

## The future of Extracellular Vesicles as Theranostics – an ISEV meeting report

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### ABSTRACT

The utilization of extracellular vesicles (EVs) in clinical theranostics has rapidly advanced in the past decade. In November 2018, the International Society for Extracellular Vesicles (ISEV) held a workshop on "EVs in Clinical Theranostic". Here, we report the conclusions of roundtable discussions on the current advancement in the analysis technologies and we provide some guidelines to researchers in the field to consider the use of EVs in clinical application. The main challenges and the requirements for EV separation and characterization strategies, quality control and clinical investigation were discussed to promote the application of EVs in future clinical studies.

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### Main text

Extracellular vesicles (EVs) are nano-sized lipid bilayer encapsulated membranes carrying proteins, lipids, and nucleic acids and sugars that are shed by the majority of the cells into extracellular milieu to mediate inter-cellular communication by transferring molecules from parental/donor cells to target cells. Recent advances have shown the multiple roles of EVs in physiological and pathophysiological processes, highlighting their

potential to serve as clinical biomarkers for disease diagnosis and monitoring, and as therapeutic agents [1,2]. However, utilizing EVs in clinical practices faces many challenges due to their diverse origins.

The biogenesis of EVs is not fully elucidated at present. Identified pathways include vesiculation at the plasma membrane; the canonical endosome pathways; or those that are formed during apoptosis and senescence-associated cellular processes. EV biogenesis and secretion are driven by constitutive secretory

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pathways or secreted as a response to growth conditions or treatments. Currently, EVs are categorized based on their origin, release mechanisms, markers, size and/or density. Efforts to standardize the methodologies for EV isolation and characterization are ongoing and remain a major research focus.

Bridging the challenges in standardizing the isolation and characterization methods that are suitable for EV studies for clinical applications, the International Society for Extracellular Vesicles (ISEV) initiated a workshop on EV-based Clinical Theranostics on 18<sup>th</sup>–20<sup>th</sup> November 2018 in Guangzhou (China). The workshop covered the state of the art and current challenges in EV-based biomarker discoveries and applications as well as EV-based therapeutics. Specifically, the workshop explored key issues in translating EV research from bench to bedside, both to achieve a consensus in best practices and to identify potential obstacles in translating clinical applications. It was attended by 66 participants who have been working extensively in basic/discovery research, clinical/translational research and technology innovations on EV-based theranostics. Participants were grouped for roundtable discussions to consider four key topics [1]: EV isolation methodologies and standardization [2]; EV omics analysis and biomarker discovery [3]; EV biomarker detection and applications in clinical diagnostics; and [4] EV-based therapies in human disease (Table 1).

## EV isolation method and standardization

MISEV2018 covers the basic categorization suitable for EVs in clinical settings [3]. It provides a guideline that can help to standardize EV research from sample collection and pre-processing, to storage, separation and characterization. Based on the MISEV2018, pre-analytical variables and standardized methods were carefully and systematically considered to enable data collection and comparison across different laboratories.

EV compositions are largely affected by multiple pre-analytic factors, such as sample storage time, temperature, the use of anticoagulants and basic centrifugation parameters [4–8]. Further assessment of the EV nomenclature and characteristics that cover their quantification and evaluation of purity needs to be discussed and systematically reported. ISEV endorsed EV-TRACK and EV-METRIC [9] as practical platforms to achieve preliminary evaluations, record parameters and receive further suggestions on best practices in reporting of results was proposed.

ISEV endorsed to use of the term EVs in the clinical field. EVs found in biofluids are not easily categorised based solely on expression markers. This is due to the limited knowledge of their biogenesis which is very difficult to trace when EVs have already been released into systemic circulation. To date, EVs have been isolated from more than 30 subtypes of biological fluids; with most research performed on plasma, serum, urine, saliva and breast milk [10–12]. Other sources of biofluids are also being investigated as source for EVs, *albeit* less frequently used due to the limited access. These include cerebrospinal fluid (CSF), ascites, pleural effusions, semen (“prostasomes” and “epididymosomes”), ocular effluent and aqueous humour, amniotic fluid, nasal secretions, bronchoalveolar lavage (BAL), synovial fluid, bile and seroma fluid. Currently, substantial improvements have been achieved on development of EV isolation methods from biofluids, however, it is understood that there are some overlaps in the expression of EV markers across EV subtypes [3]. Furthermore, recent research have evaluated tissue interstitial space, including interstitial fluid and extracellular matrix (ECM) as a potential EV reservoir. Isolation of EVs directly from tissues requires strict controls and meticulous standardization of methods to avoid cellular contamination caused by, e.g. cell disruption [10,13,14]. Although biofluids are the preferred source due to their accessibility and high abundance, tissue derived EVs harbour cell type specific information which can be utilized as valuable tools to report on the physiological and pathological conditions of organs [15–19].

New isolation methods that suitable for clinical theranostics were discussed during the workshop. These tools were initially designed to allow rapid isolation and characterization of EVs from clinical samples. Many chip-based methods are currently under development, such as the dielectrophoretic (DEP) chip, that are claimed to allow rapid analysis of plasma to detect EGFR and KRAS mutations and higher levels of miR-21, miR-192 and miR-191 in EVs from lung cancer patients in comparison with healthy individuals [20]. Other methodologies, such as chromatography or ultrafiltration in combination with strategies to enrich EV subtypes, i.e. by immunoaffinity precipitation (see also Roundtable 3 on EV clinical biomarkers), and hydrostatic filtration dialysis (HFD) [21] were also discussed. Combining isolation methods can help to improve the application of EVs for diagnostics and patient monitoring by increase the specificity and efficiency, such as demonstrated by combination of PEG precipitation and immune-capture to separate L1CAM positive EVs in periphery samples [22,23]. The

**Table 1.** Summary of topics, descriptions and questions for 4 roundtable discussions.

Roundtable #1	
Topic	EV Isolation Methods and Standardization
Descriptions	Methodologies that are used for EV isolation from different sample sources were discussed. The focus was on isolation methods suitable for EVs that are intended to be utilized for theranostics applications and pre-analytical issues which may affect EV isolation and analysis.
Questions	<ol style="list-style-type: none"> <li>1. What are the main problems in EV isolation?</li> <li>2. Are there isolation methods that are most suitable for clinical theranostics?</li> <li>3. How can we standardize the pre-analytical sample quality?</li> </ol>
Roundtable #2	
Topic	EV Omics Analysis and Biomarker Discovery
Descriptions	Omics analyses, including RNA sequencing, proteomics, lipidomics, and metabolomics, are common platforms used for biomarker screening. The focus here was on questions to be addressed when performing omics studies for EV biomarker discovery.
Questions	<ol style="list-style-type: none"> <li>1. How can we achieve most stringent quality control? Is there a recommendation on platform(s) that we can use to share omics datasets?</li> <li>2. How can we avoid false discovery or inaccurate detection during the discovery phase of EV-based protein biomarkers?</li> <li>3. Regarding RNA sequence data analysis: Should we reach a consensus on an algorithm which is suitable for RNA-seq? What are recommendations for normalization of omics datasets?</li> <li>4. Do we need to treat EVs with RNase before sequencing to confirm the RNA cargo content? Do we need to treat EVs with protease enzymes before performing proteomics analysis for protein cargo content?</li> <li>5. What volume of samples is needed for the RNA library construction?</li> <li>6. How many samples should be included for initial biomarker discovery? How can we improve the confidence for data verification?</li> <li>7. How could omics datasets be translated to clinical applications?</li> <li>8. After lipidomic analysis, what assays and platforms can be used for lipid verification?</li> </ol>
Roundtable #3	
Topic	EV Biomarker Detection and Applications in Clinical Diagnostics
Descriptions	EV biomarker detection technologies are instrumental in applications on clinical diagnostics. Here, the potential of using EV-related biomarkers, such as EV number and/or cargo, as diagnostics was discussed. Also, some novel techniques were considered as potential tools for EV-based clinical diagnostics.
Questions	<ol style="list-style-type: none"> <li>1. What kind of EV-based biomarkers are suitable for clinical diagnosis?</li> <li>2. Are there any new technologies that can be used for biomarker detection to help meet clinical requirements and standardization?</li> <li>3. What are the main challenges for biomarker detection and applications?</li> </ol>
Roundtable #4	
Topic	EV-based Therapies in Human Disease
Descriptions	EVs have been proposed as new therapeutic or delivery agents. Here, the potentials and challenges for EV-based therapies for diseases, including immunomodulatory and regenerative therapies, vaccinations and drug delivery were considered.
Questions	<ol style="list-style-type: none"> <li>1. What are the main challenges in the development of EV-based therapies?</li> <li>2. What are the requirements for manufacturing, quality control and subsequent clinical use?</li> </ol>

development of novel and combined methods is promising for clinical applications, however, further studies are needed to verify and standardize the platform to ensure the reproducibility of these new methodologies.

Biofluid are varied due to specific biophysical and chemical characteristics, such as viscosity, and cellular or tissue-derived contaminants. Most available methods showed some carry-over of non-EV molecules that are co-isolated in EV fractions. These include albumin and lipoproteins (from plasma or serum), Tamm-Horsfall protein (from urine), fat-containing vesicles (from milk), surfactant (from bronchoalveolar lavage) and bile salt (from bile). Others look into identifying biomarkers from EVs derived from conditioned medium from cells that are grown *in vitro*, in the absence of foetal calf serum (FCS or foetal bovine serum (FBS)), and/or without other complex materials such as platelet lysate and bile salts [24]; reducing the avoid co-isolation of non-EV contaminants that are hard to separate for EV derived from biofluids. However, the effects of total serum removal should be re-investigated thoroughly as serum may be essential for cell culture growth. Utilization of EVs derived from biofluid may

be enabled in the future, in parallel with maturation of isolation techniques that fulfill compliance with MISEV2018 guidelines or future updates of this topic.

The development of fast and reproducible standardized methods for EV isolation from clinical samples is critical for advancing EV research to routine clinical utility. These include but are not limited to identifying: sample sources, EV sub-population(s) of interest, methods to be utilized for downstream analysis and intended clinical applications. For example, isolation and analytical methods that are suitable for biomarker discovery may be different from the ones that are informative on fundamental EV biogenesis. Currently, no single method is suitable for all sample types and applications. The method selection will be largely determined by intended downstream analysis. As such, many methodologies could be adopted and further optimized for intended applications. For example, in biomarker discovery research, the pre-process technique and storage parameter standardization of samples is urgently needed. Biofluids that have not been processed prior to storage may contain a high amount of impurities such as platelet debris in blood, which further impede the downstream analysis and data interpretation.

Many -as yet poorly determined- pre-analytical variables should be considered, including age and gender of donors, time of sample collection, fasting or non-fasting state, exercise history and duration before immediate sample collection, diet, body mass index (BMI), presence of infectious or other diseases, and medications that the donor is taking. Depending on the study, it may be necessary to record the current and/or previous pregnancy [10]. The importance of these considerations is exemplified by the fact that, e.g. plasma-derived EVs from fasting subjects include less lipoproteins compared to samples taken post-prandially [25]. Other factors, such as diet, can affect the protein (CD9, Alix) composition of urine-derived EV [26].

Many technical variables may also alter EV profile including, but not limited to, the initial volume of sample, type of tubes used to collect samples, choice of anti-coagulant or coagulant, mixing or agitation steps, time before processing or storage, temperature during storage and processing, methods of transport, methods to isolate EVs from biofluids, haemolysis degree for plasma and platelet/lipoprotein depletion process for blood samples [7,27]. These characteristics should be recorded and taken into consideration for downstream analysis and data interpretation.

The advantages and pitfalls of currently available protocols have been extensively discussed elsewhere [28–30]. Here, we reached the consensus on protocol that needs to be adapted for standardization. A detailed methodology of EV isolation which includes the type of matrix, vials and freezing temperature, needs to be recorded. The addition of cryo-preserved such as glycerol and DMSO should be avoided as they may disrupt EVs, while reagents such as trehalose has been shown -in some cases- to help preserve EVs [25]. Isotonic buffers are recommended for storing EVs to prevent pH shifts during storage, freezing and thawing; thus, ultimately helping to maintain EVs' functional and physical properties. It is important to store purified EVs at  $-80^{\circ}\text{C}$  or lower, in order to guarantee analytical validity for up to months [12,25,31,32]. Repeated freeze-thaw cycles should be avoided to prevent EV aggregation. Some justified control experiments are essential to ensure the validity of adopted method including the analysis of EVs from healthy individuals or from patients at different treatment time points. Also, a suitable biological reference material with physical and biochemical properties equivalent to EVs will be beneficial for

calibration of analytical instruments and validation of isolation methodologies in the clinics.

## EV omics analysis and biomarker discovery

Omics analyses are widely used in the discovery of disease-specific EV biomarkers, in order to globally assess EV cargo of proteins, nucleic acids, metabolites and lipids. For instance, proteomic approach may be performed to find enriched proteins that are involved in metabolic processes or signal transduction. Moreover, biochemical assays may help to characterize the activity of enzymes. Measurement of Enzymatic activity may provide some quantifiable features to assess EV functions. The importance of this is exemplified by the fact that EVs secreted by hepatocytes carry enzymes induced by exposure to liver toxins and drugs in both *in vitro* cell lines and in mouse model of drug-induced liver damage (DILI) [33,34]. Utilizing advanced mass spectrometry to detect active enzymes in hepatocyte-secreted EVs further suggests that EVs are associated with altering metabolic profile as a response to drugs [33,35].

Metabolomic profiling of serum samples can also be utilized to assess whether EVs harbour metabolites that are potentially involved in oxidative stress metabolism and affect endothelial function [36]. Combined omics approaches were used to develop non-invasive biomarker discovery strategies for prostate cancer, where EVs from urine samples can exhibit differential physical and biological properties for prostate cancer compared to benign prostate hyperplasia (BPH) [37]. Transcriptomics and metabolomics analysis of urine-derived EVs show that numerous transcripts and metabolites are differentially expressed between prostate cancer and benign patients [37,38]. Additionally, assessment of the cargo of EV sub-populations can be beneficial for the utilization of EVs as biomarkers, which can be utilized to assess the effect of androgen deprivation therapy in advanced prostate cancer [39].

As a common approach in proteomics by untargeted mass spectrometry, EV-derived proteins from clinical samples were identified based on the relative abundance of distinct peptide profile. Potential protein biomarkers are selected according to their expression level, statistical significance and relevance. Hence, it is noted that highly abundant contaminant proteins can mask the signal of less abundant proteins, creating bias towards protein abundance, such as observed in the presence of albumin in EV samples derived from plasma, serum or urine. Next-generation mass

spectrometry-based analysis, targeted mass spectrometry or multiple reaction monitoring can offer a high level of sensitivity and precision by thoroughly measuring all ions at all abundance ranges above the noise level. Combining the untargeted and targeted methods is currently in favour to provide a more comprehensive strategy for EV-based protein biomarker discovery.

Some technical issues in proteomics-based approaches which can create variability of results, such as protein lysis methods, mass spectrometry platforms, mapping software and reference database were discussed. Mapping of proteins based on the molecular and cellular proteomics (MCP) guidelines could help to reduce variations [40]. It is also important to note that high quality of samples as indicated by the presence of EV markers, can be utilized to obtain reproducible data. Multiple parameters need to be carefully recorded for EV isolation, including the preprocessing parameters and storage conditions that can be adapted by biobanks and other centres.

RNA sequencing of EV cargo is also a useful tool to potentially detect various stages of the disease. High throughput RNA profiling of EV cargo provides a relevant approach for biomarker discovery for many diseases, as already shown for neurodegenerative diseases (Alzheimer's disease (AD) and prion disease) and others. Brain-derived EVs carry neurodegenerative disease-related proteins and small RNAs that can be used for the diagnostics of AD [13,15,19]. Similarly, the miRNA content of EVs may be useful to detect prion-related diseases in preclinical mouse model [41,42]. EV derived miRNAs could also be utilized to detect insulin resistance and staging in development of diabetic kidney disease [43].

Genomic technologies have been widely utilized to profile the DNA, mRNA, snRNA, lncRNA and small RNAs of EVs. At present, Illumina HiSeq, Roche 454 pyrosequencing and the SOLiD system (Applied Biosystems) are commonly utilized across many labs working on EV biology. Deep sequencing platforms for small datasets, such as MiSeq (Illumina), Ion Torrent Personal Genome Machine (Life Technologies) and the GS Junior (Roche), have been implemented to assess EV-derived RNA contents. Some key parameters should be included for reporting of EV RNA are the sequence depth, library preparation methods, sequencing platforms and algorithms. Deeper coverage will assist the analysis of rare sequences and give more confidence in the number of reads per transcript. Recently, it was reported that the classic ligation dependent library preparation method was biased to miRNAs [44], which calls for consideration of more recently developed methods such as capture and

amplification by tailing and switching (CATS) [45,46]. During the data analysis phase, different algorithms/calibration methods should be applied to characterize overlapping targets in order to improve the result confidence. It is also important to engage comprehensive bioinformatics platforms from early stages of all analyses.

Lipids are essential components of EV membranes and so EV lipidomics study is an interesting research area that is progressing rapidly. However, as lipids are major constituents of all biological membranes and organelles, it is necessary to exclude non-EV lipid contaminants prior to further analysis. Mass spectrometry and nuclear magnetic resonance spectroscopy are technological platforms commonly used for lipid analysis. Assessment of lipid C = C isomers may be utilized to classify EV-derived phospholipids for biomarker discovery [47,48].

Stringent verification is also critical to validate the targets recovered from data profiles. While appropriate sample numbers carry substance depending on the scientific/clinical question, it was considered that, in general, a minimum number of 30 subjects is highly recommended to provide minimal statistical power. However, it was also acknowledged that there are instances in which obtaining this number may be challenging or, indeed, impossible, e.g. in the case of rare samples from cerebrospinal fluid (CSF), joint fluid and seroma fluid, or in rare diseases. Study design for rare samples should be adjusted accordingly and to increase statistical power wherever possible.

Apart from isolation and analysis techniques, as previously mentioned different genetic backgrounds, diet, age, fasting and other factors can also influence the composition of EVs. A standard operation procedure (SOP) is needed to decrease variability and improve control of these factors. The development of normalization methodologies for omics analysis could help to address the variance among studies. One approach to standardize omics methods for biofluids is to determine the original sample volume used for EV separation. Other characters, such as the EV number and size, will provide informative data on the nature of EVs being characterized. The nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), tunable resistive pulse sensing (TRPS), microfluidic resistive pulse sensing (MRPS) and high-resolution flow cytometry are widely used techniques for vesicle quantification. However, it is important to understand that many of these methods detect all particles with properties similar to EV, including contaminants that were co-isolated during EV isolation. Thus, the analysis of the levels of some EV specific markers, RNA or protein

concentration has been investigated to standardized methodology of EVs characterization from clinical samples.

The possibility of integrating multiple omics platforms to allow a comprehensive analysis of EV compositions in different disease settings is a promising aspect discussed during the meeting. This strategy is an appropriate path to discovery biomarker panels suitable for clinical use. It was noted, however, that this strategy will lead to a vast amount of data generated from each study. As such, it is important to ensure the implementation of standardized isolation and analysis techniques, supported by adequate bioinformatics and biostatistics expertise for data processing, and to build the standardized frame for data analysis.

### EV biomarker detection and application in clinical diagnosis

This section focused on EV biomarkers that are suitable for clinical settings and new technologies that have been developed to achieve high-sensitivity, low-cost and high-throughput detection in clinical diagnosis. The potential of circulating EV counts in body fluids as an unspecific marker for diagnostics in a disease state was discussed. EV quantification could be performed by appropriate equipment designed to measure the concentration of nano-sized particles with various limitations on methodologies, including NTA, resistive pulse sensing (RPS), cryo-electron microscopy, surface plasmon resonance (SPR) or atomic force microscopy (AFM). The molecular contents of EVs could also be analysed by western blotting for proteins, PCR-based assay or sequencing to detect RNAs or gDNA, and sulpho-phospho-vanillin (SPV) assay for total lipids [49,50]. For a more detailed analysis of EV cargo, omics technology -as discussed above- allows fingerprint-type identification of EV cargo [51].

Other methods, such as fluorescence or colorimetric-based assay or enzyme-linked immunosorbent assay (ELISA), may indicate the quantity of EVs in relevance to the abundance of specific protein markers [52]. State-of-the-art equipment with multi-modality approaches have been developed, such as the combination of microfluidic technology with optical or electrical biosensors for protein detection to identify EV subtypes on a chip. A single-EV-counting enzyme-linked immunoassay (digital droplet ExoELISA) approach based on microfluidic droplet technology has been introduced that enables absolute counting of cancer-specific small EVs with a limit of detection

down to 10 EVs per microlitre for breast cancer diagnostics and therapeutic evaluation [53].

Assessment of a single EV can be performed by visualization at high resolution, to provide information on both their structure and composition. EVs can be labelled by antibodies to detect protein(s) of interest or labelled with lipid dyes. Imaging techniques are widely employed to assess single EVs including the scanning electron microscope (SEM), transmission electron microscope (TEM), cryo-EM, scanning-probe microscopy (SPM) such as the AFM; and super-resolution microscopies such as the structured illumination microscopy (SIM) and stochastic optical reconstruction microscopy (STORM). Application of these techniques, while providing great detail on EVs recovered from biofluids, may not be suitable for routine clinical diagnostic applications due to lack of expertise, cost of instruments, limited volume of samples and the duration of time required to process complete assessment of EVs [49].

The application of high-sensitivity flow cytometry for single EV analysis to identify disease-specific biomarker detection in a simple, rapid and high-throughput manner was discussed. Flow cytometry has also been developed to analyse EVs by increasing the number of parameters for analysis and optimizing the antibody-staining protocols together with the application of a panel of antibodies to characterized EV subtypes. Further improvement could also be achieved by the development of brighter fluorophores to enhance the fluorescence detection level. By optimization of these parameters, it should be possible to detect rare subpopulations of disease-associated EVs (0.01–0.1%). To improve the limitation of detection on small EVs, molecules of equivalent soluble fluorochrome (MESF) beads could be utilized to determine the sensitivity and improve the detection limit [54]. Next-generation of flow cytometry that is designed to analyse small particle, i.e. the nano-flow cytometry (nFCM) that allows light scattering detection of individual EVs down to 40 nm from the conditioned medium of HCT15 cells (~ 90% purity) was considered. This technology is reported to allow quantification, sizing and phenotyping of individual EV in a rapid manner [55].

The utilization of EV biomarkers for cancer prognosis was also discussed as cancer EVs are reported to carry distinct integrin profiles, which is associated with their preferential metastatic site [56] and functionally relevant [57,58]. Other biomarkers are currently being developed to predict clinical outcomes, such as the EV-derived PD-L1 has been suggested as a predictor for the clinical outcomes of anti-PD-1 therapy [59] and EV-tethered TGF- $\beta$  had previously shown

promise to predict response to HER2-targeted drugs [60]. Additionally, as EV cargo is protected by lipid bilayer from external proteases, detection for protein biomarkers that are sensitive to proteases is made possible, such as EV-derived phosphoproteins in breast cancer [61]. In neurodegenerative diseases, the detection of misfolded proteins (i.e. superoxide dismutase-1) on EVs provide clinical utility for diagnosis [62]. The strategy of combining several EV cargoes to increase the sensitivity and specificity of diagnosis was also discussed. As an example, a panel may consist of six EV protein markers that are involved in complement activation, lipoprotein metabolism and platelet activation showing a significant association with a post-infarction response that could benefit patients with coronary artery diseases (CADs) [63].

Characterization of EV cargo and their association with disease state for the development of biomarkers for treatment response was also discussed. Transmembrane proteins that are found at the surface of EVs are of practical use as they could be detected easier than internal EV cargo. The tetraspanins CD9, CD63 and CD81 are classically utilized as EV markers, which could provide information on the circulating EV count and subtypes [39]. Other membrane-associated proteins, such as the glypican-1 (GPC-1) was first identified as a reliable EV-derived biomarker superior to conventional marker CA19-9 for the detection of early pancreatic cancer [64]. GPC-1 positive EVs are also shown as a potential diagnostic value for the presence of colon and breast cancer [53,65].

EV-derived small RNAs have been proposed as biomarkers for various diseases. In a preclinical model, circulating EV count and the EV-derived miRNA were substantially different among plasma, CSF, and brain tissue from healthy and simian immunodeficiency virus (SIV)-infected pigtailed macaques. The correlation of plasma EV level and SIV infection showed a higher EV number after SIV infection. It was identified that EV production reached to peak upon acute infection and decreased after treatment, supporting the use of plasma EVs to evaluate the stages of infection (YH, unpublished data). EV-carried small RNAs are also proposed as diagnostic biomarkers such as for patients suffering from solitary pulmonary nodules (SPNs), neurodegenerative disease, infectious diseases (i.e. HIV infection) and cancer [66–70]. Others researchers have also proposed long non-coding RNA, EV-associated gDNA or combination of RNA species as biomarkers. For example, the expression of two mRNAs, KRTAP5-4 and MAGEA3, and BCAR4 lncRNA in EVs could be utilized for a diagnostic biomarker in colorectal cancer. Detection of a mutated

sequence of double-stranded gDNA fragments could also indicate treatment response in prostate cancer cohort [71].

EV-derived lipid species were also proposed as a clinical biomarker. Phosphatidylserine (PS) positive EVs have been shown to be involved in tumour growth and metastasis and suggest that blood PS is a biomarker for early-stage malignancies [72]. Moreover, EV-derived PS and lactosylceramide from urine are significantly elevated in prostate cancer patients. Combinations of these lipid species were reported to provide 93% sensitivity and 100% specificity, which showed for the first time the potential use of EV lipid species from urine as prostate cancer biomarkers [73].

Participants agreed that technologies play an essential role in biomarker detection, in which new technologies which can assess EVs in a limited number of samples are highly appreciated. A new generation of kit (ExoLutE<sup>®</sup>) based on the size-exclusion spun column were introduced to separate EVs from biofluids in a relatively short time and higher purity in comparison with the traditional ultracentrifugation methods [74]. Other technologies aiming to improve enrichment efficiency to isolate EVs from biofluids were introduced, including immuno-affinity enrichment, field flow fractionation and microfluidic filtering. A new generation of biosensors that are designed to detect CD145/CD9 EVs can be performed using 5  $\mu$ L serum samples in two hours [75]. Other biosensor to detect GPRC5C/CD63 is also introduced to discriminate pancreatic patients from stage II pancreatic cancer [76].

Microfluidic chips have also been developed to detect EV derived nucleic acids, improving detection limit. State-of-the-art platforms with high sensitivity are utilized, including digital droplet PCR, localized SPR (LSPR)-based biosensors and dielectrophoretic (DEP) chip. LSPR was developed to provide quantitation of target miRNA markers. The DEP chip combines a rapid 30 min isolation and detection of miRNA on an automated workflow and allows the collection of EVs for further validation by PCR. This chip has been tested to detect a panel of miRNAs for lung cancer patients with EGFR and KRAS mutations in EVs from plasma samples.

### EV-based therapy in human disease

Reliable and reproducible isolation techniques are necessary for the development of EV-based therapies. EVs were initially assessed as a therapeutic agent in immunotherapy, as EVs from antigen-presenting cells are capable of activating CD4 and CD8 positive

T cells. EVs from antigen-presenting cells contain MHC-peptide complexes that enhanced immunity to reduce tumour burden in immunocompetent mice [77]. Several cell types were utilized as donor cells to produce EVs, including the autologous monocyte-derived dendritic cells, autologous ascites and NK cell [78–80].

Mesenchymal stem/stromal cells (MSCs) derived EVs has been the most characterized and shown to exert therapeutic efficacies. MSC-derived EVs were first shown to be therapeutically potent against renal ischaemia and myocardial reperfusion injury [81,82]. Since then, MSC-EVs have been shown to be therapeutically efficacious in 30 animal models of human diseases [83]. MSC-derived EVs were also protective against toxicant-induced injury [84–86], even though the underlying mechanisms have not been well characterized. There are several advantages to the use of MSC-EVs that involves their immune-modulatory and regenerative aspects, including low-risk of embolism, immune-privileged, easy manufacture and storage [87]. However, several critical issues including identity, potency, safety and heterogeneity of MSC-EVs need to be addressed before MSC-EVs can be developed into clinical products [87]. ISEV, together with ISCT, ISBMT and SOCRATES, has proposed several metrics to define the therapeutic potential of MSC-sEVs as treatment for COVID-19 [88,89], while evaluation and clinical trials are desperately needed to confirm their potential [90].

EV from non-MSC cell sources such as endothelial colony-forming cells (ECFCs) have also been reported and are under preclinical investigation [91]. Recently, CpG oligodeoxynucleotides (ODN) activated macrophage-derived EVs have been utilized to transport ODN to induce the release of chemokine TNF- $\alpha$ . These TLR9-activated macrophage EVs transferred Cdc42, a potential target for the treatment of autoimmune diseases, to recipient inactive macrophage cells, resulting in further enhancement of their cellular uptake [92]. The transportation of ODN and Cdc42 from TLR9 activated macrophages to naive cells via EVs exerted synergetic effects in the propagation of intracellular immune responses.

EVs from human red blood cells (RBC) have been proposed as delivery vehicles for EV-based gene therapy to deliver antisense oligonucleotides (ASO) or Cas9 mRNA and gRNAs to cancer cells [93]. RBC EVs were taken up by leukaemia cells and provided better delivery of ASOs than commercial transfection reagents with no observable cytotoxicity by the EVs. Local or systemic delivery of ASO-loaded RBC-EVs provided efficient knockdown of the oncogenic miR-125b and

suppression of breast cancer or leukaemia growth in xenograft mouse models, showing their potential for utilizing RBC-EVs for therapy.

EVs has been assessed as vehicle for anti-cancer drugs, small RNA and anti-inflammatory agents. Animal studies have shown that EVs from various cells are capable of crossing tissue barriers and delivering their contents to the target cells. EVs can also be engineered, by modifying parental, or depending on the context here also referred as donor cells, to express targeting molecules; by culturing parental cells in different medium to alter the composition of the content of EVs; or by indirectly loading with drugs endogenously (by culturing cells in drug containing medium); or by exogenous loading the EVs directly with methods such as electroporation or sonoporation methods [94–98]. Similar to the design of other lipid-based drug nanoparticles, the development of EV-based drug delivery system is currently focused on improving the efficacy of drugs by altering their physical and biological properties to reach the target recipient cells and deliver their content. To improve the delivery of EVs, the use of targeting peptides or proteins on EVs has been tested [96,99–101]. Introducing targeting peptide can be performed by engineering the donor cells to express the targeted peptide. The secreted EVs will harbour the targeted peptide and can be further isolated, purified and loaded with the drug of interest. A promising report has shown that the targeting of small EVs can be improved by an association of small EVs with an Adeno-associated virus (AAV) vector for gene therapy. Small EV associated AAV vector from human 293 T cells was able to rescue partial hearing in mice by direct cochlear injection. The EV-associated AAV is also more efficient for gene therapy to the brain at lower vector doses and even more promising as it can cross the blood-brain barrier, while the conventional AAV cannot [101].

EVs exhibit cell-targeting properties, owing to their surface molecules such as tetraspanins and integrins. However, intravenously injected EVs mainly end up in the liver or spleen of mice in melanoma model which homed to specific target tissues [102]. Thus, the development of targeting strategies needs to be improved to achieve a better targeting ability of EVs, by engineering their surface molecules. A new strategy to improve the targeting properties of EVs was shown by the development of a peptide (CP05), CP05-modified dystrophin-splice-correcting phosphorodiamidate morpholino oligomer (EXOPMO), to enable targeting, cargo loading and capture of EVs from diverse origins through binding to CD63. CP05-muscle-targeting peptide increased dystrophin expression in muscle with functional improvement

without any detectable toxicity, providing a tool for EV engineering for the development of targeted therapeutic drug delivery [103].

EVs carrying pathogen-specific antigens may provide useful tools for the development of new vaccination for infectious diseases in humans and animals, or other disease in preclinical mouse models. Parasite-derived EVs can modify the profile of inflammatory mediators in the intestine of preclinical mice model, including cytokines (i.e. TNF $\alpha$ , IL-6 and IL-17), and signalling molecules (i.e. serine kinases and transcription factors) [104]. EVs secreted by the helminth parasites have shown protective and therapeutic benefits on the inflammatory response in pre-clinical models of inflammatory bowel disease (IBD) in mice, producing a great reduction in pro-inflammatory cytokines [105,106]. However, as parasite EVs carry plenty of active macromolecules, the use of the parasite EV for therapy needs to be carefully evaluated.

For clinical applications, it is vital to uphold the highest standard in EVs isolation and production and to comply with regional accreditation for investigational new drugs. European guidance published by European Medicines Agency (EMA) on biologically active substances covers the manufacturing and clinical evaluation of novel EV-based therapeutics. In the USA, EV-based therapies for human use would also consider biological products, and would be regulated by the Centre for Biologics Evaluation and Research (CBER) within the Food and Drug Administration (FDA). In Australia, the government agency Therapeutic Goods Administration (TGA) provides the rules and guidelines relating to the manufacture and use of therapeutics, which are frequently adopted from European regulations.

Additional to general requirements such as EV isolation, storage and characteristics standard, safety and efficacy evaluation of EV-based therapeutics is required. Changes in the tissue or cell culture methodologies may have profound impacts on the EVs biological properties. A quality management system should be taken into account for both donor and recipient safety. Accordingly, EV production has to be performed in conformity to good practice (GxP) regulations, which is a collection of quality guidelines and regulations created to ensure that bio/pharmaceutical products are safe, meet their intended use and adhere to quality processes during manufacturing, control, storage and distribution. good practice (GxP) standards are being harmonized between regions by “The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH”. The current safety and efficacy standards for cells and

tissues *in vitro* may serve as roadmaps for the development of EV-based therapeutics in pre-clinical studies [107].

Clinical requirements should be considered in advance for phase I clinical trials. Consequently, safety, toxicity and immunogenicity need to be monitored in the process of early phase clinical trials. Finally, reliable information concerning efficacy and long-term adverse effects of autologous or allogeneic EVs will be obtained from later phase clinical trials (phase III–IV). Interdisciplinary collaborations between academia and industry will certainly help to accelerate preclinical development and successful clinical translation. Besides, EMA and FDA in the USA offer advice to investigators and clinical trial sponsors at a national or international level, depending on the developmental stage of a new drug.

## Conclusion

EV detection as a clinical biomarker(s) is being developed for diagnosis, monitoring disease progression, treatment response and determining the prognosis of diseases. While several EV-derived molecules have been proposed as biomarkers, challenges remain in the validation process due to the variability in the methodology for sample collection, storage and analytical methods across multiple centres. Main focusses of current research in this field include identifying alternative methodologies to isolate EVs from a range of sources of biofluids, development of new techniques that provide a rapid assessment on the single EV level, as well as the availability of reference materials.

A proposed new EV-derived biomarker should have a better performance than the traditionally available biomarker as the new EV-derived biomarker should be superior to current standard-of-care. While several limitations were identified and acknowledged, the development of new technologies promises a rapid and easy to use analysis that can be utilized to analyse EVs in a limited volume of sample. As EVs carry multiple cargoes, EV assessment should be performed in a high throughput manner, providing more detailed information on diseases.

EVs may be utilized as therapeutic delivery agents due to their characteristics. Drugs such as anti-cancer drugs, small RNA and anti-inflammatory agents could potentially be delivered by EVs. Several methods are being investigated to load drugs into EVs, as well as assessment of donor cells, and attachment of targeting molecules. Understanding the guidelines and regulations applicable to drug development and administration is necessary in order to establish EVs as therapeutic agents in the clinics.

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## Disclosure statement

XY declares competing financial interests as a cofounder of NanoFCM Inc.

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