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Extracellular vesicles in hepatology: Physiological role, involvement in pathogenesis, and therapeutic opportunities

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ABSTRACT

Since the first descriptions of hepatocyte-released exosome-like vesicles in 2008, the number of publications describing Extracellular Vesicles (EVs) released by liver cells in the context of hepatic physiology and pathology has grown exponentially. This growing interest highlights both the importance that cell-to-cell communication has in the organization of multicellular organisms from a physiological point of view, as well as the opportunity that these circulating organelles offer in diagnostics and therapeutics. In the present review, we summarize systematically and comprehensively the myriad of works that appeared in the last decade and lighted the discussion about the best opportunities for using EVs in liver disease therapeutics.

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1. Introduction

The liver is the largest internal organ of the human body, and it is both functionally and structurally complex. The different types of cells that form this organ can be categorized into two groups. From one side, the parenchymal cells or hepatocytes, which are in charge of the central metabolic functions attributed to the liver; on the other hand, the non-parenchymal cells comprise a diverse group of cells from different origins that support to the parenchyma in different ways. They include liver sinusoidal endothelial cells (LSECs), cholangiocytes, hepatic stellate cells (HSCs), and liver-specific macrophages, known as Kupffer

Abbreviations: α-SMA, Alpha smooth muscle actin; ACR, Acute cellular rejection; APAP, Acetaminophen; CD40L, The cluster of differentiation 40 ligand; CTGF, Connective tissue growth factor; CYP, Cytochromes; DILI, Drug-Induced Liver Injury; EMT, Epithelial-mesenchymal transition; EVs, Extracellular Vesicles; hBM-MSC, Human bone marrow-MSC; HCC, Hepatocellular carcinoma; HSCs, Hepatic stellate cells; qHSCs, Quiescent hepatic stellate cells; aHSCs, Activated hepatic stellate cells; LSECs, Liver sinusoidal endothelial cells; MIF, Migration inhibitor factor; MSCs, Mesenchymal stem cells; NAFLD, Non-alcoholic fatty liver disease; NASH, Non-alcoholic steatohepatitis; NK, Natural killer; NVs, Nanovesicles; S1P, Sphingosine-1-phosphate.

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cells. Hepatocytes occupy as much as 80% of the total liver volume, while non-parenchymal cells, in turn, represent 6.5% of the liver volume. However, this finally corresponds to 40% of whole liver cells, making evident their importance in liver homeostasis. In fact, these cells are responsible for maintaining the physiological conditions in the hepatic microenvironment by secreting factors that influence and regulate other hepatic and immune-related cells (Blouin, Bolender, & Weibel, 1977). In such complex organ, cell-to-cell communication has been proved to be critical in both physiological and pathological events, and the cell cross-talking network system is responsible for maintaining the organ "machinery" working together (Marrone, Shah, & Gracia-Sancho, 2016).

In addition to the well-known communication system based on soluble factors secreted to the extracellular *milieu*, the signaling carried by different types of extracellular vesicles (EVs) is involved in cell-to-cell signaling (Fig. 1). EVs are cell-derived membrane structures that allow the packaging of a wide variety of molecules for their transportation to targeted cells (Vallabhaneni et al., 2015). The cargo content of these vesicles determines their function. It depends on the cell type of origin as well as its biological status, although it is also influenced by factors involving the secretory cells.

1.1. Hepatocytes

Hepatocytes are the parenchymal cells of the liver, comprise most of the organ volume, and organize in concrete structures known as hepatic lobules, with fixed distribution respect to arterial, venous and biliary vessels (Blouin et al., 1977). As many differentiated epithelial cells, hepatocytes are polarized cells implicating the formation of specific membrane domains and individual cytoskeletal and endoplasmic reticulum networks (Decaens, Durand, Grosse, & Cassio, 2008). Hepatocytes are involved in the synthesis of proteins, cholesterol, bile, phospholipids, and glycoproteins. Besides, they play a crucial role in detoxification processes and in the storage and mobilization of energy through glucose and lipid metabolism. Interestingly, hepatocytes have distinct differential metabolic gene expression and functionality along the hepatic lobules that acquire a zonal specialization (Trefts, Gannon, & Wasserman, 2017; van Liempd, Cabrera, Mato, & Falcon-Perez, 2013).

Due to the complexity of the organ, and the degree of specialization of hepatocytes, it is not surprising that finding appropriate cellular models for *in vitro* studies is extremely difficult. Nowadays, there are three strategies available; the use of primary cultures, the use of cell lines, and the generation of differentiated hepatocytes from pluripotent cells. In the case of primary culture, not only exist an explicit limitation to obtain human hepatocytes, but also freshly isolated hepatocytes from adult livers rapidly lose their function in culture (Miyajima, Tanaka, & Itoh, 2014) although, significant improvements have been made recently through 3D cell culture models (Hu et al., 2018; Huch et al., 2013; Ortega-Ribera et al., 2018). In the case of cell differentiation, two major schemes have been described; from one side obtain hepatocytes by inducing differentiation of stem cells, either from the hepatic or not hepatic origin, and from another side the use of pluripotent cells, either embryonic or chemically induced (Zeilinger, Freyer, Damm, Seehofer, & Knospel, 2016).

Given the difficulty of producing a large amount of EVs from a limited number of cells, it is not surprising that the use of established cell lines is an essential alternative for characterization studies. Tumoral cell lines such as Huh7 or HepG2 were isolated from tumors. They

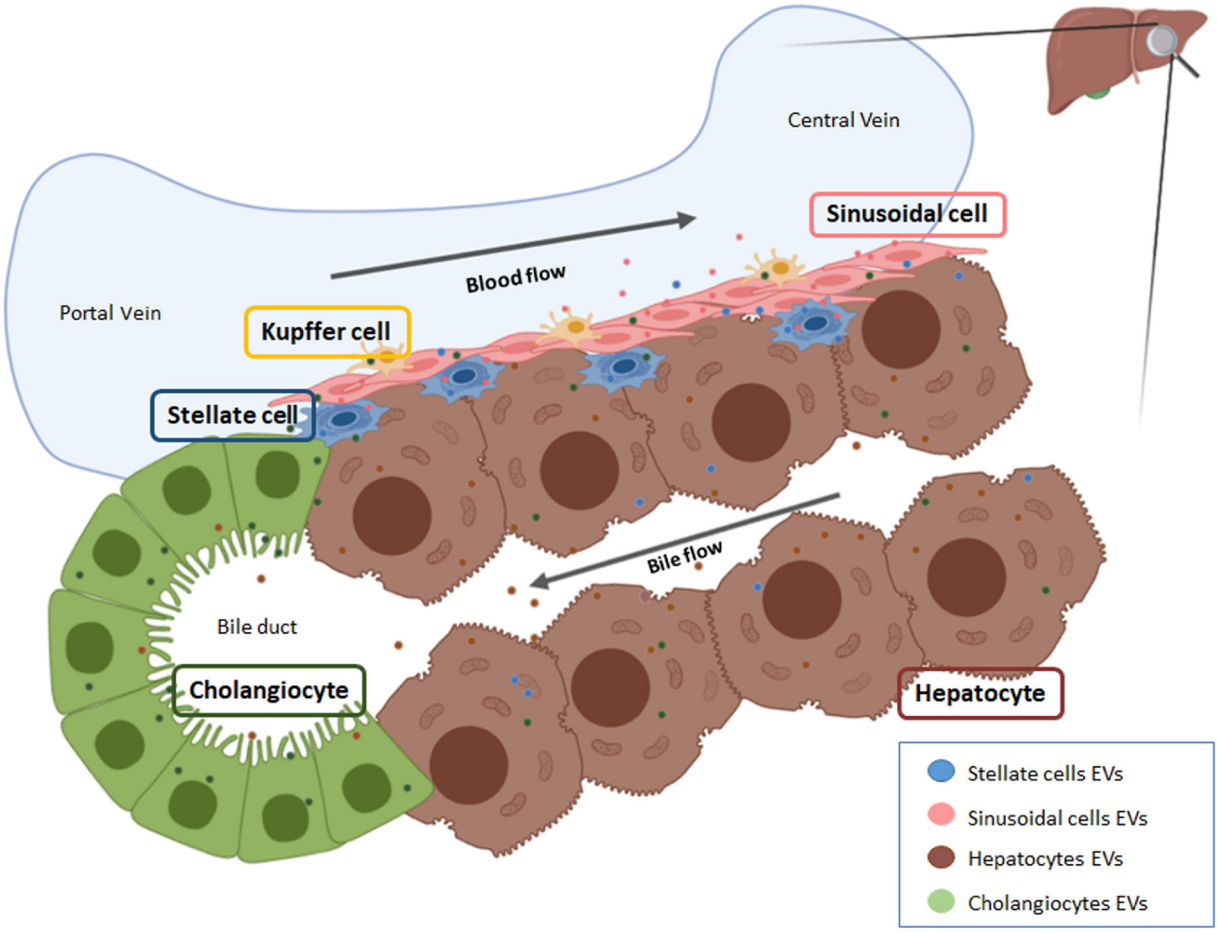

Fig. 1. Liver architecture and EVs interactions between different liver cell types.

maintain few features of the mature hepatocytes, such as the capacity of being infected by hepatitis virus C in the case of Huh7 (Lohmann et al., 1999) or the secretion of some plasmatic proteins (Sormunen, Eskelinen, & Lehto, 1993). The use of hepatic progenitor cell lines, such as MLP29, has showed important differences between their EVs and those ones released by mature primary hepatocytes (Royo et al., 2013; Royo et al., 2019). As a consequence, most characterization studies relay on EVs obtained from primary culture (Conde-Vancells et al., 2008; Herrera et al., 2010; Jiang et al., 2020; Zhang et al., 2019). An interesting alternative is the use of the HepaRG cell line, a cell line from human origin that behaves almost as a fully-functional human hepatocyte (Gripon et al., 2002), and for that reason it has been a model for extracellular studies related to drug induce liver damage (DILI) (Duan et al., 2019; Mosedale et al., 2018).

Taking advantage of those cellular models, the study of hepatocyte-derived EVs have evolved and becoming an important part of the hepatology field. In 2008, our group made the characterization of hepatocyte-derived exosomes-like vesicles, and also described their proteome that not only contain proteins previously associated with vesicles, but also proteins specifics from hepatocyte machinery, reassuring the hypothesis that EVs content reflect the characteristics of the parental cell (Conde-Vancells et al., 2008). Some years later, we also described the transcriptome of hepatocyte-derived EVs (Royo et al., 2013), observing specific mRNA from liver, which is in agreement with the observed in other cells, like cardiomyocyte-derived EVs, that contained transcripts that encode proteins involved in mitochondrial energy generation (Waldenstrom, Genneback, Hellman, & Ronquist, 2012).

The cargo of EVs also includes signaling activators. In 2016, Nojima et al. studied the role of exosomes in liver physiology and they showed that after ischemia/reperfusion injury or partial hepatectomy, hepatocytes released exosomes carry the synthetic machinery to form the signaling lipid sphingosine-1-phosphate (S1P) in target hepatocytes (Nojima et al., 2016). Specifically, exosomes delivered sphingosine kinase 2 (SK2) promoting hepatocytes survival, growth and migration. In addition, S1P alone could promote hepatocyte proliferation in culture.

It has been demonstrated that microvesicles (MVs) have the capability of targeting other cells and transferring repair toolkits (Tetta, Bruno, Fonsato, Deregibus, & Camussi, 2011). EVs activated the proliferation of remnant hepatocytes after hepatectomy transferring specific mRNA subsets through MVs derived from human liver stem cells (Herrera et al., 2010). Currently, the knowledge of the cargo of EVs and the way that this cargo can act in the receptor cells support the creation of a cell bank establishment for allogenic transplantation, knowing that the fetal liver mesenchymal stem cells and their secreted factors could be an alternative to hepatocyte transplantation in liver cell-based therapies (Chinnici et al., 2019).

Apart from proteins and RNA, EVs could also carry active enzymes, which can induce changes in the extracellular environment and in the recipient cells. Enzymes carried by hepatocyte-released EVs are metabolically active and can affect the number of serum metabolites involved in oxidative stress metabolism and the endothelial functions (Royo et al., 2017). Over the last few years many enzymes have been identified as biomarkers of different liver diseases (reviewed in (Azparren-Angulo, Royo, & Falcón-Pérez, 2019)). A clear example is the presence of blood-circulating EVs carrying active cytochromes after liver injury, a phenomenon that could be relevant in extracellular metabolism of drugs (Palomo et al., 2018). Other enzyme described in EVs is ApoE (Conde-Vancells et al., 2008), an apolipoprotein involved in the metabolism of atherogenic lipoproteins, that has been seen to interact with selenoprotein P, also present in hepatocyte-derived EVs, to regulate their secretion to the extracellular media, and to play a role in the protection of neuronal cells from amyloid β (Aβ)-induced cell death (Jin et al., 2020).

As mentioned before, EVs cargo could modify the recipient cells, generating different responses in them. Hepatocytes-derived EVs have been implicated in the proliferation of cholangiocytes through interaction with primary cilia, working as signaling and influencing intracellular regulatory mechanism (Masyuk et al., 2010).

1.2. Liver sinusoidal endothelial cells

Liver sinusoidal endothelial cells (LSECs) comprise a small fraction of the cellular component in the liver, approximately 20% of it (but 50% of non-parenchymal fraction) (Sorensen, Simon-Santamaria, McCuskey, & Smedsrod, 2015). Their morphology and function make them unique and different from other endothelial cells (Aird, 2007), as they show fenestrae and a lack of basement membrane. These characteristics added to their high receptor-mediated endocytic activity compared to other endothelial cells, facilitate their role in the elimination of macromolecules and aggregates from the blood and in immune responses (*i.e.* acting as phagocytic cells) (Sorensen et al., 2015). Moreover, LSECs maintain hepatic stellate cells in quiescent state (Deleve, Wang, & Guo, 2008), inhibiting the intrahepatic vasoconstriction and fibrosis development, inherent to a great number of hepatic diseases (Gracia-Sancho, Marrone, & Fernandez-Iglesias, 2019).

Like hepatocytes, LSECs dedifferentiate in culture, maintaining only fenestration during two days after culture (DeLeve, Wang, Hu, McCuskey, & McCuskey, 2004) and their endocytic activity does also quickly decline (Elvevold, Smedsrod, & Martinez, 2008). In fact, this limits the use of primary cultures, although studies are still published with LSECs maintained in long-term culture. However, these cells lack most of their features and only some of them partly maintain fenestrae, as most of the available LSEC cell lines. Only SK Hep1 cell line has showed to uptake the LSECs specific ligand FITC-FSA in functional assays (Cogger et al., 2008). TRP3 cell line keeps some of the LSEC origin characteristics, but also show differences with primary cells, as low level of fenestration (Parent et al., 2014). TMNK-1 is another liver endothelial cell line available that shares most features with primary hLSECs (Giugliano et al., 2015). In spite that they do not show fenestrae *in vitro*, they can recover them when engrafted *in vivo* (Filali, Hiralall, van Veen, Stolz, & Seppen, 2013). Novel methods to maintain LSECs phenotype *in vitro* are being developed, and include the use of liver-on-a-chip technology and the generation of LSECs-like cells from pluripotent stem cells (Gage et al., 2020).

Because of their anatomical proximity with hepatic stellate cells (HSC), LSEC-derived exosomes can influence them by paracrine regulation. As happened with hepatocyte-derived EVs, LSEC-derived EVs also carry active enzymes. Using the LSEC cell line TSEC (Huebert et al., 2010), Wang and colleagues demonstrated that endothelial cells release exosomes containing sphingosine kinase 1 (SK1), which also induces the formation of S1P in recipient cells and thus activates quiescent HSCs to myofibroblasts and promotes their migration (Wang et al., 2015). Fibroblastic growth factor 2 (FGF2), which is related to both angiogenesis and fibrosis, enhances both SK1 mRNA and protein levels in TSECs and in their exosomes. The uptake of TSEC-derived EVs by HSCs recipient cells has been shown to be mediated through an exosomal fibronectin-dependent initial interaction with cellular integrins and a dynamin-dependent endocytosis. Once in the cell, the EV cargo promotes AKT activation through its phosphorylation, what lead to its subsequent signaling pathways and HSC activation (R. Wang et al., 2015). Future desirable works will hopefully validate these findings and further expand the knowledge about primary LSECs derived EVs.

1.3. Cholangiocytes

The intrahepatic and extrahepatic bile ducts are lined by cholangiocytes. They comprise a minority cell population in the liver (approximately 5%) and form a complex network extending from the Canals of Hering in the liver to the duodenum, where the bile is spilled (Alpini, McGill, & Larusso, 2002). Like other epithelial cells, they are polarized leading to different plasma membrane domains and transport

functions, many involved in bile formation. These cells are critical for bile generation, a secretory fluid containing factors such as bile acids, lipids, proteins, electrolytes and endobiotic and xenobiotic compounds that aid digestion, maintain the enterohepatic circulation and help in the elimination of compounds from the body.

Cholangiocytes acquire a greater degree of differentiation along the biliary ducts (Han et al., 2013), being immature within the Canals of Hering and much more specialized and bigger in its final steps before arriving at the duodenum. Their morphological, biochemical and functional heterogeneity along the bile tract makes difficult to establish cellular models for *in vitro* studies. Regarding *in vitro* models, two are available nowadays, the differentiation into cholangiocytes from induced pluripotent stem cells (iPSCs) (Cervantes-Alvarez et al., 2017; Sampaziotis et al., 2017), and the generation of 3D organoids (Sampaziotis et al., 2017; Vyas et al., 2018). In this latter case, distinct differentiated cholangiocytes can be obtained, being representative of their physiological situation. Even though these are the mostly used models recently, there are also some immortalized cell lines at investigators disposal, as for example H69 (although immortalized, it is not malignant and does not produce tumors *in vivo* in experiments carried out in mice) (Dutta et al., 2015).

Cholangiocytes are normally quiescent cells in a healthy state, but after the accumulation of bile acids in the damaged liver, they activate the inflammatory response. In this scenario, damaged cholangiocytes release EVs containing long non-coding RNA H19 that does disrupt bile acid homeostasis in hepatocytes. Thus, the uptake of this lncRNA suppresseses *small heterodimer partner* (*SHP*) expression in hepatocytes, a nuclear receptor and transcriptional regulator involved in the regulation of bile acid, glucose and lipid metabolism, as well as in suppressing inflammation and fibrosis. The suppression of *SHP* expression has been shown to be gradual during liver diseases progression. Finally, HSCs become also activated because of the changes in the cargo of hepatocytes exosomes (Li et al., 2018). Moreover, H19-containing cholangiocyte-derived EVs can also be taken up by Kupffer cells, promoting their activation and phagocytosis activity (Li et al., 2020). H19 RNA has also been detected in cholangiocyte-derived EVs in models of Biliary Atresia, promoting autocrine proliferation by upregulating the S1P receptor 2 (S1PR2) - SK2 axis, mainly through the activation and phosphorylation of ERK1/2 (Xiao et al., 2019).

Biliary EVs, enriched in small vesicles (exosome-like) originated in both hepatocytes and cholangiocytes, act even on the proper cholangiocytes. microRNA-15A, known for its implication in cholangiocyte proliferation, is upregulated after the interaction of biliary exosomes with these type of cells in a ciliary-dependent way, as a result of a decrease of ERK1/2 phosphorylation ratio. This led to a decrease in cholangiocyte proliferation that does not happen after removal of cell cilia (Masyuk et al., 2010).

In situations of wounding and reparation of injured tissue, platelet derived growth factor (PDGF)-BB has been seen as one of the key regulators. This factor induces angiogenesis and vasculogenesis through a process where Hedgehog (Hh) family morphogens are known to be involved. Some studies have evidenced that Hh ligands (*e.g.* Shh, Ihh) are more concentrated in cholangiocyte-derived exosomes and microvesicles after their activation with PDGF-BB, and they regulate LSEC gene expression and activate them (Witek et al., 2009).

1.4. Hepatic stellate cells

Hepatic stellate cells (HSCs) are resident mesenchymal cells that represent one-third of non-parenchymal cells and 10–15% of total resident cells in the liver. These cells are found in the subendothelial space of Disse, a virtual space between the basolateral membrane of hepatocytes and the anti-luminal side of LSECs. This space is filled with permeable connective tissue that allows the exchange of biomolecules between hepatocytes and portal blood flow. HSCs are normally quiescent (qHSCs), but several stimuli can activate them. While being in a quiescent state, they act as the major reservoir of retinoids (vitamin A) (Friedman, 2008) and when activated (aHSCs), cells transdifferentiation results in proliferative, migratory and contractile myofibroblasts with profibrotic properties [enhanced expression of alpha smooth muscle actin (α-SMA) and fibrillar collagens. In fact, qHSCs express adipocyte markers (PPAR-γ, SREBP-1c, and leptin), whereas aHSCs express myogenic markers (α-SMA, c-myb, and myocyte enhancer factor-2) (Bataller & Brenner, 2005). The upregulation of the proteins resultants of these genes gives place to the different properties comparing quiescent and activated states. Several events can activate this process (Wallace, Friedman, & Mann, 2015), that leads to a complex dysregulation of molecular pathways that perpetuates their activation, thus worsening the conditions in their microenvironment.

Primary cultures from human and rodent HSCs are widely more used to study their physiology and pathology. They can be isolated by discontinuous gradient centrifugation and transdifferentiate into myofibroblast spontaneously when grown on hard plastic (Fernandez-Iglesias, Ortega-Ribera, Guixe-Muntet, & Gracia-Sancho, 2019; Higashi, Friedman, & Hoshida, 2017). Co-culture with other hepatic cells from HSCs microenvironment (*e.g.* Kupffer cells) can restore physiological processes and dysregulation mechanisms that are lost in *in vitro* mono-cultures (De Minicis et al., 2007). Apart from that, there are immortalized HSC cell lines available originated from human (LX-1, LX-2 and TWNT-4) (Shibata et al., 2003; Xu et al., 2005), mouse (JS-1, GRX and CoI-GFP) (Borojevic et al., 1985; Meurer et al., 2013) and rat (HSC-T6 and CFSC) (Greenwel et al., 1991; Y. Kim et al., 1998). They are mostly used for *in vitro* studies and therapeutic assays.

EVs-HSCs cargo highly depend on their state. For example, qHSCs release anti-fibrotic exosomes. These exosomes contain miR-214 and miR-199a-5p in higher levels when compared to those secreted by aHSCs. When being captured by aHSCs, it leads to suppression of connective tissue growth factor (CTGF) expression and, in turn, of its downstream effectors α-SMA and collagens (Chen, Chen, Velazquez, & Brigstock, 2016; Li, Chen, Kemper, & Brigstock, 2020). Exosomes containing miR-214 and miR-199-5p can also be taken up by hepatocytes, also promoting downregulation of CTFG (Handy, Castro, & Loscalzo, 2011). On the other hand, the conversion of qHSCs into aHSCs changes the cargo and properties of HSC-derived exosomes. Activated HSCs secrete exosomes containing high levels of CTGF mRNA and its protein, and these can be captured by both quiescent and activated HSCs (Charrier et al., 2014). Hence, a positive feedback loop is caused by stimulating qHSC activation and maintaining aHSCs properties.

As happens with cholangiocytes, HSCs can also be activated by PDGF-BB, transcriptionally upregulating Shh and Ihh, increasing protein concentration in their exosomes and microvesicles (Witek et al., 2009). This leads to the same consequences; the activation of LSECs to promote angiogenesis and vasculogenesis.

1.5. Kupffer cells

Kupffer cells are the resident macrophages in the liver, originated from peripheral circulating monocytes that entered the liver and matured into their characteristic phenotype (Dixon, Barnes, Tang, Pritchard, & Nagy, 2013). However, they do not only originate from hematopoietic stem cells, but also they have self-renewal capacity (Yona et al., 2013). Their proximity to both parenchymal and non-parenchymal liver cells leads to their multiple interactions with diverse cell types, both in health and pathological conditions.

These cells are involved in the innate immune response, as they are critical in the tolerance for antigens coming from the gut, maintaining an anti-inflammatory state. They present different pattern recognition receptors (PRRs) that recognize multiple pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). This fact gives more evidence of their role in innate immunity (Dixon et al., 2013). However, this signaling seems to be involved in the progression of liver injury. In some disease situations, Kupffer cells can

become pathologically activated, a characteristic feature of chronic inflammatory diseases.

As there is a lack of cell lines that can be used as a model for the study of Kupffer cells, several studies have been carried out by isolating primary cells, despite their number limitation. THP1 is the most commonly used macrophage-like cell line, although it still keeps features from monocytes and does not show the expected levels of some known markers for Kupffer cells. A recent study developed an immortalized KC (iKC) line through the transformation of primary Kupffer cells with papillomavirus (HPV) E6 and E7 protein-coding genes (Faure-Dupuy et al., 2018). This line did not show the physiological properties of Kupffer cells, but their composition and metabolism were more similar to them than is THP1 cell line.

Unlike the other liver cell types, Kupffer-derived EVs have not been described yet, although it is likely that they had the ability as different types of macrophages (Cypryk, Ohman, Eskelinen, Matikainen, & Nyman, 2014). Indeed, there is evidence of their capacity to capture and internalize EVs secreted from other cell types, promoting a cellular response. This process has been described both in physiology and pathology by means of which they change their gene expression pattern to respond to microenvironmental demands (Nguyen-Lefebvre & Horuzsko, 2015).

1.6. Differences in the EVs cargo in a functional context

Above we have introduced the characterization of EVs for each hepatic cell type, and in the next section we would focus on the implication of EVs in different diseases. However, it is important to highlight the fact that the EVs cargo it is determined by the parental cell, making the messages specific for each cell type, and therefore it is possible to distinguish the origin of each EVs. When first characterized, EVs derived from rat hepatocytes showed the presence of numerous proteins that has not been described before associated to vesicles, and include specific metabolic machinery of the hepatocyte (i.e Aspgr or ApoE) and the pathway enrichment analysis of the proteomic contents showed oxreductase activity and lipid metabolism as main enriched pathways (Conde-Vancells et al., 2008). When compared with mouse liver progenitor cells, it become clear that each EV had their own characteristic cargo, since in the progenitor cell cannot be found transcripts specific for the mature hepatocyte, but coding genes related to cell cycle control instead (Royo et al., 2013). To identify circulating EVs derived from hepatocytes after liver damage, most common targets studied have been specific transcripts such Alb or Cyp2d1 (Royo et al., 2013), miRNA-122 (Povero et al., 2014), the presence of cytochrome proteins such Cyp2d1 (Palomo et al., 2018), or the activity of EV associated enzymes such the arginase Arg1 (Royo, Moreno, et al., 2017). However, when the presence of EVs is caused by the alteration of cholagyocytes, different subset of markers has been demonstrated in circulating EVs. Indeed, analysing circulating EVs with proteins derived by cholangiocarcinoma reveal proteins with diagnostic interest such AMPN, VNN1, and PIGR (Arbelaiz et al., 2017). Similarly, in the blood circulating EVs of primary sclerosing cholangitis patients, it is possible to detect AMPN, FCN1, and neprilysin (Arbelaiz et al., 2017). Therefore, it is possible to stablish the origin of circulating EVs by their cargo in different pathologies.

Although less studied, the rest of cellular types of the liver release EVs that contain a particular cargo associated with the functional context of the parental cell. Regarding Kupffer cells, some of the observations coming from cellular models indicates that TLR3-activated macrophages release EVs containing antiviral miRNAs (*i.e.* miRNA-29), and alcohol treated monocytes release EVs loaded with miRNA-27a, that stimulate naive monocytes to polarize into M2 macrophages (Saha, Momen-Heravi, Kodys, & Szabo, 2016). Better characterized through protemics analysis, LSEC derived EVs contain a proteome profile associated with extracellular spaces or matrix, proteasome, collagens, vesicular transport, metabolic enzymes, ribosomes and chaperones. More interesting, the study of the cargo of activated LSEC *vs* non activated reveal that some of the processes occurring on the cells had a reflect on EVs composition that become able to promote fibrogenic gene expression in the HSC acceptor cells (Li, Chen, et al., 2020). In addition, sphingosine-overexpressing endothelial cells load EVs with this enzyme regulate HSC signaling and migration (Wang et al., 2015). These findings advance the understanding of EC/HSC cross-talk and identify exosomes as a potential target to attenuate pathobiology signals. Finally, the cargo of HSC-derived contain also proteins and specific proteins related to the functional role of this type of cells. For instance, CCN2 is packaged into secreted vesicles that mediate its intercellular transfer between HSC (Charrier et al., 2014). In parallel, HSC derived EVs transfer miR-214 to neighbouring HSC or hepatocytes, which inhibits CCN2 activity (Chen et al., 2014). These studies highlight that EVs from HSC represents an important mechanism by which fibrogenic signaling is controlled and play a role in the pathogenesis of hepatic fibrosis. As a conclusion, the presented studies reassure the notion of EVs as a specific messenger involved in the cellular crosstalk that occurs in the liver. More comprehensive review of the cargo content for each cell type can be found in (Zivko, Fuhrmann, & Luciani, 2020).

2. EVs as diagnostic tools in liver pathology

In the last decade, it has been observed that hepatocyte-derived EVs play an essential role in the diagnosis of different diseases (reviewed in (Sato, Meng, Glaser, & Alpini, 2016)). The causes of stress/damage to the hepatocytes due to disease led to increased release of EVs contributing to inflammation, fibrogenesis, and angiogenesis (Hernandez et al., 2020). For that reason, it is not surprising that EVs are emerging as key players in the pathogenesis of different liver diseases. In Table 1, we have summarized the different molecules described as EV-cargo associated to liver diseases, mentioned along this review. In addition, we would like to recommend to the reader other reviews more focused on miRNAs associated to cholagiopathies (Olaizola et al., 2018), hepatocellular carcinoma (Li, Yao, Xie, Liu, & Zheng, 2018) and other liver diseases (Barrera-Saldana, Fernandez-Garza, & Barrera-Barrera, 2020; Yang, Li, Zhang, & Wang, 2018), as well as related to the enzymatic activity of liver derived EVs in the context of liver pathology (Azparren-Angulo et al., 2019).

2.1. Drug-induced liver injury (DILI)

The liver has a central role in drug metabolism and is a crucial organ for most detoxification processes, being prone to xenobiotic-induced injury (Sturgill & Lambert, 1997). Drug-induced liver injury (DILI) is a potentially fatal adverse event with significant medical and economic impact. DILI accounts for more than 50% of acute liver failure, being acute hepatitis the predominant form. Liver enzymes mediate the bioactivation of drugs to chemically reactive metabolites; these metabolites could cause damage that can end up in cell death and possible liver failure (Palomo et al., 2018).

In the last years, proteomics has been used to identify a possible source of biomarkers for different biological processes and diseases, including hepatotoxicity (Rodriguez-Suarez et al., 2014). Furthermore, it has been shown that these markers could appear in EVs and being metabolically active to alter the extracellular environment (Royo et al., 2017). As a result of the metabolic activity of the molecules transported by EVs, it has also been shown that several serum metabolites involved in oxidative stress metabolism and the endothelial function were affected (Royo, Moreno, et al., 2017).

Regarding drug metabolism, cytochromes (CYP) are vital molecules. These enzymes metabolize the majority of xenobiotics, and in the last years, different members of this family have been associated with hepatotoxicity (Gerth et al., 2019; Kumar et al., 2017). The enzymatic activity of P450 cytochrome 2E1 and 2D1 (CYP2E1 and CYP2D1) is present in circulating EVs isolated from *in vivo* models of drug toxicity. Apart from DILI, CYP2E1, this cytochrome was also detected elevated in EVs after

Table 1
Involvement of EVs in major liver pathologies.

Disease	EV source	Type	Cargo	Target (cells)	Effect	Reference
NAFLD/NASH	Hepatocytes	NON CODING RNA	miR-192	Hepatocytes	SREBP1 and stearoyl-CoA desaturase 1 overexpression	(Lin et al., 2017)
	Hepatocytes		miR-192	Macrophages	Macrophage activation	(Liu et al., 2019)
	Hepatocytes		Panel of miRNAs	Adipocytes	Adipose tissue remodeling	(Mikolasevic et al., 2016; Zhao et al., 2020)
	Adipocytes	GENE/PROTEIN	TGF-β pathway mediators	Hepatocytes HSCs	TGF-β-related pathways dysregulation	(Koeck et al., 2014)
	Hepatocytes		TRAIL	Macrophages	M1 polarization	(Hirsova, Ibrahim, Krishnan, et al., 2016)
	Hepatocytes		MLK3	Macrophages	Chemotaxis	(Hirsova, Ibrahim, Krishnan, et al., 2016)
	Hepatocytes		VNN1	LSECs	Angiogenesis	(Povero et al., 2013)
ALD/ASH	Hepatocytes	NON CODING RNA	miR-122	Monocytes	Sensibilization to LPS-induced inflammatory responses	(Momen-Heravi et al., 2015)
	Monocytes		miR-27a	Naive monocytes	Differentiation and M2 polarization	(Saha et al., 2016)
	Hepatocytes	GENE/ PROTEIN	CD40L	Monocytes and macrophages	Release of pro-inflammatory cytokines	(Verma et al., 2016)
	Monocytes		HSP90	Macrophages	Activation and M1 polarization	(Saha et al., 2018)
HCC	HCC cells	NON CODING RNA	miRNAs	HCC cells	Cell growth, migration and invasion promotion	(Kogure et al., 2011)
	HCC cells		miR-93	HCC cells	Increased proliferation and invasion capacity	(Xue et al., 2018)
	Serum from HCC patients		Downregulation of miR-451a	Hepatocytes and LSECs	Reduction of apoptosis	(Zhao et al., 2019)
	HCC cells		miR-21	HSCs	Activation and conversion into CAFs Angiogenesis	(Zhou et al., 2018)
	HCC cells		miR-32-5p	Chemotherapy sensitive cell lines	Angiogenesis and EMT Horizontal transfer of multidrug resistance	(Fu et al., 2018)
	HCC cells		Downregulation of circ-0051443	HCC cells	Reduction of apoptosis and cell cycle promotion	(Chen et al., 2020)
	HCC cells		lncVLDR	HCC cells	Chemotherapy resistance	(Takahashi et al., 2014)
	HCC cells	GENE/ PROTEIN	Angiopoietin-2	HCC cells	increased angiogenesis	(Xie et al., 2020)
	HCC cells		Downregulation of Vps4A	HCC cells	tumor suppressor function	(Hirsova, Ibrahim, Krishnan, et al., 2016; Wei et al., 2015)
	HCC cells		HSP60, HSP70 and HSP90	NK cells	Enhanced cell cytotoxicity and granzyme B production	(Lv et al., 2012)
	HCC cells		KLRK1 and HSP70	LSECs and NK cells	Angiogenesis and improvement of NK cell antitumor response	(Yukawa et al., 2018)
	PDAC cells		MIF	Kupffer cells	Activation of Kupffer cells and HSCs Preparation of the pre-metastatic niche	(Costa-Silva et al., 2015)
DILI	Hepatocytes	NON CODING RNA	miR-122	–	Increased levels in serum in APAP-induced liver injury model	(Bala et al., 2012)Bala et al., 2012; (Cho, Kim, et al., 2017)
	Hepatocytes		miR-192 and miR-155	–	Increased levels in serum in APAP-induced liver injury model	(Cho, Kim, et al., 2017)
	Hepatocytes	DNA	mtDNA	Neutrophils	Uncontrolled APAP-induced neutrophil infiltration, oxidative stress and hepatotoxicity	(He et al., 2017)
Fibrosis Cirrhosis	HCV- infected hepatocytes	NON CODING RNA	miR-19a	HSCs	Fibrogenic activation	(Kogure et al., 2011)
	HSC	GENE/ PROTEIN	Suppression of Twist1	HSC	Activation of HSC	(Chen et al., 2015)
Transplantation	Hepatocyte and serum		miR-301a	–	High levels in EVs were associated with ACR	(Nakano et al., 2017)
	Serum		miR-146a	–	High levels in EVs were associated with ACR	(Hu et al., 2013)
	Serum		Galactin 9	–	High levels in EVs were associated with ACR	(Zhang, Peng, et al., 2019)

alcohol uptake (Cho et al., 2017). In this way, CYP2E1 could be considered as a biomarker for liver injury. After galactosamine-induced liver damage, CYP2D1 increases its activity in circulating EVs compared to controls. Also, it was seen an increase in enzymes previously associated with liver damage, catecholamine-methyl transferase and arginase 1 (Casal, Palomo, Cabrera, & Falcon-Perez, 2016; Palomo et al., 2018; Rodriguez-Suarez et al., 2014; Royo, Moreno, et al., 2017).

Another molecule associated with hepatocytes' EVs as a consequence of DILI is miRNAs. Acetaminophen (APAP) and carbon tetrachloride DILI showed changes in the levels of urinary miRNAs, and among them, 10 of the increased miRNAs were in common between two injuries (X. Yang et al., 2012). Liver-specific miR-122 is increased in EVs after APAP-induced liver injury, in addition to the elevated release of EVs in this context. Moreover, miR-192 and miR-155 are also increased in exosomes after APAP treatment (Bala et al., 2012; Cho, Kim, Lee, & Baek, 2017). All these findings make it easier to define potential biomarkers to detect acute or early-stage DILI. More than single biomarkers, special protein cargo in EVs, and specific miRNA profiles are being established to use them as indicators of liver injury stage and disease prognosis.

The immune system also plays a role in the pathogenesis of DILI, as the implication of miR-223 suggests. For instance, APAP-induced hepatotoxicity leads to the release of hepatocyte-derived EVs able to upregulate miR-223 expression in neutrophils in a TLR9-dependent mechanism. TLR9 is activated by mitochondrial DNA (mtDNA) contained in damaged hepatocyte-derived EVs, which activates inflammatory mediators' production and miR-223 expression in neutrophils (He et al., 2017). The removal of miR-233 results in uncontrolled APAP-induced neutrophil infiltration, oxidative stress, and hepatotoxicity.

As we can see in this section, circulating EVs have different properties and functional roles in the development and progression of DILI. Remarkably, the biogenesis of EVs from hepatic origin after DILI may be influenced by phenomena of necrosis and cell death, and it has been reported changes in the size of vesicles as well as in the presence of

protein markers after drug-induced damage in primary cultures (Palomo et al., 2018) and metabolite profile (Royo, Palomo, et al., 2017). Other drugs, such polycyclic aromatic hydrocarbons produce changes in cell membrane fluidity due to cholesterol depletion, increasing hepatocyte EV release and cell death (van Meteren et al., 2019). Cargo analysis of the preparation may indicate wheather the origin is associated to exosomes, microvesicles, or apoptotic bodies (Battistelli & Falcieri, 2020; Jeppesen et al., 2019). This also contribute to uncover mechanistic aspects of DILI, and reinforce the interest of EVs for clinical applications, as potential biomarkers (Barile & Vassalli, 2017; Cho, Song, Akbar, & Baek, 2018).

2.2. NAFLD and NASH

Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) are different stages of liver damage progression, usually accompanied by other pathologies as obesity and diabetes. Obesity-related hyperlipidemia can saturate the lipid storage capacity in adipocytes, creating an ectopic lipid accumulation in the liver. This lipid accumulation leads to a pathological condition known as NAFLD and, particularly, its inflammatory form known as NASH (Dini et al., 2020). NAFLD is defined by the accumulation of macrovesicular fat in more than 5% of hepatocytes in individuals that consume less than 20 g of alcohol per day. NASH is defined as lipid accumulation with evidence of cellular damage, inflammation, neovascularization, and different degrees of scarring or fibrosis (Brunt, 2007; Clark, Brancati, & Diehl, 2002). Around 30% of the worldwide population is estimated to have a fatty liver. Consequently, NAFLD research has been expanded to become one of the most studied diseases together with hepatocarcinoma and viral hepatitis (Browning et al., 2004).

In 2013, Povero et al. demonstrated that hepatocytes release EVs in response to fat-induced liver damage (Povero et al., 2013). One year later, the same group showed that the protein cargo from hepatocytes-derived EVs reflected the underlying disease process. Indeed, liver-specific microRNAs, miR-122, and miR-192 appeared enriched in NAFLD/NASH patients' blood-derived EVs, decreasing the expression of these microRNAs within the liver (Povero et al., 2014). Interestingly, miR-192 is involved in multiple steps of NAFLD progression. It is downregulated in lipotoxicity experimental models, thus leading to SREBP1 and stearoyl-CoA desaturase 1 (SCD-1) overexpression in target hepatocytes (Lin et al., 2017; X. L. Liu et al., 2019). These genes are involved in *de novo* lipogenesis, and their upregulation accelerates steatosis progression (Lee et al., 2017). Secondly, miR-192 is in macrophage activation through the rapamycin-insensitive companion of mammalian target of rapamycin (Rictor) – Akt – forkhead box transcription factor O1 (FoxO1) signaling pathway. Damaged hepatocytes release increased levels of miR-192 in their exosomes. This miRNA inhibits Rictor expression, thus resulting in activation of FoxO1 activity in macrophages, their M1 polarization, and the subsequent induction of the inflammatory response (X. L. Liu et al., 2019). Those changes in EVs cargo are e observed in children with NAFLD. In a study comparing children with NAFLD and control samples, eighty-two miRNAs are differentially expressed, among which miRNA122-5p, miRNA34a-5p, miRNA155-5p, and miRNA146b-3p were up-regulated in NAFLD patients (X. Zhou et al., 2020). In addition to hepatocyte-to-adipocyte EVs delivery, reverse communication has also been reported. The gene expression of an MMP inhibitor, TIMP-1, in hepatocytes and stellate cells is altered by the EVs released from adipose tissue in obese patients. As a consequence of this effect, a dysregulation of the TGF-β pathway is induced into liver cells (Koeck et al., 2014).

Also, hepatocyte-derived exosomes can send signals of metabolic stress after lipid overload. EVs trigger adipose tissue remodeling to try to ameliorate the metabolic distress generated by the excess of lipids (Mikolasevic et al., 2016; Y. Zhao et al., 2020). In a study of NASH, it was shown that lipids stimulate death receptor 5 (DR5) on hepatocytes inducing a release of exosomes, which activate an inflammatory phenotype in macrophages (Hirsova et al., 2016). In line with these pro-inflammatory effects, mixed lineage kinase 3 (MLK3) is known to induce EV-dependent macrophage chemotaxis. In this case, MLK3 enhances the presence of C-X-C motif chemokine 10 (CXCL10) in hepatocyte-EVs when treated with toxic lipids (Ibrahim et al., 2016).

Other mechanisms are related to hormones. For instance, thyroid-stimulating hormone (TSH), a hormone that plays an essential role in lipid metabolism, is involved in the development of NAFLD. In a study in which HepG2 cells were treated with TSH, EVs production was increased, and these EVs showed a specific altered spectrum of protein (Ma et al., 2020). Lipid-overloaded hepatocyte-derived EVs have been reported to influence non-parenchymal liver cells in NAFLD. In a caspase-3 – dependent mechanism, hepatocytes release microparticles that trigger proangiogenic responses in liver endothelial cells through a Vanin-1 (VNN1) – dependent manner. VNN1 can promote the migration and reorganization of LSECs, a process that may be related to the regulation of PPAR activity (Povero et al., 2013).

2.3. Alcoholic liver disease and alcoholic steatohepatitis (ALD/ASH)

Alcoholic hepatitis caused by the continuous intake of alcohol and is defined as a syndrome of progressive inflammatory liver injury (Lucey, Mathurin, & Morgan, 2009). The initiation of the inflammatory process is the result of the accumulation of different factors, such as steatosis, oxidative stress, altered gut permeability, toxic metabolites, and release of cytokines (Fung & Pyrsopoulos, 2017). Alcoholic hepatitis is also characterized by the accumulation of fat in the liver (Baraona & Lieber, 1979; Gao & Bataller, 2011). It occurs in people that consume excessive amounts of alcohol, as the liver is critical in ethanol metabolism, and its chronic consumption results in a full spectrum of hepatic lesions. It can progress from alcoholic fatty liver (AFL) to alcoholic steatohepatitis (ASH). The first case is a reversible situation if the affected person ceases drinking alcohol, but ASH is a more severe inflammatory condition (Mendez-Sanchez, Almeda-Valdes, & Uribe, 2005), with many of the features that NASH presents (hepatocellular ballooning, presence of MDBs, neutrophilic infiltration), but also shows a higher degree of periportal inflammation. Kupffer cells have a central role in the progression of ASH, as chronic alcohol exposure can switch them from the tolerogenic M2 phenotype to the pro-inflammatory M1 phenotype (Osna, Donohue Jr., & Kharbanda, 2017).

As in other cases of liver damage, EVs also increase in patients with alcoholic hepatitis or patients consuming excess ethanol (Hirsova et al., 2016). In 2016, Verma and coworkers did a study of alcoholic liver disease showing differences in caspase-3 and cluster of differentiation 40 ligand (CD40L). They showed that the activation of caspase-3, an enzyme involved in the generation of exosomes, increased significantly after ethanol treatment. In the same line of research, CD40L, a member of TNF superfamily, appeared highly packaged in exosome-like vesicles, increasing inflammatory cytokine production in recipient monocytes, and macrophages. These macrophages can be induced to release pro-inflammatory cytokines by either ethanol treatment or from CD40L-containing EVs released from alcohol-injured hepatocytes. In this situation, macrophages are induced to release TNF-α, whereas hepatocytes (normally resistant to it) become sensitized to its action, dying by apoptosis.

Moreover, apoptotic bodies and debris coming from apoptotic hepatocytes can switch Kupffer cells to M1 phenotype, enhancing the inflammatory state and the recruitment of more immune cells (T-cells, neutrophils) to the liver. This causes a positive feedback loop that promotes parenchymal cell death and the progression of the disease (Verma et al., 2016). Another study in the field of alcoholic liver disease showed that the protein cargo of EVs changed between healthy and alcohol-induced liver damage mice, finding heat shock protein 90 (Hsp90) as the originator of EV-induced activation of macrophages (Saha et al., 2018).

As well as proteins, miRNA cargos in EVs have been identified as a potentially novel diagnostic tool for early alcoholic steatohepatitis, finding three miRNAs (let7f, miR-29a, and miR-340) that were increased in circulating EVs from alcoholic steatohepatitis that did not appear in EVs from other liver injury models (Eguchi et al., 2017). Apart from those miRNAs that are specific for alcoholic hepatitis, common miRNA for liver damage were also detected. For instance, the presence of miR-122 was enriched in EVs production in hepatocytes after alcohol exposure, a typical liver miRNA that can be identified circulating after different types of liver damage (Momen-Heravi, Bala, Kodys, & Szabo, 2015). miR-122 is released in EVs from injured hepatocytes and sensitizes monocytes to LPS-induced pro-inflammatory responses, thus explaining the exacerbated immune responses seen in this disease. miR-27a contained in monocyte-derived EVs after alcohol exposure does also activate other monocytes and their cytokine secretion. However, miR-27a enables naïve monocyte differentiation and polarization into the M2 phenotype. These findings support the existence of a complex feedback loop, modulating the sick liver inflammatory response (Saha et al., 2016).

As stated above, the accumulation of fat in the liver is one of the characteristics of alcoholic hepatitis. This accumulation is not only specific to alcoholic hepatitis, but it also occurs in nonalcoholic hepatitis. For that reason, it is challenging to use lipids as a particular biomarker of the underlying condition of liver damage. Despite this statement, sphingolipids have been proposed as a marker of alcoholic hepatitis, since the sphingolipid cargo in circulating EVs is significantly enriched in patients with alcoholic hepatitis, showing differences when compared to heavy drinkers, healthy controls, cholestatic liver diseases, and non-alcoholic steatohepatitis patients. These results suggest that EVs sphingolipid concentration may be employed in the diagnosis and differentiation of alcoholic hepatitis from other liver etiologies (Sehrawat et al., 2020).

2.4. Fibrosis and cirrhosis

Most chronic diseases progress with an accumulation of extracellular matrix proteins, including collagens (I, III and IV), fibronectin, elastin, laminin, unduly, elastin, hyaluronan, and proteoglycans (Bataller & Brenner, 2005). Liver fibrosis is the consequence of different diseases such as ASH, NASH, and chronic viral hepatitis (type B or C) infection. Initially, wound-healing after hepatocyte apoptosis and liver injury is a physiological response, taking part in liver regeneration. However, when the damage persists and becomes chronic, hepatocytes lose their regeneration capacity, and large fibrous scars substitute them. Despite what it was thought some years ago, fibrosis is partly reversible if the causal agent is removed (Aydin & Akcali, 2018). However, if left untreated, inflammation and fibrogenesis processes finally distort the hepatic architecture, and its vascular system becomes compromised. Cirrhosis is the situation in which nodules of regenerating hepatocytes appear between fibrous structures. Usually, the loss of functionality is compensated in the early stages, with part of the liver parenchyma undamaged and functional enough to offset the fibrotic regions. As the disease progress though, the patient could arrive at a decompensated phase where the liver become covered by large areas of scar tissue (Bataller & Brenner, 2005).

Twist1 is related to the fibrotic liver. It is a transcription factor involved in cell fate, critical in hepatic stellate cells activation in the fibrotic disease. In the quiescent state, HSCs express Twist1 in high levels, resulting in a high miR-214 expression. Twist1 and miR-214 are packaged into exosomes and suppress CTGF translation in recipient HSCs, maintaining them in a quiescent state (L. Chen et al., 2016). Consistent with this, in a murine model of liver fibrosis, suppression of TWIST1 expression, CTGF upregulation, HSCs activation, and profibrotic processes promotion was described (Chen, Chen, Kemper, Charrier, & Brigstock, 2015).

Another type of immune cells is also involved since Kupffer cells induce HSCs to produce pro- or anti-inflammatory cytokines (Osna et al., 2017). Prostaglandin D2 (secreted by Kupffer cells) can be sensed by HSCs, promoting the production of TGF-β1 and other anti-inflammatory cytokines in these last ones. TGF-β1 can induce the activation of HSCs in the progression of fibrosis in different related pathologies. For instance, TGF-β1 is upregulated after HCV infection, as miR-19a-containing exosomes were released from infected hepatocytes and taken up by HSCs, modulating their SOCS-STAT3-TGF-β1 axis (Devhare et al., 2017).

2.5. Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy in adults (Pons-Renedo & Llovet, 2003). It usually appears as a late complication of chronic liver disease associated with cirrhosis (Anzola, 2004). As the tumor progresses, hepatic architecture and some of the characteristic features of the liver are altered. Typically, it can be observed in cirrhotic tissue around the carcinoma. This surrounding tissue can provide insights into the carcinogenesis mechanism and characterization of the diseases in each patient (Knudsen, Gopal, & Singal, 2014).

Due to the impact of the disease in the society and the recent evidence of the implication of EVs in the mechanism mediating paracrine signaling between cells, there has been an increase in the number of studies focusing on HCC-derived EVs (Pascut, Pratama, Vo, Masadah, & Tiribelli, 2020; Wortzel, Dror, Kenific, & Lyden, 2019). Indeed, tumoral cells are dynamic generators of EVs, perhaps because the depence of the tumor on establish EVs as mediators in tumor-to-stroma, stroma-to-tumor and tumor-to-tumor communication, involving different mechanism of biogenesis (Bebelman, Smit, Pegtel, & Baglio, 2018). There has been evidence suggesting that some miRNAs carried by EVs are relevant players in the dynamic crosstalk among cancerous, immune, and stromal cells in establishing the tumorigenic microenvironment (Kogure, Lin, Yan, Braconi, & Patel, 2011; Kosaka et al., 2013). One of the main targets regulated by those miRNAs is the transforming growth factor β activated kinase-1 (TAK1), appointed as a potential candidate for therapy. It has been shown that the expression of TAK1 could be modulated through the miRNA cargo carried by HCC cell-derived exosomes (Kogure et al., 2011). Also, one of the miRNAs proposed as a novel biomarker for the diagnosis and prognosis of the disease is the miR-93, which appears up-regulated in hepatocyte-derived circulating EVs in patients with hepatocellular carcinoma (Xue, Wang, Zhao, Hu, & Qin, 2018). Recently, it has been identified LPIN1 as a target of EVs-associated miR-451a to inhibit hepatocellular tumorigenesis by regulating tumor cell apoptosis and angiogenesis (Zhao et al., 2019). Furthermore, it was shown that HCC cells could convert normal hepatic stellate cells in cancer-associated fibroblasts (CAFs), promoting tumor development by angiogenesis. HCC cells secrete exosomes containing miRNA-21 that down-regulates its target PTEN and, as a consequence, the PDK1/AKT signaling pathway is activated (Y. Zhou et al., 2018). In this same line of results, experiments with multidrug-resistant HCC cell lines revealed the presence of miR-32-5p in HCC-derived exosomes. These exosomes could also activate the PI3K/Akt pathway, resulting in angiogenesis and epithelial-mesenchymal transition (EMT). This activation has been reported to be through the same mechanism as in the case of miR-21, the direct targeting of PTEN by the miRNA123 (Fu et al., 2018). By contrast, LPIN1, apart from catalyzing lipid biosynthesis reactions, does also act as a nuclear transcriptional coactivator for PPAR-α to modulate lipid metabolism gene expression. Circular RNA circ-0051443 is typically carried from normal hepatocytes to HCC cells to suppress malignancy progression. It activates cell cycle arrest and apoptosis and has been reported to be downregulated in HCC cases (Chen et al., 2020).

In addition to their use for diagnostics, EVs may also be involved in the mechanism of drug-resistance that tumoral cells develop against chemotherapies. One example is the presence of linc-VLDLR, a non-

coding RNA, enriched in cancer cells-derived EVs that generated HCC cells chemoresistance (Takahashi, Yan, Wood, Haga, & Patel, 2014). Moreover, EVs-mediated signaling is also involved in angiogenesis, and HCC-derived EVs carried angiopoietin-2. When delivered into human umbilical vein endothelium, cells increasing angiogenesis, and the recipient cells recycle this growth factor. Therefore, angiopoietin-2 has been described as an attractive therapeutic target. Besides of that, recipient cells recover angiopoietin-2 for it reused (Xie et al., 2020).

At the protein level, Vps4A, a regulator of exosomal biogenesis in the endocytic pathway, appears downregulated in HCC tissues. This reduction was associated with tumor progression and metastasis, thus granting a tumor suppressor function to Vps4A1 (Hirsova, Ibrahim, Verma, et al., 2016; Wei et al., 2015). Additionally, HSP60, HSP70, and HSP90 have been reported to be upregulated in HCC-derived exosomes. Apart from the angiogenic effects of HSP70, these HSPs were able to enhance natural killer (NK) cell cytotoxicity and granzyme B production. These results indicate a possibility to improve the defective NK cytotoxic activity described in HCC patients through EVs, thus improving prognosis perspectives. Finally, killer cell lectin-like receptor K1 (KLRK1/NKG2D) is also present in HCC-derived EVs surface and is an activator receptor for immune components such as NK, CD8+ and γδT cells (Lv et al., 2012; Yukawa et al., 2018).

Furthermore, EVs released by cancer cells from non-hepatic origin can also influence the liver microenvironment and initiate the formation of pre-metastatic niches. Pancreatic ductal adenocarcinoma (PDAC) cells produce exosomes containing migration inhibitor factor (MIF) that can be internalized by Kupffer cells, promoting their activation. Kupffer cell activation through MIF involves the production of MIF-dependent cytokines (*e.g.*, TGF-β) that activate HSCs to enhance the metastatic niche (Costa-Silva et al., 2015). Nevertheless, it has been appointed that a proper functional study of the role of EVs in cancer pathogenesis should involve the blockade of EVs biogenesis, which is a complex issue given the different routes and mechanism involved (Bebelman et al., 2018).

2.6. Transplantation

Hepatocyte-derived EVs represent an excellent method for diagnostic in acute cellular rejection (ACR) after liver transplantation. Indeed, levels of EVs could be used as a marker of liver rejection after liver transplantation (Brodsky et al., 2008). Having high levels of EVs for two weeks after transplant may be a signal of rejection. Also, different studies have identified novel predictors of rejection, such as galactin-9 (A. B. Zhang, et al., 2019), miR-301a (Nakano et al., 2017) and miR-146a (J. Hu et al., 2013). Some years ago, it was thought that galactin nine expression was an independent factor, but it was shown that it might be a marker of recipient cell immune status (Zhang et al., 2019). In the case of miR-301a, it has been observed that it has an impact on proinflammatory cytokine production, notably in IL-6, during ACR. In a study comparing the levels of miR-301a in naïve, tolerogenic, and lethal ACR, it was shown that levels of miR-301a were significantly higher. By contrast, this effect has only been seen in recipients with ACR, not in recipients with abnormal liver function (Nakano et al., 2017). Finally, it has been observed that miR-146a plays an essential role in the initiation of ACR. These results have only appeared in ACR cases and not in other liver injuries, suggesting that this elevation might not originate from damaged hepatocytes.

3. EVs as therapeutics tools in liver pathology

3.1. Stem cell-derived EVs

Experimental cell therapy research uses mostly mesenchymal stem cells (MSCs) for treating human diseases (Fig. 2). Despite the capabilities that these cells have shown, MSCs play several simultaneous roles that could interfere in the final objective of their use, such as, the release of cytokines to reduce inflammation, expression of growth factors to help to heal, secreting immuno-modulatory proteins to alter host immune responses, enhancing responses from endogenous repair cells, and as mature functional cells for some tissues (Phinney & Pittenger, 2017). However, it has been observed that some of the benefits of MSCs treatment can also be achieved with MSC-derived EVs while avoiding many risks associated with cell transplantation (Goolaerts et al., 2014; Lai et al., 2010; Timmers et al., 2007).

As in other areas, EVs released by MSCs have also been used to treat liver diseases (Phinney & Pittenger, 2017; Sato et al., 2019). Different studies comparing MSC EVs and their parent stem cells have demonstrated that vesicles have the similar effects in liver repair and regeneration (Katsuda, Kosaka, Takeshita, & Ochiya, 2013; Konala et al., 2016). Due to their cargo, those EVs improve liver function and relieve the pathological phenotype by transferring it to damaged cells (Peterson, Otoc, Sethi, Gupta, & Antes, 2015; Wen, Peng, Liu, Weizmann, & Mahato, 2016). Also, it has been shown that MSC- EVs are proper treatments during organ transplantation; their addition to the perfusion solution ameliorates the viability and functionality of donor organs (Grange, Bellucci, Bussolati, & Ranghino, 2020).

MSCs derived EVs had also been used in liver fibrosis since it has been shown that human umbilical cord mesenchymal stem cells (huc-MSCs)-derived exosomes-like EVs reduce TGF-β1 expression. TGF-β1 activated the phosphorylation of Smad2 and led to liver EMT *in vivo*. Therefore, the treatment with 250μg EVs, directly injected in the left and right lobes of the liver, inactivating the phosphorylation of Smad2 was able to reverse liver EMT *in vivo* (Li et al., 2013). Some years later, the same group showed that after liver transplantation, a single dose of huc-MSC EVs administrated through the tail vain reduces oxidative stress and apoptotic effects, restoring liver failure. This effect could be mediated by protein cargo GPX1 that upregulated ERK1/2 and Bcl-2 and downregulated the IKKB/NFkB/casp-9/−3 pathway (Yan et al., 2017). Also, they compared the benefit of huc-MSC EVs *vs.* bifendate, a frequently used hepatic protectant, in models of liver damage. The study concludes that among both treatments, the intravenous administration of huc-MSC EVs presented higher antioxidant and hepatoprotective effects than bidentate (W. Jiang et al., 2018). As a possible explanation, huc-MSC derived EVs appear enriched in miR-455-3p, which could play an essential role in the therapy of acute inflammatory liver injury. miR-455-3p inhibits the activation and the cytokine production of macrophages attenuating macrophage infiltration and local liver damage (Shao et al., 2020). We should also mention that not only stem cells, but also healthy cells derived EVs, obtained from primary cultures had protective effects against fibrosis (Li, Chen, Kemper, & Brigstock, 2019).

Besides the umbilical cord, there are also studies about human bone marrow-MSC (hBM-MSC) that show positive effects. Rong et al., have studied the impact of hBM-MSC EVs in liver fibrosis, and demonstrated that hBM-MSC- EVs alleviate liver fibrosis, enhancing the impact of the parent hBM-MSC. Furthermore, they found that in hepatic stellate cells and liver fibrosis, the expression of tissue components of the Wnt/β-catenin pathway and collagen I were inhibited by hBN-MSC EVs (Rong et al., 2019). In a different study, it has been shown that the administration of BM-MSC EVs reduced hepatic injury and modulated cytokine expression. They identified as a possible effector agent the noncoding RNA Y-RNA-1, which appears in mayor abundance in EVs than in cells of origin (Haga, Yan, Takahashi, Matsuda, & Patel, 2017).

Despite the excellent results achieved with stem cells, some studies reveal better results with a combination of therapies. In a survey about liver fibrosis, they compare two treatments, Nilotinib, a second-generation tyrosine kinase, *versus* stem cell-derived EVs. The results showed that the combination of both treatments, consisting on daily treatments of Nilotinib (20 mg/kg), and stem cell exosomes (0.5 ml/rat intravenously) during the last weeks of CCl4 intoxication had a better effect than each one alone (Shiha et al., 2020). Another example of a combination of therapies is the use of EVs obtained from pre-treated

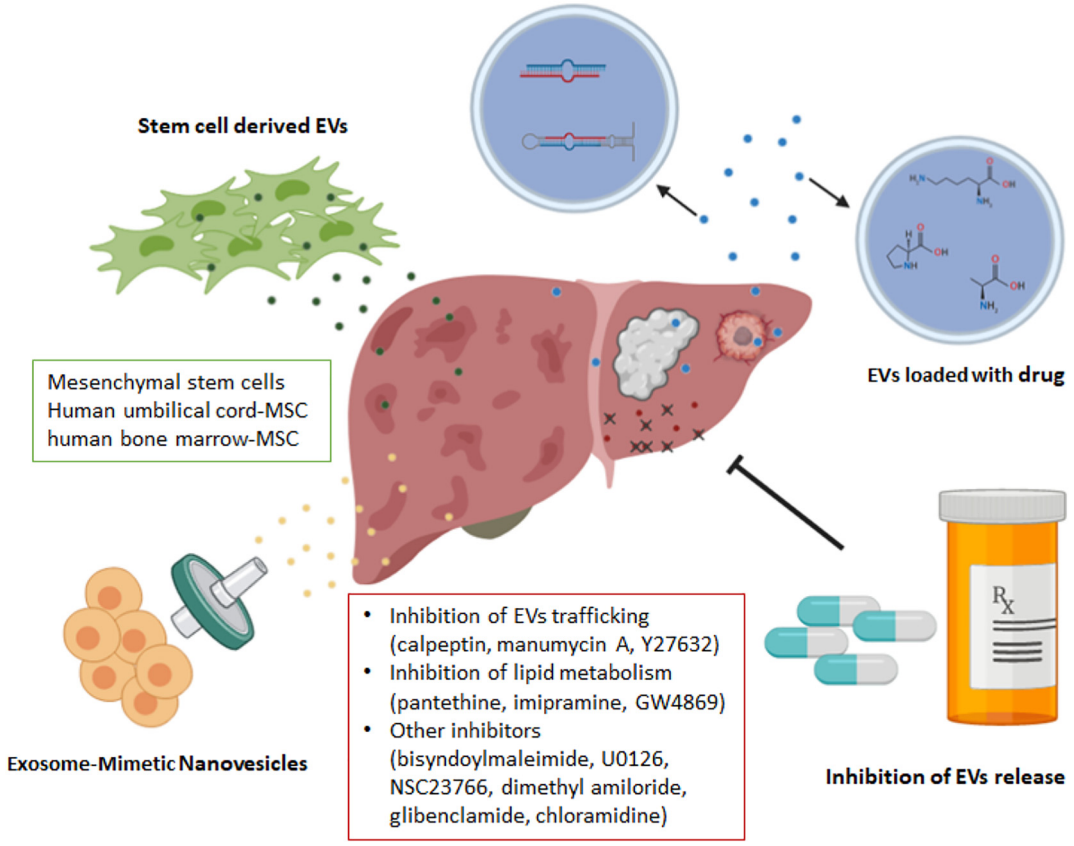

Fig. 2. Therapies with EVs used in liver pathologies.

huc-MSC with TNF-α to ameliorate acute liver failure. This combination attenuates inflammatory damage and promotes liver tissue repair by inhibiting the activation of the NLRP3 pathway (Zhang et al., 2020).

3.2. EVs loaded with drugs

EVs are circulating entities that show particular specificity on their biodistribution, according to their surface proteins (Hoshino et al., 2015; Royo, Cossio, Ruiz de Angulo, Llop, & Falcon-Perez, 2019). Moreover, there is specific selectivity for the capture of EVs, and therefore, it is possible to load EVs with chemicals, using them as a drug delivery system (Fig. 2). In that function, EVs present some advantages, such as improved *in vivo* stability, higher targeting efficiency, and targeted therapy (Armstrong & Stevens, 2018). In the case of drug loading, the biogenesis of EVs make endogenous loading easier, allowing to a pre-treatment of cells with the drug of interest and subsequently re-packaged into secreted vesicles (Alvarez-Erviti et al., 2011). The antigen-presenting cell-derived EVs give *in vivo* stability for inflammatory environments, protecting against the immune system, and offering a higher circulation time (Clayton, Harris, Court, Mason, & Morgan, 2003). In addition, some cells present uptake difficulties of synthetic systems, and for them, EVs allow higher uptake efficiency (Armstrong & Stevens, 2018).

In chemotherapy, tumor cell-derived EVs can be used to deliver therapeutic agents to tumor cells. Considering the advantages of this therapy, drug-packaging EVs were used to treat murine hepatocarcinoma and human ovarian cancer tumor cells, observing total inhibition of tumor growth (Tang et al., 2012; Urban, Mocan, Sanger, Lukacs-Kornek, & Kornek, 2019). In addition to drugs, EVs can also pack and deliver miRNAs (Fig. 2). EVs loaded with siRNAs can effectively reduce the expression of genes in the liver, gut, and kidney glomeruli (Reshke et al., 2020). For the treatment of HCC, using miRNA-loaded EVs has achieved inhibition of the proliferation and reduction of the invasion on recipient cells. Nevertheless, the transferred RNA could generate other off-target effects (Ishiguro, Yan, Lewis-Tuffin, & Patel, 2020; Wang, Li, Piontek, Sakaguchi, & Selaru, 2018).

The use of exosomes as drug delivery has also been applied in other areas of liver disease, such as viral hepatitis and fulminant hepatic failure. In the case of hepatitis B, it has been shown that interferon-α (IFN-α) pre-treated liver non-parenchymal cells release EVs with antiviral activity molecules, offering a new opportunity on antiviral treatments (Li et al., 2013). Also, a promising strategy for the hepatitis C virus vaccine has been developed with EVs. It has been shown that using DNA plasmids generating recombinant retrovirus-like particles, antigen-specific T cell responses, and antiviral immune protection are obtained (Desjardins et al., 2009). For the treatment of fulminant liver failure (FHF), EVs derived from shiitake fungi have been administered intraperitoneally at a single the dose of 1×10^{10}/g, 48 h before to induce the liver damage with galactosamine. These EVs act by blocking the formation of the NLRP3 inflammasome and protect from acute liver damage (B. Liu et al., 2020).

Despite the advantages of EVs, such as drug delivery systems, it is crucial to keep in mind that the production of EVs for therapy is a complex process. The creation of EVs has some variables that can influence their properties, for instance, cell type, cell collection process, expansion methods, culture conditions, the mechanism to trigger EV release, and EV isolation and storage methods. Variations in these parameters can completely change the characteristics of obtained EVs, creating a very heterogenous EVs sample (Burnouf, Agrahari, & Agrahari, 2019). Therefore, before starting therapy using EVs as a drug delivery system, it is essential to establish a very well-defined protocol for EVs production. There is a large potential for EVs therapy in liver diseases, but there is also big challenges to overcome. The simplest therapies, involving local administration to the target tissue, for instance, in intranasal

delivery to brain tissue (Zhuang et al., 2011) or direct injection into liver (Li, Yan, et al., 2013) would be more easy to implement. On the other hand, systemic administration presents a complex interplay of fluid dynamics, biological barriers and immune clearance. For EVs (nanoscale sized and anionic), there is entrapment in the red blood cell core and clearance by the mononuclear phagocyte system (Armstrong & Stevens, 2018). Moreover, the biochemical complexity of EVs present additional considerations beyond liposome analogy. Blood clearance for melanoma EVs in a murine model has been estimated in 2 min (Y. Takahashi et al., 2013), with accumulation at 4 h of the injected EVs in the liver (28%, spleen (1.6%), and lung (7%)(Morishita et al., 2015). However, other factors such parental cell and route of administration play also a role. It is well known that EV-glycome modifications alter their distribution, and for instance the introduction of glycoprotein-targeting moiety direct them toward acetylcholine-receptor-rich organs (Wiklander et al., 2015), or the treatment with neuraminidase increase their affinity for the lung (Royo, Cossio, et al., 2019). In addition, the route of administration and the dose of injected EVs influenced the biodistribution pattern.

A last consideration is the challenges related to manufacture. To comply with Good Manufacturing Practices (GMP), it is necessary to use controllable methods for cell culture, for instance hollow fiber-based bioreactor for cell culture is an attractive strategy for EVs production because of the advantage of reducing the volume of conditioned media (Chen, Lin, Chiou, & Harn, 2019), although it is important to remember that different culture platforms alter EV release (Palviainen et al., 2019). Regarding purification, ultrafiltration may help to reduce protein contamination *versus* ultracentrifugation, but a good set of quality control parameter, such EV markers, and properties derived from parental cells should be used to control batch quality and reproducibility. If GMP compliance and a well-developed understanding of the benefit of regulatory requirements can be applied to EV biological therapeutics development, it will create a strong bridge between pharmaceutical production and patients benefit (Gimona, Pachler, Laner-Plamberger, Schallmoser, & Rohde, 2017).

3.3. Exosome-mimetic nanovesicles

As we have seen so far, EVs could be used with a therapeutic purpose. One of the biggest problems with these vesicles is that most cells do not produce a large quantity of EVs (Jang et al., 2013; Wu et al., 2018). Also, as mentioned before, EVs production and storage have different variables that can change final EVs features. For that reason, a new therapeutic technic using exosomes-mimetic nanovesicles (NVs) has been developed (Fig. 2). Exosomes-mimetic NVs have more than a 100-fold higher yield than exosomes and are obtained through different techniques (H. Kim et al., 2020). A popular method is to subject cells to serial extrusion through filters with diminishing pore size. Through this technic, high quantities of exosome-mimetic NVs are obtained, and these NVs have some properties similar to natural exosomes such as deliver information to recipient cells (Jang et al., 2013; Wu et al., 2018). There are some exosome-mimetic NVs that are commercialized. A study performed by Lozano-Andrés et al., it was proved that using commercially available niosomes (Nio-N-GF-MAL) were able to produce tetraspanin-domain decorated nanovesicles. These vesicles present similar features with natural EVs and can detect using common EV markers (Lozano-Andres et al., 2019).

Despite being a useful technique that solves some of the problems involved in working with EVs, it still requires optimization and *in vivo* tests (Ko et al., 2020). For example, exosome-mimetic NVs have been used for research in liver disease, trying to improve liver regeneration. It has been shown that exosome-mimetic NVs from primary hepatocytes increment sphingosine kinase 2 in recipient cells promoting hepatocyte proliferation and liver regeneration. These results provide new strategies for the replacement of EVs with exosome-mimetic NVs (Wu et al., 2018). Future studies in other liver pathologies are expected.

3.4. Adeno-associated virus and EVs

Adeno-associated virus (AAV) vectors are currently a leader for direct *in vivo* gene therapy, given their safety and good ability to express genes in a variety of tissues in nonhuman models and human tissues (Coura Rdos & Nardi, 2007)(Nathwani et al., 2011). However, some limitations of certain serotypes to cross physiological boundaries such the blood brain barrier, as well as the development of neutralizing antibodies by the host immune system, had become a serious challenge to the effectivity of AAV for genetic therapy (Scallan et al., 2006). Interestingly, a portion of AAV associates with microvesicles/EVs during production, (Maguire et al., 2012). This association has shown a great potential of EVs as therapeutic delivery modalities, for instance rescuing hearing impairment (Gyorgy et al., 2017), or accomplish gene expression into the retina in a murine model (Wassmer, Carvalho, Gyorgy, Vandenberghe, & Maguire, 2017). Indeed, it had become a promising therapeutic strategy as gene vehicles supporting spread throughout the brain, reaching regions far from the injection site. This allows to reduce the number of injections required, and facilitates to deliver the dose rate needed to achieve the target concentration (Orefice et al., 2019). Accordingly, the current studies suggest that AAV associated to EVs could become a powerful tool to enhance vector spreading and increase the specificity toward the targeted tissue, offering the requested potential to address significant unmet clinical challenges.

3.5. Inhibition of EVs release

Up to this point, we have described EVs as therapeutic tools, but we have also seen along with this review that EVs may play a role in the pathogenesis of certain diseases (Hirsova, Ibrahim, Verma, et al., 2016; Povero et al., 2013). In consequence, it is crucial to consider the inhibition of EVs biogenesis or release as a therapeutic tool (Urban et al., 2019) (Fig. 2). Due to the diversity of EVs population, sometimes it is necessary to use more than one strategy for depletion (Catalano & O'Driscoll, 2020). Different approaches could be used for the reduction and release of EVs. On the one hand, it can be inhibited the trafficking of EVs using compounds as calpeptin (Yano et al., 1993), manumycin A (Datta et al., 2017), and Y27632 (Tramontano et al., 2004). Another option would be inhibiting lipid metabolism from avoiding EVs biogenesis using compounds such as pantethine (Kavian et al., 2015), imipramine (Deng et al., 2017) and GW4869 (Charrier et al., 2014). It is also possible to try to avoid EV release, for instance with the inhibitor of protein kinase C bisyndoylmaleimide (Stratton, Moore, Zheng, Lange, & Inal, 2015), the non-competitive inhibitor of MEK 1 and MEK 2 named U0126 (Li, Yu, Williams, & Liu, 2010), NSC23766 (Wang, Luo, Morgelin, & Thorlacius, 2017), dimethyl amiloride (Chalmin et al., 2010), glibenclamide (Henriksson et al., 2011) and chloramidine (Kholia et al., 2015).

Although the inhibition of EVs release is a promising tool, it is vital to take into account possible side-effects. The general side-effect is the depletion of EVs released by healthy cells, with the disruption of several pathways dependent on the cell to cell interaction (Catalano & O'Driscoll, 2020). Nevertheless, we foresight a quick development of therapeutic strategies based on EVs.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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