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WJH mainly publishes articles reporting research results and findings obtained in the field of hepatology and covering a wide range of topics including chronic cholestatic liver diseases, cirrhosis and its complications, clinical alcoholic liver disease, drug induced liver disease autoimmune, fatty liver disease, genetic and pediatric liver diseases, hepatocellular carcinoma, hepatic stellate cells and fibrosis, liver immunology, liver regeneration, hepatic surgery, liver transplantation, biliary tract pathophysiology, non-invasive markers of liver fibrosis, viral hepatitis.

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REVIEW

Contribution of extracellular vesicles to steatosis-related liver disease and their therapeutic potential

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Abstract

Extracellular vesicles (EVs) are small particles released by many cell types in different tissues, including the liver, and transfer specific cargo molecules from originating cells to receptor cells. This process generally culminates in activation of distant cells and inflammation and progression of certain diseases. The global chronic liver disease (CLD) epidemic is estimated at 1.5 billion patients world-

wide. Cirrhosis and liver cancer are the most common risk factors for CLD. However, hepatitis C and B virus infection and obesity are also highly associated with CLD. Nonetheless, the etiology of many CLD pathophysiological, cellular, and molecular events are unclear. Changes in hepatic lipid metabolism can lead to lipotoxicity events that induce EV release. Here, we aimed to present an overview of EV features, from definition to types and biogenesis, with particular focus on the molecules related to steatosis-related liver disease, diagnosis, and therapy.

Key Words: Extracellular vesicles; Exosomes; Chronic liver disease; Hepatocellular carcinoma; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis

Core Tip: Extracellular vesicles are tiny particles released by cells and transport specific molecules from one cell to another, resulting in the sending of a message. Chronic liver diseases are mainly induced by cirrhosis, liver cancer, viral hepatitis, and obesity. Alterations in hepatic lipid metabolism, as fat accumulation in liver cells, can trigger lipotoxicity events that prompt extracellular vesicle release leading to inflammation. In this context, we aimed to provide a comprehensive overview of extracellular vesicles, covering their definition, types, and biogenesis, with emphasis on extracellular vesicles associated with steatosis-related liver disease, diagnosis, treatment, and its possible therapeutic applications.

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INTRODUCTION

Extracellular vesicles (EVs) are small membrane vesicles released by cells as a communication mechanism. Due to their features, they can act as vessels for different types of bioactive molecules [DNA, RNA, microRNA (miRNA), proteins, lipids] that mediate cell-to-cell information transmission[1]. This group of vesicles is subclassified as microvesicles, exosomes, apoptotic bodies (ApoBDs), and large oncosomes, according to their biogenesis mechanism, physical properties, and cargo. As will be explored below, recent studies have implicated EVs in the diagnosis, therapy, and prognosis of several diseases.

Chronic liver disease (CLD) is defined as the progressive deterioration of liver functions for more than 6 months of evolution. This impairment derives mainly from conditions such as alcoholic liver disease, chronic viral hepatitis, and nonalcoholic fatty liver disease (NAFLD)[2]. There is a consensus of experts who have proposed renaming NAFLD as "metabolic dysfunction-associated steatotic liver disease", based on an improved clinical approach to the interplay between metabolic comorbidities associated with liver disease. Although we agree with this idea, this disease is denoted as NAFLD since most of the consulted literature is accessible with the NAFLD nomenclature[3].

The most common molecular harmful events present in CLD are lipotoxicity and oxidative stress, mainly in hepatocytes. Due to the constant damage and even cell death, or as a result of different processes induced by the damage itself, several mediators are released to announce to other cells the altered state encountered in CLD[4]. These mediators include classic cytokines, chemokines, damage-associated molecular patterns, and EVs. There are many reports that show the association of EV proteins and other molecules with CLD development, which will be discussed below in this review.

EV

There exist numerous mechanisms for cell-to-cell communication; one such mechanism is through EVs. In 1946, Chargaff and West[6] described EVs for the first time as small particles in the sediment of plasma supernatant with the capacity to regulate coagulation[5-7]. The term "extracellular vesicles" was first used by Aaronson *et al*[8] in 1971, who demonstrated their presence and features using electron microscopy. Since then, EV research has grown significantly[8,9].

The structural composition of EVs is complex and variable. They function as vessels surrounded by a membrane that contains molecules. They can carry signals from a donor to a receiving cell, thus establishing a communication system. The concentration and distribution of molecules within the EVs depends on the properties of the donor and receiving cells[10,11]. In addition, the composition can be modified by several factors such as cellular microenvironment, pathologies, wellness conditions, and other environmental factors, enabling the EVs to maintain homeostasis or to inhibit or promote the evolution of diseases[12].

The EV lipidic bilayer membrane consists mainly of complex lipids like phosphatidylserine, glycosphingolipids, sphingomyelin, phosphatidylcholine, phospholipids, and cholesterol. It also contains, in a smaller proportion, proteins and carbohydrates that help maintain its structure and mediate intercellular interactions and components destined for macrophage degradation[13,14].

EVs transport and/or contain a wide variety of components, such as organelles, cytokines, enzymes, membrane receptors, signaling factors, amino acids, peptides, and lipids and the precursors involved in their synthesis. The most studied molecules transported by EVs are the nucleic acids derived both from DNA and RNA [miRNA, small interfering RNA, circular RNA (circRNA), and messenger RNA][15-19].

EVs have a heterogeneous origin and formation. They have been reported to originate from the membrane invagination of cytosolic multivesicular bodies (MVBs) or from an apoptotic process. There is a wide variety of EVs with unique size, function, markers, and content, such as exosomes, microvesicles, ApoBDs, large oncosomes, *etc.* These features are summarized in Table 1[20-25].

EV types, biogenesis, and main features

Exosomes: Exosomes are tiny particles (40-160 nm) that originate from the intraluminal vesicles (ILVs) contained in the intracellular MVBs. Once these MVBs fuse with the cytoplasmic cell membrane and release their contents to the extracellular space, the ILVs are known as exosomes[20].

Although exosome biogenesis has not been fully elucidated, some authors have subdivided the classification of exosome biogenesis into those pathways dependent on the endosomal sorting complex required for transport (ESCRT) and those not dependent on it[26].

It is widely described that the ESCRT-dependent generation of ILVs are loaded with ubiquitinated proteins destined for degradation, but the mechanism by which they are released as exosomes is unclear. Nevertheless, it is known that ESCRT proteins (ESCRT-0, -1, -2, and -3), composed of diverse complexes, produce ILVs and deposit ubiquitinated proteins within them[26,27].

ESCRT-0 (conformed by HRas and STAM 1/2 subcomplexes) recognizes ubiquitinated proteins and recruits them into the endosomal membrane through the interaction of HRas with phosphatidyl inositol 3-phosphate. ESCRT-0 recruits ESCRT-I [conformed by tumor susceptibility gene 101 (TSG101), hVps28, Vps37, and hMvb12 subcomplexes] through interaction with TSG101. After ESCRT-I recruits ESCRT-II (conformed by EAP45, EAP30, and EAP20 subcomplexes), they finally recruit and activate ESCRT-III (conformed by CHMP-6, -4, -3, and -2). This protein senses, stabilizes, and induces the curvature of the budding vesicle to promote its formation by oligomeric assembly. ALG-2-interacting protein X (ALIX) stabilizes the assembly of ESCRT-III oligomers. Once the vesicle forms, ESCRT-III recruits the ATPase vacuolar protein sorting 4 (made up of the SKD1, CHMP5, and LIP5 complexes). This is the only ATPase that participates in ESCRT machinery and disassembles ESCRT-III oligomers. It also returns them into the cytoplasm by an ATP-dependent mechanism[27,28].

ESCRT-III orchestrates protein deubiquitylation through the recruitment of deubiquitinases. However, this is not always an essential step for exosome formation: An exosome study from human urine demonstrated that only 13% of exosome proteins were ubiquitylated with different patterns, suggesting that these proteins could be involved in exosome function and potentially be used as a biomarker and therapeutic target[29]. On the other hand, ALIX can interact directly with ESCRT-III and direct non-ubiquitinated cargo to ILVs, in a mechanism that involves structures of heparan sulphate chains of syndecan that can recruit syntenin-ALIX and support the membrane budding[30-32].

Two ESCRT-independent exosome biogenesis mechanisms have been reported. One involves ceramides and another involves the tetraspanin family. Ceramides are sphingolipids structured by a sphingosine linked to a long-chain fatty acid by an amide. This lipid is located asymmetrically in the membrane, which could affect its fluidity and curvature, taking a spontaneous curvature shape when the ceramide has a specific location. In addition, sphingomyelinases (SMases) can hydrolyze sphingomyelin to produce ceramides. In MVBs, neutral-SMase produce ceramide and induce a spontaneous negative curvature promoting the formation of ILVs[33].

Tetraspanins are a protein superfamily localized in both the cell membrane and endomembrane system of a large variety of cells. They are structured by four transmembrane domains, four to six conserved extracellular cysteine residues, polar residues within the transmembrane domain and distinct palmitoylation sites. Tetraspanins, alongside cholesterol and gangliosides, can be organized in membrane microdomains called tetraspanin-enriched microdomains. These clusters interact with a large variety of transmembrane and cytosolic signaling proteins, permitting their participation in diverse biological processes like cell adhesion, motility, invasion, membrane fusion, signaling, and protein traffic. In addition, tetraspanin-enriched microdomains can induce membrane curvature and interact with cytoskeletal proteins to induce membrane cleavage and budding of ILVs[34].

Exosomes are highly enriched with tetraspanins (7-124-fold greater than the donor cell). Cluster of differentiation (CD) 9, CD63, CD37, CD81, and CD82 tetraspanins are found in higher quantities and may serve as exosome biomarkers. However, other EV subpopulations can also express these tetraspanins, so they should be used cautiously as biomarkers [34].

A less described ESCRT-independent pathway has been recently mentioned. In this pathway, the Rab31 GTPase is recruited into ceramide and cholesterol clusters by flotillins to stimulate membrane budding and epidermal growth factor receptor packaging[35]. Rab27 GTPase controls exosome release by promoting fusion of MVBs with plasma membrane in order to release their contents to the extracellular space (Figure 1)[36].

Exosome protein contents can include the tetraspanin family, integrins, immunoglobulins, receptors, cytoskeleton proteins, proteins related to the ESCRT machinery, heat shock proteins, and proteins involved in vesicle trafficking. The proteins recognized as the main markers are the classical tetraspanins (CD9, CD63, and CD81), ALIX, and TSG101 associated with ESCRT (Figure 1)[20,21]. Nevertheless, the discovery of exosome production in the absence of some of the

Table 1 Extracellular vesicle features and markers

Name	Size	Markers	Characteristics or definition	Content
Classical exosomes	40-150 nm [20]	CD9, CD63, CD81[20,21]	EVs originated in intracellular MVBs containing ILVs released into the extracellular space[20,21]	Proteins, amino acids, metabolites, mRNA, and siRNA[20,21]
Non-classical exosomes	40-150 nm [21]	CD9-, CD63-, CD81-[21]	Exosomes lacking CD9, CD63, and CD81 expression[21]	Not yet determined
Microvesicles/ectosomes/microparticles/membrane particles	50-2000 nm[22]	ARF6, VCAMP3, Annexin A1[21]	EVs originated by budding and detachment of cell membrane[22]	Proteins, amino acids, metabolites, mRNA, siRNA, and DNA[22]
ARMM	40-100 nm [21]	ARRDC1, TSG101[23]	Small microvesicles originated by budding and detachment of cell membrane, regulated by ARRDC1 and TSG101[23]	Proteins, amino acids, metabolites, mRNA, siRNA, and DNA[23]
Large oncosomes	1-10 μm [21]	Myr-Akt1, HB-EGF, Cav-1, ARF6[24]	Atypically large EVs originated by budding and detachment of cell membrane from advanced cancer disease cells[24]	Proteins, enzymes, peptides, miRNA, mRNA, DNA, amino acids, metabolites, and lipids[24]
Apoptotic bodies	50-5000 nm[21]	TSP, C3b, ARF6 ANEXIN V[25]	EVs originated during apoptotic events[25]	DNA, miRNA, RNA, proteins, and lipids[25]

ARF6: ADP-ribosylation factor 6; ARMM: Arrestin domain-containing protein 1-mediated microvesicles; ARRDC1: Arrestin domain containing 1; C3b: Complement component 3b; Cav-1: Caveolin-1; CD: Cluster of differentiation; EVs: Extracellular vesicles; ILVs: Intraluminal vesicles; mRNA: Messenger RNA; miRNA: MicroRNA; MVBs: Multivesicular bodies; siRNA: Small interfering RNA; TSG101: Tumor susceptibility gene 101; TSP: Thrombospondin; VCAMP3: Vesicle-associated membrane protein 3.

classical tetraspanins allows for the hypothetical existence of non-classical exosomes that may not present these canonical tetraspanin markers[21,37,38].

Several molecules have been described as exosome components; many of them are involved in their biogenesis. Additionally, exosomes may express bioactive molecules that change according to their host cell type[26,39]. Some of the most important ones are included in Figure 2.

Microvesicles: Microvesicles (also known as microparticles, ectosomes, and membrane particles) are a type of EV with a size range of 50-2000 nm that originate from budding and detachment of the extracellular membrane by exocytosis[40].

Microvesicle biogenesis is less characterized than exosomes, but two ESCRT-dependent mechanisms and a third ESCRT-independent mechanism have been described to explain microvesicle formation. The first involves the ALIX, TSG101, Vps22, Chmp1/3, and vacuolar protein sorting 4 ESCRT complex proteins. A study showed that their absence reduces the secretion of Hedgehog in EVs. Another mechanism consists of the recruitment of the ESCRT subunits TSG101 and VPS4 to the plasma membrane by adapter protein arrestin domain-containing protein 1 (ARRDC1), which promotes the generation of microvesicles called ARRDC1-mediated microvesicles (Figure 1)[23].

The third mechanism is independent of ESCRT and involves the activation of acid sphingomyelinase (A-SMase) through the generation of ceramide in the membrane. In addition to the aforementioned mechanisms, it has also been seen that the small proteins GTPase ADP-rbosylation factor (ARF) 1, ARF6, and RhoA can induce the production of microvesicles (Figure 1)[23]. Likewise, lipids play an important role in microvesicle formation due to the following mechanisms: Phosphatidylinositols recruiting membrane-sculpting proteins and cone-shaped phosphatidylethanolamine inducing membrane curvature[40].

Large oncosomes: Large EVs have been described in various tumors, such as hepatic cancer, prostate cancer, breast cancer, glioblastoma, glioma, pancreatic cancer, colon cancer, melanoma, and leukemia. Large oncosomes (LO) are a type of large EV that come exclusively from cancer cells. LO can be up to a thousand times larger than exosomes (1-10 μm), allowing them to contain an extensive number of molecular compounds derived from tumoral cells and to have a different impact on the tumor microenvironment than exosomes and smaller EVs[24,41].

It is known that LO can originate from non-apoptotic plasma membrane blebbing induced through the inhibition of cytoskeletal regulator diaphanous-related formin-3, by overexpression of oncoproteins (*e.g.*, Myr-Akt1, HB-EGF, and caveolin-1) or by activation of the epidermal growth factor receptor. Nevertheless, LO has not been as widely studied as other EVs[42].

A wide range of molecules have been found in LO, including GTPase ARF6, caveolin-1, metalloproteinases-2 and -9, keratin 18 (cytokeratin type I), glyceraldehyde 3-phosphate dehydrogenase, phosphoglucose isomerase, lactate dehydrogenase B, heat shock 70 kDa protein 5, malate dehydrogenase, aspartate transaminase, glutaminase, caveolin-2, and glutathione S-transferase pi 1 gene (Figure 1)[24].

It has been reported that LOs can use autocrine and paracrine mechanisms to perform their functions, from direct proteolytic activity to the activation of protumorigenic programs into different types of target cells[24]. It has also been reported that LOs originating from an aggressive prostate cancer cell line can express integrin alpha-V on their surface,

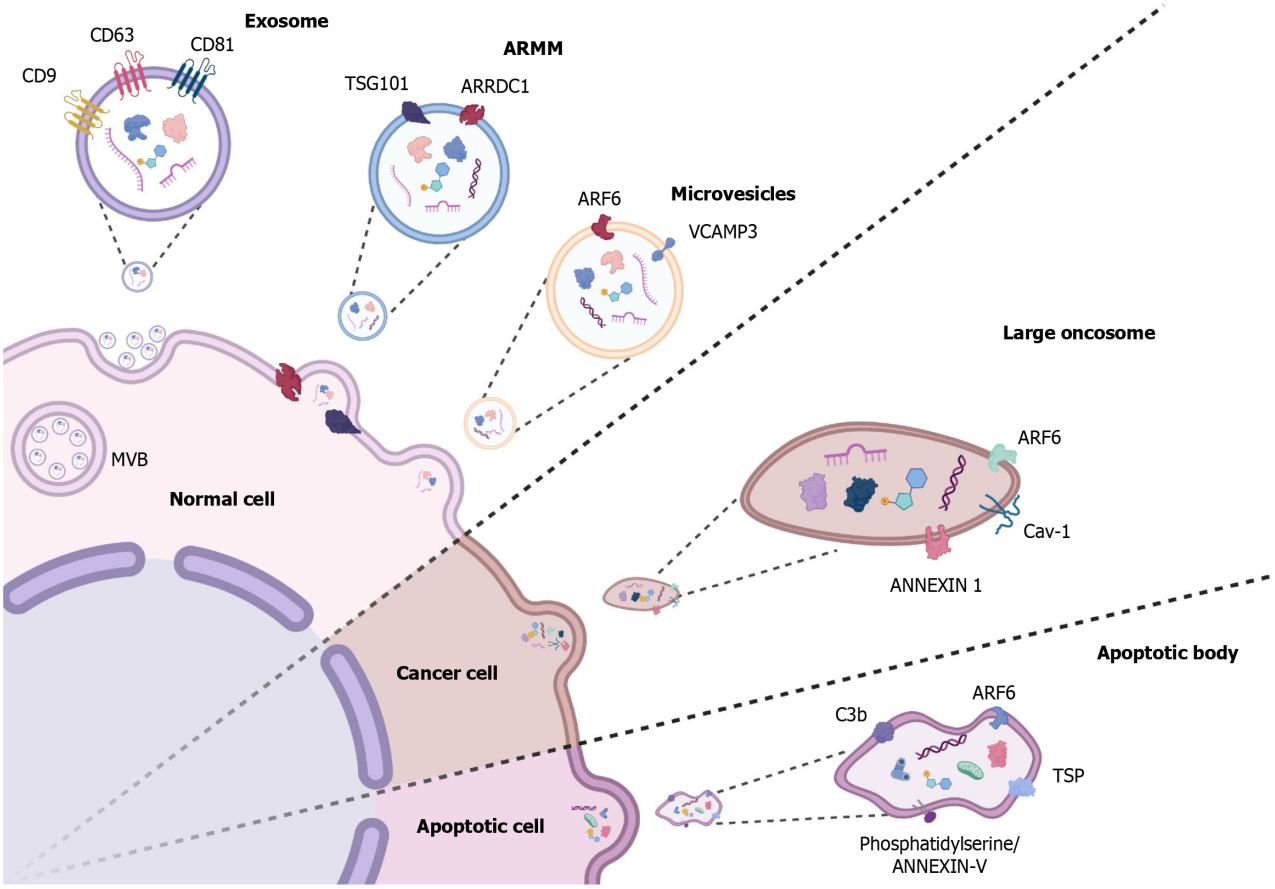

Figure 1 Extracellular vesicle biogenesis and molecular cargo. The image shows the release of exosomes and microvesicles from normal, cancer, and apoptotic cells, including their associated molecules. Exosomes from normal cells contain cluster of differentiation (CD) 9, CD63, CD81, and tumor susceptibility gene 101 (TSG101). Arrestin domain-containing protein 1-mediated microvesicles (ARMM) feature arrestin domain containing 1 (ARRDC1). Microvesicles include ADP-ribosylation factor 6 (ARF6) and vesicle-associated membrane protein 3 (VCAMP3). Large oncosomes from cancer cells contain ARF6, Caveolin-1 (Cav-1), and ANNEXIN 1. Apoptotic bodies from apoptotic cells include ARF6, complement component 3b (C3b), thrombospondin (TSP), and phosphatidylserine/ANNEXIN-V. These extracellular vesicles play critical roles in intercellular communication, carrying proteins and nucleic acids that influence recipient cell behavior. MVB: Multivesicular body. Created in BioRender.com.

which can be used to activate AKT and induce both adhesion and invasion of other prostate cancer cells[43].

ApoBD: ApoBDs, also known as apoptosomes, are derived from the division of cellular contents in late-stage apoptosis. Their structure and size (500 nm to 2 μm) are highly variable, and depending on their dimension, they may include large amounts of RNA, proteins, and lipids[25].

Once apoptosis is finished, ApoBDs are released into the extracellular space, where they can be phagocytosed by macrophages, parenchymal cells, or neoplastic cells. Phagocytosis is activated by the identification of ApoBD membrane biomarkers. Annexin V, thrombospondin, and complement component 3b are the most characteristic biomarkers (Figure 1)[22]. ApoBDs are degraded by macrophage phagolysosomes. Some ApoBDs may contain tingible bodies, which are nuclear debris of apoptotic cells[25].

Although their function has not been completely described, it is well-known that ApoBDs are also capable of transporting useful resources to healthy cells; thus, these vesicles do more than participate in phagocytosis and cellular debris degradation. Also, some biomarkers contained in the ApoBD, such as microRNA and DNA, can regulate intercellular communication[25].

In regular conditions, ApoBDs do not release inflammatory cytokines or free cellular constituents to the extracellular space because they are cleared by a fast phagocytic process, avoiding secondary necrosis. Therefore, they have been associated with inflammatory reactions only under pathological circumstances[25].

CLD

CLD is defined as the progressive deterioration of liver functions for more than 6 months[2]. In 2017, there were an estimated 1.5 billion cases of CLD worldwide[44]. The deterioration of the liver can be produced by alcoholic liver disease, which includes alcohol-fatty liver with or without hepatitis, alcohol hepatitis, and cirrhosis, chronic viral hepatitis (genetic and autoimmune causes), and NAFLD. Some of the patients with NAFLD develop non-alcoholic steato-

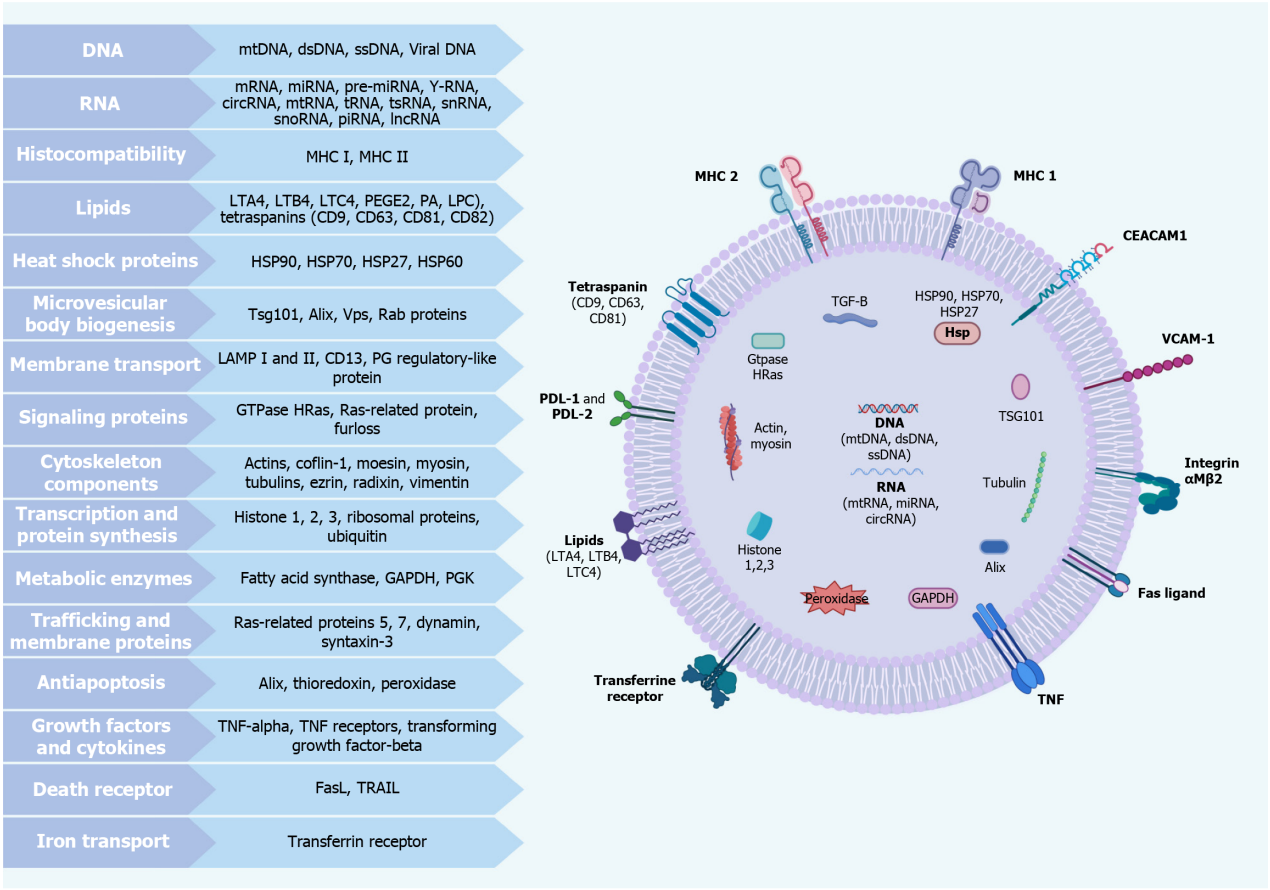

Figure 2 Exosomes: general molecular cargo. This image shows exosome components and functions. Exosomes contain various nucleic acids [mitochondrial DNA (mtDNA), double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), messenger RNA (mRNA), microRNA (miRNA), long non-coding RNA (lncRNA)], major histocompatibility complex I and II molecules, lipids [leukotriene A4 (LTA4), leukotriene D4 (LTD4), leukotriene C4 (LTC4), prostaglandin E2 (PGE2), phosphatidic acid (PA), lysophosphatidylcholine (LPC)], and tetraspanins [(cluster of differentiation) CD9, CD63, CD81, CD82]. They also include heat shock proteins (HSP90, HSP70, HSP27, HSP60), multivesicular body proteins [tumor susceptibility gene 101 (TSG101), ALG-2 interacting protein X (Alix), vacuolar protein sorting-associated proteins (Vps), Rab proteins], membrane transport proteins [lysosome-associated membrane glycoproteins 1 and 2 (LAMP1 and LAMP2), CD13], signaling proteins (GTPase HRas), cytoskeleton components (actins, tubulins), transcription and synthesis elements (histones, ribosomal proteins), metabolic enzymes [GAPDH, phosphoglycerate kinase (PGK)], trafficking proteins (dynamin, syntaxin-3), anti-apoptosis proteins (ALIX), growth factors [tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-B)], death receptors [Fas ligand (FasL)], and iron transport proteins (transferrin receptor). Created in BioRender.com.

hepatitis (NASH), which leads to cirrhosis and then hepatocellular carcinoma (HCC)[2]. In 2017, Global Health Metrics estimated that the age-standardized prevalence of NAFLD and NASH that leads to cirrhosis or liver cancer is 10935 cases per 100000. However, higher rates were found in North America and the Middle East, corresponding to a higher prevalence of obesity[45].

NAFLD is characterized by a lipidic accumulation in the liver generated by an imbalance in the acquisition and removal of triglycerides[46]. At least one risk factor, including insulin resistance, metabolic syndrome, obesity, dyslipidemia, genetic factors, and advanced age, is evident in 90% of patients with NAFLD[47-49]. When these factors persist, NAFLD may progress to an inflammation state known as NASH, which can occur in 20%-30% of patients[47,50,51].

Patients with NASH can progress to tissue fibrosis of the liver due to prolonged inflammation, producing cirrhosis. It has been described that 11% of patients with NASH will experience cirrhosis[47]. The progression to severe fibrosis has been associated with age, possibly related to accumulated metabolic alterations in elderly patients[52].

Once cirrhosis is established, the prognosis is unfavorable because of the risk of developing life-threatening complications, among which HCC stands out due to its rapid progression in NAFLD patients (11.3%). This process occurs through a procarcinogenic state, a consequence of chronic inflammation and lipid metabolism alterations[47,49].

PROTEIN AND EV EXPRESSION THROUGH CLD PROGRESSION

Certain proteins carried by different EVs show changes in their expression in different CLD stages; some of them are associated with liver damage progression. Most proteins mentioned in this review show increased levels in different CLD phases, such as apolipoprotein C-III[53], apolipoprotein C-1[53], fibulin-1[54], and fibulin-3[54] in NAFLD, protein tyrosine phosphatase receptor type G in both NAFLD and NASH[55], and fibulin-4 in cirrhosis[56] (Figure 3).

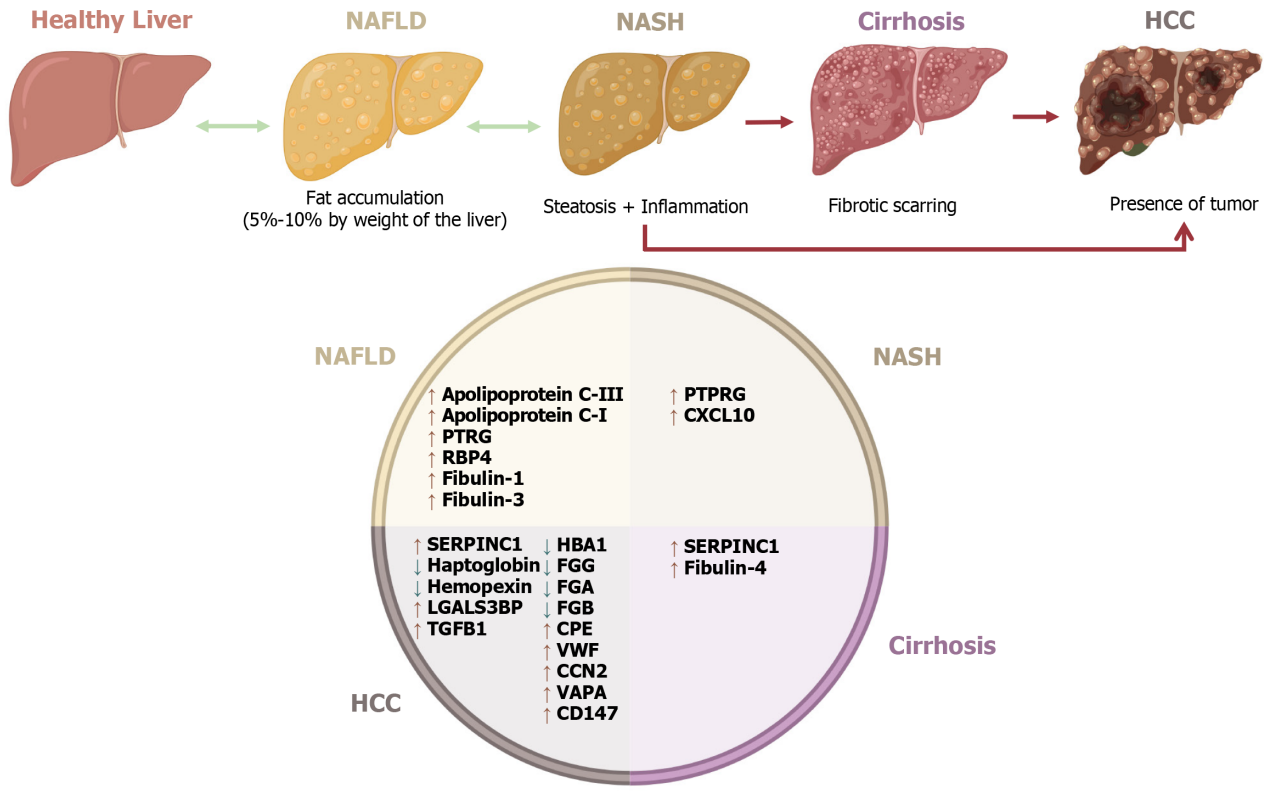

Figure 3 Extracellular vesicle protein expression in different chronic liver disease stages. The image illustrates the progression of liver disease from a healthy liver to nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma (HCC). The circular section lists proteins that increase (↑) or decrease (↓) in each stage: In NAFLD, apolipoprotein C-III (APOC3), apolipoprotein C-I (APOC1), prothymosin alpha (PTMA), retinol-binding protein 4 (RBP4), fibulin-1 (FBLN1), and fibulin-3 (FBLN3); in NASH, protein tyrosine phosphatase receptor type G (PTPRG) and C-X-C motif chemokine ligand 10 (CXCL10); in cirrhosis, serpin family C member 1 (SERPINC1) and fibulin-4 (FBLN4); in HCC, hemoglobin subunit alpha 1 (HBA1), fibrinogen gamma chain (FGC), fibrinogen alpha chain (FGA), fibrinogen beta chain (FGB), von Willebrand factor (VWF), CCN family member 2 (CCN2), vesicle-associated membrane protein associated protein A (VAPA), cluster of differentiation (CD) 147, transforming growth factor beta 1 (TGFB1), galectin-3-binding protein (LGALS3BP), haptoglobin (HP), and hemopexin (HPX). Created in BioRender.com.

Nevertheless, other relevant proteins have shown a particular association with certain types of EVs.

Exosomes

HCC-derived exosomes contain several proteins that are significantly increased when compared with healthy patient exosomes. Some of these proteins are von Willebrand factor, transforming growth factor beta 1, galectin-3-binding protein, serpin family C member 1, hemopexin, haptoglobin, hemoglobin subunit alpha 1, fibrinogen alpha chain, fibrinogen gamma chain, and fibrinogen beta chain. These proteins could be potential biomarkers in HCC diagnosis[57]. Other proteins are increased during HCC, such as carboxypeptidase-E[58] and CCN family member 2[59]. On the other hand, serpin family C member 1 decreases in cirrhosis (Table 2)[57].

It is known that glypican-3 (GPC3) is a reliable immunohistochemical marker for HCC diagnosis (Table 2)[60]. A recent study showed that GPC3 is present exclusively in HCC-derived exosomes, which provides a potential EV-mediated method for HCC early diagnosis and treatment response surveillance[61].

Exosomes promote HCC growth and motility through diverse RNA release mechanisms[62]. Exosomal miR-21/10b stimulates HCC proliferation and metastasis when found in an acidic microenvironment[63]. In addition, miR-92a-2-5p, circRNA-100, 338, and linc00511 facilitate invasiveness and angiogenesis. Furthermore, exosome circRNA-SORE prevents YBX1 degradation, which leads to kinase inhibitor resistance[64-66]. Additionally, tumor cell colonization and extrahepatic metastasis can be induced through EV-Nidogen 1, which promotes premetastatic niche formation[62].

Microvesicles

Microvesicles derived from hepatocytes or leuko-endothelial cells show higher plasma levels in patients with severe liver necroinflammatory activity, abundant liver fibrosis, and cirrhosis. For example, in a study of patients with Child-Pugh C without HCC, increased $CD31^+$ and $CD41^-$ microvesicle levels were observed. Microvesicle levels over 65 U/L predict 6-month mortality. In addition, increased microvesicle levels were associated with cirrhosis severity[67].

LO

Recent studies show that integrin alpha-V expressed on the LO surface can also interact with VAMP-associated protein A, which is then sorted into its surface. LOs enriched with VAMP-associated protein A facilitate bone-tropic metastasis of

Table 2 Extracellular vesicle proteome in liver disease

Disease	Protein	Gene name and alias symbols	Role in liver disease	Vesicle source	Expression in disease
NAFLD	Apolipoprotein C-III Composition: 79 AA MW: 10.85 kDa UA: P02656 HGNC ID: 610 Cytogenetic band: 11q23.3	Apolipoprotein C3 *APOC3* *Apo-CIII* *ApoC-III* *APOCIII* *Apo-C3* *ApoC-3*	An increased expression leads to increased steatosis in NAFLD [53]	EVs from human serum[53]	↑ NAFLD patients[53]
	Apolipoprotein C-I Composition: 83 AA MW: 9.33 kDa UA: P02654 HGNC ID: 607 Cytogenetic band: 19q13.32	Apolipoprotein C1 *APOC1*	An increased expression leads to increased steatosis in NAFLD [53]	EVs from human serum[53]	↑ NAFLD patients[53]
	Retinol-binding protein 4 Composition: 201 AA MW: 23.01 kDa UA: P02753 HGNC ID: 9922 Cytogenetic band: 10q23.33	Retinol binding protein 4 *RBP4b*	Enhances the M1-like polarization of Kupffer cells *via* promoting the activation of NOX2 and NF-κB and ROS accumulation[72]	Serum exosomes from NAFLD patients[72]	↑ NAFLD patients[72]
	Receptor-type tyrosine-protein phosphatase gamma Composition: 1445 AA MW: 162.03 kDa UA: P23470 HGNC ID: 9671 Cytogenetic band: 3p14.2	Protein tyrosine phosphatase receptor type G *PTPRG* *RPTPG*	Hepatic *PTPRG* mRNA increase proportionally to the severity of NAFLD[55]	Exosomes from human plasma and murine plasma, serum, and tissue[73]	↑ of plasmatic approximately 120 kDa protein isoform were associated with the occurrence of liver damage[73] ↑ NASH[55]
	C-X-C motif chemokine 10 Composition: 98 AA MW: 10.88 kDa UA: P02778 HGNC ID: 10637 Cytogenetic band: 4q21.1	C-X-C motif chemokine ligand 10 *CXCL10* *IFI10* *IP-10* *crg-2* *mob-1* *C7* *gIP-10*	Lipotoxic hepatocyte-derived EVs containing CXCL10 induce macrophage chemotaxis[74,75]	EVs from Mlk3-/- mice [74,75]	↑ NASH model[74,75]

	Fibulin-1 Composition: 703 AA MW: 77,214 kDa UA: P23142 HGNC ID: 3600 Cytogenetic band: 22q13.31	Fibulin-1 *FBLN1* *FBLN*	Correlate with fibrosis stage[54]	EVs from human serum[54]	↑ NAFLD patients[54]
	Fibulin-3 Composition: 493 AA MW: 54.64 UA: Q12805 HGNC ID: 3218 Cytogenetic band: 2p16.1	EGF containing fibulin extracellular matrix protein 1 *EFEMP1* *S1-5* *FBLN3* *MTLV*	Increase with liver fibrosis. Predictor of liver-related events [54]	EVs from human serum[54]	↑ NAFLD patients[54]
NASH	Antithrombin-III Composition: 464 AA MW: 52.6 kDa UA: P01008 HGNC ID: 775 Cytogenetic band: 1q25.1	Serpin family C member 1 *SERPINC1* *ATIII* *MGC22579*	Almost all of the downregulated proteins are produced in the liver[57]	Exosomes from HCC human serum samples [57]	↑ ATIII in liver cirrhosis and HCC[57]
	Von Willebrand factor Composition: 2813 AA MW: 309.26 kDa UA: P04275 HGNC ID: 12726 Cytogenetic band: 12p13.31	Von Willebrand factor *VWF*	Biomarker of severe liver fibrosis diagnosis and HCC development predictor[76]	Exosomal, from serum samples[57]	↑ HCC[57]
	Hemopexin Composition: 462 AA MW: 51.67 kDa UA: P02790 HGNC ID: 5171 Cytogenetic band: 11p15.4	Hemopexin *HPX*	↓ HPX protein develops inflammation and oxidative stress in the liver[77]	Exosomal, from serum samples[57]	↓ HCC[57]
	Galectin 3 binding protein Composition: 585 AA MW: 65.33 kDa. UA: Q08380 HGNC ID: 6564 Cytogenetic band: 17q25	Galectin 3 binding protein *LGALS3BP* *MAC-2-BP* *90K* *BTBD17B* *ANGO10B* *M2BP* *gp90*	Significant biomarker in liver fibrosis, cirrhosis, and HCC in patients with hepatitis C[78]	Exosomes from serum samples[57]	↑ HCC[57]

		CyCAP			
Cirrhosis	Transforming growth factor beta 1 Composition: 390 AA MW: 44.32 kDa UA: P01137 HGNC ID: 11766 Cytogenetic band: 19q13.2	Transforming growth factor beta 1 *TGFB1* *CED* *TGFbeta*	Promotes HSC activation and ECM production, contributing NAFLD progression[79,80]	Exosomal, from serum samples[57] Hepatoma cell lines, culture media[81] Ascites derived exosomes, from hepatic cirrhosis patients[82]	↑ HCC[57] ↑ Promote tumor metastasis[81] ↑ Promote cancer[82]
	Fibulin-4 Composition: 443 AA MW: 49.4 kDa UA: O95967 HGNC ID: 3219 Cytogenetic band: 11q13.1	EGF containing fibulin extracellular matrix protein 2 *EFEMP2*	Increased levels in cirrhosis, correlated with progression of fibrosis[56]	EVs from serum samples of patients with cirrhosis[56]	↑ Cirrhosis[56]
HCC	Haptoglobin Composition: 406AA MW: 45.20 kDa UA: P00738 HGNC ID: 5141 Cytogenetic band: 16q22.2	Haptoglobin *HP*	Patients with NAFLD with the Hp2-2 genotype had higher BMI, total cholesterol, and ferritin[83]	Exosomal, from serum samples[57]	↓ HCC[57]
	Hemoglobin subunit alpha Composition: 142 AA MW: 15.258 kDa UA: P69905 HGNC ID: 4823 Cytogenetic band: 16p13.3	Hemoglobin subunit alpha 1 *HBA1* *HBA-T3*	Hemoglobin overexpression suppresses oxidative stress[57, 84]	Exosomal, from serum samples[57] Liver biopsies from NASH patients[84]	↓ HCC[57] ↑ NASH[84]
	Fibrinogen alpha chain Composition: 866 AA, MW: 94.97 kDa UA: P02671 HGNC ID: 3661 Cytogenetic band: 4q31.3	Fibrinogen alpha chain *FGA*	α chain fragments rapid alteration in early stages in liver fibrosis[85,86]	Exosomal, from serum samples[57]	↓ HCC[57]
	Fibrinogen gamma chain Composition: 453 AA MW: 51.51 kDa UA: P02679	Fibrinogen gamma chain *FGG*	Rare cases of hypofibrinogenemia are associated with liver disease[87]	Exosomal, from serum samples[57]	↓ HCC[57]

HGNC ID: 3694 Cytogenetic band: 4q32.1				
Fibrinogen beta chain Composition: 491 AA MW: 55.928 kDa UA: P02675 HGNC ID: 3662 Cytogenetic band: 4q31.3	Fibrinogen beta chain *FGB*	Rarely, hypofibrinogenemia can present with HFSD[88]	Exosomal, from serum samples[57]	↓ HCC[57]
Carboxypeptidase E Composition: 476 AA MW: 53.15 kDa UA: P16870 HGNC ID: 2303 Cytogenetic band: 4q32.3	Carboxypeptidase E *CPE*	Promotes tumor metastasis and predicts tumor recurrence in early-stage HCC[58]	Exosomes from supernatant culture and human serum[57]	↑ HCC Promote tumor metastasis[57]
Vesicle-associated membrane protein-associated protein A Composition: 249 AA MW: 27.89 kDa UA: Q9P0L0 HGNC ID: 12648 Cytogenetic band: 18p11.22	VAMP associated protein A *VAPA* *hVAP-33* *VAP-A*	Facilitates bone-tropic metastasis of HCC by promoting osteoclastogenesis[41]	Large oncosomes from liver cancer mouse model[41]	↑ HCC[41]
Basigin Composition: 385 AA MW: 42.2 kDa UA: P35613 HGNC ID: 1116 Cytogenetic band: 19p13.3	Basigin (Ok blood group) *BSG* *EMMPRIN* *CD147* *EMPRIN*	Induces angiogenesis by stimulating VEGF production and invasiveness by stimulating MMPs Promotes the invasion and metastasis of human hepatoma cells by stimulating both tumor cells and peritumoral fibroblasts to produce elevated levels of MMPs[89]	Microvesicles from SMMC-7721 cell line (Hepatocellular carcinoma)[89]	↑ HCC *in vitro* model [89]

Information regarding protein name, composition, molecular weight, and Uniprot access was obtained from UniProt consortium[90]. The information regarding the HGNC ID, cytogenetic band, gene name and alias symbols was obtained from HUGO Gene Nomenclature Committee[91]. AA: Amino acids; BMI: Body mass index; CD: Cluster of differentiation; CXCL10: C-X-C motif chemokine ligand 10; ECM: Extracellular matrix; EVs: Extracellular vesicles; HCC: Hepatocellular carcinoma; HFSD: High-fat style diet; HGNC: HUGO Gene Nomenclature Committee; HPX: Hemopexin; HSC: Hepatic stellate cells; kDa: Kilodaltons; Mlk3: Mixed-lineage protein kinase 3; MMPs: Metalloproteinases; mRNA: Messenger RNA; MW: Molecular weight; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; PTPRG: Protein tyrosine phosphatase receptor type G; ROS: Reactive oxygen species; SERPINC1: Serpin family C member 1; UA: Uniprot access; VEGF: Vascular endothelial growth factor.

HCC by promoting osteoclastogenesis (Table 2)[41].

ApoBDs

During hepatic disease, the effectiveness of phagocytic cells is overwhelmed by a substantial number of apoptotic hepatic cells. This results in an inadequate degradation of ApoBDs, which starts a process of autolysis, where the apoptotic cells release their proinflammatory content into the extracellular space[68]. However, there are other proinflammatory mechanisms that influence hepatic injury.

Hepatic tissue-specific macrophages are known as Kupffer cells (KC) and represent the main cells involved in ApoBDs phagocytosis. KC-mediated phagocytosis is related to increased death ligand concentrations, such as TNF-α, FasL, TGF-B, and TNF-related apoptosis-inducing ligand. These death ligands are profibrogenic and produce further hepatocyte apoptosis with ApoBD formation and subsequent KC-mediated phagocytosis, repeating the cycle over and over again [69].

It has been shown that hepatic stellate cells (HSC) can also clear ApoBDs, given that both KC and HSC express phosphatidyl serine receptors, which enables them to recognize apoptotic cells[69]. When KC-mediated phagocytosis is overwhelmed, HSC initiates ApoBDs phagocytosis, which causes HSC to transition from a quiescent to a proliferative, fibrogenic phenotype known as myofibroblasts. These myofibroblasts produce extracellular matrix and scar formation in the liver. NADPH oxidase NOX2 expressed on the HSC membrane has a significant role in this process as it induces reactive oxygen species-mediated collagen production[70,71]. These characteristics are summarized in Table 2[72-91].

EVS IN LIVER DISEASE DIAGNOSIS AND THERAPY

Due to their features, EVs are attractive biomarkers, as they offer the specificity of liver biopsy samples and the non-invasiveness of peripheral blood samples[92]. Some reports support this idea. A study in two murine models of NAFLD/NASH found an increase in circulating EVs (microvesicles and exosomes). This increment was time-dependent and consistent with the progressive developmental stages of the disease. The authors also found a significant time-dependent increase in the levels of miR-122 and miR-192 (two miRNAs strongly associated with the liver and NAFLD) contained in these EVs[93]. These two miRNAs (as well as miR-19a and miR-19b, miR-125, and to a lesser extent miR-375) were upregulated in a serum argonaute2-free form in NASH patients in comparison with controls (and miR-122 was upregulated in patients with uncomplicated steatosis)[94]. Also, an association between the increase of miR-122, miR-192, and miR-375 with disease severity was observed, and miR-122 was slightly superior in predicting NASH and fibrosis than the classic markers alanine aminotransferase and aspartate aminotransferase[94].

Another study showed that overexpression of exosomal miR-500 from hepatic macrophages accelerates liver fibrosis by promoting HSC activation *in vitro* and *in vivo*[95]. This study also showed that CLD patients presented increasing levels of circulating exosomal miR-500 in accordance with disease stage; in light of this finding, the authors proposed this exosomal miRNA as a biomarker for the progression of liver fibrosis[95].

Patients with NASH (with cirrhosis and pre-cirrhosis) show an increase in circulating EVs compared to healthy individuals[96]. After a proteomic analysis, the authors of that finding proposed seven proteins upregulated in EVs from NASH patients as biomarkers: Von Willebrand factor, Wnt1-inducible signaling pathway protein-1, aminoacyl-tRNA synthetase interacting multifunctional protein 1, IL27RA, ICAM2, IL1β, serine/threonine protein kinase, and repulsive guidance molecule A precursor. Additionally, some of these proteins showed differences between cirrhotic and pre-cirrhotic NASH[96].

The percentage of hepatic exosomes (characterized by the presence of albumin) is increased in patients with NASH compared with patients with NAFLD and healthy subjects[97]. Also, the authors reported that glucose transporter 1 (GLUT1) was significantly higher in exosomes from patients with NASH compared to patients with NAFLD and with healthy subjects. In addition, exosomes and exosomal GLUT1 levels were higher in advanced stages of fibrosis (F2-4) than in early stages (F0-1). This suggests that the content of GLUT1 in exosomes may be an early marker of NAFLD and can be a prognosis tool for the severity of the disease[97].

Lipids contained in EVs have also been pointed out as potential biomarkers. Hepatocytes under lipotoxic stimuli release ceramide-rich EVs, particularly in sphingosine-1-phosphate (S1P), and can activate macrophage chemotaxis, showing that S1P in EVs can be used as a biomarker as well as a potential therapeutic target *(e.g.,* interfering with the signaling axis from S1P in macrophages)[98]. A study in patients with NAFLD and NASH revealed that hepatocyte-derived plasma EV levels decreased significantly after weight loss surgery[99]. Furthermore, pre-surgery EVs were rich in lipids like sphingosine, sphinganine, S1P, and ceramide species, which was correlated with the development of steatosis and inflammation[99]. Altogether, these results suggest the potential of EVs and their cargo molecules as a good non-invasive diagnostic tool for disease progression.

Exosomes also have potential as therapeutic agents for certain liver diseases. Due to their characteristics and cargo, they can be used either as drug delivery tools or as therapy themselves[92].

Recent research has been shedding light on the potential of mesenchymal stem cell (MSC)-EVs to treat liver diseases since they exhibit anti-inflammatory, anti-fibrotic, and regenerative properties, making them effective in treating conditions like liver fibrosis and NAFLD[100].

The intravenous injection of EVs from human liver stem cells as a therapeutic agent in a murine NASH model was shown to recover the expression of several genes involved in fibrosis and inflammation (*α-Sma, Col1α1, Tgf-β1; Tnf, IL-1β, Ifn-γ*) in the liver of NASH mice. In this study, a reduction of inflammatory cells in the liver, upregulation of IL-10 expression, a significant reduction of alanine aminotransferase in plasma, and reduced fibrosis (but not steatosis) were also observed[101]. Consistent with this result, in a murine *in vitro* model, the treatment of HSCs with exosomes released by tonsil-derived MSCs induced a reduction in the levels of proteins involved in fibrosis development and promotion (TGF-β, α-SMA, COL1α1, vimentin, and CTGF). These authors concluded that this response was due to the action of the miR-486-5p contained in these EVs[102]. According to a pooled analysis led by Fang *et al*[103], these nanovesicles can significantly boost liver function and reduce inflammation, offering new hope for treatments. They work by delivering therapeutic molecules directly to liver cells, reducing fibrosis, and promoting healing[103].

Using exosomes derived from human adipose MSCs demonstrated that these vesicles can ameliorate liver fibrosis progression by diminishing the accumulation of lipids, improving the choline-phosphatidylcholine metabolism and attenuating HSC activation[104]. Also, Ganguin *et al*[105] showed that similar vesicles from LX-2 cells can reverse liver fibrosis effectively, depending on the amount. This promising approach suggests a less invasive alternative to current treatments, opening promising applications in regenerative medicine. As research progresses, the goal is to refine these vesicles for safe and effective treatment in liver conditions[105].

In a high-fat diet mouse model, administration of human umbilical cord MSC-derived exosomes prevented hepatic steatosis. Additionally, in L02 cells, related effects are due to calcium/calmodulin-dependent protein kinase 1 increase by MSC-derived exosomes, which triggered fatty acid β-oxidation elevation and fatty acid synthesis reduction *via* an enhanced expression of p-AMPK, PPARα, and CPT-1A and the inhibition of mature-SREBP-1C and FASn, respectively [100].

The relationship between EVs, particularly exosomes, and the pathogenesis, diagnosis, and therapy of HCC has been reviewed elsewhere. Briefly, several molecules contained in exosomes, particularly miRNAs, have been found to have potential as biomarkers for liver cancer or to participate in either suppression (*e.g.*, miR-638, miR-326), development (*e.g.*, miR-21, miR-10b, miR-23a/b), or as mechanisms for tumors and metastasis (*e.g.*, miR-1237f, miR-378b), making them potential therapeutic targets for this disease[106-110].

One HCC-specific biomarker, GPC3, was found in exosomes, but not microvesicles, delivered from HCC cells. This proteoglycan, which plays a role in MVB biogenesis and release, accumulates due to the autophagy impairment present in this disease. Therefore, the detection of circulating exosomes enriched with this molecule can be used as a biomarker in patients with CLD before the development of HCC[61].

A recent study with *in vitro* and *in vivo* models pointed to exosomal formimidoyltransferase-cyclodeaminase as a potential biomarker for HCC. The high expression of this protein is associated with macrophage infiltration and polarization to the M1 type, suppressing HCC proliferation. This could lead to a better prognosis for HCC patients[111]. On the other hand, a different study showed that miR-200b-3p contained in HCC cell-derived exosomes can facilitate macrophage polarization to the M2 type, accelerating the proliferation and mediating HCC metastasis. Therefore, this exosomal miRNA can be associated with a bad prognosis[112].

Some studies underscored the use of circRNAs present in exosomes to treat HCC. For example, exosomal circ-0051443 was lower in HCC cell lines and patients with HCC compared to normal cells, and its administration to HCC cells had a suppressor effect in cell proliferation and even promoted a certain degree of apoptosis, with a corresponding reduction in tumor size in a murine model[113].

Exosomal circRNAs can also be involved in resistance to therapy, and their blockage leads to a better prognosis. An upregulation of circRNA-SORE (circRNA_104797), transported in exosomes, is critical for sorafenib resistance in certain HCC tumors, and its silencing improves the efficacy of the treatment *in vivo*[114]. Another study highlighted the importance of exosomal circUHRF1 (hsa_circ_0048677) upregulation in HCC tumors resistant to immunotherapy against PD-1, making these EVs a potential therapeutic target[115].

Apart from their participation in promoting drug resistance, EVs can be used as vessels to deliver molecules aimed for the contrary effect. The silencing of Grp78, a protein that promotes resistance to sorafenib, can be used to overcome this resistance *in vitro*. This was achieved by modifying bone marrow-derived MSCs to express the small interfering RNA siGRP78 and release it inside exosomes, which were then co-cultured with HepG2 sorafenib-resistant cells. The authors then observed a reduction in proliferation and invasion in the co-cultured cells when treated with the drug[116].

Due to limitations involved in patient staging according to liver disease degree and severity, the majority of the evidence discussed in this review has been obtained by *in vitro* and *in vivo* models. It is also not clear which specific cell types secrete distinct EV populations in patients. Thus, it is important to continue investigating and working to understand the role of EVs in the pathology and treatment of CLD.

CONCLUSION

EVs are key components for cellular communication, particularly for liver cells. This allows the expression of genes and the activation of signaling pathways in the liver microenvironment, which can ultimately impact fibrogenic and inflammatory processes.

Several studies have highlighted the role of EV-carried mediators in the development and progression of CLD as well as the role of specific proteins directly associated with CLD stages. Therefore, it is necessary to continue to investigate EV transported molecules, their presence depending on the CLD stage, and their association with disease features. As evidence continues to mount that EV expression patterns vary at differing stages of CLD, EVs hold great potential to be used as biomarkers, with the finality of improving clinical practices, including non-invasive personalized diagnosis and prognosis, and identifying potential therapeutic targets.

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