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Developing blood extracellular vesicle protein biomarkers for hepatocellular carcinoma: mechanisms, methods, and translation

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Abstract

Hepatocellular carcinoma (HCC) remains a leading cause of cancer-related mortality globally, with late-stage diagnoses predominantly driven by the absence of early clinical symptoms and the inadequate sensitivity of current diagnostic tools, highlighting a critical unmet need in liver cancer management. Circulating extracellular vesicles (EVs), which naturally carry proteins and nucleic acids, serve as both mediators of tumor-specific biology and reporters of early pathophysiological changes, offering a transformative avenue for noninvasive liquid biopsy. This review integrates mechanistic advances in understanding EV-associated proteins involved in HCC pathogenesis with state-of-the-art proteomic methodologies for EV biomarker discovery, validation, and clinical translation. We highlight how functional and mechanistic insights into EV proteins guide the identification of clinically relevant biomarkers and examine recent progress in analytical approaches for isolating and profiling blood-derived EVs in large patient cohorts. Additionally, we discuss the development of high-throughput detection platforms, such as biochip-based assays, which are accelerating the translation of EV protein biomarkers from research to routine clinical practice. Critical challenges, including the lack of standardized analytical workflows, limited demonstration of clinical utility, and the need for multicenter validation, are addressed, with a focus on strategies to ensure robust and reproducible biomarker performance. By bridging mechanistic understanding with analytical innovation and clinical application, we aim to accelerate the deployment of EV proteomics for earlier HCC detection, risk stratification, and treatment monitoring, ultimately advancing precision oncology.

Keywords Hepatocellular carcinoma, Extracellular vesicles, Proteomics, Protein biomarkers, Liquid biopsy

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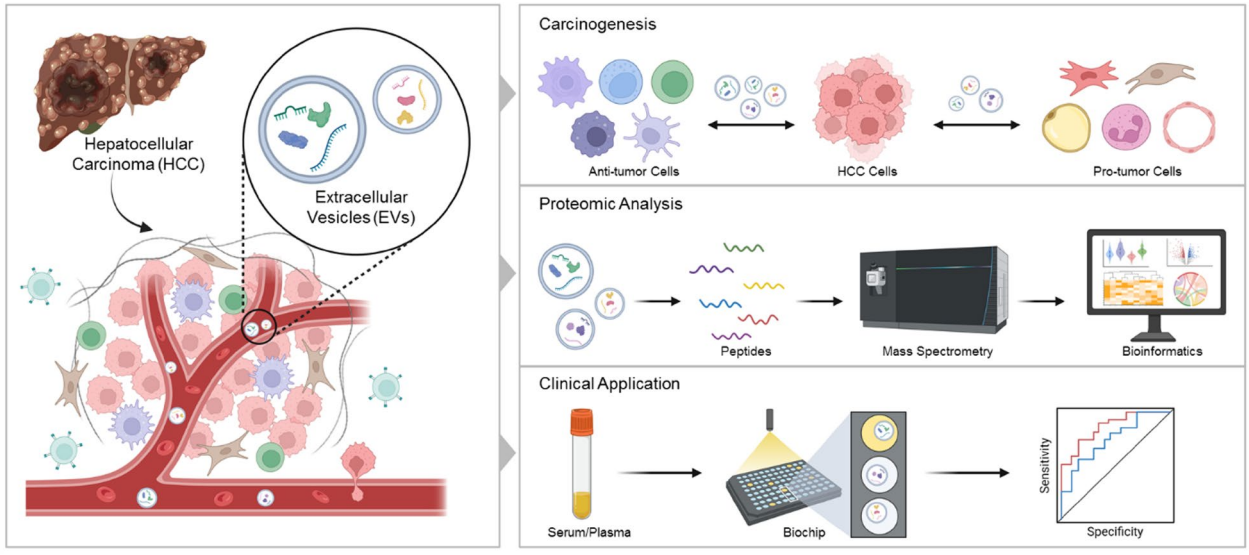
Graphical abstract



Introduction

Liver cancer ranks as the sixth most prevalent malignancy worldwide and the third leading cause of cancer-related mortality, following lung and colorectal cancers [1]. Global epidemiological projections indicate a continued rise in both incidence and death rates, with annual liver cancer deaths expected to reach 1.3 million by 2040 [2]. Primary liver cancer encompasses several histological subtypes, with hepatocellular carcinoma (HCC) representing 75%–85% of cases, followed by intrahepatic cholangiocarcinoma (ICC), combined hepatocellular-cholangiocarcinoma (cHCC-CCA), and other rare entities. The etiological landscape of HCC is shaped by chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, alcohol-related liver disease (ALD), aflatoxin exposure, obesity, smoking, and type 2 diabetes [3]. Notably, geographic variations are pronounced: over half of new HCC cases occur in China (primarily driven by HBV), while metabolic dysfunction-associated steatotic liver disease (MASLD) has emerged as the leading etiology in India (35.5%), surpassing viral hepatitis. In Western countries and Latin America, MASLD and ALD now predominate, with the incidence of MASLD-related HCC increasing by 37% over the past decade [4].

Despite advances in therapeutic modalities, the clinical outlook for HCC remains poor. Over 50% of patients are diagnosed at intermediate or advanced stages (Barcelona Clinic Liver Cancer Stage B or C), missing the window for curative intervention due to the insidious onset and lack of reliable early biomarkers [5]. Current clinical pathways for HCC management, as outlined by major international guidelines from the National Comprehensive Cancer Network (NCCN) [6], the American Association for the Study of Liver Diseases (AASLD) [7], and the European Association for the Study of the Liver (EASL) [8], predominantly rely on imaging, histopathological biopsy, and serum biomarkers. These can be broadly divided into two stages: surveillance for early detection in high-risk populations and diagnostic workup for suspected lesions. For surveillance, the AASLD recommends semiannual abdominal ultrasound (US) combined with serum alpha-fetoprotein (AFP) in patients with cirrhosis, while the EASL recommends semiannual US as the primary screening modality, noting that the addition of AFP is a reasonable option. However, this strategy has well-documented limitations. A meta-analysis cited in the AASLD guidance reported that the US alone has a sensitivity of only 53% for detecting early-stage HCC, which increases to 63% when combined with AFP [9]. The performance is operator-dependent and further compromised in patients with obesity, hepatic steatosis, or advanced cirrhosis. Serum AFP, though widely used and cost-effective, has a sensitivity of only 49% for early-stage HCC according to EASL guidelines, suffers from poor performance in HBV-negative and MASLD-related cases, and yields false positives in up to 20% of patients with benign liver conditions; thus, it is not recommended as a standalone screening test [10]. To date, no body fluid biomarker other than AFP has been incorporated into routine HCC surveillance guidelines by AASLD or EASL. For diagnostic evaluation, multiphasic computed tomography (CT) or magnetic resonance imaging (MRI) is recommended when surveillance identifies a suspicious lesion ≥ 1 cm or a rising AFP level. While these imaging

modalities provide valuable anatomical and hemodynamic detail, EASL guidelines note that their sensitivity for lesions in the 1–2 cm range remains limited, with a prospective multicenter study reporting a sensitivity of 71% for MRI and 68% for CT [8]. Histopathological biopsy, the diagnostic gold standard, is reserved for cases where imaging is inconclusive but carries inherent risks of bleeding, infection, and sampling error, especially for lesions in deep hepatic regions or adjacent to major vessels. Together, these limitations across current surveillance and diagnosis frameworks contribute to delayed detection and suboptimal treatment outcomes, with five-year survival rates remaining as low as 18% [11, 12]. This pressing clinical issue highlights the necessity for noninvasive biomarkers with high sensitivity and specificity for early HCC detection.

In response to these unmet needs, liquid biopsy technologies have gained momentum as transformative tools for cancer diagnostics. Among these, circulating extracellular vesicles (EVs), the lipid bilayer-enclosed carriers of cell-specific proteins, nucleic acids, and lipids, have emerged as a particularly promising avenue for noninvasive HCC detection. Unlike free circulating proteins, EVs offer enhanced stability in biofluids and faithfully reflect the molecular landscape of their cells of origin, including early pathological changes in tumor cells [13]. EVs mediate intercellular communication and play pivotal roles in tumorigenesis, angiogenesis, immune modulation, and pre-metastatic niche formation [13]. Their abundance, molecular diversity, and stability in circulation render them ideal candidates for biomarker development. Critically, cancer-derived EVs can signal disease-associated alterations before the onset of clinical symptoms or radiological evidence, positioning them as powerful tools for early-stage cancer diagnosis [14]. The development of robust and sensitive EV-based biomarkers holds substantial promise for advancing the early detection and risk stratification of HCC, with the potential to improve patient outcomes.

This review provides a comprehensive synthesis of the current landscape in blood EV proteomics for HCC. We delineate the mechanistic roles of EV-associated proteins in HCC pathogenesis and discuss state-of-the-art proteomic methodologies for the discovery, validation, and clinical translation of EV biomarkers. Special emphasis is placed on EV isolation strategies, optimized workflows for large-scale blood EV proteomics, and emerging targeted detection platforms, including biochip-based systems tailored for clinical implementation. We systematically consolidate evidence for EV protein biomarkers with diagnostic and prognostic utility, critically appraise analytical and biological sources of variability, and propose a roadmap for standardization, regulatory compliance, and multicenter validation. By integrating mechanistic insights with methodological rigor and clinical relevance, this review aims to accelerate the deployment of EV proteomics for earlier HCC detection, risk stratification, and personalized treatment monitoring.

EV Classification and Contents

EVs are membrane-bound particles secreted by virtually all cell types, carrying a diverse array of biomolecules. Traditionally, EVs have been classified by their biogenesis into ectosomes (100–1000 nm, originating from plasma membrane budding), exosomes (30–150 nm, formed within multivesicular bodies), and apoptotic bodies (fragments from apoptotic cells) [13]. However, the MISEV2023 guidelines, which provide the current consensus framework for EV research, recommend avoiding these biogenesis-based terms unless the subcellular origin has been experimentally confirmed. Instead, the guidelines advocate for operational classifications based on physical properties (e.g., small/large EVs), biochemical markers (e.g., $CD63^+/CD81^+$ EVs), or cell source and production context (e.g., hypoxia-induced EVs) [15], a framework we adhere to herein.

EVs encapsulate proteins, lipids, DNA, RNA, and metabolites, protected by a lipid bilayer that confers remarkable stability compared to free circulating molecules [16]. Proteins, both luminal and membrane-associated, constitute the most abundant cargo and play pivotal roles in intercellular communication and modulation of the tumor microenvironment (TME) [17]. EVs exhibit substantial heterogeneity, not only between different cell origins but also within populations from the same cell type, reflecting dynamic pathophysiological states and supporting the existence of distinct EV subpopulations [18, 19]. This heterogeneity presents both a challenge (e.g., difficulty in isolating functionally relevant subpopulations) and an opportunity (e.g., capturing disease-specific molecular signatures) for biomarker discovery, underscoring the need for robust characterization and standardization in EV research.

Functions and Applications of EVs

EVs serve as essential mediators of intercellular communication, transferring molecular cargo to recipient cells and thereby modulating signaling pathways and cellular behaviors. They are involved in a broad spectrum of physiological and pathological processes, including immune regulation, development, cancer progression, metabolic disorders, and neurodegeneration. In cancer, EV-associated proteins and nucleic acids orchestrate intricate tumor–stroma interactions, driving proliferation, angiogenesis, metastasis, and epithelial–mesenchymal transition (EMT). Concurrently, EVs influence immune responses within the TME, promoting immune evasion and contributing to drug resistance [20].

From a clinical perspective, EVs are increasingly recognized as valuable sources of biomarkers for noninvasive cancer diagnosis, treatment monitoring, and prognosis prediction [21]. Their clinical utility is underpinned by their molecular diversity, exceptional stability in biofluids, and capacity to faithfully reflect the physiological state of donor cells, particularly in response to dynamic changes within the TME such as hypoxia, acidosis, and inflammation [22]. For example, urinary EVs carrying RNA transcripts (PCA3, ERG, and SPDEF) have been clinically validated for prostate cancer risk stratification via the platform ExoDx Prostate, which has aided over 50,000 patients in their decision-making process and is now included in clinical guidelines [23]. Emerging translational applications utilize EV surface markers, such as CD147 for colorectal cancer detection and Glypican-1 for pancreatic cancer screening, as described in a recent comprehensive review [24]. Advanced proteomic and phosphoproteomic profiling of EVs derived from plasma or urine has facilitated the identification of diagnostic biomarkers for multiple cancer types, including breast [25], kidney [26], and prostate cancers [27], highlighting the translational potential of EV-based assays.

Beyond diagnostics, EVs are gaining prominence as a versatile therapeutic platform for cancer treatment. Current preclinical efforts have primarily explored three directions: EV-based drug delivery, immunotherapy, and overcoming therapeutic resistance. A distinctive advantage of EVs for HCC treatment is the natural hepatic tropism of systemically administered EVs, particularly their preferential uptake by Kupffer cells and hepatic sinusoidal endothelial cells, making the liver an ideal target organ for EV-based therapeutic strategies. Compared with synthetic nanocarriers, EVs possess several intrinsic advantages as drug delivery vehicles. Their endogenous origin confers low immunogenicity, high biocompatibility, and minimal toxicity, while the lipid bilayer protects encapsulated cargo from enzymatic degradation, and their nanoscale dimensions enable prolonged circulation and passive hepatic accumulation [28–31]. These collectively contribute to reduced immune clearance and efficient cellular uptake. To date, engineered EVs have been loaded with diverse therapeutic payloads, including chemotherapeutic agents (e.g., methotrexate, doxorubicin, paclitaxel), nucleic acids (e.g., siRNA, miRNA), and even proteins such as the Cas9 nuclease for CRISPR-based gene editing [32, 33]. EVs also hold considerable promise for cancer immunotherapy. Currently, cancer vaccines have been developed using immune cell-derived EVs [34]. Dendritic cell-derived EVs (Dex) naturally harbor MHC molecules and costimulatory proteins, and can be engineered to present tumor-associated antigens via peptide loading, enabling the priming of antigen-specific T cell and natural killer (NK) cell responses. Such Dex-based cell-free vaccines have been demonstrated in phase I and phase II clinical trials for advanced malignancies [35–37]. Beyond these peptide-loaded Dex platforms, further engineered EVs functionalized with tumor-targeting ligands, immunoadjuvants, and tumor antigens offer a versatile and universally applicable strategy for personalized immunotherapy, particularly against genetically heterogeneous tumors. Notably, this modular approach can be readily adapted to different solid tumor types by exchanging the targeting moiety [32, 38]. In addition, EVs can help overcome therapeutic resistance, a critical challenge given the high recurrence rate of HCC. For instance, knockdown of an oncogenic lncRNA in HCC cells correspondingly depleted its levels in secreted EVs, thereby reducing the ability of these EVs to transfer chemoresistance to recipient tumor cells and restoring their drug sensitivity [20]. Co-delivery of chemotherapeutic agents with siRNAs targeting resistance-associated pathways via a single EV platform also represents a promising strategy to re-sensitize resistant HCC cells to drugs such as sorafenib [39]. Despite these encouraging preclinical advances, the translation of EV-based therapeutics into clinical practice faces significant hurdles, most notably the establishment of scalable Good Manufacturing Practice (GMP)-grade production processes and standardized quality control criteria to ensure batch-to-batch consistency, as well as rigorous clinical validation of safety and efficacy in HCC patients.

The Role of EV-Associated Proteins in HCC Pathogenesis

The TME of HCC consists of a complex mixture of extracellular matrix (ECM) and diverse stromal cells. These include cancer-associated fibroblasts (CAFs), hepatic stellate cells (HSCs), mesenchymal stem cells (MSCs), adipocytes, tumor-associated macrophages (TAMs), dendritic cells (DCs), and myeloid-derived suppressor cells. EVs serve as critical mediators of communication between these stromal components and tumor cells, thereby playing a pivotal role in hepatocarcinogenesis (illustrated in Fig. 1). Growing evidence demonstrates that EV-associated proteins, both those encapsulated within EVs and those whose expression is modulated in recipient cells, profoundly influence oncogenic processes, including cell proliferation and apoptosis, tumor invasion and metastasis, EMT, immune evasion, angiogenesis, TME remodeling and drug resistance [40]. These proteins, acting through diverse signaling pathways, facilitate dynamic bidirectional interactions between tumor and stromal cells, thereby regulating HCC progression (Table 1).

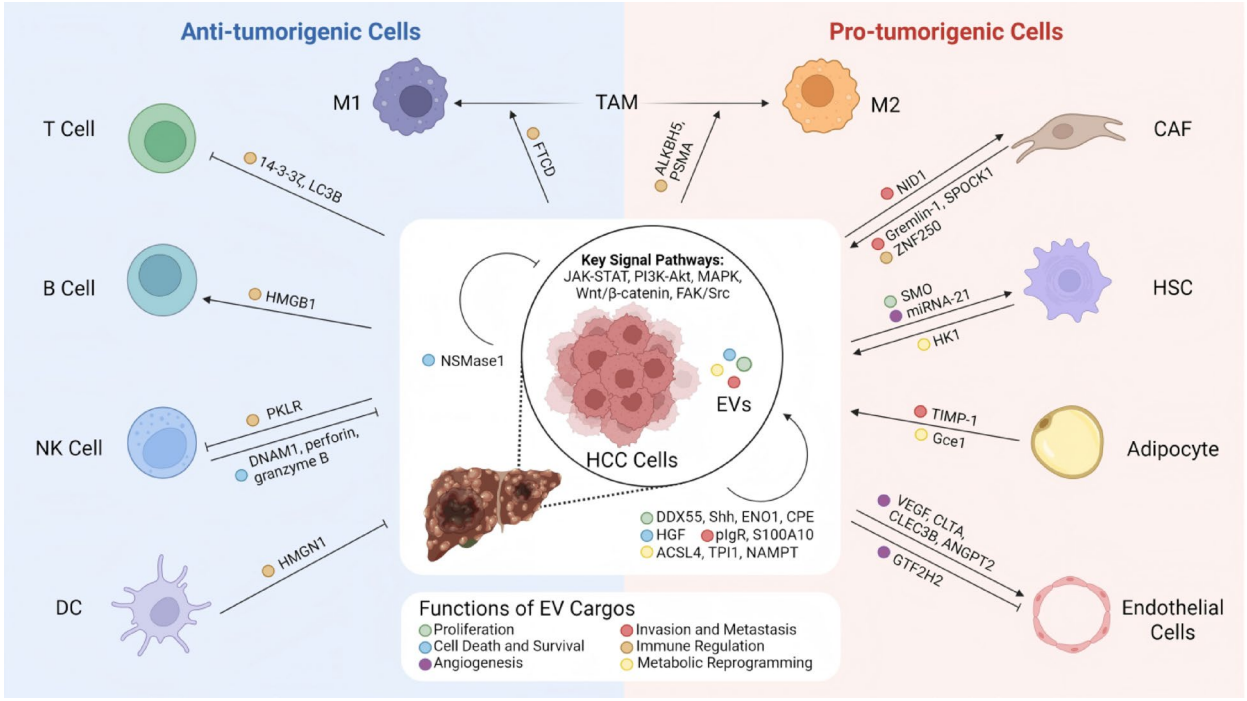


Fig. 1 EV-based interaction between HCC cells and stromal cells, including anti-tumorigenic cells and pro-tumorigenic cells. EVs with different colors indicate distinct functions, including orchestrating uncontrolled proliferation, modulating cell death and survival, promoting angiogenesis, driving invasion and metastasis, immune regulation, and metabolic reprogramming. The figure was created in BioRender.com, with authorized permission

Table 1 Functional roles and mechanisms of representative EV proteins in HCC

EV Protein	EV Source	Level	Functions	Key Mechanisms	Reference
GPC3	HCC cells	↑	tumor cell proliferation, metastasis	Wnt/β-catenin, Hedgehog signaling pathway	[41]
DDX55	HCC cells	↑	HCC cell proliferation, migration and invasion, angiogenesis, EMT	PI3K/Akt/GSK-3β/β-catenin pathway	[42]
ENO1	HCC cells	↑	EMT, metastasis	upregulation of integrin α6β4, FAK/Src-p38MAPK signaling pathway	[46]
CPE	HCC cells	↑	tumor cell proliferation, metastasis	Cyclin D1, C-MYC, ERK signaling pathway	[47]
Gremlin1	CAFs	↑	EMT, tumor cell stemness, metastasis, sorafenib resistance	Wnt/β-catenin, PDGF-AB/Rab27a, BMP signaling pathway	[64]
LOXL4	HCC cells	↑	EMT, metastasis, immune escape	FAK/Src, TGF-β/SMAD signaling pathway	[67]
LGALS3BP	HCC cells	↑	tumor cell proliferation, metastasis, invasion, immune escape	RhoA/MLCK signaling pathway	[68]
pIgR	HCC cells	↑	tumor cell stemness, proliferation, metastasis, EMT	PDK1/Akt/GSK3β/β-catenin signaling pathway	[69]
S100A10	HCC cells	↑	tumor cell stemness, drug resistance, EMT	EGFR/AKT/ERK signaling pathway	[70]
NID1	HCC cells	↑	angiogenesis, pulmonary endothelial permeability, metastasis	activate fibroblasts in pre-metastatic niche formation to secrete TNFR1	[71]
FTCD	HCC cells	↓	M1 polarization, glycolytic reprogramming	HIF-1α, PI3K-AKT signaling pathway	[73]
PKM2	HCC cells	↑	monocyte-to-macrophage differentiation, TME remodeling	CCL1-CCR8 axis, glycolytic metabolic reprogramming, STAT3 phosphorylation	[77]
14-3-3ζ	HCC cells	↑	inhibit anti-tumor functions and regulate differentiation of T cells	down-regulation of CD69, Ki67 and anti-tumor cytokines, up-regulation of PD-1, TIM-3 and immunosuppressive factors	[83]
HK1	HSCs	↑	glycolytic reprogramming	TGF-β/Akt/Nur77/ABHD17B axis	[86]

Orchestrating Uncontrolled Proliferation

EVs act as potent mitogenic effectors in the TME by shuttling oncogenic proteins that reactivate developmental pathways, including the Wnt/β-catenin and Hedgehog axes, and amplify kinase cascades. For instance, EV-encapsulated Glypican-3 (GPC3) [41] and DEAD-box helicase 55 (DDX55) [42] have been identified to converge on β-catenin signaling, thereby driving cell cycle

progression. In parallel, the Hedgehog pathway is fueled by the EV-mediated transfer of Sonic Hedgehog (Shh) [43] and the transducer Smoothened (SMO) [44]. Notably, SMO is not only retained in tumor cells but also transferred to HSCs, creating a pro-tumorigenic feedback loop.

Beyond developmental pathways, EV proteins directly manipulate kinase-driven growth signals. Vasorin (VASN) [45] and α-Enolase (ENO1) [46] delivered by EVs have been shown to trigger STAT3 and MAPK pathways, respectively, utilizing integrin-mediated signaling to bypass normal growth checkpoints. Similarly, Carboxypeptidase E (CPE) promotes proliferation by modulating the c-MYC axis [47]. While the majority of identified cargo is pro-tumorigenic, EVs also maintain a complex balance by transporting tumor suppressors like p120-catenin, which inhibits STAT signaling [48].

It is important to note that this protein-driven network acts in concert with non-coding RNAs. While proteins often serve as direct mediators of mitogenic signaling, EV-associated miRNAs (e.g., miR-125a/b [49]) and lncRNAs (e.g., C5orf66-AS1 [50]) function as upstream regulators that fine-tune the expression of these key proliferative drivers. However, from a translational perspective, the high abundance and stability of EV-associated proteins such as GPC3 and VASN in circulation make them more robust candidates for proteomic-based liquid biopsy.

Modulating Cell Death and Survival

EV proteins act as a double-edged sword in the TME, either fueling therapeutic resistance or triggering tumor cell elimination depending on their cellular origin. On the pro-tumorigenic side, HCC cells utilize EVs to evade apoptosis and sustain survival under stress. A key mechanism involves the transfer of Hepatocyte Growth Factor (HGF), which activates the c-Met/Akt signaling in recipient cells, suppresses caspase-9 and caspase-3 activation, and thereby attenuates apoptosis and promotes sorafenib resistance [51]. Conversely, evasion of cell death may also be facilitated by downregulation of pro-apoptotic EV cargo; for instance, reduced levels of Neutral Sphingomyelinase 1 (NSMase1) in HCC-derived EVs may limit ceramide accumulation, thereby shifting lipid signaling away from apoptosis [52].

In contrast, EVs derived from cytotoxic immune and stromal cells carry a lethal protein cargo that can be harnessed for therapy. NK cell-derived EVs exert potent antitumor effects against Hep3B cells by delivering perforin and granzyme B [53]. This cytotoxic delivery is facilitated by EV surface DNAM-1, which engages CD155/CD112 on tumor cells and promotes EV binding and internalization [54]. Following uptake, perforin and granzyme B activate the intrinsic apoptotic pathway. Beyond classical apoptosis, MSC-derived EVs have been reported to induce a non-canonical cell death program reminiscent of neutrophil extracellular trap (NET) formation [55]. This effect appears to be mediated by a unique proteomic payload enriched in complement components, ECM proteins, and serine protease inhibitors, and is associated with activation of the PI3K-Akt and Toll-like receptor signaling together with complement and coagulation cascades. Translationally, EV-associated molecules, such as surface DNAM-1, and abundant circulating proteins, such as HGF, may serve as accessible blood-based biomarkers for longitudinal monitoring of the evolving balance between tumor progression and immune-mediated antitumor responses.

Promoting Angiogenesis and Vascular Remodeling

EVs promote tumor neovascularization by delivering a diverse repertoire of pro-angiogenic factors that stimulate endothelial cell proliferation, migration, and tube formation.

HCC-derived EVs directly enhance angiogenesis by transferring molecules such as vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs), thereby activating the PI3K/AKT and MAPK pathways in endothelial cells [56]. They can also indirectly remodel the vascular niche by delivering miR-21 to HSCs, where PTEN suppression activates the PDK1/AKT pathway and drives HSC differentiation into CAFs that secrete VEGF, MMP2, MMP9, bFGF, and TGF-β [57]. Additional EV-associated proteins further reinforce this program. Angiopoietin-2 (ANGPT2) is internalized by endothelial cells and activates the AKT/eNOS and AKT/β-catenin axes, driving endothelial activation [58]. In parallel, vWF establishes a pro-angiogenic feedback loop by inducing endothelial secretion of VEGF-A and FGF2, thereby creating a cytokine-rich microenvironment [59]. Likewise, Clathrin Light Chain A (CLTA) enhances vascular permeability and tube formation by stabilizing the transmembrane protein Basigin (BSG) [60], while EV-packaged CCR6 drives angiogenesis through activation of the CCL20-CCR6 axis [61]. Conversely, angiogenesis may also be facilitated by depletion of inhibitory cargos. For instance, the downregulation of C-type lectin domain family 3 member B (CLEC3B) in HCC-EVs reduces AMPK pathway activation, leading to unchecked VEGF expression and enhanced neovascularization [62]. While less common, specific cargos such as general transcription factor IIH subunit 2 (GTF2H2) can exert anti-angiogenic effects [63], illustrating the complexity of EV-mediated vascular remodeling. Clinically, enrichment of angiogenic drivers like ANGPT2 and CLTA within circulating EVs offers a distinct advantage for liquid biopsy, as these encapsulated forms may reflect

the active state of the tumor microvasculature more faithfully than free-circulating factors.

Driving Invasion and Metastasis

EVs orchestrate the metastatic cascade via a coordinated 'seed and soil' strategy: they empower tumor cells ('seeds') with invasive properties through EMT, while simultaneously fertilizing distant microenvironments ('soil') to support colonization.

The induction of an invasive phenotype is frequently mediated by stromal-derived cues. For instance, CAF-derived EVs transfer Gremlin-1, which activates Wnt/β-catenin and BMP signaling to drive EMT [64]. Similarly, CAF-derived SPOCK1, a matricellular proteoglycan, enhances tumor cell invasiveness [65], while adipocyte-derived EVs promote a pro-metastatic state via integrin αvβ5 and tissue inhibitor of metalloproteinase-1 (TIMP-1), which amplifies TGF-β signaling and inhibits MMPs, respectively [66]. Within tumor cells, autocrine EV signaling further reinforces this plasticity. Lysyl oxidase-like 4 (LOXL4) and Galectin-3 binding protein (LGALS3BP) are key cargos that activate FAK/Src and RhoA/MLCK pathways, respectively, thereby promoting cytoskeletal reorganization and cell migration [67, 68]. Furthermore, stemness-associated cargos such as polymeric immunoglobulin receptor (pIgR) and S100 calcium-binding protein A10 (S100A10) sustain metastatic competence by activating the PDK1/Akt/β-catenin and AKT/ERK axes [69, 70].

Beyond local invasion, EVs act as long-distance messengers to establish pre-metastatic niches. A prime example is Nidogen-1 (NID1), which is enriched in EVs from metastatic HCC cells. NID1 activates pulmonary fibroblasts to secrete tumor necrosis factor receptor 1 (TNFR1), thereby enhancing pulmonary vascular permeability and establishing a lung pre-metastatic niche that supports the colonization and growth of disseminated tumor cells [71]. Additionally, EVs carrying specific ECM components, such as modified Fibronectin 1 (FN1) and Fibrinogen γ chain (FGG), have been implicated in conditioning the microenvironment to support dissemination [72]. Clinically, detection of niche-promoting factors such as NID1 and invasion drivers like LOXL4 in plasma EVs may serve as a specific indicator of metastatic risk, potentially preceding radiological evidence.

Immune Regulation

EVs act as pivotal mediators in the dynamic crosstalk between the tumor and the immune system, exerting context-dependent effects that either restrain or fuel HCC progression. In the context of tumor suppression, specific EV cargo functions as an immune booster to enhance surveillance. For instance, Formimidoyltransferase-cyclodeaminase (FTCD) transferred to macrophages promotes their polarization toward the pro-inflammatory M1 phenotype, thereby reinvigorating anti-tumor immunity [73]. Similarly, DC-derived EVs are equipped with functional MHC molecules and immunostimulatory proteins that augment the cytotoxicity of T cells and NK cells [74]. Leveraging this mechanism, engineered EVs loaded with the immunoadjuvant HMGN1 have demonstrated potential as cancer vaccines capable of inhibiting HCC growth and inducing durable immune memory [38].

Conversely, tumor-derived EVs more commonly orchestrate systemic immune escape, primarily by paralyzing innate defenders and exhausting adaptive effectors. To subvert innate immunity, HCC-derived EVs skew macrophages toward the immunosuppressive M2 phenotype. Key drivers include AlkB homolog H5 (ALKBH5), which mediates M2 polarization via the SOX4/SHH axis [75], proteasome subunit α5 (PSMA5), which activates JAK2/STAT3 signaling [76], and pyruvate kinase M2 (PKM2), which induces metabolic reprogramming and monocyte-to-macrophage differentiation [77]. Simultaneously, NK cell surveillance is compromised by the delivery of pyruvate kinase L/R (PKLR), which disrupts metabolism [78], and the checkpoint ligand CD155, which causes exhaustion via TIGIT signaling [79].

To evade adaptive elimination, EVs establish a multilayered suppression of $CD8^+$ T cells, with programmed cell death ligand 1 (PD-L1) serving as a central hub. Golgi membrane protein 1 (GOLM1) facilitates the sorting of PD-L1 into EVs, enabling suppression of $CD8^+$ T cell function when transferred to TAMs [80], while LOXL4 and zinc finger protein 250 (ZNF250) upregulate PD-L1 expression in recipient macrophages and tumor cells, respectively, thereby suppressing $CD8^+$ T cell cytotoxicity via binding to PD-1 [81, 82]. Beyond checkpoints, direct T cell exhaustion is driven by EV-associated 14-3-3ζ [83] and by light chain 3 beta (LC3B)-positive EVs carrying HSP90α, which trigger a suppressive IL-6/IL-8 loop [84]. Additionally, HMGB1 fosters the expansion of regulatory B cells, further dampening $CD8^+$ T cell responses [85]. From a clinical perspective, these EV-encapsulated regulators, particularly PD-L1 and CD155, offer a promising liquid biopsy biomarker for dynamic monitoring of the immune landscape and prediction of immunotherapy response.

Metabolic Reprogramming

To support rapid tumor growth, EVs facilitate metabolic symbiosis between tumor and stromal cells, primarily by redistributing key regulators of glycolysis and lipid metabolism. Glycolytic reprogramming is a prominent feature of this process and is driven in part by the horizontal transfer of rate-limiting enzymes. For instance, Hexokinase 1 (HK1) is secreted by HSCs in EVs and

subsequently taken up by HCC cells, where it accelerates glycolysis and tumor growth [86]. Conversely, HCC cells secrete PKM2-enriched EVs to reprogram monocytes towards a glycolytic phenotype that supports a tumor-friendly niche [77]. In addition to cargo delivery, metabolic rewiring may also result from selective cargo loss; the reduced level of Triosephosphate Isomerase 1 (TPI1) in EVs internalized by HCC cells leads to substrate accumulation that fuels the glycolytic bypass [87].

Lipid metabolism is likewise hijacked to meet the bioenergetic and biosynthetic demands of tumor progression. Adipocyte-derived EVs transfer Gce1, a phosphodiesterase that accelerates triglyceride synthesis and free fatty acid release, thereby promoting lipid accumulation in the TME [88]. In parallel, acyl-CoA synthetase long-chain family member 4 (ACSL4), delivered by HCC-EVs, induces lipid peroxidation and senescence in neighboring hepatocytes, thereby driving disease progression [89]. The transfer of nicotinamide phosphoribosyltransferase (NAMPT) also represents a cross-talk mechanism linking inflammation to metabolic signaling. It activates the TLR4/NF-κB pathway, leading to upregulation of SLC27A4, a lipid transporter and acyl-CoA synthetase, whose downstream products feed into glycolytic pathways, thereby supporting tumor progression [90]. Collectively, these findings suggest that EV-encapsulated metabolic enzymes represent a distinct class of functional biomarkers that reflect the metabolic plasticity and energetic state of HCC.

Challenges and Future Perspectives in Deciphering EVs in HCC Pathogenesis

Despite substantial progress, several major challenges continue to hinder a comprehensive understanding of EVs in HCC. First, EV heterogeneity and functional specificity remain incompletely defined. Although EVs derived from different cellular sources have been implicated in diverse pathological processes, such as fibrosis [64, 86] and metabolic reprogramming [87], the lack of standardized single-EV proteomic and multi-omic profiling methods limits accurate discrimination between pro-tumorigenic and anti-tumorigenic EV subpopulations. For instance, the bidirectional effects observed with MSC-derived EVs may result from variations in EV subgroups [50, 91, 92], yet current techniques remain insufficient to validate this possibility in clinical samples. Second, the absence of robust dynamic network-modeling approaches constrains our ability to quantify the multilayered interactions mediated by EVs among tumor, stromal, and immune cells. Most studies still focus on individual signaling pathways [59, 75, 93], thereby hampering a systematic understanding of EV-driven communication networks within the TME. Third, translational validation remains inadequate. While several EV-associated proteins have been proposed as candidate markers of immunotherapy resistance [77, 84, 85], currently available liquid-biopsy technologies often lack the sensitivity and reproducibility required for reliable clinical implementation.

To address these challenges, recent technological advances are reshaping the field. Notably, EV analysis has progressed from bulk population measurements toward single-vesicle resolution, enabling more refined characterization of vesicle heterogeneity. Innovative platforms, including microfluidic chips, digital biochips, and nanoplasmonic sensors, offer increasingly high-throughput and high-resolution profiling of EVs, directly addressing the inherent heterogeneity within vesicle populations [94]. These emerging tools may facilitate the identification of clinically relevant EV subtypes and improve the diagnostic performance of EV-based assays.

Looking forward, future research should advance along three strategic directions. First, continued technological innovation will be crucial, particularly the development of single-EV multi-omics platforms and advanced organoid models to map functional EV networks and simulate key processes such as vascular invasion and therapeutic resistance. Second, improving clinical translation will require establishing comprehensive EV protein panels for multidimensional prognostic assessment and developing engineered dual-functional EV therapeutics that combine antigen presentation with targeted delivery of therapeutic agents. Third, cross-scale integration will be essential for linking EV-driven metabolic reprogramming with systemic metabolomics [87, 90] and synchronizing autocrine signaling with circadian regulation, which will be essential for refining treatment strategies, including chronotherapy-guided EV targeting.

Collectively, this framework highlights the urgent need for standardized detection technologies, dynamic modeling tools, and rigorous translation validation, while paving the way for clinically actionable EV-based diagnostics and therapies to ultimately improve outcomes for patients with HCC.

Proteomic Analysis of Blood EVs

Proteomic analysis of blood-derived EVs is rapidly emerging as a transformative approach for biomarker discovery and for generating mechanistic insights into HCC and other cancers. MS-based proteomics, particularly data-independent acquisition (DIA) strategies, now provide high accuracy and reproducibility in protein quantification, which is essential given the marked heterogeneity of clinical samples [95]. The development of advanced instrumentation, such as the Orbitrap Astral and TimsTOF, has further enabled deep proteome coverage and precise qualitative and quantitative analyses with minimal sample input, helping to address a longstanding

challenge in EV research due to limited sample amounts [95, 96]. A typical EV proteomics workflow encompasses blood sample collection, EV isolation, protein extraction and preparation, liquid chromatography-mass spectrometry (LC-MS) analysis, and comprehensive bioinformatics interpretation (see Fig. 2).

Unlike conventional cell- or tissue-based proteomics, EVs present unique analytical challenges: their low abundance, encapsulation within lipid bilayers, and pronounced heterogeneity necessitate specialized optimization at each step. This includes rigorous attention to the selection, collection, and storage of biofluids, as well as tailored protocols for EV isolation and protein preparation [15]. Blood, particularly plasma and serum, remains the most accessible and clinically relevant source for EV-based liquid biopsy, making it a focal point for translational cancer research [97]. The following subsections critically examine the current state, key technical bottlenecks, and future directions of blood EV proteomics.

Blood Sample Collection and Storage

Reproducibility and interpretability in EV proteomics depend fundamentally on standardizing sample collection and storage, as pre-analytical variables can significantly influence downstream analyses. Donor-related factors, including age, sex, physical activity, pregnancy, fasting status, circadian rhythm, and medication use, should be carefully documented and, where possible, controlled [98]. In addition, pre-analytical variables such as collection time and method, tube type, processing intervals, and transport conditions require strict attention [99]. While serum often contains a higher number of EVs, many are generated in vitro during coagulation, potentially introducing analytical artifacts; for this reason, plasma is generally preferred for EV studies [100]. To minimize platelet activation and artifactual EV release, blood samples should be handled gently, maintained under appropriate temperatures, and processed promptly, ideally by early centrifugation to separate plasma or serum [101].

If immediate analysis is not feasible, cryopreservation of plasma or serum is generally recommended over storage of isolated EVs, as this approach better preserves EV integrity [102]. However, despite the recognized importance of storage conditions, no international consensus has yet been established regarding optimal protocols, a major unmet need in the field [103]. For long-term preservation, storage at −80 °C is most commonly recommended, while ultra-low temperatures (such as −196 °C) are typically reserved for purified EVs, although some evidence suggests that liquid nitrogen may be less effective than −80 °C for maintaining EV function [104, 105]. Storage at −20 °C may be acceptable for limited periods, whereas 4 °C is generally suitable only for short-term preservation, typically within one week [102, 106]. Since the timelines of most studies range from a few days to several weeks, and typically do not exceed 12 months, the effects of prolonged storage remain insufficiently characterized [106]. Repeated freeze-thaw cycles should be avoided, as they can significantly compromise EV abundance, structural integrity, and biological activity [107]. Additionally, the choice of anticoagulant (e.g., sodium citrate, lithium heparin, EDTA) for plasma collection can impact EV preservation and downstream analyses [102].

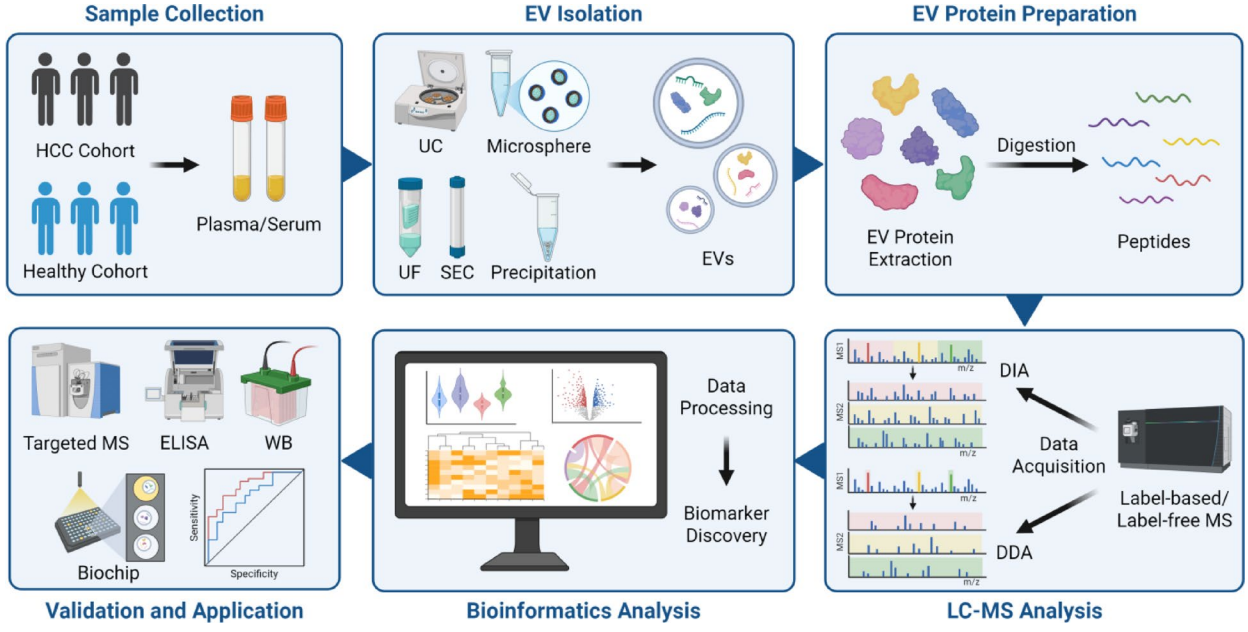


Fig. 2 Workflow for the proteomic analysis of blood EVs. The figure was created in BioRender.com, with authorized permission

In summary, freshly processed blood samples are ideal for EV proteomics. When storage is unavoidable, plasma or serum should preferentially be stored at −80 °C, with freeze-thaw cycles minimized and storage duration kept as short as practicable to preserve EV quality and biomarker reliability.

Isolation and Characterization of Blood EVs

The isolation of EVs from blood remains a major technical bottleneck, complicated by the presence of abundant proteins, nucleic acids, metabolites, lipoproteins, and protein aggregates that share physicochemical properties similar to those of EVs [108]. The choice of isolation method directly impacts yield, purity, integrity, and downstream detectability of EV-derived proteins. At present, no universally accepted or standardized isolation method is available, as the optimal approach depends on both sample characteristics and the intended downstream applications [15, 39]. Key criteria for evaluating isolation methods include yield, purity, recovery, EV integrity, processing time, equipment requirements, starting sample volume, automation potential, throughput, and reproducibility (Table 2).

Ultracentrifugation (UC) and density gradient centrifugation (DGC) are widely used methods that leverage sedimentation and density differences. UC accommodates large sample volumes but often exhibits low yield, requires specialized instrumentation, and involves labor-intensive procedures, with risks of EV aggregation and structural destruction [109]. DGC generally provides improved purity by separating EVs based on buoyant density, yet it adds operational complexity and typically results in reduced recovery [110]. Size-exclusion chromatography (SEC) based on size separation offers high recovery and maintains EV integrity, but its utility may be limited by sample dilution, expensive column, and moderate throughput [109, 111]. Ultrafiltration (UF) employs a porous membrane to retain EVs based on size, provides straightforward processing, and preserves EV integrity, but is prone to membrane adsorption and clogging, thereby restricting its use to relatively simple matrices or as a concentration step [112]. Precipitation methods that utilize polymers to co-precipitate EVs offer convenience and high throughput, but they often co-isolate non-vesicular contaminants, reducing purity [113]. Immunoaffinity capture uses antibodies against EV surface markers, such as tetraspanins, to enrich specific subpopulations; however, its utility is constrained by antibody availability, cost, incubation time, and potential epitope-dependent bias [114, 115].

More recently, alternative approaches have been developed to improve EV enrichment from complex biofluids. Microsphere-based chemical affinity capture methods exploit specific interactions between functionalized microspheres and EV surface components, including electrostatic attraction, hydrophobic lipid interactions, and chelation of Ti4 + ions with EV phosphate groups on the EV membrane, thereby enabling rapid and efficient EV capture [27, 55, 116, 117]. Microfluidic technologies also offer precise and potentially high-throughput EV isolation based on features such as size, density, or surface charge, and hold considerable promise for clinical applications [118]. For example, the EXODUS platform uses negative-pressure oscillation and nanoporous membrane vibration to reduce clogging and aggregation, thereby improving EV purification efficiency [119]. Given the complexity of plasma and serum, combining orthogonal isolation techniques is increasingly recognized as a practical strategy to balance EV purity and yield [15, 120]. Among these methods, chemical affinity capture is particularly attractive for its rapid processing, high throughput, low cost, and low sample consumption, making it well-suited to large clinical cohorts [116]. When coupled with advanced mass spectrometry platforms, this system can support sensitive analysis of trace EV samples and facilitate biomarker discovery.

Following enrichment, comprehensive EV characterization is essential to confirm vesicle identity and assess sample quality [121, 122]. Morphological features are visualized via scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM) [123]. Size distribution and particle concentration are commonly assessed using nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), and tunable resistive pulse sensing (TRPS). EV-associated protein markers are typically detected by western blotting (WB), enzyme-linked immunosorbent assay (ELISA), and nanoflow cytometry [124]. Importantly, conventional characterization methods generally operate at the bulk-population level and therefore have limited ability to resolve EV heterogeneity. Recent advances in single-particle analysis are helping to overcome this limitation and driving the field toward higher resolution and more precise biomarker discovery [125].

Preparation of EV Proteins

Efficient and reproducible protein preparation from isolated EVs is crucial for successful proteomic analysis. This process generally includes protein extraction, denaturation, reduction, alkylation, denaturant removal or dilution, proteolytic digestion, and peptide desalting. The lipid bilayer that encapsulates EVs requires careful selection of lysis reagents and protocols to ensure complete protein extraction while maintaining compatibility with downstream digestion and MS analysis [126]. In addition, the typically low protein yield from EV samples (often < 50 μg) necessitates strategies that minimize sample loss and optimize processing volumes.

Table 2 Comparison of common EV isolation methods [98, 116, 177, 178]

Isolation Method	UC	DGC	UF	SEC	Precipitation	Immuno-affinity Capture	Chemical Affinity Capture	Microfluidics	EXODUS
Principle	Sedimentation (size/density)	Density	Size	Size	Solubility	Antigen-antibody interaction	Chemical interactions	Size/Density/Charge	Size
Recovery Rate	2%–80%	10%–50%	10%–80%	40%–90%	90%	10%–50%	60%–95%	>80%	90%
Purity	Medium	High	Medium	High	Low	High	Medium-High	High	High
EV Integrity	Low	High	Medium	High	High	High	High	Medium-High	High
Throughput	Low	Low	Medium	Medium	High	High	High	High	Low
Processing Time	5–9 h	16–90 h	0.5–4 h	0.5 h	0.25–12 h	4–20 h	5 min–1 h	10 min–1 h	10 min
Equipment & Material	Ultracentrifuge; ultracentrifuge tubes	Ultracentrifuge; gradient medium	Ultrafiltration tubes; centrifuge	SEC column	Centrifuge; precipitation reagent	Antibody-coated beads/plates; centrifuge	Chemically functionalized beads; centrifuge	PDMS chip; silicon chip; micropump; magnet; electrode	EXODUS workstation; vibration motor
Sample Volume	mL-L	µL-mL	µL-L	µL-mL	µL-mL	µL-mL	µL-mL	µL-mL	µL-mL
Automation Potential	Low	Low	Medium	Medium	High	High	High	High	High
Equipment & Material Cost	High	High	Low-Medium	Medium	Low-Medium	Medium-High	Low-Medium	Medium-High	High
Approximate Cost Per Sample	Medium	Medium-High	Low-Medium	Medium	Low-Medium	Medium-High	Low-Medium	Low-Medium	Medium
Advantages	Large sample volume; wide range of sample types	High purity	Rapid concentration; simple operation	Preservation of EV integrity	High yield; simple operation; high throughput; no expensive instrument	High specificity; simple operation; high throughput; no expensive instrument	High specificity; good recovery; simple procedure; high throughput; no expensive instrument	High specificity; automation potential; high throughput	High level of automation; high yield; good purity; good integrity
Limitations	EV aggregation; expensive instrument; laborious and time-consuming; low throughput	Low recovery; complex operation; expensive instrument; laborious and time-consuming; low throughput	Membrane adsorption and clogging; limited to samples with simple matrix	EV dilution; expensive column; limited throughput	Low purity	Expensive beads; specific marker/antibody-dependent; low recovery; difficult to elute EVs from beads	Specific EV marker-dependent; difficult to elute EVs from beads	Specific EV marker-dependent; clogging risk; external force required	Specific instrument; low throughput

Current approaches fall into three main categories: in-solution digestion, filter-aided sample preparation (FASP), and in-gel digestion [127]. In-solution digestion utilizes denaturants (e.g., urea [19, 116]) or surfactants (e.g., sodium deoxycholate, sodium lauroyl sarcosinate [27], sodium 2-(4-hexylphenyl) azo-1-sulfonate [128]) to lyse EVs and denature proteins, followed by buffer dilution and proteolytic digestion. Surfactants are subsequently removed by organic extraction or photodegradation before MS analysis. FASP employs strong lysis reagents (e.g., SDS, dithiothreitol, and urea) and ultrafiltration devices to remove denaturants and enable buffer exchange, thereby facilitating efficient digestion and direct MS analysis. In-gel digestion involves SDS-PAGE

separation, in-gel digestion of excised protein bands, and subsequent peptide extraction.

Each method has distinct advantages and limitations. In-solution digestion is simple and scalable, but it may suffer from reduced digestion efficiency and usually requires desalting. FASP often provides superior digestion and clean peptide recovery, but it requires additional consumables and may result in protein loss during multiple filtration steps. In-gel digestion, while historically important, is labor-intensive and less efficient and is therefore used less frequently. For large-scale clinical samples, in-solution digestion is generally considered the most practical method because of its operational simplicity and high throughput, and it has been adopted in many EV proteomic studies.

Liquid Chromatography-Mass Spectrometry Detection of the EV Proteome

LC-MS has emerged as the pivotal technology for comprehensive proteomic analysis of EVs. By integrating high-resolution chromatographic separation with sensitive mass spectrometric detection, LC-MS enables robust identification and quantification of proteins in complex biological samples, such as blood. The development of LC-MS has been driven by advances in both quantitative strategies and data-acquisition modes, resulting in diverse technical frameworks tailored to specific research and clinical needs.

Proteomic quantification strategies based on LC-MS are broadly classified into label-based and label-free approaches. Label-based quantification employs stable isotope tags to enable relative quantification across multiple samples, thereby minimizing batch effects and often improving proteome coverage. MS1-based methods (e.g., SILAC, dimethyl labeling) rely on precursor ion intensities, whereas MS2-based approaches (e.g., TMT, iTRAQ) utilize reporter ion signals detected in the MS2 stage. Despite their advantages, label-based methods are often hampered by complex sample preparation, high reagent costs, and limited multiplexing flexibility [129]. In contrast, label-free quantification bypasses the need for chemical labeling and directly compares peptide precursor or fragment ion intensities across samples. This approach offers a streamlined workflow and greater scalability, making it particularly suitable for large-scale clinical proteomics. Nevertheless, label-free quantification places stringent demands on instrument stability and necessitates rigorous standardization and quality control to ensure reproducibility across runs [130].

The choice of data acquisition mode further influences the depth and reliability of EV proteome analysis. Data-dependent acquisition (DDA) prioritizes fragmentation of the most intense parent ions, potentially overlooking low-abundance proteins that may be clinically relevant. DIA. By contrast, DIA segments and scans all parent ions, ensuring unbiased spectral coverage and improved detection of low-abundance proteins [131]. However, the highly mixed MS2 spectra generated by DIA require sophisticated computational approaches for accurate interpretation. Historically, DDA was favored due to instrumentation/software limitations, but recent advances in acquisition speed, sensitivity, and database-based search algorithms have made DIA a leading method for large-cohort studies [132]. In addition, parallel reaction monitoring (PRM) is increasingly applied for targeted quantification of specific proteins, offering high sensitivity and multiplexing capability without reliance on antibodies, thereby supporting high-throughput biomarker validation [133].

Proteomic Data Processing and Bioinformatic Analysis

Following mass spectrometric data acquisition, rigorous data processing and bioinformatic analysis are essential to extract meaningful biological insights from EV proteomic datasets. The initial step involves database searching using specialized software, both open-source (e.g., MaxQuant, DIA-NN) and commercial platforms (e.g., Spectronaut, PEAKS, Protein Discoverer), which match experimental peptide spectra against theoretical databases or spectral libraries to enable accurate protein identification and inference. Integration of machine learning algorithms has further improved sensitivity and specificity in protein identification, supporting a shift toward more intelligent, automated workflows [134]. Quantitative information is subsequently derived through computational transformation, correction, and normalization of MS signal intensities. Preprocessing steps, including data normalization, missing-value filtering, and imputation, are critical for minimizing technical variation and preparing the protein matrix for downstream analysis [135, 136].

Statistical analyses typically encompass hypothesis testing to identify differentially expressed proteins, principal component analysis (PCA) to discern group-specific patterns, and hierarchical clustering analysis (HCA) to visualize protein expression profiles. Functional annotation commonly leverages Gene Ontology (GO) enrichment analysis to characterize the biological processes, molecular functions, and cellular components associated with the identified proteins. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis can reveal enriched signaling cascades, while the STRING database facilitates the elucidation of protein-protein interaction networks. EV-specific databases, such as ExoCarta and Vesiclepedia, are also valuable for assessing whether identified proteins are consistent with EV-related signatures, and tissue enrichment analyses using the Human Protein Atlas (HPA) may provide insights into the likely tissue

origin of EVs. For biomarker discovery, receiver operating characteristic (ROC) analysis and advanced machine learning algorithms are typically employed to evaluate candidate markers, identify informative proteins, and develop predictive models with potential clinical relevance [134, 137]. More broadly, integration of multi-omic datasets with advanced artificial intelligence holds promise for improving biomarker discovery and clinical translation.

Validation of Key Proteins

Targeted detection and validation of key proteins identified through EV proteomics are essential steps toward clinical application. Conventional validation methods include targeted mass spectrometry, such as PRM, and immunological assays, including ELISA, Western blotting, and immunohistochemistry. However, these approaches are often constrained by labor-intensive workflows, limited throughput, or challenges in scalability, which impede their utility in large-scale clinical settings [133]. Recent advances in biochip technology offer compelling solutions to address these limitations. Diverse biochip formats, including electrochemical sensors, microfluidic devices, and plasmonic optical sensing platforms, have been developed for the rapid and sensitive detection of blood-derived EV proteins [138–140]. These technologies employ immobilized bioactive molecules, such as antibodies, ligands, nucleic acid aptamers, on miniaturized substrates to enable efficient in situ capture and detection of target proteins. Innovative signal amplification strategies and device integration have enhanced sensitivity, throughput, and portability, positioning biochips as promising tools for point-of-care diagnostics and clinical validation.

Applications of EV Proteins in HCC Diagnosis, Prognosis, and Treatment Response Monitoring

EV-associated proteins represent an emerging area in non-invasive biomarker research for HCC, offering a liquid biopsy approach that captures the molecular heterogeneity and dynamic interactions within the TME. By reflecting the proteomic state of their parent tumor cells, circulating EV proteins provide a molecular snapshot with the potential to inform clinical decision-making from early detection to therapeutic response monitoring. This section synthesizes the current evidence for the application of EV-associated proteins in HCC diagnosis, prognosis, and treatment monitoring.

Diagnostic Applications

Blood-derived EV proteins enable non-invasive discrimination of HCC from benign liver lesions, as summarized in Table 3. Several individual protein biomarkers, including pIgR, LGALS3BP, and GPC3, have demonstrated high sensitivity and specificity for distinguishing HCC from healthy controls and other liver diseases [41, 68]. Of note, GPC3 is already used as a histopathological biomarker for HCC but has shown limited diagnostic performance when measured in serum. In this context, the EV-bound fraction of GPC3 shows superior diagnostic performance relative to total serum GPC3, suggesting that EV enrichment concentrates tumor-derived GPC-3 while reducing background signals from non-tumor sources, thereby amplifying the diagnostic signal-to-noise ratio. This finding suggests that the biological compartment in which a biomarker is measured may be as critical as the biomarker itself in determining diagnostic performance. NID1, alone or in combination with TNFR1 or AFP, has also shown improved accuracy for early HCC detection, with area under the curve (AUC) values exceeding those of conventional markers [71]. Emerging evidence further highlights the diagnostic potential of autoantibody signatures against EV proteins, including anti-ATIC, anti-BRD2, and anti-EIF3A, which may complement existing biomarker panels and further enhance diagnostic yield [141–143]. To overcome tumor heterogeneity and improve diagnostic power, multi-marker panels and integrated scoring systems are increasingly being developed. For example, the HCC EV ECG score, which integrates three EV subpopulations defined by surface marker combinations ($EpCAM^+CD63^+$, $CD147^+CD63^+$, and $GPC3^+CD63^+$), achieved an AUC of 0.93 for early-stage HCC detection in a validation cohort and significantly outperformed AFP alone [144]. These findings underscore the value of capturing cell-of-origin information through combinations of EV surface proteins.

Despite these encouraging findings, many reported biomarkers come from single-center studies with limited sample sizes, which may overestimate diagnostic efficacy and reduce statistical robustness. In addition, cohorts dominated by HBV-related HCC may not reflect biomarker performance in regions where MASLD is more prevalent. Future studies should therefore include multicenter cohorts with etiological stratification, such as HBV-, MASLD-, alcohol-related HCC, and sufficiently large sample sizes, ideally ≥ 100 per group, to improve generalizability. Continued advances in EV isolation, proteomic technologies, and biomarker validation are expected to facilitate the clinical translation of EV-based diagnostics.

Prognostic Applications

EV-associated proteins involved in key pathological processes, including metastasis, immunosuppression, and treatment resistance, may have prognostic value by correlating with clinical outcomes, including progression, recurrence, and overall survival.

Table 3 Diagnostic performance of blood-based EV protein candidate biomarkers in HCC

Sample	Cohort Information	Isolation Method	Detection Method	Candidate Biomarker	Diagnostic Efficacy	Reference
Serum	HCC ($n=25$) LC ($n=25$) HC ($n=25$) Other tumors ($n=10$)	EV isolation reagent, Streptavidin latex beads	LC-MS, ELISA	GPC3	HCC vs. HC: AUC = 1.00; HCC vs. LC: AUC = 0.95; HCC vs. Other tumors: AUC = 1.00	[41]
Plasma	Early HCC ($n=43$) HC ($n=12$)	UC	LC-MS	NID1	AUC = 0.769	[71]
				NID1 + TNFR1	AUC = 0.822	
				NID1 + AFP	AUC = 0.919	
Serum	HCC ($n=29$) CCA ($n=43$) PSC ($n=30$) HC ($n=32$)	UC	LC-MS	pIgR	HCC vs. HC: AUC = 0.837, SEN = 82.8%, SPE = 71.8%; CCA vs. HC: AUC = 0.844, SEN = 83.7%, SPE = 71.8%; PSC vs. HC: AUC = 0.742, SEN = 76.7%, SPE = 71.8%	[68]
				LGALS3BP	HCC vs. HC: AUC = 0.904, SEN = 96.9%, SPE = 71.8%	
Serum	CHB ($n=64$) LC ($n=64$) HCC ($n=84$) HC ($n=42$)	Test kit (System Biosciences)	LC-MS, WB, ELISA	Anti-ATIC	HCC vs. Non-HCC (CHB + LC + HC): AUC = 0.87, SEN = 70.83%, SPE = 90.68%	[141]
Serum	HCC ($n=118$) HC ($n=91$)	Test kit (ExoQuick-TC, System Biosciences)	LC-MS, WB	Anti-BRD2	AUC = 0.7761, SEN = 64.41%, SPE = 82.42%	[142]
Serum	HCC ($n=102$) Normal ($n=85$)	UC	LC-MS, WB, ELISA, IP	Anti-EIF3A	AUC = 0.87, SEN = 79.4%, SPE = 83.5%	[143]
Plasma	Training cohort: Early HCC ($n=45$) LC ($n=61$) Validation cohort: Early HCC ($n=35$) LC ($n=37$)	Click beads	TMA, WB	HCC EV ECG score: $EpCAM^+$ $CD63^+$, $CD147^+$ $CD63^+$, $GPC3^+$ $CD63^+$	Training cohort: AUC = 0.95, SEN = 91%, SPE = 90% Validation cohort: AUC = 0.93, SEN = 94% (cutoff = −0.65) or 91% (cutoff = −0.40), SPE = 81%	[144]
Plasma	HCC ($n=155$) LC ($n=59$) HC ($n=82$)	UC	IHC, WB, nFCM	CD147	HCC vs. HC: AUC = 0.821, SEN = 80.00%, SPE = 70.73%; LC vs. HCC: AUC = 0.796	[145]
				CD147 + AFP	HCC vs. HC: AUC = 0.959, SEN = 88.39%, SPE = 100.00% LC vs. HCC: AUC = 0.947	
Serum	Test cohort: HC ($n=14$) HCC ($n=15$) Validation cohort: HC ($n=34$) CHB ($n=25$) LC ($n=33$) HCC ($n=132$)	Test kit (ExoQuick)	LC-MS, WB, ELISA, qRT-PCR	CCT8	Test cohort: AUC = 0.748 Validation cohort: HCC vs. Non-HCC (HC + CHB + LC): AUC = 0.698, SEN = 46.21%, SPE = 93.48%	[146]
				CFL1	Test cohort: AUC = 0.790 Validation cohort: HCC vs. Non-HCC (HC + CHB + LC): AUC = 0.677, SEN = 40.91%, SPE = 94.57%	
Plasma	HCC ($n=7$) CHB ($n=10$) LC ($n=7$) HC ($n=4$)	SEC	Label-free LC-MS	CO9	CHB vs. HC: AUC = 0.925, SEN = 90.0%, SPE = 100.0%; LC vs. HC: AUC = 1.000, SEN = 100.0%, SPE = 100.0%; HCC vs. HC: AUC = 0.929, SEN = 85.7%, SPE = 100.0%; HCC vs. CHB: AUC = 0.857, SEN = 85.7%, SPE = 90.0%	[147]
				LBP	CHB vs. HC: AUC = 0.950, SEN = 92.9%, SPE = 100.0%; LC vs. HC: AUC = 0.964, SEN = 100.0%, SPE = 75.0%; HCC vs. HC: AUC = 0.964, SEN = 100.0%, SPE = 75.0%	
				vWF	CHB vs. HC: AUC = 0.875, SEN = 90.0%, SPE = 75.0%; LC vs. HC: AUC = 1.000, SEN = 100.0%, SPE = 100.0%; HCC vs. HC: AUC = 0.964, SEN = 100.0%, SPE = 75.0%; LC vs. CHB: AUC = 0.914, SEN = 85.7%, SPE = 90.0%	
				SVEP1	CHB vs. HC: AUC = 0.900, SEN = 80.0%, SPE = 100.0%; LC vs. HC: AUC = 1.000, SEN = 100.0%, SPE = 100.0%; HCC vs. HC: AUC = 1.000, SEN = 100.0%, SPE = 100.0%	
				KV311	HCC vs. LC: AUC = 0.857, SEN = 100.0%, SPE = 71.4%	

Table 3 (continued)

Sample	Cohort Information	Isolation Method	Detection Method	Candidate Biomarker	Diagnostic Efficacy	Reference
Serum	Training cohort: Normal ($n=20$) CHB ($n=24$) LC ($n=23$) HCC ($n=29$) Validation cohort: Normal ($n=18$) CHB ($n=26$) LC ($n=26$) HCC ($n=26$)	Polysaccharide chitosan-based magnetic beads	Label free LC-DIA-MS	KNG1, F11, KLKB1, CAPNS1, CDH1, CPN2, NME2	Validation cohort: HCC vs. CHB: AUC = 0.978, SEN = 88.5%, SPE = 92.3%; HCC vs. LC: AUC = 0.943, SEN = 76.9%, SPE = 96.2%; HCC vs. Non-HCC (HC + CHB + LC): AUC = 0.953, SEN = 76.9%, SPE = 100.0%	[148]
Serum	HC ($n=30$) BLD ($n=30$) HCC ($n=30$) ICC ($n=30$)	Antibody-conjugated meta-plasmonic biosensor	Surface plasma resonance	CD63	BLD vs. HCC: AUC = 0.882; HCC vs. ICC: AUC = 0.519; HCC vs. Non-HCC: AUC = 0.839	[149]
				GPC3	BLD vs. HCC: AUC = 0.782; HCC vs. ICC: AUC = 0.961; HCC vs. Non-HCC: AUC = 0.991	

BLD, benign liver disease; CAPNS1, calpain small subunit 1; CCA, cholangiocarcinoma; CCT8, chaperonin containing TCP1 subunit 8; CDH1, cadherin 1; CFL1, cofilin 1; CHB, chronic hepatitis B; CHC, chronic hepatitis C; CO9, complement component 9; CPN2, carboxypeptidase N subunit 2; F11, coagulation factor XI; HC, healthy control; IF, immunofluorescence; IHC, immunohistochemistry; IP, immunoprecipitation; KLKB1, kallikrein B1; KNG1, kininogen 1; KV311, kappa variable 3–11; LBP, lipopolysaccharide binding protein; LC, liver cirrhosis; nFCM, nano-flow cytometry; NME2, NME/NM23 nucleoside diphosphate kinase 2; PSC, primary sclerosing cholangitis; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; SEN, sensitivity; SPE, specificity; SVEP1, sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1; TMA, tissue microarray

DDX55 upregulation has been identified as an independent prognostic indicator for recurrence-free survival and overall survival in HCC patients, regardless of ethnicity. It appears to show prominent prognostic relevance within the DEAD-box protein family [42]. Increased CCR6 expression has been associated with higher histological grade and shorter overall survival [61]. LOXL4 is significantly upregulated in HCC and correlates with aggressive clinicopathological features, including poor differentiation, vascular invasion, advanced TNM stage, and reduced overall and disease-free survival [67]. Elevated EV-associated S100A4 is also strongly associated with aggressive disease features and poor postoperative survival. Notably, the combination of EV-S100A4 and plasma osteopontin provides superior prognostic stratification compared to either marker alone, highlighting its potential utility for predicting HCC recurrence and patient outcomes [150]. The abundance of plasma SMAD3-containing EVs has been associated with advanced disease stage, higher pathological grade, and shorter disease-free survival in postoperative HCC patients, supporting their potential as indicators of poor prognosis [151]. In contrast, high FTCD expression is associated with favorable clinical features, including lower tumor grade and reduced vascular invasion, and has been identified as an independent protective factor for overall survival. FTCD expression also positively correlates with infiltration of anti-tumor M1 macrophage, suggesting a role in shaping a less aggressive tumor immune microenvironment [73]. Analysis of TCGA data together with an independent cohort further showed that high CLTA expression is associated with larger tumor size, later-stage disease, and shorter overall survival in HCC patients [152]. In addition, upregulation of vacuolar protein sorting-associated protein 35 (VPS35) promotes EV secretion and HCC metastasis, and correlates with higher metastasis risk and poorer survival [153].

Despite these findings, the prognostic performance and predictive robustness of many EV-associate proteins remain insufficiently validated. Larger, well-characterized clinical cohorts and independent external validation studies are still needed to clarify their value in risk stratification and outcome prediction.

Treatment Response Monitoring

Current treatment options for HCC include surgery, locoregional therapies, chemotherapy, radiotherapy, immunotherapy, and targeted therapy [11]. Given the marked heterogeneity and high recurrence rate of HCC, robust methods for longitudinal assessment of therapeutic efficacy are critically needed. Because EVs are released dynamically and can be sampled via minimally invasive procedures, they may serve as useful indicators for assessing treatment response, detecting residual disease, and early identifying therapeutic failure.

Recent advances have highlighted the potential of EV-associated proteins as dynamic biomarkers across multiple treatment modalities. In immunotherapy, EV-derived PD-L1 levels have been shown to fluctuate in response to anti-PD-1 therapy, reflecting adaptive immune evasion and offering a potential real-time metric for predicting and monitoring resistance to immune checkpoint inhibitors [154]. In the context of chemotherapy, drug-resistant HCC cells secrete EVs enriched in specific heat shock

proteins (HSPs). These HSP-bearing EVs can modulate the TME by enhancing NK cell cytotoxicity, and their abundance correlates with the degree of drug resistance, suggesting a possible novel strategy for profiling therapy-induced immunogenic stress [155]. Furthermore, a proteomics-driven machine learning model, TSExs, based on a 14-eV-protein signature, has been developed to stratify patient risk and predict responses to both sorafenib and PD-1 inhibitors. This model also identifies patients with high immune-evasion potential and may help guide the selection of alternative therapeutic avenues [156]. Although these findings are encouraging, substantial challenges remain before such approaches can be translated into routine clinical use. These include the need to validate the underlying mechanisms and predictive signatures in human patient cohorts, as well as the development of scalable, standardized, and cost-effective technologies for EV isolation and detection.

When EV proteins identified in mechanistic studies are compared with candidate biomarkers discovered in clinical biofluids, only a limited subset, including GPC3, pIgR, NID1, LOXL4, LGALS3BP, and PD-L1, emerges in both contexts as functional mediators of HCC pathogenesis and promising liquid biopsy candidates. This limited overlap indicates a substantial translational gap. This discrepancy is likely attributed to several converging factors. Most fundamentally, mechanistic studies and biomarker discovery are conducted in different biological contexts. Mechanistic investigations rely largely on conditioned media from cell lines or animal models under controlled experimental conditions, whereas biomarker studies analyze patient-derived biofluids with far greater biological complexity. As a result, the EV populations sampled in each context differ substantially in cellular origin, purity, and molecular composition [157]. Beyond this systemic divergence, many functionally validated proteins may be inherently difficult to detect in circulation due to low tissue expression, inefficient secretion, stage-specific expression, or short biological half-lives, all of which can reduce their abundance in blood EVs. Even when these proteins are detectable, they may lack sufficient specificity to distinguish HCC from other chronic liver diseases, thereby being excluded from biomarker candidate lists. Another major limitation is that tumor-derived EVs constitute only a minor fraction of the total circulating EV pool, which is dominated by vesicles derived from platelets, erythrocytes, and other non-malignant cells. In conventional bulk EV analysis, this substantial background can dilute or mask tumor-specific cargo, making it difficult to detect proteins mechanistically linked to HCC progression [158]. Technical barriers related to reproducible EV isolation and sensitive protein detection from complex biofluids further exacerbate this issue [159]. Consequently, proteins that are both functionally implicated in HCC biology and consistently detectable in circulating EVs should be considered high-priority targets for clinical development.

To help bridge this translational gap, the field is increasingly shifting from bulk EV analysis toward high-resolution single-vesicle platforms, including nanoflow cytometry and microfluidic imaging systems. By dissecting individual vesicles, these technologies have the potential to distinguish tumor-derived from non-tumor-derived EV subpopulations in clinical samples, thereby improving the recovery of functionally relevant proteins that are obscured in bulk measurements [158, 160]. Such advances may help narrow the current disconnect between mechanistic insight and biomarker translation and support more precise strategies for EV-based marker discovery and validation in HCC.

Clinical Translation of EV Biomarkers

The clinical value of EV protein biomarkers for HCC will depend on their successful translation into routine practice. However, the path from biomarker discovery to clinical application remains challenging, resulting in a substantial gap between the large number of EV biomarkers reported in the literature and the few that have progressed to clinical use. Drawing on successful examples of proteomic biomarker translation, particularly the OVA1 test for ovarian cancer [161] and the PrecivityAD test for Alzheimer's disease [162], we outline a structured roadmap for advancing HCC EV protein biomarkers from laboratory to clinic (Fig. 3). The preceding sections of this review have addressed Phase I of this roadmap, namely, the deep discovery of candidate EV proteins and their targeted verification (Steps 1–2, Fig. 3). The following subsections focus on the subsequent translational phases, including large-scale validation and standardization (Phase II), clinical evidence generation and regulatory approval (Phase III, Step 5), as well as clinical implementation and cost-effectiveness evaluation (Phase III, Step 6).

Large-Scale Validation and Process Standardization

The transition from single-center discovery to robust multicenter validation constitutes the first major translational hurdle (Phase II, Steps 3–4, Fig. 3). Large-scale clinical validation requires testing candidate biomarkers in multicenter cohorts that represent the intended-use population, including patients with diverse etiologies such as HBV, HCV, MASLD, and ALD, as well as the full disease spectrum from cirrhosis to early- and advanced-stage HCC. The primary objective of this phase is to determine whether EV biomarker panels provide diagnostic performance that is superior to, or at least complementary to, the current standard of care, namely AFP combined with ultrasound [7], and to emerging

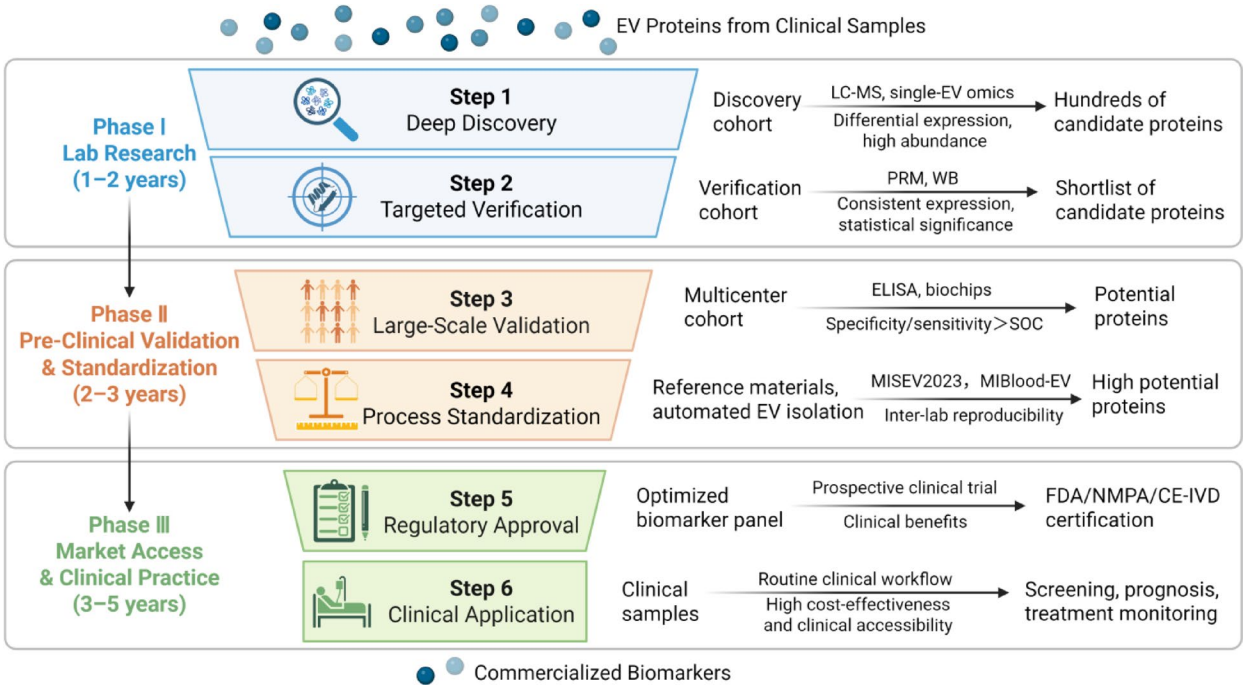


Fig. 3 The clinical translation roadmap of EV biomarkers. This figure illustrates the critical, sequential stages required to bridge the gap between the discovery of EV protein biomarkers for HCC and their implementation in clinical practice, which involve three main phases and six key steps. The figure was created in BioRender.com, with authorized permission

comparator models such as the GALAD score (Gender, Age, AFP-L3%, AFP, and DCP) [163]. The decision to advance a candidate beyond this phase should be guided by pre-specified performance criteria, such as an AUC exceeding 0.85 in an independent verification cohort.

Meanwhile, meaningful multicenter validation is difficult to achieve without parallel pre-analytical standardization. Results generated at one center must be reproducible at another; otherwise, data cannot be reliably compared or pooled across sites. Pre-analytical standardization should adhere to established international guidelines, particularly MISEV2023 [98] and MIBlood-EV [99], and should encompass the entire biofluid handling process from sample collection to measurement, including anticoagulant selection, centrifugation procedures, storage conditions, and freeze-thaw cycles. It is also strategically important to incorporate samples from multiple sites during the discovery phase, so that biomarkers advanced to later-stage validation are less susceptible to pre-analytical variability and therefore more suitable for multicenter studies.

Beyond pre-analytical sample handling, EV isolation remains a major source of inter-laboratory variability. Currently, no universally accepted isolation method is available, and commonly used approaches differ substantially in yield, purity, and the EV subpopulation recovered, making cross-study comparison inherently challenging [164]. Establishing standardized, fit-for-purpose isolation protocols or kit-based workflows that balance simplicity, speed, cost-efficiency, reproducibility, and throughput is therefore a prerequisite for multicenter validation. Similarly, the detection and quantification of EV-associated proteins require analytical platforms that combine high sensitivity with adequate throughput. Among established methods, ELISA remains a practical and clinically accessible option for targeted validation of defined EV protein panels [165]. For higher-order multiplexing, emerging electrochemical and optical biosensing platforms offer complementary capabilities, enabling rapid, sensitive profiling from minimal sample volumes [166]. The convergence of standardized sample collection, EV isolation, and sensitive detection workflows will be essential for generating reproducible, multicenter-comparable data to support downstream clinical validation.

A closely related challenge is the current scarcity of universally accepted reference materials [167]. Without certified reference standards, assay performance cannot be benchmarked reliably, instruments cannot be calibrated consistently across platforms, and data cannot be harmonized across laboratories [168]. The development and dissemination of such materials, including synthetic liposomes with defined surface protein composition, lyophilized EV preparations, and well-characterized pooled plasma standards, therefore represent essential enabling infrastructure for the field [169, 170]. Encouragingly, the ISEV and several national metrology institutes

are actively pursuing EV reference material programs, which may provide a critical foundation for future standardization efforts.

Clinical Evidence and Regulatory Pathway

Once a candidate biomarker has demonstrated robust performance in multicenter validation studies, the next critical phase is to generate the level of evidence required for regulatory approval (Phase III, Step 5, Fig. 3). This process involves two sequential yet overlapping components: formal analytical validation and demonstration of prospective clinical utility.

Analytical validation rigorously defines the assay's measurement performance characteristics, including accuracy, precision, limit of detection, limit of quantitation, analytical specificity, linearity, and reportable range. These parameters should be established under conditions representative of routine clinical laboratory practice rather than optimized research settings alone.

Clinical validation and utility assessment must then move beyond retrospective case-control designs toward prospective, multicenter clinical trials with clearly defined endpoints. At this stage, the critical question is no longer whether the test can distinguish HCC from non-HCC samples, but whether its use leads to clinically meaningful benefit, such as increased detection of early-stage (BCLC 0/A) tumors eligible for curative intervention, or improved overall survival compared to standard AFP-plus-ultrasound surveillance as recommended by AASLD [7] and EASL [8]. A head-to-head comparison with the GALAD score, which has recently been validated in a prospective multicenter cohort, would further clarify the incremental value of EV-based assays within the evolving surveillance landscape [171]. To ensure generalizability, such trials should be sufficiently powered, include patients with diverse etiologies, and reflect real-world clinical settings [157]. In the context of HCC surveillance in cirrhotic patients, high sensitivity is paramount because missed early-stage tumors may reduce opportunities for curative intervention [168].

Navigating the regulatory pathway is an integral part of this phase. Translation typically proceeds from a laboratory-developed test (LDT), designed and validated within a single Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory, to a commercially manufactured and broadly distributed In Vitro Diagnostic (IVD) device. For a novel cancer diagnostic classified as a Class III medical device, regulatory agencies such as the FDA, NMPA, and EMA generally require a comprehensive demonstration of both analytical and clinical validity based on prospective utility trials [168]. Currently, no EV protein-specific IVD has been approved by any major regulatory authority for HCC. The ExoDx Prostate test remains the first and most commercially mature CLIA-validated EV-based cancer risk assessment tool, providing a valuable precedent that demonstrates both the feasibility of EV biomarker translation and substantial time and evidence required to achieve it [157].

Clinical Implementation and Cost-Effectiveness

The final phase of the roadmap addresses the real-world factors that determine whether a test that has received regulatory approval will be adopted and sustainably integrated into healthcare systems (Phase III, Step 6, Fig. 3). Regulatory approval alone does not guarantee clinical adoption, and the test must also demonstrate operational feasibility, compatibility with existing workflows, and favorable health economics.

While Sect. 7.1 addressed the need to standardize EV isolation and detection workflows for multicenter validation, routine clinical implementation poses additional operational demands. Assays intended for population-level surveillance must be robust, high-throughput, and amenable to automation [172]. Accordingly, the field requires a deliberate technological evolution toward platforms suitable for clinical settings, such as automated EV isolation instruments, in situ EV-capture and detection biochips, automated immunoassay systems, and multiplexed nanoflow cytometry panels. The viability of these platforms will depend on their ability to minimize manual operations, reduce technical variability, and achieve turnaround times compatible with clinical decision-making [173]. Ideally, the test must integrate seamlessly into the existing semiannual surveillance workflow recommended by AASLD and EASL and require no more than a standard blood draw obtained during routine clinical visits.

From a health-economic perspective, the cost-effectiveness of a new EV-based surveillance test must be benchmarked against the current AFP-plus-ultrasound paradigm, considering not only the direct assay cost but also the downstream economic impact of improved diagnostic accuracy [174]. Potential benefits include fewer unnecessary cross-sectional imaging and liver biopsies triggered by indeterminate ultrasound findings, as well as earlier therapeutic intervention that may reduce the burden and cost of late-stage disease management. A useful precedent is the ExoDx Prostate test, for which published economic analyses have demonstrated that incorporation into clinical decision-making can reduce unnecessary biopsies and associated healthcare expenditures [175, 176]. Similar health-economic modeling frameworks should be developed for EV-based HCC surveillance to support reimbursement negotiations and facilitate adoption by payers and healthcare systems.

Perspective

While no EV protein-specific IVD has yet been approved for HCC, the field is actively moving toward this goal. Based on the current translational landscape, EV protein biomarkers are most likely to achieve initial clinical adoption for early HCC screening and surveillance in high-risk cirrhotic populations. This represents a well-defined target population that is already surveilled at standardized semiannual intervals, making it amenable to a reproducible testing workflow. The clinical entry point is further reinforced by both AASLD and EASL guidelines, which have explicitly acknowledged the suboptimal sensitivity of the current AFP-plus-ultrasound paradigm, particularly in patients with MASLD/ALD-related cirrhosis and obesity, thereby endorsing the need for complementary blood-based surveillance tools. Notably, this is not merely a theoretical proposition: several EV-based candidates, including the HCC EV ECG score [144] and the EvoLiver test [173], are already progressing through clinical validation pipelines in this setting.

To realize this vision over the coming years, two breakthroughs appear particularly important. The first is the establishment of internationally recognized EV reference materials and standardized operating procedures, without which multicenter validation studies are unlikely to generate the reproducible evidence required for regulatory approval. The second is completion of at least one adequately powered, prospective, multicenter clinical trial demonstrating that an EV protein-based assay has superior or complementary diagnostic performance compared with classical surveillance in cirrhotic patients. By advancing through the staged roadmap described above, through standardization, generation of regulatory-grade evidence, and demonstration of health-economic value, the field can transform EV protein biomarkers from promising research targets into indispensable components of clinical practice for earlier HCC detection, risk stratification, and personalized management.

Conclusion and Future Perspectives

Precision medicine for HCC requires reliable and minimally invasive strategies for early detection, given the insidious onset of the disease and the limitations of current diagnostic modalities. EVs have emerged as important elements in liquid biopsy, serving as ubiquitous carriers of molecular information that reflect the dynamic interplay between tumor cells and their microenvironment [14]. By encapsulating diverse biomolecules, EVs offer a promising source of biomarkers that could identify HCC at earlier, more treatable stages [13]. Recent advances in blood EV proteomics have significantly deepened our understanding of HCC pathogenesis and expanded the repertoire of candidate biomarkers for diagnosis and prognosis [148]. Nonetheless, translation into clinical practice remains limited by several critical challenges, including the lack of standardization for EV isolation, characterization, and storage, and detection, as well as insufficient harmonization of analytical workflows from sample collection to data processing. These challenges align with the current guideline expectations, which emphasize rigorous validation, standardization, and quality control before liquid biopsy-based assays can be incorporated into routine care [7, 8].

To facilitate clinical translation of EV-derived biomarkers, standardized procedures for EV isolation, characterization, and storage should be established, specifically tailored to blood samples and compatible with high-throughput clinical workflows. For large cohorts, automated EV isolation and sample preparation workflows, combined with high-sensitivity mass spectrometry technologies such as chemical affinity capture coupled with Orbitrap Astral mass spectrometry, will be important for stable and in-depth discovery of protein markers. Rigorous quality control measures, including particle-size analysis, concentration determination, morphological imaging, and MS assays, should be implemented for comprehensive evaluation of EVs and their protein cargos. Harmonization of data acquisition and processing pipelines, particularly through DIA technology, is crucial to ensure reliability and reproducibility, while minimizing missing values. Multicenter collaboration through shared protocols and centralized data repositories will enable cross-cohort comparisons and meta-analyses. Prospective multicenter clinical trials with clearly defined endpoints are necessary to validate candidate EV protein biomarkers across diverse patient populations. Additionally, integrating mechanistic insights into EV subgroups and their associated pathways will help prioritize the biomarkers most relevant to HCC pathophysiology. Continued innovation in single-EV multi-omics, high-throughput biochips, and other emerging technologies, supported by methodological rigor, will be essential to unlock the full clinical potential of blood EV proteomics.

In conclusion, blood-based EV proteomics represents a versatile platform for biomarker discovery. With continued progress in methodological standardization, multicenter validation, and clinically adaptable detection technologies, EV-derived protein biomarkers may become increasingly useful for noninvasive HCC detection, risk stratification, and disease monitoring. Although substantial translational challenges remain, the field is steadily moving toward more practical and clinically relevant applications.

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Author contributions
Yijia Zhuang, Tingting Long, Nan Gao, Weitao Zhong and Hongyu Mu were responsible for the literature search and writing of the original draft. Yijia Zhuang and Tingting Long drafted the figures and tables. Wenjuan Zeng and Hao Yang contributed to the conceptualization, writing, review and editing, and supervision. Xinyuan Wang and Hao Yang provided technical guidance of EV isolation and analysis, and funded. All authors have read and approved the final manuscript for publication.

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Data availability
No datasets were generated or analyzed during the current study.

Declarations

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Not applicable.

Consent for publication
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