
















Extracellular vesicles in metabolic dysfunction associated fatty liver disease: mechanisms, diagnostic and therapeutic implications

Zongmei Wu^{1†}, Mengmeng Xia^{1†}, Sandra Serna Salas^{1†}, Maria Camila Trillos-Almanza^{1†}, Magnolia Martinez Aguilar^{1†}, Johanna C. Arroyave-Ospina^{1†}, Junyu Wang^{1†}, Marco Arrese², Svenja Sydor³, Lars P. Bechmann³, Frederike GI van Vilsteren¹, Hans Blokzijl¹, Han Moshage^{1*}

¹Department of Gastroenterology and Hepatology, University Medical Center Groningen, University of Groningen, 9700 RB Groningen, Netherlands

²Departamento de Gastroenterología, Escuela de Medicina, Pontificia Universidad Católica de Chile, Santiago 6510260, Chile

³Department of Internal Medicine, University Hospital Knappschaftskrankenhaus, Ruhr-University Bochum, 44892 Bochum, Germany

[†]These authors contributed equally to this work.

***Correspondence:** Han Moshage, Department of Gastroenterology and Hepatology, University Medical Center Groningen, University of Groningen, 9700 RB Groningen, Netherlands. a.j.moshage@umcg.nl

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Abstract

The prevalence of metabolic dysfunction-associated fatty liver disease (MAFLD) is increasing rapidly worldwide due to the obesity epidemic. Advanced stages of the MAFLD, such as non-alcoholic steatohepatitis (NASH) with advanced fibrosis or cirrhosis are affecting global health. Extracellular vesicles (EVs) are released by all cell types and are important in cell-to-cell communication and maintaining homeostasis, but they also play a role in the pathogenesis of various diseases. EVs contain biological information such as lipids, proteins, messenger RNAs (mRNAs), small RNAs, and DNA, and they act on (distant) target cells. The cargo of EVs is dependent on the type and the state of the releasing cell. EVs have been proposed as biomarkers, prognostic, and even therapeutic agents, also in the context of liver diseases. This review aims to give an overview of the current knowledge on EVs in MAFLD, including the role and interaction of EVs with different cell types in the liver. Several aspects of EVs, including their origin, characteristics, cargo, and functions are reviewed. Moreover, the potential of EVs as targets for the treatment of MAFLD is discussed.

Keywords

Extracellular vesicles, exosomes, metabolic dysfunction-associated fatty liver disease, non-alcoholic steatohepatitis, lipotoxicity, liver fibrosis, biomarkers, therapeutic application

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Introduction

Metabolic dysfunction-associated fatty liver disease (MAFLD) is considered one of the most important causes of chronic liver disease worldwide. The prevalence in Western countries ranges from 20% to 30% in the general population. It is estimated that in the near future the number of cases will continue to rise because of the worldwide increased prevalence of obesity [1]. MAFLD is the result of impaired lipid metabolism resulting in an excessive accumulation of lipids in hepatocytes. Furthermore, it is associated with manifestations of metabolic syndrome such as type 2 diabetes, atherosclerosis, dyslipidemia, and hypertension [2]. About 3% to 5% of patients with MAFLD have non-alcoholic steatohepatitis (NASH), which is characterized by chronic inflammation, ballooning of hepatocytes, and progression to fibrosis [3]. NASH is also an important risk factor for the development of cirrhosis and even hepatocellular carcinoma (HCC). Drug-based therapy for MAFLD and its advanced stages is lacking. Currently, the only effective treatment for advanced MAFLD (cirrhosis) is liver transplantation or bariatric surgery [4]. Non-invasive, accurate tools to determine the stage of MAFLD are lacking but urgently needed. Major efforts have been made to identify novel non-invasive biomarkers to diagnose and stage the MAFLD. In the past decades, research on extracellular vesicles (EVs) has evolved from basic research to clinical application. EVs were first described in 1946 by Chargaff and West [5] as pellets resulting from ultracentrifugation of blood plasma. EVs may have significant biological and clinical value as they reflect the stage of the cell releasing the EVs. EVs are nanometer to micrometer-sized particles and can be subdivided into three groups: exosomes, microvesicles, and apoptotic bodies (see section 2 for more details). The role of EVs in intercellular transport and communication with the discovery that EVs contain RNA was reported in 2006 [6]. The cargo of EVs is composed of messenger RNAs (mRNAs), microRNAs (miRNAs), DNA, proteins, and lipids that may contribute to (patho)physiological effects in target cells [7]. Proteins identified in EVs include pro-inflammatory cytokines, regulatory proteins, active peptides, and growth factors [8, 9]. The identification of biologically active components in EVs has sparked interest not only in their (patho)physiological role but also in their application in diagnosis and even therapy, including MAFLD, chronic hepatitis B virus (HBV) and C (HCV), HCC, and acute liver failure [10–13]. EVs released from injured hepatocytes can have effects on nearby healthy hepatocytes, non-parenchymal liver cells, e.g., Kupffer cells (KCs) and hepatic stellate cells (HSCs), as well as more distant non-hepatic cells [13–15]. These findings are particularly relevant for MAFLD, where growing evidence suggests that metabolic reprogramming and oxidative stress are key mechanisms that affect liver function in these conditions. This review aims to discuss the role of EVs in the context of MAFLD, with special emphasis on their role in intercellular communication and their potential application for diagnosis and treatment.

Classification and biogenesis of EVs

EVs are spherical particles enclosed by a lipid membrane [16]. They are generated by all cell types and can be detected in diverse biological fluids such as blood, saliva, urine, semen, breast milk, and others [17]. They are important mediators in cell-cell communication due to their biological characteristics: they are composed of membranes and cytosolic content also known as ‘molecular cargo’, containing bioactive molecules that can serve as messengers. The molecular cargo varies according to the cell of origin and the condition of the releasing cell. These bioactive molecules contain proteins [e.g., tetraspanins, cytoskeletal proteins, cytosolic proteins, cytokines, extracellular matrix (ECM) proteins, and plasma membrane proteins], lipids, and nucleic acids [e.g., miRNA, mRNA, ribosomal RNA, and cell-free DNA, including double-stranded (ds), and single-stranded (ss) fragments, mitochondrial DNA (mtDNA), extrachromosomal circular DNA and viral, bacterial and plasmid DNA] [13, 18–21], which can be transferred to target cells by membrane fusion. The molecular cargo of EVs can then cause (patho)physiological changes in target cells. Target cells can be nearby cells but also distant cells, generating responses at distant sites without the need for direct contact between cells [8].

EVs can be classified according to their size and biogenesis (Table 1). Three distinct groups are recognized: large EVs also known as microvesicles, ranging in size from 100 nm to 1 µm (diameter);

small EVs or exosomes, with a diameter from 30 nm to 100 nm and apoptotic bodies ranging in size from 500 nm to 5,000 nm. Microvesicles are produced by the protrusion or outward budding of the plasma membrane into the extracellular space by multiple mechanisms involving evagination of the plasma membrane via the endosomal sorting complex required for transport-I (ESCRT-I) subcomplex [22], Rho family-induced cytoskeletal rearrangements [23], redistribution of phospholipids via intracellular calcium signaling [24], membrane curvature proteins (e.g., ESCRT-III proteins) [25] and phosphatidylserine aggregation [26]. Exosomes are derived from endosomal multivesicular bodies (MVBs) that, upon fusion with the plasma membrane, release intraluminal vesicles into the extracellular environment regulated in part by Rab subfamily of small GTPases (Rab protein) [27, 28]. This process is mediated by two mechanisms: the ESCRT-dependent vesicular pathway and an ESCRT-independent pathway [28, 29]. The first pathway is triggered by protein ubiquitination with ESCRT proteins guiding cargo selection, clustering, and membrane fusion to form intraluminal vesicles. The second pathway is driven by a process involving ceramides and tetraspanins [28, 29]. The last group of vesicles is the apoptotic bodies that are membrane-surrounded fragments of cells undergoing apoptosis [17], which are released from dying cells into the extracellular space [30].

Table 1. Three major types of EVs and their characterization

EVs classification	Size	Biogenesis	Markers	Physical methods
Microvesicles	100–1,000 nm	Plasma membrane budding	Integrins, selectins, and CD40	SEM, TEM, AFM, NTA, TRPS, flow cytometry
Exosomes	30–100 nm	Endosomal MVBs	ESCRT proteins, ALIX, TSG101, HSP90, tetraspanins (CD81, CD63, CD9), Rab proteins, flotillin	SEM, TEM, Cryo-EM, AFM, DLS, NTA, TRPS, SEA, flow cytometry
Apoptotic bodies	500–5,000 nm	Membrane-surrounded cell fragments	Annexin V, thrombospondin, C3b	SEM, TEM, flow cytometry

SEM: scanning electron microscopy; TEM: transmission electron microscopy; AFM: atomic force microscopy; NTA: nanoparticle tracking analysis; TRPS: tunable resistive pulse sensing; Cryo-EM: cryo-electron microscopy; DLS: dynamic light scattering; SEA: single EV analysis; C3b: activated fragment of complement component C3; ALIX: apoptosis-linked gene 2-interacting protein X; TSG101: tumor susceptibility gene 101; HSP90: heat shock protein 90

To characterize and classify the EVs, specific markers have been identified, e.g., tetraspanins in exosomes and integrins in microvesicles. In addition, multiple analytical methods, notably electron microscopy and size determination, have been used to identify different types of EVs (see [Table 1](#)) [31].

EVs mediate cell-to-cell communication in MAFLD

In recent years, increasing evidence has been collected on the role of EVs in MAFLD and many studies support the hypothesis that EVs are involved in inflammation and fibrosis in MAFLD [7]. The excessive lipid accumulation can cause lipotoxicity in hepatocytes, triggering pro-inflammatory signaling and cell death [32]. Furthermore, in response to stress hepatocytes release large numbers of EVs, leading to the activation of non-parenchymal inflammatory cells in the liver, further promoting inflammation and fibrosis [29]. This is supported by the fact that in patients with NASH increased levels of circulating EVs have been observed [33].

The liver contains multiple cell types, including parenchymal cells (hepatocytes) and non-parenchymal cells (NPCs) such as HSCs, liver-specific macrophages, or KCs and liver sinusoidal endothelial cells (LSECs). Effective communication between different liver cell types ensures normal liver function and maintains hepatic homeostasis [34]. Likewise, impaired intercellular communication is involved in the pathogenesis of various liver diseases, including MAFLD [35]. EVs have been demonstrated to play an important role in intercellular crosstalk in the liver also in the context of MAFLD [34–36]. In this section, we will discuss the role of EVs in intercellular communication in the context of MAFLD ([Figure 1](#) and [Table 2](#)).

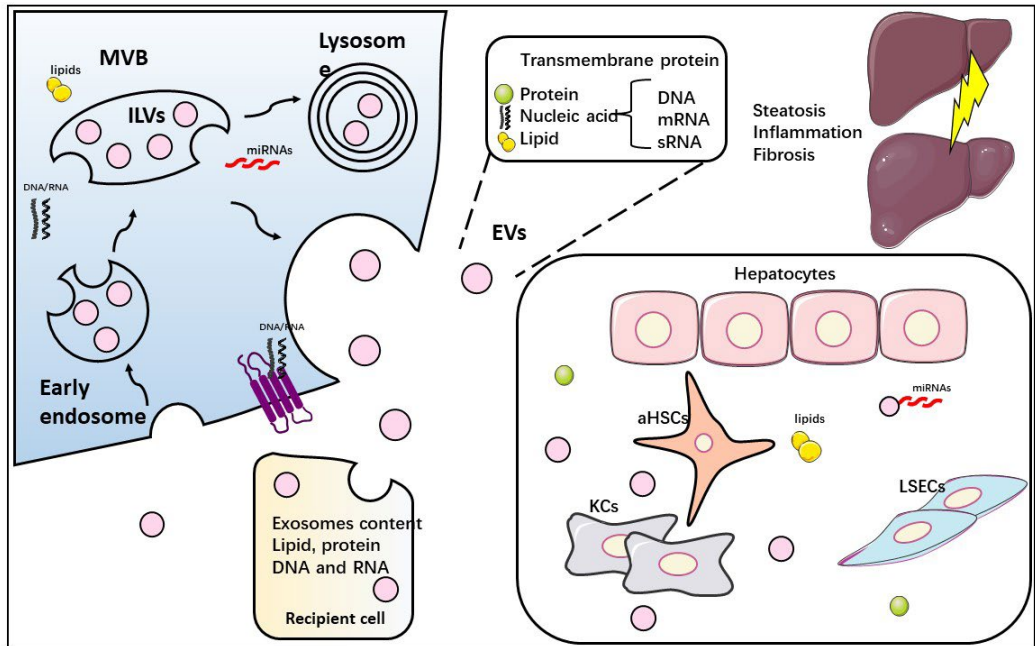


Figure 1. Schematic depiction of biosynthesis and release of exosomes and their role in cell-to-cell communication in the liver. Early endosomes are formed from the plasma membrane and subsequently transformed into MVBs (MVBs are formed by the invagination of endosomal membranes). They then fuse with lysosomes or with the plasma membrane. The resulting vesicles are degraded by lysosomes or secreted as exosomes. The cargo of exosomes is dependent on the cell type and the state of the cell. In liver injury, stressed hepatocytes release EVs containing DNA, RNA, proteins, and lipids that have a potent pro-inflammatory and pro-fibrogenic effect, activating both KCs as well as aHSCs. ILVs: intraluminal vesicles; aHSCs: activated HSCs; sRNA: small RNA

Table 2. Overview of studies demonstrating EV-mediated cell-to-cell communication in the liver in MAFLD

Cell source	Cargo	Target cell	Associated molecules and/or signaling pathways	Role in MAFLD	References
Hepatocytes	Not evaluated	KCs	Not evaluated	Pro-inflammatory	[37]
	TRAIL	Bone marrow-derived macrophages (BMDM) and THP-1 macrophages	CHOP-DR5-Caspase-3/ Caspase-8-ROCK1 NF-κB RIP1	Promotes M1 polarization of BMDM and THP-1 macrophages	[14]
	ITGβ1	THP-1 macrophages	Not evaluated	Promotes THP-1 inflammatory phenotype and increases THP-1-LSEC adhesion	[38]
	Ceramides	Macrophages	IRE1α-XBP1-Sptlc1 and Sptlc2	Promotes activation and recruitment of macrophages	[11, 39]
	S1P	Macrophages	SphK1 and SphK2 S1P ₁ receptor	Promotes activation and recruitment of macrophages	[40]
	mtDNA	KCs and RAW 264.7 cells	TLR-9	Promotes activation of KCs and RAW 264.7 cells	[41, 42]
HSCs	Proteins	Not evaluated	ECM components, proteasome, collagens, vesicular transport, metabolic enzymes, ribosomes, and chaperones	Regulation of HSC activation and fibrogenic pathways <i>in vivo</i>	[43]
	Fibrogenic proteins	HSCs, macrophages, and endothelial cells (ECs)	PDGF; SHP2; mTOR signaling; ROCK signaling	Autophagy plays a critical role in the release of pro-fibrogenic EVs and HSC function	[44]

Table 2. Overview of studies demonstrating EV-mediated cell-to-cell communication in the liver in MAFLD (*continued*)

Cell source	Cargo	Target cell	Associated molecules and/or signaling pathways	Role in MAFLD	References
LSECs	IGFBP7; ADAMTS1	Fibrotic Th17 cells	Histone deacetylase 2 (HDAC2) DNA methyltransferase 1 (DNMT1)	Epigenetic dysregulation of LSEC exacerbates liver disease	[45]
	Tropomyosin-1 (TPM1)	Rat hepatocytes, HSCs, human macrophages	ESCRT-dependent pathway (Mvb12-a, Mvb12-b), lipid-rafts structures such as Flot2, tetraspanins CD9 and CD81, and the Rab3 small GTPase	Deactivation of HSCs	[46]

TRAIL: tumor necrosis factor-related apoptosis-inducing ligand; ITG β 1: integrin β 1; IGFBP7: insulin-like growth factor-binding protein-7; Th17: T helper type 17; ROCK1: Rho-associated, coiled-coil-containing protein kinase 1; NF- κ B: nuclear factor- κ B; RIP1: receptor-interacting protein kinase 1; IRE1 α : inositol-requiring transmembrane kinase/endoribonuclease 1 α ; SphK1: sphingosine kinase 1; TLR-9: Toll-like receptor-9; PDGF: platelet-derived growth factor; SHP2: src-homology-2-domain-containing PTP 2; mTOR: mechanistic target of rapamycin; CHOP: C/EBP homologous protein; DR5: death receptor 5; ADAMTS1: ADAM metalloproteinase with thrombospondin type 1 motif 1; XBP1: X-box binding protein 1; Sptlc1: serine palmitoyltransferase long chain base subunit 1; S1P: sphingosine 1-phosphate; Flot2: flotillin 2; Mvb12-a/b: multivesicular body subunit 12A/B

Hepatocyte-derived EVs in MAFLD

Hepatocytes are the most abundant cells within the liver [34] and play an important role in liver-specific functions like glycogen metabolism, bile secretion, lipid metabolism, steroid hormone metabolism, plasma protein synthesis, and detoxification.

Many studies have shown that EVs released from lipotoxic hepatocytes promote inflammation in MAFLD [11, 14, 37–42, 47–49]. Moreover, hypoxic conditions, present in MAFLD, aggravate the pro-inflammatory effect of EVs derived from lipotoxic hepatocytes on KCs [37]. Palmitate and lysophosphatidyl choline (LPC) treatment increased the release of EVs enriched with TRAIL from hepatocytes. This release was mediated by the CHOP-DR5-Caspase-8/Caspase-3-ROCK1 signaling cascade. TRAIL-enriched EVs induced the activation of various types of macrophages. On the one hand, they change the polarization of mouse BMDM towards a classical M1 macrophage phenotype with increased expression of interleukin-1 β (IL-1 β) and IL-6 in a DR5-dependent manner. On the other hand, human THP-1 macrophages treated with TRAIL-containing EVs were activated in an NF- κ B and RIP1-dependent manner, and activation was inhibited by RIP1 inhibitor or loss of RIP1. Moreover, the application of a ROCK1 inhibitor in murine NASH models reduced the release of proinflammatory EVs and thus ameliorated NASH [14]. LPC-treated hepatocytes release active ITG β 1-enriched EVs that were shown to induce macrophage chemotaxis. ITG β 1-containing EVs mediate THP-1 cell adhesion to LSECs [38]. In addition, EVs enriched with ITG β 1 promote the inflammatory phenotype of monocytes, underscoring their proinflammatory role in MAFLD [38]. Several reports highlighted the role of lipotoxic ceramides and S1P in macrophage chemotaxis and inflammation in MAFLD [11, 39, 40]. EVs from lipotoxic hepatocytes enriched in ceramides induce the recruitment of macrophages into the liver and promote inflammation. The release of EVs enriched in ceramides is mediated by IRE1 α and decreasing levels of IRE1 α in hepatocytes decrease their release of EVs [11]. EVs released from palmitic acid-treated hepatocytes also promote the recruitment of macrophages into the liver via the interaction of exosomal S1P with the S1P receptor in macrophages. Inactivation of S1P signaling by preventing sphingosine phosphorylation via inhibition of SphK1 and SphK2 or deletion of the S1P receptor in macrophages results in the loss of the chemoattractive effects of EVs derived from palmitate-treated hepatocytes on macrophages [40].

Finally, EVs from hydrogen peroxide-damaged hepatocytes are enriched in mtDNA that increase the expression of pro-inflammatory cytokines on murine RAW 264.7 cells in a TLR-9 dependent manner, whereas IL-22Fc pretreatment of these hepatocytes decrease the pro-inflammatory potential of these EVs [42].

EVs released from non-parenchymal liver cells in MAFLD

Hepatic fibrosis is characterized by the excessive deposition of ECM in the liver. The main producers of ECM in the liver are aHSCs. The HSCs reside in the space of Disse. Under normal conditions, HSCs are quiescent pericyte-like cells. In chronic liver injury, HSCs transdifferentiate into an activated phenotype, characterized by loss of lipid stores, increased synthesis and secretion of ECM components, and increased proliferation. Activation is also associated with a 4.5-fold increase in EV production [43, 44]. Recent studies have shown that exosomes containing specific miRNAs from lipotoxic hepatocytes can promote stellate cell activation, proliferation, and migration [13, 15]. A comparative proteomic analysis of mouse HSC-derived EVs indicated that EVs from HSCs may play a key role in the regulation of HSC function and activation *in vivo* [43]. The internalization of hepatocyte-derived EVs by HSCs is dependent on the presence of either vanin-1 (VNN-1) or fibronectin (FN) [43, 50, 51]. Once internalized, these EVs stimulate the expression of pro-fibrotic mediators in HSCs, as also observed in our studies: hypoxic, palmitate-treated HepG2 cells release EVs that increase the expression of transforming growth factor- β (TGF- β), connective tissue growth factor (CCN2), collagen type 1 and α -smooth muscle actin (α -SMA) in HSCs [43, 52]. In contrast, EVs secreted by quiescent HSCs contain high levels of Twist 1, which drives miR-214 expression and results in the suppression of fibrogenic signaling. It is therefore relevant to consider the activation state when investigating HSC-derived EVs [43, 53, 54]. EVs can also act in an autocrine manner on HSCs: CCN2 promotes activation of HSCs and was detected in EVs released by HSCs [55].

LSECs are a major component of liver NPCs [56, 57]. Whereas vascular ECs form a continuous lining in arteries and veins, LSECs form a discontinuous layer in liver sinusoids. Moreover, in contrast to vascular ECs, LSECs are fenestrated [45, 46]. In chronic liver diseases like NASH, the LSECs dedifferentiate and form a continuous lining and lose their fenestrae. One study [45], using single-cell omics analysis, demonstrated that epigenetic dysregulation of LSECs promotes the dedifferentiation of these cells from a sinusoidal to a vascular phenotype. This was associated with increased production of EVs containing IGFBP7 and ADAMTS1, which further promoted the recruitment of pro-fibrogenic Th17 cells to the liver and exacerbation of hepatic fibrosis [45]. Investigation of the proteome of LSEC-derived EVs during the progression of chronic liver disease demonstrated that LSEC-derived EVs contain high levels of angiocrine effectors and tropomyosin 1. Subsequently, tropomyosin was shown to have a de-activating effect on HSCs [46].

KCs are the liver-specific macrophages. They play an important role as inflammatory cells in the progression of chronic liver diseases and secrete immunosuppressive factors and metabolites, however, not much is known about the role of KC-derived EVs in MAFLD [58, 59].

Signaling pathways regulated by EVs in the context of MAFLD

Recent evidence suggests that EVs are important regulators of the inflammatory response (Table 2) and it has been proposed that proinflammatory EVs contribute to MAFLD progression [33]. The role of EVs in the pathophysiological mechanisms during MAFLD progression is discussed in this section.

Hepatic steatosis and lipotoxicity

Steatosis is the accumulation of lipids in hepatocytes and is the earliest and defining feature of MAFLD. Studies in mice have shown that EVs are involved in the response to lipid overload in the liver and that the liver can control lipid remodeling in adipose tissue via EVs in conditions of steatosis [60]. Recent findings suggest that EVs can be mediators of lipotoxicity during MAFLD [32]. Lipid accumulation and in particular lipotoxicity can trigger cell signaling pathways that favor the release of EVs from hepatocytes [7]. *In vitro* studies using primary mouse hepatocytes and human Huh7 cells have shown that palmitate activates DR5 signaling, which induces the release of EVs that promote the inflammatory phenotype of macrophages [14]. Lipotoxicity induced by saturated fatty acids like palmitate increases membrane lipid saturation causing endoplasmic reticulum (ER) stress. Lipotoxicity has also been shown to induce membrane alterations in the EV membrane and EV lipid cargo, suggesting that these EVs might be useful to track lipotoxic stress in the liver [61].

NASH, the inflammatory stage of MAFLD, is characterized by increased levels of pro-inflammatory and chemotactic cytokines, oxidative stress, mitochondrial and ER stress, lipid peroxidation, and eventually cell death. In particular oxidative stress is considered a central mechanism in the pathophysiology of MAFLD [62]. Lipotoxicity can induce oxidative stress and inflammatory response by increasing lipid peroxidation and the formation of neo-epitopes such as oxidation-specific epitopes (OSEs) [63]. These OSEs have been demonstrated to be present in EVs as well as in circulating lipoproteins and can contribute to the activation of other liver cells such as KCs, promoting inflammation [64]. Lipotoxic stress is also related to organelle dysfunction, including ER stress and mitochondrial dysfunction. In a murine model of diet-induced NASH, mitochondrial stress and oxidative stress correlate with increased release of EVs, containing oxidatively damaged mitochondrial particles. These EVs can contribute to an antioxidant response to protect from acute oxidative stress but they can also be deleterious by enhancing the inflammatory response [65].

Inflammatory response

The innate immune response plays an important role in inflammation. Classical innate immune receptors include pattern recognition receptors (PRRs) like the TLRs, which detect damage-associated molecular patterns (DAMPs). Activation of TLRs has been demonstrated in MAFLD [66]. mtDNA in EVs released from damaged hepatocytes activates TLR-9 on target cells [41, 42]. Loss of TLR-9 attenuates NASH and specific deletion of TLR-9 in KCs reduced the expression of inflammatory cytokines such as IL- β , IL-6, and tumour necrosis factor α (TNF α) in a high-fat diet (HFD) model of MAFLD [41]. Hepatocytes from HFD hepatocytes release EVs enriched in mtDNA, resulting in TLR-9 activation and increased expression of the TLR-9 downstream target TNF α in KCs. Plasma from obese patients with liver injury contained higher levels of mtDNA and TLR-9 ligand activity, suggesting that mtDNA from damaged hepatocytes and TLR-9 activation is involved in the inflammatory response in NASH, possibly mediated via EVs [41]. EVs derived from hepatocytes mediate the activation of TLR-3 in HSCs. This may aggravate fibrosis by enhancing IL-17 production by $\gamma\delta$ T cells at the early stages of liver fibrosis [67].

Other pro-inflammatory PRRs associated with the development of MAFLD include the inflammasome sensor molecule typically a NOD-like receptor (NLR) family pyrin domain containing protein 3 (NLRP3), which is a component of inflammasomes and has been shown to promote inflammation and fibrogenesis during the progression of MAFLD [68]. EVs modulate the activation of NLRP3 [69]. In the choline deficient L-amino acid (CDA) and HFD mice models of MAFLD, altered levels of proteins related to cell death, stress, and angiogenesis were demonstrated in plasma-derived EVs [69]. Other studies showed the enrichment of miR-7 in hepatic EVs that promote lysosomal membrane barrier permeability, inducing the activation of NLRP3 and microvascular hyperpermeability [70]. Furthermore, it has been shown that steatotic hepatic cells release EVs that induce the expression of NLRP3 and inflammasome activity in target cells, including macrophages. Moreover, in this study increased hepatic expression of NLRP3 was detected in mice with methionine-choline deficient diet-induced NASH [71].

In addition to macrophages, ECs are also a target for EVs. It has been demonstrated that human umbilical vein ECs (HUVECs) can internalize EVs derived from palmitic acid-treated human HepG2 cells and this process appeared to be dependent on the presence of lipid rafts and VNN-1. In fact, VNN-1 is not only involved in the internalization of EVs, but also in EC migration [72]. Another study demonstrated uptake of EVs derived from palmitic acid-treated human Huh7 cells by HUVEC cells, leading to increased expression of inflammation-related genes in HUVECs like *E-selectin*, intercellular adhesion molecule-1 (*ICAM-1*), vascular cell adhesion molecule-1 (*VCAM-1*), *IL-1 β* and matrix metalloproteinase-1 (*MMP-1*) [73].

To achieve novel interventional and non-interventional strategies to treat patients with MAFLD, a thorough understanding of the mechanisms leading to MAFLD is necessary. It is clear from the available evidence that both paracrine and autocrine communication mediated by EVs play an important role in the pathogenesis of MAFLD. Therefore, more studies, focusing on analyzing the content and effects of the cargo of EVs are necessary.

Diagnostic and therapeutic application

EVs in MAFLD: interventional implications

Research on EVs has given us insights into the possible application of EVs as diagnostic tools. In 2011, the presence of small EVs in plasma from patients with active hepatitis C was reported [74]. Interestingly, these microparticles were derived from CD4⁺ and CD8⁺ T cells. Moreover, these EVs induced the expression of several metalloproteases such as MMP-1, MMP-3, MMP-9, and MMP-13 and reduced the expression of procollagen α 1(I) mRNA in the human HSC cell line LX-2 [74].

In 2012, it was demonstrated that patients with NASH have increased plasma levels of circulating EVs derived from invariant natural killer T (iNKT) cells and monocytes (CD14⁺), which were also postulated to play a role in the pathogenesis of NASH [10]. Taken together, these results indicate that the amount and content of circulating EVs can provide information on the clinical status.

Since the pathophysiology of MAFLD involves hepatocyte lipotoxicity and concomitant pro-inflammatory and profibrogenic responses, the macrophage (KC) and the HSC are attractive targets for intervention. Adipose-derived stem cells (ADSCs) secrete EVs that promote M2 polarization in macrophages (high Arg-1 phenotype), with beneficial effects on inflammation, systemic insulin resistance, dyslipidemia, and hepatic steatosis in an *in vivo* model of NASH [75]. Stem cells (SCs) may be a suitable medium to deliver EVs to target cells. SCs are cell types that can self-renew and differentiate into other cell types. Furthermore, it has been proposed that EVs derived from SCs may induce regenerative programs in injured organs such as the liver by transcriptional or translational modification [76]. EVs from human liver SC (HLSC; HLSC-EVs) improved liver function in a murine diet-induced NASH model [77]. In addition, the transcriptomic analysis demonstrated 29 pro-fibrogenic genes (α -SMA, Col1 α 1, Ltbp-1, and TGF- β 1) and pro-inflammatory genes [TNF α , IL-1 β , interferon- γ (IFN- γ)] to be upregulated in hepatic tissue in NASH. Interestingly, the expression of 28 of these genes was significantly reduced after treatment with the HLSC-EVs [77]. Similarly, in a genetic model of NASH (Mc4r knockout mice), treatment with human adipose tissue-derived mesenchymal SC EVs (AT-MSC-EVs) significantly attenuated liver fibrosis. Moreover, different metabolic parameters such as triglyceride (TG), free fatty acids (FFAs), aspartate transaminase (AST), alanine transaminase (ALT), and albumin (ALB), glucose (Glu) or total bilirubin (T-bil) levels were also improved [78].

Since EVs have been shown to have therapeutic value in the resolution of MAFLD and since miRNAs are components of the cargo of EVs and miRNAs are involved in various biological functions, we will discuss the application of EV-derived miRNAs in more detail in the next section.

miRNAs loaded EVs as the potential therapeutic application in MAFLD

miRNAs have been implicated in the epigenetic regulation of cell proliferation, fat metabolism, and cell death [79]. Moreover, the role of miRNAs in liver inflammation, fibrosis, and cirrhosis has been acknowledged [80]. miRNAs are detected in biological fluids like blood, urine, tears, saliva, and breastmilk and can be used as diagnostic biomarkers, e.g., in type 2 diabetes, HCC, and MAFLD [81, 82]. miR-122 is released from damaged hepatocytes in alcoholic liver disease, viral hepatitis, and drug-induced liver injury [83–85]. miRNAs constitute an important class of gene-regulatory molecules in both humans and mice [86]. They are small, non-coding RNAs (18–25 nucleotides) and can be incorporated into exosomes and secreted into the extracellular environment [87]. miRNAs are involved in a number of biological functions such as cellular differentiation, proliferation, metabolism, and apoptosis [88, 89]. They regulate gene expression in a posttranscriptional manner via binding complementary mRNA transcripts, suppressing gene expression via mRNA degradation and repression of translation. miRNAs not only interact with mRNAs but also with long non-coding RNAs (lncRNAs), pseudogenes, and circular RNAs (circRNAs) [90]. Several miRNAs have been detected in liver-derived exosomes, e.g., miR-21, miR-125, miR-146, miR-192, miR-223, and miR-378 [91]. Exosomal miRNAs originating from lipotoxic hepatocytes are also crucial in inflammation during the progression of MAFLD [47, 48]. EVs from lipotoxic hepatocytes contain miR-192-5p. This miRNA was able to polarize THP-1 macrophages towards the pro-inflammatory M1 phenotype via targeting the 3' untranslated region (3' UTR) of Rictor and suppressing its expression, subsequently inhibiting

downstream protein kinase B/forkhead box O1 (Akt/FoxO1) factors. Overexpression of miR-192-5p and lack of Rictor in THP-1 cells further underscored the key role of miR-192-5p in M1 macrophage polarization [47]. Oxidized low-density lipoprotein (ox-LDL) and cholesterol impaired degradation of MVBs in Huh7 cells, leading to increased release of EVs containing miR-122-5p, promoting M1 polarization of THP-1 macrophages [49]. The M1 polarization proved to be miR-122-5p dependent since downregulation of miR-122-5p in Huh7 cells abrogated the exosome-induced M1 polarization of THP-1 macrophages [49]. miR-128-3p was identified as the exosomal messenger that reduced the expression of the stellate cell quiescence marker peroxisome proliferator-activated receptor- γ (PPAR- γ) [15].

The expression of specific miRNAs was increased in EVs released from hepatocytes treated with toxic lipids, including miR-24, 19b, 34a, 122, and 192. High levels of exosomal miR-192 promote HSC proliferation and activation [13]. Increased circulating exosomal miR-192 and miR-122 in MAFLD patients further demonstrate that the miRNA profile may be a representative marker in the diagnosis of fibrosis in MAFLD [13].

Several studies indicated the therapeutic potential of exosomal miRNAs. Thus, miR-627-5p overexpressed in EVs from human umbilical cord-derived mesenchymal SCs (HUC-MSC-EVs) effectively reduced serum levels of ALT, AST, TG, and total cholesterol as well as lipid deposition in a high fat-high fructose diet rat model of NASH [92]. Moreover, in *in vitro* studies, miR-627-5p HUC-MSC-EVs improved cell viability and lipid homeostasis and inhibited apoptosis and fatty acid synthesis in palmitate-treated L-02 cells [92]. Another relevant miRNA is miRNA-96-5p. Its presence in EVs from bone marrow-derived mesenchymal SCs (BM-MSC-EVs) downregulated fatty acid synthesis and lipid uptake in an HFD rat model of NASH, whereas mitophagy genes [*Parkin*, phosphatase and tensin homologue (PTEN)-induced putative kinase 1 (*PINK1*), unc-51 like autophagy activating kinase 1 (*ULK1*), B-cell lymphoma 2/adenovirus E1B 19 kDa protein-interacting protein 3 (*BNIP3L*)] and autophagic genes [autophagy related gene 5 (*ATG5*), *ATG7*, *ATG12*] were induced [93]. Liver-derived EVs also have an important role in lipid metabolism. Wu et al. [94] demonstrated, using a murine diet model of MAFLD, that miR-130a-3p regulates Glu metabolism, suppressing PH domain leucine-rich repeat protein phosphatase 2 (PHLPP2) expression and activating Akt-A160-Glu transporter type 4 (GLUT4) signaling in adipocytes, thus increasing Glu uptake. The role of the liver in regulating lipid metabolism, in particular, whether hepatic EVs containing miRNAs play a role in the communication between liver and adipose tissue, was also investigated in a murine MAFLD model [60]. In this study it was shown that Let-7e-5p enhances adipocyte lipid deposition by increasing lipogenesis and inhibiting lipid oxidation via PPAR- γ co-activator 1-alpha (PGC1 α), confirming that the liver orchestrates an integrated response involving EVs to modulate lipid metabolism.

In conclusion, EVs as an interventional therapy still represent a largely unexplored field. Nevertheless, promising results in the treatment of MAFLD as well as other liver diseases have been reported. Due to the heterogeneity of EVs, diverse effects on target cells have been reported. This stresses the need to standardize the use of EVs in therapeutic applications (isolation, characterization, standardization of administration, etc.) and to take into account the type of the EV-releasing cell, the state of the releasing cell, the stimulus that led to their secretion, their cargo, as well as the target cell, in order to be able to carry out clinical trials in the near future (see Table 3).

Table 3. Diagnostic and therapeutic potential of EVs in MAFLD

Cell source	Target cell	EVs markers	Biological effects	References
Hepatocytes	HSCs	TLR-3	Pro-fibrotic response at early stages of liver fibrosis	[67]
Liver (circulating EVs)	Not evaluated	miR-122, miR-192	Increased levels of circulating EVs are associated with the severity of MAFLD	[69]
Hepatocytes	ECs (HUVECs)	VNN-1	FFA-treated hepatocytes released EVs with proangiogenic effects	[72]

Table 3. Diagnostic and therapeutic potential of EVs in MAFLD (*continued*)

Cell source	Target cell	EVs markers	Biological effects	References
ECs	HSCs	FN, integrin	HSC adherence and migration	[51]
HSCs	Not evaluated	Twist 1	Limits the magnitude of the fibrotic response	[54]
CD4 ⁺ and CD8 ⁺ T from hepatitis C patient	HSCs	Not evaluated	Induce the upregulation of fibrolytic genes in HSCs	[74]
Activated and quiescent HSCs	Not evaluated	CCN2	HSC activation	[55]
Adipocytes	HepG2 cells	Adipokines	Modulate insulin resistance	[95]
ADSCs	KCs	Not evaluated	Polarize KCs toward Arg-1 M2 phenotype	[75]
AMSCs	KCs	Not evaluated	Decreased number of Kupffer cells and decreased expression of inflammatory genes in NASH rat model	[96]
HLSC	NASH rats	Not evaluated	Improved liver function with downregulation of pro-inflammatory and profibrotic genes	[78]
Human adipose tissue-derived mesenchymal SCs	NASH mice	Not evaluated	Decreased liver fibrosis	[77]
Hepatocytes	Macrophages	miR-192-5p	Pro-inflammatory	[47, 49]
	THP-1 cells	miR-122-5p		
Hepatocytes	HSCs and LX2 cells	miR-128-3p miR-192	Pro-fibrotic	[13, 15]
Human umbilical cord-derived mesenchymal SCs	Rat NASH model	miR-627-5p	Improved liver function and reduced lipid accumulation	[92]
Hepatocytes	Adipocytes	miR-130a-3p Let-7e-5p	Increased Glu uptake, lipogenesis, and decreased lipid oxidation	[60]
KCs	Huh7 cells	miRNA-29	Anti-HCV	[97]

AMSCs: amnion-derived mesenchymal SCs

Conclusions and future prospects

MAFLD is a major global public health problem. It ranges from simple steatosis to advanced steatohepatitis (NASH) and can progress to fibrosis, cirrhosis, and even HCC. Much progress has been made in recent years to elucidate the mechanisms underlying the pathogenesis of MAFLD. Nevertheless, there is still an urgent need to develop (non-invasive) diagnostic, prognostic and therapeutic tools. The role and application of EVs is an emerging area of research. Not only the pathophysiological role of EVs in diseases is currently being studied, but also their application in diagnosis and as a potential tool for drug delivery.

In our review, we provide an overview of existing literature on EVs and their use as a therapeutic tool in the context of MAFLD. We conclude that EVs are intimately involved in the pathogenesis of MAFLD. They play an important role in cell-to-cell communication, not only within the liver but also at more distant sites (e.g., adipose tissue). EVs are crucial in the transfer of biological information and/or activity (e.g., mRNAs, miRNAs, proteins, enzymes, etc.) from the cell releasing the EV to the cell internalizing the EV. Since the cargo is dependent on the type of releasing cell and the state of these cells (e.g., lipotoxic vs. normal hepatocytes), the cargo and effect of EVs on targets cell will vary according to the type and state of the releasing cell.

There are still gaps in our knowledge on the role of EVs in general and in the context of MAFLD in particular. The effect of EVs from extra-hepatic tissues on liver cells in the context of MAFLD is largely unexplored (e.g., EVs from adipose tissue or atherosclerotic ECs). The use of SC-derived EVs as treatment needs to be further investigated and needs confirmation in clinical trials. The application of EVs in clinical trials also needs further exploration on the preparation, characterization, and administration of EVs as well as the manipulation of the cargo of EVs, e.g., by overexpressing specific therapeutic components (miRNAs). In this regard, it is also important to develop methods to identify the cellular source of EVs

found in circulating plasma. So far, identification of the cellular source, using cell-specific markers in EVs, is only possible to some extent. Based on the existing knowledge and the high potential of EVs, as summarized in this review, we expect that research on EVs and their therapeutic application will expand significantly in the near future.

Abbreviations

ADAMTS1: ADAM metalloproteinase with thrombospondin type 1 motif 1

aHSCs: activated hepatic stellate cells

ATG5: autophagy related gene 5

BMDM: bone marrow-derived macrophages

CCN2: connective tissue growth factor

CHOP: C/EBP homologous protein

DR5: death receptor 5

ECM: extracellular matrix

ECs: endothelial cells

ER: endoplasmic reticulum

ESCRT-I: endosomal sorting complex required for transport-I

EVs: extracellular vesicles

Glu: glucose

HCC: hepatocellular carcinoma

HFD: high-fat diet

HLSC: human liver stem cell

HSCs: hepatic stellate cells

HUVECs: human umbilical vein endothelial cells

IGFBP7: insulin-like growth factor-binding protein-7

IL-1 β : interleukin-1 β

IRE1 α : inositol-requiring transmembrane kinase/endoribonuclease 1 α

ITG β 1: integrin β 1

KCs: Kupffer cells

LSECs: liver sinusoidal endothelial cells

MAFLD: metabolic dysfunction-associated fatty liver disease

miRNAs: microRNAs

MMP-1: matrix metalloproteinase-1

mRNAs: messenger RNAs

mtDNA: mitochondrial DNA

MVBs: multivesicular bodies

NASH: non-alcoholic steatohepatitis

NF- κ B: nuclear factor- κ B

NLRP3: NOD-like receptor family pyrin domain containing protein 3

Rab protein: Rab subfamily of small GTPases

RIP1: receptor-interacting protein kinase 1

ROCK1: Rho-associated, coiled-coil-containing protein kinase 1

S1P: sphingosine 1-phosphate

SCs: stem cells

SphK1: sphingosine kinase 1

Th17: T helper type 17

TLR-9: Toll-like receptor-9

TNF α : tumor necrosis factor α

VNN-1: vanin-1

Declarations

Author contributions

ZW contributed to the abstract, sections 1, 2, 5.2, conclusions and Figure 1. MX contributed to the Tables 1, 2 and 3, sections 2 and 3. SSS contributed to sections 1 and 2. MCTA contributed to sections 2, 4, and 5.1. MMA contributed to section 4. JAO contributed to section 4.1 and Table 3. JW contributed to section 3.2 and Table 2. MA, SS, LPB, FGvV, HB, and HM contributed to overall lay-out and content of the manuscript. All authors discussed the results and commented on the manuscript.

Conflicts of interest

The authors declare that they have no known competing financial interests of personal relationships that could have appeared to influence the work reported in this research paper.

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Not applicable.

Consent to participate

Not applicable.

Consent to publication

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References

1. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology*. 2016;64:73–84.
2. Brunt EM. Pathology of nonalcoholic fatty liver disease. *Nat Rev Gastroenterol Hepatol*. 2010;7:195–203.
3. Lakhani HV, Sharma D, Dodrill MW, Nawab A, Sharma N, Cottrill CL, et al. Phenotypic alteration of hepatocytes in non-alcoholic fatty liver disease. *Int J Med Sci*. 2018;15:1591–9.

4. Kutlu O, Kaleli HN, Ozer E. Molecular pathogenesis of nonalcoholic steatohepatitis- (NASH-) related hepatocellular carcinoma. *Can J Gastroenterol Hepatol*. 2018;2018:8543763.
5. Chargaff E, West R. The biological significance of the thromboplastic protein of blood. *J Biol Chem*. 1946;166:189–97.
6. Ratajczak J, Miekus K, Kucia M, Zhang J, Reca R, Dvorak P, et al. Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia*. 2006;20:847–56.
7. Hernández A, Arab JP, Reyes D, Lapitz A, Moshage H, Bañales JM, et al. Extracellular vesicles in NAFLD/ALD: from pathobiology to therapy. *Cells*. 2020;9:817.
8. Hafiane A, Daskalopoulou SS. Extracellular vesicles characteristics and emerging roles in atherosclerotic cardiovascular disease. *Metabolism*. 2018;85:213–22.
9. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol*. 2013;200:373–83.
10. Kornek M, Lynch M, Mehta SH, Lai M, Exley M, Afdhal NH, et al. Circulating microparticles as disease-specific biomarkers of severity of inflammation in patients with hepatitis C or nonalcoholic steatohepatitis. *Gastroenterology*. 2012;143:448–58.
11. Kakazu E, Mauer AS, Yin M, Malhi H. Hepatocytes release ceramide-enriched pro-inflammatory extracellular vesicles in an IRE1 α -dependent manner. *J Lipid Res*. 2016;57:233–45.
12. Murakami Y, Toyoda H, Tanahashi T, Tanaka J, Kumada T, Yoshioka Y, et al. Comprehensive miRNA expression analysis in peripheral blood can diagnose liver disease. *PLoS One*. 2012;7:e48366.
13. Lee YS, Kim SY, Ko E, Lee JH, Yi HS, Yoo YJ, et al. Exosomes derived from palmitic acid-treated hepatocytes induce fibrotic activation of hepatic stellate cells. *Sci Rep*. 2017;7:3710.
14. Hirsova P, Ibrahim SH, Krishnan A, Verma VK, Bronk SF, Werneburg NW, et al. Lipid-induced signaling causes release of inflammatory extracellular vesicles from hepatocytes. *Gastroenterology*. 2016;150:956–67.
15. Povero D, Panera N, Eguchi A, Johnson CD, Papouchado BG, de Araujo Horcel L, et al. Lipid-induced hepatocyte-derived extracellular vesicles regulate hepatic stellate cell via microRNAs targeting PPAR- γ . *Cell Mol Gastroenterol Hepatol*. 2015;1:646–63.e4.
16. Doyle LM, Wang MZ. Overview of extracellular vesicles, their origin, composition, purpose, and methods for exosome isolation and analysis. *Cells*. 2019;8:727.
17. Zha QB, Yao YF, Ren ZJ, Li XJ, Tang JH. Extracellular vesicles: an overview of biogenesis, function, and role in breast cancer. *Tumour Biol*. 2017;39:1010428317691182.
18. Kowal J, Arras G, Colombo M, Jouve M, Morath JP, Primdal-Bengtson B, et al. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proc Natl Acad Sci U S A*. 2016;113:E968–77.
19. Akbar N, Azzimato V, Choudhury RP, Aouadi M. Extracellular vesicles in metabolic disease. *Diabetologia*. 2019;62:2179–87.
20. Kanada M, Bachmann MH, Hardy JW, Frimannson DO, Bronsart L, Wang A, et al. Differential fates of biomolecules delivered to target cells via extracellular vesicles. *Proc Natl Acad Sci U S A*. 2015;112:E1433–42.
21. Elzanowska J, Semira C, Costa-Silva B. DNA in extracellular vesicles: biological and clinical aspects. *Mol Oncol*. 2021;15:1701–14.
22. Nabhan JF, Hu R, Oh RS, Cohen SN, Lu Q. Formation and release of arrestin domain-containing protein 1-mediated microvesicles (ARMMs) at plasma membrane by recruitment of TSG101 protein. *Proc Natl Acad Sci U S A*. 2012;109:4146–51.
23. Latifkar A, Hur YH, Sanchez JC, Cerione RA, Antonyak MA. New insights into extracellular vesicle biogenesis and function. *J Cell Sci*. 2019;132:jcs222406.
24. Abels ER, Breakefield XO. Introduction to extracellular vesicles: biogenesis, RNA cargo selection, content, release, and uptake. *Cell Mol Neurobiol*. 2016;36:301–12.

25. Chiaruttini N, Redondo-Morata L, Colom A, Humbert F, Lenz M, Scheuring S, et al. Relaxation of loaded ESCRT-III spiral springs drives membrane deformation. *Cell*. 2015;163:866–79.
26. Muralidharan-Chari V, Clancy JW, Sedgwick A, D'Souza-Schorey C. Microvesicles: mediators of extracellular communication during cancer progression. *J Cell Sci*. 2010;123:1603–11.
27. Morán L, Cubero FJ. Extracellular vesicles in liver disease and beyond. *World J Gastroenterol*. 2018;24:4519–26.
28. Hirsova P, Ibrahim SH, Verma VK, Morton LA, Shah VH, LaRusso NF, et al. Extracellular vesicles in liver pathobiology: small particles with big impact. *Hepatology*. 2016;64:2219–33.
29. Wu D, Zhu H, Wang H. Extracellular vesicles in non-alcoholic fatty liver disease and alcoholic liver disease. *Front Physiol*. 2021;12:707429.
30. Wickman G, Julian L, Olson MF. How apoptotic cells aid in the removal of their own cold dead bodies. *Cell Death Differ*. 2012;19:735–42.
31. Shao H, Im H, Castro CM, Breakefield X, Weissleder R, Lee H. New technologies for analysis of extracellular vesicles. *Chem Rev*. 2018;118:1917–50.
32. Geng Y, Faber KN, de Meijer VE, Blokzijl H, Moshage H. How does hepatic lipid accumulation lead to lipotoxicity in non-alcoholic fatty liver disease? *Hepatol Int*. 2021;15:21–35.
33. Srinivas AN, Suresh D, Santhekadur PK, Suvarna D, Kumar DP. Extracellular vesicles as inflammatory drivers in NAFLD. *Front Immunol*. 2021;11:627424.
34. Newman LA, Sorich MJ, Rowland A. Role of extracellular vesicles in the pathophysiology, diagnosis and tracking of non-alcoholic fatty liver disease. *J Clin Med*. 2020;9:2032.
35. Devhare PB, Ray RB. Extracellular vesicles: novel mediator for cell to cell communications in liver pathogenesis. *Mol Aspects Med*. 2018;60:115–22.
36. Kostallari E, Valainathan S, Biquard L, Shah VH, Rautou PE. Role of extracellular vesicles in liver diseases and their therapeutic potential. *Adv Drug Deliv Rev*. 2021;175:113816.
37. Hernández A, Geng Y, Sepúlveda R, Solís N, Torres J, Arab JP, et al. Chemical hypoxia induces pro-inflammatory signals in fat-laden hepatocytes and contributes to cellular crosstalk with Kupffer cells through extracellular vesicles. *Biochim Biophys Acta Mol Basis Dis*. 2020;1866:165753.
38. Guo Q, Furuta K, Lucien F, Gutierrez Sanchez LH, Hirsova P, Krishnan A, et al. Integrin β_1 -enriched extracellular vesicles mediate monocyte adhesion and promote liver inflammation in murine NASH. *J Hepatol*. 2019;71:1193–205.
39. Dasgupta D, Nakao Y, Mauer AS, Thompson JM, Sehrawat TS, Liao CY, et al. IRE1A stimulates hepatocyte-derived extracellular vesicles that promote inflammation in mice with steatohepatitis. *Gastroenterology*. 2020;159:1487–503.e17. Erratum in: *Gastroenterology*. 2022;162:1363–5.
40. Liao CY, Song MJ, Gao Y, Mauer AS, Revzin A, Malhi H. Hepatocyte-derived lipotoxic extracellular vesicle sphingosine 1-phosphate induces macrophage chemotaxis. *Front Immunol*. 2018;9:2980.
41. Garcia-Martinez I, Santoro N, Chen Y, Hoque R, Ouyang X, Caprio S, et al. Hepatocyte mitochondrial DNA drives nonalcoholic steatohepatitis by activation of TLR9. *J Clin Invest*. 2016;126:859–64.
42. Hwang S, He Y, Xiang X, Seo W, Kim SJ, Ma J, et al. Interleukin-22 ameliorates neutrophil-driven nonalcoholic steatohepatitis through multiple targets. *Hepatology*. 2020;72:412–29.
43. Li X, Chen R, Kemper S, Brigstock DR. Dynamic changes in function and proteomic composition of extracellular vesicles from hepatic stellate cells during cellular activation. *Cells*. 2020;9:290.
44. Gao J, Wei B, de Assuncao TM, Liu Z, Hu X, Ibrahim S, et al. Hepatic stellate cell autophagy inhibits extracellular vesicle release to attenuate liver fibrosis. *J Hepatol*. 2020;73:1144–54.
45. Zhang H, Ma Y, Cheng X, Wu D, Huang X, Chen B, et al. Targeting epigenetically maladapted vascular niche alleviates liver fibrosis in nonalcoholic steatohepatitis. *Sci Transl Med*. 2021;13:eabd1206.
46. Manicardi N, Fernández-Iglesias A, Abad-Jordà L, Royo F, Azkargorta M, Ortega-Ribera M, et al. Transcriptomic profiling of the liver sinusoidal endothelium during cirrhosis reveals stage-specific secretory signature. *Cancers (Basel)*. 2021;13:2688.

47. Liu XL, Pan Q, Cao HX, Xin FZ, Zhao ZH, Yang RX, et al. Lipotoxic hepatocyte-derived exosomal microRNA 192-5p activates macrophages through Rictor/Akt/Forkhead box transcription factor O1 signaling in nonalcoholic fatty liver disease. *Hepatology*. 2020;72:454–69.
48. Liu H, Niu Q, Wang T, Dong H, Bian C. Lipotoxic hepatocytes promote nonalcoholic fatty liver disease progression by delivering microRNA-9-5p and activating macrophages. *Int J Biol Sci*. 2021;17:3745–59.
49. Zhao Z, Zhong L, Li P, He K, Qiu C, Zhao L, et al. Cholesterol impairs hepatocyte lysosomal function causing M1 polarization of macrophages via exosomal miR-122-5p. *Exp Cell Res*. 2020;387:111738.
50. Tasin FR, Halder D, Mandal C. Possible therapeutic uses of extracellular vesicles for reversion of activated hepatic stellate cells: context and future perspectives. *Curr Mol Med*. 2022;22:151–64.
51. Wang R, Ding Q, Yaqoob U, de Assuncao TM, Verma VK, Hirsova P, et al. Exosome adherence and internalization by hepatic stellate cells triggers sphingosine 1-phosphate-dependent migration. *J Biol Chem*. 2015;290:30684–96.
52. Hernández A, Reyes D, Geng Y, Arab JP, Cabrera D, Sepulveda R, et al. Extracellular vesicles derived from fat-laden hepatocytes undergoing chemical hypoxia promote a pro-fibrotic phenotype in hepatic stellate cells. *Biochim Biophys Acta Mol Basis Dis*. 2020;1866:165857.
53. Chen L, Charrier A, Zhou Y, Chen R, Yu B, Agarwal K, et al. Epigenetic regulation of connective tissue growth factor by microRNA-214 delivery in exosomes from mouse or human hepatic stellate cells. *Hepatology*. 2014;59:1118–29.
54. Chen L, Chen R, Kemper S, Charrier A, Brigstock DR. Suppression of fibrogenic signaling in hepatic stellate cells by Twist1-dependent microRNA-214 expression: role of exosomes in horizontal transfer of Twist1. *Am J Physiol Gastrointest Liver Physiol*. 2015;309:G491–9.
55. Charrier A, Chen R, Chen L, Kemper S, Hattori T, Takigawa M, et al. Exosomes mediate intercellular transfer of pro-fibrogenic connective tissue growth factor (CCN2) between hepatic stellate cells, the principal fibrotic cells in the liver. *Surgery*. 2014;156:548–55.
56. Shetty S, Lalor PF, Adams DH. Liver sinusoidal endothelial cells-gatekeepers of hepatic immunity. *Nat Rev Gastroenterol Hepatol*. 2018;15:555–67.
57. Xu M, Wang X, Zou Y, Zhong Y. Key role of liver sinusoidal endothelial cells in liver fibrosis. *Biosci Trends*. 2017;11:163–8.
58. Yang P, Zhou W, Li C, Zhang M, Jiang Y, Jiang R, et al. Kupffer-cell-expressed transmembrane TNF- α is a major contributor to lipopolysaccharide and D-galactosamine-induced liver injury. *Cell Tissue Res*. 2016;363:371–83.
59. Wang GX, Pan JY, Wang YJ, Huang TC, Li XF. MiR-640 inhibition alleviates acute liver injury via regulating WNT signaling pathway and LRP1. *Eur Rev Med Pharmacol Sci*. 2020;24:8988–96.
60. Zhao Y, Zhao MF, Jiang S, Wu J, Liu J, Yuan XW, et al. Liver governs adipose remodelling via extracellular vesicles in response to lipid overload. *Nat Commun*. 2020;11:719.
61. Buratta S, Shimanaka Y, Costanzi E, Ni S, Urbanelli L, Kono N, et al. Lipotoxic stress alters the membrane lipid profile of extracellular vesicles released by Huh-7 hepatocarcinoma cells. *Sci Rep*. 2021;11:4613.
62. Arroyave-Ospina JC, Wu Z, Geng Y, Moshage H. Role of oxidative stress in the pathogenesis of non-alcoholic fatty liver disease: implications for prevention and therapy. *Antioxidants (Basel)*. 2021;10:174.
63. Hendrikx T, Binder CJ. Oxidation-specific Epitopes in non-alcoholic fatty liver disease. *Front Endocrinol (Lausanne)*. 2020;11:607011.
64. Tsiantoulas D, Perkmann T, Afonyushkin T, Mangold A, Prohaska TA, Papac-Milicevic N, et al. Circulating microparticles carry oxidation-specific epitopes and are recognized by natural IgM antibodies. *J Lipid Res*. 2015;56:440–8.
65. Crewe C, Funcke JB, Li S, Joffin N, Gliniak CM, Ghaben AL, et al. Extracellular vesicle-based interorgan transport of mitochondria from energetically stressed adipocytes. *Cell Metab*. 2021;33:1853–68.e11.
66. Miura K, Seki E, Ohnishi H, Brenner DA. Role of toll-like receptors and their downstream molecules in the development of nonalcoholic fatty liver disease. *Gastroenterol Res Pract*. 2010;2010:362847.

67. Seo W, Eun HS, Kim SY, Yi HS, Lee YS, Park SH, et al. Exosome-mediated activation of toll-like receptor 3 in stellate cells stimulates interleukin-17 production by $\gamma\delta$ T cells in liver fibrosis. *Hepatology*. 2016;64:616–31.
68. Wan X, Xu C, Yu C, Li Y. Role of NLRP3 inflammasome in the progression of NAFLD to NASH. *Can J Gastroenterol Hepatol*. 2016;2016:6489012.
69. Povero D, Eguchi A, Li H, Johnson CD, Papouchado BG, Wree A, et al. Circulating extracellular vesicles with specific proteome and liver microRNAs are potential biomarkers for liver injury in experimental fatty liver disease. *PLoS One*. 2014;9:e113651.
70. Zuo R, Ye LF, Huang Y, Song ZQ, Wang L, Zhi H, et al. Hepatic small extracellular vesicles promote microvascular endothelial hyperpermeability during NAFLD via novel-miRNA-7. *J Nanobiotechnology*. 2021;19:396.
71. Cannito S, Morello E, Bocca C, Foglia B, Benetti E, Novo E, et al. Microvesicles released from fat-laden cells promote activation of hepatocellular NLRP3 inflammasome: a pro-inflammatory link between lipotoxicity and non-alcoholic steatohepatitis. *PLoS One*. 2017;12:e0172575.
72. Povero D, Eguchi A, Niesman IR, Andronikou N, de Mollerat du Jeu X, Mulya A, et al. Lipid-induced toxicity stimulates hepatocytes to release angiogenic microparticles that require Vanin-1 for uptake by endothelial cells. *Sci Signal*. 2013;6:ra88.
73. Jiang F, Chen Q, Wang W, Ling Y, Yan Y, Xia P. Hepatocyte-derived extracellular vesicles promote endothelial inflammation and atherogenesis via microRNA-1. *J Hepatol*. 2020;72:156–66.
74. Kornek M, Popov Y, Libermann TA, Afdhal NH, Schuppan D. Human T cell microparticles circulate in blood of hepatitis patients and induce fibrolytic activation of hepatic stellate cells. *Hepatology*. 2011;53:230–42.
75. Zhao H, Shang Q, Pan Z, Bai Y, Li Z, Zhang H, et al. Exosomes from adipose-derived stem cells attenuate adipose inflammation and obesity through polarizing M2 macrophages and Beiging in white adipose tissue. *Diabetes*. 2018;67:235–47.
76. Nazarie Ignat SR, Gharbia S, Hermenean A, Dinescu S, Costache M. Regenerative potential of mesenchymal stem cells' (MSCs) secretome for liver fibrosis therapies. *Int J Mol Sci*. 2021;22:13292.
77. Bruno S, Pasquino C, Herrera Sanchez MB, Tapparo M, Figliolini F, Grange C, et al. HLSC-derived extracellular vesicles attenuate liver fibrosis and inflammation in a murine model of non-alcoholic steatohepatitis. *Mol Ther*. 2020;28:479–89.
78. Watanabe T, Tsuchiya A, Takeuchi S, Nojiri S, Yoshida T, Ogawa M, et al. Development of a non-alcoholic steatohepatitis model with rapid accumulation of fibrosis, and its treatment using mesenchymal stem cells and their small extracellular vesicles. *Regen Ther*. 2020;14:252–61.
79. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116:281–97.
80. Szabo G, Csak T. Role of microRNAs in NAFLD/NASH. *Dig Dis Sci*. 2016;61:1314–24.
81. Pérez-García A, Torrecilla-Parra M, Fernández-de Frutos M, Martín-Martín Y, Pardo-Marqués V, Ramírez CM. Posttranscriptional regulation of insulin resistance: implications for metabolic diseases. *Biomolecules*. 2022;12:208.
82. Fluitt MB, Kumari N, Nunlee-Bland G, Nekhai S, Gambhir KK. miRNA-15a, miRNA-15b, and miRNA-499 are reduced in erythrocytes of pre-diabetic African-American adults. *J Diabetes Endocrinol*. 2016;2:014.
83. Bala S, Petrasek J, Mundkur S, Catalano D, Levin I, Ward J, et al. Circulating microRNAs in exosomes indicate hepatocyte injury and inflammation in alcoholic, drug-induced, and inflammatory liver diseases. *Hepatology*. 2012;56:1946–57.
84. Zhang Y, Jia Y, Zheng R, Guo Y, Wang Y, Guo H, et al. Plasma microRNA-122 as a biomarker for viral-, alcohol-, and chemical-related hepatic diseases. *Clin Chem*. 2010;56:1830–8.
85. Wang K, Zhang S, Marzolf B, Troisch P, Brightman A, Hu Z, et al. Circulating microRNAs, potential biomarkers for drug-induced liver injury. *Proc Natl Acad Sci U S A*. 2009;106:4402–7.
86. Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, et al. A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell*. 2007;129:1401–14.

87. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, et al. The microRNA spectrum in 12 body fluids. *Clin Chem*. 2010;56:1733–41.
88. Leidinger P, Backes C, Dahmke IN, Galata V, Huwer H, Stehle I, et al. What makes a blood cell based miRNA expression pattern disease specific?--a miRNome analysis of blood cell subsets in lung cancer patients and healthy controls. *Oncotarget*. 2014;5:9484–97.
89. O'Connell TM, Markunas CA. DNA methylation and microRNA-based biomarkers for risk of type 2 diabetes. *Curr Diabetes Rev*. 2016;12:20–9.
90. Tay Y, Rinn J, Pandolfi PP. The multilayered complexity of ceRNA crosstalk and competition. *Nature*. 2014;505:344–52.
91. Zhang Z, Moon R, Thorne JL, Moore JB. NAFLD and vitamin D: evidence for intersection of microRNA-regulated pathways. *Nutr Res Rev*. 2021:1–20.
92. Cheng L, Yu P, Li F, Jiang X, Jiao X, Shen Y, et al. Human umbilical cord-derived mesenchymal stem cell-exosomal miR-627-5p ameliorates non-alcoholic fatty liver disease by repressing FTO expression. *Hum Cell*. 2021;34:1697–708.
93. El-Derany MO, AbdelHamid SG. Upregulation of miR-96-5p by bone marrow mesenchymal stem cells and their exosomes alleviate non-alcoholic steatohepatitis: emphasis on caspase-2 signaling inhibition. *Biochem Pharmacol*. 2021;190:114624.
94. Wu J, Dong T, Chen T, Sun J, Luo J, He J, et al. Hepatic exosome-derived miR-130a-3p attenuates glucose intolerance via suppressing PHLPP2 gene in adipocyte. *Metabolism*. 2020;103:154006.
95. Kranendonk ME, Visseren FL, van Herwaarden JA, Nolte-'t Hoen EN, de Jager W, Wauben MH, et al. Effect of extracellular vesicles of human adipose tissue on insulin signaling in liver and muscle cells. *Obesity (Silver Spring)*. 2014;22:2216–23.
96. Ohara M, Ohnishi S, Hosono H, Yamamoto K, Yuyama K, Nakamura H, et al. Extracellular vesicles from amnion-derived mesenchymal stem cells ameliorate hepatic inflammation and fibrosis in rats. *Stem Cells Int*. 2018;2018:3212643.
97. Zhou Y, Wang X, Sun L, Zhou L, Ma TC, Song L, et al. Toll-like receptor 3-activated macrophages confer anti-HCV activity to hepatocytes through exosomes. *FASEB J*. 2016;30:4132–40.