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体液外泌体代谢组学研究进展和挑战

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摘要　外泌体作为疾病诊断、监测和治疗的非侵入性生物标志物，在体液中广泛存在. 作为细胞间信使，外泌体携带有丰富的蛋白质、核酸和代谢物，目前大多数研究集中在蛋白质和RNA上. 最近，外泌体代谢组学在疾病检测和疾病病理生理学研究中展示出临床价值和潜在优势，但仍存在诸多挑战，特别是外泌体分离和代谢物检测. 本文综合评述了外泌体分离和代谢物检测方面的技术进展和挑战，并通过案例研究，展示了不同类型体液的外泌体代谢物作为生物标志物在疾病早期诊断和治疗中的潜力.

关键词　外泌体；代谢组学；体液；生物标志物；疾病诊断
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Advances and Challenges of Exosome Metabolomics in Body Fluids

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Abstract　Exosomes, ubiquitously present in body fluids, serve as non-invasive biomarkers for disease diagnosis, monitoring, and treatment. As intercellular messengers, exosomes encapsulate a rich array of proteins, nucleic acids, and metabolites, although most studies have primarily focused on proteins and RNA. Recently, exosome metabolomics has demonstrated clinical value and potential advantages in disease detection and pathophysiology, despite significant challenges, particularly in exosome isolation and metabolite detection. This review discusses the significant technical challenges in exosome isolation and metabolite detection, highlighting the advancements in these areas that support the clinical application of exosome metabolomics, and illustrates the potential of exosomal metabolites from various body fluids as biomarkers for early disease diagnosis and treatment.

Keywords　Exosome; Metabolomics; Body fluid; Biomarker; Disease diagnosis

1　Introduction

Exosomes are extracellular vesicles with a lipid bilayer, secreted by living cells through exocytosis, measuring approximately 30—150 nm in diameter[1,2]. The most widely accepted mechanism for their formation involves the endosomal pathway[3,4]: extracellular soluble proteins and cell membrane proteins are internalized, leading to the formation of early sorting endosomes(ESEs); these migrate near the nucleus and mature into late sorting endosomes(LSEs), which then form multivesicular bodies(MVBs) containing multiple intraluminal vesicles(ILVs). Some MVBs migrate to the cell membrane and merge with it, releasing ILVs as exosomes through exocytosis. Alternative biogenetic pathways, such as plasma membrane budding, are also recognized[5]. Virtually all cell types, including tumor cells, mesenchymal stem cells, dendritic cells, and

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macrophages, can secrete exosomes[6]. After formation, exosomes are ubiquitously present in various body fluids such as blood, urine, breast milk, cerebrospinal fluid, saliva, and pleural effusion[7,8]. Moreover, exosomes can be absorbed and released into tissues[9]. As recorded in major databases like ExoCarta, Vesiclepedia, and EVpedia, exosomes carry numerous biomolecules, including proteins, RNA, DNA, lipids, polysaccharides, metabolites, and other bioactive substances[10—13]. Initially considered merely cellular waste[14—16], exosomes are increasingly acknowledged as crucial mediators of intercellular communication[10,17,18]. They interact directly with receptors on target cells[19—21], initiating downstream signaling cascades, or merge with target cell plasma membranes[22—24] or are internalized[25—27] to transfer their molecular cargo. This facilitates roles in tumor development and metastasis, stem cell maintenance, antigen presentation, cell migration, and proliferation[4,10,28]. Exosomes and their contents are finely tuned according to the parent cell's type and physiological or pathological states, such as differentiation, transformation, stimulation, or stress[29], allowing for precise assessments of the parent cell's function and status.

Exosomes and their molecular contents offer diagnostic and therapeutic insights for a range of diseases, such as cardiovascular diseases, neurodegenerative disorders, inflammation, and cancers[10,17,18]. Particularly in oncology, with the advent of precision medicine, exosomes together with circulating tumor cells, tumor DNA, and microRNAs, have become key biomarkers in liquid biopsies[29—31]. Recently, due to their greater abundance, stable membrane structure, higher marker specificity, and higher concentrations in cancer patient fluids[32], exosomes are progressively supplanting circulating tumor cells in cancer diagnostics, prognosis, and treatment[10,17,18].

The introduction of precision medicine strategies in 2015 provided new avenues for accurate disease monitoring and diagnosis. Precision medicine relies on modern molecular detection technologies such as genomics, transcriptomics, proteomics, and metabolomics for precise identification of disease causes and therapeutic targets. Changes in genes may not necessarily be expressed, and altered proteins may not be active, with compensatory mechanisms potentially negating any substantial effects on the organism. The high-quality RNA samples required for transcriptomics are often challenging to procure. Metabolomics, the closest technology to phenotypic changes, accurately reflects the ultimate functional or state changes in biological systems upon external stimuli and is extensively used for disease diagnosis and biomarker discovery[33—35]. Exosome metabolomics offers several advantages over direct fluid metabolomics: it can act as a "snapshot" reflecting the pathophysiological state of the source cell; the double lipid layer of exosomes protects metabolites from dilution and degradation, enhancing sensitivity and reducing individual variations; and the discovery that changes in exosomal metabolites are more pronounced, making them sensitive indicators of abnormalities[36,37].

Reports have utilized the rich nucleic acid content within exosomes for the discovery of biomarkers and mechanistic studies in various cancers; numerous transmembrane and intracellular proteins have been employed in exosomal proteomics research, aiding early cancer screening and prognostic evaluations in clinical studies. Although exosome metabolomics research is still in its infancy, it shows tremendous potential and promising prospects in tumor research, presenting numerous challenges that remain to be addressed. The basic workflow for exosome metabolomics research involves the isolation and characterization of exosomes, extraction, detection and identification of metabolites within exosomes, data processing, and the discovery of differential metabolites(Fig.1). The main challenges in exosome metabolomics research lie in the isolation of exosomes and the detection of metabolites. Nevertheless, exosome metabolomics is increasingly attracting researchers' interest, partly because metabolites are involved in all cellular processes, offering significant potential in exploring new biomarkers for tumor diagnosis; and partly because metabolites are the final and

most direct manifestation of gene and protein changes, reflecting the impacts of environmental changes, thus accurately mirroring the current state of the biological system and guiding disease diagnosis and drug screening.

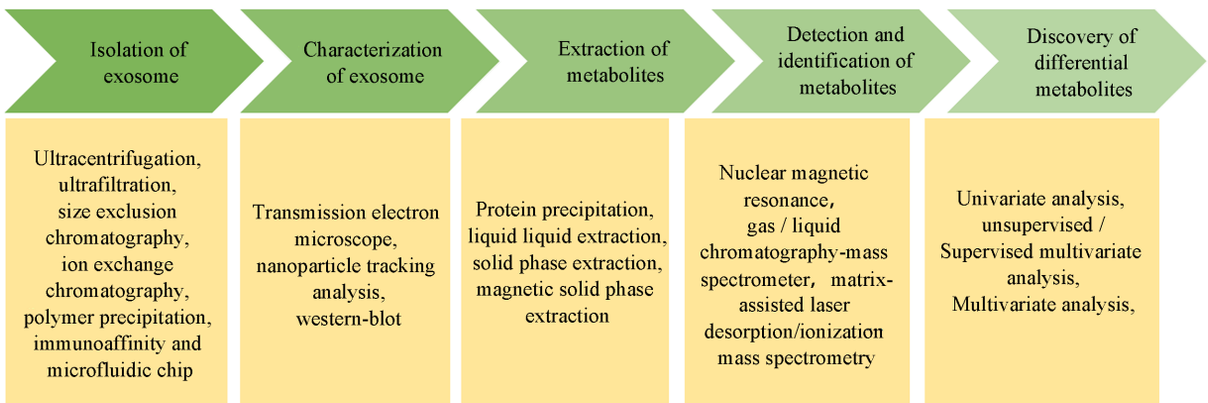

Fig. 1 Basic workflow for exosome metabolomics research

This review will focus on exosome isolation techniques and metabolite detection methods, as well as the significant role of exosome metabolomics in disease diagnosis and treatment.

2 Challenges of Exosome Metabolomics in Body Fluids

2.1 Exosome Isolation Techniques from Body Fluids

Due to the low abundance of exosomes, their small size, and interference from high-abundance proteins and other types of vesicles, as well as heterogeneity in size, density, and marker protein expression, efficient and specific isolation of exosomes from body fluids is a prerequisite and challenge for exosome metabolomics research. Current methods for isolating exosomes from circulation are based on their size, density, and immunophenotype, including ultracentrifugation, ultrafiltration, polymer precipitation, size exclusion chromatography, ion exchange chromatography, immunoaffinity and microfluidic chip(Table 1).

Table 1 Methods for separating exosomes from body fluids

Separation technique	Principle	Advantage	Disadvantage
Ultracentrifugation	Density	Mature method, "gold standard"	Expensive equipment, time-consuming(>4 h)
Ultrafiltration	Size	Simple operation, suitable for small volumes	Low purity, affects exosome integrity
Polymer precipitation	Solubility	Simple, no expensive equipment required	Low purity, affects exosome integrity
Size exclusion chromatography	Size	High purity, good reproducibility	High maintenance cost, low concentration, requires additional concentration steps
Ion exchange chromatography	Charge	High purity	Requires multiple optimizations, affects exosome integrity
Immunoaffinity	Affinity	High purity, high specificity	High cost, not suitable for large-scale
Microfluidic chip	Fluid dynamics	Automation and high throughput	High equipment cost, technical complexity

2.1.1 *Traditional Exosome Isolation Methods* Traditional methods for isolating exosomes include requirements, yield, and purity of the separated exosomes. Comparing common exosome isolation methods used in 2015 and 2019, it is evident that all methods are continuously evolving, yet no universally optimized exosome isolation method has been established[38].

2.1.2 *Exosome Isolation Method Based on Chromatography and Microfluidic Chip* Size Exclusion Chromatography(SEC) is a column-based separation method based on particle size. When a liquid sample passes through a stationary phase composed of porous polymer beads, particles smaller than the pore size enter

the pores and are slowed down, eluting later from the column; particles larger than the pore size bypass the beads and elute earlier. SEC can achieve high purity and intact membrane structure of exosomes, with good reproducibility and short separation time. However, SEC also has drawbacks, such as high maintenance costs for the required high-performance liquid chromatography (HPLC) system, inability to completely remove contaminants with sizes similar to exosomes, and low concentration of isolated exosomes, necessitating additional enrichment or concentration steps, which limit its application in high-throughput exosome isolation[39].

Ion Exchange Chromatography (IEC) is another column-based separation method based on surface charge differences of molecules. Utilizing the negative charge of exosomes and the positive charge of the resin in the column, reversible charge interactions occur, and different molecules are gradually exchanged and eluted by changing the ionic environment with gradient salt solutions or pH changes. IEC can achieve high purity of exosomes; however, the separation effectiveness is highly dependent on operating conditions such as pH and ionic strength, requiring multiple optimizations and precise control. Additionally, strong charge interactions and high ionic concentrations during ion exchange may damage the integrity and biological activity of exosomes.

Microfluidic-based exosome isolation techniques utilize the biochemical and physical properties of exosomes on microchips. These techniques can be broadly classified into several categories: modified microfluidic channels based on immunoaffinity, microfluidic chips based on size exclusion, dynamic microfluidic techniques facilitated by fluid properties or external forces, and orthogonal combinations of the aforementioned methods[40]. Microfluidic technology, known as "lab on a chip", operates fluids at the microliter or nanoliter scale, reducing sample and reagent usage. High-precision fluid control aids in the precise separation of exosomes, while the high throughput and automation of the devices enhance separation efficiency[41,42]. However, there are drawbacks such as the complexity of design and fabrication, as well as the propensity for channel clogging, making these methods unsuitable for large-scale exosome separation.

2.1.3 *Novel Exosome Isolation Methods Based on Nanomaterials* Immunoaffinity is a method for isolating exosomes that exploits the interaction between highly expressed biomarkers on the exosome surface and corresponding antibodies. This technique primarily includes immunological affinity based on exosomal proteins, as well as membrane affinity which targets other components on the exosome surface.

In immunoaffinity methods, commonly used markers for exosomal proteins and aptamers are transmembrane proteins such as CD81, CD63, CD9, and CD82. Antibody-based immunoaffinity methods offer strong affinity, high purity, and high specificity, but their high selectivity also implies limitations in separation. Specifically, exosomes that do not express certain marker proteins cannot be isolated, resulting in low exosome yield. Therefore, in addition to protein-based immunoaffinity methods, membrane affinity methods that target other components on the exosome surface are also utilized for exosome separation. For example, common techniques include the interaction between phosphatidylserine (PS) on the exosome lipid membrane and proteins (like Tim4 protein[43]), peptides (such as CLIKKPF[44]), or metals or metal oxides (like Ti^{4+}[45], Zr-O[46]). Additionally, interactions between heparin and heparan sulfate proteoglycans on the exosome membrane surface[47], lectins and polysaccharides on the membrane surface[48], as well as overexpressed glycan chains like mannose and poly-N-acetylglucosamine[49], are also employed for exosome isolation.

For effective immunoaffinity-based exosome isolation, antibodies are typically fixed on solid substrates, with columns, plates, beads, and microfluidic chips being gradually developed and utilized[50]. In recent years, magnetic nanomaterials have also been widely used as solid substrates for exosome isolation (Table 2).

Their advantages include: (1) size matching: the size of nanomaterials matches that of exosomes (30—200 nm), greatly improving contact efficiency and enhancing interactions, thereby increasing separation efficiency; (2) high specific surface area: nanomaterials often have a high specific surface area, providing more sites for modification and exosome capture, increasing yield; (3) easy modification: the surface of nanomaterials can be chemically or physically modified with specific functional groups, allowing for specific recognition and binding of exosomes, improving selectivity; (4) easy removal: magnetic nanomaterials do not require complex centrifugation steps and can achieve rapid solid-liquid separation through an external magnetic field, simplifying the separation process and increasing efficiency; (5) easy detection: nanomaterials used for exosome capture and isolation can be integrated into various analytical techniques, such as microfluidics, biosensing, and surface-enhanced Raman scattering (SERS), combining separation and detection. Some nanomaterials have inherent signal enhancement effects, increasing the sensitivity of detection methods. Nanomaterials for immunoaffinity can be broadly categorized into antigen-antibody affinity nanomaterials, aptamer affinity nanomaterials, and other affinity nanomaterials. Additionally, nanomaterials based on size exclusion have also been used for exosome isolation. The following sections will introduce representative studies for these four categories of nanomaterials.

Table 2 Nanomaterials for exosome isolation

Nanomaterial	Mechanism of exosome separation	Target	Source of exosome	Analyte	Analysis platform	Ref.
CD9-HPLC-IAC	Antibody affinity	CD9	Serum	Protein	LC-MS/MS	[51]
CoMPC@Au-Apt	Aptamer affinity	CD63	Urine	Metabolite	MALDI-MS	[52]
TiO_2	Ti-O and phosphate groups interaction	Lipid bilayer	Serum	Protein	LC-MS/MS	[53,54]
Fe_3O_4@PDA@UiO-66-NH_2	Zr-O and phosphate groups interaction	Lipid bilayer	Urine	Phosphoryl-peptide	LC-MS/MS	[55]
Fe_3O_4@SiO_2@Eu_2O_3	Eu-O and phosphate groups interaction	Lipid bilayer	Plasma	Metabolite	LC-MS/MS	[56]
$CaTiO_3$/Al^{3+}/Pr^{3+}/Sm^{3+}	$CaTiO_3$, Al^{3+}, Pr^{3+}, Sm^{3+} and phosphate groups interaction	Lipid bilayer	Serum	Protein	MALDI-MS	[57]
Phospholipid-MIP	MIP recognition for phosphatidylserine (PS)	Lipid bilayer	Plasma	Protein	LC-MS/MS	[58]
Heparin-agarose beads	Heparin and proteoglycans interaction	Lipid bilayer	Plasma	RNA	RT-qPCR	[47]
EXODUS	Size exclusion	—	Plasma	Metabolite	LC-MS/MS	[59]
SNAPs	Size exclusion	—	Urine	Protein	LC-MS/MS	[60]

Cai *et al.* [61] successfully developed antibody CD63-based superparamagnetic affinity nanomaterials IS-NPs. Through the host-guest interaction between β-cyclodextrin (β-CD) and 4-aminoazobenzene (AAB), the antibody CD63 is linked to superparamagnetic nanoparticles, enabling efficient capture of exosomes from cell supernatants (4T1 and MCF-7 cell culture mediums) and bodily fluids (serum, saliva, and urine). Exosomes are then gently eluted using the competitive host molecule α-CD, achieving capture rates up to 80% and release rates up to 86.5% after elution. Compared to traditional ultracentrifugation, polyethylene glycol precipitation methods, and commercial kits, IS-NPs provide higher purity and quantity of captured exosomes.

Chang *et al.* [62] synthesized aptamer CD63-based affinity magnetic nanomaterials MagG@PEI@DSP@aptamer, achieving rapid capture, efficient enrichment, and non-destructive elution of exosomes for metabolomic analysis. This composite material consists of Fe_3O_4 nanoparticles doped with graphene and modified with polyetherimide (PEI) as the base, aptamer CD63 as the exosome capture unit, and 3,3′-dithiobis (succinimidyl propionate) (DSP) as a cleavable cross-linker between them. The material's exosome capture capacity reaches up to 450 μg/mg, and exosomes can be released non-destructively by cleaving the internal disulfide bonds of the DSP molecule. Researchers used this material to capture exosomes derived from the breast cancer cell line MCF-7 and conducted non-targeted metabolomic analysis on the exosome contents,

revealing a significantly higher number of metabolite features detected compared to traditional ultracentrifugation(4528 in positive charge mode *vs*. 3967, and 3785 in negative charge mode).

Gao *et al*. [53] first discovered and utilized the specific interaction between TiO_2 and the phosphate groups on the exosomal lipid bilayer, isolating exosomes from human serum for downstream proteomic analysis. The high affinity interaction between TiO_2 and exosomes not only allows for efficient separation within 5 min but also enables reversible recovery of exosomes, with a recovery rate of 93.4%. Subsequent proteomic analysis identified 59 upregulated proteins, 30 of which have been reported to be closely related to pancreatic cancer, and 29 previously unreported, indicating the potential of this method for disease diagnosis and health monitoring.

Li *et al*. [60] proposed a novel negative separation method for extracellular vesicles(EVs) based on surface nanosieve polyethersulfone particles(SNAPs). Impurities such as proteins in the sample are irreversibly adsorbed by graphene oxide(GO) in the SNAPs, while larger EVs(30—150 nm) are excluded from SNAPs with surface pore sizes of 10—40 nm. This method achieved an exosome purity of $(4.91\pm1.01)e^{10}$ particles/μg from urine, which is 40.9 234 times higher than ultracentrifugation, size exclusion, and PEG precipitation methods; the recovery rate ranged from 90.4%—93.8%, which is 1.8—4.3 times higher than ultracentrifugation and size exclusion methods, and comparable to PEG precipitation methods. Researchers then used this method to isolate urine EVs from IgA nephropathy patients and healthy volunteers for proteomic analysis.

2.2 Metabolite Detection Techniques

Due to the diversity in molecular polarity, molecular weight, concentration, and functional groups of metabolomic analysis targets, and the generally low concentration of endogenous metabolites in biological samples, the analytical methods used must meet the requirements of high throughput, high sensitivity, and unbiased analysis. The main platforms for metabolite detection in metabolomics are as follows:

2.2.1 *Nuclear Magnetic Resonance (NMR)* NMR has advantages in metabolomics analysis such as being non-destructive, highly reproducible, providing structural information, and having strong quantitative analysis capabilities, making it a powerful tool for metabolite detection. Agudiez *et al*. [63] first established a new method for urine exosome metabolomics using NMR, with cardiovascular disease as a disease model. This method is compatible with LC-MS/MS analysis and has been successfully applied to clinical samples. Exosome metabolomics results showed significant changes in the levels of 4-aminocyclopropane-1-carboxylic acid, *N*-1-methylnicotinamide, and citric acid in urine exosomes from patients at risk of cardiovascular disease compared to healthy individuals.

2.2.2 *Mass Spectrometry (MS)* MS exhibits high selectivity and sensitivity in metabolomics, especially in determining metabolite types. However, MS alone cannot fully meet metabolomics requirements, necessitating the coupling of multiple analytical platforms. Common combinations include GC-MS, LC-MS, and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). High-performance liquid chromatography-mass spectrometry(HPLC-MS), with its high resolution and sensitivity, is the most common technique for exosome metabolite detection. HPLC-MS can effectively separate and detect low-concentration metabolites in complex biological samples; chromatographic separation reduces ion suppression effects from complex sample matrices in MS detection, improving qualitative and quantitative accuracy, and can identify more low-concentration metabolites in exosomes. HPLC-MS is suitable for comprehensive non-targeted and targeted exosome metabolomics profiling, differential metabolite discovery, and metabolic pathway analysis. In LC-MS, the commonly used acquisition modes include Data Dependent Acquisition (DDA), Data Independent Acquisition(DIA), and Full Scan. In DDA, the mass spectrometer alternates between scanning

for precursor ions (typically in MS1) and selecting specific precursor ions for fragmentation (in MS2). This approach selects ions based on intensity during the MS1 scan, and only a certain number of the most intense ions are selected for MS2 analysis. In DIA, the mass spectrometer systematically fragments all ions within a predefined mass range, without pre-selection based on intensity. This method generates MS2 spectra for every ion, providing a comprehensive dataset. In a Full Scan mode, the mass spectrometer continuously scans across a wide mass range (MS1) without selecting specific ions for fragmentation. This approach generates a comprehensive MS1 profile of all ions present in the sample. The choice between these acquisition modes depends on the specific goals of the study. DDA is best suited for detailed structural analysis of selected metabolites, but it may miss low-abundance compounds and has limited coverage. DIA offers comprehensive coverage and improved quantification across all metabolites, but requires sophisticated data analysis tools due to its complexity. Full Scan is efficient for obtaining an overall metabolomic profile with high throughput, but lacks the specificity and structural information provided by MS2 data. GC-MS in metabolomics offers high sensitivity and specificity for analyzing volatile and thermally stable metabolites, making it ideal for small molecules like sugars, carboxylic acids, amino acids, alcohols, and amines. GC-MS is particularly effective for analyzing volatile and thermally stable metabolites, offering robustness and quantitative accuracy. However, GC-MS requires chemical derivatization, limiting its applicability to non-volatile or thermally labile compounds. MALDI-MS offers simple sample preparation, rapid detection, and high throughput, increasingly applied in metabolite detection in recent years.

Furthermore, in metabolomics, addressing the challenges of extracting low concentrations of metabolites, optimizing detection methods, and standardizing data is critical to obtaining reliable results. To extract low-abundance metabolites effectively, advanced sample preparation techniques such as solid-phase extraction (SPE) and liquid-Liquid extraction (LLE) are employed to concentrate metabolites, enhancing their detectability. Additionally, enrichment strategies like chemical derivatization and affinity-based methods selectively isolate metabolites, further improving detection. Optimizing detection methods is equally important; high-resolution mass spectrometry (HRMS), including Orbitrap and TOF analyzers, offers high sensitivity and accuracy, crucial for identifying low-abundance metabolites. Complementary techniques like ion mobility spectrometry (IMS) and ultra-high-performance liquid chromatography (UHPLC) enhance separation and detection in complex samples. To ensure the reliability and reproducibility of data, standardization is achieved through the establishment of standard operating procedures (SOPs), the use of certified reference materials, and the implementation of data normalization techniques. quality control (QC) samples are routinely included to monitor instrument performance and data quality. Finally, data harmonization is facilitated by adhering to reporting standards and utilizing metabolomics databases, ensuring consistency and comparability across studies. Together, these approaches enhance the precision and reliability of metabolomic analyses, enabling the accurate detection and quantification of metabolites even at low concentrations.

2.3 Metabolite Analysis Techniques

In metabolomics, various analytical strategies are employed to detect and quantify metabolites, each tailored to address specific research objectives and challenges. These strategies not only differ in their methodological approaches but also in their application contexts, sensitivity, specificity, and the type of data they generate. Understanding the strengths and limitations of each strategy is crucial for selecting the appropriate method to achieve reliable and meaningful results. Below, we discuss the three main analytical strategies used in exosome metabolomics: targeted, non-targeted, and pseudotargeted metabolomics, highlighting how each approach contributes to the overall understanding of the metabolomics.

2.3.1 *Targeted Metabolomics* Targeted metabolomics is highly specific and sensitive, allowing for the precise quantification of a predefined set of metabolites. This method is particularly advantageous when the metabolites of interest are well-known and relevant to a specific biological pathway or disease state. It offers high reproducibility and is effective in quantitative studies where the absolute concentration of metabolites is critical. However, targeted metabolomics is limited by its focus on a predefined set of metabolites, which may result in the exclusion of novel or unexpected metabolites. Additionally, the need for extensive method development and optimization, including the use of internal standards for each targeted metabolite, can be resource-intensive. Clos-Garcia *et al.* [64] conducted targeted metabolomics analysis of metabolites in the urine of patients with benign prostatic hyperplasia (BPH) and prostate cancer (PCa), and mapped the differential metabolites to corresponding cellular pathways. Among them, 76 differential metabolites showed significant differences between BPH and PCa, and reproduced many metabolic changes reported in PCa.

2.3.2 *Non-Targeted Metabolomics* Non-targeted metabolomics provides a comprehensive overview of the metabolome, enabling the discovery of a wide range of metabolites without prior knowledge. This approach is particularly useful for hypothesis generation, biomarker discovery, and understanding the broad metabolic landscape of exosomes. It allows researchers to identify unexpected or novel metabolites that may be relevant to disease processes. At present, exosome metabolomics analysis mainly focuses on exosome non-targeted metabolomics. Yang *et al.* [65] conducted a non-targeted metabolomics analysis of urine exosomes from 75 patients with early-stage lung cancer and 27 healthy volunteers, and identified a diagnostic model consisting of markers such as xanthine nucleoside. This model has a high accuracy in distinguishing and predicting early-stage lung cancer with an AUC >0.84.

2.3.3 *Pseudotargeted Metabolomics* Pseudo-targeted metabolomics offers a middle ground between targeted and non-targeted approaches. It involves the use of a broad set of multiple reaction monitoring (MRM) transitions that cover a wide range of metabolites, enabling both the comprehensive coverage of non-targeted metabolomics and the quantification accuracy of targeted approaches. This method enhances the sensitivity and reproducibility of metabolite detection, making it particularly useful in studies where a balance between coverage and quantification is needed. The pseudo-targeted approach still requires extensive method development, including the selection and validation of MRM transitions, which can be time-consuming. Additionally, while it offers broader coverage than purely targeted methods, it may still miss some metabolites that fall outside the predefined transition list, thus limiting its comprehensiveness compared to a fully non-targeted approach.

3 Applications of Exosome Metabolomics in Body Fluids

Exosomes are widely present in various human body fluids, and detecting changes in exosomal metabolites in patient fluids can provide crucial clinical information for disease diagnosis and treatment. This section aims to demonstrate the significant role of exosome metabolomics in disease research across different types of body fluids (serum, plasma, urine, and other fluids)(Table 3).

3.1 Plasma Exosome Metabolomics

Non-alcoholic fatty liver disease (NAFLD) is considered a hepatic manifestation of metabolic syndrome. Metabolomic analysis of circulating exosomes can provide early signals of metabolic disorders and complications associated with NAFLD. Jiang *et al.* [66] extracted plasma exosomes from NAFLD patients with or without impaired fasting glucose using differential centrifugation. Metabolomic profiling using ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) revealed significant changes in linoleic acid metabolism and fatty acid pathways in NAFLD patients with impaired

fasting glucose. These preliminary results provide a foundation for further studies on exosome biology in patients and may help elucidate the intrinsic nature of metabolic dysregulation in the pathogenesis of NAFLD. Liu *et al.* [67] conducted a comprehensive proteomic and metabolomic analysis of plasma-derived exosomes from patients with no tumor (TFC), prostate cancer (PCa), and castration-resistant prostate cancer (CRPC). Metabolomic evaluation using LC-MS/MS identified a series of differentially expressed metabolites, with a combination of these metabolites showing ROC values of 0.94 for distinguishing PCa from TFC and 0.97 for distinguishing CRPC from PCa. This suggests the potential of these metabolites as biomarkers for CRPC. Neoadjuvant chemotherapy (NAC) is commonly used for breast cancer (BC) patients, with only 30% achieving pathological complete response (pCR), while residual disease (RD) is associated with poor long-term prognosis. A key challenge in improving NAC outcomes is the limited understanding of the mechanisms underlying differential treatment responses. Joshi *et al.* [68] identified differential metabolic pathways and metabolites in plasma exosomes from RD compared to pCR patients. Results showed significant upregulation of succinate and L-lactate in RD patients' plasma exosomes after NAC, highlighting their importance in understanding the disease course. Identifying exosomal metabolic features associated with RD or pCR may provide insights into metabolic pathways regulating NAC response in BC patients.

3.2 Serum Exosome Metabolomics

Tang *et al.* [69] conducted a comprehensive metabolomic analysis of serum exosome samples from high-altitude cerebral edema (HACE) patients and healthy controls (HC) using UHPLC-MS/MS, along with KEGG pathway enrichment analysis to identify affected metabolic pathways in HACE patients. Differentially abundant metabolites were identified as potential biomarkers for HACE. KEGG pathway enrichment analysis revealed several pathways significantly impacting energy metabolism regulation, providing potential targets for early diagnosis and therapeutic intervention in HACE patients. Fan *et al.* [70] isolated exosomes from hypoxia-preconditioned and control serum samples using qEV columns, followed by metabolomic analysis with UHPLC-MS/MS. The analysis revealed 136 differentially expressed metabolites with significant performance in distinguishing hypoxia-preconditioned subjects from controls. Bioinformatics analysis identified five metabolites with excellent performance in this differentiation. Yang *et al.* [71] extracted serum exosomes from patients with diabetic retinopathy (DR) and diabetic nephropathy (DN) and used glomerular endothelial cells (HGECs) as a cell model to examine differential proteins and metabolites in exosomes using quantitative proteomics and metabolomics. The results indicated that endothelial dysfunction might be primarily caused by upregulating factor fibrinogen (FIBA) and downregulating 1-methylhistidine (1-MH). FIBA related to 1-MH might reduce excessive cysteine and methionine metabolism pathways, with taste transduction possibly playing a role. This helps understand the molecular mechanisms of exosome involvement in diabetic macrovascular endothelial dysfunction.

3.3 Urine Exosome Metabolomics

As a major route for the excretion of metabolites, the composition of urine is closely related to the physiological and pathological states of the body. Due to its non-invasive nature, ease of collection, and minimal endogenous interference, urine samples have become increasingly popular in exosome metabolomics research, especially for diseases directly related to the urinary system such as bladder cancer[72], prostate cancer[73,74], and chronic kidney disease[75]. Skotland's group[76] used sequential centrifugation to extract exosomes from the urine of healthy subjects and prostate cancer patients and analyzed the lipidome of the exosomes using high-throughput mass spectrometry. The experiment analyzed 107 lipids, with significant differences found in 9 lipids, particularly lactosyl-*N*-palmitoyl-sphingosine (d18∶1/16∶0), phosphatidylserine (18∶1/18∶1), and phosphatidylserine (16∶0—18∶1), which allowed the researchers to accurately and

sensitively differentiate between the two groups. Lactosyl-*N*-palmitoyl-sphingosine(d18∶1/16∶0), phosphatidylserine(18∶1/18∶1), and phosphatidylserine(16∶0—18∶1) show promise as biomarkers for diagnosing prostate cancer. Clos-Garcia and others[64] used ultracentrifugation to extract exosomes from the urine of 14 patients with benign prostatic hyperplasia and 31 prostate cancer patients and conducted metabolomics analysis using UHPLC-MS. The results showed significant differences in 76 out of the 248 analyzed metabolites, mainly phosphatidylcholines, acetylcholine, and sterols. Samples from patients with benign prostatic hyperplasia had higher levels of phosphatidylcholines, while those from prostate cancer patients had higher levels of acetylcholine and sterols. The experiment also compared the metabolite differences at different stages of prostate cancer, with stage 3 prostate cancer patients showing reduced levels of sphingolipids such as ceramides, monohexosylceramides, and sphingomyelins compared to stage 2 patients. The differences in metabolite levels may be related to the expression of enzymes under pathological conditions. These findings help in diagnosing the progression of prostate cancer. Shi *et al.* [77] took healthy individuals, patients with type 2 diabetes(T2D), and patients with type 2 diabetes and diabetic kidney disease(DKD) as study subjects. They extracted exosomes from the samples using high-speed centrifugation, enriched exosomes expressing CD13 using immunomagnetic beads, and targeted quantification of metabolites in the samples using UPLC-MS. The experimental results showed that the median area under the ROC curve for fifteen metabolites including myristoylglycine, N-acetylalanine, valine, leucine, and undecanoic acid was greater than 0.7. *N*-acetylalanine had an area under the curve of 0.840, myristoylglycine 0.854, valine 0.812, and leucine 0.806, indicating their potential to differentiate between T2D and DKD.

3.4 Other Exosome Metabolomics

In addition to blood and urine, exosomes are also prevalent in other types of bodily fluids such as tears, pleural effusion, and cerebrospinal fluid. Rohit and others[78] compared the lipid composition of three types of tears: basal tears, reflex tears, and emotional tears. The results indicated that reflex and emotional tears contain lower levels of various lipids, thus basal tears are preferred for tear metabolomics studies. Metabolomics studies using tears as samples have made some progress in the diagnosis of ophthalmic diseases such as glaucoma[79] and dry eye syndrome[80,81], but research in exosome metabolomics in these areas is still limited. As exosome extraction and separation techniques improve and sample collection issues are resolved, tear exosome metabolomics is expected to offer unique advantages in the diagnosis of eye diseases. Pleural effusions are a common respiratory system condition. Lam *et al.* [82] employed untargeted mass spectrometry metabolomics analysis and found that fatty acids in pleural effusions could distinguish between malignant pleural effusions from lung cancer and benign pleural effusions from tuberculosis. Li *et al.* [83] studied patients with lung cancer who had acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI) and those who had not received EGFR-TKI treatment. They conducted metabolomics analysis of the pleural effusions from both groups using LC-MS. The results showed significant changes in the metabolic characteristics of the EGFR-TKI resistant patients' pleural effusion, with increased levels of *L*-lysine, ornithine, citrulline and taurine, and decreased levels of *L*-tryptophan, uric acid, *L*-phenylalanine, *L*-leucine and *N*-formyl-L-methionine, indicating abnormalities in amino acid metabolism in these patients. Luo *et al.* [84] performed differential centrifugation on pleural effusions from lung cancer patients(malignancy pleural effusion, MPE) and tuberculosis patients (tuberculosis pleural effusion, TPE), extracting large and small extracellular vesicles. They analyzed 579 metabolites using LC-MS/MS. The results suggested that extracellular vesicles could serve as biomarkers for MPE and TPE. Cerebrospinal fluid(CSF) is a clear, colorless fluid that circulates around the brain and spinal cord and holds immense potential in diagnosing central nervous system diseases. van der Velpen *et al.* [85] conducted metabolomics analysis on plasma and

Table 3 Applications of exosome metabolomics in disease research across different types of body fluids

Exosome source	Isolation method	Metabolomic profiling	Disease type	Application	Ref.
Plasma	Differential ultracentrifugation	UHPLC-Q-TOF-MS/MS	Nonalcoholic fatty liver disease(NAFLD)	A distinct change in fatty acids and related pathways in nonalcoholic fatty liver disease patients	[66]
Plasma	Ultracentrifugation	LC-MS	Breast cancer	Targeting succinic acid and *L*-lactic acid in patients with RD after NAC to improve their disease course	[68]
Plasma	A home-constructed device called EXODUS	UPLC-MS/MS	Esophageal squamous cell carcinoma	Novel biomarkers for diagnosis and prognosis of ESCC	[87]
Plasma	Invitrogen total exosome isolation kit	UPLC-TOF-MS/MS	COVID-19	GM3-enriched exosomes may partake in pathological processes related to COVID-19 pathogenesis	[88]
Serum	Ultracentrifugation	LC-MS/MS	Castration resistant prostate cancer(CRPC)	Diagnostic TFC, PCa and CRPC by differential metabolites	[67]
Serum	Size exclusion chromatography	UPLC-MS/MS	High-altitude cerebral edema (HACE)	Distinguish the HCs and HACE patients	[69]
Serum	qEV column	UPLC-MS/MS	Acute mountain sicknesses	Identified 5 metabolites to distinguish hypoxic preconditioning participants and control subjects	[70]
Serum	ExoQuick, a fast-acting exosome precipitation solution	UPLC-MS/MS	Diabetic nephropathy(DN) and diabetic retinopathy (DR)	1-MH loss may be linked to the pathogenicity of diabetic endothelial dysfunction in DR/DN	[71]
Serum	qEV column	UPLC-MS/MS	Bipolar disorder	15 Exosomal metabolites to distinguish BD patients and other major psychiatric diseases	[89]
Urine	MXene@TiO_2/Fe_3O_4	LDI MS	Bladder cancer	Three biomarkers are indication of treatment in individual patients	[90]
Urine	HPL-SEC	MALDI-TOF MS	Systemic lupus erythematosus(SLE)	Screen differential expressions of metabolite signals in the HC and SLE groups	[91]
Urine	Serial centrifugation	MS	Prostate cancer	Potential prostate cancer biomarkers	[76]
Urine	Ultracentrifuged	UHPLC-MS	Prostate cancer	Distinguish prostate cancer pathogenesis and progression	[64]
Urine	Ultracentrifuged	^{1}H NMR	Cardiovascular risk	Three metabolites can be CV risk biomarkers	[63]
Tissue and urine	Ultrafiltration and ultracentrifugation	LC-ESI-MS/MS	Prostate cancer	Prove uEVs are potential prostate cancer biomarkers	[92]
Pleural effusions	Differential ultracentrifugation	LC-MS/MS	Tuberculosis and malignancy	Identifying novel biomarkers for diagnosing TPE and MPE	[84]
Frontal cortex tissues	Ultracentrifuged	nESI- UHRAMS and HCD-MS/MS	AD	AD BDEVs have a unique lipid signature	[86]
Follicular fluid	Exosomes Isolation Reagent and Ultracentrifugation	GC-TOFMS	—	Reveal age-related changes in ovarian follicular fluid	[93]
Bile juice	EX-03 kit	UPLC-Orbitrap-MS	Gallbladder cancers (GBCCs)	Activation of PI3K/AKT pathway is found in the gallbladder cancer group	[94]
Femoral bone tissue	Ultracentrifugation	UPLC-MS/MS	Osteonecrosis of the femoral head (ONFH)	Lipid metabolism disorder is an important pathological factor in ONFH	[95]

CSF samples from Alzheimer's disease(AD) patients and healthy subjects. The results showed that changes in metabolites in plasma samples were consistent with changes in intermediate metabolites in CSF, with concentrations of amino acids and other metabolic products in CSF being related to AD pathology. Su *et al.*[86] extracted exosomes from the frontal cortex tissues of healthy subjects and AD patients and conducted lipidome analysis using nESI-UHRAMS and HCD-MS/MS shotgun approach. The results revealed significant

differences in glycerophospholipids and sphingolipids content between AD patients and healthy subjects, with an increase in glycerophospholipids and a decrease in sphingolipids in AD patients, which may be related to the pathogenesis of AD. This experiment was the first to reveal the lipidomic characteristics of exosomes in AD patients. Given the inconvenience and potential harm to patients from tissue sampling, CSF exosomes hold the potential for consistency with brain tissue samples and could be used in the future for AD diagnosis through CSF exosomes.

4 Summary and Outlook

As carriers of intercellular information and material exchange, exosomes contain vast amounts of information and have the potential to serve as biomarkers. Metabolites, as the results of multiple reactions involving carbohydrates, lipids, nucleic acids, and proteins, can trace entire metabolic pathways through metabolomic studies, providing a broader perspective for exploration at the molecular level. Comprehensive, precise, and high-throughput analysis of metabolites in exosomes through metabolomics is of great significance and value for discovering and screening disease biomarkers and exploring mechanisms of disease occurrence. However, exosome metabolomics, as a relatively new research field, still has many challenges to explore and address:

(1) Exosome isolation challenges: efficient and reliable isolation of exosomes from biological fluids is one of the most persistent challenges. Current methods such as ultracentrifugation, ultrafiltration, and immunoaffinity separation, although widely used, often fail to achieve the desired balance between purity, recovery, and scalability. These limitations not only impact the downstream metabolite extraction and detection processes but also introduce significant variability in research findings. The lack of standardized isolation protocols further exacerbates this issue, leading to inconsistencies across studies. It is imperative to prioritize the development of novel, standardized, and more effective isolation techniques that can ensure reproducibility and comparability of results.

(2) Technological limitations in detection: although MS has become a powerful tool for exosome metabolomics due to its high sensitivity, wide dynamic range, and high mass resolution, most detected metabolites in body fluid exosome metabolomics are lipids, possibly due to their higher abundance in exosomes. Improving the sensitivity and accuracy of detecting low-abundance metabolites in exosomes remains a pressing issue.

(3) Integration with other omics: as an important part of omics research, exosome metabolomics can directly reflect the current state of the organism. Combining it with other upstream omics can allow researchers to observe and analyze biological systems from multiple dimensions, such as gene expression, protein activity, and metabolite levels, aiding in the discovery of more accurate biomarkers and a deeper understanding of disease mechanisms. However, the complexity of multi-omics data integration requires sophisticated bioinformatics tools and expertise, which are not yet fully developed or widely accessible. Furthermore, the interpretation of such complex datasets poses significant challenges, often leading to oversimplified conclusions or misinterpretations. There is an urgent need for the development of more advanced computational tools and collaborative efforts to enhance our ability to integrate and interpret multi-omics data effectively.

(4) Biomarker validation: the majority of biomarkers identified in exosome metabolomics studies stem from exploratory research with limited sample sizes. While these studies provide valuable insights, they often lack the rigor needed for clinical application. The translation of these potential biomarkers into clinically relevant tools requires extensive validation through large-scale mechanistic studies and clinical trials. The

field must move beyond exploratory research and invest in long-term, collaborative studies that can validate these findings in diverse populations and clinical settings. Without such efforts, the clinical applicability of exosome-based biomarkers remains speculative.

(5) Standardization and reproducibility: the field of exosome metabolomics suffers from a lack of uniformity in experimental methodologies, including sample collection, exosome isolation, metabolite extraction, detection platforms, and data processing. This variability leads to poor comparability and reproducibility of results across different studies, undermining the credibility of research findings. Establishing standardized protocols and achieving consensus is crucial for the advancement of the field. This standardization should extend to every aspect of the experimental workflow to ensure that findings are robust, reproducible, and widely applicable.

(6) Ethical and regulatory challenges: as exosome metabolomics advances towards clinical applications, ethical and regulatory considerations become increasingly critical. Issues such as patient privacy, data security, and the ethical use of biological samples must be rigorously addressed. The development of regulatory frameworks that govern the use of exosomal data in clinical settings is essential to ensure ethical compliance and protect patient rights. Additionally, public trust in exosome-based diagnostics and therapeutics will depend on transparent communication and stringent oversight of these ethical considerations.

In conclusion, while exosome metabolomics holds transformative potential for biomarker discovery and understanding disease mechanisms, the field is still in its infancy and faces numerous challenges. Addressing these challenges requires not only technological innovation but also a concerted effort towards standardization, validation, and ethical governance. By embracing interdisciplinary collaboration and rigorously addressing the current limitations, exosome metabolomics can transition from a promising research field to a cornerstone of precision medicine, with the potential to revolutionize disease diagnosis, monitoring, and treatment on a global scale.

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