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# Extracellular Vesicles in Liver Diseases: Meeting Report from the International Liver Congress 2018

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## Keywords

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# Extracellular Vesicles in Liver Diseases: Meeting Report from the International Liver Congress 2018

Jesus M. Banales,<sup>1</sup> Ariel E. Feldstein,<sup>2</sup> Hanna Sanger,<sup>3</sup> Veronika Lukacs-Kornek,<sup>4</sup> Gyongyi Szabo,<sup>5</sup> and Miroslaw Kornek<sup>6,7</sup>

Extracellular vesicles (EVs) are small and heterogeneous membrane-bound structures released by cells and found in all biological fluids. They are effective intercellular communicators, acting on a number of close and/or distant target cells. EV cargo may reflect the cell of origin as well as the specific stress that induces their formation and release. They transport a variety of bioactive molecules, including messenger RNA, noncoding RNAs, proteins, lipids, and metabolites, that can be transferred among cells, regulating various cell responses. Alteration in the concentration and composition of EVs in biological fluids is a typical hallmark of pathologies in different liver diseases. Circulating EVs can serve as biomarkers or as messengers following uptake by other cells. This review is a meeting report from the International Liver Congress 2018 (European Association for the Study of the Liver) celebrated in Paris (Symposium: Extracellular vesicles and signal transmission) that discusses the role of EVs in several liver diseases, highlighting their potential value as disease biomarkers and therapeutic opportunities. (*Hepatology Communications* 2019;3:305-315).

Extracellular vesicles (EVs) are small cell-derived structures enveloped by a double-layer membrane that are shed by cells as a mechanism of horizontal communication. First described as an outgrowth of platelet activity or sample contamination, the role of EVs remained largely unexplored<sup>(1,2)</sup> until the early 2000s when growing enthusiasm for the field of EV biology and pathobiology resulted in

increasing numbers of new publications each year. The potential of EVs as diagnostic and prognostic tools is being increasingly recognized by the scientific community and awaits translation into human medicine.

Currently, three major types of EVs are recognized: exosomes, microvesicles (MVs), and apoptotic bodies. Despite some disagreements over their exact

*Abbreviations: AH, alcoholic hepatitis; ALD, alcoholic liver disease; AMPN, aminopeptidase N; ASGPR1, asialoglycoprotein receptor 1; AUROC, area under the receiver operating characteristic curve; CA19.9, carbohydrate antigen 19-9; CCA, cholangiocarcinoma; CD, clusters of differentiation; EpCAM, epithelial cell adhesion molecule; EV, extracellular vesicle; FCN, ficolin; FIBG, fibrogenin; GPC, glypican; HCC, hepatocellular carcinoma; HSC, hepatic stellate cell; IL, interleukin; lncRNA, long noncoding RNA; LPS, lipopolysaccharide; miR, microRNA; MP, microparticle; MV, microvesicle; MVB, multivesicular body; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PIGR, polymeric immunoglobulin receptor; PPAR- $\gamma$ , peroxisome proliferator-activated receptor gamma; PSC, primary sclerosing cholangitis; SEN, sensitivity; SPE, specificity; ta, tumor-associated; VNN1, vanin-1.*

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definitions, essentially these three types of EVs differ in size and mode of production. Exosomes, which are up to 150 to 200 nm in diameter, represent the smallest type.<sup>(3)</sup> They are produced within the endosomal membrane system of their parental cells and are transported toward the plasma membrane inside endosomal vesicles, also known as multivesicular bodies (MVBs).<sup>(3)</sup> These MVBs merge with the cell plasma membrane and shed exosomes into the extracellular space by exocytosis.<sup>(4)</sup> In contrast, MVs, also referred to as microparticles (MPs) or ectosomes, are released through a coordinated budding process of the cell plasma membrane, resulting in their membrane composition mirroring that of the parental cells.<sup>(5,6)</sup> Characterization of MVs can be used to identify the cell type that was activated to release a particular population of MVs.<sup>(7)</sup> MVs range from 0.1 to 1  $\mu\text{m}$  in diameter and are characterized by a bilayer membrane containing externalized phosphatidylserine.<sup>(8,9)</sup> In cells undergoing regulated death, apoptotic signals induce their fragmentation into apoptotic bodies,<sup>(10)</sup> which represent the largest EVs and range from 1 to 5  $\mu\text{m}$  in diameter.<sup>(11)</sup>

It is believed that all cell types of the human body are capable of releasing EVs either constitutively or as adaptive cellular responses.<sup>(8)</sup> Based on their ability to travel through biological fluids, EVs function as messengers, communicating between distant sites while maintaining a high specificity (SPE) to their destination.<sup>(12-14)</sup> Their cargo can comprise various types of molecules, making them ideal regulators of biological processes. EVs can transport short and long nucleic acids (microRNAs [miRs/miRNAs], small interfering RNAs, messenger RNAs,

or long noncoding RNAs [lncRNAs]), proteins (cytosolic, cytoskeletal, membrane-bound transporters and receptors, enzymes, adhesion molecules), lipids (sphingomyelin, phosphatidylserine, cholesterol, ceramide), and metabolites.<sup>(15)</sup> Recently, their potential as vehicles for effective and site-specific drug delivery has been implemented.<sup>(16)</sup> By fusing with a recipient cell, EVs integrate their membrane into the phospholipid bilayer of the receiving cell, thereby transferring their content into the recipient and modulating intracellular pathways.<sup>(5)</sup> EVs can also be taken up by recipient cells through protein and/or lipid interactions, further leading to their endocytosis.<sup>(13)</sup>

In earlier years research focused on detection and quantification of EVs; however, since the mid-2000s the analysis of their composition and the study of their biological functions have become the center of attention. As EV composition and content reflect features of the parental cells, EVs represent invaluable indicators of mechanisms driving pathobiological processes.<sup>(5,6)</sup> By isolating circulating exosomes or MVs from patient blood, it is possible to create disease-specific EV profiles with respect to their surface antigens and/or whole RNA, protein, and lipidomic content. Apart from peripheral blood, various other body fluids have been used for EV characterization, including saliva,<sup>(17)</sup> urine,<sup>(18)</sup> breast milk,<sup>(19)</sup> cerebrospinal fluid,<sup>(20)</sup> and bile.<sup>(21)</sup> Accordingly, numerous liver conditions have been evaluated in terms of their EV profile and pathobiological relevance, including, among others, nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), alcoholic hepatitis (AH), cirrhosis, hepatocellular carcinoma (HCC),

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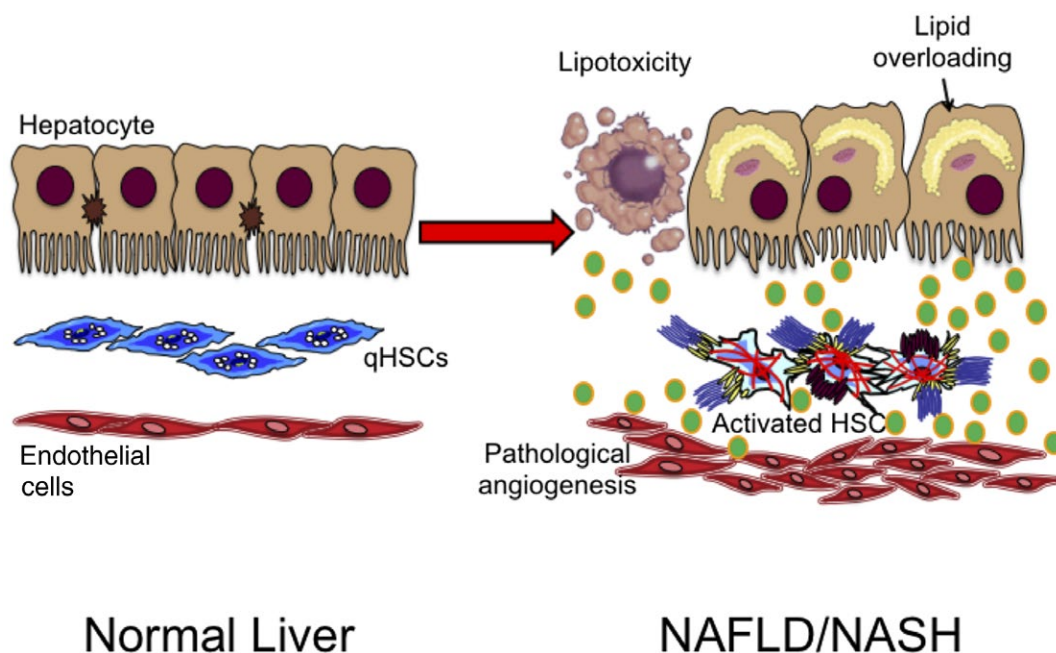
cholangiocarcinoma (CCA), and other cholangiopathies (such as primary sclerosing cholangitis [PSC]). In the liver, hepatocytes, immune cells, endothelial cells, hepatic stellate cells (HSCs), and cholangiocytes all contribute to EV production.<sup>(22-25)</sup>

## NAFLD and NASH

Lipotoxicity, a process by which accumulation of certain toxic lipids (e.g., saturated free fatty acids) in hepatocytes triggers various molecular pathways of cell stress and eventually results in cell death, has evolved as a key event along NAFLD progression to NASH and eventually to HCC.<sup>(4)</sup> During the process of lipotoxicity, hepatocytes release large numbers of EVs that may act on different target cells and contribute to key processes involved in NAFLD pathogenesis, including immune modulation, angiogenesis, and fibrosis (Fig. 1). The EVs produced and released during NAFLD progression have specific antigenic composition, reflecting the pathologic alterations typical of its progression, and

present miRs and proteins abundantly found in the liver. Additionally, the EV levels are dynamic and change over time, correlating with changes in liver histopathology.

Proteome analysis of circulating EVs from mouse models of NAFLD demonstrated that these vesicles carry a selective antigenic composition.<sup>(26)</sup> Hierarchical clustering analysis allowed for discrimination of mice with established NASH from those with isolated steatosis and normal livers, identifying a proteomic signature in serum EVs that might be used to noninvasively diagnose NASH. Notably, the level of circulating EVs released during diet-induced mouse models of NAFLD strongly correlated with histopathologic features of NAFLD (i.e., fat content, fibrosis, cell death, and pathologic angiogenesis) and were enriched in miR-122 and miR-192, two miRs highly expressed in hepatocytes. The level of these miRNAs increased in EVs and decreased in livers over time during NAFLD progression. The release of these miRs from stressed or damaged hepatocytes in EVs during NAFLD progression may provide an attractive explanation for the decreased expression



**FIG. 1.** Extracellular vesicles in the pathogenesis of fatty liver disease. During the process of lipotoxicity, hepatocytes release a large amount of EVs that may then act on various target cells in the local environment, contributing to key processes involved in NAFLD pathogenesis, including immune modulation, angiogenesis, and fibrosis. Additionally, EVs may be released to the systemic circulation and can be potentially used to noninvasively monitor the extent of liver injury. Abbreviation: qHSC, quiescent HSC.



level of miR-122 found in the livers of patients with NASH.<sup>(27)</sup> In contrast to healthy individuals where miR-122 is present in circulation only in the Argonaut 2 (Ago2) complex fraction, the majority of serum miR-122 circulates in Ago2-free forms in patients with NAFLD,<sup>(28)</sup> although its specific compartment is still unknown.

Patients with NASH might also present changes in the immune-derived EV composition because increased circulating levels of leukoendothelial-derived clusters of differentiation (CD)31<sup>+</sup>/41<sup>-</sup>, pan-leukocyte-derived CD11a<sup>+</sup>, lymphocyte-derived CD4<sup>+</sup>, and erythrocyte-derived CD235a<sup>+</sup> EVs occur in patients with liver cirrhosis.<sup>(29,30)</sup> Additionally, MVs positive for inflammatory cell markers, such as CD4<sup>+</sup> cells, CD8<sup>+</sup> T cells, or CD14<sup>+</sup> monocytes/invariant natural killer cells, are detected in plasma of patients with various liver conditions.<sup>(7)</sup> However, EVs derived from inflammatory cells are not specific and may be elevated in a number of immune and inflammatory conditions associated with their activation, thus limiting their utility as biomarkers of liver disease.

At the pathobiological level, it has been shown that MPs from plasma of both mice and patients with NASH contain high levels of mitochondrial DNA and intact mitochondria that are able to trigger toll-like receptor 9 signaling, which is critical for NASH development.<sup>(31)</sup> Moreover, serum EVs originated from the liver of mice with diet-induced NASH triggered migration and tubular structure formation when applied to endothelial cells *in vitro*.<sup>(32)</sup> The angiogenic effects of hepatocyte-derived MVs underexposed to saturated fatty acids involved a vanin-1 (VNN1)-dependent uptake of EVs by endothelial cells (i.e., an enzyme located on the surface of the vesicles). Similarly, EVs released by hepatocytes exposed to lipotoxicity are efficiently internalized into HSCs through a VNN1-dependent mechanism.<sup>(33)</sup> The internalization of vesicles induced a phenotypic switch of HSCs from quiescent to activated collagen-producing myofibroblasts.<sup>(33)</sup> These data suggest that pathologic angiogenesis and fibrosis could be reduced by preventing endothelial cells and HSCs from internalizing VNN1-positive EVs from lipotoxic hepatocytes.

On the other hand, miR-128-3p was found enriched in EVs derived from fat-laden hepatocytes in the liver of two murine diet-induced NAFLD/NASH models<sup>(33)</sup> and in the liver of patients with

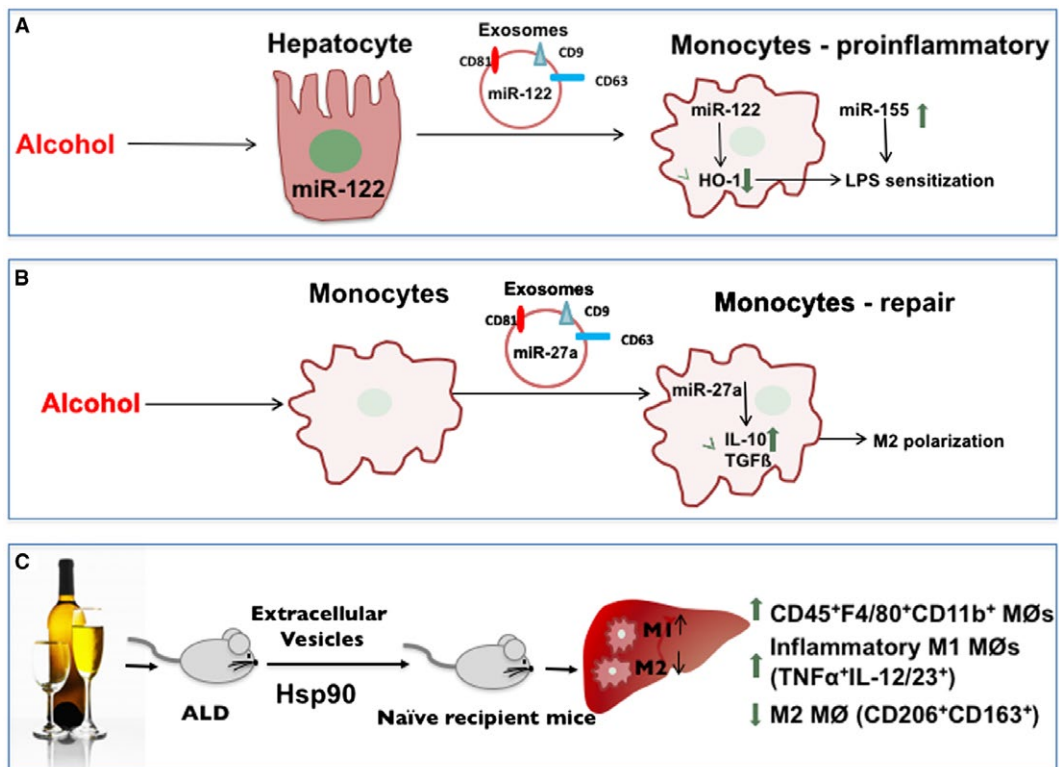
NASH.<sup>(27)</sup> This miR is selectively transferred through hepatocyte-derived EVs into HSCs, promoting HSC activation through direct targeting of peroxisome proliferator-activated receptor gamma (PPAR- $\gamma$ ) expression. PPAR- $\gamma$  is a member of the nuclear hormone-receptor superfamily that modulates HSC quiescence. PPAR- $\gamma$  expression progressively decreases along HSC activation and is completely depleted in fully activated HSCs,<sup>(34)</sup> highlighting the important role of the EV transfer of miRs in the pathogenesis of NAFLD/NASH.

Regarding the mechanisms of EV biogenesis in NASH, mixed lineage kinase 3 was shown to mediate the release of chemokine (C-X-C motif) ligand 10 in exosomes from lipotoxic hepatocytes, and these exosomes induced macrophage chemotaxis in NASH.<sup>(35)</sup> Lipid-induced signaling can also cause the release of EVs with inflammatory potential into hepatocytes,<sup>(36)</sup> while the ceramide transport protein steroidogenic acute regulatory protein-related lipid transfer domain 11 has been recently uncovered as a novel modulator of exosome biogenesis during lipotoxic insult to hepatocytes.<sup>(37)</sup>

## Alcoholic Liver Disease and Alcoholic Hepatitis

AH, the clinically severe manifestation of alcoholic liver disease (ALD), represents one of the most common etiologies of progressive liver diseases leading to cirrhosis and predisposing to HCC. EVs have been explored as biomarkers for ALD and AH. Moreover, their biodistribution, cellular sources, biological effects, and therapeutic potential have been investigated (Fig. 2).

The numbers of circulating EVs were found to be increased in mouse models of ALD as well as in patients with AH.<sup>(22)</sup> The majority of these EVs are exosomes (40 and 200 nm) and some MVs (200 and 1,000 nm). Screening of the EV cargo revealed specific changes in miR composition in a mouse model of ALD compared to control mice. Specifically, the concentration of miR-122, miR-192, and miR-30a was found increased in EVs of alcohol-fed mice compared to controls, presenting diagnostic capacity.<sup>(22)</sup> Notably, the levels of these miRs were also increased in serum EVs of patients with AH, indicating the



**FIG. 2.** Extracellular vesicles in ALD. (A) EVs are released from different cell types in the liver and provide communication between hepatocytes and immune cells as well as within immune cells, through miRs. (B) miR signals that promote M1 (classically activated) or M2 (alternatively activated) macrophage polarization contribute to the mixed M1/M2 phenotype of macrophages in the liver in ALD. (C) Circulating EVs from chronic alcohol-fed mice induce liver inflammation and macrophage polarization in alcohol-naïve recipient mice. Abbreviations: HO, Heme oxygenase; Hsp, heat shock protein; TGF $\beta$ , transforming growth factor  $\beta$ .

potential value as disease biomarkers.<sup>(22)</sup> Although the mechanistic explanation for the increased levels of these miRs in EVs of ALD and AH needs to be revealed, it is important to note that miR-122 and miR-192 are highly abundant in hepatocytes and the increased levels of these miRs may be indicative of ongoing hepatocyte damage.<sup>(38-40)</sup> Furthermore, miR-30a regulates different steps of the autophagy process and MVB formation.<sup>(41)</sup> Thus, it is tempting to speculate that the miR-30a concentration in EVs may be the result of changes in autophagy and/or exosome production in ALD. Another study also found increased EV levels in a mouse model of AH.<sup>(42)</sup> This study identified mitochondrial DNA in the cargo of MVs that showed a biological effect in mediating neutrophil activation in AH.

Mass spectrometry analysis of serum EVs from a mouse model of ALD revealed specific changes in the EV protein cargo compared to controls.<sup>(43)</sup> Expression

of proteins related to immune cell movement, pattern recognition receptors, interleukin-8 (IL-8) signaling, endoplasmic reticulum stress, unfolded protein response, and hypoxia inducible factor signaling were different between ALD and control EVs. There were also specific proteins exclusively identified in ALD EVs and not in control EVs, indicating a potential disease-specific protein signature.<sup>(43)</sup> Of note, transfer of ALD EVs to naïve healthy mice resulted in recruitment of inflammatory monocytes and neutrophil leukocytes in the recipient livers, with these immune cells showing a proinflammatory phenotype.<sup>(43)</sup> Heat shock protein 90, a major regulator of multiple cellular functions, was identified as uniquely increased in ALD EVs and as a regulator of these biological effects.<sup>(43)</sup> These results indicate that circulating EVs have potential to modulate cell recruitment and function in distant organs. In a biodistribution study using miR-155 as an indicator of biodistribution, intravenously

administered EVs from wild-type mice rapidly (10 minutes) reached many organs, including the liver.<sup>(44)</sup> In miR-155-deficient recipient mice, miR-155 transferred in EVs was found most abundantly in the liver and adipose tissue and to a lesser extent in lung, kidney, and muscle tissue.<sup>(44)</sup> miR-155 transferred by EVs was taken up by specific cells in the liver, such as hepatocytes and macrophages, further regulating biological activities in these cells.<sup>(44)</sup> These results indicate a rapid biodistribution and cellular uptake of miRNAs in recipient mice and tissues *in vivo*.

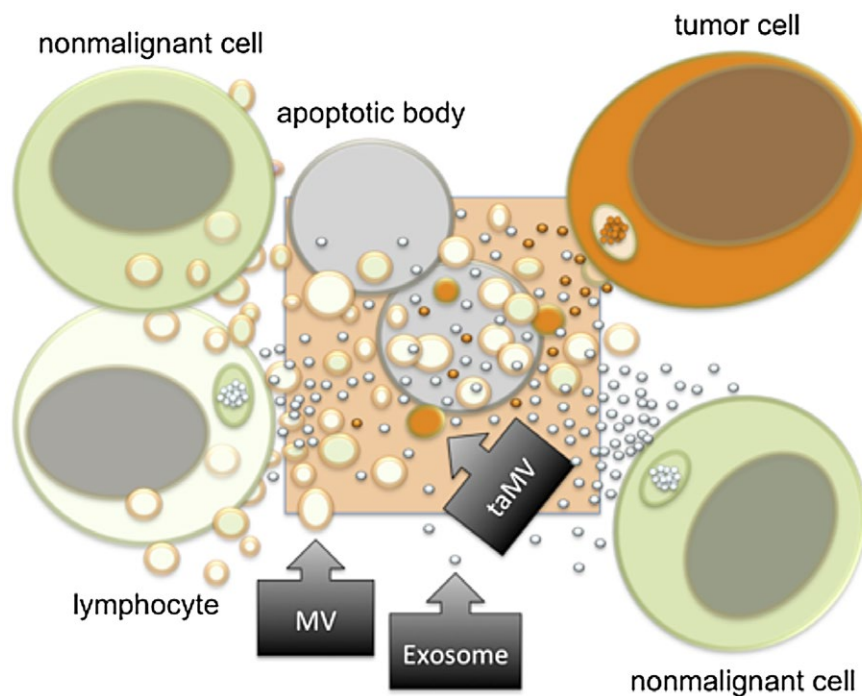
The exposure of hepatocytes, liver macrophages, and Kupffer cells to alcohol was found to increase EV release in all cell types *in vitro*.<sup>(45,46)</sup> Functionally, these EVs produced by different liver cells act as messengers on target cells. This is supported by observations where hepatocyte-derived EVs that were enriched in miR-122 were taken up by monocytes that normally have no or very low levels of miR-122, modifying the phenotype of the recipient monocytes and resulting in lipopolysaccharide (LPS) sensitization and increased LPS-dependent proinflammatory cytokine production compared to monocytes that received control EVs.<sup>(45)</sup> Furthermore, EVs derived from monocytes after alcohol exposure also had functional capacity to modulate naive monocyte function to result in an M2-like repair phenotype.<sup>(46)</sup> Alcohol exposure also increased miR-27a content in monocyte-derived EVs, and their transfer to recipient naive monocytes induced an M2-like phenotype.<sup>(46)</sup> EVs can be loaded with specific miR inhibitors or precursors, being potential therapeutic vehicles for liver diseases.<sup>(45)</sup> Alcohol was also shown to stimulate macrophage activation through hepatocyte-derived release of CD40L-containing EVs in a caspase-dependent manner.<sup>(25)</sup>

## HCC

The correct numeration and phenotypic characterization of EVs can be regarded as tracers for the presence of specific cells, i.e., immunocytes associated with NAFLD<sup>(7)</sup> or malignant tumor cells,<sup>(47)</sup> among others. Therefore, the tumor-associated (ta) EVs, also known as taMVs/taMPs, might help in the detection/diagnosis of cancer. Evidence suggests that EV shedding is not dependent on a metastatic

cancer phenotype.<sup>(48)</sup> Therefore, the use of EVs as an advanced tool for a novel kind of liquid biopsy marker eventually revealing tumor presence, entity, location, and stage/diameter might be possible. However, a main challenge is the quest for specific antigens on EVs that could help in the identification of specific EVs derived from the cancer cells (Fig. 3). A pioneering study identified glypican (GPC)-1 on the surface of pancreatic cancer-derived EVs, which showed 100% sensitivity (SEN) and SPE for the diagnosis of early and late-stage pancreatic cancer compared to healthy subjects and to patients with a benign pancreatic disease.<sup>(49)</sup> However, GPC1<sup>+</sup> EVs were also found elevated in patients with breast cancer, suggesting that this could be a potential pan-cancer biomarker. Moreover, patients with hepatitis C virus presented a differential immune cell-derived MV pattern (i.e., from lymphocytes [CD4<sup>+</sup>, CD8<sup>+</sup>] or natural killer T cells and macrophages/monocytes [CD14<sup>+</sup>]) compared to patients with NAFLD.<sup>(7)</sup> These data suggest that the simultaneous combination of cancer cell-derived antigens in MVs might be useful in the diagnosis of specific cancer entities. This approach resulted first in the use of a pan-cancer marker based on epithelial cell adhesion molecule (EpCAM)<sup>+</sup>CD147<sup>+</sup>MPs/MVs<sup>(48)</sup> that was further refined and validated for the diagnosis of hepatobiliary tumors, such as HCC and CCA.<sup>(47)</sup> In fact, it was confirmed that AnnexinV<sup>+</sup>EpCAM<sup>+</sup>CD147<sup>+</sup> MVs/MPs were elevated in HCC and CCA as in other investigated tumor entities and that asialoglycoprotein receptor 1 (ASGPR1)<sup>+</sup>AnnexinV<sup>+</sup>EpCAM<sup>+</sup>CD133<sup>+</sup> was more specific for hepatic disorders, such as nonmalignant cirrhosis (liver fibrosis F4 stage), HCC, and CCA. This was expected because ASGPR1 is a commonly used hepatocyte marker and therefore added hepatoma specificity to the antigen combination of the MVs. Patients with liver cancer (HCC or CCA), particularly early stage tumors, presented higher serum concentration of AnnexinV<sup>+</sup>EpCAM<sup>+</sup>ASGPR1<sup>+</sup> taMPs compared to patients with nonmalignant hepatic cirrhosis (SEN, 75%; SPE, 47%),<sup>(47)</sup> indicating the potential value of taMPs for the early diagnosis of liver cancer in patients with cirrhosis. However, a main question is whether taEVs bear the potential to be applied as a minimally invasive liquid biopsy marker. In this regard, proteomic analysis of serum EV from patients with HCC revealed





**FIG. 3.** Potential cellular origins of EVs. Lymphocytes and nonmalignant cells eventually secrete exosomes and MVs, whereas malignant tumor cells can also secrete taMVs/taMPs and ta-EXOs). The center box represents the typical EV content usually found in the full blood of patients with cancer, including the few taMVs/taMPs/taEXOs (inferior numbers). Abbreviation: EXO, exosome.

good potential candidates that must be investigated in future studies.<sup>(50)</sup>

## Cholangiopathies

The presence of EVs in bile was described in 2010 as a consequence of the secretory activities from both hepatocytes and cholangiocytes.<sup>(21)</sup> Contrary to the initial idea in which EVs were considered as a release mechanism of waste molecules, EVs in bile regulate the biology of cholangiocytes. Under physiological conditions, bile EVs are able to bind to the primary cilium of cholangiocytes, inhibiting their proliferation and promoting the characteristic quiescent status of the biliary epithelium.<sup>(21)</sup>

EVs are also important in cholangiopathies, regulating different pathologic processes and containing potentially helpful biomarkers for the noninvasive diagnosis of these diseases. The value of bile and serum EVs as a source of biomarkers (e.g., miRs, lncRNAs, or proteins) for cholangiopathies has been investigated. Up-regulation of an miR panel (i.e., 191,

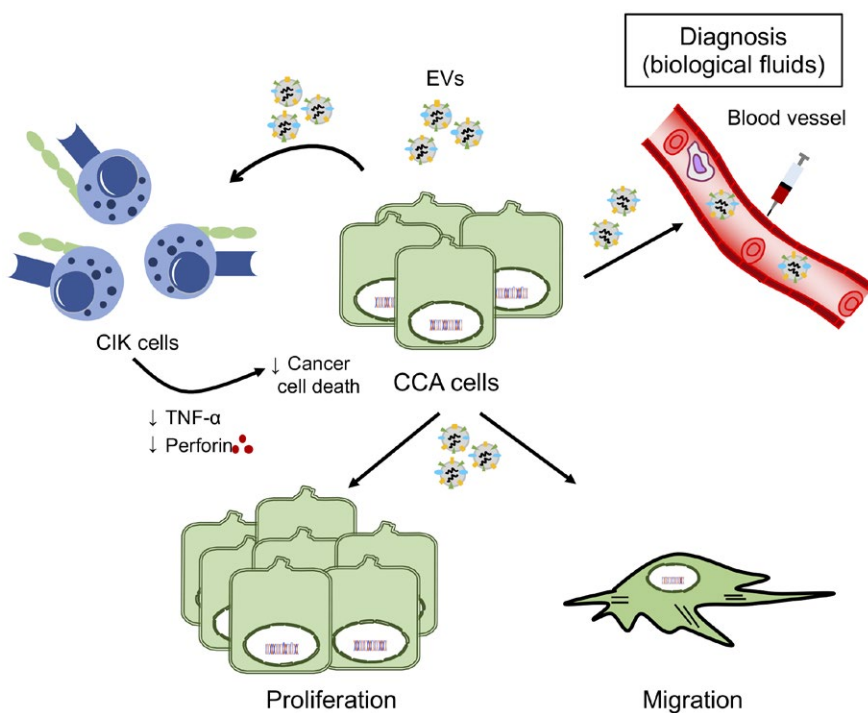
486-3p, 1274b, 16, 484) was described in bile EVs from patients with CCA compared to control individuals (i.e., patients with PSC, a known risk factor for CCA, biliary obstruction, or bile leak syndrome) with diagnostic significance (SEN, 67%; SPE, 96%).<sup>(51)</sup> In addition, two lncRNAs (ENST00000588480.1 and ENST00000517758.1) were reported up-regulated in bile EVs from patients with CCA compared to biliary obstruction conditions (SEN, 83%; SPE, 59%; area under the receiver operating characteristic curve [AUROC], 0.709).<sup>(52)</sup>

Different protein profiles were found in serum EVs from patients with CCA, PSC, HCC, or healthy individuals (control group).<sup>(50)</sup> Patients with HCC presented higher EV concentrations compared to the other three groups in the study. Single protein analysis provided several proteins with diagnostic value and maximum AUROC values of 0.878 for aminopeptidase N (AMPN; SEN, 91%; SPE, 66%) in CCA versus control and 0.905 for polymeric immunoglobulin receptor (PIGR; SEN, 75%; SPE, 95%) in CCA stage I-II versus control. These two AUROC values are similar to the results obtained for the nonspecific

tumor marker carbohydrate antigen 19-9 (CA19.9), commonly used to help in the diagnosis of CCA (AUROC, 0.907 and 0.916, respectively). Notably, the combination of six biomarkers (AMPN, VNN1, PIGR, C-reactive protein, gamma-glutamyltransferase 1 [GGT1], and fibrogenin [FIBG]) resulted in an AUROC value of 0.991 (SEN, 98%; SPE, 97%) in CCA versus control. Likewise, AMPN provided a maximum area under the curve value of 0.789 for the diagnosis of PSC versus control (SEN, 83%; SPE, 63%), and its combination with six additional biomarkers (ficolin [FCN]-1, nuclear export protein, PIGR, VNN1, GPC5C, immunoglobulin kappa variable 3-01) increased this value to 0.989 (SEN, 93%; SPE, 100%). A maximum AUROC value of 0.796 for FIBG was reported for CCA versus PSC, and the combination of seven biomarkers (alpha-1-acid glycoprotein 1, S100A8, A10A9, small archaeal modifier proteins, GGT1, FCN2, immunoglobulin heavy constant alpha 1) increased this value to 0.956 (SEN, 88%; SPE, 93%) compared to 0.819 of CA19.9. Moreover, the stratification of patients with CCA resulted in a maximum AUROC value of 0.956 for FCN2 in CCA

stage I-II versus patients with PSC compared to the 0.736 value of CA19.9. When comparing HCC versus control, galectin-3-binding protein provided a maximum AUROC value of 0.904 (SEN, 97%; SPE, 72%) compared to 0.802 for alpha-fetoprotein (AFP; commonly used to help in the diagnosis of HCC). Furthermore, FIBG provided a maximum AUROC value of 0.894 (SEN, 83%; SPE, 90%) for the diagnosis of intrahepatic CCA versus HCC compared to CA19.9 (0.801) and AFP (0.753) values. Remarkably, although these biomarkers are present in EVs, they can be equally detected by immunoblot using total serum, which could be helpful for future validation studies and potential translation of the results into a clinical setting.<sup>(50)</sup>

EVs in serum can be secreted by multiples cells, including cancer cells. An orthotopic mouse model of human CCA showed the presence of specific human proteins in serum EVs of these mice, pointing out the role of tumor-derived EVs for biomarker discovery and as potential regulators of disease progression (i.e., metastasis, immune regulation).<sup>(50)</sup> Furthermore, total EV concentration could also help in the diagnosis of



**FIG. 4.** Cholangiocarcinoma-derived EVs regulate tumor growth and the microenvironment and are a source of biomarkers in biological fluids. Abbreviations: CIK, cytokine-induced killer; TNF, tumor necrosis factor.

malignant common bile duct stenosis (i.e., pancreatic cancer and CCA) compared to nonmalignant conditions (i.e., chronic pancreatitis or biliary stones) as a higher concentration of EVs was reported in bile (AUROC, 1.000) and serum (AUROC, 0.813) of the malignant group.<sup>(53)</sup>

EVs may also regulate the pathogenesis of biliary diseases (Fig. 4). EVs secreted by human CCA cells contain oncogenic proteins (i.e., epidermal growth factor receptor, integrin- $\beta$ 4, agrin, disintegrin, and metalloproteinase domain-containing protein 10, among others) involved in the promotion of cancer cell proliferation, survival, and migration.<sup>(50,54,55)</sup> These cancer EVs are able to regulate the tumor microenvironment. CCA-derived EVs promote the differentiation of mesenchymal stem cells into fibroblasts and the secretion of IL-6, which ultimately stimulates CCA proliferation.<sup>(56)</sup> CCA-derived EVs are also able to inhibit the antitumor capacity of cytokine-induced killer cells by down-regulating the secretion of tumor necrosis factor alpha and perforin.<sup>(57)</sup> On the other hand, the serum concentration of the cholangiocyte-derived exosomal lncRNA H19 increases under certain cholestatic conditions, further promoting hepatocellular injury and fibrosis.<sup>(58)</sup>

## Conclusions and Future Directions

Increasing evidence points to the significant role of EVs in liver pathobiology, participating in intercellular and interorgan communications, and emerging as a new opportunity for biomarker discovery and therapy. It is now evident that EVs participate in the development and progression of certain liver diseases, including NAFLD/NASH, ALD/AH, HCC, CCA, and other cholangiopathies, which are being postulated as potential targets for therapy. However, EVs also participate in the pathogenesis of other types of liver diseases/conditions not discussed in the EV Symposium of the International Liver Conference 2018 (e.g., drug-induced liver injury, viral hepatitis, and liver fibrosis, among others). Several inhibitors of EV biogenesis, release, and/or uptake are available, but their safety, efficacy, and selectivity need to be further evaluated at the preclinical and clinical level. The different concentration and/or composition of

EVs in certain liver diseases provide a unique opportunity for biomarker discovery. Moreover, all studies involving EVs require their adequate experimental characterization<sup>(59)</sup> as indicated by the International Society of Extracellular Vesicles. In sum, EVs represent a new and promising field of research in liver diseases that deserves future investigation.

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