or "platelet-poor plasma" as they lack clarity and scientific definition. Instead, precisely define the starting material by reporting the concentration of remaining platelets. 2. **Evaluate Lipoprotein Residuals:** After establishing a laboratory method, assess the number of residual lipoproteins, ensuring a comprehensive understanding of potential contaminants. 3. **Provide Detailed Information:** Furnish comprehensive details about sample properties, preanalytical handling, and the level of detection of the instrumentation used in your manuscript. Utilize the latest checklists, such as those designed for flow cytometry or biobank samples, to guide reporting. 4. **Stay Informed:** Keep updated with the latest educational materials provided by ISEV to enhance your understanding and adherence to current guidelines and best practices in blood EV research. Regular updates contribute to maintaining high standards in study design and reporting.	1. MISEV2018 recommendations remain valid. 2. MiBlood tool for reporting the traceability of blood-derived samples used for EV studies[a] (9). Categorized based on confounding factors in blood EV research: a. General study information. b. Blood collection, processing, storage. c. Qualitative and quantitative evaluation of hemolysis, platelets, and lipoproteins.	Standardization and reporting: • Donor variables[b] • Pre-analytical processing variables[c]

[a] Created by the ISEV Blood Task Force to report the traceability of blood-derived samples used for EV studies
[b] Age, biological sex, circadian rhythm, diet, exercise level, and medication
[c] Blood collection, preparation, handling, storage, anticoagulants, centrifugation protocol, and handling time

5.2.4. Cerebrospinal fluid

Leveraging cerebrospinal fluid (CSF) as a biofluid source of EVs is more relevant in detecting diseases of the central nervous system (CNS) due to its contact with the brain and spinal cord [19]. Previous renditions of the MISEV guidelines endorsed the importance of pre-analytical variables without specific biofluid-related considerations, but the updated 2023 MISEV guidelines exhibit a more nuanced and tailored approach to addressing the complexities of CSF EV studies. An essential aspect highlighted by the updated guidelines is the rostro-caudal gradient established by the continuous flow of CSF through the brain and spinal cord. The gradient results in fluctuations of specific brain proteins across distinct regions, underlining the significance of documenting the collection site (e.g., lumbar/spinal canal vs. brain) and volume, as they may influence the composition of CSF [7].

MISEV2023 points out common challenges encountered in CSF studies, including issues such as residual cells and blood contamination, given the notably higher concentrations of blood proteins compared to CSF [7]. A review article from the ISEV CSF Task Force, acknowledges that the inadequate reporting of pre-analytical variables pertaining to CSF collection, processing, and storage in numerous publications hinders reproducibility and comparability among laboratories [20]. The guidelines recommend measuring specific co-isolates/contaminants and establishing exclusion criteria, such as excluding CSF samples with more than 500 erythrocytes/μL from biomarker studies. Another challenge highlighted stems from the remarkably low concentration of EVs in CSF and the limited total volume of CSF samples. Given the precious nature of CSF samples, there is a notable emphasis on the necessity for high-yield separation approaches and high-sensitivity characterization assays in CSF EV studies. To overcome these challenges, the guidelines propose strategically pooling samples from multiple donors to optimize new protocols or facilitate omics characterization, with or without subsequent higher-sensitivity specific molecular assays for individual samples. In contrast to the 2018 guidelines, the 2023 version takes a more tailored approach to tackle the intricacies of CSF EV studies, aligning with the evolving understanding of biofluid-specific challenges in EV research.

5.2.5. Saliva

MISEV2023 introduces a new section focused on saliva studies, recognizing saliva's significance as a non-invasive source of biomarkers, including EVs. This addition features the practical appeal of saliva, especially in the context of oral and periodontal conditions [21]. The guidelines stress considering diverse components in saliva studies, including eukaryotic cells, proteins, electrolytes, food debris, bacterial cells, and bacterial EVs. Saliva's composition depends on the activity and contributions of major and minor salivary glands, each potentially secreting different amounts of enzymes and mucins. Specific parameters for reporting in saliva studies are detailed, including the type of saliva collected (whole or from a specific gland), the method of collection, and any salivation stimulus used. Standardizing food and drink intake before collection is stressed for consistency in results or, at the very least, assessing these factors at the time of collection.

Furthermore, the guidelines delve into factors influencing saliva composition, such as age, biological sex, smoking, stress, exercise, oral hygiene, medical conditions, medications, and mental health status. While these factors have been associated with differences in viscosity, pH, concentrations of different proteins, and saliva flow rate in studies of whole saliva, the guidelines acknowledge that their impact on saliva EV concentration and composition remains unclear. As such, the inclusion of this section underscores the need for additional research in this specific domain.

5.2.6. Synovial fluid

Another novel section in MISEV2023 focuses on synovial fluid (SF) studies, highlighting the potential of SF EV as biomarkers and therapeutic agents for joint disorders. This addition underscores SF's relevance in studying joint-related conditions by highlighting its direct contact with affected tissues within joint spaces [22]. The unique viscosity of SF, attributed to substantial protein content and the glycosaminoglycan hyaluronic acid, poses challenges in reproducible SF EV studies. These challenges include difficulties in pelleting cells and debris before freezing, as well as hindering EV recovery. The guidelines acknowledge that many reported samples have undergone freezing and thawing before EV separation and characterization, leading to inconsistent removal of cells and debris pre-freezing. To address these challenges, the guidelines recommend using hyaluronidase to reduce SF viscosity, ensuring a more homogenized synovial fluid before EV separation and characterization. However, it is noted that the use of hyaluronidase varies among research groups. Additionally, MISEV2023 highlights SEC's potential superiority over UC in removing proteins such as albumin, fibronectin, and apolipoprotein A-I. They draw attention to donor characteristics that may impact SF variables and possibly EVs, including biological sex and the identity and stage of joint-related diseases. This section emphasizes the need for standardized approaches in SF EV studies and recognizes the complexities associated with SF viscosity and its influence on sample processing and characterization.

5.2.7. Milk

Renowned for its rich and complex composition, milk stands as a valuable source of nutritional and immunological components, playing a more prominent role in the 2023 guidelines. MISEV2018 revealed the ISEV community's interest in further research to formulate more specific recommendations for the study of EVs collected from this biofluid. Both MISEV2018 and 2023 guidelines recognize the challenges inherent in studying milk EVs. The presence of components sharing EV characteristics, such as milk fat globules and cellular debris, necessitates careful pre-processing steps like centrifugation to ensure relatively pure EV isolation. A significant challenge highlighted is the interference posed by casein micelles, especially in ruminant species' milk, for which strategies like micelle precipitation, enzymatic treatment, and calcium sequestration are recommended.

MISEV2018 acknowledged the importance of considering pre-analytical variables in milk EV studies but lacked specific recommendations for biofluids. In contrast, MISEV2023 offers a dedicated section for milk EV studies, providing detailed recommendations and methodologies. Notably, both guidelines stress the need for removing common EV co-isolates and emphasize the importance of tracking their presence throughout the EV separation process. Most importantly, the incorporation of specific recommendations in the 2023 guidelines reflects the field's progress and the demand for more tailored methodologies to advance research in milk EVs.

5.3. Solid tissue and other sources

Comparing the guidelines for isolating and characterizing tissue derived EVs in the 2018 and 2023 editions reveals notable shifts and expansions in focus. While the 2018 guidelines recognized challenges in ensuring the authenticity of vesicles from tissues, the 2023 guidelines, acknowledging complexities in tissue harvesting methods, cellular composition, and physical properties, delineate two fundamental approaches for tissue EV studies: maintaining tissues/cells alive in culture or harvesting EVs directly from tissues.

In the ex vivo culturing approach, the 2023 guidelines emphasize maintaining tissues as close as possible to their native conditions, considering factors such as hydration, nutrition, and the influence of cell death processes on EV preparation. In contrast, the direct harvesting approach involves meticulous consideration of specific practices for tissue harvesting, storage, physical and enzymatic tissue separation, and the impact of various EV separation and concentration methods. These recommendations intend to guide researchers in ensuring robust and reproducible tissue EV studies. Additionally, the 2023 guidelines introduce a comprehensive focus on tissue EV characterizations,

accentuating the need to trace the presence of cellular components that may be expected to be depleted in EVs. This heightened scrutiny aims to address potential contaminants, including cells and cellular artifacts, in tissue EV preparations.

In essence, the evolution from the 2018 to the 2023 guidelines reflects a deepening understanding of the intricacies surrounding tissue EV studies. The expanded recommendations in the 2023 guidelines provide a more detailed framework to guide researchers in navigating the challenges associated with tissue harvesting, processing, and storage, ultimately promoting rigorous and standardized practices in the evolving field of tissue-derived EV research.

5.4. Storage and processing

The 2018 guidelines extensively focused on the storage and retrieval conditions of both matrix and isolated EVs, emphasizing the impact on EV characteristics. The guidelines questioned the preparation and storage of biofluids, tissues, or media, urging detailed reporting on variables such as storage container, temperature, freezing/thawing, buffer, cryoprotectant use, and freeze-thaw cycles. Despite a consensus on the importance of pre-analytical variables, biofluid-specific considerations, and standard operating procedures were not extensively covered. MISEV2018 also addressed challenges in achieving absolute purification during EV separation and concentration, introducing terms like "separation" and "concentration" instead of "purification" or "isolation." It highlighted various techniques, with differential ultracentrifugation being most used. The guidelines emphasized the need for detailed reporting of methods and cautioned against proprietary kits, reflecting the collaborative spirit to ensure reproducibility.

In contrast, the 2023 guidelines highlight the evolving understanding of biofluid-specific challenges, advocating for the development of biofluid-specific reporting guidelines (Table 3). Both guidelines acknowledge the importance of considering diverse biophysical and chemical characteristics, but MISEV2023 takes a more targeted approach in line with evolving insights into distinct considerations in EV isolation.

Table 3
Summary of Novel Aspects in Various Biofluids for EV Research.

Biofluid	Novel Aspects
Blood	• Emphasis on pre-analytical variables' effects on various EV classes • Comprehensive exploration of blood collection and pre-processing methods • Transparency regarding unrecorded variables in archived samples is emphasized • Specific recommendations for addressing blood composition variations and lipoprotein confounders are provided
Urine	• Specific considerations for urine collection and storage • Strategies for mitigating the impact of common co-isolates/contaminants on uEV purity and yield • Recommendations for normalization approaches considering inter- and intra-individual variation
Cerebrospinal Fluid (CSF)	• Tailored approach to addressing the complexities of CSF EV studies • Emphasis on documenting collection site and volume to account for rostro-caudal gradient • Strategies for overcoming challenges related to low EV concentration and limited CSF sample volume
Saliva	• Introduction of new guidelines recognizing saliva's significance as a non-invasive source of biomarkers • Recommendations for standardizing saliva collection methods and reporting parameters • Exploration of factors influencing saliva composition and their potential impact on saliva EV concentration
Synovial Fluid	• Recognition of SF's potential for biomarkers and therapeutic agents for joint disorders • Recommendations for addressing challenges related to SF viscosity through hyaluronidase treatment and SEC • Emphasis on standardized approaches in SF EV studies and recognition of complexities associated with SF viscosity
Milk	• Specific recommendations for addressing challenges in milk EV studies, including interference from casein micelles • Importance of removing common EV co-isolates and tracking their presence throughout the EV separation process
Solid Tissue and Other Sources	• Two fundamental approaches delineated for tissue EV studies: maintaining tissues/cells alive in culture or harvesting EVs directly from tissues • Recommendations for robust and reproducible tissue EV studies, including considerations for tissue harvesting and EV separation methods • Comprehensive focus on tissue EV characterizations and addressing potential contaminants

Abbreviations: EV (Extracellular Vesicle), uEVs (Urine Extracellular Vesicles), CSF (Cerebrospinal Fluid), SF (Synovial Fluid), and SEC (Size Exclusion Chromatography).

6. Characterization techniques

Characterizing EVs is crucial for estimating their quantity, confirming their presence, and assessing non-EV components. Challenges, such as small size and heterogeneity, exist due to the absence of universal identification methods and non-specific measurement techniques. The recommended approach is to use orthogonal methods, employing diverse and independent techniques that offer complementary information to overcome inherent challenges in EV analysis.

6.1. Quantifying particle number concentration and particle size

In comparing the 2023 and 2018 guidelines on EV quantification, both emphasize challenges in measuring EV characteristics. The 2023 guidelines focus on particle number concentration, recognizing its limitations in specificity and sensitivity. They introduce traceable measurements and considerations for well-characterized EV reference materials. MISEV2023 recommends reporting assay limits of detection, advocating for orthogonal methods and distinguishing between "particle" and "EV concentration." In contrast, the 2018 guidelines discuss quantifying EVs based on individual components, emphasizing techniques like light scattering and cryogenic electron microscopy. Both express concerns about specificity, but the 2023 guidelines provide a more detailed and updated perspective, addressing traceability, orthogonal methods, and nuances in EV size quantification.

6.2. Quantifying total protein, lipids, and RNA

In MISEV2018, the guidelines discussed methods for quantifying EV components, such as total protein, lipids, and RNA. Total protein was measured using colorimetric assays like Bradford or BCA, fluorometric assays, or global protein stains on SDS-PAGE. Challenges included potential overestimation due to co-isolated contaminants and variability based on the specificity of EV separation methods. Lipid quantification methods involved assays, fluorescence of membrane intercalating dyes, total reflection FTIR spectroscopy, or chromatography. Quantifying total RNA faced challenges due to the association with other entities and potential interference from co-separating particles. Specific molecule quantification, such as using ELISA or flow cytometry, was recommended for estimating the amount of EVs containing particular components.

6.2.1. Total protein

The 2018 guidelines highlighted colorimetric assays like Bradford or BCA, fluorometric assays, and global protein stains on SDS-PAGE for total protein quantification. Identified challenges included potential overestimation due to co-isolated contaminants, especially when using less specific EV separation methods. Fast-forward to 2023, the updated

guidelines caution against relying solely on protein concentration as a direct surrogate for EV concentration. Emphasis is placed on potential discrepancies arising from different cellular phenotypes or stimulations. Recommendations in 2023 include reporting absolute protein and particle concentrations separately, providing details on whether intact or disrupted preparations are measured, and considering the lower concentration limit of detection for each assay. Caution in interpreting particles-to-protein ratio is stressed, aligning with the broader goal of ensuring transparent and meaningful interpretation of results.

6.2.2. Total lipids

In 2018, total lipid quantification methods included colorimetric assays, fluorescence of membrane intercalating dyes, total reflection FTIR spectroscopy, or chromatography. Challenges included potential insensitivity for small amounts of EVs and uncertainties about detecting all EVs regardless of lipid composition. The 2023 guidelines provide nuanced recommendations, advising consideration of assay limits of detection, sensitivity to co-isolated NVEPs like lipoproteins, and caution in interpreting results to avoid overestimation of EVs due to co-isolated NVEPs.

6.2.3. Total RNA

In MISEV2018, total RNA quantification methods, including capillary electrophoresis, faced challenges, particularly in using RNA as a reliable surrogate for EV concentration due to the abundance of extra-EV RNA. The 2023 guidelines recognize these issues and emphasize a discerning approach. They stress evaluating the assay's ability to differentiate RNA from DNA and recommend reporting enzymatic pretreatments, like DNase, for accurate RNA quantification. While acknowledging the challenges, the guidelines suggest using total RNA quantification for basic EV characterization purposes, such as quality control or normalization. Caution is advised against relying solely on total RNA as a surrogate for EV concentration or purity, considering challenges like differentiation between RNA and DNA, potential impacts of isolation kits, and variability in sensitivity across methods.

6.3. Characterizing EV morphology

MISEV2018 briefly mentioned imaging techniques like electron microscopy and density gradients, noting challenges such as artifacts in desiccated conditions and throughput limitations. In contrast, the 2023 guidelines offer a detailed framework, urging researchers to report experimental specifics for any imaging technique used. Emphasis is on disclosing instrument specifications, software versions, sample preparation, and controls. Limitations, including low throughput and bias in some techniques, are acknowledged to enhance the reliability of EV morphology assessment.

6.4. Characterizing protein composition

The 2018 guidelines acknowledged the heterogeneous nature of EVs, avoiding specific molecular markers for each subtype and suggesting three categories of markers for analysing bulk EV preparations. MISEV2023 maintains a similar stance but presents a more detailed five-component framework for reporting claims about EV protein content. Emphasis is on EV features, purity from contaminants, and additional information on intracellular origins. Researchers are cautioned about the non-specificity of certain proteins across different EV analysis methods, reflecting a nuanced and evolving understanding of EV protein composition.

6.5. Characterizing non-protein markers

The 2018 guidelines briefly mentioned non-protein markers like phospholipids as potential positive controls for EV presence but cautioned about their non-specificity, recommending appropriate negative controls. In contrast, MISEV2023 offers more comprehensive recommendations for non-protein markers, including considerations for phosphatidylserine, glycans, and specific nucleic acids. The guidelines emphasize caution regarding non-specificity and advocate for protein colocalization when using non-protein markers, providing a nuanced understanding of complexities, and highlighting the importance of careful interpretation in EV research.

6.6. Localization of EV-associated components

Both the 2018 and 2023 guidelines stress determining the topology of EV-associated components like proteins, nucleic acids, and glycans. In 2018, a new recommendation endorsed investigating luminal versus surface topology, considering theoretical expectations based on the cytosol of EV-secreting cells. The guidelines highlighted potential unexpected surface topology, raising questions about the source. Methods involving mild digestions, permeabilizations, or antibody studies were suggested. In 2023, the guidelines take a proactive stance, explicitly recommending considering topology during method design, proposing specific methodologies, and encouraging a nuanced perspective. While both agree on the necessity of determining topology, the 2023 guidelines provide more detailed recommendations, fostering a deeper understanding of biological implications associated with the spatial organization of EV cargo.

7. Reporting standards

As the literature on EV detection assays and instrumentation continues to expand, MISEV guidelines must adapt to meet the demand for robust reporting standards [23]. Given the diverse applications and increasing expertise in EV research, these guidelines provide comprehensive minimal reporting criteria designed to be applicable across a variety of experimental designs. While the techniques listed are not exhaustive, they include commercially available methods with established literature, representing a collaborative effort to establish reliable and reproducible standards. Recognizing the dynamic nature of the field, the guidelines acknowledge ongoing developments and research in detection technologies, suggesting that additional criteria may be necessary to address subjective experimental parameters.

The 2018 guidelines presented more generalized recommendations for reporting standards in EV research. During collection and preprocessing, MISEV2018 emphasized the need for researchers to devise a plan for collection and experimental procedures to maximize the identification of known, reportable parameters and encouraged reporting as many pre-analytical parameters as possible. In the context of EV isolation/characterization from conditioned media, MISEV2018 advocated for reporting basic characterization of releasing cells, details about culture and harvesting conditions, identification of cell lineage, the percentage of dead, apoptotic, or necrotic cells at EV harvest, and other relevant characteristics such as the state of activation, malignancy, and senescence. Due to potential alterations in EV conditions caused by storage and retrieval conditions, MISEV2018 advised researchers to provide detailed information about the storage process and a procedure for critically evaluating the implications of storage method and time on EV activity and other properties, where applicable. Although the 2018 guidelines offered sample questions for researchers to critically analyze and report their findings, it lacked an exhaustive reporting guide or checklist. Additionally, MISEV2018 introduced the EV-TRACK knowledgebase, a web tool with seven facilitating elements designed to guide researchers in using the EV-METRIC, consolidating information on EV characteristics and methodologies, searching through research articles, and involving researchers in decisions regarding ongoing enhancements [24–26]. Authors were also prompted to submit EV profiling data to public databases, including those curated by the European Bioinformatics Institute, the US National Center for Biotechnology Information, and the Japanese Center for Information Biology. Field-specific

databases such as EVpedia, Vesiclepedia (formerly ExoCarta), and the exRNA Atlas were also recommended for data submission. By strategically planning and reporting collection and experimental procedures, prioritizing transparency, and facilitating reliable replication, these recommendations sought to strengthen the robustness of EV research.

The inclusion of the question "Have you shared data and reported methods in sufficient detail to enable others to replicate or reproduce your results?" in the MISEV2023 is emblematic of the evolving spirit of these guidelines, reflecting a deliberate effort to enhance reporting standards. This question underscores the increased focus on methodological transparency and data sharing in MISEV2023. Unlike prior versions, these guidelines now distinctly outline “recommendations” and/or “reporting recommendations” at the conclusion of each subsection, enhancing clarity. This strategic organization facilitates a clearer understanding of the detailed reporting requirements, reinforcing MISEV2023’s commitment to fostering a research environment marked by precision, transparency, and replicability. Reporting recommendations cover key parts of MISEV2023, including defining EVs and their subtypes, pre-analytical variables, EV separation and concentration, EV characterization, technique-specific reporting requirements, EV release and uptake studies, EV functional studies, and EV *in vivo* studies.

New sections introduced since MISEV2018 enrich and refine the guidelines in several crucial aspects. Firstly, the guidance on NVEPs provides valuable insights into working with entities beyond traditional EVs, fostering a comprehensive understanding of the extracellular environment. This inclusion improves standardization of nomenclature within the literature and acknowledges evolving complexities in EV studies. Additionally, the incorporation of new pre-analytical variables, spanning EV sources such as bacteria, biofluids, and tissues, demonstrates MISEV’s commitment to adapting recommendations. This expansion enables researchers to consider a wider array of influential factors that may impact EV studies, reinforcing the guidelines’ dedication to addressing the evolving challenges and nuances in the field. Detailed biofluid-specific reporting guidelines were absent in MISEV2018 but are now included in the 2023 update. MISEV2023 now features technique-specific reporting for EV characterization, tailoring recommendations to the subtleties of different analytical methods. This approach promotes standardized reporting across diverse techniques, contributing to the overall robustness and reproducibility of EV research. Lastly, the inclusion of sections on EV release and uptake, as well as *in vivo* EV studies, delves into critical stages of EV biology and application. However, it should be noted that the intention for the “*in vivo*” section of the guidelines is intended to raise awareness and foster innovation, rather than provide prescriptive rules. This offers targeted guidance for researchers engaged in these specific areas, further enhancing the comprehensiveness and applicability of the MISEV recommendations.

8. Biological and potential clinical relevance

One of MISEV’s pillars asserts its relevance to translational and clinical research, encompassing applications such as the production and initial evaluation of therapeutic EVs (Table 4) [23]. The 2018 guidelines explore the critical consideration of EV purity, acknowledging its variability based on the experimental question and intended EV use. The level of purity needed depends on whether the focusis on basic research or clinical applications. Situations prioritizing attributing a function or identifying a biomarker specific to vesicles necessitates highly purified EVs. Conversely, in contexts where a biomarker is valuable even without pre-enrichment of EVs or in therapeutic scenarios prioritizing functionality over a definitive association with EVs, less pure EVs may suffice. It is worth noting that some presumed contaminants may co-isolate with EVs, and intriguingly, might even contribute to their function. Hence, the method chosen for separation and concentration must be guided by factors that can vary between studies, underscoring the absence of a one-size-fits-all approach.

Table 4
Implementation of EV Guidelines in Clinical Translation.

Aspect	Implementation in Clinical Setting
EV Purity Considerations	• Critical consideration of EV purity based on intended use • Situational dependency on required purity level • Method choice guided by study-specific factors
Technique-Reporting Considerations	• Utilization of MS for protein analysis • Targeted and untargeted proteomic approaches • Inclusion of SIL peptides for absolute quantification
Functional Study Recommendations	• Multiplexing for enhanced sensitivity • Emphasis on physiologically informed studies • Importance of selection of EV negative controls
In Vivo Study Recommendations	• Inclusion of appropriate controls for discerning specific EV functions • Focus on EV release, biodistribution, and function in model organisms • Use of genetically tractable organisms for specific labeling
Therapeutic translation challenges	• Reporting all details of labeling and detection technologies • Standardization of isolation and purification methods • Addressing challenges in EV-based drug delivery • Ensuring reproducibility in EV analysis
Standardization efforts	• Establishment of consistent methodologies and reporting criteria • Balancing standardization and innovation for scientific progress • Potential future customization of guidelines based on research objectives

Abbreviations: EV (Extracellular Vesicle), MS (Mass Spectrometry), SIL (Stable Isotope Labelled).

The MISEV2023 guidelines detail technique-reporting considerations, such as mass spectrometry (MS) as a widely employed tool for detecting and characterizing EV-associated proteins in both discovery and targeted clinical applications. Proteomic analysis employs two primary methods, targeted and untargeted, each presenting specific applications, benefits, and constraints. Targeted analyses, executed on a triple quadrupole liquid chromatography (LC)-MS platform, excel in meticulous investigations. Conversely, untargeted proteomics, carried out on Time-of-Flight (ToF) or Orbitrap MS platforms, delivers a thorough grasp of the sample’s protein makeup by identifying all discernible ions, encompassing those originating from EV-related proteins or matrix contaminants. These proteomic approaches can cater to biomarker discovery through untargeted studies, offering insights into the biological relevance of EVs. Targeted peptide analysis is more relevant for assessing EV purity, enabling the quantification of absolute protein abundance. This ensures a detailed understanding of protein changes, especially in the context of diseases or therapeutic interventions. Furthermore, inclusion of stable isotope labelled (SIL) peptide standards in targeted analysis allows for absolute quantification of endogenous analytes, improving the precision of measurements. In the context of enhancing sensitivity, multiplexing (as seen in LC-MS workflows) is more suitable for limited sample volumes, such as those from clinical trials.

Functional study recommendations persist in the 2023 guidelines, offering overarching suggestions for the diverse nature of *in vivo* and *in vitro* studies. The key points include a continued emphasis on physiologically informed dose-response and time-course studies as an essential aspect of experimental design. Furthermore, the guidelines stress the importance of selecting EV negative controls meticulously. This involves evaluating ’background’ EV activity by considering factors such as EVs present in culture medium components or non-specific activity of EVs beyond those of primary interest. Examples of negative controls are

outlined in MISEV2023, such as using unconditioned medium processed similarly to conditioned medium for cell culture derived EVs. Engineered EVs should be compared with those from unmanipulated cells or cells engineered with irrelevant components. For disease studies, it is recommended to use EVs sourced from healthy, matched, or untreated donors. The guidelines suggest the inclusion of controls involving non-EV-containing, EV-depleted, or enzymatically treated EV separation fractions. This aims to discern whether a particular function is specific to EVs or associated with co-isolating materials. Notably, the guidelines acknowledge emerging evidence since MISEV2018 regarding the functional role of loosely tethered coronal elements, which may complicate the analysis. These elements, along with EV co-isolates, may contribute additively or synergistically to observed effects. Lastly, the guidelines highlight the significance of investigating the influence of EV separation/concentration, storage, and formulation factors on EV activity. The goal is to maximize the reliability and relevance of experimental outcomes. Acknowledging the impracticality of studying all conceivable controls simultaneously, the guidelines propose the use of potency assays to identify the most informative controls for both pre-clinical and clinical studies.

The MISEV2023 guidelines maintain recommendations for *in vivo* studies of EVs, focusing on their release, biodistribution, pharmacokinetics, and function. These studies offer valuable mechanistic insights into EV behavior and are conducted across a diverse range of species, including clinically relevant model organisms that mirror aspects of human health and disease. The guidelines underscore the advantages of genetically tractable organisms, facilitating hypothesis testing and allowing for specific EV labeling approaches. The ease of genetic manipulation in both invertebrate and vertebrate model organisms supports the investigation of EV subtype-specific mechanisms. The guidelines present diverse *in vivo* models for EV studies, each with specific strengths and limitations. Examples include Drosophila melanogaster, allowing visualization of vesicle biogenesis through endosomal compartments, and Caenorhabditis elegans, providing insights into EV roles in various cellular and developmental processes. While recognizing challenges in separating and concentrating EVs for small invertebrates, the guidelines highlight reported success in nematode worms and fruit flies. Larger mammalian models are acknowledged as essential to replicate specific aspects of human physiology and disease. *In vivo* models provide a unique opportunity to assess the release of physiological levels of EVs and their interaction with target cells. The guidelines discuss various *in vivo* study approaches, ranging from examining endogenous EVs using tags like fluorescence or bioluminescence to introducing exogenous EVs into organisms. Preclinical studies with syngeneic models and human cancer cell line xenograft models enable specific labeling and tracing of tumor and other EVs. However, the guidelines acknowledge potential caveats in exogenous approaches, such as label effects on biodistribution patterns and detectability thresholds. MISEV2023 emphasizes the importance of reporting all details of labeling and detection/imaging technologies for replication studies. For exogenous EV administration, researchers are encouraged to provide comprehensive details, including anatomical site, timing, and dose. Consideration of potential effects of EV labeling on biodistribution, pharmacokinetics, and function is recommended, along with awareness of unintended consequences of blocking EV production. Researchers are also advised to consider potential differences in behavior between endogenous and exogenous EVs.

An exciting realm of EV therapeutic translation involves leveraging the natural biogenesis process of EVs. EVs are inherently produced with high biocompatibility, enhanced stability, and limited immunogenicity, offering numerous advantages as drug delivery systems [27]. Despite these benefits, challenges persist for implementing EV-based drug delivery, including the lack of a standardized isolation and purification method, limited drug loading efficacy potentially due to loading method, and insufficient clinical grade production [28]. Analyzing EVs encounters difficulties due to the multitude of assessment methods with varying reproducibility. The challenge is further compounded by the presence of other materials sharing similar biochemical and/or biophysical characteristics.

MISEV's ultimate objective is to facilitate communication, collaboration, and advancement in the field of EV research by establishing a common framework for reporting, interpretation, and comparison of results. Standardization involves establishing consistent and well-defined methodologies, reporting criteria, and experimental practices across different studies. This helps ensure that researchers adhere to a common set of guidelines when conducting experiments, characterizing EVs, and reporting their findings. However, there is a potential trade-off between standardization and innovation. Standardization aims to provide a uniform framework for conducting and reporting research, which can enhance reproducibility and comparability of results. On the other hand, innovation often involves exploring new and novel approaches, methodologies, or techniques that may not align perfectly with established standards. Researchers may face challenges in balancing the need for standardization, which contributes to reliability and comparability, with the desire for innovation, which drives scientific progress. Striking the right balance is essential to ensure that the field benefits from both the advantages of standardized practices and the exploration of innovative ideas. Perhaps the future direction of MISEV guidelines might include customization based on specific research objectives, allowing for flexibility in the guidelines as needed. Another suggestion is for journals to offer checklists to authors submitting their research, potentially fostering greater adherence to the guidelines.

9. Integration with other guidelines

The updated MISEV2023 guidelines have been integrated with several other guidelines to facilitate reporting methods. Although the 2018 guidelines reference position papers, they do not specifically direct authors to supplementary guidelines that may guide their reporting strategies. In the 2023 update of the guidelines, ISEV recommends the adoption of additional reporting and atlas tools, such as the 'Minimum Information for the Publication of Quantitative Real-Time PCR Experiments' (MIQE) designed for real-time reverse transcriptase-quantitative polymerase chain reaction (qPCR) analyses [29]. These databases serve as repositories for detailed information on experimental methodologies, characterization techniques, and other essential parameters, ensuring transparency and reproducibility in EV research. Moreover, these structured resources facilitate the identification of best practices, methodological nuances, and potential pitfalls. In this way, the databases effectively guide researchers through a checklist-like format, aiding them in adhering to established standards, avoiding oversights, and contributing to the overall rigor and reproducibility of their work.

10. Community feedback and involvement

The collaborative development of MISEV2023, akin to MISEV2018, commenced with a 2020 pre-drafting survey, garnering feedback from over 1000 respondents to identify key areas for emphasis and exploration. A committee of five authors was then tasked with preparing a draft, which underwent numerous refinements based on feedback from the ISEV board and approximately 70 contributing authors. Subsequent community engagement in 2022, with over 1000 responses, further shaped the guidelines. The final manuscript involved a total of 1051 co-authors from at least 53 countries.

Acknowledging MISEV's limitations, the guidelines clarify their role as a tool for building rigor and reproducibility in EV research. They aim to educate newcomers without presenting an unreasonable barrier or hindering innovation. While not a comprehensive literature review, MISEV incorporates over 500 citations, highlighting the challenge of categorizing the primacy and quality of thousands of annual EV publications.

The MISEV2023 document invites critique and debate, recognizing

that such discussions contribute to the refinement of EV science. For future editions, questions arise due to the current process's complexity and duration. Proposed solutions advocate for no field-wide renewal but a more structured approach, potentially concentrating on specific methods, reagents, experimental design, or applications. The potential integration of artificial intelligence, if accessible, and heightened engagement of ISEV task forces and special interest groups are suggested. These groups could focus on specific elements such as methods, reagents, experimental designs, or applications without necessitating a comprehensive field-wide revision of MISEV. Another option entails establishing more frequent review cycles at shorter intervals to systematically assess and integrate relevant advancements or creating a dynamic online platform or a "living" document to allow real-time updates to individual sections as new information emerges.

These multifaceted mechanisms collectively contribute to maintaining the guidelines' currency, facilitating their role as a valuable resource for the scientific community engaged in EV research. MISEV encourages ongoing awareness and dissemination efforts, aiming to engage the community in the pursuit of continuous improvement.

11. Conclusion

In conclusion, the MISEV guidelines, introduced by ISEV in 2014 and subsequently updated in 2018 and 2023, have been instrumental in shaping the landscape of EV research. Initially designed to establish standards for enhancing reliability and reproducibility of EV studies, the guidelines have evolved to adapt to the dynamic nature of the field. The 2018 update, driven by community engagement and feedback, demonstrated a commitment to inclusivity and flexibility, recognizing the diverse perspectives within the ISEV community. This evolution has been widely embraced by researchers globally, fostering adherence to best practices and elevating the quality and comparability of EV studies worldwide. As a set of recommendations, the MISEV guidelines serve as a guiding framework for crucial aspects of EV research, including separation, characterization, and functional studies. The guidelines actively engage the EV research community through surveys and outreach efforts, reflecting a commitment to achieving consensus on essential requirements and practices. Researchers are encouraged to transparently report their methods and results, contributing to the overall reliability of EV research.

This comparative exploration has meticulously explored the nuanced differences between the 2018 and 2023 versions of the MISEV guidelines. The 2023 update built upon the foundation laid by its predecessor, offers enhanced principles, new recommendations, and guidance to address emerging areas of development. The primary objective of this study was to systematically assess the advancements in methodologies, changes in recommendations, and the overall impact on the standardization and progress of EV research. Through this in-depth examination, the transformative journey within the field of EV research is illuminated, highlighting the pivotal role played by MISEV in steering the course of this scientific domain. The MISEV guidelines' evolution vividly reflects the resilience and adaptability of the scientific community in meeting evolving challenges, ensuring their continued relevance and effectiveness.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Data Availability

No data was used for the research described in the article.

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