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REVIEW

Integrating mass spectrometry-based multi-omic signatures of extracellular vesicles: from discovery to clinical translation

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Abstract

Extracellular vesicles (EVs) are increasingly recognised as key mediators of intercellular communication and disease progression. Their capacity to carry bioactive molecules, namely proteins, lipids and metabolites, reflects the physiological and pathological states of their cells of origin, making them surrogates for diagnostic, prognostic and therapeutic endpoints. Recent advances in mass spectrometry have enabled comprehensive, high-resolution profiling of EVs across multiple omics layers. Proteomics has uncovered both conserved and disease-specific protein markers; lipidomics has revealed structurally distinct membrane compositions influencing EV stability and function; and metabolomics has captured dynamic snapshots of cellular metabolism. However, significant challenges persist for standardisation and interpretation of EVs, which include variation in EV isolation purity, scalability, EV heterogeneity and cross-study comparability. This perspective critically synthesises findings from recent EV multi-omics studies and proposes a conceptual framework for integrating these omics layers to better define EV identity and functionality. We highlight emerging clinical applications and outline future directions involving single-vesicle omics and the rational engineering of therapeutic EVs. The integration of multi-omics approaches with translational aims holds promise for advancing EVs from experimental tools to new pillars of precision medicine.

Keywords: exosomes, extracellular vesicles, liquid biopsy, mass spectrometry, multi-omics

INTRODUCTION

Extracellular vesicles (EVs), encompassing exosomes and microvesicles, are nanoscale, lipid bilayer-enclosed vesicles secreted by most cell types and organisms.[1–3] Once considered cellular waste products, EVs are now understood to be functional conveyors of molecular information, mediating processes as diverse as immune modulation, cancer metastasis, neurodegeneration and tissue repair.[4–6] Their cargos, including proteins, lipids, metabolites and nucleic acids, are

reflective of the biological state of their parent cells, and their presence in accessible biofluids, such as plasma, urine and saliva enables non-invasive disease monitoring (Figure 1a).[7–10]

Recent advances in mass spectrometry (MS)-based multi-omics have accelerated the functional exploration of EVs by enabling simultaneous profiling of proteomes, metabolomes and lipidomes. Proteomics has uncovered subtype-specific cargos in cancer-derived EVs, such as epithelial cell adhesion molecule (EpCAM) and ALIX in glioblastoma stem-like cell vesicles, underscoring their roles in tumor heterogeneity.[11] Metabolomics has demonstrated that vesicles carry disease-relevant metabolic signatures, including nucleotide and amino acid alterations in pancreatic cancer EVs[12] and bile acid perturbations in bipolar disorder EVs.[13] Lipidomics has revealed how vesicle lipid remodelling mirrors and potentially mediates disease, with urinary EV lipids distinguishing nonalcoholic fatty liver (NAFL) from nonalcoholic steatohepatitis (NASH)[14] and COVID-19 plasma EVs showing dynamic sterol and phospholipid shifts.[15]

Despite these advances, challenges persist in translating EV omics into translational studies. Heterogeneity in isolation methods, variability in sample preparation and fragmentation across MS platforms complicate reproducibility and biomarker validation. Moreover, while high-resolution MS workflows remain the discovery gold standard, their translation into scalable diagnostics requires bridging towards clinically proven platforms, such as flow cytometry and ELISA. This review synthesises the current state of MS-based multi-omics of EVs, highlighting biological sources, methodological advances, functional insights, translational applications and the urgent need for standardisation to realise their translational and clinical potential.

CONCEPTUAL OVERVIEW OF MULTI-OMICS IN EV RESEARCH

Mass spectrometry underpins the multi-omics exploration of EVs, with different analytical platforms optimised for distinct cargo classes and research aims.[16–18] Liquid chromatography–tandem mass spectrometry (LC–MS/MS) is the most widely applied, providing high sensitivity and dynamic range for EV proteomics and lipidomics, while recent data-independent acquisition (DIA) strategies enhance reproducibility and scalability across cohorts. High-resolution analysers such as Orbitraps and time-of-flight (TOF) systems enable confident identification of complex lipid and metabolite species, crucial for untargeted omics where isobaric compounds are common. Complementary approaches such as matrix-assisted laser desorption/ionisation (MALDI-MS) offer rapid, high-throughput screening of intact proteins and lipids, whereas targeted methods like selected reaction monitoring (SRM) and parallel reaction monitoring (PRM) support sensitive validation of candidate EV biomarkers in clinical biofluids.[19,20] Specialised applications, including elemental MS (ICP-MS) for metal-associated cargos and emerging single-vesicle MS for resolving intra-population heterogeneity, expand the analytical landscape further.[21] Together, these complementary MS modalities provide a conceptual framework for linking EV proteomic, lipidomic and metabolomic signatures, underscoring the necessity of multi-omics integration to capture the full biological and translational potential of EVs.

Proteomics has been the most extensively applied strategy in EV research, offering high-resolution insights into the protein composition of vesicles.[22–24] These studies not only chart vesicle composition but also identify pathway enrichments, such as signalling proteins in prostate cancer urinary EVs ([24]) or subtype-specific protein cargos in glioblastoma EVs.[11] Canonical markers such as CD9, CD63, CD81, ALIX and TSG101 are routinely used for vesicle validation.[25–27] More importantly, proteomic profiling has identified functionally significant proteins reflective of the parent cell type and pathological condition, including immune checkpoint proteins, pro-angiogenic factors and components of cell death pathways. Techniques such as label-free LC–MS/MS, tandem mass tag (TMT) labelling and SRM have enabled both unbiased discovery and targeted quantitation in diverse sample types.[28–30]

Lipidomics uniquely captures the structural and signalling functions of EV membranes. Urinary EV lipidomics discriminated NAFL from NASH using class-specific lipid biomarkers,[14] while breast milk EV lipidomics identified phospholipids relevant to neonatal immune development.[31] Plasma EV lipidomics in COVID-19 patients revealed temporal remodelling of sterols and phospholipids, offering

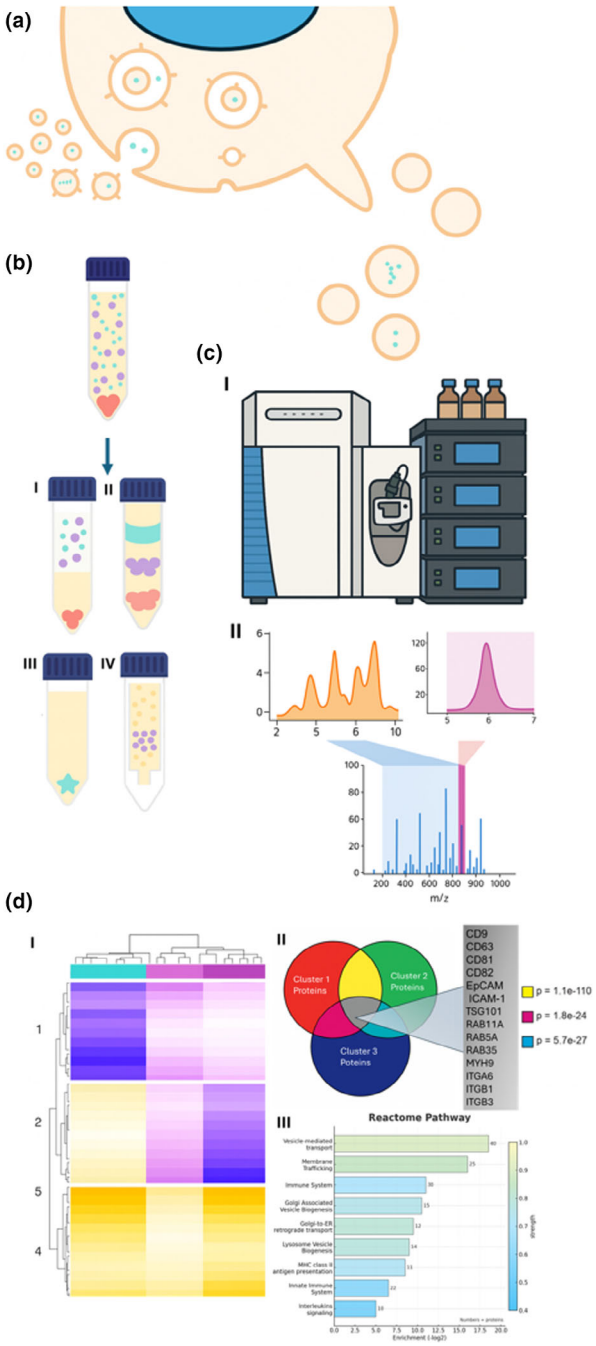

Figure 1. Workflow for mass spectrometry-based multi-omics of extracellular vesicles (EV). **(a)** EV biogenesis and release. EVs originate from endosomal and plasma membrane pathways. Schematic illustrating intracellular vesicle formation, multivesicular body (MVB) trafficking and release of heterogeneous extracellular vesicle populations into the extracellular space. **(b)** EV isolation strategies. Multiple complementary techniques exist for EV enrichment, each with distinct separation principles. Common approaches included the following: (I) ultrafiltration/tangential flow filtration for size-based concentration, (II) density gradient ultracentrifugation for buoyant density separation, (III) polymer-based precipitation for rapid bulk isolation and (IV) size-exclusion chromatography for high-purity separation based on hydrodynamic radius. **(c)** Mass spectrometry-based multi-omics acquisition. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) enables comprehensive molecular profiling of EV cargo. (I) Schematic of liquid chromatography–tandem mass spectrometry (LC–MS/MS) instrumentation used for proteomics, lipidomics and metabolomics profiling of isolated EVs. (II) Representative raw data outputs, including extracted ion chromatograms, MS1 precursor spectra and MS2 fragmentation spectra used for molecular identification and quantification. **(d)** Bioinformatics downstream data analysis. Computational approaches reveal EV molecular signatures and functional associations. (I) Hierarchical clustering heatmap illustrating sample grouping and differential abundance patterns based on top protein signatures. (II) Venn diagram depicting the intersection of differentially expressed proteins across experimental conditions, highlighting shared and unique molecular markers. (III) Reactome pathway over-representation analysis revealing functional enrichment of EV-associated proteins in vesicle-mediated transport, membrane trafficking and immune-related pathways.

mechanistic insights into infection and recovery dynamics.[15] Lipidomics complements proteomics by characterising the structural and signalling lipid components of EV membranes.[32] Unlike cellular membranes, EVs are enriched in sphingomyelin (SM), cholesterol, ceramides and phosphatidylserine (PS), lipids known to modulate membrane rigidity, cargo sorting and cell entry.[32–34] Mass spectrometry-based lipidomics, whether untargeted or targeted via multiple reaction monitoring (MRM), reveals lipid class distributions and identifies disease-specific lipid species.[28,35,36] These lipids not only maintain vesicle stability but also mediate immune recognition, organotropism and intercellular signalling.

Metabolomics expands the functional lens to small molecules that reflect cellular metabolic rewiring. Multi-platform metabolomics of pancreatic cancer EVs captured hydrophilic metabolites spanning nucleotide and amino acid metabolism,[12] while bipolar disorder EV metabolomics revealed altered bile acid and amino acid profiles.[13] These findings emphasise the diagnostic promise of EV metabolites as accessible indicators of systemic biochemical states. Metabolomics, though relatively underexplored, provides a sensitive readout of biochemical pathway flux and cellular stress states. Small metabolites including amino acids, nucleotides

and lipid intermediates encapsulated within EVs are increasingly recognised as functional effectors rather than metabolic waste. Metabolomics is especially valuable for its temporal resolution, capturing short-lived changes in cell physiology reflected in EV cargo.

EV analysis achieves its full explanatory value when proteomic, lipidomic and metabolomic data are integrated, enabling detection of regulatory modules, co-enrichment patterns and functional redundancies.[15,37,38] For example, concordant changes in immune regulatory proteins and pro-inflammatory lipid species may indicate vesicle-mediated immunomodulation. Integrating metabolomics with protein transporters or lipid membrane composition can provide mechanistic insights into cargo sorting or vesicle targeting.[39,40] Multi-omics integration thus enables the mapping of molecular networks that are otherwise obscured when each omics layer is examined in isolation.[15,37,38] Importantly, emerging analytical frameworks such as data fusion pipelines and machine learning-based multi-omics modelling are now being deployed to extract biologically and clinically meaningful patterns from complex datasets. These integrative approaches are already informing biomarker discovery, vesicle engineering and therapeutic development.[41–43] As EV research matures, adopting a multi-omics strategy is no longer optional but essential. It bridges molecular detail with functional understanding and opens the door to transformative applications in diagnostics, regenerative medicine and targeted drug delivery.

BIOLOGICAL AND CLINICAL SOURCES OF EVS IN OMICS STUDIES

Extracellular vesicle omics research has drawn upon an increasingly diverse repertoire of biological and clinical sources, each contributing unique insights into EV biology and translational biomarker potential.[44–46] The choice of source material determines vesicle yield, purity and biological interpretability, as EV composition is highly context-dependent, reflecting both cell type and pathological state.[47–50]

Clinical biofluids remain the most widely utilised source because of their minimally invasive collection and direct disease relevance. Serum and plasma EVs have been extensively profiled, particularly in oncology and neurology.[22,51] Similarly, urinary EVs have been positioned as attractive biomarkers for urological malignancies and renal dysfunction.[24]

Beyond traditional plasma and urine, specialised biofluids are emerging as underexploited reservoirs of EVs with unique functional relevance. Breast milk EVs, for example, were shown to carry distinct lipidomic profiles with potential roles in neonatal immune programming.[31] *In vitro* cell culture models complement clinical studies by enabling controlled interrogation of EV biology under defined conditions. Glioblastoma stem-like cell–derived EVs have been leveraged to identify subtype-specific proteomic signatures and mechanistic insights into tumor heterogeneity.[11] Likewise, melanoma cancer stem cell exosomes provided the first metabolic blueprint of tumor stemness-associated EV cargo.[52] Fibroblast-derived EVs have been used to benchmark proteomic methods.[53] However, these models often lack the complexity of physiological microenvironments and may over-represent abundant cellular proteins, requiring careful contextualisation when extrapolating to patient-derived biofluids and translational biomarker studies.

Despite this diversity, certain gaps are evident. Most studies are anchored to serum/plasma, which creates a bias towards systemic readouts while potentially overlooking tissue-specific EV signals. By contrast, *in vitro* models, though mechanistically informative, rarely translate directly into clinically actionable biomarkers without validation in patient-derived EVs.[54]

Moving forward, integrative approaches coupling patient-derived EVs with complementary *in vitro* systems may bridge this gap, enabling both mechanistic discovery and translational application. Expanding into underexplored fluids such as saliva, CSF and urine-derived EVs offers opportunities to diversify biomarker discovery beyond the systemic plasma compartment.

ISOLATION AND CHARACTERISATION STRATEGIES FOR MULTI-OMICS

Isolation and characterisation of EVs represent foundational steps in multi-omics workflows. The strategies chosen not only influence vesicle yield and purity but also dictate downstream proteomic, metabolomic and lipidomic data quality. A critical comparison of approaches across recent studies reveals both methodological strengths and persistent challenges.

Differential ultracentrifugation continues to serve as the backbone of EV isolation in many omics studies (Figure 1b). Its appeal lies in widespread accessibility and historical precedence, as exemplified in fibroblast EV proteomics where stepwise centrifugation and nanoparticle tracking analysis provided a baseline for proteome mapping.[53] Similarly, plasma EVs for proteomic–metabolomic integration were isolated via differential ultracentrifugation at sequential g-forces.[51] However, this approach often co-isolates protein aggregates and lipoproteins, contributing to background contamination and reducing the detectability of low-abundance EV cargo.

To address purity limitations, size-based enrichment methods have gained traction. Automated magnetic bead-based enrichment (EVrich) was deployed in urinary EV proteomics, enabling reproducible high-throughput profiling and phosphoproteomic characterisation.[23] Similarly, size-exclusion chromatography (SEC) has been adopted in metabolomic workflows, with serum EVs from amyotrophic lateral sclerosis patients successfully profiled following SEC-based enrichment.[55] SEC provides improved purity relative to ultracentrifugation but can be limited by dilution effects, impacting downstream sensitivity.

Emerging next-generation isolation methods demonstrate improved recovery and reproducibility. The EXODUS system, applied to urinary EV lipidomics, yielded high-purity vesicles and enabled biomarker discovery distinguishing NAFL from NASH.[14] Such automated platforms hold promise for clinical translation, where consistency across cohorts is paramount. Likewise, polymer precipitation combined with differential centrifugation was leveraged in COVID-19 plasma EV lipidomics, facilitating high-coverage sterol and phospholipid profiling despite complex inflammatory backgrounds.[15] These approaches illustrate the drive towards scalable and standardised pipelines, though polymer precipitation carries the risk of coprecipitating non-EV components.

Characterisation strategies are equally central to validating EV identity prior to omics profiling. Conventional marker analysis remains the norm, with proteins such as CD9, CD63, CD81, ALIX and TSG101 frequently used to confirm exosomal enrichment. In glioblastoma stem-like cell EVs, ALIX, CD63 and EPCAM served as positive markers, while calnexin provided a negative control to exclude endoplasmic reticulum contamination.[11] Lipidomics studies similarly validated vesicles through immunoblotting of CD63, CD81, TSG101 and ALIX.[14] However, marker reliance risks circular validation, as not all EV subtypes express canonical proteins, and overemphasis on tetraspanins may obscure biologically relevant heterogeneity.[56–58]

Methodological innovations are also reshaping sample preparation for omics integration. Proteomics studies have introduced efficient workflows, such as filter-aided sample preparation (FASP) in serum EV proteomics[22] and one-pot photocleavable surfactant-based lysis in fibroblast EVs.[53] Metabolomics has advanced through comparative optimisation of solvent extraction protocols, where prostate cancer EV studies systematically benchmarked methanol-only versus biphasic approaches to maximise metabolite recovery.[59] Lipidomics workflows have incorporated gold-standard MTBE or Folch extractions, supplemented by internal standards, such as SPLASH® Lipidomix, to ensure accurate quantification in plasma and breast milk EV studies.[31,60] Collectively, these innovations demonstrate how methodological refinements in preparation can dramatically extend the depth and reliability of multi-omics coverage.

However, contemporary characterisation increasingly extends beyond these foundational approaches to encompass post-translational modifications that reflect dynamic biological states. Phosphoproteomic profiling of EVs has emerged as a clinically relevant layer of characterisation, revealing diagnostic signatures such as phosphorylated PD-L1 as a tissue-proxy biomarker in lung cancer and glycan modifications distinguishing early malignant transformation.[61,62] Such phospho-EV analyses enable liquid biopsy snapshots of key signalling pathway activation states, offering utility for both diagnosis and real-time therapy monitoring.[63] These advances exemplify the evolution towards functional, rather than purely compositional, EV characterisation—integrating seamlessly with multi-omics frameworks to provide biomarker depth that conventional protein abundance measurements cannot achieve.

MASS SPECTROMETRY STRATEGIES IN MULTI-OMICS OF EVS

The evolution of MS strategies has been central to unlocking the multi-omics potential of EVs. Different omics layers impose distinct analytical

demands, driving innovation in MS platforms and workflows. The increasing body of literature, including curated case studies summarised in Supplementary table 1 (Proteomics), Supplementary table 2 (Metabolomics) and Supplementary table 3 (Lipidomics) demonstrates the practical and translational value of MS in resolving EV complexity and functional specificity.

Proteomics has traditionally relied on LC–MS/MS as the cornerstone for EV protein characterisation. Discovery-based, shotgun LC–MS/MS workflows using Orbitrap systems remain dominant, as seen in serum EV profiling where the FASP pipeline enabled robust proteome coverage.[22] More specialised platforms expand this repertoire: glioblastoma EVs were resolved using 2D electrophoresis coupled to MALDI-TOF/TOF for subtype-specific protein mapping,[11] while mammary fibroblast EVs benefited from LC-TIMS-Q-TOF strategies, which coupled ion mobility with TOF MS to maximise proteome depth and resolution.[53] Importantly, phosphoproteomic extensions using LC–MS/MS demonstrated the feasibility of post-translational modification profiling in urinary EVs.[23,24] Despite these advances, discovery proteomics remains vulnerable to undersampling in data-dependent acquisition modes; DIA approaches are addressing this limitation.

Metabolomics strategies have expanded the methodological toolkit by integrating multiple separation modalities with high-resolution MS. In pancreatic cancer EVs, multi-platform analysis combined capillary ion chromatography-MS, LC–MS and GC–MS to comprehensively map hydrophilic metabolites.[12] Other studies optimised chromatographic conditions directly, with prostate cancer EV metabolomics benchmarking ZIC-pHILIC versus C18 LC–MS/MS separations to maximise coverage of polar versus hydrophobic metabolites.[59] Targeted approaches also feature prominently: bipolar disorder serum EVs were profiled using widely targeted UPLC–MS/MS, enabling quantification of metabolites,[64,65] while untargeted HRMS workflows (e.g. Q-TOF MS in melanoma CSC-derived EVs).[52]

Lipidomics has perhaps seen the most rapid methodological diversification. Discovery lipidomics has been performed with LC–MS/MS platforms optimised for broad lipid class coverage, such as high-resolution LC-Q-TOF systems in plasma EVs from psoriasis patients.[60] The EXODUS-isolated urinary EVs were subjected to UPLC–MS/MS combined with machine learning classification, yielding lipid biomarkers for NAFL versus NASH.[14] Breast milk EV lipidomics utilised Folch extraction and ultraperformance supercritical fluid chromatography-Q-TOF-MS, which proved particularly effective for resolving complex phospholipid classes.[31] In infectious disease contexts, COVID-19 plasma EVs analysed by HPLC–MS/MS revealed temporal remodelling of sterols and phospholipids.[15] Overall, lipidomics benefits from integration of high-resolution untargeted discovery and computational tools (e.g. machine learning), though class-specific ionisation biases and matrix effects remain barriers to full lipidome capture.

Our integrated multi-omics analysis of 229 molecules (80 proteins, 77 metabolites, 72 lipids) across 67 independent studies (Figure 1c and d; summarised in Supplementary table 1. Proteomics, Supplementary table 2. Metabolomics and Supplementary table 3. Lipidomics) revealed three pathways with full multi-omics convergence: exosome biogenesis, membrane trafficking and the ESCRT pathway (Figure 2a). Network topology analysis identified ATP as the central metabolic hub with the highest degree centrality, demonstrating unprecedented connectivity across all three molecular layers (Figure 2b). Specifically, ATP coordinates energy-dependent processes linking tetraspanin proteins (CD63, CD81 and CD9) to membrane phospholipids (PE, PS, PC) and ESCRT machinery (TSG101, ALIX and PDCD6IP), revealing a previously underappreciated metabolic checkpoint in EV formation. This ATP-centric node represents a tractable therapeutic target, as metabolic perturbation at this nexus would simultaneously disrupt protein sorting, membrane remodelling and vesicle budding. Furthermore, the pathway-based circos visualisation (Figure 2b) demonstrates that cross-omics connections (red ribbons) predominantly emanate from ATP and cholesterol, suggesting these molecules function as master coordinators bridging cellular metabolism to membrane architecture. The identification of PS and phosphatidylethanolamine (PE) as secondary hubs with high betweenness centrality indicates these lipids serve as critical signalling platforms, potentially explaining their enrichment in exosome membranes beyond mere structural requirements. Notably, our approach revealed that SM and ceramides cluster with protein machinery in community analysis, suggesting lipid

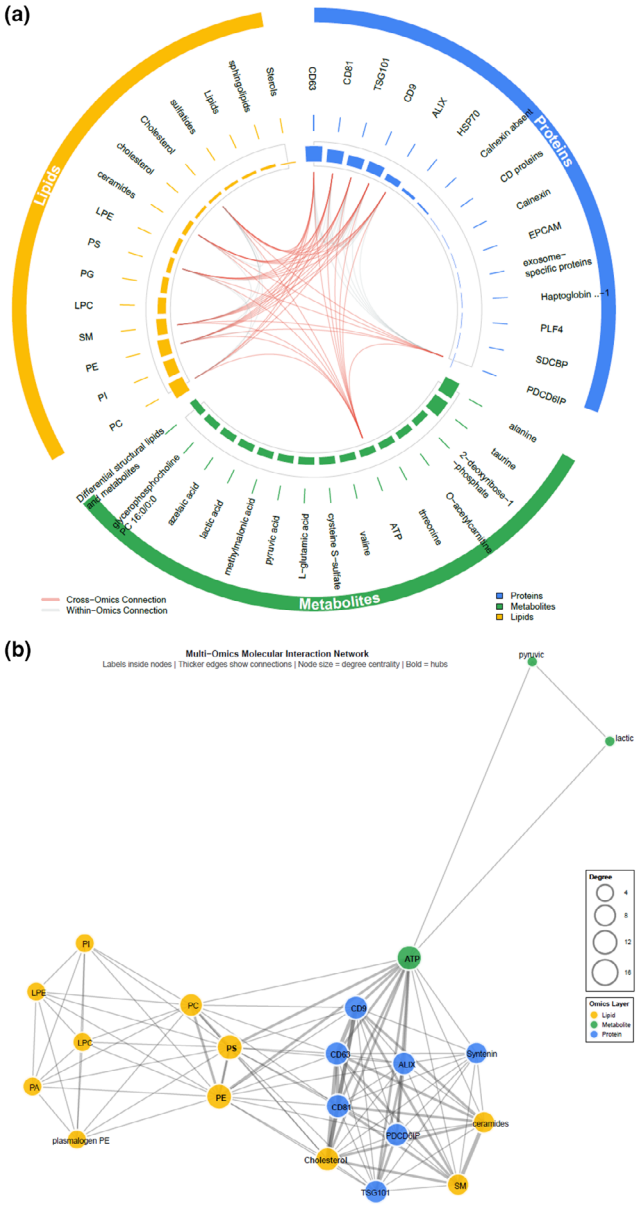

Figure 2. Multi-omics network analysis reveals ATP as the central metabolic hub coordinating extracellular vesicle biogenesis. **(a)** Circos plot visualisation of multi-omics integration. The top 15 molecules from each omics layer are displayed in three sectors: proteins (blue arc), metabolites (green arc) and lipids (yellow arc). Inner bar charts represent detection frequency across 67 independent studies. Ribbons connect molecules participating in shared biological pathways: pink ribbons indicate cross-omics connections (between different molecular types); grey ribbons indicate within-omics connections. **(b)** Molecular interaction network. Network topology of 25 highly connected molecules across three omics layers: proteins (blue nodes), metabolites (green nodes) and lipids (yellow nodes). Node size represents degree centrality. Edge thickness represents connection strength based on shared pathway membership. ATP emerged as the highest degree hub with extensive cross-omics connectivity to tetraspanins (CD63, CD81 and CD9), ESCRT machinery (TSG101, ALIX and PDCD6IP) and membrane lipids (PE, PS, PC, cholesterol). Secondary lipid hubs (PE, PS, cholesterol) bridge metabolic and structural functions. ALIX, ALG-2-interacting protein X; ATP, adenosine triphosphate; CD9/CD63/CD81, cluster of differentiation 9/63/81 (tetraspanins); LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PDCD6IP, programmed cell death 6-interacting protein; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; TSG101, tumor susceptibility gene 101. Data sources and analysis: Multi-omics data were curated from 67 independent studies identifying proteins (n = 80), metabolites (n = 77) and lipids (n = 72) in extracellular vesicles. Pathway annotations were derived from established biological databases, including KEGG, Reactome and Gene Ontology.

raft-mediated protein sorting operates as a discrete functional module. These mechanistic insights, which would be obscured in single-omics analyses, position ATP synthase inhibitors and sphingolipid metabolism modulators as rational candidates for therapeutic intervention in diseases characterised by dysregulated EV secretion, including cancer metastasis and neurodegenerative disorders. The robust detection of these molecules across diverse biological contexts (as shown by frequency bars in Figure 2b) further validates their universal importance in EV biology, strengthening the translational potential of targeting this multi-molecular axis.

FUNCTIONAL INSIGHTS DISEASE ASSOCIATIONS AND BIOLOGICAL PATHWAYS

Proteomic analyses have revealed the breadth of EV cargo in both physiological and pathological contexts. Serum EV proteomics distinguished proteomic signatures associated with inflammatory pathways and platelet activation, suggesting potential utility in monitoring systemic disease processes.[66–72] Glioblastoma stem-like cell EVs displayed subtype-specific protein cargos, with enrichment of oncogenic markers, such as EPCAM and ALIX, underscoring EVs as mediators of tumor heterogeneity and possible therapeutic resistance.[11] Fibroblast-derived EVs demonstrated overlapping markers with ExoCarta and Vesiclepedia databases, validating methodological approaches while also exposing vesicular roles in extracellular matrix remodelling.[53] In prostate cancer, urinary EV phosphoproteomics identified signalling networks relevant to tumor progression, illustrating the feasibility of phospho-EV analysis for pathway mapping.[23] These findings collectively highlight how EV proteomics maps directly onto functional pathways implicated in cancer, immune regulation and cell–cell communication.

Metabolomic profiling has extended these functional insights to cellular metabolism and disease-specific biochemical alterations.[7,73] Pancreatic cancer EV metabolomics uncovered perturbations in nucleotide and amino acid metabolism, consistent with the metabolic rewiring characteristic of tumor cells.[12] In prostate cancer EVs, methodological optimisation revealed robust recovery of TCA cycle metabolites and amino acid intermediates, validating EVs as carriers of central carbon metabolic signatures.[59] Metabolomic signatures of melanoma cancer stem cell–derived EVs highlighted differential lipid and energy metabolites, implicating EVs in sustaining stemness and metastatic potential.[52] Beyond oncology, serum EVs from patients with bipolar disorder displayed altered bile acid metabolism, suggesting vesicular metabolites may serve as windows into neuropsychiatric biochemical dysregulation.[13,64,74–76] Early-stage ALS patient EVs exhibited changes in citrate cycle intermediates and complement pathway metabolites, implicating EVs in neuroinflammatory and energy dysfunction pathways.[55] Together, these studies demonstrate how EV metabolomics bridges cellular metabolism with systemic disease states, though questions remain about the stability and specificity of these signatures across cohorts.

Lipidomic studies further enrich this picture by linking EV lipid content to membrane biology and disease-specific lipid remodelling.[70,77–80] Plasma EVs from COVID-19 patients demonstrated temporal shifts in sterols and phospholipids, reflecting immune-metabolic reprogramming during infection and recovery.[15] Breast milk EV lipidomics identified unique phospholipid classes with potential roles in neonatal immune maturation and gut development.[31,81–84] Urinary EV lipid profiles distinguished NAFL from NASH, pinpointing lipid biomarkers with diagnostic value for liver disease progression.[14] Macrophage-derived EVs showed altered glycerophospholipids and ceramides in response to immune activation, illustrating how lipid cargos act as functional mediators of inflammatory responses.[85] Plasma EV lipidomics in psoriasis revealed distinct differences between exosomal and microvesicular lipid profiles, implicating sphingolipid metabolism in inflammatory skin disease.[60] These lipidomic findings emphasise that EVs not only mirror but also potentially propagate lipid-driven signalling pathways central to immunity, metabolism and disease pathogenesis.

Integration of functional insights across omics layers underscores both convergence and divergence in pathway associations. Cancer-related studies consistently implicate EV proteins and metabolites in metabolic rewiring and signalling pathways that sustain proliferation, invasion and stemness.[86–89] Neuropsychiatric and neurodegenerative disease studies highlight vesicular metabolites as proxies for altered

neurotransmitter and energy metabolism, suggesting systemic accessibility to central nervous system processes.[74,76,90–93] Infectious and inflammatory conditions reveal EV lipid remodelling as an indicator of host–pathogen interactions and immune dysregulation.[69,72,85,94,95] Yet, the diversity of sample types, analytical platforms and data normalisation strategies complicates direct comparison across studies. Furthermore, functional enrichment analyses often over-represent canonical pathways, necessitating validation with orthogonal biological assays.

Taken together, functional insights from EV multi-omics reveal strong disease associations across cancer, neurodegeneration, psychiatric disorders, infectious diseases and chronic inflammation. Proteins highlight signalling and structural pathways, metabolites map central biochemical circuits and lipids uncover membrane dynamics and metabolic remodelling.

TRANSLATIONAL APPLICATIONS AND CLINICAL POTENTIAL

The integration of multi-omics with EV biology has opened a translational frontier, where EVs are increasingly positioned as minimally invasive biomarkers and potential therapeutic tools. Yet, their journey towards clinical application is shaped by methodological constraints and the challenge of translating omics signatures into reproducible, actionable endpoints.

Proteomic profiling of EVs has provided some of the strongest evidence for biomarker potential in oncology and clinical diagnostics. Serum-derived EVs demonstrated differentiated proteomic signatures linked to disease states, establishing the feasibility of EV proteomics for monitoring systemic pathophysiology.[22,96–99] In prostate cancer, tumor-specific EV protein profiles have been delineated, revealing clinically relevant endpoints. Differential enrichment of proteins, such as PD-L1, ERG, Integrin-β5, Survivin, TGF-β, phosphorylated TSC2 and key components of MAPK and mTOR pathways underscores the ability of EV proteomics to capture oncogenic signalling landscapes.[99] In multiple myeloma, EVs derived from both patient serum and resistant cell lines were enriched in MHC I and CD44, implicating these cargos in disease progression and treatment resistance.[96] Similarly, in COVID-19, proteomic analysis revealed 414 EV proteins, with 48 uniquely associated with moderate to severe disease; these proteins mapped to platelet degranulation, complement activation and immune effector functions, reflecting systemic immune dysregulation.[98]

EV proteomics has broadened insight into solid-tumor biology by revealing how vesicle cargo reflects disease heterogeneity and signalling states. In glioblastoma, subtype-specific EV signatures enriched in markers such as EPCAM and ALIX delineate intratumoral diversity and align with patterns of therapeutic responsiveness.[11] Similar advances are evident in prostate cancer, where urinary EV phosphoproteomes capture signalling proteins central to tumor progression, underscoring the diagnostic value of phosphorylation-dependent vesicle cargo.[23] This expanding analytical capacity is strengthened by integrated workflows that extract both proteomic and metabolomic data from the same plasma EV preparations, reducing sample demands while enabling more comprehensive biomarker discovery.[51] The versatility of these approaches is further illustrated in reproductive biology, where EVs released by endometrial epithelial cells carry implantation-associated proteins, including HSP70, TSG101, CD9 and CD81, demonstrating the broad applicability of EV proteomics across physiological and pathological contexts.[100]

Metabolomic studies of EVs reinforce their role as disease sentinels across oncology, psychiatry and neurodegeneration. Pancreatic cancer EV metabolomics uncovered nucleotide and amino acid alterations consistent with tumor metabolic reprogramming, reinforcing their biomarker promise.[12] Method optimisation in prostate cancer EVs demonstrated robust recovery of metabolic intermediates, underscoring the importance of standardised protocols for clinical reproducibility.[59] Melanoma cancer stem cell–derived EVs provided the first metabolic signatures of tumor stemness, suggesting EV metabolomics could stratify cancer subpopulations.[52] Beyond oncology, serum EVs from bipolar disorder patients revealed dysregulated bile acid metabolism, pointing to psychiatric applications where conventional biomarkers are lacking.[13,64] Similarly, early ALS patient EVs revealed perturbations in energy metabolism and complement pathways, suggesting EV metabolomics may offer early diagnostic windows in neurodegenerative disease.[55]

Lipidomic analyses have underscored EVs as indicators of host metabolic status and immune function. In COVID-19, plasma EV lipidomics

revealed dynamic remodelling of sterols and phospholipids across disease progression, suggesting utility in tracking infectious disease trajectories.[15] Breast milk EV lipidomics identified unique phospholipids with potential applications in neonatal nutrition and immune support.[31] Urinary EV lipid profiles distinguished NAFL from NASH with high diagnostic accuracy, underscoring the promise of lipid-based biomarkers for liver disease progression.[14,101,102] In inflammatory contexts, macrophage-derived EV lipidomics revealed ceramide and phospholipid remodelling upon immune activation,[85] while plasma EVs in psoriasis showed distinct lipid patterns between exosomes and microvesicles, pointing to their role in stratifying chronic inflammatory diseases.[60]

These studies highlight that EV omics holds clear clinical promise but remains at a transitional stage. On the one hand, EVs enable minimally invasive, repeatable access to molecular information across oncology, neurodegeneration, psychiatry, infectious disease and metabolic disorders. On the other hand, translational potential is tempered by methodological heterogeneity, lack of validated reference ranges and the scarcity of large-scale, multi-centre validation cohorts. Furthermore, most studies remain discovery-oriented, with limited progression to targeted assays or clinical-grade diagnostic pipelines.

CHALLENGES AND STANDARDISATION NEEDS

EV multi-omics requires exceptionally pure preparations, as abundant plasma proteins and lipoproteins can severely compromise analytical outcomes. Among the most problematic contaminants are serum albumin and lipoprotein particles carrying apolipoproteins A (apoA) and B (apoB). Albumin can dominate MS spectra, masking signals from low-abundance proteins. Similarly, lipoproteins contribute their own protein cargo and carry substantial lipid and metabolite content, confounding both lipidomics and metabolomics datasets.[103] In particular, the lipid signatures of apoA- and apoB-associated lipoproteins overlap substantially with those of EV membranes, making it difficult to discriminate between vesicle-derived and contaminant-derived signals.[104] These interferences risk both false positives and the loss of sensitivity needed to detect rare but functionally significant lipid species or metabolic intermediates. Therefore, rigorous depletion of albumin and lipoproteins is a prerequisite to unlock the full potential of EV omics.

To address the pervasive problem of co-isolated contaminants in EV preparations, several purification strategies have been developed, the most widely used being SEC and ultracentrifugation (UC).[105–108] SEC has proven particularly effective at separating EVs from soluble proteins such as albumin, as well as from smaller lipoprotein particles, by exploiting differences in particle size. It offers the advantages of preserving vesicle integrity and being compatible with downstream omics workflows, but its resolution can be limited when EVs overlap in size with high-density lipoproteins (HDL), making complete depletion of apoA-containing particles challenging. UC, in contrast, separates vesicles based on buoyant density and has long been considered a gold standard for plasma EV enrichment (Figure 3b).

However, high-speed spins can co-pellet low-density lipoproteins (LDL/VLDL), while repeated centrifugation steps risk vesicle aggregation, loss of yield and changes in vesicle surface composition. Mult-step methods, such as density gradient UC or SEC coupled with density cushions, can substantially reduce contaminants but are labour intensive, time consuming and often not scalable to large cohorts. Moreover, no method to date guarantees absolute removal of albumin or lipoproteins without also impacting EV yield. These technical hurdles highlight the ongoing need for improved purification platforms that can deliver high recovery of intact EVs with minimal contamination, thereby enabling more reliable multi-omics profiling and ensuring that the data obtained reflects the biology of EVs rather than artefacts of co-isolated plasma components.

Taken together, these challenges highlight the urgent need for internationally recognised guidelines for EV multi-omics, similar to the minimal information for studies of extracellular vesicles (MISEV) recommendations for EV research.[54] Standardisation must encompass: (i) isolation protocols tailored for purity without sacrificing scalability, (ii) sample preparation consensus with harmonised extraction and digestion workflows, (iii) benchmarking of MS platforms against shared reference materials and (iv) multi-omics integration frameworks that ensure data comparability.

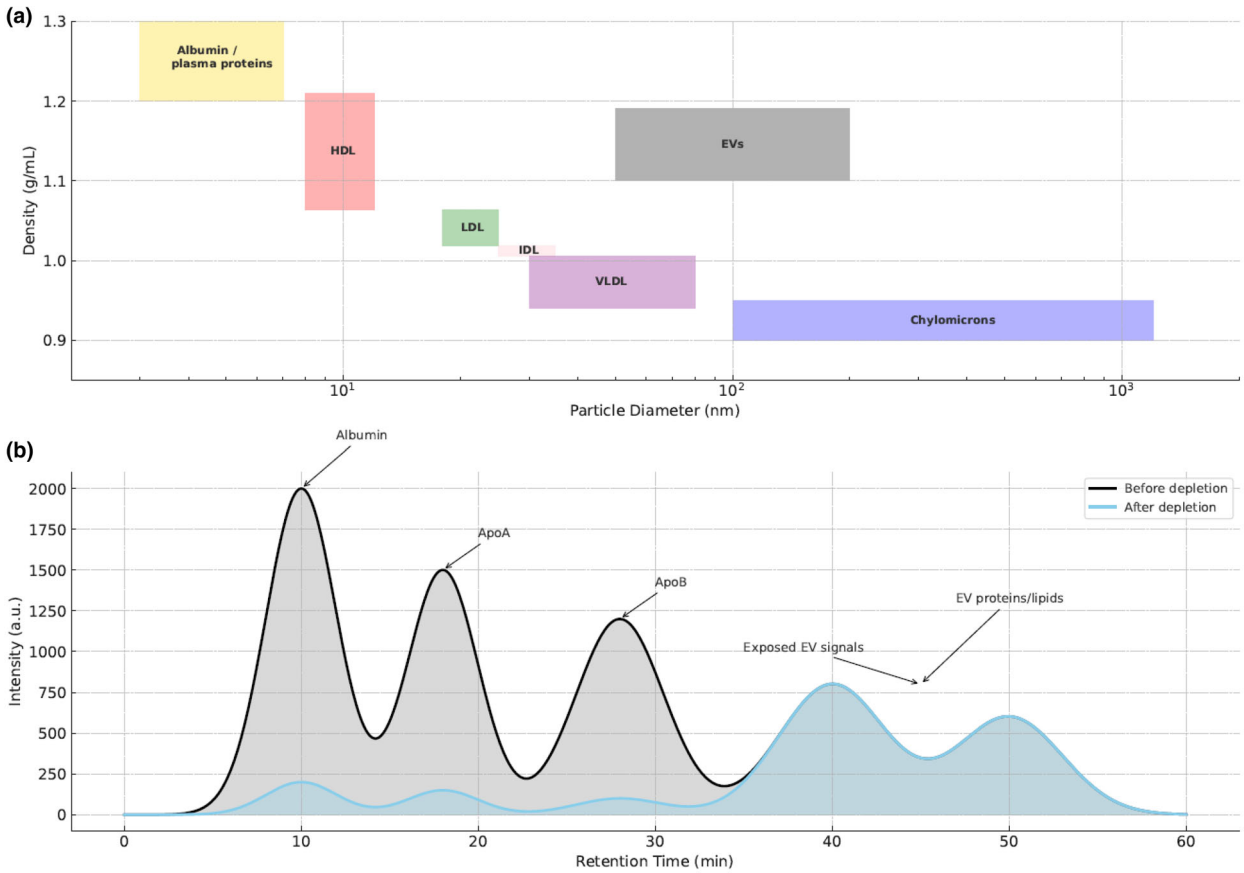

Figure 3. Schematic of lipoprotein/extracellular vesicles (EV) overlap and effect of depletion on LC–MS analysis. **(a)** Size and density distributions of major plasma lipoproteins, albumin/plasma proteins and EVs. Albumin and abundant plasma proteins are small (< 7 nm) but highly dense (1.20–1.30 g/mL). High-density lipoproteins (HDL; 8–12 nm, 1.063–1.210 g/mL) overlap extensively with the EV buoyant density window (1.10–1.19 g/mL), making them the most problematic contaminants. Low-density lipoproteins (LDL; 18–25 nm), intermediate-density lipoproteins (IDL; 25–35 nm), very-low-density lipoproteins (VLDL; 30–80 nm) and chylomicrons (> 100 nm) overlap with EVs in size but not density. **(b)** Simulated LC–MS chromatograms before (grey) and after (blue) depletion of albumin and apolipoprotein-containing lipoproteins (ApoA and ApoB). Prior to depletion, albumin and ApoA/B dominate the chromatogram, masking EV-derived peaks. Following depletion, contaminants are reduced, allowing EV-associated signals to emerge.

Bridging omics discoveries to clinical diagnostics also requires pragmatic consideration of existing, validated technologies. While MS-based platforms provide unparalleled resolution, their cost, complexity and need for specialised expertise limit immediate clinical adoption. To translate EV-derived biomarker candidates into routine diagnostics, omics discoveries must ultimately be adapted into scalable assays that can be performed in standard hospital or laboratory settings. Flow cytometry, for instance, has already been adapted to characterise surface proteins, such as CD9, CD63 and CD81 on EVs, and its widespread clinical use makes it a natural bridge between discovery proteomics and routine diagnostic implementation.[27,109,110] Similarly, ELISA-based assays targeting EV proteins or lipid-associated epitopes can provide cost-effective, reproducible quantification at scale. Studies that identify disease-specific proteomic or lipidomic EV markers[14,22,60] should therefore be designed with downstream translatability in mind, prioritising markers that can be validated with antibody-based assays. For metabolomics, targeted panels measuring key metabolites identified in discovery studies[13,55,64] can be adapted into clinical-grade kits if supported by robust standardisation and validation. In this way, the most effective route to clinical implementation is likely a hybrid approach; discovery-driven multi-omics for

candidate identification, followed by translation into flow cytometry or ELISA-based diagnostics for clinical scalability.

CONCLUSION

Mass spectrometry-based multi-omics has transformed our understanding of EVs from passive cellular debris to dynamic carriers of diagnostically actionable information. EVs isolated from diverse biofluids consistently mirror pathological states, with proteomic, metabolomic and lipidomic signatures revealing functional associations across oncology, neurodegeneration and inflammatory disease.

Yet, while discovery-driven studies underscore the immense potential of EV omics, several barriers remain before translation into routine clinical practice. Isolation strategies vary widely, with ultracentrifugation, bead enrichment, SEC and next-generation systems like EXODUS each offering different trade-offs between yield and purity. Sample preparation workflows, extraction chemistries and the diversity of MS platforms further fragment the field, often confounding biological variability with methodological inconsistency. Without harmonisation, reproducibility across studies and thus clinical reliability remains a major obstacle.

Moving forward, standardisation and integration will define the trajectory of EV omics. Establishing consensus protocols for isolation, sample preparation and data processing will be critical to ensure comparability across laboratories and patient cohorts. Equally important is the translation of omics discoveries into clinically scalable assays. While MS-based workflows remain indispensable for discovery, their complexity and cost limit widespread deployment. Bridging this gap will require the adaptation of omics-identified biomarkers into validated clinical platforms, such as flow cytometry and ELISA, which already underpin diagnostic workflows in hospitals worldwide. Targeted assays and antibody-based platforms provide the most immediate opportunity to transform EV multi-omics into diagnostic and prognostic tools, particularly when coupled with rigorous validation in multi-faceted studies.

Ultimately, the clinical promise of EV multi-omics lies in its ability to deliver integrated molecular portraits that capture proteins, metabolites and lipids from a single vesicular preparation, offering a window into disease biology that is both minimally invasive and dynamically responsive. By coupling methodological rigour with translational foresight, the field is poised to shift from proof-of-concept studies towards clinically actionable biomarkers and therapeutic strategies. The next phase of EV omics research must therefore balance innovation with standardisation, ensuring that technological sophistication is matched by clinical feasibility. In doing so, EV-based multi-omics has the potential to redefine precision medicine, turning EVs into robust sentinels and active participants in human health and disease.

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AUTHOR CONTRIBUTIONS

Dimitri Aubert: Data curation; writing – original draft; writing – review and editing; resources; methodology; validation. **Arutha Kulasinghe:** Data curation; writing – original draft; writing – review and editing; supervision; resources; validation; investigation. **Akila Wijerathna-Yapa:** Conceptualization; methodology; software; data curation; supervision; formal analysis; validation; investigation; writing – original draft; writing – review and editing; project administration; resources.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Statistical analyses and visualisation scripts are freely available at GitHub: https://github.com/akila-wijerathna-yapa/MultiOmics-EV-Analysis/.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.