Microchemical Journal 171 (2021) 106816

Contents lists available at ScienceDirect

Microchemical Journal

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

Recent technical advances to study metabolomics of extracellular vesicles

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ARTICLE INFO

Keywords:
Extracellular vesicle
Metabolomics
Metabolome profiling
Biomarker discovery
Cancer diagnostics

ABSTRACT

Extracellular vesicles (EVs) serving as cell-to-cell communication mediators have rapidly attracted attention from researchers for biomarker discovery, diagnostics and therapeutics. EVs participate in multiple physiological processes and can incorporate various biological molecules. The metabolite is an important molecular type carried by EVs, yet the least researched component compared to proteins and RNAs. Metabolomics is used to investigate the downstream metabolic alternations which reflect cell phenotypes and provide unique insights into disease pathogenesis and biological mechanisms. Recently, the interest of the research community in EV metabolomics has been increasing for its promising diagnostic values of various diseases. The knowledge about the composition and function of the EV metabolome is accumulating. But the clinical applications are still limited by the lack of robust and reproducible methods for obtaining high purity EV samples. In this short review, we will summarize the recent technical advances of EV isolation and purification and the downstream analytical methods for the related metabolic analysis. We will also discuss the new techniques that are needed to move this field forward and the future directions of EV metabolomics.

1. Introduction

Extracellular vesicles (EVs) are lipid bilayer membrane nanoparticles released in the extracellular environment by almost all living cells [1,2]. As the mediators of cell-to-cell communications, EVs carry various biological message materials, including transmembrane proteins on lipid membrane, incorporated cytosolic proteins, RNAs, and metabolites [3,4]. EVs are a heterogeneous group, mainly composed of 3 types of membrane vesicles formed with different cellular origins, *i.e.* microvesicles (MVs), exosomes, and apoptotic bodies [5,6] (Fig. 1a). MVs in a size of 100–1000 nm are formed via cell membrane budding, while exosomes are the smallest vesicles (30–150 nm), generated inside multivesicular endosomes or multivesicular bodies (MVBs). The latter can be directed to lysosomes for degradation or to the plasma membrane for exosome particle releasing after membrane fusion. Moreover, cells undergoing apoptosis can form apoptotic bodies with a relatively large size over 1000 nm in diameter. All EVs express specific surface molecules with different types or different enriching levels, which allows them to be targeted by recipient cells for delivering their contents and further modifying the physiological state of the recipient cells.

EVs are thought to play multiple intercellular communication roles in various physiological and pathological processes, such as regulating cellular communication, cell growth, angiogenesis, and immune modulation [7–9]. Tumor-derived EVs have gained intensive interest in the research field. They are released into cellular microenvironments and circulate in all body fluids, which can promote tumor progression and metastasis by inducing matrix remodeling, angiogenesis, inflammation, and metastatic niche formation [10]. Increasing evidence suggests that EVs could be used as potential markers for cancer detection, cancer prognosis, and for evaluating the treatment outcomes and guide therapy [8,11,12]. The molecules packing mechanism by EV is not clear till now. It is speculated that they may either be packed into vesicles by indirect

Abbreviations: APCI, atmospheric pressure chemical ionization; EI, electron ionization; ESI, electrospray ionization; EVs, extracellular vesicles; EXODUS, exosome detection method via the ultrafast-isolation system; GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry; MVs, microvesicles; MVBs, multivesicular bodies; NMR, nuclear magnetic resonance; PEG, polyethylene glycol; SEC, size exclusion chromatography; TFF, tangential flow filtration; UC, ultracentrifugation.

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https://doi.org/10.1016/j.microc.2021.106816
Received 10 August 2021; Accepted 3 September 2021
Available online 8 September 2021

sorting during the vesicle formation process of cytosol encapsulation or be direct sorting by integrations between biomolecules [13–15]. Moreover, EV content is dynamic and changes in response to different physiological conditions and cell phenotypes, and thus can be, to some extent, considered as a snapshot of the parent cell's physiology.

Metabolites are a type of small molecules containing a large range of species with a molecule weight < 2 kDa, such as steroid hormones, amino acids, the metabolic intermediates of nutrient anabolism and lipids, etc. Unlike exosomal components such as proteins and nucleic acids (miRNA, mRNA, and lncRNA) that have been extensively studied, the exosomal metabolites are the least researched vesicle components so far, possibly because of the following two challenges, including (1) lacking effective EV isolation method to obtain high purity EVs to exclude the non-exosomal metabolites existing in the biofluidic matrix. (2) technique limits to accurately determine and validate the entire EV metabolome. Since metabolites are involved in all cellular processes, EV metabolomics holds great potentials to explore novel biomarkers for cancer diagnosis [16,17]. Considered the unique role of EVs in a biological system, EV metabolomic investigation offers the significant advantage to measure the direct functional readout of phenotype encoded in the genome [18]. As the metabolome is also influenced by the environment, EV metabolomics may also reflect dynamic changes of environmental alternations and are very likely to reveal the impact of non-genetic factors and discover cancer makers at early stages [16,19].

The metabolites are essential components carried by EVs, and the knowledge about the composition and function of these compounds in vesicles is accumulating. This review will focus on the advances of state-of-the-art approaches for EV isolation and metabolome determination, as well as the important and innovative roles that EV metabolomes can play in unlocking their functions in disease diagnosis. Since the terms "EV" and "exosome" are sometimes used interchangeably in literatures, we will keep the nomenclature that is used in the cited references in the present review.

2. EV preparation techniques

EV sample preparation is essential, yet still challenging for EV-related studies in the research community [20,21]. Compared to the conditioned culture medium, the biofluids usually contain a complex matrix making the isolation process very difficult to obtain high purity EV product. The most applied EV isolation methods are summarized in Fig. 1b, including ultracentrifugation (UC), polymer-based precipitation, size exclusion chromatography (SEC), and magnetic beads-based affinity capture. Sequential UC applying 100,000–120,000 g to pellet vesicles is the most popular way to isolate EVs from various biological sources [22]. The optimized method based on UC, namely density

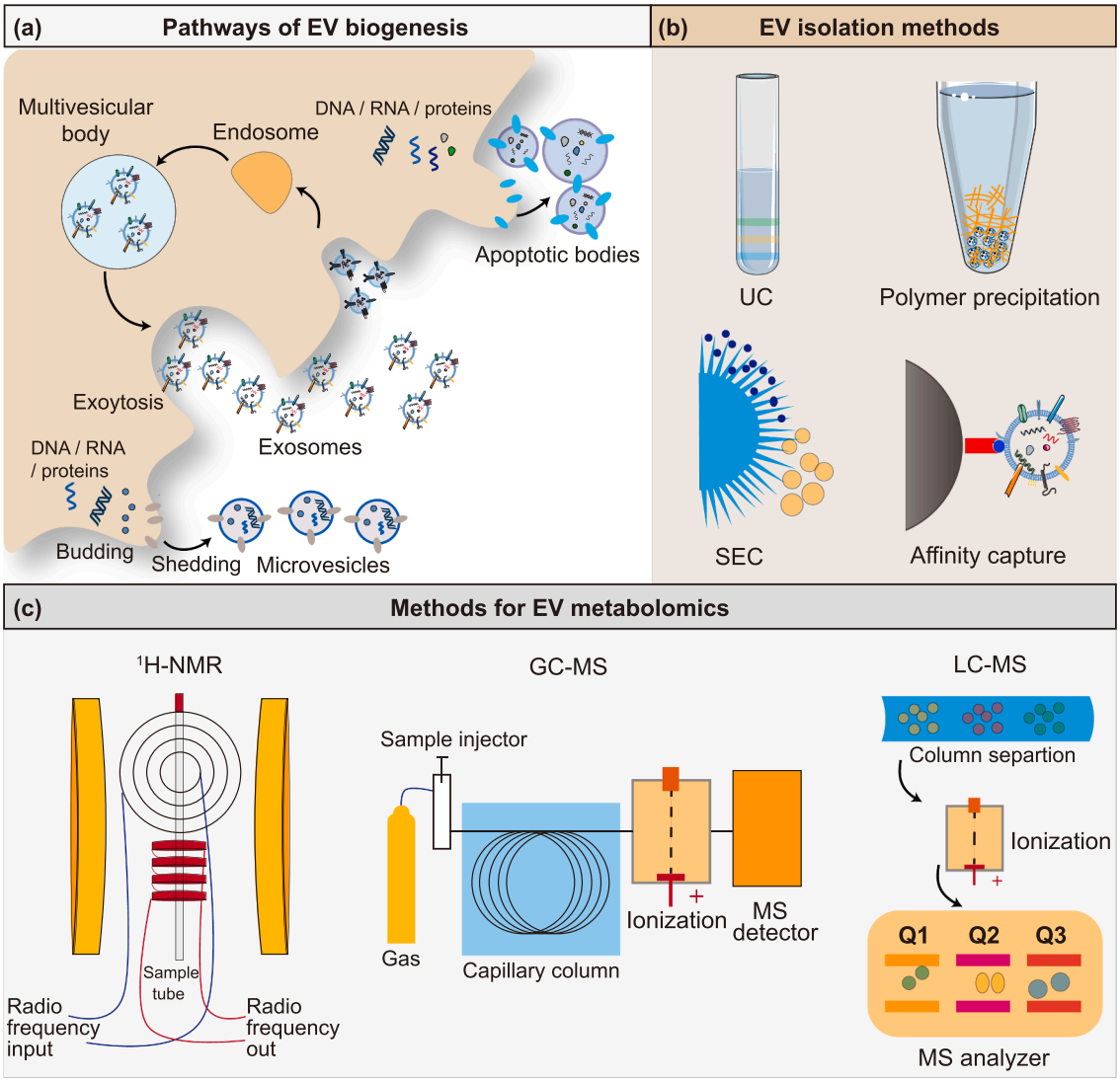

Fig. 1. Methods for EV metabolomic analysis. (a) Biogenesis pathways of different EV subtypes. (b) Illustrations of typical EV isolation methods. (c) Current methods for metabolome characterization, including NMR, GC–MS, and LC-MS.

gradient centrifugation, further allows separating vesicles with different molecular content [23,24]. The resultant EV purity obtained by UC generally meets the requirement for the metabolome analysis. UC is processable for a wide volume range of samples from hundreds of microliters to liters, but it requires time-consuming steps and often results in a low yield [22,25]. Also, one should note that the particle structures such as lipoproteins and amphisomes with similar densities to EVs might be co-isolated and cause contaminations to EV metabolite analysis [26,27].

Polymer-based precipitation is also an often applied approach for EV isolation and purification [28]. This method relies on water-excluding polymers for instance polyethylene glycol (PEG), which can tie up water molecules and force the less soluble vesicles out of solution. The method is easy to perform and does not require expensive instruments. Although the high yield is often reported by the precipitation method, it suffers from low product purity caused by co-precipitation of proteins from the sample matrix as the polymer networks could decrease the solubilities of both EVs and proteins. Therefore, the polymer precipitation method may not be suitable for the studies that require high EV purity such as proteomics and metabolomics, otherwise, the EV sample must be further purified.

SEC applying a porous stationary phase is effective for removing soluble proteins. Components with a small hydrodynamic radius can pass through the pores, resulting in a late elution, while the large particles are excluded from entering the pore becoming early eluted fractions. SEC method could generate relatively high purity EV product and is often used for EVs metabolomic analysis. However, it fails in the separation of particles with similar sizes, for example separating small EVs from lipoproteins [29]. Also, one should be aware that the vesicles might be retained on the columns and cause the loss of product.

Affinity methods including immune affinity and phospholipid membrane affinity have also been applied for EV isolation [30–34]. Immunocapture methods relying on epitope on vesicle surface (such as CD9, CD63, CD81, etc.) may result in homogenous isolation and exclude subtypes from the same origin but enriching different membrane proteins, and thus cause loss of vesicle overall information. Other affinity strategies, such as targeting vesicle membrane via annexin [35], metal [33], or diacyl lipid (DSPE) [36] (usually on magnetic beads) for EV isolation have also been developed, and such methods are reported to have a relatively high EV yield and can capture all types of membrane vesicles.

The new emerging methods based on nanotechnologies and microfluidics have been recently reported, such as tangential flow filtration (TFF) [37], asymmetric flow field-flow fractionation (AF4) [3,8], and exosome detection method via the ultrafast-isolation system (EXODUS) [39]. TFF is a size-based microfluidic method relying on fluid that flows tangentially across a filter to avoid pore-clogging and filter cake formation [37], while the AF4 uses the cross-flow for nanoparticles separation, that induced via carrier liquid generated by the semipermeable wall on the bottom of the channel [38]. Both TFF and AF4 can be performed in a continuous flow manner, thus they are scalable for processing samples with large volumes. EXODUS is an advanced ultrafiltration method based on two facing standing membranes [39]. It uses the negative pressure oscillation and double coupled harmonic oscillators to inhibit fouling effects, which leads to a decrease in the permeated flux and improves sample processing efficiency [39]. The EXODUS may be highly suitable for handing clinical samples for its automation and high consistency features.

The existing EV isolation methods usually need to balance the product purity and yield as the two factors often show an inverse relationship. There is a strong need to develop novel isolation and analytical methods to obtain high purity EV samples in an efficient manner. The throughput should be improved to obtain EV samples on a large scale to meet the requirements of downstream analysis. Also, the importance of precise characterization of vesicles with different subpopulations should be noted, which is essential to understand the mechanism and functionality of these nanoscale vesicles involved in the physiological and pathological process.

3. Metabolite characterization methods

For characterizing metabolites in biological systems, two main analytical techniques are currently applied, *i.e.* nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (MS) (Fig. 1). NMR is a highly reproducible spectroscopic method based on the energy absorption and re-emission of the atom nuclei in an external magnetic field [40]. Hydrogen (1H NMR) is the most commonly targeted nucleus for its natural abundance in biological samples [41]. The carbon (13C NMR) and phosphorus (31P NMR) are less frequently targeted, which add additional NMR information on specific metabolite types [42]. One-dimensional NMR (1D-NMR) spectra using a single frequency axis is the most often applied method in high-throughput metabolomics studies. To have a better peak resolution, the two-dimensional NMR (2D-NMR) method is sometimes applied to separate overlapping spectral peaks that cannot be identified with 1D-NMR spectra [43].

MS acquires spectral data in the form of a mass-to-charge ratio (m/z) and a relative abundance of the measured compounds, which has been extensively studied for metabolomics [44]. Compared to NMR, the MS method is considered with higher specificity and sensitivity given a wide range of instrumental and technical variants that are currently available for metabolite characterization [45]. The modern separation tools such as liquid chromatography (LC) and gas chromatography (GC) are often coupled with MS for pre-resolving compounds, which reduces the high complexity of the biological samples and allows to determine different types of separated analytes at different times. Currently, there are several types of commercially available ionization methods such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and electron ionization (EI), which work effectively for analytes with certain physical and chemical properties. LC-MS utilizing an ESI source is probably the most popular approach that works for polar metabolites, while EI is suitable for non-polar compounds.

In general, metabolomic profiling can be classified into targeted and untargeted methods [46,47]. The targeted metabolomics focus on the identification and quantitation of a defined set of metabolites which are predetermined by the research community, or the metabolite library is available for data analysis. For the untargeted assay, detection and measuring as many signals as possible across a sample set is the aim, and the signals are assigned according to metabolite ID from metabolomics databases. Thus, the target method aims to identify known metabolites accurately, while the untargeted method is highly interested in all detectable signals to find out unknown metabolites. Both methods need to take further steps to confirm identified metabolic signatures to avoid mistaken identifications, which is especially important in clinical studies related to biomarker discoveries.

4. Recent applications of EV metabolomics

EVs function as carriers of various important biomolecules in their unique biogenesis process. They can actively interact with metabolites and directly reflect the phenotypes of a cellular system. This has also been successfully proven by the recent studies in the aspect of biomarker exploring and fundamental EV biology via metabolomic analysis [16,18,48]. To now, the metabolites incorporated in EVs from various biofluids such as human plasma, urine, cell culture medium, etc., have been frequently investigated (Table 1). In the following part, we will exemplify the typical studies (not review all literatures) to show the current research interests of EV metabolomics.

Urinary EVs comprise a wide range of biologically distinct structures and are considered a promising non-invasive diagnostic source for urological diseases. Our group recently developed a method that combines ultrafiltration and phospholipid affinity for obtaining high purity urine EVs for their metabolome profiling using both untargeted and

Table 1
Examples of recent EV metabolomic studies.

EV source	Isolation methods	Metabolite detection	Disease type	Study type
Urine	Ultrafiltration plus TiO_2 beads	LC-MS	–	Method development; Metabolomic profiling [32]
Urine	UC	LC-MS, NMR	Cardiovascular disease	Cardiometabolic biomarker discovery [49]
Urine	UC	LC-MS	Prostate cancer	Markers for cancer and benign hyperplasia [50]
Urine	Flow field-flow fractionation	LC-MS	Prostate cancer	Method development; Biomarker discovery [51]
Cell model	UC	LC-MS	Prostate cancer cell lines	Growth conditions affect EV metabolome [52]
Cell model	UC	LC-MS	Malignant melanoma	Biomarker discovery [53]
Cell model	MagG@PEI@DSP@aptamer	LC-MS	Breast cancer cells	Method development;Differential metabolite [54]
Cell model	UC	NMR	Glioblastoma	Metabolic difference of cells, EVs and media [55]
Cell model	UC	GC–MS	Insect infection	Hexanal compound produced in infection [56]
Cell model	Total exosomes isolation	GC–MS	Prostate cancer	CDEs promoting tumor growth [57]
Serum	UC	LC-MS	Pancreatic cancer	Method development; Biomarker discovery [58]
Serum	SEC	LC-MS	Schizophrenia	Biomarker discovery [59]
Serum/Cell culture	UC	GC–MS; LC-MS	Colorectal cancer	Joint pathways for colorectal cancer [60]
Plasma/Urine	UC	LC-MS	Prostate cancer	Biomarker discovery [61]
Plasma	UC	LC-MS	–	Comparing exosomes and MVs [62]
Plasma/Cell culture	SEC	LC-MS	Head and neckcancer	Purine markers and related pathways [63]
Pleural effusion	UC	LC-MS	Tuberculosis; malignancy	Biomarker discovery [64]
Intestinal microbiota	UC	GC–MS; LC-MS	–	Comparing strains NTBF and EBTF

lipid-targeted LC-MS method [32]. The effective capture of EVs by TiO_2 coated magnetic beads relies on the phospholipid affinity between metal and phosphate groups on the lipid bilayers, which allows to analyze metabolites from the membrane vesicles and exclude contaminants from the urine matrix. Further untargeted metabolomic profiling detected 433 metabolites and the targeted method identifies 467 lipids, indicating the urine EVs carry abundant small compounds with various types. Another study isolates urine EVs by UC and characterizes their metabolites by both ^{1}H NMR and targeted LC-MS method [49]. The metabolic alterations in exosomes are observed for the detection of cardiovascular disease. The added value of the exosomes compared to urine is reported about disease early diagnosis. Using a similar method, researchers have identified metabolic change of urinary EVs for prostate cancer compared to benign prostate hyperplasia, demonstrating that urine EVs can be a non-invasive source of biomarkers closely related to prostate cancer initiation and progression [50].

Cell culture is a very important model to study EV biology as an *in vitro* system. A recent work studies the cell culture conditions, including conventional cell culture dishes and two-chamber bioreactors to elucidate how the growth environment affects the EV characteristics [52]. They report the bioreactor can increase the EV yield more than100 times compared to culture dishes. Furthermore, significant differences in both polar and non-polar metabolites are observed with two different cell culture conditions. This work suggests that the culture conditions should be standardized and thoroughly detailed in publications in terms of EV-related studies, especially for EV metabolomics. Another study attempts to investigate potential biomarkers of melanoma cancer by comparing EV metabolome patterns from the primary patient-derived melanoma cancer cell line enriched in cancer stem cells and patient's serum [53]. The metabolomic profiles are analyzed by LC-MS with an untargeted approach using univariate and multivariate statistical analyses. The authors conclude that the metabolomic characterization of cancer stem cell-derived exosomes might facilitate the discovery of clinically useful biomarkers in neoplasia. Instead of applying the traditional method, a research group uses aptamer-functionalized magnetic graphene oxide for highly efficient enrichment of exosomes from cell culture medium for their metabolomics analysis [54]. The CD63 positive exosomes are isolated from MCF-7 and normal breast cells (MCF-10A) followed by a comprehensive analysis of metabolites using LC-MS. Moreover, an interesting work investigates a possible flux of EV metabolic process in glioblastoma cells using constraint-based modeling based on simulations, showing that the active metabolisms are involved within EVs including enzymatic reactions and the transfer of metabolites through the EV membrane [55].

Peripheral blood provides a source of vesicles for investigating circulating EV metabolites. However, the complexity of the blood matrix and lipoprotein contaminants make EV isolation extremely challenging [65,66]. A recent study explores the potential role of exosomal metabolites in the diagnostics of schizophrenia with over 600 individual participants [59]. The exosomes are isolated from serum samples using a commercial kit called "qEV" based on the principles of SEC. They identify 25 "perturbed molecules" that might be related to neurological diseases by LC-MS analysis. This finding supports the role of exosomal metabolite dysregulation in the pathophysiology of schizophrenia. Researchers also apply the UC method for isolation of serum or plasma EVs for obtaining purity vesicles for metabolomic analysis [60–62]. The colorectal cancer exosomes are studied both from patient's serums and cell lines for multi-omic studies, and the joint pathways show the fatty acid and amino acid metabolism-related pathways are changed significantly in cancer patients [60]. In another assay, for the comparison of microvesicles and small EVs, the authors develop a tandem extraction method based on UC to obtain metabolites from such two types of vesicles simultaneously from the same sample [62]. They observe 50 differential metabolites between small EVs and MVs, demonstrating the variety of EV subpopulations about their metabolomes.

The pleural effusion sample has also been studied as an EV source to investigate their diagnostic potency for tuberculosis pleural effusion and malignancy pleural effusion [64]. The authors isolate large EVs and small EVs via differential centrifugation and characterized the difference between these two subpopulations with metabolomics and lipidomics analysis. Data shows the large EVs display significant alterations between tuberculosis and malignancy groups, and their differential metabolites are more closely related to clinical parameters than those of small EVs. The study of EV metabolomics has been extended to the intestinal microbiota. An interesting study tries to tack the question of whether *toxigenic B. fragilis* (ETBF) vesicles can be utilized for potential pathogenic implementation via vesicle metabolomics [67]. The comparative metabolomic analysis is performed for the vesicles isolated from strains of *nontoxigenic B. fragilis* (NTBF) and ETBF by both LC-MS and GC–MS with subsequent reconstruction of the vesicle metabolic pathways. They conclude that the metabolic activity of ETBF vesicles showing similarity to microreactors are likely to be used for long-term persistence and implementing pathogenic potential in the host.

Unlike exploring EV metabolome in a global view, focusing on a specific type of metabolite that is closely related to diseases has also been studied. The gene expression levels of the purine synthesis pathway in exosomes are investigated using the Cancer Genome Atlas Head and Neck Cancer database [63]. Exosomes are isolated from UMSCC47 cells and patient plasma using SEC and the metabolites are characterized by LC-MS. The results show evidence that the head and neck squamous cell carcinoma (HNSCC) cells shuttle purine metabolites in exosomes with immunosuppressive adenosine being the most prominent purine.

Changes in the content and levels of purine metabolites in circulating exosomes may reflect disease progression in HNSCC patients. Another study focuses on the volatile organic compounds in the EV metabolome to understand how insects are attracted to humans [56]. The authors purify EV samples using UC from *plasmodium falciparum*-infected cultures with high and low parasitemias, which are then analyzed by GC–MS for metabolome determination. Hexanal (insect attractant) has been observed in all samples from infected erythrocytes but not in uninfected ones, suggesting it may be originated during parasite infection.

From the above-mentioned examples, we can see that EVs have shown exciting opportunities for metabolomic investigations. A large part of relevant studies focuses on the topic related to biomarker discoveries with the investigation of EVs derived from various biofluids. This may be largely because EVs are considered a promising source of potential biomarkers. Compared to the well-established metabolomic studies of biofluids such as serum, plasma and urine [68–70], investigation of metabolome from EV is a newly emerging approach and the database of EV metabolites is still accumulating.

5. Conclusions and future directions

The downstream metabolites can directly reflect the phenotype change and serve as signaling molecules in the processes of gene and protein regulations. EV metabolome profiling is extremely important for providing unique insights into the pathogenesis of disease and EV biological pathways. However, the research efforts have yet made viable clinical translations. Currently, there are intractable practical difficulties involved in EV metabolome analysis mainly due to the lack of robust methods for vesicle isolation and purification. The most existing methods represent only proof-of-concept approaches and need to be refined to demonstrate the speed, throughput, and automation for clinical applications. Also, more specific and reproducible methods are expected to develop to enable independent laboratories to achieve highly similar results.

The conventional analytical platforms for metabolomics are NMR and MS, and not a single method could capture all metabolites. Thus, suitable methods should be selected to meet the experimental purpose. In general, most published EV metabolomic studies have focused on biomarker discoveries, and machine learning is the most applied strategy to identify differential metabolites. However, less attention has been paid to the validation of the detected metabolic signatures to exclude false-positive results. The metabolic biomarkers have rarely been validated given the fact that the individual metabolite targeting is difficult to be precisely conducted in complex biological samples.

For future studies, the cross-validation of the potential metabolic markers is needed. Additionally, EV metabolomics might also be performed and analyzed in combination with EV proteomics and transcriptomics to provide more evidence for understanding the biological mechanism, and thus to better understand the flow of information that underlies diseases.

CRediT authorship contribution statement

Qingfu Zhu: Conceptualization, Funding acquisition, Investigation, Writing - review & editing. **Yijiang Huang:** Funding acquisition, Investigation, Writing - review & editing. **Qinsi Yang:** Investigation, Writing - review & editing. **Fei Liu:** Conceptualization, Funding acquisition, Investigation, Writing - review & editing.

Declaration of Competing Interest

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

Acknowledgments

This work was supported by research funding provided by Wenzhou Basic Research Projects (grant numbers: Y2020916, Y2020987), Affiliated Eye Hospital of Wenzhou Medical University (grant numbers: KYQD20191101).

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