

Chapter 9

Extracellular Vesicles in Non-alcoholic Fatty Liver Disease: Key Players in Disease Pathogenesis and Promising Biomarker Tools



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Abbreviations

2D-SWE	2-dimensional shear wave elastography
ADSCs	Adipose-derived stem cells
AFM	Atomic force microscopy
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AUC	Area under the receiver operating curve
cryo-EM	Cryo-electron microscopy
CAP	Controlled attenuation parameter
CCN2	Connective tissue growth factor 2
CDAAs	Choline-deficient L-amino acid defined
CHC	Chronic hepatitis C
CK18	Cytokeratin 18
CXCL10	C-X-C motif ligand 10
DLS	Dynamic light scattering
ELISA	Enzyme-linked immunosorbent assay
EVs	Extracellular vesicles
FFAs	Free fatty acids
FGF21	Fibroblast growth factor 21
GPI	Glycosylphosphatidylinositol
hrFC	High-resolution flow cytometer
HCC	Hepatocellular carcinoma
HFS	Hepamet fibrosis score
HMGB1	High mobility group box 1
HSCs	Hepatic stellate cells
iNKT	Invariant natural killer T cells
Ihh	Indian hedgehog
ILVs	Intraluminal vesicles
ISEV	International society for extracellular vesicles
LFS	Liver fibrosis score
miRNAs	microRNAs
mtDNA	Mitochondrial DNA
MLK3	Mixed lineage kinase 3
MRE	Magnetic resonance elastography
MRI-PDFF	Magnetic resonance imaging-derived proton density fat fraction
MVBs	Multivesicular bodies
MVEs	Multivesicular endosomes
MVs	Microvesicles
ncRNAs	Non-coding RNAs
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Non-alcoholic steatohepatitis

NFS	NAFLD fibrosis score
NTA	Nanoparticle tracking analysis
pSWE	Point shear wave elastography
PA	Palmitic acid
PPARs	Peroxisome proliferator-associated receptors
ROCK1	Rho-associated, coiled-coil-containing protein kinase 1
S1P	Spingosine-1-phosphate
SAF	Steatosis activity fibrosis
SEM	Scanning electron microscopy
Shh	Sonic hedgehog
TE	Transient elastography
TEM	Transmission electron microscopy
TGF β	Transforming growth factor β
TLR9	Toll-like receptor 9
TRAIL	TNF-related apoptosis inducing ligand
TRPS	Tunable resistive pulse sensing
VCAM1	Vascular cell adhesion molecule 1

Introduction

Non-alcoholic fatty liver disease (NAFLD) represents a common condition characterized by the pathologic accumulation of fat within more than 5% of hepatocytes, in the absence of other forms of liver disease, such as viral infections or excessive alcohol intake, among others [1]. NAFLD encompasses a spectrum of liver lesions ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). Hepatic or simple steatosis is usually benign and considered one of the earliest and less severe stages of NAFLD, exhibiting a relatively favorable clinical course. In about 5–10% of patients, hepatic steatosis may progress to NASH, a more malignant state that is prone to further progress to cirrhosis and hepatocellular carcinoma (HCC), substantially contributing to liver-related morbidity and mortality [1].

NAFLD is currently the most prevalent liver disease worldwide, affecting around one-fourth of the general population [2]. In fact, several NAFLD epidemiological studies have been conducted in both the United States and Europe, indicating that over than 64 million people in the United States are believed to possess NAFLD [3–5]. In Europe, the prevalence of NAFLD varies between countries. In Spain, a 2010 multicenter cross-sectional population study revealed that the overall prevalence of NAFLD was 25.8% [6]. In Romania, the estimated prevalence was 20% [7] while a Greek study stated that 31.3 and 39.8% of liver biopsies from 498 individuals showed hepatic steatosis or NASH, respectively [8]. In this regard, it was predicted that over 52 million individuals would be affected by NAFLD in Germany, France, Italy and the United Kingdom [3]. Although NAFLD incidence is increasing worldwide, it is most noticeable in specific population groups, particularly those

with metabolic comorbidities. NAFLD prevalence is increased in patients with type 2 diabetes, who also display an increased risk for development of NASH [9, 10]. Two major European studies reported a NAFLD prevalence rate between 42.6 and 69.5% in patients with type 2 diabetes [11, 12]. Obese individuals are also more likely to develop NAFLD. In particular, 65.7% of obese patients undergoing bariatric surgery have NAFLD, with 33.6% also evidencing NASH [13]. Overall, patients with metabolic syndrome, pathological conditions characterized by abdominal obesity, hypertension, dyslipidemia and glucose intolerance are at increased risk of developing NAFLD [14].

NAFLD is thought to increase cardiovascular risk, as most NAFLD patients die from cardiovascular-related problems [15–19]. Indeed, the number of NAFLD patients with advanced disease (i.e., cirrhosis, end-stage liver disease, HCC), a major cause of liver disease-related morbidity and mortality, is alarmingly increasing [20, 21]. Both liver-specific and overall mortality among NAFLD patients is estimated at 0.77 and 11.77 per 1000 person-year, respectively, further increasing in patients with NASH (15.44 and 25.56 per 1000 person-year, respectively) [2]. Of note, NASH is currently the second indication for liver transplantation among all chronic liver diseases and is expected to become the leading indication for transplantation in the next decades [22, 23]. In parallel with NAFLD pathogenesis remaining incompletely understood, one of the major clinical challenges is the difficulty of obtaining differential diagnosis between the different disease severity stages, and also the identification of patients that might be at higher risk for disease progression. As such, there is a substantial unmet need for novel non-invasive and accurate tools that might allow the diagnosis and risk stratification of NAFLD patients. In this regard, extracellular vesicles (EVs) are emerging as promising molecular targets and biomarkers, being involved in disease pathogenesis and harboring diagnostic and prognostic potential. In this chapter, we will review the most recent findings concerning the role of EVs in NAFLD pathogenesis and in diagnosis/prognosis.

NASH Diagnosis and Monitoring: Current Approaches

Liver biopsy is still the gold standard procedure to undoubtedly identify and stage NAFLD. Kleiner and Blunt proposed a scoring system, named the NAFLD Activity Score (NAS), which is calculated by the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3) and hepatocyte ballooning (0–2) [24]. Consequently, NAS ranges from 0 to 8 and a total score of 0–2 is considered a non-NASH diagnosis while scores greater or equal to 5 are diagnosed as NASH. Of note, fibrosis was not included as a component of the activity score due to its irreversible nature and because it was thought to only result from disease progression. Still, fibrosis is often present in NASH; to that matter, the Steatosis Activity Fibrosis (SAF) score, which does not sub-classifies NAFLD based solely in NASH, was proposed. This score evaluates three variables: steatosis, on a scale of 0–3 (S0: <5%, S1: 5–33%, S2: 34–66%, S3: >67%); ballooning and lobular inflammation, each graded between 0 and 2 and then summed, representing activity (A0–A4), and fibro-

sis, scored between 0 and 4 (F0: none, F1: perisinusoidal or periportal, F2: perisinusoidal and periportal, F3: bridging or F4: cirrhosis) [25]. This new score was shown to decrease intra-observer variation among pathologists and includes the fibrosis component in the final decision which, although not required for the diagnosis of NASH, is now considered to represent the best predictor of advanced liver disease and mortality [26]. Nonetheless, the SAF score still requires a liver biopsy to be performed, carrying several intrinsic limitations, including invasiveness, poor acceptability, variability and cost [27]. Therefore, alternative non-invasive strategies have been proposed in order to achieve a more accurate diagnosis and to assist in the management of NAFLD patients [27, 28]. For instance, the Hepamet Fibrosis Score (HFS), mainly based on serum markers, was recently developed and validated in a cohort of 2453 patients with NAFLD, presenting area under the receiver operating characteristic (AUC) values greater than other fibrosis score systems (the NAFLD fibrosis score [NFS] and FIB-4) [29]. Currently, 2 different complementary approaches are being used as non-invasive methods in the diagnosis and management of NAFLD: a “biological” approach, which mainly relies on the quantification of serum biomarkers, and a “physical” approach, related with the measurement of the intrinsic physical properties of the liver parenchyma (liver stiffness) by different imaging techniques [27].

Although the vast majority of NAFLD patients are clinically asymptomatic, approximately 20% display elevated liver enzymes [30]. Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) may present normal to moderate elevations—about 1.5–2 times the upper limit—although these are considered poor markers of fatty liver disease [31, 32]. Several predictive models (SteatoTest [33], Fatty Liver Index [34], Hepatic Steatosis Index [35], lipid accumulation product [36], the Index of NASH [37] or the NAFLD Liver Fat Score [38], based on the differential combination of serum transaminases, triglyceride levels and clinical information have been developed (reviewed in [39]) to help in the diagnosis of steatosis. It should be noted that diagnostic performances of each test are difficult to compare since they are based against different standards, namely liver biopsy or imaging techniques. Still, in a cohort of 324 patients with suspected NAFLD and liver biopsy, the Fatty Liver Index, NAFLD Fat Score and Hepatic Steatosis Index were retrospectively evaluated, providing similar AUC values (0.83, 0.80 and 0.81, respectively) [40]. However, these scores are still not widely used as they fail to provide substantial additional information when compared with current clinical, laboratory and imaging studies, and await further validation studies. Cytokeratin-18 (CK18) fragments have been extensively studied as biomarkers for NASH diagnosis. CK18 fragments are released from apoptotic hepatocytes and the caspase-cleaved fragment M30 is detected by enzyme-linked immunosorbent assay (ELISA) in the serum of patients with NASH, with a AUC value of 0.83 [41]. In two subsequent meta-analyses, the pooled AUC for CK18 to predict NASH was 0.82 (median sensitivity and specificity of 66–78% and 82–87%, respectively) [42, 43]. Nonetheless, there are still several drawbacks with regards to using CK18 as a diagnosis biomarker for NASH, restraining its translation into clinical practice. In particular, there is significant variability in the suggested cutoffs and diagnostic accuracy among studies [44], no commercially available clinical tests [45] and a rather limited sensitivity when used alone [46]. On

this note, the diagnostic accuracy of CK18 is increased when used in combination with sFas levels [47], uric acid [48], adiponectin and resistin [49, 50], ALT or the presence of metabolic syndrome [51], among others. Some models have been proposed but most of them were studied in small and highly selected populations (morbidly obese patients) and, as such, need to be further validated. Serum metabolomics was also shown to be of great value in the diagnosis of NAFLD/NASH and recent studies have reported specific serum metabolomic signatures that allowed the specific diagnosis of NAFLD (OWL Liver Care) and the differential diagnosis of steatosis and NASH (OWL Liver Test) [52, 53] as well as between NASH subtypes [54], that are currently commercialized by OWL Metabolomics.

Imaging techniques, either ultrasound- or magnetic resonance-based, are widely used in NAFLD diagnosis. Conventional ultrasonography is currently the most commonly used imaging technique for the diagnosis of steatosis due to its general availability [27]. With ordinal ultrasonography scores, steatosis may be subjectively categorized as mild, moderate and severe [55, 56]. Importantly, in a large meta-analysis, including 34 studies and 2815 patients with suspected/diagnosed liver diseases, pooled sensitivities and specificities of ultrasonography to differentiate moderate/severe fatty liver and absence of steatosis, were 85% and 93%, respectively [57]. Still, in daily practice, ultrasonography is only used to provide a diagnosis of presence or absence of steatosis, only being capable of detecting liver fat amounts greater than 2.5–20% [58] and displaying lower accuracy in obese patients with concomitant renal disease [59, 60]. According to the European Guidelines for the management of NAFLD [20], ultrasonography constitutes the first choice imaging technique to monitor steatosis in adults at risk for disease development. Controlled Attenuation Parameter (CAP) is a new non-invasive transient elastography (TE)-based imaging technique for the staging of steatosis. Good inter-observer reproducibility was reported (concordance rates between observers of 0.82–0.84) [55, 56], and in a cohort of patients with chronic liver diseases (15% with NAFLD), steatosis was accurately detected by CAP. Still, this technique was not precise enough to discern between the different stages of steatosis [61]. Furthermore, CAP determinations might be influenced by the presence of covariates, such as the body mass index or diabetes [27]. Finally, magnetic resonance-based imaging techniques have been reported as the most accurate available imaging techniques to quantify liver fat and fibrosis. Magnetic resonance imaging proton density fat fraction (MRI-PDFF) is highly accurate (AUC: 0.950) [62], and reproducible, fast, and allows for evaluation of the entire liver, quantification of fat content, and stratification of steatosis (with excellent sensitivity for detecting mild steatosis) [63]. This technique has been validated in several studies [64, 65] and is emerging as the gold standard for the quantification of liver fat. However, it is not widely available, it is rather expensive and measurements are presumed to be affected by food intake [66]. Novel methodologies, including point shear wave elastography (pSWE) and 2-dimensional shear wave elastography (2D-SWE) are now being evaluated [67–69].

Of note, the differential diagnosis of simple steatosis and NASH by imaging techniques remains challenging, as MRI-PDFF cannot effectively detect liver inflammation and ballooning, nor NASH resolution or fibrosis improvements [70]. The same holds true for magnetic resonance elastography (MRE) [71–75] and TE [72, 76–78].

Overall, there is still no biomarker or imaging method capable of accurately diagnosing, staging and performing the follow-up of NAFLD (including fibrosis) for which new approaches are eagerly awaited. In this regard, EVs are emerging as novel potential NAFLD biomarkers, while also participating in disease pathogenesis.

Extracellular Vesicles

EVs are a heterogeneous population of membrane vesicles ranging from 30 nm to 2 μ m in diameter, secreted by diverse cell types and containing distinct biomolecules, including proteins, nucleic acids and lipids [79–82]. The first reports on EVs considered them membrane debris with no biological significance, and a way to eliminate needless compounds by the cells [83]. However, new evidence demonstrating their potential to stimulate adaptative immune responses [84, 85], opened their role in intercellular communication. In the last decade, this is an emerging field which is exponentially increasing, with special interest in their capacity to exchange components between cells and acting as signaling vehicles (Fig. 9.1).

Based on the current knowledge of their biogenesis, EVs are classified as exosomes, microvesicles (MV) and apoptotic bodies (Fig. 9.2) [79–82, 85, 86]. **Exosomes** (30–200 nm) are formed as intraluminal vesicles (ILV) by the inward

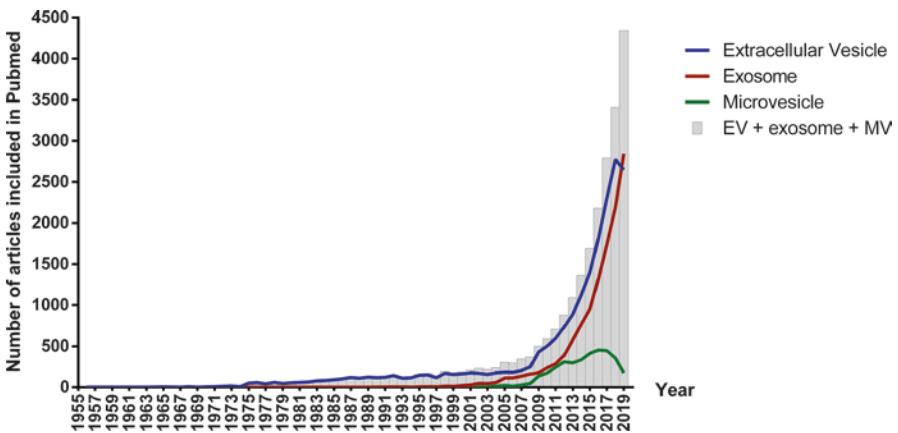


Fig. 9.1 Timeline (1955–2019) of articles referring to extracellular vesicles, microvesicles and exosomes in PubMed. (<https://www.ncbi.nlm.nih.gov/pubmed/>). Accession date: December 2019. Abbreviations: *EV* extracellular vesicle; *MV* microvesicle

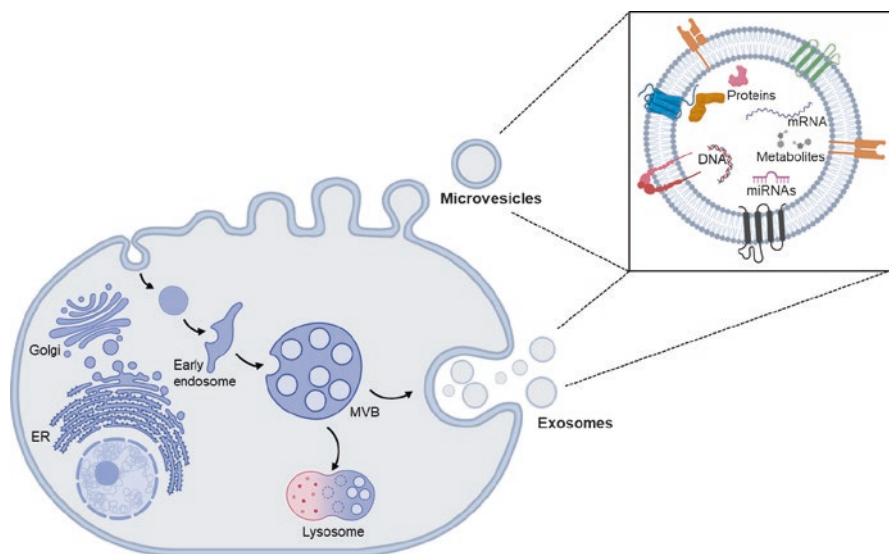


Fig. 9.2 Biogenesis, overall composition and release of EVs. MVs are produced by the outward budding and fission of the plasma membrane, whereas exosomes are formed by the inward budding of the multivesicular body and released upon fusion of multi-vesicular bodies with the plasma membrane. EVs are lipid-bilayer membrane vesicles which contain cytoplasmic proteins, lipid raft-interacting proteins, membrane proteins, lipids, metabolites, DNA and different types of RNA. Abbreviations: *ER* endoplasmic reticulum; *MVB* multivesicular body

budding of the endosomal membrane during maturation of multivesicular endosomes (MVE). They are secreted from the lumen of late endosomes, also called multivesicular bodies (MVBs), to the extracellular space by fusion of MVBs with the plasma membrane. **Microvesicles** (50–1000 nm) are generated by the outward budding and fission of the plasma membrane and the subsequent release of vesicles into the extracellular space. Finally, **apoptotic bodies** (800–5000 nm) are released by cells when plasma membrane blebbing occurs during programmed cell death. All EVs may contain cytoplasmic proteins, lipid raft-interacting proteins, membrane proteins, lipids, metabolites, DNA and different types of RNA, including mRNAs, microRNAs (miRNAs) and other non-coding RNAs (ncRNAs) [85, 87]. Their molecular cargo varies widely among cell types and conditions (e.g., physiology/pathology), directly affecting the fate and function of these membrane vesicles [80]. EVs are found in all biological fluids, including serum, plasma, urine, saliva, and bile, among others, as well as in culture supernatants [81, 82]. However, despite their different biogenesis, once they reach the extracellular zone, exosomes and microvesicles display a similar appearance, overlapping in size, and often presenting a common biomolecular composition [80]. Therefore, it is difficult to ascertain the origin of EVs when they are isolated from the extracellular medium or from diverse biological fluids.

A wide variety of methods have been proposed for the isolation of EVs from extracellular fluids: differential centrifugation/ultracentrifugation, flotation on density gradients, separation by size exclusion chromatography, precipitation with different polymers, filtration and antibody-based purification (immuno-affinity) [79, 88]. These methods allow the separation of EVs from non-vesicular entities, such as protein aggregates, lipoparticles, viruses and cell debris, with different rates of success [80]. Given the heterogeneous EV population, each purification method will result in enrichment of specific EV subpopulations, with distinct recovery/specificity rates [88]. Nonetheless, combination of multiple isolation procedures is capable of specifically separating subpopulations of vesicles based on their size, density, surface proteins, sugar, lipid composition or other biophysical properties, such as surface charge. Even so, considering that a single optimal separation method or a gold standard is not yet defined, the isolation method should be chosen based on the downstream application and on the scientific question that is being addressed [79, 88]. Once EVs are obtained, they should be properly characterized using multiple, complementary techniques, in order to obtain reproducible results. In this regard, the International Society for Extracellular Vesicles (ISEV) regularly publishes minimal requirement guidelines for the study of EVs, focusing on adequate and standardized characterization [79, 88]. In parallel, a consortium called EV-TRACK (transparent reporting and centralizing knowledge in extracellular vesicle research) has been gathered to build a crowdsourcing knowledgebase (<http://evtrack.org/>) that centralizes EV biology and methodology with the goal of stimulating authors, reviewers, editors and funders to put experimental guidelines into practice [89].

The characterization of EVs should include determination of its morphology, size and concentration, as well as reporting of the components typically associated with EVs, particularly membrane proteins [79, 88]. Scanning electron microscopy (SEM), transmission electron microscopy (TEM), cryo-electron microscopy (cryo-EM) and atomic force microscopy (AFM) images enable the analysis of EV morphology and size, while also providing information on the heterogeneity of the EV preparation. Particle number and size can be measured quantitatively by analyzing large numbers of single EVs with light scattering technologies, such as nanoparticle tracking analysis (NTA) and dynamic light scattering (DLS), as well as with high resolution flow cytometry (hrFC) or tunable resistive pulse sensing (TRPS). The analysis of EV-associated proteins is typically performed through immunoblotting, flow cytometry and/or mass spectrometry. It has been described that transmembrane or glycosylphosphatidylinositol (GPI)-anchored proteins localized in plasma membrane and/or endosomes, such as tetraspanins (CD63, CD81, others) or integrins, may be considered as markers of any type of EVs, as their presence demonstrates the existence of the lipid-bilayer structure characteristic of EVs. Additionally, cytosolic proteins with membrane-binding ability, such as ESCRT-I/II/III (e.g., TSG101), heat shock proteins, ALIX and ARF6 are also commonly found in EVs, given the nature of its biogenesis. Besides proteins, phospholipids found in lipid bilayers are

also potential positive controls for identifying EVs. Still, these might be non-specific, as other particles such as lipoproteins can also contain phospholipids. It remains important to clarify the ratios of cholesterol, sphingomyelin, ceramide, and phosphatidylcholine/ethanolamine/inositol found in EVs, and how these differ from the ratios found in lipoproteins. In fact, there are still no markers capable of accurately distinguishing between every different EV subtype [80, 85, 88].

The mechanisms involved in EV-mediated cell-to-cell communication are vast and still incompletely understood. When interacting with target cells, EVs may modulate cellular signaling pathways in a pleiotropic manner, including the direct activation of cell surface receptors *via* protein and bioactive lipid ligands, or by merging their membrane contents into the recipient cell [80]. This type of communication is believed to occur in both physiological conditions as well as in pathological states [85]. In physiological conditions, EVs participate in the maintenance of stemness [90], tissue repair [91], blood coagulation [92], immune surveillance [93], neuronal plasticity [94] and several other physiological functions [85]. In turn, EVs may contribute to tumorigenesis by inducing abnormal cell proliferation [95], stimulating tumor growth [96], promoting extracellular matrix remodeling [97], and facilitating tumor metastasis [98] and immune escape [93]. Beyond cancer, EVs appear to also play a role in the spread of different pathogens, in the local propagation of neurodegenerative diseases, and in several liver diseases, including NAFLD (reviewed in [99, 100]).

Role of Extracellular Vesicles in NAFLD Pathogenesis

As central mediators of cell-to-cell communication, EVs have recently arisen as novel players in NAFLD pathogenesis and progression (Fig. 9.3; Table 9.1). Data from different diet-induced animal models of NASH have shown that EV concentration increases with disease progression, in a time-dependent manner [101–103]. This may result from accumulation of lipotoxic lipids and their downstream mediators in the liver, already shown to increase the capacity of hepatocytes to form and release different types of EVs [102–105]. EVs can then be internalized by macrophages, neutrophils and monocytes, leading to their activation and recruitment to the liver, promoting and exacerbating the inflammatory responses observed in NASH. In fact, palmitic acid (PA) and lysophosphatidylcholine were shown to increase the release of microvesicles carrying TNF-related apoptosis inducing ligand (TRAIL) ligand from both mouse and human hepatocytes [105]. In mice, this promotes the expression of pro-inflammatory cytokines IL-1 β and IL-6 in bone marrow-derived macrophages, in a rho-associated, coiled-coil-containing protein kinase 1 (ROCK1)-dependent manner [105]. Of note, administration of fasudil, a ROCK1 inhibitor, to mice with NASH decreases the amount of EVs in the serum as well as liver injury, inflammation and fibrosis. PA-stimulated hepatocytes have also been shown to release EVs enriched in ceramide, which increase macrophage recruitment to the liver *via* sphingosine-1-phosphate (S1P). Indeed, increased levels

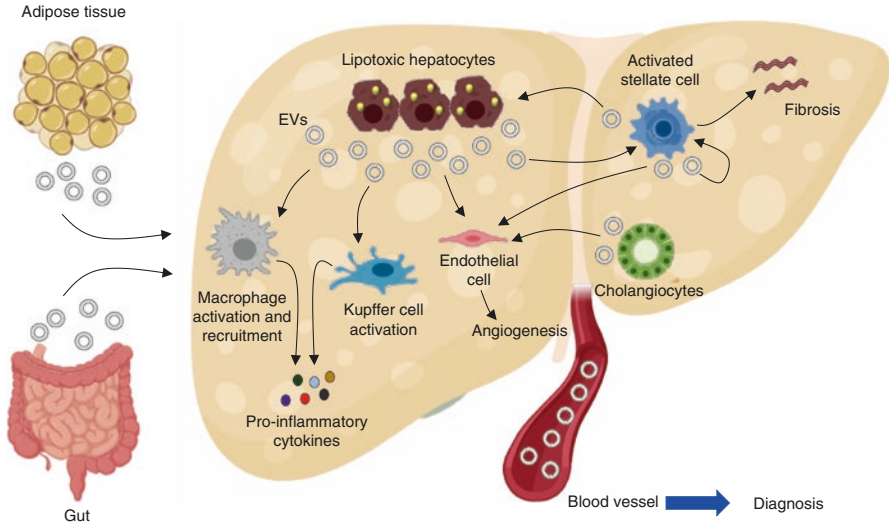


Fig. 9.3 Role of EVs in NAFLD pathogenesis. EVs are released by lipotoxic hepatocytes, thus contributing to the recruitment and activation of macrophages, Kupffer and stellate cell activation, as well as angiogenesis, through targeting of endothelial cells. Hepatic stellate cells and cholangiocytes might also secrete EVs, thus also contributing for disease progression. In addition, adipose tissue- and gut-derived EVs are also known to target the liver and contribute to NAFLD. Finally, increased blood concentration of EVs might help in NAFLD diagnosis. Abbreviations: *EVs* extracellular vesicles

Table 9.1 Extracellular vesicles (EVs) involved in cell-to-cell and organ crosstalk during NAFLD pathogenesis

EV type	Source cells	Target cells	EV cargo	Modulated targets in recipient cells	Models	Ref.
EVs	Liver (hepatocytes)	Bone marrow-derived macrophages	Ceramide	S1P	<i>In vitro</i> ; <i>In vivo</i> (mouse); patients	[102]
Microvesicles	Liver (hepatocytes)	Liver (endothelial cells)	Vanin-1	–	<i>In vitro</i> ; <i>In vivo</i> (mice)	[103]
EVs (exosomes)	Liver (hepatocytes)	Bone marrow-derived macrophages	CXCL10	–	<i>In vitro</i> ; <i>In vivo</i> (mice)	[104]
Microvesicles	Liver (hepatocytes)	Bone marrow-derived macrophages	TRAIL	IL-1 β and IL-6	<i>In vitro</i> ; <i>In vivo</i> (mouse); patients	[105]
EVs	Liver (hepatocytes)	Neutrophils and Kupfer cells	mtDNA	TLR9	<i>In vivo</i> (mice); patients	[107]

(continued)

Table 9.1 (continued)

EV type	Source cells	Target cells	EV cargo	Modulated targets in recipient cells	Models	Ref.
Exosomes	Liver (hepatocytes)	Liver (HSCs)	miR-192	Fibrogenic markers (α -SMA, TGF- β , and Col1 α 1)	<i>In vitro</i>	[110]
Exosomes	Liver (hepatocytes)	Liver (HSCs)	–	Fibrogenic markers (Col1 α 1, Col3 α 1, MMP-2, and TIMP-1)	<i>In vitro</i> ; <i>In vivo</i> (mice)	[111]
EVs	Liver (hepatocytes)	Liver (HSCs)	miR-128-3p	PPAR- γ	<i>In vitro</i> ; <i>In vivo</i> (mice)	[112]
Exosomes	Liver (HSCs)	Liver (hepatocytes and HSCs)	miR-214	CCN2	<i>In vitro</i>	[113]
Exosomes	Liver (HSCs)	Liver (HSCs)	CCN2	–	<i>In vitro</i>	[114]
Exosomes	Liver (HSCs and hepatocytes)	Liver (HSCs)	Twist1	miR-214 (CCN2)	<i>In vitro</i>	[115]
Exosomes	VAT (adipocytes)	Liver (hepatocytes and HSCs)	–	TGF- β pathway genes (TIMP-1, TIMP-4, integrin $\alpha\beta$ -5, integrin $\alpha\beta$ -8, PAI-1, Smad-3, MMP-7 and MMP-9)	<i>In vitro</i> ; patients	[117]
Exosomes	BAT (adipocytes)	Liver	miR-99b	FGF-21	<i>In vivo</i> (mouse)	[118]
Exosomes	ADSC (adipose-derived stem cells)	Macrophages	STAT3	Arginase-1	<i>In vivo</i> (mouse)	[119]
Exosomes	Intestine	Liver	HMGB1	–	<i>In vivo</i> (mouse)	[121]
Exosomes	Liver (MF-HSC and cholangiocytes)	Liver (endothelial cells)	Shh and Ihh	–	<i>In vitro</i> ; <i>In vivo</i> (mice)	[122]

EV extracellular vesicles; VAT visceral adipose tissue; HSC hepatic stellate cells; TGF- β transforming growth factor- β ; TIMP-1 tissue inhibitor of matrix metalloproteinase-1; TIMP-4 tissue inhibitor of matrix metalloproteinase-4; PAI-1 plasminogen activator inhibitor-1; MMP-7 matrix metalloproteinase-7; MMP-9 matrix metalloproteinase-9; BAT brown adipose tissue; FGF21 fibroblast growth factor-21; STAT3 signal transducer and activator of transcription 3; HMCB1 high mobility group box-1; TRAIL tumor necrosis factor-related apoptosis-inducing ligand; *Il-1 β* interleukin 1 beta; *Il-6* interleukin 6; *SIP* sphingosine-1-phosphate; *CXCL10* C-X-C motif ligand 10; *mtDNA* mitochondrial DNA; *TLR9* toll-like receptor 9; α -SMA α -smooth muscle actin; *Col1 α 1* Collagen, type I, alpha 1; *Col3 α 1* Collagen, type III, alpha 1; *MMP-2* matrix metalloproteinase-2; *PPAR- γ* peroxisome proliferator-activated receptor- γ ; *CCN2* pro-fibrogenic connective tissue growth factor; *MF-HSC* myofibroblastic hepatic stellate cells; *Shh* Sonic hedgehog; *Ihh* Indian hedgehog

of ceramide- and S1P-enriched EVs were reported in plasma from both mice and patients with NASH [102]. Importantly, in mice fed a high-fructose, -saturated fat and -cholesterol diet, blocking of S1P was shown to improve liver histology, namely reducing hepatocyte ballooning and inflammatory foci, in parallel with reduced hepatomegaly, serum transaminases and accumulation of hepatic macrophages [106]. Finally, EVs carrying C-X-C motif ligand 10 (CXCL10), a potent chemokine that is released by lipotoxic hepatocytes in a Mixed Lineage Kinase 3 (MLK3)-dependent manner, have also been shown to associate with macrophage recruitment [104]. In addition, the majority of mitochondrial DNA (mtDNA) released from hepatocytes circulate within microparticles and promote activation of neutrophils and kupffer cells through toll-like receptor 9 (TLR9) [107]. More recently, it was shown that the NLRP3 inflammasome is activated by microvesicles released from fat-laden cells undergoing lipotoxicity, either in hepatocytes and macrophages, further reinforcing the role of EVs in disease progression from simple steatosis to NASH [108]. On that note, the transplant of circulating EVs from high fat-fed mice to chow-fed mice was shown to induce accumulation and consequent activation of myeloid cells in the liver, thus promoting liver inflammation and injury [109].

Injured hepatocytes are able to communicate with other liver cell types, such as hepatic stellate cells (HSCs), thus triggering the expression of fibrosis-related genes [110, 111]. In fact, EVs released from PA-stimulated hepatocytes have been shown to enhance the expression of fibrosis markers in HSCs. Among its cargo, these EVs were shown to carry several miRNAs, including miR-192, already associated with NAFLD progression and liver fibrosis [110]. Interestingly, incubation of HSC cultures with plasma EVs from mice fed a high-fat diet, triggered their activation, again underscoring EVs as key cell-to-cell communication mediators. In addition, in a dietary murine model of NASH, mice treated with thiazolidinediones, a group of insulin sensitizers that activate peroxisome proliferator-activated receptors (PPARs), exhibited reduced secretion of EVs from hepatocytes, thereby impairing HSCs activation [111]. This emphasizes the crucial role of PPARs inhibition, especially PPAR- γ , in the phenotypical switch of HSCs from quiescent to their active form. In agreement, hepatocyte-derived EVs released during lipotoxicity are enriched with miR-128-3p, a miRNA that direct targets PPAR- γ and promote HSCs migration, proliferation and activation, therefore contributing to fibrosis and NAFLD progression [112].

Hepatocytes are not the only source of EVs in the liver that actively contribute to this type of paracrine communication. In fact, both mouse and human HSCs release EVs that target hepatocytes and HSCs themselves [113–115]. For instance, it was shown that under fibrotic conditions, HSCs release EVs carrying lower levels of miR-214, when compared with physiological situations, which directly target connective tissue growth factor 2 (CCN2), a pivotal activator of HSCs, thereby promoting fibrosis [113]. Curiously, CCN2 itself can be present within HSCs-derived EVs, thus enhancing expression of several pro-fibrotic genes in quiescent HSCs, contributing to their activation [114]. On the other hand, EVs secreted by quiescent HSCs were shown to carry high levels of Twist1, a transcription factor that binds to the

miR-214 promoter, increasing its expression. In turn, miR-214 expression is stimulated in receptor cells, thus suppressing the expression CCN2 and its downstream effectors [115].

Current evidence indicates that accumulation of fat in the adipose tissue actively contributes to hepatic steatosis, mainly through the release of adipokines and free fatty acids (FFAs) into circulation, which will lastly end up in the liver [116]. EVs, more specifically exosomes, have already been implicated in this process, representing an important component of the adipose tissue-liver axis. Visceral adipose tissue-derived exosomes obtained from obese patients were shown to dysregulate pro-fibrogenic transforming growth factor- β (TGF- β)-related pathways in both hepatocytes and HSCs [117]. Furthermore, exosomal miRNAs isolated from human and mice adipose tissue constitute the majority of circulating exosomal miRNAs [118]. Of note, under physiologic conditions, exosomal miR-99b targets the hepatic fibroblast growth factor-21 (FGF-21) and represses its expression. Interestingly, only exosomal, but not free miR-99b, is able to regulate FGF-21 in the liver [118]. Finally, transfer of exosomes from adipose-derived stem cells (ADSC) to obese mice improved insulin resistance and reduced obesity, in parallel with reduction of hepatic steatosis. Furthermore, the authors reported that ADSC-derived exosomes induced a M2 anti-inflammatory phenotype when transferred into macrophages, a process that was dependent on the activation of arginase-1 by STAT3-carrying exosomes [119]. In parallel with the adipose tissue-liver axis, several studies support the notion of a gut-liver axis in the pathogenesis of several liver diseases [120]. In fact, high-fat diet-induced dysbiosis in mice leads to the release of gut-derived exosomes carrying injury-related high mobility group box 1 (HMGB1) protein, thus contributing to the development and progression of hepatic steatosis [121]. Last but not least, EVs have also been reported to have pro-angiogenic properties in the liver through targeting of endothelial cells [103, 122]. In particular, hepatocytes undergoing lipoapoptosis release microvesicles exhibiting Vanin-1 on its surface, thus allowing interaction with lipid raft domains of endothelial cells, resulting in cell migration and tube formation [103]. Interestingly, treating primary rat endothelial cells with plasma-derived exosomes from high-fat diet-fed rats markedly increased oxidative stress and the expression of vascular cell adhesion molecule 1 (VCAM1), thus contributing to a pro-inflammatory and pro-angiogenic environment [123]. In addition, exosomes carrying Sonic Hedgehog (Shh) and Indian Hedgehog ligands (Ihh), released by HSCs and cholangiocytes, engage a pro-angiogenic switch in endothelial cells during cirrhosis [122]. However, the role of these EVs in angiogenesis in the NAFLD context needs further exploration.

Extracellular Vesicles as Non-invasive Biomarkers for NAFLD

It is now widely established that circulating EVs are remarkably stable, thus constituting promising non-invasive biomarkers [100]. A pioneer study on this field compared the EV blood profile from patients with simple steatosis (NAFL) or NASH, to

patients with chronic hepatitis C (CHC) or healthy controls. In order to ascertain the possible cells of origin, the authors measured the presence of EVs with leuko-endothelial surface markers by flow cytometry [124]. Serum EVs derived from CD4⁺ and CD8⁺ T cells were found increased in patients with NAFL/NASH and CHC, compared to healthy individuals, but they were unable to specifically differentiate between these disease conditions (AUC: 0.57 and 0.65, respectively). On the other hand, patients with NAFL/NASH displayed marked increases in serum EVs containing surface markers from invariant natural killer T cells (iNKT) and monocytes/macrophages (CD14⁺), as well as lower levels of neutrophil- (CD15⁺) and endothelial cell- (CD41⁺) derived EVs. Noteworthy, CD14⁺- and iNKT-derived EVs positively correlated with serum ALT levels and NAS score, and allowed the differential diagnosis of NAFL/NASH and CHC (AUC:0.999 and 0.97, respectively). Importantly, these two type of EV populations were reported to be key players in liver fibrosis during NAFLD pathogenesis [125, 126]. Further, the release of EVs from immune cells might be involved in liver inflammation and, consequently, in the progression of NAFL to NASH. The levels of adipose tissue-derived EVs in obese patients also correlate with the levels of liver transaminases and were shown to contribute to insulin resistance, interfering with the insulin signaling pathway in hepatocytes [127]. C16:0 ceramide- and S1P-enriched EVs might also embody promising diagnostic biomarkers for NALFD/NASH, since they were shown to progressively increase in the plasma of obese patients with simple steatosis, and further in NASH patients with early fibrosis (F1), when compared with control obese patients [102]. However, these findings were obtained from a small cohort of patients (n = 43) and their diagnostic accuracy remains incompletely explored. As such, further studies, including larger cohorts of patients, should ideally be performed. Specifically concerning fibrosis, a previous study found that CD14⁺ and CD16⁺ EVs count could predict fibrosis severity, being inversely associated with NAFLD-related liver fibrosis, while also increasing the diagnostic capability of the enhanced liver fibrosis score (LFS) in patients with NAFLD (AUC: 0.948 and 0.967 for CD14⁺ and CD16⁺ EVs, respectively, vs. 0.915 for LFS alone) [128].

Increased concentration of serum EVs has been described in dietary murine models of NASH. In mice fed a choline-deficient L-amino acid defined (CDAA) diet, EV levels were shown to increase early in disease progression, further increasing with time and correlating with hepatocyte cell death, fibrosis and neo-angiogenesis [101]. Furthermore, proteomic analysis of blood EVs from CDAA-fed mice revealed a distinct protein cargo, when compared with EVs isolated from control mice, with most of the identified proteins being already described as players in NASH pathogenesis, namely affecting cell death and inflammatory pathways, among others. Although no AUC values were reported, the authors stated that this proteomic signature allowed for the discrimination of diseased mice compared to controls. In addition, miR-122, a liver-specific miRNA, was found enriched in blood EVs from mice fed a CDAA diet compared to controls, while its hepatic levels were reduced, pinpointing for a potential diagnostic capability of miR-122-EVs for NAFLD [101]. However, future validation studies should be performed, in order to clearly assess its accuracy for NAFLD. Similarly, increased amounts of hepatocyte-derived EVs were detected in blood from diet-induced

NASH mice, and found to correlate with disease severity [103]. In a similar model, endothelial-derived EVs (CD144⁺) were found increased, an effect that could be reverted by treatment with atorvastatin [129]. EV-derived hepatocyte mtDNA is also increased in the plasma of both mice and human patients with NASH, contributing to the activation of the TLR9 pathway, and the activation of sterile inflammation [107]. Nevertheless, the mechanisms by which mtDNA is targeted in EVs, as well as the accuracy of these particles to diagnose NAFLD remains to be clarified.

Conclusions and Future Directions

In the past decade, the number of reports addressing the role of EVs in human disease has grown exponentially. Particularly for liver diseases, including NAFLD, hundreds of papers have already been published, showcasing promising results that might translate into the clinics in a near future. Nonetheless, several aspects still need to be addressed before this jump can be made, particularly those related with methodological aspects. For instance, many studies use the terms “exosome” and “microvesicles” indiscriminately, without proper characterization of the isolated EV fraction. Further, considering that EV biogenesis is not entirely understood, and purification protocols are not homogeneous, many inconsistencies are still found in the literature [79, 80, 85]. In order to bypass these problems, and aid in the rapid translation of EVs into the clinics, standardized, large-scale and cost-effective protocols are urgently needed. In this regard, the EV-TRACK knowledgebase constitutes a key resource that researchers should consult in order to standardize research in this field and increase reproducibility of EV-related reports [80].

NAFLD is a complex metabolic multisystem and multicellular disease, involving extra-hepatic organs and several cell types in liver, which encompasses different degrees of autocrine, paracrine and endocrine communication. Although the investigation on this field is still scarce and inconclusive, there is still much to discover, it is now clear that inter-cell and inter-organ communication in NAFLD might be of pivotal importance and is mediated in part, by circulating EVs. Mostly due to the accumulation of toxic lipid species within hepatocytes, these liver cells are currently considered as one of the major sources of EVs in NAFLD, consubstantiating a key mechanism for disease progression into more nefarious stages. Although the role of EVs in NAFLD pathogenesis is unquestionable, there are still unsolved questions that should be addressed in the future: what are the major EV contents that directly contribute for disease progression? And how can we specifically target these EVs? Furthermore, deeply studying the molecular mechanisms underlying EV biogenesis will contribute with key concepts that will hopefully allow the manipulation of EV generation in patients, thus opening a new window for therapeutic interventions. Still, this idea should be approached with caution, as manipulation of the machinery involved in EV biogenesis might hold potential secondary effects on healthy tissues [80]. In this regard, the role of EVs as biomarkers for NAFLD is probably more close to make this translational jump. In fact, many reports have already illustrated the

potential of these vesicles to act as either diagnostic or prognostic biomarkers. In order to advance the field, future studies should assess the diagnostic value of EVs in larger cohorts of patients, including properly characterized individuals (biopsy-proven). Further, stratifying patients according to their metabolic status (presence/absence of diabetes, obesity, hypercholesterolemia, among others), as well as to the presence/absence of fibrosis, might reveal interesting and could provide decisive results. Lastly, it is widely known that patients with advanced NASH are at higher risk of progressing to HCC. It will also be important to query whether EVs might help in the prediction of patients who might be at risk for experiencing disease progression to HCC. In this regard, we have recently described a specific proteomic profile in serum EVs that allowed the specific diagnosis of HCC, when compared with healthy controls or patients with intrahepatic cholangiocarcinoma [130]. It will now be imperative to conduct studies in order to address the diagnostic capacity of these EVs, in this context.

In the next decade, several EV-related studies are envisioned in the NAFLD field, which might contribute with new concepts that will help in deciphering disease pathogenesis and possibly provide new diagnostic and prognostic tools to be applied into daily clinics [81, 85, 131]. The wide-range cellular and biological functions of EVs, as well as their ability of encapsulating and protecting biological and artificial therapeutic compounds, support the idea that EVs and their components may also be used as novel therapeutic targets, therapeutic agents and/or drug delivery vehicles to treat NAFLD.

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