Reproducibility of extracellular vesicle research

Nieuwland, Rienk

2022-08

Nieuwland , R , Siljander , P R M , Falcón-Pérez , J M & Witwer , K W 2022 , ' Reproducibility of extracellular vesicle research ' , European Journal of Cell Biology , vol. 101 , no. 3 , 151226 . https://doi.org/10.1016/j.ejcb.2022.151226

http://hdl.handle.net/10138/343676
https://doi.org/10.1016/j.ejcb.2022.151226

European Journal of Cell Biology 101 (2022) 151226

Contents lists available at ScienceDirect

European Journal of Cell Biology

journal homepage: www.elsevier.com/locate/ejcb

Reproducibility of extracellular vesicle research

Rienk Nieuwland [a,b,*], Pia R.-M. Siljander [c], Juan M. Falcón-Pérez [d,e,f,**], Kenneth W. Witwer [g,h,***]

[a] Laboratory of Experimental Clinical Chemistry, Department of Clinical Chemistry, Amsterdam UMC location University of Amsterdam, Meibergdreef 9, Amsterdam, The Netherlands
[b] Vesicle Observation Centre, Amsterdam UMC location University of Amsterdam, Meibergdreef 9, Amsterdam, The Netherlands
[c] EV Group, Molecular and Integrative Biosciences Research Programme, Faculty of Biological and Environmental Sciences, University of Helsinki, Helsinki, Finland
[d] Center for Cooperative Research in Biosciences (CIC bioGUNE), Basque Research and Technology Alliance (BRTA), Exosomes Laboratory, Derio, Spain
[e] Centro de Investigación Biomédica en Red de enfermedades hepáticas y digestivas (CIBERehd), 28029 Madrid, Spain
[f] IKERBASQUE, Basque Foundation for Science, 48013 Bilbao, Spain
[g] Department of Molecular and Comparative Pathobiology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA
[h] Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

A B S T R A C T

Cells release membrane-delimited particles into the environment. These particles are called "extracellular vesicles" (EVs), and EVs are present in fluids contacting cells, including body fluids and conditioned culture media. Because EVs change and contribute to health and disease, EVs have become a hot topic. From the thousands of papers now published on EVs annually, one easily gets the impression that EVs provide biomarkers for all diseases, and that EVs are carriers of all relevant biomolecules and are omnipotent therapeutics. At the same time, EVs are heterogeneous, elusive and difficult to study due to their physical properties and the complex composition of their environment. This overview addresses the current challenges encountered when working with EVs, and how we envision that most of these challenges will be overcome in the near future. Right now, an infrastructure is being developed to improve the reproducibility of EV measurement results. This infrastructure comprises expert task forces of the International Society of Extracellular Vesicles (ISEV) developing guidelines and recommendations, instrument calibration, standardized and transparent reporting, and education. Altogether, these developments will support the credibility of EV research by introducing robust reproducibility, which is a prerequisite for understanding their biological significance and biomarker potential.

1. Introduction

Extracellular vesicles (EVs) is an umbrella term for different types of vesicles that are released by cells, including the endosome-origin exosomes and the plasma membrane-origin ectosomes or microvesicles of living cells, and the apoptotic bodies of apoptotic cells (Yáñez-Mó et al., 2015). The term "extracellular vesicles" was introduced by the International Society for Extracellular Vesicles (ISEV) because the different types of EVs are often indistinguishable (Théry et al., 2018). Studying EVs is challenging for several reasons, which will be briefly outlined in Part 2: Challenges. These challenges will be illustrated by explaining relevant examples, which include the physical properties of EVs underlying their heterogeneity and how this heterogeneity affects isolation and detection, the complexity of blood to illustrate the difficulties encountered when studying EVs in a body fluid, and flow cytometry as an EV detection method that likely will produce reproducible measurement results in the near future.

2. Challenges

2.1. Heterogeneity of extracellular vesicles

The first size distributions of EVs that were published for human plasma and urine in 2014, showed that EVs range in diameter from less than < 100 nm to 1 μm or larger (van der Pol et al., 2014; Arraud et al., 2014). These publications were important for several reasons. Firstly, the size distributions showed that there are no distinct peaks of "small EVs" and "large EVs" (as was once assumed of exosomes and

* Corresponding author at: Laboratory of Experimental Clinical Chemistry, Department of Clinical Chemistry, Amsterdam UMC location University of Amsterdam, Meibergdreef 9, Amsterdam, The Netherlands.
** Corresponding author at: Center for Cooperative Research in Biosciences (CIC bioGUNE), Basque Research and Technology Alliance (BRTA), Exosomes Laboratory, Derio, Spain.
*** Corresponding author at: Department of Molecular and Comparative Pathobiology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.
E-mail addresses: r.nieuwland@amsterdamumc.nl (R. Nieuwland), jfalcon@cicbiogune.es (J.M. Falcón-Pérez), kwitwer1@jhmi.edu (K.W. Witwer).

https://doi.org/10.1016/j.ejcb.2022.151226
Received 8 March 2022; Received in revised form 12 April 2022; Accepted 12 April 2022
Available online 15 April 2022

microvesicles), but rather that EVs can be of almost any size or diameter in a continuum. In other words, based on size, there are no distinguishable populations of small and large EVs. Thus, the size of EVs contained in a preparation will depend on the physical basis of the chosen method(s) employed for separation or enrichment of the sample, and different methods will provide different EV preparations starting from the very same material (Veerman et al., 2021).

Secondly, the size distribution of EVs hampers single-step isolation of all EVs. For example, when EVs are isolated by Sepharose 2B size-exclusion chromatography, EVs with a diameter of ~70 nm and larger will be isolated, but smaller EVs will not be isolated and co-migrate with lipoproteins and soluble proteins (Böing et al., 2014).

Thirdly, EV size distributions follow a power-law function, meaning that there is a high concentration of small EVs and a low concentration of large EVs. From the published size distributions and concentrations one can estimate the *total surface area* and *total volume* of EVs, assuming that all EVs are spherical and keeping in mind that below about 100 nm we have at the moment insufficient measurement data about the size distribution and number of EVs (van der Pol et al., 2013). The total surface area of EVs is estimated at about 1600 mm (Théry et al., 2018) per mL of body fluid. This large surface area derives from the high concentration of EVs with a diameter < 200 nm. In contrast, for the same total population of EVs, the total volume is about 53 nL. This volume is due to the fact that the concentration of EVs with a diameter > 200 nm is low. As a result, the total volume of EVs is about 0.005% of the volume of body fluids such as blood plasma or urine, and thus purification of EVs requires special attention.

Fourthly, whereas the total surface area of EVs is substantial, the surface area of a single EV is limited. Thus, EVs expose a limited number of epitopes compared with cells, and after staining with e.g. fluorescently-labeled antibodies, the fluorescence of single EVs is dim and often below the detection threshold of fluorescence detectors used in single particle detection methods such as flow cytometry (Welsh et al., 2020). Furthermore, EVs also have a relatively low refractive index, which may depend on the EV size, membrane thickness and its molecular composition (van der Pol et al., 2021). In principle, an EV consists mainly of intravesicular fluid that is surrounded by a thin (about 4 nm) phospholipid bilayer membrane. When EVs are detected by optical, i.e. light-based methods, the EV will scatter light, which (like fluorescence) can be measured and quantified by single-particle detection methods. Because EVs scatter less light than similarly sized particles of silica or polystyrene, which often are incorrectly used as a reference material for optical detection methods such as for example flow cytometry, the amount of scattered light per EV is small and thus difficult to detect (van der Pol et al., 2018).

Apart from their heterogeneity in size, the density of EVs also causes challenges, especially when isolating EVs. For example in the case of blood plasma and serum, the density of EVs hardly differs from the density of their environment, and this low "density contrast" makes it difficult to isolate EVs by centrifugation (Rikkert et al., 2020). Importantly, fluids like blood plasma and serum also contain non-EV particles such as high-density lipoprotein particles (Yuana et al., 2014) and platelets (Rikkert et al., 2020) which overlap in density with EVs. Thus, isolation of blood plasma EVs by density gradient centrifugation is complex (Zhang et al., 2020).

Regarding the biochemical composition, EVs contain lipids, nucleic acids, metabolites and proteins (Yáñez-Mó et al., 2015). Thus far, the compositional knowledge has been obtained using techniques that analyze the bulk composition of multiple EVs, and more, often insufficient attention has been paid to critical confounders as they are not yet all recognized. Some techniques provide information on the global biochemical composition at the level of single EVs, such as Raman spectroscopy (Enciso-Martinez et al., 2020), but these techniques are in their infancy regarding EV analyses, and more research is needed to determine the real combination and stoichiometry of the molecules forming a vesicle. Also, the current techniques poorly enable analysis of time-dependent changes in the EV populations.

Taken together, it is clear that the physical properties of EVs cause challenges for (optical) detection and isolation, but we have come to a point where technological innovations in detection and isolation of EVs, combined with standardization efforts and robust reporting, will improve reproducibility to such an extent that multicenter-studies will soon become possible (Nieuwland et al., 2020). In the next sections, we will focus on challenges caused by the complex environments in which EVs are often present. Current challenges are summarized in Fig. 1.

2.2. Complexity of fluids containing extracellular vesicles

Often, EVs are present in complex (body) fluids with high concentrations of cells, non-EV particles, and soluble proteins. Commonly, EVs are separated from cells by differential centrifugation. There is at least one challenge, and that is the separation of EVs from platelets in blood plasma. Because platelets are small cells (2–4 μm), lack a nucleus and have a density close to EVs, it is difficult to separate platelets from EVs by centrifugation (Rikkert et al., 2018). Consequently, "platelet-free" plasma will still contain platelets (Rikkert et al., 2021). Soluble proteins present less of a problem, because the bulk of proteins can be separated from EVs with differential ultracentrifugation combined with washes or by size exclusion chromatography (Böing et al., 2014). However, the presence of non-EV particles, including lipoproteins (plasma), protein aggregates and even viruses, cause more problems since they may overlap in size and density with EVs (Zhang et al., 2020). This is illustrated in Fig. 2, which shows the presence of a few EVs in a multitude of lipoproteins. Similarly, conditioned culture medium often contains EVs, lipoproteins (including chylomicrons) and soluble proteins from the serum used to culture cells (Zhang et al., 2020), and milk contains not only EVs but also casein particles, milk fat globules, and possibly lipoproteins that co-isolate with EVs (Hu et al., 2021). Thus, the presence of non-EV particles may interfere with separation and characterization of EVs.

A second challenge with regard to body fluids is that the composition of the biospecimen will also be donor-dependent (Clayton et al., 2019). For example, tumor-derived vesicles may be present in body fluids collected from cancer patients, but such vesicles should be absent in body fluids from healthy individuals. Although in this example the difference depends on the presence or absence of a disease, it is largely unknown how other variables affect the presence and function of EVs. These variables include age, body mass index, circadian rhythm, gender, health status, lifestyle, and the use of medication (Yates et al., 2022). Thus, within the term "biospecimen," many still unknown variables may be present that affect the presence, composition and thereby ultimately the function of EVs (Clayton et al., 2018).

2.3. Collection, handling and storage of fluids containing extracellular vesicles

Before EVs can be isolated and measured, EV-containing fluids are collected, handled and often stored in biobanks. This "pre-analytical phase" of collection, handling and storage is likely to affect the presence, composition and function of EVs (Clayton et al., 2018). As with the biospecimen, the applied pre-analytical procedures should be described in detail, but it remains a challenge to identify, standardize and report all relevant variables. For example, in the pre-analytical workflow of blood collection, blood plasma preparation and storage, at least forty different pre-analytical variables are present, and to which extent any of these variables affect the presence and/or function of EVs, is largely unexplored (Clayton et al., 2019). Furthermore, recently we observed that independent researchers using the same blood collection and plasma preparation procedure, yet produced blood plasma with different concentrations of (non-removed) platelets. Since none of the presently available isolation procedures completely separate platelets from EVs, such differences may affect the downstream analysis of EVs.

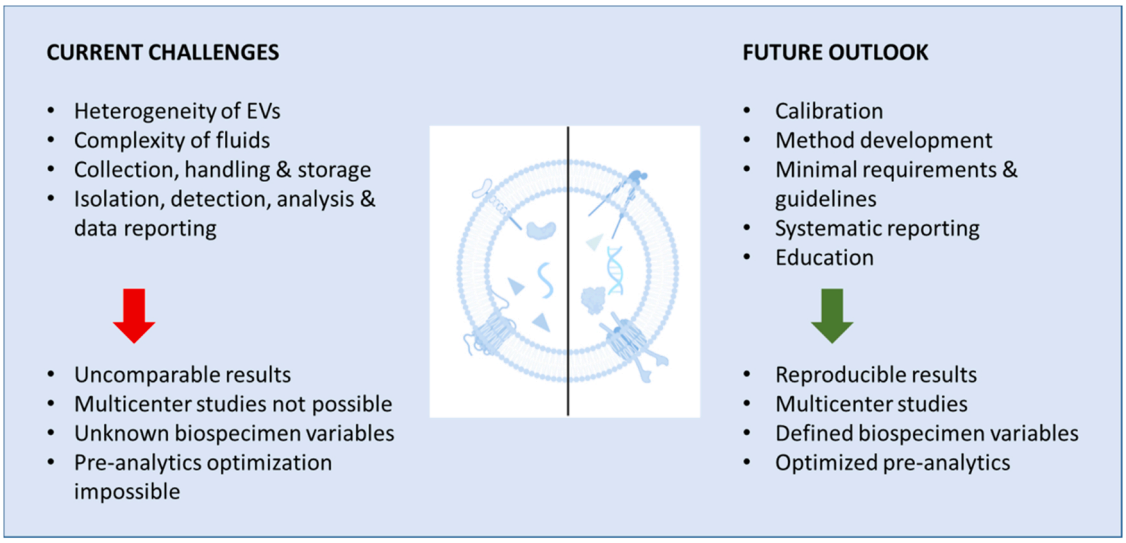

Fig. 1. Towards reproducible measurements of extracellular vesicles. The current lack of reproducibility in extracellular vesicle (EV) research hinders progress in understanding their biological role and theranostic applicability. Isolation and analyses are hampered by the physical and biochemical heterogeneity of EVs, the complex composition of tissues and biofluids containing the EVs, unidentified variables that affect the presence and function of EVs in a biospecimen, and the lack of both instrument calibration and standardized reporting of methods and results. In the near future, based on ongoing developments, reproducibility will be greatly improved by guidelines for collection, handling and storage of biofluids (pre-analytics), minimal development of novel separation and characterization methods, instrument calibration, requirements for systematic reporting of relevant biospecimen variables, methods and results, defining minimal requirements relevant to each step, and education effort. Comparable and reproducible results will then enable multicenter studies required for biomarker discovery and clinical utility.

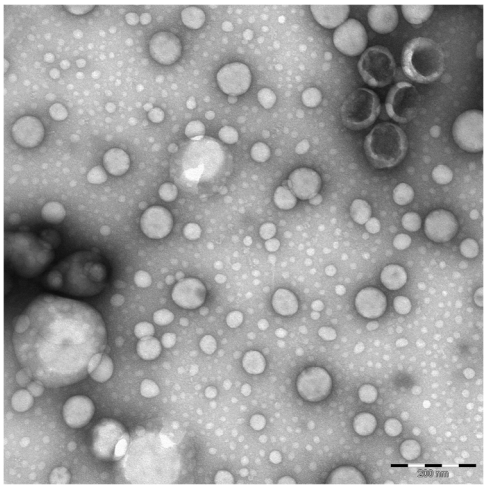

Fig. 2. Extracellular vesicles and lipoproteins in human plasma. A transmission electron microscopy image showing five extracellular vesicles (EVs) in the upper right corner. EVs collapse and appear often as cup-shaped structures due to fixation and dehydration. This image illustrates that EVs in human plasma are a small fraction compared to lipoproteins, which appear as white circular particles of various diameter. Scale bar: 200 nm.

Thus, the introduction of quality control parameters, such as measuring the concentration of platelets in the prepared blood plasma, will give insight into the presence of confounders and may help to interpret EV measurement results (Clayton et al., 2019). Finally, also the effects of storage on EVs are incompletely known, although the field is now actively addressing this issue (Gelibter et al., 2022; Barreiro et al., 2021).

2.4. Isolation, detection, analysis and data reporting

After the collection of EV-containing fluids, most downstream methods require isolation of EVs prior to analysis, for example to perform proteomics or lipidomics. The choice of an isolation method depends on the EV-containing (body) fluid studied, the downstream assay, whether or not the presence of particular confounders or reagents (e.g. anticoagulants added to blood to prevent clotting) may interfere with the downstream assay results, and the final application, which can range from basic research assays to routine diagnostics. As explained in the previous sections, the physical properties of EVs and the complexity of the EV-containing fluids present challenges to EV isolation. In principle, the currently available methods used to isolate EVs separate particles essentially based on either size, charge, density, or biochemical composition. As a rule of thumb, one can state that when EVs are isolated by one method, i.e. a method isolating EVs based on size, charge, density, or biochemical composition, the isolated EVs are likely to be impure and contain confounders. Therefore, combinations of separation methods are now being explored. For example, plasma EVs can be purified by separation based on size followed by separation based on density. In the first step, EVs are separated from soluble proteins and small lipoproteins such as HDL by size-exclusion chromatography, and in the second step EVs are separated from chylomicrons and LDL (larger lipoprotein particles) by density gradient centrifugation (Karimi et al., 2018).

Detection methods such as flow cytometry are able to detect single submicrometer particles directly in suspension. Direct detection offers the advantage that isolation is not required, and therefore exclusion of (sub)populations and inclusion of confounders is at least in part mitigated. However, the sensitivity of many single particle detection methods, i.e. the smallest EVs that can be detected, is not known because size gates are set using inadequate reference particles (Welsh et al., 2020), and within a method such as flow cytometry, the detection sensitivity varies per instrument due to their differences in the optical configuration, and differences in maintenance and operator skill (van der Pol et al., 2018). For this reason, the concentration of EVs in a given sample measured on different flow cytometers differs $>$ 100-fold between instruments (van der Pol et al., 2018). To some extent, these problems can be overcome by calibration procedures with relevant reference materials (Welsh et al., 2020). Over the years, flow cytometers have become more sensitive, meaning they are able to detect smaller EVs than previously. This causes an unexpected problem, because also

the size distribution of lipoproteins follows a powerlaw function, meaning that there is a much higher concentration of small lipoproteins than large lipoproteins (Ala-Korpela et al., 2021). Because the total concentration of lipoproteins and protein aggregates is $>10^5$-fold higher than the estimated total concentration of plasma EVs (Zhang et al., 2020), sensitive flow cytometers will detect predominantly lipoproteins and not EVs, and for a statistically reliable data set of EVs a large number of particles has to be detected, which will take an unreasonable amount of time making the analysis unfeasible.

As explained, there are multiple known and probably even more unknown variables that may affect EV measurement results. As long as we do not know all variables and have limited tools to assess and quantitate the effects of such variables in a reproducible manner, detailed reporting of characteristics of the collected biospecimen and the applied pre-analytical procedures remain important.

3. Towards reproducible measurements

In this section, we review several ongoing initiatives that are aimed to generate an "infrastructure" for reproducible measurement results of EVs and related reporting. Please see Table 1 for an overview and the main goal of these initiatives, and Fig. 1 for future outlook.

3.1. MISEV

In 2014 and 2018, ISEV published position statements on the "Minimal information for studies of extracellular vesicles" (MISEV). The first manuscript was a product of field experts serving on the ISEV Board of Directors (Lötvall et al., 2014), but the second, more expansive effort was based on crowd-sourcing i.e. information-gathering surveys of the ISEV members. "MISEV2018″ included recommendations in six domains: nomenclature, pre-analytical variables, EV separation, EV characterization, functional studies, and reporting (Théry et al., 2018). The goal of the MISEV initiative is to provide a useful framework for rigorous EV studies, whether one is purifying or detecting EVs or seeking to attribute specific biological cargo or functions to EVs. A new MISEV update is scheduled for 2022 (Witwer et al., 2021). Importantly, MISEV provides a useful starting point to new researchers entering the EV field and helps to avoid several methodological pitfalls.

Table 1
Towards reproducible measurements of extracellular vesicles. This table provides an overview of the main events, most organized by the International Society on Extracellular Vesicles (ISEV), mentioned in this manuscript to promote education, reproducibility and standardization in the field of extracellular vesicles (EVs); MISEV: minimal information for studies on EVs; MOOC: massive open online course; *Since 2009, interlaboratory comparison studies have been organized to standardize EV concentration measurements by flow cytometry (ongoing in 2022); #Expected to be performed, completed and/or published in 2022.

2022	• Compendium on EV flow cytometry (Education)[#]
	• Standardization EV concentration measurements* (Standardization)[#]
	• MISEV (Reproducibility)[#]
	• MOOC III - Detection and isolation of EVs (Education)[#]
2021	• MIFlowCyt-EV (Reporting)
2019	• Rigor & Standardization Subcommittee ISEV (Reproducibility, standardization)
	• MOOC II - EVs in health and disease (Education)
	• ISEV Survey - Methods used to isolate and characterize EVs (Methods)
2018	• MISEV (Reproducibility)
	• Standardization of EV concentration measurements* (Standardization)
2017	• EV-TRACK (Reporting)
2016	• MOOC I - Basics of EVs (Education)
	• ExoCarta (Online database)
2015	• EV Working group on flow cytometry (Education, Reproducibility, Standardization)
	• ISEV Survey - Methods used to isolate and characterize EVs (Methods)
2014	• MISEV (Reproducibility)
2012	• Vesiclepedia (Online database)

3.2. Position papers and guidelines

Since EVs are a ubiquitous cell biological phenomenon and relevant for many fields from basic cell biology to theranostic applications and method development, ISEV has published multiple "position papers". Many of these papers have arisen from ISEV workshops and co-sponsored events. Topics include standardization of sample collection and processing (Clayton et al., 2018), EV RNA (Hill et al., 2013), non-mammalian EVs (Soares et al., 2017), membranes (Russell et al., 2019) and biomarker considerations (Clayton et al., 2018). Most of these manuscripts have been published in the *Journal of Extracellular Vesicles* (JEV), the first ISEV journal, which in 2021 was joined by a second publication, the *Journal of Extracellular Biology* (JExBio). Upon invitation by the American Heart Association (AHA), a manuscript was published on "*Methodological Guidelines to Study Extracellular Vesicles*", which was a co-production of (board) members of ISEV, the International Society on Thrombosis and Hemostasis (ISTH), and the International Society on Advancement of Cytometry (ISAC) (Coumans et al., 2017).

3.3. Collaboration between societies

In 2015, these three international scientific organizations realized that they were working independently on detection of EVs by flow cytometry, and they founded and endorsed the Extracellular Vesicle Flow Cytometry Working Group (www.evflowcytometry.org). Together, dedicated members of ISEV, ISTH and ISAC collaborate to improve the reproducibility of flow cytometry measurements of EVs, and recently the working group published a position paper on standardized reporting parallel in JEV (Welsh et al., 2020), Cytometry A (Welsh et al., 2021) and the Journal of Thrombosis and Haemostasis (van der Pol et al., 2022), the journals of ISEV, ISAC and ISTH, respectively. Also, an educational compendium is in preparation by the same authors about detection of single EVs by flow cytometry. ISEV reaches out to other (inter)national societies to provide the most up-to-date information to enable a sound entry for newcomers into the field with the aim to boost reproducibility of EV research.

3.4. Rigor and Standardization Subcommittee

In 2019, ISEV launched a "Rigor and Standardization Subcommittee" to improve the quality, reproducibility and thereby the credibility of EV research. Within this subcommittee, there are several task forces working on specific topics. An overview of task forces is available at https://www.isev.org/rigor-standardization. Several task forces focus on a particular EV-containing (body) fluid, and currently there are task forces on blood, cerebrospinal fluid, conditioned medium, milk, saliva, synovial fluid, and urine. Other task forces focus on bacterial EVs, reference materials, regulatory affairs, the clinical use of EV-based therapeutics, and reproduction. ISEV members can propose task forces through an online application system, and proposals are reviewed by the subcommittee. Task forces are ad hoc groups devoted to specific questions, which are supported by ISEV, and are expected to produce products such as position papers, methodologic protocols, literature reviews and online educational materials. Examples of task force products that have been completed are a roadmap to study blood EVs (Clayton et al., 2019), a paper on reference materials for EV research (Welsh et al., 2020), a position paper on urinary EVs (Erdbrügger et al., 2021), and a workshop on EV standardization and reference materials (Ghent, Belgium 2019).

The Rigor and Standardization Subcommittee also monitors method and technology development. From a 2019 ISEV survey on the methods used to isolate and characterize EVs, more than 600 responses revealed changes in the used methods compared to 2015 (Royo et al., 2020). Importantly, an increasing number of EV researchers use new and more dedicated separation and characterization methods, have started measuring quality control parameters, and are applying more EV

characterization methods in parallel according to MISEV guidelines (Théry et al., 2018). Thus, there is a growing awareness amongst EV researchers that "rigor and standardization" are essential to improve reproducibility and quality of EV research.

3.5. Towards standardization

Metrology is the science of measurements and their applications, and comprises traceable accuracy, precision and repeatability of a measurement, often with a help of a "standard", to help data interpretation and comparison between different measuring systems. Although biological systems are difficult if not impossible to standardize, principles of metrology are now being explored in the EV field to pave way to reproducibility.

As explained at the end of section 2, a "catch 22″ situation governs EV standardization. Specifically, calibration of EV detection instruments require dedicated reference materials and standardized biological samples. At present, such reference materials and biological standards are being developed for flow cytometry, together with the help from European national metrology institutes and the industry. Flow cytometry is probably the most commonly used instrument worldwide to measure and identify single EVs (Kuiper et al., 2021). The reference materials are designed to share physical properties as small diameter, low refractive index and dim fluorescence with EVs (www.metves.eu) (Welsh et al., 2020), and the biological standard samples contain purified, stabilized and pre-labeled but still heterogeneous EVs (e.g. regarding cellular origin and size) from normal human body fluids to validate developed calibration procedures and reference materials. Together, the reference materials and biological standards will be used to calibrate flow cytometers participating in an interlaboratory comparison study, scheduled for 2022. The goal of this study is to compare concentration measurements of EVs in the biological standard sample, which will be already the fifth interlaboratory study of this kind. In the most recent standardization study, light scattering of flow cytometers was calibrated using reference materials not resembling EVs and modeling, but two aspects were not calibrated, i.e. fluorescence and flow rate and without stable biological standard samples (van der Pol et al., 2018). The expectation is that in the upcoming comparison study, the variation in the detected concentration of cell-type specific EVs in the biological test sample will differ less than about 20%. If so, then calibration is likely to become a cornerstone of EV research because comparable data become available on different instruments and in different institutes, thus enabling multi-center studies. Due to this progress, manufacturers of flow cytometers have become interested in developing instruments with improved sensitivity to detect particles as small as EVs, which were previously merely considered as "noise" when measured with common flow *cyto*meters. Together, these developments are likely to allow traceable and reproducible quantification of concentrations of cell-type specific EVs, which in turn will enable multi-center studies, establishment of reference ranges, but also may enable to optimize pre-analytical procedures and storage.

3.6. Data reporting

In 2012, Vesiclepedia (www.microvesicles.org) was founded, which is "*a manually curated compendium that contains molecular data identified in all classes of EVs*" (Kalra et al., 2012). In 2017, the EV-TRACK consortium launched a knowledgebase (www.evtrack.org) with the goal to improve transparent reporting and experimental design of EV research. This initiative is endorsed by ISEV and JEV. The key feature is the EV-metric, which is calculated as a percentage of fulfilled requirements from a list of nine, which according to the EV-TRACK consortium are indispensable for the unambiguous interpretation and independent reproduction of EV experiments. Since 2017, the EV-metric has risen from 17% to 50% in 2021, which tells about the usefulness of a community compiled set of criteria in increasing systematic reporting. In addition, in 2016 the ExoCarta (www.exocarta.org), was founded, which is an online database for molecular data (proteins, RNA, and lipids) identified exclusively in exosomes (Keerthikumar et al., 2016). Also the before mentioned MiFlowCyt-EV (Section 3.3) is an example, which "incorporates the MISEV guidelines and Minimum Information about a FC experiment (MIFlowCyt) standard in an EV-flow cytometry-specific reporting framework (MIFlowCyt-EV)" to standardize reporting (Welsh et al., 2020).

3.7. Education

The field of EV research is growing fast, and it is not easy to keep pace with all new developments. There are high expectations in regard to EVs for applications in theranostics and for example in understanding disease pathogenesis. The ubiquitous nature of EVs in all biological realms from bacteria, fungi and plants to mammalian cells makes the field at the same time lucrative but also treacherous to newcomers. The Educational Committee of ISEV strongly supports education that is widely accessible and free of charge, so that the information is also available to researchers in low-income countries. With this goal in mind, massive open online courses (MOOCs) were developed consisting of recorded presentations of experts in the EV field. The first MOOC (I) on the "*Basics of extracellular vesicles*" appeared in 2016 (www.coursera.org/learn/extracellular-vesicles), which was followed by a second MOOC (II) on "*Extracellular vesicles in health and disease*" in 2019 (www.coursera.org/learn/extracellular-vesicles-health-disease). A MOOC on "*Detection and isolation of extracellular vesicles*" is in preparation and expected to go online in 2022. Since the SARS-CoV-2-pandemia, educational webinars have become popular, including the #EVClub and #WebEVTalks.

4. Summary

In the first part we summarized the challenges of studying EVs, and in the following part how the field is actively moving towards traceable and reproducible measurements via a community-built infrastructure. Instrument calibration is expected to pave the way towards monitoring possible variations caused by the biospecimen and pre-analytical procedures, screening the efficacy of isolation procedures, performing multi-center studies, and finally, establishing reference ranges of cell-type specific EVs in body fluids for clinical use. Furthermore, by initiating the Rigor and Standardization Subcommittee and promoting task force activities, education and transparent reporting, there is an already proven and growing awareness amongst EV researchers about the relevance of producing and reporting traceable and reproducible results (Nieuwland et al., 2020). Importantly, the developed infrastructure may place EV research at a pole position in the field of (bio)medical research by producing robust and reproducible data, which in turn may contribute to the overcoming of reproducibility problems in science.

Data availability

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

Acknowledgments

RN and PRMS are supported by the European Association of National Metrology Institutes, Grant/Award Number: 18HLT01 METVES II. PRMS is supported by The Academy of Finland (grant #330486, #337641), Magnus Ehrnrooth Foundation, Finnish Red Cross Blood Service Foundation and Business Finland (EVE consortium).

References

Ala-Korpela, M., Zhao, S., Järvelin, M.-R., Mäkinen, V.-P., Ohukainen, P., 2021. Apt interpretation of comprehensive lipoprotein data in large-scale epidemiology:

disclosure of fundamental structural and metabolic relationships. Int. J. Epidemiol. 1–16. https://doi.org/10.1093/ije/dyab156.
Arraud, N., Linares, R., Tan, S., et al., 2014. Extracellular vesicles from blood plasma: determination of their morphology, size, phenotype and concentration. J. Thromb. Haemost. 12 (5), 614–627. https://doi.org/10.1111/jth.12554.
Barreiro, K., Dwivedi, O.P., Valkonen, S., et al., 2021. Urinary extracellular vesicles: assessment of pre-analytical variables and development of a quality control with focus on transcriptomic biomarker research. J. Extra Vesicles 10 (12). https://doi.org/10.1002/jev2.12158.
Böing, A.N., van der Pol, E., Grootemaat, A.E., Coumans, F.A.W., Sturk, A., Nieuwland, R., 2014. Single-step isolation of extracellular vesicles by size-exclusion chromatography. J. Extra Vesicles 3 (1). https://doi.org/10.3402/jev.v3.23430.
Clayton, A., Boilard, E., Buzas, E.I., et al., 2019. Considerations towards a roadmap for collection, handling and storage of blood extracellular vesicles. J. Extra Vesicles 8 (1). https://doi.org/10.1080/20013078.2019.1647027.
Clayton, A., Buschmann, D., Byrd, J.B., et al., 2018. Summary of the ISEV workshop on extracellular vesicles as disease biomarkers, held in Birmingham, UK, during December 2017. J. Extra Vesicles 7 (1). https://doi.org/10.1080/20013078.2018.1473707.
Coumans, F.A.W., Brisson, A.R., Buzas, E.I., et al., 2017. Methodological guidelines to study extracellular vesicles. Circ. Res. 120 (10), 1632–1648. https://doi.org/10.1161/CIRCRESAHA.117.309417.
Enciso-Martinez, A., van der Pol, E., Lenferink, A.T.M., Terstappen, L.W.M.M., van Leeuwen, T.G., Otto, C., 2020. Synchronized Rayleigh and Raman scattering for the characterization of single optically trapped extracellular vesicles. Nanomed. Nanotechnol. Biol. Med. 24, 102109 https://doi.org/10.1016/j.nano.2019.102109.
Erdbrügger, U., Blijdorp, C.J., Bijnsdorp, I.V., et al., 2021. Urinary extracellular vesicles: a position paper by the Urine Task Force of the International Society for Extracellular Vesicles. J. Extra Vesicles 10 (7). https://doi.org/10.1002/jev2.12093.
Gelibter, S., Marostica, G., Mandelli, A., et al., 2022. The impact of storage on extracellular vesicles: a systematic study. J. Extra Vesicles 11 (2), e12162. https://doi.org/10.1002/jev2.12162.
Hill, A.F., Pegtel, D.M., Lambertz, U., et al., 2013. ISEV position paper: extracellular vesicle RNA analysis and bioinformatics. J. Extra Vesicles 2 (1), 22859. https://doi.org/10.3402/jev.v2i0.22859.
Hu, Y., Thaler, J., Nieuwland, R., 2021. Extracellular vesicles in human milk. Pharmaceuticals 14 (10), 1–20. https://doi.org/10.3390/ph14101050.
Kalra, H., Simpson, R.J., Ji, H., et al., 2012. Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. PLoS Biol. 10 (12), 8–12. https://doi.org/10.1371/journal.pbio.1001450.
Karimi, N., Cvjetkovic, A., Jang, S.C., et al., 2018. Detailed analysis of the plasma extracellular vesicle proteome after separation from lipoproteins. Cell Mol. Life Sci. 75 (15), 2873–2886. https://doi.org/10.1007/s00018-018-2773-4.
Keerthikumar, S., Chisanga, D., Ariyaratne, D., et al., 2016. ExoCarta: a web-based compendium of exosomal cargo. J. Mol. Biol. 428 (4), 688–692. https://doi.org/10.1016/j.jmb.2015.09.019.
Kuiper, M., van de Nes, A., Nieuwland, R., Varga, Z., van der Pol, E., 2021. Reliable measurements of extracellular vesicles by clinical flow cytometry. Am. J. Reprod. Immunol. 85 (2), 1–11. https://doi.org/10.1111/aji.13350.
Lötvall, J., Hill, A.F., Hochberg, F., et al., 2014. Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. J. Extra Vesicles 3 (1), 1–6. https://doi.org/10.3402/jev.v3.26913.
Nieuwland, R., Falcón-Pérez, J.M., Théry, C., Witwer, K.W., 2020. Rigor and standardization of extracellular vesicle research: paving the road towards robustness. J. Extra Vesicles 10 (2). https://doi.org/10.1002/jev2.12037.
van der Pol, E., Coumans, F.A.W., Grootemaat, A.E., et al., 2014. Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing. J. Thromb. Haemost. 12 (7), 1182–1192. https://doi.org/10.1111/jth.12602.
van der Pol, E., Coumans, F., Varga, Z., Krumrey, M., Nieuwland, R., 2013. Innovation in detection of microparticles and exosomes. J. Thromb. Haemost. 11 (SUPPL. 1), 36–45 doi:10.1111/jth.12254.
van der Pol, E., Sturk, A., van Leeuwen, T., et al., 2018. Standardization of extracellular vesicle measurements by flow cytometry through vesicle diameter approximation. J. Thromb. Haemost. 16 (6), 1236–1245. https://doi.org/10.1111/jth.14009.
van der Pol, E., van Leeuwen, T.G., Yan, X., 2021. Misinterpretation of solid sphere equivalent refractive index measurements and smallest detectable diameters of extracellular vesicles by flow cytometry. Sci. Rep. 11 (1), 1–6. https://doi.org/10.1038/s41598-021-03015-2.
van der Pol, E., Welsh, J.A., Nieuwland, R., 2022. Minimum information to report about a flow cytometry experiment on extracellular vesicles: communication from the ISTH SSC subcommittee on vascular biology. J. Thromb. Haemost. 20 (1), 245–251. https://doi.org/10.1111/jth.15540.
Rikkert, L.G., Coumans, F.A.W., Hau, C.M., Terstappen, L.W.M.M., Nieuwland, R., 2021. Platelet removal by single-step centrifugation. Platelets 32 (4), 440–443. https://doi.org/10.1080/09537104.2020.1779924.
Rikkert, L.G., Engelaer, M., Hau, C.M., Terstappen, L.W.M.M., Nieuwland, R., Coumans, F.A.W., 2020. Rate zonal centrifugation can partially separate platelets from platelet-derived vesicles. Res Pract. Thromb. Haemost. 4 (6), 1053–1059. https://doi.org/10.1002/rth2.12366.
Rikkert, L.G., van der Pol, E., van Leeuwen, T.G., Nieuwland, R., Coumans, F.A.W., 2018. Centrifugation affects the purity of liquid biopsy-based tumor biomarkers. Cytometry Part A 93 (12), 1207–1212. https://doi.org/10.1002/cyto.a.23641.
Royo, F., Théry, C., Falcón-Pérez, J.M., Nieuwland, R., Witwer, K.W., 2020. Methods for separation and characterization of extracellular vesicles: results of a worldwide survey performed by the ISEV rigor and standardization subcommittee. Cells 9 (9). https://doi.org/10.3390/cells9091955.
Russell, A.E., Sneider, A., Witwer, K.W., et al., 2019. Biological membranes in EV biogenesis, stability, uptake, and cargo transfer: an ISEV position paper arising from the ISEV membranes and EVs workshop. J. Extra Vesicles 8 (1). https://doi.org/10.1080/20013078.2019.1684862.
Soares, R.P., Xander, P., Costa, A.O., et al., 2017. Highlights of the São Paulo ISEV workshop on extracellular vesicles in cross-kingdom communication. J. Extra Vesicles 6 (1). https://doi.org/10.1080/20013078.2017.1407213.
Théry, C., Witwer, K.W., Aikawa, E., et al., 2018. 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. Extra Vesicles 7 (1). https://doi.org/10.1080/20013078.2018.1535750.
Veerman, R.E., Teeuwen, L., Czarnewski, P., et al., 2021. Molecular evaluation of five different isolation methods for extracellular vesicles reveals different clinical applicability and subcellular origin. J. Extra Vesicles 10 (9). https://doi.org/10.1002/jev2.12128.
Welsh, J.A., Tang, V.A., van der Pol, E., Görgens, A., 2021. MIFlowCyt-EV: the next chapter in the reporting and reliability of single extracellular vesicle flow cytometry experiments. Cytometry Part A 99 (4), 365–368. https://doi.org/10.1002/cyto.a.24268.
Welsh, J.A., Van Der Pol, E., Arkesteijn, G.J.A., et al., 2020. MIFlowCyt-EV: a framework for standardized reporting of extracellular vesicle flow cytometry experiments. J. Extra Vesicles 9 (1). https://doi.org/10.1080/20013078.2020.1713526.
Welsh, J.A., Van Der, Pol E., Bettin, B.A., et al., 2020. Towards defining reference materials for extracellular vesicle size, concentration, refractive index and epitope abundance. J. Extracell. Vesicles 24 (9), 1–17. https://doi.org/10.1080/20013078.2020.1816641.
Witwer, K.W., Goberdhan, D.C.I., O'Driscoll, L., et al., 2021. Updating MISEV: Evolving the minimal requirements for studies of extracellular vesicles. J. Extra Vesicles 10 (14). https://doi.org/10.1002/jev2.12182.
Yáñez-Mó, M., Siljander, P.R.M., Andreu, Z., et al., 2015. Biological properties of extracellular vesicles and their physiological functions. J. Extra Vesicles 4 (2015), 1–60. https://doi.org/10.3402/jev.v4.27066.
Yates, A.G., Pink, R.C., Erdbrügger, U., et al., 2022. In sickness and in health: the functional role of extracellular vesicles in physiology and pathology in vivo: part I: health and normal physiology. J. Extra Vesicles 11 (1). https://doi.org/10.1002/jev2.12151.
Yuana, Y., Levels, J., Grootemaat, A., Sturk, A., Nieuwland, R., 2014. Co-isolation of extracellular vesicles and high-density lipoproteins using density gradient ultracentrifugation. J. Extra Vesicles 3 (1), 1–5. https://doi.org/10.3402/jev.v3.23262.
Zhang, X., Borg, E.G.F., Liaci, A.M., Vos, H.R., Stoorvogel, W., 2020. A novel three step protocol to isolate extracellular vesicles from plasma or cell culture medium with both high yield and purity. J. Extra Vesicles 9 (1). https://doi.org/10.1080/20013078.2020.1791450.