Review

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Consensus statement on extracellular vesicles in liquid biopsy for advancing laboratory medicine

https://doi.org/10.1515/cclm-2024-0188
Received February 7, 2024; accepted May 10, 2024;
published online June 19, 2024

Abstract: Extracellular vesicles (EVs) represent a diverse class of nanoscale membrane vesicles actively released by cells. These EVs can be further subdivided into categories like exosomes and microvesicles, based on their origins, sizes, and physical attributes. Significantly, disease-derived EVs have been detected in virtually all types of body fluids, providing a comprehensive molecular profile of their cellular origins. As a result, EVs are emerging as a valuable addition to liquid biopsy techniques. In this collective statement, the authors share their current perspectives on EV-related research and product development, with a shared commitment to translating this newfound knowledge into clinical applications for cancer and other diseases, particularly as disease biomarkers. The consensus within this document revolves around the overarching recognition of the merits, unresolved questions,

All of the authors are key members of the Chinese Anti-Cancer Association (CACA) TBM Society for Exosomes and Microvesicles (CSEMV).

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and existing challenges surrounding EVs. This consensus manuscript is a collaborative effort led by the Committee of Exosomes, Society of Tumor Markers, Chinese anti-Cancer Association, aimed at expediting the cultivation of robust scientific and clinically applicable breakthroughs and propelling the field forward with greater swiftness and efficacy.

Keywords: extracellular vesicles; cancer biology; clinical oncology; liquid biopsy diagnostics; therapeutics

Introduction

Extracellular vesicles (EVs) are a catch-all term for various tiny vesicles enclosed by a lipid-bilayer membrane that carry cell-based contents, secreted by all types of cells, which serve as messengers by carrying lipids, proteins, and nucleic acids. They play vital roles in physiological and pathophysiological processes in both recipient and parental cells [1]. There are many types of EVs which have particular attributes and subcellular or cellular origin. Based on dimensions, EVs are categorized as small extracellular vesicles (sEVs)<200 nm and medium/large EVs (m/lEVs)>200 nm [2]. The identification of EV markers is capable of dynamically mirroring the medical situation of patients in real time [3–5]. Hence, EVs possess vast potential in the areas of disease screening and early detection, non-invasive diagnosis, and real-time monitoring of treatment response [6–9]. Moreover, as a prospective carrier for drug delivery systems, EVs hold promise in therapy option for tumors and other illnesses [10–13].

In recent years, research related to EVs has experienced exponential growth. Academic groups, such as the International Society for Extracellular Vesicles (ISEV), have made extensive efforts and collaborations to publish the guidelines on the key issues within this research field [2, 14, 15]. In the *13th Five-Year Plan for Bioindustry Development*, the National Development and Reform Commission of China, and related institutions, proposed that bioindustry should accelerate its extensive application in various fields to serve the people. In response to this, experts from the Chinese Anti-Cancer Association (CACA) TBM Society for Exosomes and Microvesicles (CSEMV) published the Chinese version “Consensus statement on exosomes in translational research and clinical practice” in 2018. We are now updating this consensus statement based on the evolution of knowledge related to EVs in the last six years. Our main goal is to promote collaboration between research institutions, universities, and industry in the study of EVs as tumor markers or therapeutic carriers (targets). We aim to establish relevant standards and norms to guide the translation of EV-related technology and research findings from the bench to the bedside for patient diagnosis and treatment.

Advancements in EV research for successful clinical applications in cancer early detection and treatment

EVs were first identified in 1985 and were initially believed to be mere cellular debris [16]. However, subsequent research has revealed their crucial role in intercellular communication due to their diverse and specific cargo contents. Recent landmark findings have highlighted the tremendous potential of EVs in clinical applications for cancer diagnosis and treatment, ushering in a new era of EV research (Figure 1).

In 1983, two independent studies reported the release of small vesicles with transferrin receptors into the extracellular space by reticulocytes [17, 18]. Later in 1987, these vesicles were named ‘exosomes’ [19]. Further studies demonstrated that exosomes secreted by B lymphocytes carry molecules crucial for antigen presentation and eliciting T cell responses [20]. Dendritic cells were also found to secrete exosomes capable of presenting antigens [21]. It was later discovered that exosomes facilitate intercellular communication by transporting RNA molecules [22]. In 2011, exosomes were shown to safely deliver siRNA, showcasing their potential for gene therapies targeting chronic neurodegenerative disorders [23]. The Nobel Prize in Physiology or Medicine was awarded to Rothman, Schekman, and Südhof in 2013 for the discovery of the machinery regulating vesicle traffic in cells. A study in 2015 conducted by researchers at the MD Anderson Cancer Center in Houston, TX, revealed that the abundance of exosomal glypican-1 in the serum of early pancreatic cancer patients is significantly higher than that in control individuals [24]. In January 2016, Exosome Diagnostics (Waltham, MA) launched the ExoDx Lung (ALK), the first test utilizing exosomal markers to detect lung cancer. Tumor-derived exosomes carrying bioactive PD-L1 were discovered to suppress the immune response, posing challenges for cancer immunotherapy in 2018 [25]. The ExoDxTM Prostate (IntelliScore), the first exosome-based test for prostate cancer diagnosis, received FDA Breakthrough Designation in June 2019. Additionally, the National Comprehensive Cancer Network (NCCN) included ExoDx in its May 2019 Prostate Cancer Screening Guidelines (NCCN V2.2019) for early detection of prostate cancer for both initial and follow-up prostate biopsy. Clinical trials for exoIL-12 in cutaneous T cell lymphoma and exoSTING in solid tumors began in September 2020.

The rapid development of EV research is attributed to their important biological functions and the continuous progress in analysis and detection technologies. Despite this

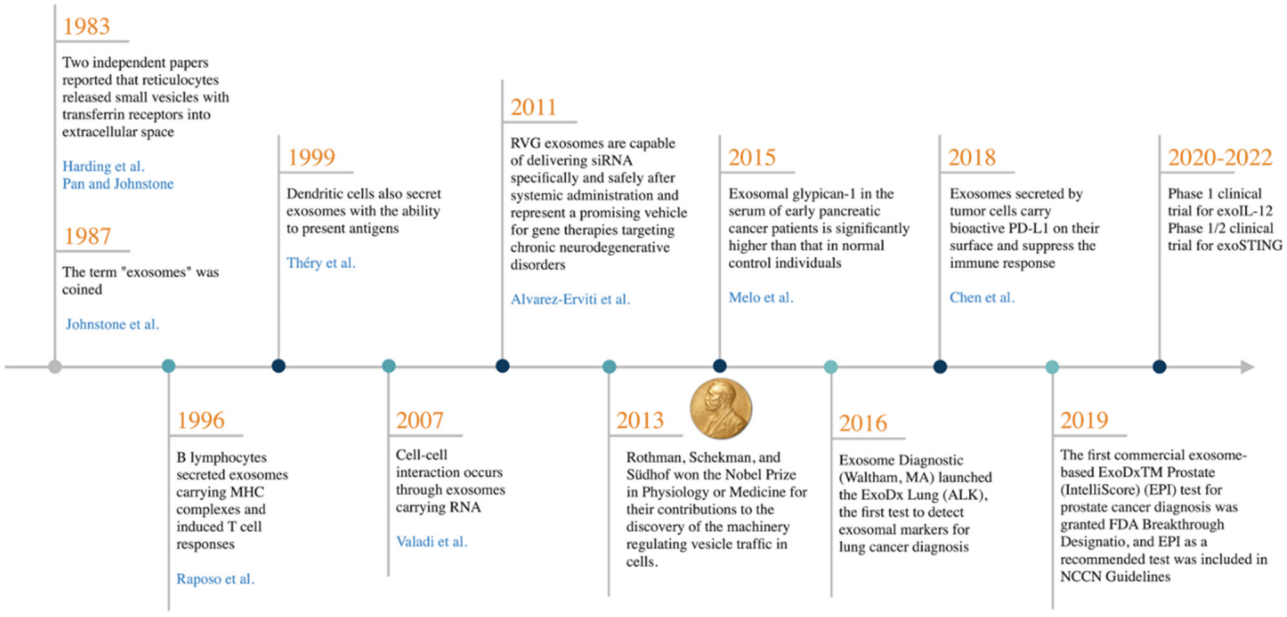

Figure 1: The evolution of EV research: from bench to successful clinical applications in early cancer detection and treatment. Created with BioRender.com.

progress, the current understanding of EVs remains limited in many aspects. Experts in the present consensus believe that the results of existing single-center studies with small sample sizes need to be verified by further research and multicenter clinical trials.

EV-based liquid biopsy in personalized medicine: advancements and future directions

The rapid development of liquid biopsy techniques has demonstrated their convenience and the abundance of data that can be obtained in clinical practice. Given their ability to transport proteins, RNAs, DNAs, and metabolites, EVs offer a new avenue for dynamic disease monitoring in real-time. As such, EVs have emerged as the third major tool in the field of liquid biopsies, following ctDNA and CTCs [26, 27].

CTCs shed from solid tumors invade the peripheral blood circulation, carrying the molecular features and morphological information of tumor cells. On the other hand, ctDNA refers to the tumor- or CTC-derived DNA fragments present in the blood. Compared to CTCs and ctDNA, EVs possess distinct advantages – as highlighted in Table 1 – with at least three key benefits: (1) more ubiquitous, as EVs are present in almost all body fluid samples, while CTCs and ctDNA are mainly found in peripheral blood samples; (2) preserving the content through the phospholipid bilayer membrane, providing more stability compared to ctDNA; and (3) high abundance (10^{8-13} exosomes/mL in plasma) and stability (can be stored at −80 °C for months or even years).

While the three main tools for liquid biopsy (CTCs, ctDNA, and EVs) have been used internationally for cancer diagnosis, the absence of a unified standard remains a common issue in the field. However, through the joint efforts of multiple disciplines, technical barriers related to standardization may soon be resolved.

Discovery of EV-based biomarkers: clinical application and challenge

The discovery of novel EV markers has become increasingly challenging due to the intricate and time-consuming process. Standardized screening and verification strategies are paramount when it comes to discovering new EV markers and transitioning them into clinical practice. Key steps for the translation of EV markers include (Figure 2): (1) Determining the purpose and application scenarios of EV marker screening; (2) Screening potential EV markers based on specific disease characteristics and specimen types; (3) Establishing appropriate detection methods according to EV marker types, verifying them using a small set of clinical samples, and comparing them with common or gold standard methods; (4) Conducting

Table 1: Characteristics and comparative analysis of liquid biopsy techniques in oncology.

	CTC	ctDNA	sEV/exosomes
Origin	Detaches from solid tumors and invades peripheral blood	Apoptotic or necrotic tumor cells, may also be actively secreted by tumor cells	Actively secreted by living cells
Size	Micron scale (12–25 μm)	Molecular scale (~167 bp)	Nanoscale (40–100 nm)
Information	Have cell morphology and contain full molecular information of the tumor	Contain only the genomic information of the tumor	Contain tumor genome, transcriptome, proteome, and metabolome information
Clinical significance	Can be used for some cancer prognosis assessment, clinical staging, recurrence monitoring and medication guidance, and can also be cultured *in vitro* for functional studies	Can be used for early diagnosis of tumors, recurrence monitoring, drug resistance detection, and medication guidance	Can be used for early diagnosis of tumors, recurrence monitoring, drug resistance detection, and medication guidance, etc., and can also be used as drug preparations or drug carriers for tumor treatment
Stability	Short half-life of 1–2.4 h in circulating blood	With a circulating time of 16 min up to 2.5 h. Stable storage after separation and extraction	Protected by a phospholipid bilayer, the contents are relatively stable
Limitation	Low abundance, difficult detection, many separation and detection techniques but lack of standards	Most of them are derived from dead cells and only contain the genomic information of the tumor; it is difficult to standardize the pre-analytical conditions	There is interference from other EVs subgroups, and there are many isolation and detection techniques but lack of standards

multi-center clinical trials to validate the external clinical value of EV markers using a larger set of clinical samples; (5) Establishing a quality management system for the clinical detection of EV markers; (6) Overcoming tumor heterogeneity through the use of multi-omics techniques; (7) Analyzing data using advanced methods such as machine learning models.

The successful discovery and development of novel EV markers necessitates a comprehensive understanding of clinical requirements, design principles, and research methodologies. For instance, various bioinformatics screening techniques, sample collection, and storage conditions could potentially yield different results. As a result, the

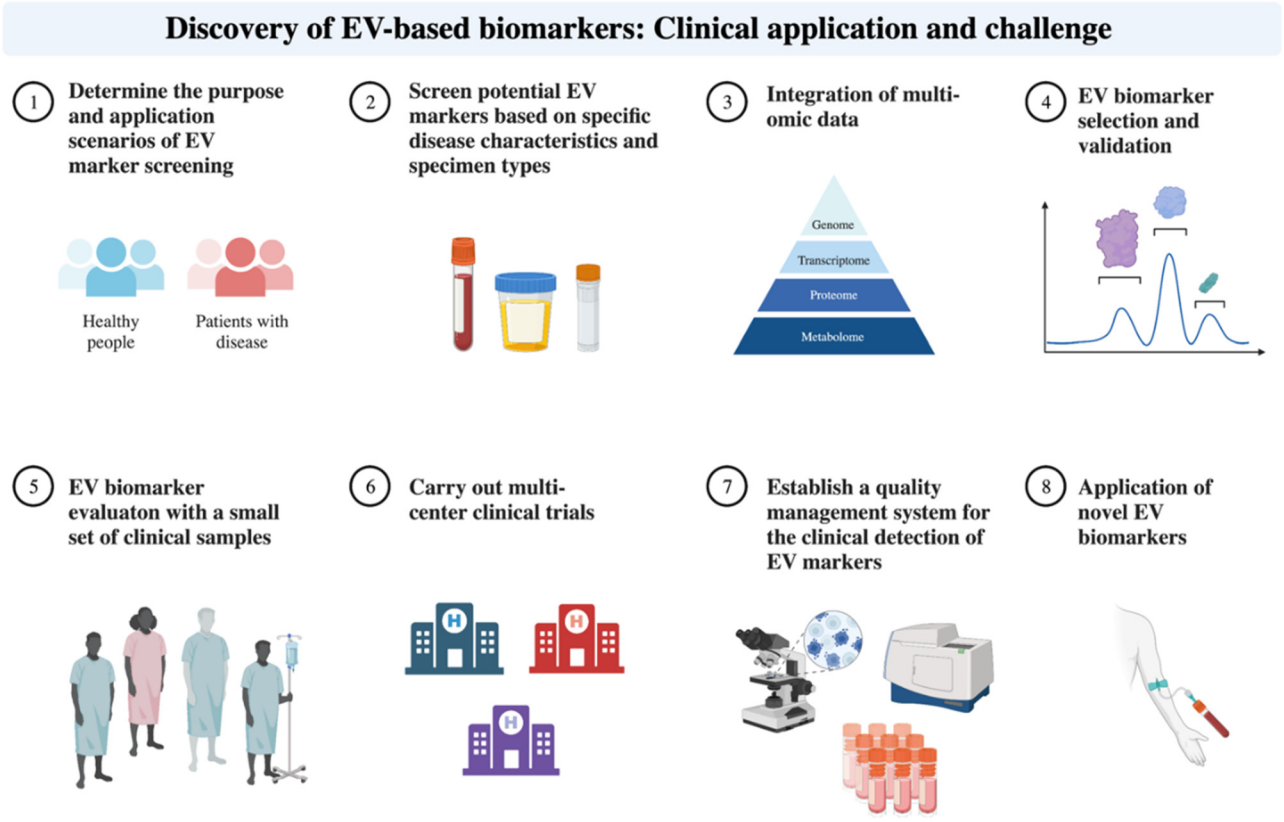

Figure 2: Key steps for the discovery of novel EV markers and their translation into clinical practice. Created with BioRender.com.

absence of standardized procedures poses a significant obstacle to the further development of EV markers. In order to advance the research in this field, it is crucial to learn from previous experiences in developing existing markers, to comprehend the characteristics of each EV type, and to bolster cooperation between clinicians and basic research scientists.

EV collection and pre-processing

EVs are ubiquitous in various human body fluids, such as serum, plasma, urine, saliva, and cerebrospinal fluid, as well as in the cell culture supernatant obtained from cultured cells. The analysis of EVs isolated from these body fluids or liquids holds promise for the diagnosis and prognosis of various diseases, including cancer, cardiovascular and cerebrovascular diseases, urinary system diseases. Given that plasma, serum, and cell culture supernatant are most frequently employed in both clinical and experimental settings, this section mainly focuses on the collection and pre-processing steps that precede EV separation and subsequent analysis.

(1) Plasma sample: To collect plasma samples, 8–10 mL of peripheral blood is obtained from participants using vacuum blood collection tubes or blood lancets containing the anticoagulant EDTA to prevent the effects of heparin on RNA analysis in EVs. The blood cells are then removed by low-speed centrifugation (below 2,000×*g*) at 4 °C or room temperature (RT) for 15–20 min. The resulting supernatant (more than 4 mL) is collected and subjected to further centrifugation before the isolation of EVs using appropriate methods or storage at −80 °C [14, 28].

(2) Serum sample: To collect serum samples, 8–10 mL of peripheral blood is collected from participants using vacuum blood collection tubes or blood lancets without any anticoagulant. The blood samples are then left to precipitate at RT for about 30 min or at 4 °C for 3–4 h, or even overnight to remove blood cells. The serum samples are collected using the same method as plasma samples [14, 28].

(3) Cell culture supernatant: Adherent cells with 70–80 % confluency or suspension cells with 60–70 % cell density are cultured with medium supplemented with EV-depleted fetal bovine serum (FBS) for an appropriate time. The resulting cell culture medium (more than 10 mL) is then centrifuged at 300×*g* and 4 °C for 10–20 min to remove dead cells, followed by further centrifugation at 3,000×*g* and 4 °C for 10–20 min to eliminate cell debris. If possible, the supernatant is further filtered using 0.22 μm microfiltration membranes. The cell culture supernatant is then processed for EV separation and analysis or stored at −80 °C [14].

(4) Urine sample: Urine is a convenient and non-invasive source of EVs. First, the subject should avoid consuming any food or drink that may affect urine composition for at least 2 h before sample collection. Second, mid-stream urine is collected into a sterile tube and centrifuged at 2,000×*g* for 10–20 min at 4 °C to remove cells and debris. The supernatant is then filtered through a 0.22 μm microfiltration membrane. The filtered urine sample can then be used for EV separation and analysis or stored at −80 °C [14, 29].
It should be noted that urine samples are generally considered to have low EV concentrations compared to other body fluids, and their analysis may require additional concentration steps before EV separation. Additionally, urine composition may be influenced by a range of factors, including diet, hydration status, and the presence of urinary tract infections. Standardized and reproducible protocols for urine sample collection and processing are essential for reliable and accurate EV research.

(5) Saliva sample: Saliva samples are obtained in the morning (between 8 and 10 AM) from all subjects. The participants are instructed to avoid eating, drinking, or using oral hygiene products for at least 1 h before sample collection. No stimulation of salivation is provided, and after rinsing the mouth with water, the subjects spit 3–5 mL saliva into a 35 mm dish. The participants are reminded not to cough up mucus during sample collection, and the entire process is completed within 30 min. The saliva sample is transferred to a 1.5 mL tube and centrifuged at 3,000×*g* for 15 min at 4 °C to remove cells and cellular fragments. The supernatant is transferred to a new 1.5 mL tube and stored at −80 °C [6–9, 30–32].

(6) Cerebrospinal fluid (CSF): CSF is a clear bodily fluid found in the brain and spinal cord and can be used as a diagnostic tool for various neurological diseases. To collect CSF samples, the lumbar puncture technique is utilized, whereby the area is sterilized with antiseptic and local anesthesia is administered to minimize discomfort. Subsequently, a needle is inserted into the spinal canal to extract the CSF using a syringe. Once collected, CSF samples need to undergo centrifugation at 300–2,000×*g* for 10–15 min at 4 °C to eliminate cells, protein aggregates, and debris [33, 34]. Further purification of EVs can be accomplished using ultracentrifugation, density gradient centrifugation, size exclusion chromatography, or immunoprecipitation

assays, depending on the downstream analysis method. Careful attention should be given to the possible contamination of blood-derived EVs in CSF samples, which may require additional separation or purification steps to reduce interference. It is important to note that due to the potential to transmit infectious agents, CSF samples should be handled with care and in accordance with specific biosafety standards. Establishing standardized protocols for the collection, processing, and storage of CSF samples for EV analysis promotes reproducibility and ensures the reliability of results. Additionally, while CSF is an ideal source of EVs for studying neurological diseases, the low yield of EVs and potential contamination from blood necessitate strict collection and processing procedures to avoid artifacts. Robust quality control measures can help minimize variability in EV isolation and maximize the accuracy and clinical value of EV-based biomarkers for neurological diseases.

It is worth noting that EV separation and analysis in body fluids can be influenced by a range of biological factors such as age, gender, pregnancy, food intake, weight, smoking, and the presence of specific communicable or non-communicable diseases. The use of certain medications may also impact EV analysis. Additionally, technical factors, including sample volume, container type, selection of an appropriate anticoagulant, mixing uniformity, centrifugation parameters, and storage and processing conditions, can have critical impacts on EV isolation and analysis. While biological factors are often uncontrollable due to individual differences, mitigating technical factors through standardized collection and pre-processing of body fluids and liquids for EV separation and analysis is crucial. Standardized EV research methods enable accurate disease diagnosis and prognosis, facilitate personalized medicine, and guide clinicians towards effective treatment options.

EV separation methods

EVs can be broadly categorized into several subgroups, each with distinct biogenic pathways, sizes, compositions, and functions, thereby underscoring the heterogeneity within the EV landscape. These subgroups include exosomes, which are typically small (30–150 nm in diameter) vesicles formed inside endosomal compartments known as multivesicular bodies and released upon fusion with the plasma membrane. Ectosomes, also referred to as microparticles or microvesicles, are larger (up to 1 μm in diameter) vesicles that bud directly from the plasma membrane. Oncosomes are a subtype of EVs, notably larger than exosomes, and are often associated with cancer, being released in large quantities by cancer cells. Apoptotic bodies are another category, which are relatively large (1–5 μm in diameter) and released by cells undergoing programmed cell death or apoptosis.

The challenge in the field of EV research lies in the proper characterization of these biophysically and biochemically similar EV subtypes. This difficulty is compounded by the overlapping size ranges of these vesicles and the lack of specific markers to distinguish them conclusively. Further complicating the matter is our limited understanding of the specific pathways involved in their biogenesis, the precise composition of their cargo (including proteins, lipids, and nucleic acids), and the distinct functions they serve both in homeostatic conditions and in the context of disease.

To address these challenges, a variety of methods have been developed for the isolation and extraction of EVs, while each leveraging only one or several physical and chemical properties unique to EVs. These methods include:

Ultracentrifugation

Ultracentrifugation is a widely used method for EV separation, which typically involves differential centrifugation and density gradient centrifugation. Differential centrifugation utilizes multiple centrifugation steps to remove cells, large vesicles, and debris before precipitating small EVs or exosomes. This method is suitable for isolating sEVs from diverse samples for functional studies and marker screening, but its efficiency can be influenced by several factors such as acceleration, rotor type, centrifugation time, and sample viscosity. Analyzing biological samples with high viscosity, like plasma and serum, may require more ultracentrifugation steps and higher centrifugation speeds. Despite its advantages, ultracentrifugation can cause sEV destruction and aggregation, as well as contamination with aggregated protein and nucleic acid, due to the ultra-high centrifugal forces applied (up to 200,000 *g*).

Density gradient centrifugation is another technique that combines ultracentrifugation with a sucrose or iodixanol density gradient [35]. This method can attain higher sEV purity but is more time-consuming (around 8–30 h) and yields lower amounts of sEVs. Moreover, neither differential centrifugation nor density gradient centrifugation can achieve standardization across laboratories.

Precipitation

Polymer-based precipitation is a technique that utilizes polymers to reduce the solubility of EVs by “hijacking” water

molecules, followed by sedimenting EVs under low-speed centrifugation conditions. This method is straightforward to execute. However, hybrid proteins, free nucleic acids, and lipoproteins can aggregate and precipitate with EVs during centrifugation due to the presence of water molecules on their surfaces, resulting in low EV purity.

Ultrafiltration

Ultrafiltration membranes are commonly utilized for EV isolation, allowing for the separation of EVs from proteins and other macromolecules based on microvesicle size. The most used filtration membranes have pore sizes of 0.8 μm, 0.45 μm, or 0.22 μm, collecting EVs that are larger than 800 nm, 400 nm, or 200 nm, respectively. Ultrafiltration is a simple and efficient concentration method often used in conjunction with other separation techniques. One main limitation of this method is that EVs may clog the filter pores, causing shortened membrane life and reduced separation efficiency. Additionally, sEV/exosomes may adhere to the filtration membranes, and long-term centrifugal force can damage the membrane structure of EVs.

The label-free exosome purification system (EXODUS) is an innovative ultrafiltration technology that uses double-coupled harmonic oscillations in a double-membrane filter configuration to produce transverse waves. The oscillation of the nanoporous membrane and filter cartridge strongly suppresses fouling effects through acoustically controlled flow, avoiding flow rate reduction. As a result, EV isolation and purification yield, purity, and processing speed are all improved. However, the current EXODUS platform is restricted to single-channel isolation, and high-throughput biological studies would benefit from an array of EXODUS devices with automatic reagent distribution and sample collection.

Tangential flow filtration

Tangential flow filtration (TFF), similar to ultrafiltration, uses membrane retention to enrich EVs by molecular size. However, there are key differences between the two methods. In TFF, the liquid sample flows parallel to the membrane surface. Application of pressure causes a portion of the flow to pass through the membrane based on filter size, while the remainder is recycled back to the feed tank for repeated filtration, eventually achieving complete filtration. Due to its high sample throughput, TFF is well-suited for large-scale industrial EV preparation.

Size-exclusion chromatography

Size-exclusion chromatography (SEC) separates macromolecules based on their size, rather than their molecular weight, by employing a column packed with porous polymeric beads [36]. This method utilizes gravity instead of centrifugal force to isolate EVs, avoiding any potential damage to the structure of EVs caused by centrifugal force. SEC can produce EVs with a higher purity than other methods, but the EVs obtained are often diluted and require concentration by ultrafiltration. This process is time-consuming and not ideal for processing large-volume samples.

Magnetic bead method

The separation of EVs can be achieved by coating specific proteins or nucleic acids onto the surface of magnetic beads to specifically bind to proteins or phosphatidylserine (PS) on the surface of EVs. This method improves the separation efficiency and specificity, as magnetic beads provide a larger surface area to capture EVs. However, this method is not suitable for obtaining EVs from large-volume samples, as it is heavily dependent on antibodies.

Microfluidic chip technology

Using microfluidic chip technology, various operations such as EV isolation, recovery, and detection are integrated into micron-scale chips. These methods rapidly isolate EVs from small amounts of body fluids and perform real-time EV characterization analysis for *in situ* detection. However, these methods are only suitable for small sample separation, and the separation efficiency mainly depends on the principle adopted by the device, making the selectivity and specificity of these methods verified.

None of the methods mentioned above can separate EVs into different subpopulations. To confirm that inclusion molecules or biological functions described in studies are exclusively derived from EVs, the ISEV proposed the minimal information for studies of extracellular vesicles (MISEV2014) and the updated “MISEV2018” and “MISEV2023”, which recommend essential identification, characterization, and functional experiments of EVs [2, 37, 38]. However, the MISEV guidelines lack emphasis on identifying non-EV impurities, highlighting the need to design experiments to quantify non-EV impurities in the product or to deplete non-EV impurities and their biological activities to improve research findings’ reliability.

EV characterization and single-EV analysis

Conventional methods used for EV characterization are mainly based on ensemble-averaged approaches, such as western blotting, ELISA, bead-based flow cytometry, and PCR. However, since EVs in body fluids are highly heterogeneous in size and composition owing to their differences in parent cell types, secretion pathways, and pathophysiologic statuses, the importance of single EV analysis is gradually recognized and appreciated. Techniques applied for single EV analysis are classified into two categories, unlabeled and labeled (Figure 3). These techniques can be used to quantify EV subsets expression of specific protein markers in body fluids for clinical diagnosis and prognosis. Therefore, single EV analysis is a promising method to advance the field of EV research and provide new insights into disease diagnosis and treatment.

Unlabeled analysis of single EVs

1) Nanoparticle tracking analysis (NTA) is a technique used to characterize the size and concentration of nanoparticles in liquid suspension by analyzing their Brownian motion. However, accurate particle size distribution assessment requires specific trace length, stable temperature, and a large number of repeated measurements. In the case of EV analysis, NTA has limitations in measuring the light-scattering intensity of EVs smaller than 70 nm [39]. Furthermore, this method cannot specifically distinguish EVs from other particles, such as protein aggregates or viral particles, and the expression of proteins on EVs cannot be directly assessed.
2) Tunable resistive pulse sensing (TRPS) is a precise technique for measuring the size, concentration, and charge of nanoparticles in suspension, based on the conductivity change of a porous membrane. However, similar to NTA, TRPS does not provide information on surface-expressed proteins, and cannot distinguish EVs from other micro-particles.
3) Transmission electron microscopy (TEM), including cryo-TEM, is commonly used for morphological characterization of single EVs [40]. In cryo-TEM, samples are rapidly frozen to preserve the native structure of EVs. While high resolution visualization of the phospholipid bilayer, lumen structure, and interior features of single

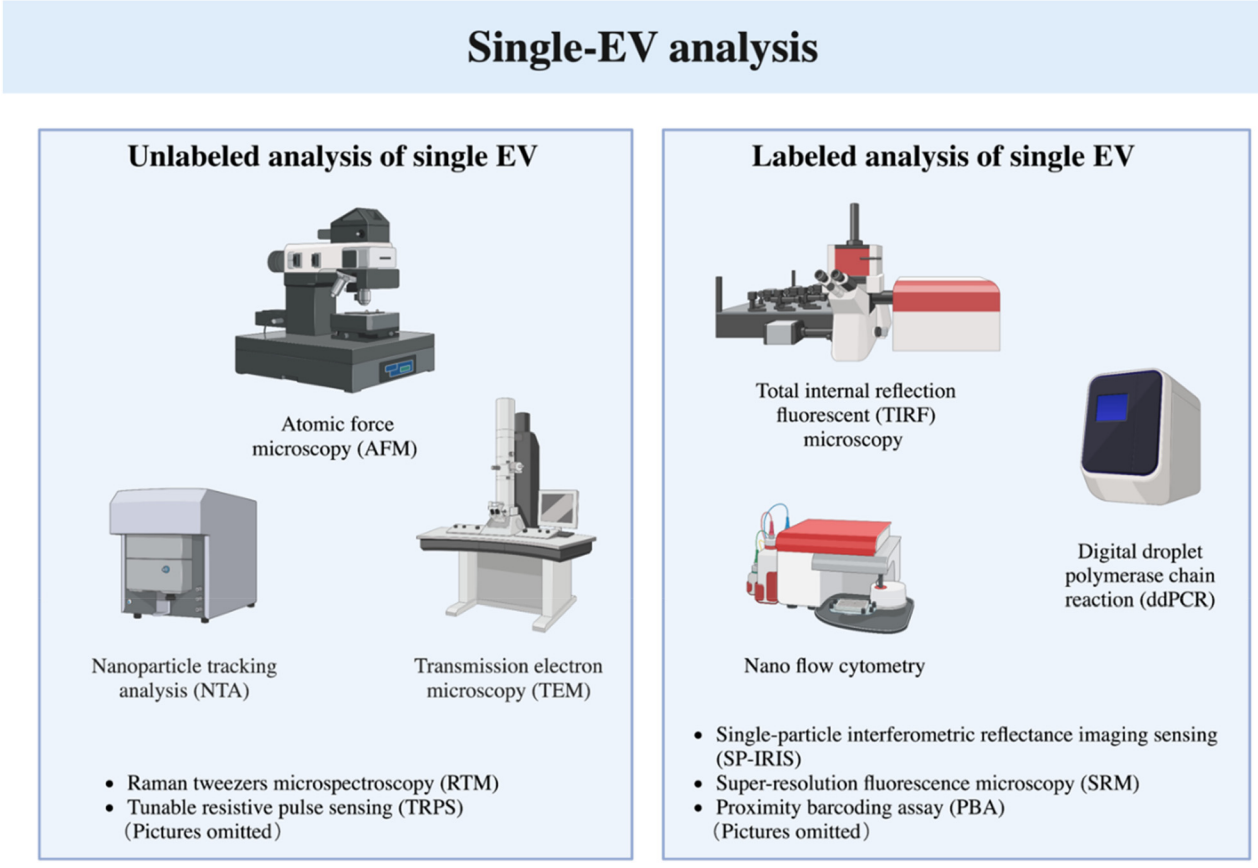

Figure 3: Technologies for analysis of single EV. Created with BioRender.com.

EVs is possible, cryo-TEM has low throughput and making it difficult to measure particle size distribution of EV samples rapidly.

4) Atomic force microscopy (AFM) utilizes interaction forces between the probing tip and EVs to reflect the morphology and size of single EVs [41]. While a high-throughput method based on AFM has been developed to measure size and stiffness distribution of 100 vesicles per hour, it still remains time-consuming and labor-intensive.
5) Raman tweezers microspectroscopy (RTM) uses a tightly focused laser beam to both optically trap single nanoparticles in liquid suspension and to excite subsequent Raman scattering detection, thus providing a vibrational fingerprint unique to the constituent biomolecules of the captured particles [42]. The greatest advantage of this method is signal linearity, which enables qualitative and quantitative analysis of the biochemical characteristics of single EVs without labeling. The main disadvantage is that scattering efficiency is usually low, requiring extended data acquisition.

Labeled analysis of single EVs

1) Total internal reflection fluorescent (TIRF) microscopy utilizes total internal reflection to excite fluorescent molecules located in the vicinity of high-refractive solid materials for the purpose of imaging single EVs. This technique generates an evanescent wave, parallel to the substrate surface, which provides limited illumination of the sample. As a result, TIRF has an enhanced signal-to-noise ratio and is capable of realizing single fluorescent molecule imaging. TIRF has been used for real-time secretion studies of single EVs and for *in situ* protein or RNA detection [43, 44]. However, the limitation of TIRF is that the fluorophore is only excited within a few hundred nanometers of the solid substrate and the instability and photobleaching of the fluorophores may lead to the misinterpretation of data in long-term measurements.
2) Super-resolution fluorescence microscopy (SRM), including techniques such as photoactivation localization microscopy (PALM) [45], stochastic optical reconstruction microscopy (STORM) [46], and fluorescence lifetime imaging microscopy (FLIM) [47], presents higher resolution compared to traditional light microscopy and is used to visualize targets much smaller than the optical diffraction limit. Therefore, it offers significant advantages in identifying the precise location of single EVs and studying their physiological processes via imaging. However, these techniques often require sophisticated equipment and data processing and are incapable of rapidly characterizing the concentration and particle size of EVs.
3) Single-particle interferometric reflectance imaging sensing (SP-IRIS) is a novel technology that detects multiple surface biomarkers and the size of single EVs through interferometric imaging [48]. The ExoView platform, launched by NanoView Biosciences, employs a multiplexed microarray chip for the immuno-capture of CD9, CD63, and CD81-positive EVs [49]. The captured EV is probed with additional fluorescent antibodies under fluorescence mode, enabling the assessment of up to three surface markers on single EVs. However, highly concentrated EV samples should be avoided as multiple EVs within the detection volume may lead to misclassification of the signal as a larger vesicle.
4) Digital droplet polymerase chain reaction (ddPCR) is an advanced technology capable of high-throughput and quantitative analysis of a small amount of protein or miRNA on single EVs with high sensitivity [50, 51]. In ddPCR, the EV sample is first incubated with specific antibody-DNA conjugates, followed by stochastic microfluidic incorporation of one or zero EVs into each droplet. The anchored DNA on EVs is then *in situ* amplified upon PCR with fluorescent reporter probes, and the signals from each droplet are read out through a simple fluorescence imaging setup. Using different barcode sequences and fluorophores, ddPCR achieves multiple mutation analysis to examine specific mutations in distinct populations of EVs. Therefore, ddPCR allows accurate quantification of rare EV subpopulations in complex biofluids and has demonstrated great potential for broad biomedical applications.
5) Proximity barcoding assay (PBA) is a digital method for multiparameter analysis of single EVs that has made a breakthrough in recent years [52]. EVs are first incubated with different antibody-DNA conjugates. Single-stranded DNA clusters, each with a unique DNA motif, are then generated via rolling circle amplification and added into the EV samples. Individual DNA clusters, similar in size to sEVs, bind to the antibody-DNA conjugates on the surface of single EVs. Enzyme hybridization reaction and PCR amplifications are performed, converting membrane protein composition into DNA sequence information. Finally, the proteomic information of single EVs is decoded through the next-generation sequencing technology.
6) High-resolution flow cytometry has been applied to analyze surface proteins of individual EVs using fluorescence threshold triggering [53, 54]. Conventional

flow cytometry typically has minimum detectable vesicle sizes of 300–1,200 nm, while dedicated FCM has minimum detectable vesicle sizes of 150–190 nm, based on scattered light. Recently, Yan's group has developed nano-flow cytometry (nFCM), which enables light scattering detection of individual EVs as small as 40 nm in diameter with an analysis rate of up to 10,000 particles per minute [55]. By using monodisperse silica nanoparticles as a reference standard and refractive index mismatch correction based on the Mie theory, statistically representative particle size distribution of EVs can be acquired in minutes with the resolution and accuracy comparable to those of cryo-TEM. nFCM provides a sensitive and rapid platform for biomolecule characterization and sizing of individual EVs. This technology greatly advances our understanding of EV-mediated intercellular communication and the development of advanced diagnostic and therapeutic strategies.

Despite the advanced development of EV isolation and single-EV characterization techniques, there are still concerns that need to be addressed. Firstly, some EV isolation methods are not directly applicable to all sources of samples. Secondly, except for PBA, most of the single-EV analysis techniques hardly provide large-scale screening of biomarkers on single EVs. Lastly, the diversity of technologies for EV isolation and characterization poses great challenges in drawing unanimous conclusions in this field. Further technology development in EV isolation and single EV analysis is needed to improve the convenience and accuracy of EV-based liquid biopsy. To facilitate transparent reporting and replication of experiments, EV research should be conducted in accordance with guidelines and minimal requirements for sample collection, processing, and EV identification and characterization [2].

Advanced high-throughput technologies and machine learning models for big data analysis

For diseases with complex etiology and pathological features, such as cancer, autoimmune diseases, and neurodegenerative diseases, achieving satisfactory accuracy in disease diagnosis and prognosis analysis using a single or a few EV biomarkers is generally challenging. The development of high-throughput sequencing technologies and microarrays has greatly advanced biomarker studies. Machine learning analysis of omics data has led to identifying composite EV biomarkers. Multidimensional information integration and multivariate data correlation fitting for the analysis of EV-related research, especially the intelligent diagnostic analysis of disease imaging and extensive mining of potential disease-diagnostic signatures in EV multi-omics big data requires multiplex analysis and deep mining of EV-related biomarkers and cargoes. With the rapid development of machine learning techniques, complex algorithms are constantly updated and iterated, such as linear discriminant analysis (LDA), principal component analysis (PCA), random forests (RF), sure independence screening and sparsifying operator (SISSO), and convolutional neural network (CNN) [56]. Experts recommend the use of machine learning for efficient multivariate data integration and correlation fitting for EV-related research, particularly for intelligent diagnostic analysis and extensive mining of potential disease-diagnostic signatures in EV multi-omics big data. However, there are concerns regarding the adoption of machine learning for EV-related research and analysis, particularly in improving the accuracy of machine learning-based information mining, disease diagnosis, and disease prediction, while trying to circumvent the black boxes and overfitting problems that accompany the increased complexity of algorithms.

1) Adherence to the MISEV guideline manual is essential during EV isolation and purification to ensure the purity of obtained EV samples and credibility of subsequent multi-omics identification of EV-related cargoes. Strict adherence to these standards will avoid interference from non-EV impurities in the products, thus minimizing the interference during machine learning modeling.
2) Novel high-throughput techniques should be developed to provide machine learning algorithms with high-quality, multidimensional, and time-series explicit read-in data. For instance, the membrane proteome, intraluminal proteome, lipidome, nucleic acidome, and metabolome of EV, derived from patients at various stages of multiple disease subtypes, as well as the corresponding imaging data of pathological samples. However, the overwhelming amount of data generated from single-EV sequencing and mass spectrometry may pose a great challenge to the computational power of machine learning algorithms.
3) We suggest researchers validate the results using different algorithms with different underlying logic, or use reliable experimental techniques to dissect and validate disease prediction algorithms and biomarker signatures derived from machine learning algorithms to enhance the interpretability and credibility of these algorithms. Therefore, validating the results after using

one algorithm for model construction and information concatenation is crucial, even if it involves significant effort to avoid potential threats in the algorithmic black box.

EVs in clinical applications

Studies are currently underway to investigate the clinical eligibility of EV-based liquid biopsy for various diseases by using different EV-carrying genetic materials derived from body fluids, such as plasma, serum, urine, saliva, cerebrospinal fluid, and ascites.

Currently, EV-based diagnostic research mostly focuses on circulating EV miRNAs, which have been shown to be clinically feasible biomarkers in a variety of solid tumors. For example, let-7, miR-21, and -146b-5p have been found to be pan-cancer markers, miR-181-5p for lung adenocarcinoma and thyroid cancer. Additionally, EV mRNAs provide opportunities to monitor the disease state/progression. For instance, high levels of exo-hTERT mRNA, which have been found in diverse cancers, along with EV WASF2 and ARF6 mRNAs, represent an alternative approach to identifying cancer-associated biomarkers (Table 2). Nonetheless, the evaluation of EV mRNA mutations, such as alternatively spliced isoform ARv7, skipping of MET exon 14, fused mRNA transcripts such as EML4-ALK and BCR-ABL, and chimeric RNA GOLM1-NAA35, underscores the importance of EV long RNA in cancer diagnosis. EV DNA, in addition to RNA, has also been shown to have potential for biomarker development and clinical application. In patients with pancreatic cancer, the mutated KRAS and p53 DNA in the serum sEVs have been found, along with copy number variations of genes such as MYC, AKT1, PTK2, KLF10, and PTEN, which are regularly found in plasma lEVs of prostate cancer patients. Recently, the clinical significance of genomic rearrangements of MGMT in EVs has also been revealed in gliomas.

Similar to nucleic acids, proteins carried by EVs are currently being investigated for their potential in discriminating between different types of cancer. For instance, serum EV CD147, CA19-9 and CEA have been explored in colorectal cancer, EGFRvIII in brain cancer, PSA in prostate cancer, and HER2 in breast cancer. Other approaches, such as the use of specific enrichment methods (e.g., antibodies against EpCAM that is universally expressed in epithelial malignancies) against α-insulin-like growth factor 1 receptor in lung cancer and CA-125 in ovarian cancer, have also shown clinical promise in diagnosis.

Despite the vast effort in developing EV biomarkers for cancer diagnosis, research studies have expanded to the areas of transplant rejection, cardiovascular diseases, neurodegenerative disorders, and immunological diseases for circulating biomarkers. A number of clinical diagnostic trials of EVs in cancers are currently underway (Table 3). However, standardizing appropriate detection methods according to different types of EV biomarkers, verifying with a relatively large set of clinical samples, comparing potentials with common clinical methods and gold standards, and conducting multi-center clinical trials to verify external clinical values are still urgently needed to accelerate the translation of this promising technique from bench-side to bed-side.

Apart from diagnosis, there are also active EV-based clinical trials listed on Clinicaltrials.gov that utilize EVs as therapeutic drugs to alleviate medical conditions related to COVID-19 pneumonia and autoimmune diseases (Table 4). MSC-derived EVs are mostly used, and some studies have progressed to phase III trials, indicating the promising future of EVs as an effective adjuvant therapy in hyperimmunity. EVs are also attracting more research interest as a drug delivery vehicle, capable of carrying small therapeutic molecules such as siRNA, tumor neoantigens, and chemicals due to their high histocompatibility, enhanced loading capacity, low immunogenicity, and possible tumor or tissue tropism nature.

Despite great progress in the study of EVs, there are still many scientific and practical challenges in using EVs as therapeutic drugs, such as industrial production, selection of parent cells, purification, efficient loading of bioactive molecules, and EV heterogeneity. Further understanding of EV formation and mechanism through basic research, continuous breakthroughs in EV drug design, EV engineering, EV delivery technology development, and EV large-scale production will accelerate the process of EV drugs from bench to bedside.

Outlook

As a new research focus, EVs have become potentially effective biomarkers for disease diagnosis and treatment due to their extensive distribution in the body and ease of access, showing promise in precision therapy.

The potential of EVs extends beyond their initial promise in oncology, offering new insights and therapeutic avenues in a variety of diseases. For instance, in neurology, EVs have been identified as key players in neural function and pathology. They are involved in regulating neuronal activity, synaptic plasticity, and the integrity of the myelin sheath, which are critical for the normal functioning of the nervous system [96]. Their role has been further implicated

Table 2: Representative EV RNA markers for cancers in studies.

Biomarker	Type of biomarker	Cancer type	Type of body fluid	Role	Reference
hTERT	mRNA	Solid tumors and hematological malignancy	Serum	Early diagnosis	[57]
hnRNPH1	mRNA	Hepatocellular carcinoma	Serum	Diagnosis + prognosis	[58]
ARF6, WASF2	mRNA	Pancreatic cancer	Serum	Early diagnosis	[59]
miR-1246, miR-4644, miR-3976 and miR-4306	miRNA	Pancreatic cancer	Serum	Diagnosis	[60, 61]
miR-10b, miR-21, miR-30c, miR-181a and miR-let7a	miRNA	Pancreatic cancer	Plasma	Early diagnosis	[62]
let-7d-3p and miR-30d-5p	miRNA	Cervical cancer	Plasma	Early diagnosis	[63]
miR-125a-3p	miRNA	Colon cancer	Plasma	Early diagnosis	[64]
miR125a-5p and miR-1343-3p	miRNA	Colorectal cancer, prostate cancer and pancreatic cancer	Plasma	Diagnosis	[65]
let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, and miR-23a	miRNA	Colorectal cancer	Serum	Early diagnosis	[66]
miR-378a, miR-379, miR-139-5p and miR-200b-5p	miRNA	Lung adenocarcinoma	Plasma	Diagnosis	[67]
miR-21-5p, miR-126-3p, miR-141-3p, miR-146a-5p, miR-155-5p, miR-222-3p, miR-223-3p, and miR-486-5p	miRNA	NSCLC	Plasma	Diagnosis	[68]
miR-181-5p, miR-30a-3p, miR-30e-3p and miR-361-5p	miRNA	NSCLC (adenocarcinoma-specific)	Plasma	Early diagnosis	[69]
miR-10b-5p, miR-15b-5p, and miR-320b	miRNA	NSCLC (SCC-specific)	Plasma	Early diagnosis	[69]
miR-21-5p	miRNA	Breast cancer	Serum	Diagnosis	[70]
let-7b-5p, miR-122-5p, miR-146b-5p, miR-210-3p and miR-215-5p	miRNA	Breast cancer	Plasma	Diagnosis	[71]
miR-21 and miR-200c	miRNA	Metastatic breast cancer	Tear	Diagnosis	[72]
miR-17-5p	miRNA	Breast cancer	Plasma	Diagnosis	[73]
miR-340-5p, miR-17-5p, miR-130a-3p, and miR-93-5p	miRNA	Breast cancer	Serum	Prognosis	[74]
miR-21	miRNA	Esophageal squamous cell carcinoma	Serum	Diagnosis + prognosis	[75]
miR-941	miRNA	Laryngeal squamous cell carcinoma	Serum	Diagnosis	[76]
miR-1246	miRNA	Oesophageal squamous cell carcinoma	Serum	Diagnosis + prognosis	[77]
tRNA-GlyGCC-5	tsRNA	Esophageal squamous cell carcinoma	Saliva	Diagnosis + predict treatment benefit	[8]
GOLM1-NAA35	Chimeric RNA	Esophageal squamous cell carcinoma	Saliva	Early diagnosis + recurrence monitoring + predict chemoradiation response	[6]
miR-10b-5p	miRNA	Gastric cancer	Serum	Diagnosis	[78]
miR-10b-5p, miR-101-3p and miR-143-5p	miRNA	Metastatic gastric cancer	Plasma	Diagnosis	[79]
miR-146a-5p, miR-151a-3p, and miR-2110	miRNA	Cervical cancer	Plasma	Diagnosis	[80]
miR-125a-5p	miRNA	Cervical cancer	Plasma	Diagnosis	[81]
miR-625-5p	miRNA	Glioblastoma	Plasma	Prognosis	[82]
miR-21 and miR-92a-3p	miRNA	Hepatocellular carcinoma	Plasma	Diagnosis + prognosis	[83]
miR-718	miRNA	Hepatocellular carcinoma	Serum	Diagnosis	[84]
miR-375 and miR-141	miRNA	Prostate cancer	Serum	Diagnosis + prognosis	[85]
miR-1246	miRNA	Prostate cancer	Serum	Diagnosis + prognosis	[86]
miR-1290 and miR-375	miRNA	Castration-resistant prostate cancer	Plasma	Prognosis	[87]
miR-200c-3p and miR-21-5p	miRNA	Prostate cancer	Urine	Diagnosis	[88]
PCA3, SPDEF and ERG	mRNA	Prostate cancer	Urine	Diagnosis	[89, 90]

Table 2: (continued)

Biomarker	Type of biomarker	Cancer type	Type of body fluid	Role	Reference
miR-196a-5p and miR-501-3p	miRNA	Prostate cancer	Urine	Diagnosis	[91]
miRNA-21 and miR-375	miRNA	Prostate cancer	Urine	Diagnosis	[92]
miR-145	miRNA	Prostate cancer	Urine	Diagnosis	[93]
miR-21	miRNA	Glioblastoma	Cerebrospinal fluid	Diagnosis	[94]
miR-29a-3p, miR-29b-3p and miR-29c-3p	miRNA	Peritoneal recurrence of gastric cancer	Peritoneal fluid	Diagnosis	[95]

Table 3: Clinical trials relevant to EV-based diagnosis.

NCT number	Study title	Country
NCT05270174	A prospective, multicenter cohort study of urinary exosome lncRNAs for preoperative diagnosis of lymphatic metastasis in patients with bladder cancer	China
NCT03738319	Non-coding RNA in the exosome of the epithelia ovarian cancer	China
NCT03542253	Combined diagnosis of CT and exosome in early lung cancer	China
ChiCTR2000031507	A prospective study of diagnosis biomarkers of esophageal squamous cell carcinoma	China
NCT04182893	Clinical study of ctDNA and exosome combined detection to identify benign and malignant pulmonary nodules (ctDNA)	China
NCT04053855	Evaluation of urinary exosomes presence from clear cell renal cell carcinoma (PEP-C)	France
NCT03102268	ncRNAs in exosomes of cholangiocarcinoma	China
NCT04894695	Urine exosomes to identify biomarkers for lupus nephritis	China
NCT02147418	Exosome testing as a screening modality for human papillomavirus-positive oropharyngeal squamous cell carcinoma	Mexico
NCT03581435	A study of circulating exosome proteomics in gallbladder carcinoma patients (EXOGBC001)	China
NCT04394572	Identification of new diagnostic protein markers for colorectal cancer (EXOSCOL01)	France
NCT04529915	Multicenter clinical research for early diagnosis of lung cancer using blood plasma derived exosome	Korea
NCT03562715	Role of mesenchymal stem cells exosomes derived microRNAs; miR-136, miR-494 and miR-495 in pre-eclampsia diagnosis and evaluation	Egypt
NCT04629079	Lung cancer detection using blood exosomes and HRCT-improving the early detection of lung cancer by combining exosomal analysis of hypoxia with standard of care imaging	U.K.
NCT03830619	Serum exosomal long noncoding RNAs as potential biomarkers for lung cancer diagnosis	China
NCT04636788	Circulating extracellular exosomal small RNA as potential biomarker for human pancreatic cancer	China
NCT03264976	Role of the serum exosomal miRNA in diabetic retinopathy (DR)	China
NCT05463107	Correlation between various urinary exosomal protein biomarkers and pathological manifestation in thyroid follicular neoplasm: early and pre-operative diagnosis of follicular thyroid cancer	Taiwan

in the pathogenesis of various neurological disorders such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis, where EVs can serve as both biomarkers for early diagnosis and targets for therapeutic intervention. The potential of EVs in these contexts has been highlighted by numerous studies, indicating their value in understanding disease mechanisms, improving diagnosis, and evaluating treatment outcomes [97, 98].

The impact of EVs is also significant in the study of infectious diseases, where they play a crucial role in modulating immune responses and facilitating the spread of pathogens [99, 100]. Studies have shown that EVs can carry and transfer infectious agents like viruses and bacteria, impacting the course of infections such as HIV, tuberculosis, and COVID-19. Their ability to influence the host-pathogen dynamic makes them promising tools for diagnosis, vaccine development, and therapeutic strategies aimed at controlling infections and mitigating their effects [101–103].

Consequently, the potential of EVs to act as biomarkers extends well beyond oncology, encompassing a broad spectrum of medical fields. They are poised to revolutionize the landscape of vaccine development, immunotherapy, gene therapy, and precision-targeted drug delivery, addressing a diverse range of diseases [104–106]. As research progresses, the expectation is that EVs will prove instrumental in addressing a plethora of health challenges.

Recently, researchers have found that mature EVs in interstitial tissues are more likely to reflect the original

Table 4: Clinical trials relevant to EV-based intervention.

NCT number	Conditions	Interventions	Phases	Locations
NCT05375604	Advanced hepatocellular carcinoma (HCC)	exoASO-STAT6 (CDK-004)	1	USA
NCT04592484	Advanced solid tumor	CDK-002 (exoSTING)	1	USA
NCT05523011	Psoriasis	Mesenchymal stem cell derived exosomes	1	Singapore
NCT05499156	Perianal fistula with Crohn's disease	Placenal mesenchymal stem cell derived exosomes	1|2	Iran
NCT05490173	Neurodevelopmental disorders	Mesenchymal stem cell derived exosomes	NA	Russia
NCT05475418	Wounds and injuries	Adipose tissue derived exosomes	NA	China
NCT05413148	Retinitis pigmentosa	Umbilical mesenchymal stem cell derived exosomes	2|3	Turkey
NCT05402748	Fistula perianal	Placenta mesenchymal stem cell derived exosomes	1|2	Iran
NCT05387278	Covid19 acute respiratory distress syndrome	Placental derived exosomes and umbilical cord mesenchymal stem cells	1	USA
NCT05261360	Knee injuries	Synovial fluid mesenchymal stem cells derived exosomes|Synovial fluid-derived mesenchymal stem cell	2	Turkey
NCT05216562	Covid19	Mesenchymal stem cell derived exosomes	2|3	Indonesia
NCT05191381	Covid19|Critical illness |Hypercytokinemia| Lung fibrosis	Mesenchymal stem cell derived exosomes	NA	Germany
NCT05060107	Osteoarthritis, knee	Mesenchymal stem cell derived exosomes	1	Chile
NCT05043181	Familial hypercholesterolemia	Bone marrow MSCs derived exosomes	1	China
NCT04969172	Covid19	Human embryonic kidney T-293 cell derived exosomes overexpressing CD24	2	Israel
NCT04902183	Covid19	Human embryonic kidney T-293 cell derived exosomes overexpressing CD24	2	Greece
NCT04879810	Irritable bowel disease	Ginger derived exosomes	NA	USA
NCT04849429	Chronic low back pain|Degenerative disc disease	Mesenchymal stem cell derived exosomes	1	India
NCT04798716	Covid19|Pneumonia|Acute respiratory distress syndrome	Mesenchymal stem cell derived exosomes	1|2	USA
NCT04747574	Covid19	Human embryonic kidney T-293 cell derived exosomes overexpressing CD24	1	Israel
NCT04544215	Pulmonary infection	Mesenchymal stem cell derived exosomes	1|2	China
NCT04493242	Covid19|ARDS|Pneumonia	Bone marrow mesenchymal stem cell derived extracellular vesicles	2	USA
NCT04491240	Covid19|Pneumonia	Mesenchymal stem cell derived exosomes	1|2	Russia
NCT04356300	Multiple organ failure	Mesenchymal stem cell derived exosomes	NA	China
NCT04313647	Healthy	Exosomes derived from allogenic adipose mesenchymal stem cells	1	China
NCT04276987	Covid19	Exosomes derived from allogenic adipose mesenchymal stem cells	1	China
NCT04213248	Dry eye	Umbilical mesenchymal stem cells derived exosomes	1|2	China
NCT03608631	KRAS NP_004976.2:p.G12D|Metastatic pancreatic cancer	Mesenchymal stromal cells-derived exosomes with KRAS G12D siRNA	1	USA
NCT03437759	Macular holes	Umbilical mesenchymal stem cells derived exosomes	1	China
NCT03384433	Cerebrovascular disorders	Mesenchymal stem cell derived exosomes	1|2	Iran
NCT01668849	Head and neck cancer|Oral mucositis	Grape exosomes	1	USA
NCT01294072	Colon cancer	Curcumin conjugated with plant exosomes	1	USA
NCT01159288	Non-small cell lung cancer	Tumor antigen-loaded dendritic cell-derived exosomes	2	France

disease state of the tissue, making tissue-derived EVs potentially the most effective diagnostic biomarkers. The isolation of tissue-derived EVs can provide a better understanding of the complexity of cell-to-cell communication and further understanding of the role of various factors in disease development, thus giving rise to innovative theragnostic strategies.

In addition, two new extracellular particles (EPs) have recently been defined: supermeres and exomeres, which are also functional agents of intercellular communication that are efficiently taken up by multiple organs. Thus, supermeres and exomeres join EVs as a rich source of circulating cargo for candidate biomarkers and therapeutic targets in several disease states. The newly defined small EPs have sparked great interest in EV research and require further investigation in the future.

The clinical application of EV technology is still in its infancy, facing many challenges that are accompanied

by the possibilities of countless new discoveries and technologies. Functional research, as well as diagnostic and therapeutic applications of EVs in major diseases such as cancer, have shown great potential and possibilities. New understandings and consensuses regarding EV research in emerging fields will continue to arise and even subvert. Although standardization in EV research may be a long and difficult process, further studies in this field will likely lead to surprises.

Acknowledgments: We thank all members from the CSEMV, for contributing to this consensus.
Research ethics: Not applicable.
Informed consent: Not applicable.
Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.
Competing interests: The authors state no conflict of interest.
Research funding: The work was supported in part by grants from the National Natural Science Foundation of China (82273183 and 82072683 to Hao Zhang); Natural Science Foundation of Guangdong Province of China (2022A1515010925 to Hao Zhang); Major Project of the Open Joint Fund of the National Clinical Key Specialty Construction of Oncology, The First Affiliated Hospital of Henan University of Science and Technology (ZLKFJJ20230104 to Hao Zhang); St. George and Sutherland Medical Research Foundation (RG220652 to Yong Li), Australia; the Prostate and Breast Cancer Foundation (PBCF), Australia; St George Hospital Cancer Care Research Trust Fund (190481 to Yong Li), Australia; the National Natural Science Foundation of China (82272885 to Xingli Dong); the Key Project of Department of Education of Guangdong Province (No. 2021ZDZX2020 to Xingli Dong); Shenzhen Key Laboratory Foundation (ZDSYS20200811143757022 to Xingli Dong).
Data availability: Not applicable.

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