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Technical challenges of working with extracellular vesicles

Marcel I. Ramirez,†[a,b] Maria G. Amorim,†[c] Catarina Gadelha,[d] Ivana Milic,[e] Joshua A. Welsh,[f] Vanessa M. Freitas,[g] Muhammad Nawaz,[g,h] Naveed Akbar,[i] Yvonne Couch,[j] Laura Makin,[k] Fiona Cooke,[l] Andre L. Vettore,[m] Patricia X. Batista,[m] Roberta Freezor,[n] Julia A. Pezuk,[o] Lívia Rosa-Fernandes,[g,p] Ana Claudia O. Carreira,[g] Andrew Devitt,[e] Laura Jacobs,[q] Israel T. Silva,[c] Gillian Coakley,[r] Diana N. Nunes,[c] Dave Carter,[s] Giuseppe Palmisano[g,t] and Emmanuel Dias-Neto *[c,g]

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Extracellular Vesicles (EVs) are gaining interest as central players in liquid biopsies, with potential applications in diagnosis, prognosis and therapeutic guidance in most pathological conditions. These nano-sized particles transmit signals determined by their protein, lipid, nucleic acid and sugar content, and the unique molecular pattern of EVs dictates the type of signal to be transmitted to recipient cells. However, their small sizes and the limited quantities that can usually be obtained from patient-derived samples pose a number of challenges to their isolation, study and characterization. These challenges and some possible options to overcome them are discussed in this review.

1 Introduction

Extracellular Vesicles (EVs) are involved in the transmission of biological signals between populations of prokaryotic and eukaryotic cells. As central mediators of intercellular communication, EVs are involved in many cellular processes, such as compensating for stress conditions, triggering physiological responses that contribute to the maintenance of cellular integrity, organismal homeostasis[1–3] and regulating a range of biological activities. Their potential applications for diagnosis and guiding therapeutics, as well as determining prognosis of pathological conditions has allowed the field of EV-studies to grow steadily in recent years.

The term EVs, coined by the International Society for Extracellular Vesicles (ISEV, http://www.isev.org), categorizes vesicles based on their biogenesis or release pathway, and include exosomes (50–100 nm in diameter), originated from the endocytic pathway and with buoyant densities of 1.11–1.19 g mL^{-1},[4] shedding microparticles/microvesicles (100–1000 nm) released directly from the plasma membrane,[5] apoptotic blebs (50 nm–2 μm; produced as a consequence of indiscriminate apoptotic disintegration),[6] large oncosomes (1–10 μm)[7] and other miscellaneous EV subsets.[8] As the sizes of the different EVs-subsets overlap, as well as their cargo, several groups have now started characterizing the composition of EV-subtypes. Recent papers claim successful subclassification of EVs based on general surface proteomic profiling,[9,10] or on transcriptional profiles of individual EV populations.[11,12] EV subtypes have been isolated by a number of means, including recovery at different centrifugation forces, different filters, at slightly different positions in density gradients, *via* immuno-isolation by different surface molecules, chromatography or by flow cytometric sorting.

At present, functions of EVs have not been fully elucidated. However, they appear to be able to modulate host–pathogen

[a]*Fundação Instituto Oswaldo Cruz, Rio de Janeiro, RJ, Brazil*
[b]*Universidade Federal do Paraná, Curitiba, PR, Brazil*
[c]*CIPE, A.C.Camargo Cancer Center, São Paulo, SP, Brazil.*
E-mail: emmanuel@cipe.accamargo.org.br
[d]*School of Life Sciences, University of Nottingham, Nottingham, UK*
[e]*School of Life and Health Sciences, Aston University, England, UK*
[f]*University of Southampton, Southampton, England, UK*
[g]*Universidade de São Paulo, São Paulo, SP, Brazil*
[h]*University of Gothenburg, Sweden*
[i]*Division of Cardiovascular Medicine, University of Oxford, Oxford, England, UK*
[j]*Acute Stroke Programme, RDM-Investigative Medicine, University of Oxford, Oxford, England, UK*
[k]*Sir William Dunn School of Pathology, University of Oxford, Oxford, England, UK*
[l]*University of St Andrews, St Andrews, Fife, Scotland, UK*
[m]*Federal University of São Paulo campus Diadema, Diadema, Brazil*
[n]*London Metropolitan University, London, England, UK*
[o]*Universidade Anhanguera de São Paulo, São Paulo, Brazil*
[p]*University of Southern Denmark, Odense, Denmark*
[q]*Technische Universität München, Munich, Germany*
[r]*Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, Scotland, UK*
[s]*Oxford Brookes University, Oxford, England, UK*
[t]*IRCCS, Fondazione Santa Lucia, Rome, Italy*
† Equal contribution.

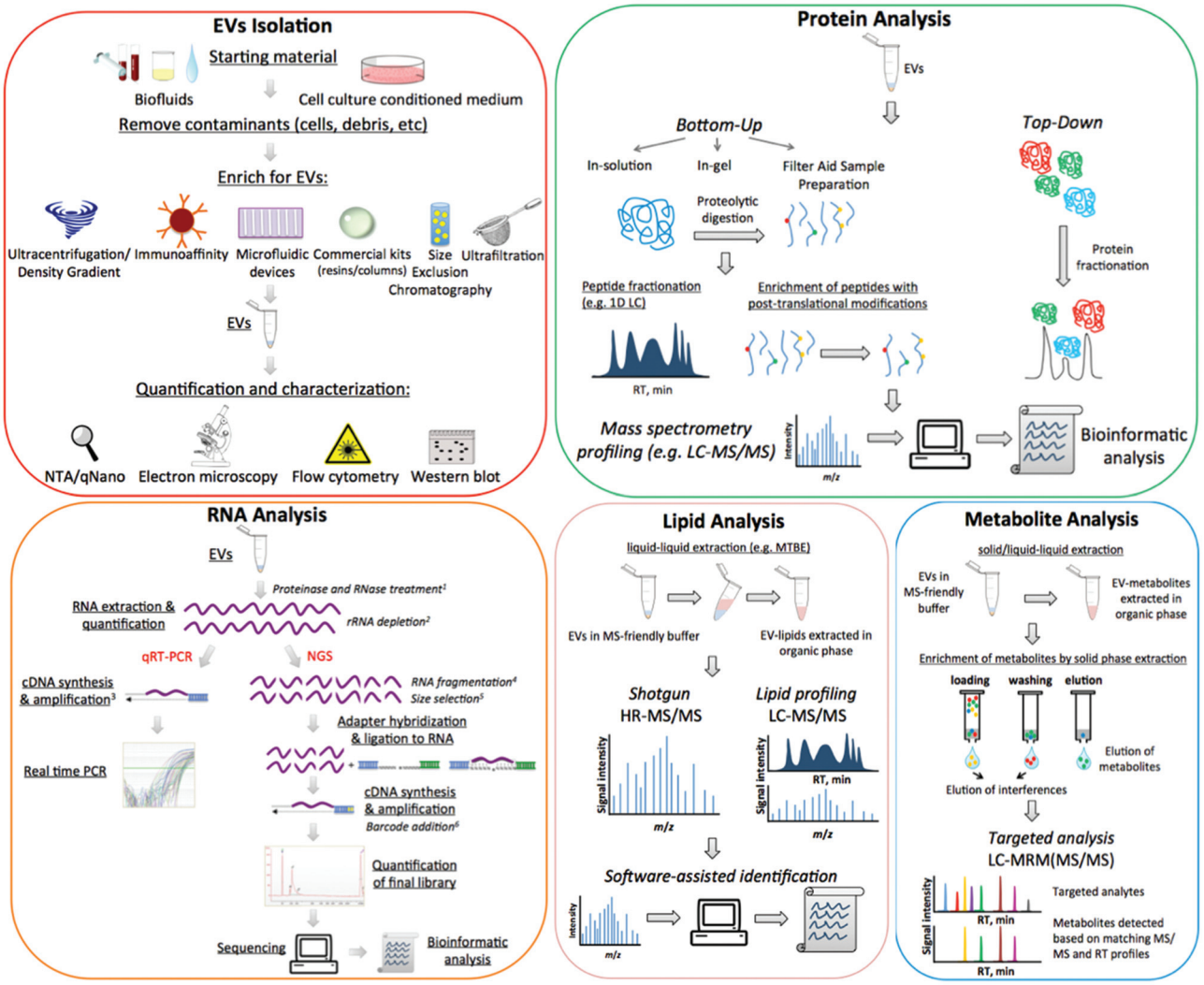

Fig. 1 Scheme of EVs isolation, quantification and characterization, including the analysis of EVs molecular content. The superscript numbers on the figures indicate the need of each step as follows: 1 – to ensure intravesicular origin of RNAs; 2 – to remove abundant ribosomal RNAs that may be present as fragments in EVs, and therefore not visible by Bioanalyzer analysis; 3 – cDNA synthesis may be performed with transcript-specific or general primers; cDNA pre-amplification before real time PCR is optional; 4 – to break down long RNA molecules; 5 – to separate small RNAs (<200 nt) from long RNAs (>200 nt); 6 – to tag molecules from different samples and enable multiplex analysis.

interactions[13] and to contribute to several pathological conditions such as infectious and inflammatory diseases, neurological disorders and cancer. EVs are particularly important in clinical settings, largely because they contain a wealth of biomarkers that can be used to monitor clinical status, chemotherapy-response,[14] disease progression,[15] and many relevant and diverse clinical conditions. The composition of EVs is not random and each EV-cargo delivers specific molecular messages. Indeed these nanosized membrane vesicles transmit signals by proteins, lipids, nucleic acids, and sugars, and the unique molecular pattern of this package dictates the type of extracellular signal to be transmitted to recipient cells.[1] Despite the promise of revealing informative markers of medical interest, working with these small particles poses many technical challenges. In addition to the concerns over data analysis, there are further uncertainties over protocol standardisation and how to define the pre-analytical and analytical variables that impact upon outcome measures.[16] The aim of this review is to outline the most relevant challenges related to working with EVs, specifically discussing the advantages and disadvantages of different approaches and suggesting alternative routes to better overcome frequent issues. A detailed scheme of EV purification and characterization methods is presented in Fig. 1.

2 Pre-analytical factors

2.1 EVs derived from biofluids

EVs have been isolated from diverse biofluids including blood,[17] urine,[18] saliva,[19] breast milk,[20] cerebrospinal[21] and ascitic fluids,[22] gastric juice,[23] bile,[24] sputum,[25] bronchoalveo-

lar lavage,[26] semen[27] and tears.[28] The viscosity of these fluids, as well as their fat and protein content are highly variable, which may affect EVs purity and yield and, therefore require the protocols to be adjusted according to the biofluid of interest. Standardized pre-analytical steps are crucial to minimizing artefacts in EV-analysis, particularly when EVs are derived from complex body fluids such as blood. The amount, purity and content of biofluid-derived EVs may be affected by numerous factors including age, gender, ethnicity, body mass index, disease, use of medications, general lifestyle, and dietary habits.[29] Ideally, these factors should be taken into account and normalized across all study subjects (patients and any relevant controls).

Other important considerations include methods of blood collection, as shear force may induce hemolysis, which has been shown to alter the expression of some plasma miRNA molecules[30] and may also affect EV-derived content. Whereas EV counts appear to remain stable overtime in samples stored with no agitation, gentle agitation (used to simulate blood transportation) leads to an artificial and strong release of platelet-derived EVs.[31] Additionally, anticoagulants have been shown to be capable of preventing the formation of EV-blood cell aggregates (aggregation was reduced in EDTA-preserved blood).[32] Blood preserved with EDTA also appears to reduce the above-mentioned agitation effects on platelet-derived EVs if compared to citrate or heparin. Storage time is another important factor: Fendl *et al.* 2016 found comparable amounts of EVs in freshly drawn blood (collected in heparin, EDTA or citrate) but increased amounts of EVs were observed after 3 h storage, in an anticoagulant-dependent fashion, with increments varying from 2× (EDTA) to 10× (citrate), primarily due to platelet-derived EVs.[31] Also critical is the careful separation of platelets from plasma with adequate centrifugations; the International Society on Thrombosis and Haemostasis recommends blood collection in citrated tubes and two successive centrifugations at 2500*g* for 15 minutes at room temperature.[33] However, EDTA has been recently suggested as a valuable option in clinical settings.[32]

Another factor that needs to be considered when selecting EVs isolation protocols across different biofluids is the volume of starting material, as some biofluids may need to be concentrated prior to EVs isolation such as urine, an important source for patients diagnosed with renal pathologies, prostate and bladder cancers,[18] and other conditions including the parasitic infection schistosomiasis.[34] A recent study compared several filters and concluded that the best for recovering EVs from plasma, urine and EV-spiked PBS was a regenerated cellulose membrane with pores capable of retaining particles above 10 kDa.[35] Liang *et al.*, have also demonstrated the feasibility of concentrating the EVs using a double-filtration microfluidic device capable of isolating, concentrating and quantifying urinary EVs, using 8 mL of the pre-filtered (0.22 μm filter) and 20 000*g* centrifuged urine-supernatant collected from bladder cancer patients and controls.[36] Using this approach the authors demonstrated that the principle of size-exclusion using two polycarbonate membranes (with pore sizes of 200 and 30 nm) permitted the concentration of EVs within this size-range and the later study of CD63 expression using ELISA. Urine-derived CD9-positive exosomes have also been isolated using magnetic beads[37] and Gilani *et al.* have also shown that digital flow cytometry can be a good approach for quantifying the expression of markers of interest in urine-derived EVs from renal-injury patients.[38]

Besides biofluid-volume, specific methodological parameters need to be optimised during EVs isolation according to their inherent biochemical properties. For example, the pellet of EVs isolated from breast milk by 100 000*g* ultracentrifugation was found to be too solid to resuspend, because of the high abundance of whey and casein protein in milk.[39] One proposed solution was to purify the 10 000*g* supernatant through an overnight sucrose density gradient, allowing separation of EVs from protein complexes.[39]

2.2 Cell culture conditioned medium

The use of cell culture conditioned medium enables a more controlled environment for EV isolation. Nonetheless, there are important aspects that may directly influence EV yields that should be considered. The choice between culture medium containing EV-depleted fetal bovine serum (FBS) *versus* culture medium with no FBS (serum starvation of cells) needs to be considered. This choice implies that an abrupt change to serum-free medium will likely cause a major stress to cells and lead to altered EV secretion.[40] On the other hand, rigorous EV depletion of FBS needs to be thoroughly performed, using long ultracentrifugation protocols (16 h at ≥100 000*g*) in order to maximize the removal of FBS-derived EVs.[41–43] Recent evidence, however, suggests that even this extended ultracentrifugation cannot remove contamination from bovine small RNAs, some of which can be mis-annotated as human RNAs.[44] Apoptosis-induction control experiments can also be performed to exclude the possibility that EVs, and consequently the putative isolated EV-RNAs, are due to cell death,[45] especially as many experiments report changes in RNAs upon EV induction as opposed to the steady-cell state. Other factors that need to be carefully considered include cell culture matrices and plastics, exact culture medium composition/volume, cell passage, cell confluency and viability, mycoplasma-status and other microbial contamination. If conditioned medium will be stored before EVs isolation, it is crucial to clear it of cells and cellular debris by centrifugation before freezing, and to minimize subsequent thaws.

2.3 Storage of EVs

Lőrincz *et al.* performed a detailed analysis of the effect of storage in different conditions (+20 °C, +4 °C, −20 °C, −80 °C for 1, 7 or 28 days) on the physical and functional properties of EVs derived from human neutrophilic granulocytes.[46] Flow cytometry, dynamic light scattering and electron microscopy were used for measuring EVs physical properties (number, size and morphology), and an antibacterial assay was used to test the functional property of EVs. They found that storage (even at −20 or −80 °C) significantly altered the functional properties of EVs, although their number and morphology stayed

constant. In another study, a 7-fold increase in the number of phosphatidylserine-exposing EVs was found in EVs isolated from plasma samples that had undergone a single freeze–thaw cycle, suggesting changes in the vesicle membrane phospholipids, whereas in urine-derived EVs the same effect was not observed.[47] Kalra *et al.* spiked EVs isolated from a colorectal cancer cell line into plasma samples and measured TSG101 protein expression after 10, 30 and 90 days in storage at 4 °C, −20 °C or −80 °C, as well as PKH67 dye labeling and uptake by cells (−20 °C for 30 days).[48] They found that all storage conditions yielded EVs with detectable TSG101 and capable of being transferred into cells.

Although several studies have investigated whether storage impacts specific characteristics of EVs, there is a lack of large-scale analysis of EVs cargo changes that may be caused by storage. To the best of our knowledge, no study has evaluated transcriptomic, proteomic and lipidomics alterations that may be induced upon EVs storage. Until more in-depth "omics" studies are performed, in addition to functional assays, the understanding of the true stability of EVs remains incomplete. In this sense, we recommend EVs to be evaluated as soon as possible after their isolation, especially for functional studies, avoiding repeated freezing and thawing cycles.

3 EV isolation protocols

There are several EV isolation and characterization techniques available, and each has its own set of pre-analytical factors that may influence yields. The ISEV has issued several position papers highlighting the importance of standardization of sample collection and EV isolation and characterization methods, which summarize all the detailed information that should be recorded and provided in publications.[40,49,50]

Launched recently, the EV-TRACK database (http://evtrack.org) is an excellent initiative in the EV-research field to encourage standardization of vesicle isolation and characterization methods.[51] This initiative was created by an international consortium of 92 researchers from 12 countries, who evaluated and scored the experimental parameters of 1226 recently published EV-related articles. The EV-METRIC is a summary score of the article's adherence to and detailed reporting nine experimental parameters. The fact that the average EV-METRIC across all biofluid studies is only 20% indicates how the heterogeneous EV-field is currently deficient in methodological reporting. The online database is searchable, allowing easy identification and comparison of EV-related articles. Another interesting feature is that the authors can annotate their study's database entries, adding experimental details that may have been overlooked during publication. Hopefully, EV-TRACK will contribute to better transparency and reporting in EVs publications, allowing improved interpretation and reproducibility of experiments.

3.1 Ultracentrifugation (UC)

The selection of an EV-isolation method largely depends on the source of sample. For less complex samples obtained from cell cultures, it is beneficial to use differential UC with no preliminary steps, an approach that has long been considered the most efficient EV isolation technique. New isolation methods appear often, usually involving column enrichment or precipitation. However, the most popular primary isolation methods still in use are based on UC.[52] On the other hand, isolation of EVs derived from complex biological fluids may benefit from the use of multiple methods to remove specific components (*e.g.* platelets, lipoproteins or protein complexes) before UC,[53] or even alternative isolation methods, involving column enrichment, sequential filtering or precipitation. However, as a means of first EV-isolation step, UC is a cost-efficient and widely accepted way to isolate EVs.[52]

The isolation of EVs through differential UC relies on several centrifugation steps with increased centrifugal force to produce sequential pellets of cells and cell-debris (300–1000*g*), microvesicles (10 000–20 000*g*) and exosomes (100 000*g*). Some have described the use of serial ultrafiltration through 0.22 and 0.45 μm filters before pelleting[54] and variation of the centrifugation forces have been implemented in different protocols.

Advantages of UC include low cost – as ultracentrifuges are readily available in many labs – and the capacity to spin a wide range of volumes from a few millilitres up to >100 mL. However, there are a number of problems associated with isolating EVs by UC. One of the major caveats is the co-purification of non EV-associated proteins, particularly protein aggregates and lipoproteins,[55] which may be interpreted as integral or enriched EV components in downstream analyses. Ultrafiltration can be coupled with UC to reduce EV-protein aggregates.[56] Attention should be given to centrifugation forces applied to EVs. Nordin *et al.*, have shown some degree of EV disruption after UC for 70 min at 120 000*g*.[56] Therefore, when the recovery of intact EVs using UC is an aim, we recommend the use of a maximum of 100 000*g*.

3.2 Density gradients (DG)

An approach that is commonly used to overcome the problem of co-purification is to follow UC with further purification of EVs in a sucrose DG which makes use of EV density for better separation (26). EVs of a particular size and origin have a particular floatation density, usually in the range of 1.08–1.22 g mL^{-1}.[57] Several protocols have been described,[58] the majority of which involve resuspending an EV-enriched pellet following UC, overlaying a sucrose gradient in various buffers which may also contain deuterium oxide, performing UC again and collecting the appropriate fractions which are enriched in EVs. Differential UC shows the presence of larger vesicles by laser particle size measurement, and protein concentration studies show that DG UC yields higher protein concentration and more target exosome proteins.[59]

Unfortunately, differential UC can be a lengthy process with the most time taken for the UC itself. For clinical grade data or highly enriched samples it is recommended to use multiple centrifugation speeds that will considerably lengthen the process, reducing its applicability for clinical settings.[60]

Whereas sucrose gradient UC is relatively time consuming, it undoubtedly results in the isolation of purer EV samples than UC alone.[59] However, due to fractionation a relatively high starting concentration of EVs is required.[61] Besides being able to isolate purer EVs, it should be noted that contaminants that have the same density of EVs, such as some viruses,[62] plasma-derived high- and low-density lipoproteins[63,64] and uromodulin and albumin[65] from urine, are likely to contaminate the EVs isolated by DGs. It has been shown that the similar floatation densities of HIV virus particles and EVs make them indistinguishable by conventional gradient separation.[66] Presumably, the co-purification of EVs and virus particles could be considered a universal problem due to common viral contaminations of cell cultures which go undetected. Cantin *et al.* found that it was possible to separate HIV particles and EVs using commercially available OptiPrep DG solution.[66] This employs a gradient of iodixanol rather than sucrose, and has been used for isolating fractions of pure EVs for proteomic analysis from conditioned cell culture medium[67] and plasma.[48]

3.3 Immunoaffinity

An approach to improve the purity of the EVs population is to use immunoisolation, whereby EVs preparations are incubated with antibody-coated latex[17] or magnetic beads,[68] allowing their separation based on the expression of markers of interest. This technique has the potential to specifically pull-down EVs with a particular surface marker while excluding contaminating particles or other EVs populations. A study comparing immunoisolation with DG separation and UC for the isolation of human colon cancer-derived EVs showed that immunoisolation produced the highest EVs yield as measured by quantitative protein mass spectrometry.[61]

As immunoisolation is not compatible with large-volume samples, samples with low EV content must be concentrated prior to incubation with antibody-coated beads. Therefore, immunoisolation is commonly used as an additional purification step following conventional EVs isolation from large sample volumes using UC and possibly DG,[17] making this a lengthy procedure. When small volumes of starting material contain sufficient EVs to isolate and analyse (as with serum), immunoisolation can be used to isolate EVs directly.[69] Importantly, when isolating EVs from serum, it is essential to pre-coat the beads with a molecule such as sulfobetaine to reduce the non-specific adsorption of abundant serum proteins. This single-step EV isolation technique may prove to be a valuable diagnostic tool for identifying disease markers in EVs.[69,70]

While one of the key advantages of immunoisolation is its specificity based on the chosen antibody, this approach may also be limiting since it is likely to isolate merely a sub-population of EVs. There is still poor understanding of which EVs markers are present on different EVs subpopulations, and what the subcellular and cellular origins of these EVs are. For example, there are no accepted, exclusive markers to distinguish between exosomes and microvesicles despite their distinct mechanisms of biogenesis.[71] In a diagnostic setting, the best case scenario would be to have a cell-type specific surface markers which are differentially regulated during disease and released in EVs as recently reported.[68]

While immunoisolation has been successfully used for analysis of EVs by mass spectrometry[61] and RNA sequencing,[72] the use of immunoisolated EVs in functional studies is more difficult due to the challenge of dissociating high yields of active EVs from the beads. Chaotropic agents such as NaCl have been successfully used to elute EVs from beads where EVs have been shown to retain at least some functionality.[73] However, in spite of successful elution of EVs using mild conditions, small changes in EVs size and surface structure can occur.[74,75] Thus, care must be taken when interpreting results from functional analyses using immunoisolated EVs.

3.4 Microfluidics

Microfluidics technologies can be used to isolate EVs populations of interest. Here, molecules enriched in the EVs membrane, such as specific lipids and proteins, can be used to isolate them.[69] Many groups have already described the use of customised on-chip devices to isolate EVs, partly because this approach enables the use of low input sample. Another important point is the feasibility of EVs evaluation directly from bodily fluids (without the need for prior extraction steps), which can be of particular interest in the diagnostic setting.[76] Combining microfluidics with previously described immunoaffinity has resulted in an immunoaffinity microfluidic device based on CD63, an abundant tetraspanin present in the EVs membrane.[77] More recently, the Exochip, another on-chip device allows not only EVs isolation based on CD63 immunoaffinity, but also their quantification by fluorescent dye staining.[78]

EV features, such as electric properties, shape, size and density, can also be exploited in the development of customised chips.[79] As a note of caution we must state that it remains to be demonstrated if antibody-isolation approaches somehow modify the cargo or the functionality of EVs and if their biological activity is dependent on the simultaneous signalling given by a more diverse EVs set, that can not be isolated by specific antibodies.

3.5 Size exclusion chromatography (SEC)

EVs with different sizes can be separated using Size Exclusion Chromatography (SEC), a technique that has been more and more used currently, allowing the recovery of pure vesicles with increased functionality, purity and integrity. Advantages of SEC include the reduction of EVs-aggregation during the isolation procedure,[56] the efficient separation of EV from soluble proteins and capability of isolating pure, intact and biologically active EVs.[80] SEC removes 99% of the soluble plasma proteins and >95% of HDL from the purest fraction of EVs, does not induce aggregation of EVs, and retains the integrity and biological activity of EVs.[56,80–82] Contaminants such as von Willebrand factor and LDL are unexpected based on particle size, but these can still be found possibly complexed

to EVs.[63,83] SEC allows the EVs environment to be changed, such as from plasma to buffer, with no or minimal detrimental effects on EVs themselves. Also, it is fast, 10 to 20 minutes per sample, and relatively inexpensive,[84] which makes it more applicable in a clinical setting. A disadvantage of SEC is the dilution of EVs sample, which often requires second step *i.e.* re-concentration of EVs by ultrafiltration.[56,80,81,83–86] Compared with DC, EVs isolated by SEC have a high yield of biophysically intact EVs although at the expense of dilution.[56,86] A single-step plasma EVs isolation using SEC has been published, based on the use of qEV SEC columns, an efficient system to isolate EVs from plasma proteins.[60,84] Also, instead of pelleting EVs by UC, as used by some, the application of protein concentrating devices allows the fast concentration of EV fractions.[60,84] This provides an efficient means of isolating and concentrating EVs from human plasma, while avoiding some negative issues related to UC as previously mentioned. SEC and ultrafiltration can be coupled to provide higher EVs purity and good recovery rates, while still preserving their biophysical and functional properties.[56] Ultrafiltration of cultured media results in higher recovery of EVs after DG purification, and is a faster alternative to UC, whereas the SEC outperforms precipitation of EVs isolated from human plasma.[60]

3.6 Ultrafiltration (UF)

Filtration-based EVs isolation methods can be used independently or in conjunction with other techniques such as UC. Advantages of filtration-based methods are the ability to use variable sample input volumes, simplicity and low cost. Davies *et al.* developed a microfluidic filtration system for isolation of EVs directly from unprocessed whole blood[55] which allowed the study of very limited amounts of blood from melanoma-bearing mice. After being isolated from a few microliters of blood by this pressure-based device, the EVs were shown to retain their intact morphology and to express Melan A mRNA, a melanoma tumor marker, as well as other classic EV-markers (as evidenced by Western blot of CD9, CD63 and CD81). Interestingly, a direct comparison with EVs isolated by UC was performed, and TEM analysis showed that UC caused many EVs to aggregate and to deform, which did not occur in the filtration-derived EVs, even under pressure.[55] A three-step sequential filtration-based protocol allowed the isolation of EVs from larger volumes, such as 150 mL of cell culture conditioned medium.[87] The authors conclude that sequential filtration with a 100 nm cut off final filtering step enriches for exosome-sized vesicles (81% compared to 23% for UC), suggesting that UC may lead to co-purification of larger vesicles and/or protein aggregates. UF is more appropriate with volumes in excess of 400 mL due to the higher flow rate, and that EVs loss is only observed with the first 50–100 mL of cultured media.[60] Whereas it is far more time efficient than centrifugation methods, taking only 20 minutes to concentrate over 150 mL of sample compared to 2 rounds of UC for 90 minutes each.[60] Importantly, UF can have a recovery of up to 80% and may concentrate EVs up to 240-fold.[60] This implies that UF-based methods are effective to concentrate EVs. However, UF has some limitations such as it may result into non-specific binding of EVs to membranes[35,56] and thus may present some loss of EV yield owing to trapping in filter pores.

3.7 General aspects of EVs isolation methods

Coincident with the EV-TRACK initiative, several groups have compared methodologies to improve the efficiency of the isolation and characterization of EVs. Even being considered gold standard for purification, differential centrifugation can provide technical difficulties to process large volumes of conditioned medium and it has been suggested that repeated UC steps can damage vesicles and reduce yield, impacting proteomic and RNA studies.[56] In this sense, the group of Dr Andreas Moeller (QIMR Berghofer Medical Research Institute, Australia) compared UC to UF for the isolation of exosomes from cell culture conditioned medium and concluded that the later provided faster and better recovery.[60] They then further compared four alternative methodologies (two precipitation-based methods: ExoQuick and Exo-spin; one SEC-based method: Izon qEV columns; and one DG purification-based method: Opti-Prep), concluding that precipitation-based methods led to higher particle yield but less purity (measured by particle to protein ratio), while qEV columns provided the purest preparations, including also plasma-derived exosomes.

A recent work has compared four exosome isolation protocols (single-step UC) and density-gradient UC-based protocols using iodixanol (Optiprep) and two commercially available precipitation-solution based protocols EXO-Quick (EQ) and total exosome isolation (TEI).[88] The results showed Optiprep to be the method of choice in terms of removing EV-associated protein/RNA complexes contaminants, allowing the recovery of exosome-specific proteins and RNA.[88] This was reinforced in a clinical study on plasma exosomes where Optiprep gradient centrifugation was the single method capable of removing contaminating plasma proteins.[48] Regarding the quality of the RNA, Van Deun *et al.* found several differences of RNA profiles between the methods, and strongly recommend to validate isolation methods prior to consider exosome-specific content, functions and biomarkers.

Moreover the group of Dr Pieter Vader (University Medical Center Utrecht, The Netherlands) and Samir El Andaloussi (Karolinska Institutet, Stockholm, Sweden), provided evidence that SEC-isolated EVs better retain their functional activities, as compared to UC-isolated EVs.[89] They investigated differences in functionality of cardiomyocyte progenitor cell derived EVs isolated using UC and SEC, concluding that SEC-isolated EVs were more efficient in stimulating the migration of endothelial cells, having EV-induced ERK1/2 phosphorylation as a measurement to compare the UC-EVs and SEC-EVs functionality.

Reinforcing the importance of UF the group of Dr An Hendrix (Ghent University, Belgium) compared five commonly used filters for their efficiency to recover exosomes from clinical samples.[35] Regenerated cellulose membrane filters, with

pores of 10 kDa, showed higher efficiency. They also compared colorimetric and fluorimetric kits to measure EV protein content, and found the Qubit fluorometric assay to be the most sensitive (considering the lower BSA standard of 200 $\mu g\ mL^{-1}$) and the more consistent in terms of quantification among technical and biological replicates. This group also suggested the Optiprep (Sigma Aldrich) to be the best method to isolate EVs and proposed a protocol to remove Optiprep leftovers from EVs. Other efforts have been done by Dr Marca Wauben's group (Utrecht University, The Netherlands) who developed a fluorescence-based flow cytometric high-throughput method for quantitative and qualitative analysis of nano-sized cell-derived membrane vesicles (50–100 nm) which cannot be visualized by conventional scatter-based analysis.[90,91]

In order to ensure how pure are the EVs isolated by any of the possible protocols, the ratio of EVs and non-EV components can be assessed. One of the most used methods is the determination of the number of EVs per microgram of measured proteins in the isolated EVs. EVs purified using sucrose DG from conditioned cell culture media provided highly purified EVs: 3.3×10^{10} particles per μg protein. However, when isolated from biofluids EVs are much more contaminated by proteins giving ratios around 6.5×10^{9} particles per μg protein for fresh serum and 1.1×10^{9} particles per μg protein for fresh urine.[92] This calculation should be interpreted with caution as the ratio depends on the source of the vesicles as well as the method of extraction.[88]

4 Characterization of the isolated EVs

After isolation, EV populations need to be characterized for intended downstream applications, for which there is a variety of techniques available. This section will discuss some of the available options in light of the instrumental parameters of each technique.

4.1 Electron microscopy (EM)

The use of electron microscopy (EM) has considerably pushed the field of EVs and enabled the discovery of apoptotic vesicles, microvesicles and exosomes. The nanometer-scale resolving power of transmission electron microscopes has, however, its drawbacks, which include lengthy sample preparation, lack of multi-parametric phenotyping, and low throughput capacity. These characteristics make large EM studies rather challenging, with the amount of starting EVs material being particularly critical. Yet, morphological characterization using parameters such lipid and protein compositions, cellular origin, size, density and morphology[4] is a key step for classification of isolated EVs. Thus, efforts to mitigate the challenges in the use of electron microscopy to study EVs are most welcome in the field.

One of these common challenges relates to the amount of available material for transmission EM preparation, microtomy and visualisation. Some tissues and body fluids release an abundant amount of vesicles that are easily harvested by differential centrifugation or affinity purification chromatography. However, most often than not, isolated EVs are few and far between; and sample preparation itself can at times cause further loss of material. In an attempt to overcome this issue, recently there have been informal discussions, in conferences and focused meetings, about the use of proteinaceous material (*e.g.* matrigel, bovine serum albumin, or other inert protein) to 'encapsulate' EVs in a rich protein surrounding that is extensively cross-linked during fixation. This is because the protein around EVs, when cross-linked together, will form a firm matrix around the vesicles, protecting them from falling apart. Alternatively, the use of inert polysaccharides (*e.g.* agarose, agar, methylcellulose) to embed isolated EVs in a small piece of gel prior to EM processing has also been reported by individual laboratories with success.

A routine EM method that has been used with great success for the study of EVs is that of negatively-stained whole mount preparation.[4] Here EVs are adsorbed onto a filmed metal grid, chemically fixed and negatively stained prior to observation using a transmission electron microscope. Whole mounts are extremely useful for morphological analysis (*e.g.* EV size, shape, density), and can be combined with immunolabelling techniques. This method is particularly useful for the identification and localisation of immunological epitopes on the external surface of EVs, whereas EV-internal epitopes are better visualised by positive staining methods.

Nevertheless, the visualisation of EVs by transmission electron microscopy, coupled or not with immunolocalisation techniques, suffers mostly from two major challenges: lack of contrast and preservation (of vesicle morphology and/or molecular epitopes). This is particularly critical when considering the potential of EVs as biomarkers in medical applications. Some whole-mount preparations yield cup-shaped exosomes, which has been suggested to be an artefact caused by sample dehydration.[93] Indeed, the preparation steps of chemical fixation, dehydration, observation under vacuum, and electron beam radiation damage could all interfere with an important feature of exosomes, that of size.[94,95]

Cryo-electron microscopy (cryo-EM) comes at hand here, and hydrated exosomes studied by cryo-EM have been reported as close-to-spherical nanoparticles.[96,97] As fixation tends to be physical (cryogenic) rather than chemical, cryo-EM is thought to preserve EVs closer to their native state,[98] in particular in terms of their morphology and immunological epitopes used in the identification of vesicle biomarkers. An interesting feature described in cryo-EM of exosomes from prion-infected cells was the presence of a second, internal membrane-bound region of denser core and, in many cases, of hexagonal shape.[97] Vesicles derived from human plasma and studied by the same method also show a variety of forms and sizes, suggesting multiple membrane profiles in EVs. Elongated vesicles, empty or not, were also observed, and so were EVs surrounded by smaller spherical structures.[99] Yet, because most cryo-EM procedures do not include the use of heavy metals, contrast of the material could be compromised if not for

various optical and diffraction techniques – the work of colleagues such as Alain Brisson from the Institut de Chimie & Biologie des Membranes & des Nano-objets (CBMN, Bordeaux, France) in the development of suitable imaging analysis methods, and of diagnostic assays using EVs, holds much promise here.

4.2 Nanoparticle tracking analysis (NTA)

NTA is a light scattering technique that makes use of both laser light scattering and the Brownian motion of particles to obtain the particle size distribution and concentration. This approach takes into consideration the particles' speed in a medium of known temperature and viscosity and allows the calculation of its hydrodynamic diameter. The first studies of EVs using NTA were published in 2011.[100] NTA is currently the most popular quantitative method of EV analysis.[52] This is because few other high-throughput techniques have the appropriate resolution for single EV particle analysis. Whilst NTA allows relatively high throughput, and claims to have resolution down to small diameters, its ability to size and determine concentration accurately is however debatable.[101] Moreover, when performing NTA measurements, before dilution of samples, attention should be given towards the lack of particulate matter in suspension buffers (usually phosphate buffered saline) to avoid artificial inflation of EV counts. The addition of a detergent such a Triton X to NTA preparations allows for confirmation of EV-lipid moieties. Its fluorescent mode is also capable of providing specific results for labelled particles. In a recent application of fluorescent NTA, it was shown the use of miRNA-specific molecular beacons encapsulated in cationic lipoplex nanoparticles that fuse non-specifically with negatively charged EVs and this allowed the precise quantification of EVs carrying an specific miRNA and how many copies of this miRNA could be found in these EVs.[102] This paves the way to future therapeutic applications that rely in the identification/quantification of specific markers.

4.3 Resistive pulse sensing (RPS)

RPS utilises the Coulter principle to determine the absolute diameter and distribution of particles in a suspension in the range of ~50–10 000 nm.[101,103] RPS utilised in the EV field is generally carried out using the qNano (Izon Science Ltd, Christchurch, New Zealand). The qNano consists of two fluid cells separated by a non-conductive membrane. An electric current is passed through a single pore in the membrane and, as particles pass through this pore, a transient attenuation of signal occurs that is approximately proportional to the particle volume. This system is calibrated using beads of a known diameter and concentration.[104] Sample volumes in the qNano can be as low as 10 μL. By applying pressure differences between the fluidic cells, pressure-driven flow overcomes the flow produced by diffusion, electrophoresis and electro-osmosis. Disadvantages of this technique include: multiple pore sizes are required to measure the full EV size range; pores are prone to clogging; little phenotypic information regarding the EV's derivation is gained; identifying EVs from similar sized contaminants is not possible.

4.4 Flow cytometry (FCM)

Although mostly used for cellular analysis, FCM is currently one of the most popular techniques used to study EVs, in particular microvesicles.[105] Flow cytometers that have been developed for dedicated EV analysis, referred to as dedicated FCM (dFCM), have been shown to be capable of resolving particles consistent with biological vesicles to <30 nm. dFCM are usually conventional flow cytometers that have been customised by a laboratory, although more recently they have also become commercially available.[106–108] A persistent concern in FCM is the ability to reliably distinguish between EVs that carry a specific protein marker from those that do not, and thus to accurately measure the proportion of EVs of a certain type. Quantitative and qualitative analysis of EVs populations present within a sample are needed. Although being a prerequisite for a correct data interpretation, the use of proper controls that robustly validate the test samples and establish background levels are generally missing.[109] Fluorescently-stained polystyrene microspheres have been used as a standardisation method for EV analysis, yet their high refractive index compared to EVs means that they cannot reliably be used to directly approximate the size of EVs using FCM scatter parameters. Furthermore, due to FCM collecting light at different angles, scatter resolution cannot be compared using polystyrene microspheres without the use of refractive index normalisation using laser scatter physics modelling.[110] Fluorescent sensitivity can be quantified using microspheres that have a known quantity of molecules of equivalent soluble fluorophore (MESF) that are recommended for standardising EV analysis. Reviews dedicated to EV standardisation using FCM can be found elsewhere.[105]

5 Characterization of EV content

5.1 Characterization of EVs content: RNA

5.1.1 RNA purification. Several sources of bias due to differences in experimental methodologies may explain discrepancies between results of EV-studies, including EV-RNA related analysis. The two main RNA purification approaches are silica membrane column-based kits and/or organic extraction and precipitation-based kits. One of the earliest studies that systemically compared methods evaluated seven different extraction protocols and concluded that there were major differences in yield, purity and size distribution of the RNAs extracted by the kits.[111] Nowadays there is a plethora of commercial kits available from many different companies, specifically tailored for EV isolation and subsequent RNA extraction. Each method has its own peculiarities and attention must be paid when comparing results from different studies, taking into account the EVs and RNA isolation strategies employed. Another important source of bias relates to the possible co-purification of non-vesicular nucleic acids bound to the exter-

nal surface of EVs. To avoid this and isolate only intra-vesicular RNAs protected by the lipid membrane, ISEV recommends before RNA extraction performing a proteinase treatment first to eliminate protein complexes that may also protect RNAs from enzymatic degradation, followed by RNAse treatment.[112] However, residual RNase activity may also damage RNA upon extraction of the luminal contents. Consequently careful consideration of the aim of the study should be first taken into account prior to RNase treatments.

The major drawback of working with EV-derived RNA, especially in the case of those isolated from biofluids, is the poor RNA yield, which is often below the detection limit of current quantification techniques such as fluorimetry (Qubit) and capillary electrophoresis (Bioanalyzer). One possible way of overcoming this limitation is normalizing all samples by the initial biofluid input volume used for extraction, and vacuum-concentrating all the extracted RNA for downstream analysis. Suggestions to improve EV-RNA yield include diluting biological fluid prior to extraction[113] and also the observation that more starting material is not necessarily advantageous due to potential over-loading of sample in column based extraction methods.[114] Despite the fact that more EVs are present in serum, plasma is more commonly studied due to the absence of EVs released by platelets during the clotting response.[115]

5.1.2 Microarray analysis. Microarray technology enabled the seminal discovery in 2007/2008 of RNAs contained in vesicles derived from mast cell lines[116] and glioblastoma primary cells as well as patient serum samples.[117] These studies used gene expression arrays and first identified thousands of transcripts in EVs, including miRNAs and mRNAs.[116,117] The mRNA molecules were shown to be functional, leading to protein production in the recipient cells upon transfer of the EV-cargo.[117]

5.1.3 Quantitative reverse transcriptase-PCR (qRT-PCR) and PCR arrays. Similarly, several studies used quantitative real time PCR-based arrays to profile the RNA content of EVs.[118–121] This is by far the most widely used method to evaluate and quantify the miRNA content of EVs, due to the low requirements of starting RNA (as little as 1 ng total RNA, using a pre-amplification protocol), the ability to evaluate hundreds of miRNAs of interest and the straightforward analysis of the data that requires simple calculations and no bioinformatics. As most studies that evaluate the RNA-content of EVs aim to identify biomarkers, qRT-PCR is a good, low-cost and reliable tool. The major drawback of this approach is the need defining *a priori* the list of possible markers, as their evaluation depends on the probes/primers available in the selected platform.

During the qRT-PCR procedure *per se*, one of the most important factors is the amount of starting molecules. When direct quantification of EVs cannot be performed, the normalization of the input to volume of sample input is recommended.[122] Moreover, in an attempt to enhance the amount of amplifiable molecules, pre-amplification methods can be used,[123] with no apparent bias. Despite the qRT-PCR approach used (specific probes or non-specific DNA dyes), results can be strongly biased according to the selected normalization method. A major issue is the absence of known endogenous controls to be used for qRT-PCR. In this sense, the Sample and Assay Standards Working Group of the Extracellular RNA Communication Consortium (ERCC), recently suggested the use of spike-in controls to properly normalize the RNA populations[43] an approach that has been successfully used.[124]

5.1.4 Next generation sequencing (NGS). Recently, the RNA content of EVs from diverse sources has been characterised by next generation sequencing (NGS), allowing a more comprehensive analysis of the EV-RNA repertoire.[125–128] The predominating view is that EVs are enriched in small non-coding RNAs, such as miRNAs, tRNAs and miscellaneous structural RNAs such as Y-RNAs, vault RNAs and SRP-RNAs.[129] This may be due to the fact that the majority of studies have focused on the small RNA fraction of the transcriptome, by performing either a gel- or bead-based size selection step that limits the analysis to small RNAs (<200 nucleotides).[129–132] In this way, researchers have bypassed the issue of sequencing abundant rRNAs, but have consequently missed out on the identification of mRNAs and long RNAs that offer great potential as biomarkers. One study that performed whole transcriptome analysis of EVs derived from breast cancer cell lines found 97% of reads were derived from rRNA and argued that EVs are enriched in rRNA molecules that are fragmented and therefore do not appear as peaks on Bioanalyzer analysis.[133] Likewise, in another study with EVs derived from epidermoid and hepatocellular carcinoma cell lines, the authors found 92% rRNA, but as they chose not to perform any size selection step they only analysed RNAs greater than 50 nt, which therefore precluded the identification of miRNA molecules and some other small RNAs.[134] In another recent study with EVs isolated from human pleural effusion and plasma, mRNA amplification was performed using oligo dT primers, restricting the analysis to the poly-adenylated fraction of transcriptome.[135] An unbiased characterization of the complete vesicular transcriptome is currently lacking, mainly due to methodological constraints that require different library construction protocols for small or long RNA analysis wherein size selections limit the analysis to specific transcript fractions. The limited RNA yield that is obtainable from EVs, particularly those derived from biofluids, oftentimes prohibits the construction of more than one library per sample, not to mention the high costs involved. The depletion of rRNA before sequencing has not been possible also due to limited RNA yield, as the majority of commercial kits require at least 100 ng of RNA input, or has been attempted but was not successful to the fact that fragmented rRNA hinders probe-based capture.[133]

Crescitelli *et al.* compared the RNA profiles of apoptotic bodies, microvesicles and exosomes isolated by UC from the conditioned medium of three different cell lines.[11] However, authors only undertook a Bioanalyzer analysis, which limits the results to RNA size profiles. This could be misleading as typical 28S and 18S rRNA peaks will not be visible if the rRNAs are present as fragments inside EVs, which has been reported

by NGS data.[133,134] Nonetheless, Crescitelli *et al.* found that rRNAs are more prevalent in apoptotic bodies, and increases after TRAIL-induced apoptosis. This observation may perhaps explain the variability of rRNA content of EVs reported in the literature, and highlights the importance of reporting cell viability in studies, when applicable. The variability in quantities of rRNA reported is discussed by Mateescu *et al.* in an ISEV position paper, and they mention that ribosomes present in extracellular fluids may become associated with EVs during centrifugation and co-isolate at high *g*-force.[136] They conclude that the only way to effectively prove if a particular RNA species is truly inside EVs is by RNAse protection assays after proteinase treatment. Therefore, it is our view that the best description of the whole EV-transcriptome would require the depletion of apoptotic bodies by several steps of lower-speed centrifugations followed by pelleting of EVs at higher *g*-forces and RNase digestion of intact EVs.

Despite the above-mentioned technical hurdles, NGS is the most comprehensive tool for the analysis of transcriptome, as it allows the unbiased view of all molecule types in a sample and permits not only quantification, but also the identification of mutations, and structural variations such as alternative splicing isoforms, circular-RNAs and translocations.[128] However, the limitations of this method include its relatively high-cost, the requirement of large amounts of starting RNA (usually above 100 ng), and the dynamic bioinformatics pipelines to evaluate the results.

5.1.5 Digital droplet PCR (ddPCR). Digital droplet PCR (ddPCR) is a relatively new technology that allows the absolute quantification of gene expression. By partioning PCR amplifications into thousands of fractions, this methodology allows very sensitive detection of RNA expression levels and DNA variants without the use of standard curves. Recently ddPCR has been applied to the study of plasma-derived exosomal RNA from prostate cancer patients[137] and EVs derived from serum and CSF of glioma patients.[138] ddPCR is also very useful to validate NGS findings, even more so in the case of EV transcriptome analysis, as standard qRT-PCR depends on normalization to the expression of levels of endogenous controls, and in the case of vesicle-derived RNAs such controls are not ubiquitous.

5.1.6 Bioinformatics analysis of the EV-transcriptome. After generating the sequences by NGS, the computational analysis is straightforward with no relevant deviations from routine bioinformatics protocols. Normalised count-based differential expression analysis is performed over the RNA sequencing data, after removing artifacts and low quality reads usually using the FastQC[139] tool, which is able to report information related to the GC content, the presence of adaptors, over represented k-mers and duplicated reads, PCR artifacts or contaminations. Reads derived from fragments of ribosomal RNAs are usually removed in this step as well. Next, one key aspect is to deal with distinct read-length distribution. To overcome this issue, the alignment step could be performed by using one or the combination of the mapping tools, such as Burrows–Wheeler Aligner (BWA),[140] Bowtie2[141] or STAR.[142] After the alignment is carried out, the mapped reads are used to estimate gene and transcript expression and, the simplest approach is to quantify raw counts of mapped reads using programs such as FeatureCounts[143] or HTSeq-count.[144] To facilitate transparent comparison of transcript levels both within and between samples it is of utmost importance to use metrics that attempt to normalize for sequencing depth and gene length. The fragments per kilobase of exon per million reads mapped (FPKM)[145] and RNASeq by Expectation Maximization (RSEM) software[146] are the most used ways to normalize the count data from RNA-seq platforms. The analysis of differentially expressed genes (DEG) follows the above-mentioned steps and the popular methods for DEG are DESeq[147] and EdegR[148] DGE data can be visualized as MAPplots (log ratio × abundance), Volcano plots (fold change × statistical significance), heatmaps and scatterplots. Further analysis steps include the functional profiling in which DEG are involved. Nevertheless, the characterization of the molecular function requires the availability of functional annotation data for the transcriptome under study. Gene Set Enrichment Analysis (GSEA) is one the most common knowledge-based methods for interpreting genome-wide expression profiles.[149] Many of the tasks shown in this section are embedded in the Bioconductor[150] a flexible software-engineering environment that enable genome-scale analysis of high-throughput data.

5.2 Characterisation of EVs content: protein

The protein cargo of EVs is cell- and disease-type dependent, and confers particular features to these vesicles influencing their biological properties.[71,151,152] Protein components of EVs that are derived from different cell types and biofluids have been widely described using Western blotting, proteomic technologies and fluorescent-activated cell-sorting.[153–155]

5.2.1 Western blotting. Western blotting for the so-called EV markers is often used as a confirmation of EVs presence in samples of interest.[96,156] Commonly used markers include CD63, Alix, Tsg101 and HSP70.[157,158] A common mistake in the literature is the attempt to classify isolated EVs according to the presence of certain markers, in particular the presence of markers such as CD63, to annotate exosomes. There are two major problems with this approach: firstly, most EV isolation methods will isolate a mixed population of exosomes and microvesicles, and secondly, there are no widely accepted, reliable markers to distinguish between these two populations.[71] In addition to the fact that there are further subpopulations of EVs within these two subsets. Indeed, Kowal *et al.* found that CD63 is present in EVs, which are larger than exosomes and that the common exosome markers HSP70, flotillin-1 and actin are present in all EVs sizes.[158] Furthermore, they found that other markers, including Tsg101, were present in only a subset of exosome-sized EVs, suggesting that these are not suitable as generic 'exosome' markers. One limitation of this study, however, was that it focused only on dendritic cell EVs, and it is impossible to draw conclusions about universal EV markers without more extensive studies. Indeed, Yoshioka *et al.* found that there was variation in the presence

of EV markers such as CD63, flotillin-1 and HSP70 even within different prostate cell lines[157] while CD9 and CD81 were uniformly present. The variability among different prostate cell lines may reflect prostate-cancer heterogeneity and it is well known that cancerous cell lines can secrete EVs with different cargo from their non-cancerous counterparts,[68,159] yet many commonly used cell lines are cancer cell lines and information taken from these cells taken to infer universal EV-markers will probably be misleading. More research is needed to identify reliable EV markers. However, it remains to be seen whether universal EV markers in fact exist and could be used across all mammalian cells and, furthermore, whether markers will be found to reliably distinguish exosome and microvesicle populations. Showing the relative enrichment of several marker proteins in isolated EVs compared to their corresponding whole cells is probably a good way of confirming the presence of EVs in the absence of better tools.

Another point to note is that most of the common EV markers are also abundant or detectable in whole cells[157] so confirming their presence in isolated EVs is not necessarily a good method of demonstrating sample purity. It is therefore essential to also show negative control Western blots[96,156] for proteins such as calnexin or histones which can be confidently expected not to be secreted in EVs; showing the absence of abundant whole cell proteins is an important step towards proving that isolated EVs are not contaminated by cell debris.

A more general problem is that all the research discussed above fails to address EV markers in non-mammalian cells, which may have an entirely different set of EV markers even if mammalian markers are present in the genome. EVs are studied from bacteria,[160] to algae,[161] helminths[162] and protozoa[163] and have important implications in basic biology. An alternate, unbiased way of showing protein loading and distinct protein composition from whole cells without the need for specific markers is to use protein staining instead of Western blotting, such as silver or SYPRO Ruby staining.[164]

5.2.2 Proteomics. Undeniably, mass spectrometry based proteomic analysis has increased our knowledge about the protein content of EVs. In particular, bottom up mass spectrometry-based proteomics has been used worldwide as the strategy of choice. In this approach proteins are extracted from a biological source, digested into peptides that are subsequently separated by 1D or 2D gel electrophoresis (gel-based) or liquid chromatography (gel-free) and analysed by mass spectrometry. Peptide ions are fragmented in the gas phase and their sequence and post-translational modifications (PTMs) can be deduced. Protein quantification can be achieved using different strategies depending on the study aims.[165] In particular, shotgun proteomics approach allows a discovery-driven protein identification and quantification where peptide ions are measured and heuristically selected for fragmentation using a data-dependent mode.[166] In targeted proteomics, only predetermined peptide ions are selected for fragmentation allowing a hypothesis-driven protein detection and quantification. Several acquisition methods have been implemented for targeted proteomics such as selected reaction monitoring (SRM)[167] pseudo selected reaction monitoring (pSRM)[168] and parallel reaction monitoring (PRM).[169,170] Another approach is the data-independent acquisition in which no precursor ion selection occurs and all precursors are fragmented.[171–173] The resulting MS/MS spectra are commonly searched using spectral libraries[171] or novel computational frameworks.[174] These proteomics strategies have been used in several EVs characterization studies and excellent reviews on EVs isolation and proteomics have been published.[175–177] As such, we will focus on the use of mass spectrometry-based protein analysis and proteomics technologies to better understand the more specific differences in the EV proteome such as post-translational modification (phosphorylation and glycosylation).

5.2.3 Post-translational modifications in EVs: a path to explore. Mass spectrometry analysis allows quantitative protein profile of EVs in different conditions. Still, there are additional layers of protein regulation, such as PTMs that modulate protein structure and function changing its physicochemical characteristics, interaction partners and activity.[178]

Protein glycosylation is one of most widespread PTMs, which involves the enzymatic attachment of a glycan moiety to a protein, occurring mostly in the ER and Golgi. Indeed, protein glycosylation has been shown to play an important role in cancer, diabetes, neurodegenerative and cardiovascular diseases.[179] Several glycan binding proteins were identified in EVs such as CD62, found in isolates from activated platelet[5] and several members of the galectin family.[180,181]

Protein phosphorylation is a very dynamic PTM catalysed by kinases and reversed by phosphatases that regulates several signalling events both extra- and intracellularly. Even though most reports have applied immunodetection techniques to study protein phosphorylation in EVs, Gonzales *et al.* have described a large-scale phosphoproteomic approach to exosomes isolated from urine that allowed the identification of 14 phosphoproteins, such as AQP2, which was confirmed by immunoblotting.[182]

Since regulation of cell function is achieved through an intricate network of PTMs, it would be desirable to study several PTMs simultaneously. Although mass spectrometry-based proteomics has allowed the comprehensive identification of PTMs and their crosstalk,[183–185] bioinformatics analysis and functional interpretation of multiple PTMs remains a challenge. However, we believe that increasing comprehensive multiple PTMs studies will help shed some light in EVs PTMs cross-talk and burst the discovery of novel signalling mechanisms.

5.3 Characterization of EVs content: lipids

Structurally and functionally, lipids represent an extremely heterogeneous group of molecules, defined by various permutations of head-groups and fatty acid chains, which together define lipid structure, head-group polarity, and hydrophobicity. Due to often limiting amounts of sample available and its high complexity, mass spectrometry-based approaches offer high sensitivity and specificity that is essential for the analysis of EV-lipidome. Modern mass spectrometers offer high resolu-

tion and high mass accuracy of survey scan, thereby providing the required accurate mass measurement of the lipid molecular weight. However, partial or complete overlap in the elemental compositions between lipids of different classes imposes a great challenge for their confident identification.[186]

Lipid content can be analysed from EV pellets reconstituted in aqueous environment after their UC, or from highly concentrated EVs size exclusion chromatography fractions. Typically, the lipid amount used for analysis is expressed relative to protein quantity, and may vary from 100 μg protein equivalent or more.[187,188] EV lipids are most commonly extracted using Bligh and Dyer[189] and Folch[190] liquid–liquid extraction, where lipids are enriched in the lower organic layer of chloroform/methanol/water phase. However, both methods carry a risk of contamination by protein from the upper aqueous phase during the collection of organic phase, which may have deleterious effects to the downstream nLC/LC-MS/MS analysis. Additionally, due to the hydrophobic nature of chloroform, both methods may discriminate extraction of partially polar lipids and additionally challenge their detection. Recently methyl-*tert*-butyl-ether (MTBE) extraction of lipids was demonstrated to extract almost all lipid classes form highly complex biological samples with equivalent or better recoveries than the "gold standard" Folch extraction.[191] To date, lipidomic profiling of EVs derived from different cells,[187,192] species[193] and biological tissues[194,195] have been published, together with recent reviews on lipidomics of EVs.[176] Since EVs are cell-derived particles, their lipid composition is related to its parent cell. Eventhough, a direct comparison of the lipid profile between exosomes and plasma membrane of the releasing cells allowed the identification of specific lipid classes that appear to be enriched in the exosomal fraction. Membranes of typical EVs are mainly enriched in fully saturated glycerophospatidylcholines, glycerophospatidylethanolamines and glycerophospatidylserines, that are responsible for membrane rigidity and integrity, and sphingomyelin, cholesterol lipids and ganglioside GM3.[196] A study characterized more than 280 lipid species from metastatic prostate cancer cell-line derived exosomes. Exosomes were enriched in glycosphingolipids, sphingomyelin, cholesterol, and phosphatidylserine indicating a particular lipid sorting in the exosome membrane.[197] Although lipid content of EVs attracts increasing attention, not much is known about their biological activity. Sphingomyelin is reported to mediate antigenic activity of tumour-derived EVs both *in vivo* and *in vitro*.[74] Lysophosphatidylcholines in EVs derived from mature dendritic cells appear to act *via* G-protein coupled receptors and trigger lymphocyte chemotaxis.[198]

5.4 Characterization of EVs content: metabolites

During EV formation, small metabolites such as sugars, amino acids, nucleotides, different enzymatic cofactors, lipid metabolites and other redox regulatory molecules can be packed into EVs. Sub-nM concentrations of metabolites and low sample amounts impose a great challenge for the metabolomics of EVs, which may be one of the reasons why this type of analysis is only sporadically reported.[199–201] Metabolites comprise a broad array of small molecular weight analytes, derived as intermediates and products of enzymatically-driven cellular processes. Due to their smaller size, metabolites are characterized by their lower hydrophobicity compared to lipids. Thus, they are usually enriched *via* extraction from vesicle pellets with methanol or methanol/water. However, co-extraction of highly abundant lipids may suppress ionization of metabolites of interest and greatly challenge their detection even with the most sensitive targeted mass spectrometric detection. Thus, metabolites of interest are further enriched using solid phase extraction (SPE) cartridges. The choice of SPE chemistry depends solely on the analyte chemical nature and eluting condition. The most common conditions include loading of sample in 10% aqueous methanol (pH 2), hexane washing of hydrophobic lipids, and elution of metabolites in methanolic ethylacetate or butylaceta.[199,201] Eluates are further vacuum dried and can be stored at −20 °C, or analysed immediately by mass spectrometry.

Although information of the metabolome of EVs is limited, all published studies to date support the significance of EVs as carriers of important metabolome fingerprints which can be used for defining specific changes in cellular homeostasis. Increasing evidence suggests that, during defined stages of the acute inflammatory process, neutrophil-derived EVs carry specific enzymatically-derived polyunsaturated fatty acid (PUFA) metabolites such as 4-, 7-, 14- and 17-hydroxy-docosahexaenoic acid, 5-, 12-, 15-hydroxy-arachidonic acid, 12-, 15- and 18-hydroxy-eicosapentaenoic acid and others.[199,201] These metabolites are of particular importance because they serve as direct precursors of anti-inflammatory and pro-resolving lipid mediators,[202] such as resolvins, maresins, protectins and lipoxins. Prostaglandin (PG) rich vesicles trigger PG-dependent intracellular pathways in the host cells.[203] Thus, depending on the metabolome cargo, EVs in target cells can induce formation of pro-inflammatory, or anti-inflammatory and pro-resolving lipid mediators, and thereby influence inflammatory outcomes and tissue homeostasis.

Metabolomics is an emerging technique with focus on identification and quantification of a wide variety of small molecules which may be indicative of physiological status in health and disease. Metabolites are produced *in vivo*, usually *via* enzymatically-driven processes; thus their circulating levels are very low, and often out of the reach even for the most sensitive detection techniques. Thus, analysis of EV-metabolome relies on a careful and well-designed sample enrichment, and sensitive MS-detection. Recently some authors[200] for the first time described methodology for the LC-MS/MS profiling of blood plasma EVs, and EVs derived from cultured cells. Coenzyme Q10, ubiquinone 9,25-hydroxy-hexadehydrovitamin D3, malonyl-CoA, deoxyvitamin D3 and others, are only some of identified metabolites that belong to the wide range of metabolite classes. However, MS-profiling often does not offer the sensitivity necessary for detection of very low abundance metabolites. Targeted mass spectrometry (*e.g.* multiple reaction monitoring, MRM) provides necessary sensitivity and specificity, however it discriminates detection and identifi-

cation of those metabolites that are not "targeted" by designed acquisition methods, and cannot be used for wide-band metabolome profiling.[204]

5.4.1 Bioinformatic tools used in lipidomics and metabolomics. Comprehensive analysis of large lipidomics and metabolomics data-sets is a challenging and time consuming task. Although significant improvement in the field has been made over the past few years, identification of lipids and metabolites is still not as straightforward as protein identification.[205] The LIPID MAPS (http://www.lipidmaps.org) is a free of charge online platform for the analysis of MS data based on their accurate mass measurement.[206] Recently, LIPID MAPS have implemented a search tool that uses precursor or product ion lists and matches them with thousands of different lipids with *in silico*-generated structures or those present in the database. The Human Metabolome Database (http://www.hmdb.ca) is a freely available database that accounts for 42 003 metabolites found in the human body and supports analysis of MS, MS/MS and GC-MS data uploaded in a form of peak lists. LipidXplorer-assisted analysis of large shotgun lipidomics data sets is used routinely for lipid identification, independently of any resource of reference mass spectra, and solely based on assumed molecular fragmentation pathways.[207] The software and MFQL library are free for download (https://wiki.mpi-cbg.de/lipidx/Main_Page) and can be used regardless of the mass spectrometer type. It is important to note that the mass resolution and accuracy influence strongly unequivocal lipid identification. Lipid View (ABSciex) and Progenesis QI for small molecules (Waters) are commercially available platforms. Both softwares can use MS and MS/MS that they match against publicly available and in-house databases.

Although many different MS platforms for lipid analyses are available nowadays, analysis of EV-bound metabolites and lipids remains a significant challenge. Very low amount of sample still imposes the greatest challenge for EVs wide-band profiling. To date, both analyses have already advanced our knowledge on biomolecular composition of EVs and helped us better define their structure-to-function relationship. Nevertheless, further advances in lipidomics and metabolomics are necessary to allow application of MS for the analysis of EVs derived from various different sources and biological tissues. The main technical pitfalls of working with EVs and potential ways to avoid them are summarized in Table 1.

6 *In vivo* studies of EV-function

The ability to discriminate vesicle populations of interest from the general EV pool in tissue compartments and bodily fluids remains technically challenging. The cellular origins of EVs, their route of administration, their concentration and their mode of targeting significantly impacts on EV bio-distribution.[224] However, non-traditional routes of EV administration, such as orally administered EVs have shown uptake *via* the intestinal mucosal surface and subsequent accumulation in the liver and spleen.[225] Irrespective of administration route, a key outcome measure is biodistribution, to trace where exogenously applied EVs go after they have been introduced into an animal.

Fluorescent labelling of cellular proteins have revolutionized *in vivo* studies of cells. However, similar protein marker tracing of EV through EV-associated proteins like CD63-GFP (green fluorescent protein) have been shown to be an effective detection approach. A fraction of EVs is CD63-positive and further work is required to establish the cellular processing of CD63 on EV populations. Numerous protein markers (CD63, TSG101, Alix and heat shock proteins) are widely reported as associated to EVs but their overall contribution to the total EV population remains contentious. Therefore, utilising these proteins as imaging or labelling moieties requires caution. The nanometer-size scale of EVs presents challenges in studies that require spatiotemporal resolution. In addition, exosomes may be as small as 50 nm in size and tagging them with a 5 nm GFP molecule may significantly affect both their biodistribution, kinetics and their functional effects.

Secondary labelling of isolated EV using lipophilic dyes (DiR (1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide, D12731, Invitrogen, Life Technologies, PKH26/67 Sigma-Aldrich or Dil Stain (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate ('DiI'; DiIC18(3))) Thermo)) have shown distribution to the spleen, liver, lungs, pancreas, kidneys and gastrointestinal tract. These lipophilic methods are limited in scope and require the harvest of tissues of interest for analysis, or the use of highly sensitive cameras. Lipophilic dyes may persist in organs and tissues after EV degradation or may associate with other lipids. Injected liposomes that model the EV-population are rapidly cleared from the circulation by the spleen and liver <1 hour after injection, suggesting differences for EV/liposomes clearance based on lipid profiles;[226] whereas Gaussia luciferase combined with metabolic biotinylation greatly enhances the spatiotemporal resolution of EV tracing *in vivo*.[227]

Whilst labelling and re-applying EVs *in vivo* enables the investigation of distribution, there is still a paucity of knowledge regarding the downstream effects of these EVs, or even whether the numbers injected are physiologically relevant. Indeed, the rapid generation time of both species, combined with an easily manipulated genome, makes them ideal for studying both the role of EVs in development, as well as the mechanistic underpinnings of their release. It is even possible to study the effect of EVs on stereotypical behaviours in invertebrates; for example Wang *et al.*, 2014[228] have shown that ciliary EVs are responsible for the tail-chasing behaviour seen in some worm species.

However, detailed investigations utilising vertebrates are needed to further test the biodistribution of EVs and ascertain the optimal route of administration for target organs or tissues. Rodents, which are traditionally used in preclinical research, provide rapid breeding cycle, complete genome sequence and some physiological similarities to humans. Two key biodistribution studies in rodents were carried out by Xandra Breakefield's and Samir El Andaloussi's group, both

Table 1 Potential technical pitfalls, recommendations and remaining challenges according to different techniques of EVs isolation and characterization

	Sample type/technique	Potential pitfalls	Recommendations	Remaining challenges
Pre-analytical factors	Cell culture conditioned medium	Presence of contaminating bovine vesicles and proteins from FBS leading to misinterpretation of EV quantification/characterisation	The use of EV-depleted FBS is recommended[208]	Much needed standardisation of protocols by the EV-community; current lack of knowledge regarding serum starvation stress over optimal duration of EVs production, concentration and content
		Use of EV-depleted FBS[209] or serum starvation[210] may stress cells leading to alterations in EVs concentration and content	In the case of complete serum removal, cells should be assessed for stress induction that could alter EVs characteristics. Autophagy, apoptosis and necrosis should be monitored	
	Blood	Artificial inflation of EVs counts *ex vivo* as a result of platelet activation due to blood collection, blood transportation and excessive delay in plasma separation	Follow guidelines such as those from the International Society on Thrombosis and Haemostasis, including citrated tubes for blood collection, and adequate separation of platelet-free plasma using two rounds of centrifugations immediately after blood collection. Abundant proteins (such as albumin and immunoglobulins) should be depleted	Difficulty applying rigorous blood collection and plasma separation/storage in a large-scale clinical setting
		Changes in EVs counts/cargo observed are not related to particular disease/phenotype being investigated, but are rather due to other clinical and environmental factors that influence EVs counts such as age, gender, circadian-clock, medications, fasting state, hormonal status, presence of infection, *etc.*; abundant proteins may hamper the identification of bona fide EV protein cargo	Compare with a matched control group without disease and try to normalize as much as possible the differences between individuals within a group, controlling for all factors cited	Unmet need for technological advances that allow disease-specific EVs to be isolated, quantified and characterized separately from all other normal-cell-derived EVs
	Urine	Membrane-bound proteins could be affected by proteases in the urine; EVs may remain attached to the collection tubes	Urine should be collected in tubes containing protease-inhibitors; containers should be vortexed to avoid EVs being adhered to tube wall; abundant proteins (such as uromodulin) should be depleted	Handling of large volume samples and the need to concentrate urine for the adequate isolation of EVs challenges its use in a clinical setting
EV isolation protocols	Ultracentrifugation (UC)	Variability in sedimentation efficiencies, no absolute separation of EVs subpopulations. High centrifugal forces may lead to EVs aggregation and disruption of larger EVs; lower centrifugal forces reduce recovery of smaller EVs	Low-angle fixed-angle rotors provide better separation efficiency[211] and reducing biofluid viscosity prior to centrifugation improves recovery;[113,212] protocols need to be adjusted according to the targeted EV-subtypes	There is a lack of methods to allow the precise assessment of EV damage and the recovery of the full range of EVs
		Co-sedimentation of protein, lipoprotein and RNA aggregates as well as viral particles	Combine ultracentrifugation with density gradients to further purify samples[66]	
	Density gradients (DG)	Sample fractionation leads to EVs being divided into numerous sub-samples	Ensure higher initial EV counts due to substantial dilution of EVs among the sub-samples	Ultracentrifugation steps as well as the preparation of the gradient are time consuming; the process is laborious and difficult to be implemented in a clinical setting
	Immunoaffinity	Concentrated EV samples are required in small volumes	Spin columns, precipitation and other methods can be used to reduce sample volume	Clearer definition of EV subpopulations and their characteristic markers. Less aggressive dissociation of EVs from beads to avoid affecting functionality.
		Markers must be pre-selected by the user	Characterise markers specific to EVs in the system of interest, it has however been demonstrated that markers vary between EVs of interest[213]	Minimizing antibody contamination and buffers' interference

Table 1 *(Contd.)*

	Sample type/technique	Potential pitfalls	Recommendations	Remaining challenges
		Saturation of beads with abundant, non-EV proteins found in biofluids. Elution of EVs from beads might damage the structure of vesicles. Captured vesicles might not retain functionality after elution. Eluted antibodies or antibody remnants and harsh elution buffers might interfere with downstream analysis	Coat beads to minimise non-specific adsorption of abundant proteins from serum samples[214]	
	Commercial kits	Contaminating non-vesicular molecules (such as protein, protein aggregates, lipids and free nucleic acids) may occur in all isolation methods, but may be more predominant here due to precipitation process	Evaluate the more prevalent non-vesicular contaminants of the sample under investigation and test kit to establish optimal protocol for experimental aim	The unknown biochemical basis of many kits restricts our ability to fully understand EV populations extracted and the non-vesicular contaminants
		Potential interference by elution buffers in downstream applications	Include controls in functional tests checking the effects of kit elution buffers on cells receiving EVs	
	Size exclusion chromatography (SEC)	Inability to concentrate diluted EV sample, which, often requires second step *i.e.* re-concentration of EVs by ultrafiltration[56,80,81,83–86]	To rapidly concentrate EV fractions the application of protein concentrating devices is recommended	Concentrating devices need to be standardized in order to allow results to be comparable across studies
	Ultrafiltration (UF)	Non-specific binding of EVs to column membranes leading to reduced yield owing to trapping in filter pores[35,56]	Ultrafiltration coupled with SEC is recommended for enhanced EV purity, preserving their properties[56,60]	Forcing vesicles through filters might damage larger EVs whereas the use of less force, through gravity or by using less pressure makes the process longer and less adequate in some cases[40]
Characterisation of isolated EVs	Electron microscopy (EM)	Dehydration and fixation of EVs during processing may disrupt membranes leading to artefacts	Use cryo-EM in combination with immunogold labelling, where samples are not dehydrated[215]	Development of new methods both quantifying and characterising EVs. No currently available EM approaches for routine analysis of EVs in a clinical scenario
		Inability to readily discern heterogeneous EV populations	Use of alternative super-resolution microscopy techniques *i.e.* PALM[216] or STORM[217]	
	Nanoparticle tracking analysis (NTA)/resistive pulse sensing (RPS)	Resuspension buffers may contain particles misinterpreted as vesicles	Include controls to ensure that EV suspension buffers analysed in order to establish a control/background	Polystyrene bead refractive index not identical to that of EVs. Current issues in discerning clustered from non-clustered vesicles, especially in samples of higher EV-concentration (NTA). Establishment of lower detection limit for RPS
		Accuracy in reporting size and concentration is debatable	EVs or their contents can be GFP labelled to ensure proper analysis.[102] Bead calibration should be used[218]	
	Flow cytometry (FCM)	Distinction between single particle positive and negative EV samples	Use reciprocal dilution to ensure single particle detection or use multicolour and multiplex approaches to resolve individual vesicles[219]	High refractive index of commonly used reference beads compared to EVs
		Scatter resolution	Use scatter beads of known size and refractive index. Use fluorescence beads with known molecules of equivalent soluble fluorophore (MESF) units[220]	
Characterisation of EV contents	RNA purification	Low RNA yields	Normalize all samples by the initial biofluid input volume used for extraction, and vacuum-concentrate extracted RNA for downstream analysis	Difficulty in eliminating residual RNase that would reduce the available luminal EVs RNA
		DNA contamination in EV RNA samples	DNase treatment of RNA samples should be performed prior to downstream analysis	
		Non-vesicular RNA complexed with proteins may be bound to the outside of the EV membrane	Use proteinase followed by RNase treatment of intact vesicles to eliminate protein complexes protecting non-vesicular	

Table 1 *(Contd.)*

	Sample type/technique	Potential pitfalls	Recommendations	Remaining challenges
			extracellular RNAs from enzymatic degradation[221]	
	RNA analysis by qRT-PCR	Low RNA yield, especially from clinical samples, may hamper the quantification and analysis of less abundant RNA populations	Pre-amplification can enhance the amount of amplifiable molecules with no apparent bias.[123] Use spike-in controls to properly normalize the RNA populations.[124] If possible, use multiple endogenous controls based on EV-transcriptomic data, or use absolute quantification (by digital PCR, for example) for validation	The technique is intrinsically limited to a very specific set of pre-selected RNAs, not allowing a broader view of the EV-transcriptome
	RNA analysis by NGS	Library preparation issues: adapter dimers, ligation and size-selection bias	Ribosomal RNA (rRNA) depletion may be unsuitable due to low starting input of RNA and presence of fragmented rRNA. Extensive fragmentation of RNA may be used to generate total transcriptome library and analyse small and long RNAs simultaneously[128] Reduce adapter dimers using specific kits[50] or use fragmented RNA to minimize artefactual adapter dimer formation and preclude size selection[128]	Fragmentation of rRNA species hinders efficient rRNA removal.[133] Excessive RNA fragmentation can lead to difficulties in properly mapping the short reads. Comprehensive EV-transcriptional databases are lacking, especially from NGS data derived from clinical samples
	Protein analysis	Co-purification of protein, protein aggregates and lipoprotein contaminants	Remove abundant proteins such as immunoglobulins by combining ultracentrifugation and protein G agarose bead incubation,[222] especially when working with EVs derived from biofluids	The depletion of contaminant proteins requires a significant mass which is not always available from clinical samples. Proteomics methods need to be developed to accommodate the analysis of reduced mass of protein
		Heterogeneity of protein markers on different EV populations, for example presence of CD63, HSP70, flotillin-1, and actin present in both exosomes and microvesicles	Use a range of markers or non-marker-specific methods to distinguish differences between cells and EVs such as silver staining or SYPRO Ruby staining[164]	Identification of protein markers capable of differentiating specific EVs subtypes
		Contamination of samples with cellular components	Non-EVs proteins, such as calnexin, GM130, cytochrome C and histones, should be used as negative controls to ensure purity of isolation[158]	
		Protein degradation during vesicle manipulation	Use of protease and phosphatase inhibitors is highly recommended during EV protein extraction	Protease treatment needs to be standardized according to the biofluid-origin of the EVs under study
	Lipid analysis	Contamination by proteins during extraction Difficulty in extracting and representing all lipid classes	Liquid–liquid extraction is preferred method for EV lipid analysis.[189,190] Use of internal standards is recommended to ensure high mass accuracy and confident identification. If possible use LC to ensure separation of lipids prior to sensitive MS/MS. MTBE extraction was shown to extract all lipid classes with better recovery than the Folch method[191]	In liquid–liquid extraction protein contamination from the aqueous phase and bias towards extraction of partially polar lipids may occur. Public databases of lipids are still far from complete
	Metabolome analysis	Low metabolite yields and reproducibility (LC/MS)	Enrich sample using solid phase extraction prior to MS.[199,201] Major lipid and protein species should be removed in this process to ensure sensitive MS analysis	Development of better bioinformatic analysis pipelines for metabolites. Public databases of metabolites are still far from complete
		Cell culture metabolite contamination	Use of a cell-free medium control[223]	

showing that by using vertebrate species and specific labelling techniques, it is possible to demonstrate that distribution of EVs is determined by cell source and route of administration.[224,227]

Tracing experiments to observe **EVs** biodistribution are important but further emphasis must be placed on the role of **EVs** in physiological systems. **EVs** cargo such as miRNAs have shown regulation of physiological barriers such as the blood brain barrier[229] to promote tissue invasion and progression[230] and descriptions detailing the physiological relevance of **EVs** at sites of accumulation or deposition are needed.

Functional studies are much more varied and outcome measures are group specific and will largely depend on the scientific question at hand. Despite this, an extremely broad range of disciplines, from basic molecular neuroscience, to complex integrated cardiovascular physiology, are now beginning to study the role of EVs in their model systems.

7 General recommendations for working with EVs

The following general recommendations are made regarding pre-analytical factors, EVs-isolation methods as well as the characterization of EVs and of their content.

7.1 Pre-analytical factors

For human samples, variables such as gender, age, use of medications, circadian-clock, fasting, *etc.*, should be controlled.[231,232] For blood-derived EVs, attention should be given to reduce platelet activation and the release of platelet-EVs. In this sense, excessive shaking of collected blood should be avoided and plasma is preferred over serum. Anti-coagulant should be chosen based on the capability to reduce the release of platelet EVs and impact on downstream applications, helping to preserve RNA and/or vesicle integrity for *in vitro* or *in vivo* studies: citrated tubes have been suggested for most applications.[33] Viscous fluids such as plasma and saliva should be diluted in PBS to improve EV recovery. EVs-depleted FBS, obtained by overnight UC (100 000*g*), should be preferentially used for cell culture medium.[41–43] Non-conditioned medium should always be used as a negative control. For urine samples, it is recommended that protease inhibitors are present in the collection containers to preserve the sample, in addition to extensive vortexing, required to recover the highest amount of vesicles that could remain attached to the tubes during storage.[233] If not processed immediately, urine samples should be stored at −80 °C to avoid bacterial contamination. Pathological conditions that affect glomeruli filtration and lead to elevated urine protein loss, together with the high levels of uromodulin, can magnify issues of protein contamination and protein aggregates that make downstream EV-studies challenging. To reduce uromodulin and protein aggregates that interfere with downstream EV-studies we suggest the use of CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic), a mild detergent that preserves protein conformation and enzymatic activity,[234] allowing the functional studies of urinary EVs. As a general rule, the characterization of the molecular content of EVs isolated from biofluids will always benefit from depletion of high abundant non-EV proteins. Albumin can be removed from plasma by the use of the Protein Organic Solvent Precipitation (PROSPR) protocol,[235] immunoglobulins in CSF can be depleted by protein G agarose beads followed by UC.[222]

7.2 EV-isolation

The need of robust, standardized and reproducible EVs-isolation methods is an essential requirement. As the EV isolation method employed directly impacts the results obtained, all samples of a study should be processed by the same method. UC, the classic approach for EV-isolation, is not suitable for clinical applications and may disrupt EVs when high *g*-forces (>100 000*g*) are used.[56] DG is a laborious and time-consuming approach that does not supply high yields but usually provides purer EVs, especially when iodixanol-based gradients are used. In general, we recommend the use of DG when highly purified EVs are required, *e.g.* for characterization of EVs subtypes, or to determine their nucleic acid or protein content. UF is a good approach to concentrate EVs and it is much faster than UC, but has not been broadly evaluated. For immunocapture we recommend the use of magnetic beads or microfluidics, to improve the interactions between EVs and the capture antibodies. However, it is not currently known if the antibody-binding would affect the functionality or the cargo of the captured EVs or whether it would introduce bias to subpopulations of EVs and non-EVs contaminants. Alternative methods such as chromatography, flow field-flow fractionation, polymer or solvent-based precipitation, immunoaffinity capture and microfluidics are useful methods to avoid the co-isolation of lipoparticles and lipid droplets from biofluids. After isolation the purity of the EVs should be assessed. As a reference, the isolation of EVs using sucrose density ultracentrifugation provided about 3.3×10^{10} EVs per μg protein from conditioned culture media (prostate, breast, bladder and mesothelioma cells) and about 2×10^{9} EVs per μg from biofluids (urine and serum from healthy donors).[92] To conclude, the selection of the method of choice strongly depends on the type of EVs-source and the downstream analyses and applications.

7.3 EV characterization

NTA is a valuable method for determining the concentration and size distribution profiles of EVs. Due to its non-specific nature, special care should be taken to ensure the particles measured are indeed EVs. We recommend the buffer in which the EV sample are suspended to be analysed separately, to establish a background count. In addition, the use of a lipid dye or a fluorescently labelled EV marker in combination with the NTA fluorescence mode will reduce the impact of non-EV particles. Similarly, efforts should be taken to reduce the contribution of non-EV contaminants when analyzing particles by RPS; this could include measurement of the background particle count present in the EV buffer. For RPS filtration and SEC

can prevent clogging and multiple counts are needed to reduce the impact of variability. Furthermore, the size distribution of EVs observed using either whole-mount Electron microscopy EM, NTA or RPS should be compared to check that they yielded similar results. For flow cytometry protocols should be optimised and these parameters checked with routine calibration, as well as the use of robust negative controls, used to minimize the impact of false-positive signals arising from antibodies/protein aggregates. EM is of great help to characterize the EVs and negatively stained whole mount EM is a relatively quick and simple technique that greatly helps evaluating the presence, morphology and size distribution of EVs. Further imaging by cryo-EM is more technically challenging but the ability of this technique to resolve lipid bilayers makes it the most direct way to directly confirm the presence of bona fide membrane-bound vesicles.

7.4 Characterization of EV content

We perceive the major challenge in establishing EV content to be related to the limited amounts of EVs that can be recovered from biofluids. The precise description of the transcriptome of EVs, a mixture of few copies of a complex transcriptome combined with high copies of fragmented ribosomal RNA, is challenging. In order to uncover the total transcriptome of EVs, non-EV RNAs must be removed by RNAse treatment. Following, we recommend the use of Next Generation Sequencing of pre-fragmented EVs-derived RNA that allows the identification and quantification of all RNA biotypes.[128] If the aim is to simply describe the EV miRNAs a comprehensive qRT-PCR arrays can be used, but these are limited to a set of pre-defined molecules.

The observation of classic EV-markers in Western blots is used to reinforce that *bona fide* EVs have been isolated. However, due to the limited knowledge regarding consistent EV-specific proteins, there is no single protein or combination of proteins that can be recommended as universal EVs markers. Nevertheless, we recommend the evaluation and reporting of at least three classic EV-proteins, as their presence or absence may be informative to the EVs characterisation. We also suggest evaluating proteins that are not expected to be present in EVs, as controls of contamination, such as proteins from the endoplasmic reticulum (*e.g.*: calnexin), Golgi (*e.g.*: GM130), mitochondria (*e.g.*: cytochrome C), and nucleus (*e.g.*: histones).[49] The addition of a protease in the presence or absence of a membrane-disrupting detergent should help to establish whether a protein is present inside the EVs, and therefore protected by the EV membrane, or a secreted factor or a co-extracted protein. Finally, the presence or absence of particular proteins should be regarded as supporting evidence, rather than interpreted to mean that the sample definitely contains EVs or a particular EV sub-population. Large-scale shotgun proteomics is the method of choice to characterize more broadly the EVs-content. The application of native mass spectrometry at the protein level characterization and cross-linking, H/D exchange and covalent labelling at the peptide level characterization allows understanding the exosomal protein–protein interaction and structural modulation in different conditions.

LDL and HDL can be co-extracted from plasma by some EV isolation protocols and, due to their content of cholesterol and its fatty acid esters, as well as phospholipids, this can lead to misleading lipid profiles, including suppressing the identification of less abundant EVs lipids. Therefore, before performing the lipidomic profiling of plasma/serum-derived EVs, we recommend the use of size exclusion chromatography for isolation of EVs distinctly from HDL/LDL particles, which might also be helpful to reduce the contamination with other circulating lipids in biological fluids. The chemical diversity of lipids and metabolites compounds requires different extraction methods to be applied for lipids and metabolites. Recently, the promising SIMPLEX protocol was used for the simultaneous isolation of proteins, lipids and metabolites reducing the sample amount needed for a comprehensive biomolecular characterization.[236] The combination of different front-end separation techniques such as GC and HPLC using a variety of stationary phases with high resolution and accuracy mass spectrometers will improve the number of identified and quantified lipids and metabolites in EVs.

8 Towards deciphering "EVome" complexity: future directions

EVs are central elements in cell–cell communication and play a key role in the field of liquid biopsies. These two aspects makes their study extremely promising, and future directions include improvements in characterizing their cargo to better define their roles in tissue homeostasis and to better identify the biomarkers that they carry. The full determination of the RNA-content of vesicles will benefit from the fast growing field of genomics, especially from the technologies that handle minute amounts of nucleic acids, including single-cell genomics and barcodes to identify individual molecules. Regarding proteomics, the amount of protein mass has deeply decreased allowing the identification of thousands of proteins from low abundant samples, such as FACS-sorted cells[237,238] and fine-needle aspiration biopsies.[239] Straightforward sample preparation methods and novel mass spectrometers can improve the sensitivity and quality of protein quantitation.[240] Nonetheless, functional validation of EV proteins is needed, linking proteomic information and functional studies and providing greater overview of EV function. Moreover, combining information on multiple omics will help in understanding the complex biological network of EVs.[175,241–243]

The field of EVs has gained increasing interest and knowledge is rapidly accumulating. This has helped this area to move from bench top science towards translational and clinical research, including *in vivo* experiments as well as diagnosis. Nevertheless, our perception is that the field is not yet ready for clinical applications. Therefore, we believe it is too early to state that we can confidently interpret EV-data or administer/manipulate EVs for clinical purposes.

Conflicts of interest

There are no conflicts to declare.

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