

Extracellular vesicles as biomarkers in liver diseases: A clinician's point of view

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Summary

Extracellular vesicles are membrane-bound vesicles containing proteins, lipids, RNAs and microRNAs. They can originate from both healthy and stressed cells, and provide a snapshot of the cell of origin in physiological and pathological circumstances. Various processes that may give rise to the release of extracellular vesicles occur in liver diseases, including hepatocyte apoptosis, hepatic stellate cell activation, liver innate immune system activation, systemic inflammation, and organelle dysfunction (mitochondrial dysfunction and endoplasmic reticulum stress). Numerous studies have therefore investigated the potential role of extracellular vesicles as biomarkers in liver diseases. This review provides an overview of the methods that can be used to measure extracellular vesicle concentrations in clinical settings, ranging from plasma preparation to extracellular vesicle measurement techniques, as well as looking at the challenges of using extracellular vesicles as biomarkers. We also provide a comprehensive review of studies that test extracellular vesicles as diagnostic, severity and prognostic biomarkers in various liver diseases, including non-alcoholic and alcoholic steatohepatitis, viral hepatitis B and C infections, cirrhosis, primary liver cancers, primary sclerosing cholangitis and acute liver failure. In particular, extracellular vesicles could be useful tools to evaluate activity and fibrosis in non-alcoholic fatty liver disease, predict risk of hepatitis B virus reactivation, predict complications and mortality in cirrhosis, detect early hepatocellular carcinoma, detect malignant transformation in primary sclerosing cholangitis and predict outcomes in acute liver failure. While most studies draw on data derived from pilot studies, which still require clinical validation, some extracellular vesicle subpopulations have already been evaluated in solid prospective studies.

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What are extracellular vesicles?

Extracellular vesicles form a heterogeneous group of membrane-bound vesicles that contain cell-derived biomolecules, such as proteins, lipids, RNAs and microRNAs (miRNAs).^{1–3} They also sometimes contain organelles, such as mitochondria.³ They can be released into the extracellular compartment by both healthy and stressed cells.⁴ All cells can form extracellular vesicles, including malignant cells. Extracellular vesicles can be found in various body fluids and tissues.

Extracellular vesicles are classified into several subtypes, each with different mechanisms of biogenesis, which are thoroughly reviewed elsewhere.¹ Briefly, exosomes (size ranging from 40 to 100–200 nm) are generated in intraluminal vesicles by inward budding of the limiting membrane of early endosomes during the maturation process of multivesicular endosomes. Exosomes are then released into the extracellular milieu by fusion of multivesicular bodies with the plasma membrane.¹ “Microvesicles” (previously called “microparticles”, with sizes ranging from 100–200 nm to 1 µm) are generated by plasma membrane budding, which is induced by a transient phospholipid imbalance caused by externalisation of phosphatidylserine

(a negatively charged phospholipid), calcium-dependent proteolysis of the cytoskeleton and local potassium efflux (responsible for cellular contraction).⁴ Apoptotic bodies (size ranging from ~1 to 4 µm), which are released by dying cells, are formed by separation of the plasma membrane from the cytoskeleton.⁵ Apoptotic bodies will not be discussed in this review, as they are the same size as platelets, and are therefore eliminated during the first steps of platelet-free plasma processing.^{6,7}

Due to overlapping size, density and composition, a precise definition of these subgroups of extracellular vesicles has not yet been firmly established (Box 1).⁸ It is now recommended that extracellular vesicles be classified by size, density, biochemical composition or cellular origin and not by biogenesis.⁸ In this review, we therefore use the terms “small”, and “larger” extracellular vesicles, using a size cut-off of 100 to 200 nm (the smallest size of extracellular vesicle that can be detected using flow cytometry).

Once released into the extracellular compartment, extracellular vesicles can reach target cells for intercellular communication. The methods by

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Box 1. How to name extracellular vesicles?

Over the last 20 years, the terms used to name different types of extracellular vesicles varied. Extracellular vesicle subtypes were previously defined by their biogenesis pathway, “exosomes” having an endosomal origin, “microvesicles” deriving from plasma membrane and “apoptotic bodies” being released by dying cells. However, no specific marker of biogenesis pathway has been found, and extracellular vesicle size and protein composition overlap between “exosomes” and “microvesicles”. Accordingly, both “Minimal information for studies of extracellular vesicles” (MISEV) guidelines 2018 and American Heart Association guidelines 2017 recommend using the term “extracellular vesicles” to avoid confusion.^{6,8} Sub-classification of these extracellular vesicles can be based on operational aspects including size (small, *i.e.* inferior to 100 or 200 nm vs. medium/large, *i.e.* superior to 100 or 200 nm), density, biochemical composition, and cell condition or origin (*e.g.* hepatocyte extracellular vesicle, apoptotic body, hypoxic extracellular vesicle).

which extracellular vesicles communicate are varied and include activation of surface receptors, transfer of vesicle content into the target cell by vesicle internalisation (phagocytosis, endocytosis) or membrane fusion.¹ The role of extracellular vesicles in cell-cell communication in liver diseases has been thoroughly reviewed elsewhere.^{9,10}

Extracellular vesicles are released following cell activation or apoptosis. Their composition depends on their cellular origin and on the stimulus responsible for their formation.⁹ Circulating extracellular vesicle concentrations and composition thus vary with disease conditions, with some specificity for the ongoing pathological process. These characteristics make extracellular vesicles useful tools for personalised medicine, as they can be used to diagnose disorders, and evaluate prognosis, disease progression and response to treatment. Circulating extracellular vesicles have therefore emerged as attractive biomarkers in liver diseases, as discussed in this review.

Measurement methods in clinical use

This section provides the reader with an overview of the pre-analytical and analytical requirements for high-throughput measurement of extracellular vesicles in a clinical setting. Therefore, we do not discuss techniques for the analysis of extracellular vesicles in research laboratories (which are reviewed extensively elsewhere^{8,11}).

Key point

Extracellular vesicles are membrane-bound vesicles, containing cell-derived biomolecules, such as proteins, lipids and RNAs.

Patient requirements

Like most routinely used blood tests, plasma extracellular vesicle concentrations can be influenced by factors such as age, sex, pregnancy, menopausal status, fasting, circadian variations, exercise, body mass index, diet, comorbidities, and medication.¹² It is therefore important to collect these data and use matched control groups in order to manage this variability.

Pre-analytical requirements

Several pre-analytical factors can influence extracellular vesicle measurement. Hence, standardisation of plasma sample collection and processing is necessary to ensure comparability among studies and, ultimately, to permit the correct interpretation of the results at an individual level.¹³

Plasma sample collection, preparation, and storage

Many factors can induce the release of extracellular vesicles by blood cells during or after blood draw.^{14–18} Consequently, over the past few years, each step of sample collection and preparation has been standardised to limit this unwanted release of extracellular vesicles by blood cells in test tubes. It could be assumed that extracellular vesicles derived from non-circulating cells, such as hepatocytes, would be less likely to be subject to additional extracellular vesicle release in the test tube, since mother cells are not present in the tube. In any case, each step – from sample collection to plasma preparation and storage – should be meticulously described.⁸ Researchers can upload experimental protocols from a crowd-sourcing knowledge base called EV-TRACK (<http://evtrack.org>).¹⁹

Recommendations on blood sample collection are summarised in Box 2.^{6,8,11,12,20} The International Society on Thrombosis and Haemostasis²¹ and the International Society for Advancement of Cytometry²⁰ recommend the use of citrate tubes (as opposed to ethylene diamine tetraacetic acid and heparin), as their use better prevents platelet activation and formation of extracellular vesicles.^{8,22} The American Heart Association's (AHA) 2017 guidelines¹¹ and the International Society for Extracellular Vesicles' (ISEV) 2017 position paper⁷ recommend that the choice of anticoagulants be adapted to downstream analysis. For example, acid citrate dextrose is considered to be more suitable for RNA analysis.^{7,11,23}

Plasma is stored at -80°C , avoiding repeated freeze-thaw cycles and thawed at 37°C . However, a unique freeze-thaw cycle does not alter plasma extracellular vesicles.²¹ It has been shown that small extracellular vesicle miRNAs could still be assessed from samples frozen for up to 8 years.²⁴

Separation methods

Most techniques for measurement of extracellular vesicle concentration require separation of extracellular vesicles from the rest of the plasma.

Currently, no extracellular vesicle separation method can entirely separate extracellular vesicles from the remaining plasma. A balance must be found between maximising recovery of extracellular vesicles (to avoid loss of information) and minimising contaminants (soluble proteins, protein aggregates, lipoproteins, nucleic acids and

viruses). Some authors use combinations of techniques such as ultrafiltration, density gradients or washing with extracellular vesicle-free buffer. This increases purity, but is more labour intensive.^{8,25} Consideration should also be given to the fact that separation methods can influence important extracellular vesicle characteristics, such as size, integrity, composition and functional properties.^{18,26,27} Separation methods can isolate non-vesicular circulating miRNAs or RNAs together with extracellular vesicles.^{28,29} Since treatment of extracellular vesicles with deoxyribonuclease/ribonuclease (DNase/RNase) does not interfere with RNA analysis and degrades externally bound RNA, its use is recommended by the ISEV and AHA.^{7,11,30} Interpretation of data should take into account isolation techniques and use of ribonuclease treatment. For small cohorts, it is useful to verify the reliability of separation methods, by quantifying contaminants (measuring albumin, ApoB100, ApoA1 and ApoB48) and/or by characterising the aspect of separate extracellular vesicles (for example, by using transmission electron microscopy).^{11,31} A ratio of at least 3×10^{10} vesicles per μg of protein is proposed to define high purity.³²

The main extracellular vesicle separation methods applicable for biomarker use are summarised in [Table 1](#).^{6,7,11,12,22,24,29,33–42} Some separation methods are unlikely to be suitable for use in clinical routines, including differential centrifugation, density gradient centrifugation and immunoaffinity capture. These methods are described in the [supplementary text](#).^{6,11,12,19,25,33–35,42} Other separation methods seem to be better adapted to clinical settings: (a) Size-exclusion chromatography separates extracellular vesicles from other components based on size.⁴³ It uses a column containing porous beads: proteins are small enough to pass through the pores, but extracellular vesicles are not. Extracellular vesicles therefore migrate at a higher speed than soluble proteins ([Fig. 1](#)). However, if the molecule (such as a lipoprotein) is the same size as the extracellular vesicle, both will migrate at the same speed and can therefore be co-isolated.³¹ This technique has been shown to give intact and functional vesicles, as well as fewer protein and lipoprotein contaminants than other methods, although it achieves suboptimal purity.^{7,26,31,35,37,44} Automated acquisition methods have recently been developed, which make this technique less labour intensive and more useful in clinical settings. (b) Filtration can be used to separate smaller-sized soluble components (which can pass through pores) from extracellular vesicles which are retained on the filter. The filter reference number is important, as filter type and pore size have been shown to heavily influence recovery.^{8,38} The effect on vesicle disruption of the forces applied to push samples through the filters is unknown.³⁴ (c) Precipitation kits (such as, Exo-QuickTM, miRCURYTM Exosome Isolation Kit or

Box 2. Blood sample collection, platelet-free plasma preparation and storage, adapted from ISEV 2013, AHA 2017, ISAC 2016 and ISTH 2015 guidelines.^{6,11,12,20}

- Prefer fasting blood samples.
- If possible, collect samples during same time period.
- Adapt anticoagulant to downstream analysis: citrate, EDTA or sodium fluoride/potassium.
- Avoid heparin-based anticoagulants.
- For venipuncture, use 21-gauge needles or larger. Avoid butterfly systems.
- Remove cuff rapidly after venipuncture and discard first 2–3 ml.
- Properly fill tubes to obtain correct blood/anticoagulant ratio. Invert tubes 8–10 times without shaking.
- Stabilize tubes and store vertically. Minimize bench-top storage time <30–60 minutes.
- Avoid analyzing hemolyzed samples.
- Double centrifugate at 2500 g for 15 minutes at room temperature, set the lowest deceleration of centrifuge.
- Use a clean plastic tube for second centrifugation. Do not collect the last 0.5 cm of plasma above the buffy coat.
- Freeze and store at -80 °C.

AHA, American Heart Society; EDTA, ethylene diamine tetraacetic acid; ISAC, International Society for Advancement of Cytometry; ISEV, International Society of Extracellular Vesicles; ISTH, International Society of Thrombosis and Hemostasis.

InvitrogenTM Total Exosome Isolation Kit) recover large quantities of extracellular vesicles, but are associated with poor purity, as they also recover protein complexes and extracellular vesicle aggregates.^{8,29} Their application should be limited to samples known to be rich in small extracellular vesicles, for the study of RNA, or as a concentration method after using another separation method.^{12,24,34} Thus, as a general rule, precipitation kits should be considered with caution in a biomarker setting.¹¹

Other biofluid collection and separation methods

In addition to plasma, extracellular vesicles have been detected in other biological fluids, including serum, urine, ascites and bile. Methods to collect these biofluids and separate extracellular vesicles are described in the [supplementary text](#).^{6,11,12,20,24,45–55}

Analytical techniques

From a biomarker perspective, measurement of plasma extracellular vesicle concentrations involves a compromise between very accurate characterisation of extracellular vesicles, as proposed by MISEV 2018 guidelines,⁸ and operational strategies that can be adapted to routine laboratory settings in a rapid and inexpensive manner.

Accurate characterisation

Several steps are proposed in the MISEV 2018 guidelines⁸ in order to establish an extracellular vesicle separation method. These steps include quantification, general characterisation, single vesicle characterisation and are described in the [supplementary text](#).^{8,11,56–60} Available technologies that may be used to perform all these steps are listed in [Table 2](#).^{7,8,11,56,61–72} These steps should be considered when setting up the detection of an

Table 1. Main separation methods of extracellular vesicles.

| Method | Type of EV recovered | EV recovery | Purity | Comment | Applicability in clinical settings | Ref. |
|--|---|---|---|--|--|----------------|
| Methods unlikely to be used in clinical routine | | | | | | |
| Differential centrifugation | Small & larger EVs | Intermediate (2–80%); depends on size and density (<i>i.e.</i> EV cargo) | Intermediate: co-recovery of organelles and viruses | High variability (depending on dilution method, sample viscosity) Risk of EV aggregation or damage | No: Time consuming (2–9 h), laborious, low-throughput | 11,12,22,33,36 |
| Density gradient centrifugation | Small & larger EVs | Low (10–50%); High for small EVs | Intermediate: co-recovery with lipoproteins, High for small EVs | Risk of damage and loss of biological activity. | No: time consuming (6–48 h), laborious, low-throughput | 6,11,29,34 |
| Immunocapture assays | Small & larger EVs Specific subpopulations | Intermediate | Intermediate, poor on plasma | Variability depending on antibody panel. Low hand-on time. | Only for media with few protein contaminants. Not for plasma. High-throughput when using multiwell plates. | 11,12,34,35,42 |
| Methods adapted to clinical routine | | | | | | |
| Size-exclusion chromatography | Small & larger EVs | High Depends on pore size. | High Depends on pore size and column height. | Important dilution Commercialized columns available | Maybe: easy and fast (30 min) but labour intensive | 6,7,11,35,37 |
| Filtration | Small & larger EVs | Variable: Depends on membrane type and pore size. | High | High variability depending on filter type | Yes, for larger EVs: easy, fast (20 min) and reproducible if same filter is used. Not for small EVs. | 11,24,38–41 |
| Precipitation kits | Small EVs | High (90%) | Poor: co-recovery of protein complexes and non-EV particles | Should be used as an EV concentration method or for studying EV RNAs | Yes: inexpensive, fast, small volumes | 11,24 |

EV(s), extracellular vesicle(s).

extracellular vesicle marker, to assess the results of separation methods and to establish the likelihood that the biomarker is associated with extracellular vesicles and not with other co-separated materials.⁸ These steps do not need to be repeated, once the separation and routine measurement method have been validated.

Routine measurement of extracellular vesicles

For extracellular vesicles to be used as biomarkers in routine clinical settings, methods of measurement should exhibit the following characteristics: they should be able to detect small events as well as specific subpopulations; to have low inter-laboratory variability; and to use widely available devices, with quick acquisition time and limited costs. The 3 main detection methods commonly used for extracellular vesicles in this setting are summarised in Table 3.^{7,11,39,71–76}

High sensitivity-flow cytometry on a dedicated device can simultaneously detect light scatter (*i.e.* diameter) and fluorescence signal (*i.e.* an extracellular vesicle subpopulation) from extracellular vesicles passing one by one in front of a laser beam.⁶ It is the most commonly used technique for measuring concentrations of total extracellular vesicles, as well as subpopulations of extracellular vesicles, and can also determine cellular origin. Dedicated high-resolution flow cytometers are required, using bead-based calibrations or a scatter-diameter relationship model to detect small extracellular vesicles.^{75,77} Yet, a major limitation is the variation in the smallest detectable size with different instruments, which affects the measured concentrations, and is responsible for differing sensitivities between instruments.^{6,73} In response to this high inter-laboratory variability, efforts to standardise the technique are currently underway.^{73,77,78}

Sandwich enzyme-linked immunosorbent assay (ELISA) quantifies specific extracellular vesicle-bound proteins.^{39,71} It is a reproducible detection method, using colorimetric or fluorescent detection, that gives results in standard units. Extracellular vesicles must be separated from soluble components. Our team has proposed an in-house filtration/ELISA method, which is highly reproducible with a variation coefficient <10%, and has proven its applicability in large cohorts.^{39–41} The filtration/ELISA technique is described in Fig. 2.

Quantitative reverse transcription PCR (qRT-PCR) is a method that quantifies a specific RNA transcript.⁷ It is the most widely used method in biomarker identification as it is robust, low cost, and has high analytical sensitivity.⁷² The check list of experimental details for RNA analysis has been described.³⁰ Other RNA quantification methods validated for extracellular vesicles are outlined in Table 2.^{7,8,11,56,61–72} Northern blot is a robust and sensitive method to measure specific RNA

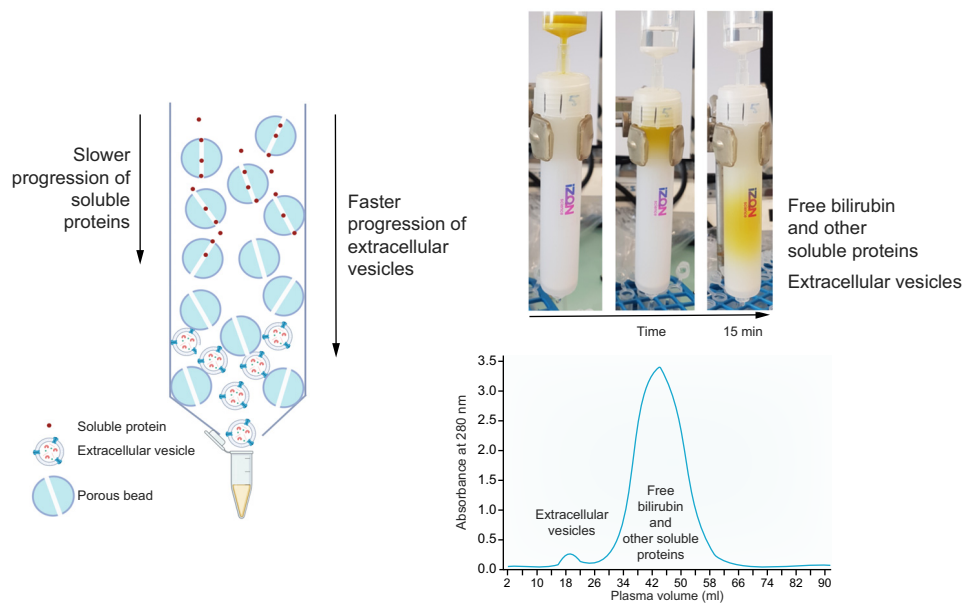


Fig. 1. Size-exclusion chromatography. Left. The column contains porous beads separating soluble proteins (which migrate at lower speed, as they are small enough to go through the pores) from extracellular vesicles. Right. As an example, plasma from a patient with Child-Pugh C cirrhosis goes through a size-exclusion chromatography column, progressively separating the extracellular vesicle compartment from the bilirubin-rich protein compartment. The chromatogram shows absorbance at 280 nm of each 2 ml fraction of plasma: extracellular vesicles are detected first, followed by soluble proteins.

transcripts, but it is time consuming and thus not adapted to clinical laboratories.

Other detection methods that are often used in research settings and are less applicable in clinical settings are summarised in [Table 2](#). Nanoparticle tracking analysis, which has outperformed dynamic light scattering, is a method that determines size and concentration of extracellular vesicles by measuring their Brownian motion. However, these techniques are limited by the fact that: they are unable to detect specific extracellular vesicle subpopulations; exact concentrations can only be estimated with a risk of high inter-laboratory variability; and they require special equipment.¹¹ Current development of fluorescent detection using nanoparticle tracking analysis devices may enable measurement of specific extracellular vesicle subpopulations in the future.^{79,80}

Extracellular vesicles in liver diseases

Extracellular vesicles appear to be attractive biomarkers for diagnosis, as well as for estimating severity and prognosis in liver diseases, including non-alcoholic and alcoholic steatohepatitis, chronic viral hepatitis B and C infections, cirrhosis, primary liver cancers, primary sclerosing cholangitis and acute liver failure. The main clinical studies evaluating extracellular vesicle subpopulations as biomarkers in liver diseases are summarised in [Table 4](#) (review criteria are summarised in the [supplementary text](#)). Unless otherwise mentioned, the extracellular vesicles studied were all taken from plasma.

Non-alcoholic fatty liver disease

Non-invasive tools to assess steatohepatitis and fibrosis in non-alcoholic fatty liver disease (NAFLD) do not entirely reflect the variety of liver histological changes in these patients.⁸¹ Extracellular vesicles that reflect ongoing cell damage might be able to fill this gap and better capture the disease's complexity. [Table S1](#) summarises extracellular subpopulations as potential biomarkers in NAFLD.^{2,82–89}

Diagnosis of non-alcoholic fatty liver disease

The largest study investigating the potential diagnostic utility of extracellular vesicles in NAFLD reported higher concentrations of T cell- and monocyte-derived extracellular vesicles in 65 patients with NAFLD compared to healthy controls.⁸² A pilot study identified increased mitochondria-rich extracellular vesicles in obese patients with increased serum transaminase levels, but sample sizes were small and no histology or imaging were used to characterise NAFLD.²

One study showed that a pattern of 12 miRNAs carried by serum extracellular vesicles could discriminate 12 patients with non-alcoholic steatohepatitis from patients with chronic hepatitis B and C infections and from healthy controls, although limited access to microarrays in clinical routine limits the applicability of this approach.⁸⁴

Marker of activity and fibrosis

Regarding liver NAFLD activity, extracellular vesicles originating from natural killer T cells and monocytes (CD14+) have been shown to correlate

Key point

Extracellular vesicles are promising biomarkers for diagnosis of liver diseases and prediction of disease progression, complications, response to treatment and mortality. Different extracellular vesicle subpopulations have been studied, using total extracellular vesicle count, proteins, lipids, RNAs and microRNAs.

Table 2. Summary of available technologies to perform extracellular vesicle characterisation in research settings.

| | Quantification | General characterisation | Single vesicle characterisation |
|------------|---|--|--|
| Aim | <ul style="list-style-type: none"> • Evaluation of EV recovery • Evaluation of purity • Quantification of EV subtype | Confirmation of EV presence (protein markers) | <ul style="list-style-type: none"> • Evaluation of EV integrity • Confirmation of EV presence • Evaluation of EV subtype (size) |
| Technology | <p>Particle number:⁸</p> <ul style="list-style-type: none"> • Nanoparticle tracking analysis⁶¹ • High-resolution bead-based flow cytometry • Resistive pulse sensing • Cryo-electron microscopy⁵⁶ • Surface plasmon resonance coupled to atomic force microscopy⁶² <p>RNA quantification:⁷</p> <ul style="list-style-type: none"> • Bioanalyzer pico chip • Quant-iT RiboGreen RNA Assay • Quantitative reverse transcription PCR <p>Total protein count :⁸</p> <ul style="list-style-type: none"> • Colorimetric assays • Fluoremetric assays • Protein stain on SDS-PAGE <p>Specific protein count:^{8,11}</p> <ul style="list-style-type: none"> • ELISA • Bead-based flow cytometry • Aptamer- carbon nanotubes colorimetric assays • Nanoplasmon-enhanced scattering assay⁶³ | <ul style="list-style-type: none"> • Bead-based flow cytometry⁸ • Western blot (mainly for cell culture media)⁸ • Multiplex bead-based platforms^{64,65} • Surface plasmon resonance⁶⁶ • Fluorescence scanning⁶⁷ • More assays are described in⁷¹ | <p>High-resolution imaging technique:^{8,68}</p> <ul style="list-style-type: none"> • Transmission electron microscopy • Scanning electron microscopy • Cryo-electron microscopy • Atomic force microscopy • Super-resolution microscopy <p>Estimation of biophysical features:^{8,69,70,72}</p> <ul style="list-style-type: none"> • Resistive pulse sensing • Nanoparticle tracking analysis • High-resolution flow cytometry • Asymmetric flow field fractionation • Raman spectroscopy |

ELISA, enzyme-linked immunosorbent assay; EV, extracellular vesicle.

Table 3. Detection methods of extracellular vesicles having potential for clinical use as biomarkers.

| Method | Type of detected EV | Advantages | Disadvantages | Ref. |
|---------------------------------|--|---|--|----------|
| High sensitivity-flow cytometry | Total and EV subpopulation Minimal diameter: 100 nm | Detection of size and protein Detection of cellular origin Capacity of sorting different subpopulations Efforts in standardisation | Only detects larger extracellular vesicles Inter-laboratory variability Dedicated cytometers Labour intensive Expensive Swarming: false negatives and altered linearity False positives: antibody aggregates, inorganic precipitates, lipoproteins | 11,73–75 |
| Filtration/ELISA | EV subpopulation Size: depends on filter size | Reproducible High-throughput Absolute number and standard units | Use of transmembrane or membrane-anchored proteins Filter saturation | 39,71,76 |
| qRT-PCR | EV subpopulation | Robust and sensitive Detects intact and fragmented RNA Low cost | Time consuming Absence of endogenous controls for validation (use EV-transcriptomic data or absolute quantification) Average RNA copy number from total extracellular vesicles | 7,72 |

ELISA, enzyme-linked immunosorbent assay; EV, extracellular vesicle; qRT-PCR, quantitative reverse transcription PCR.

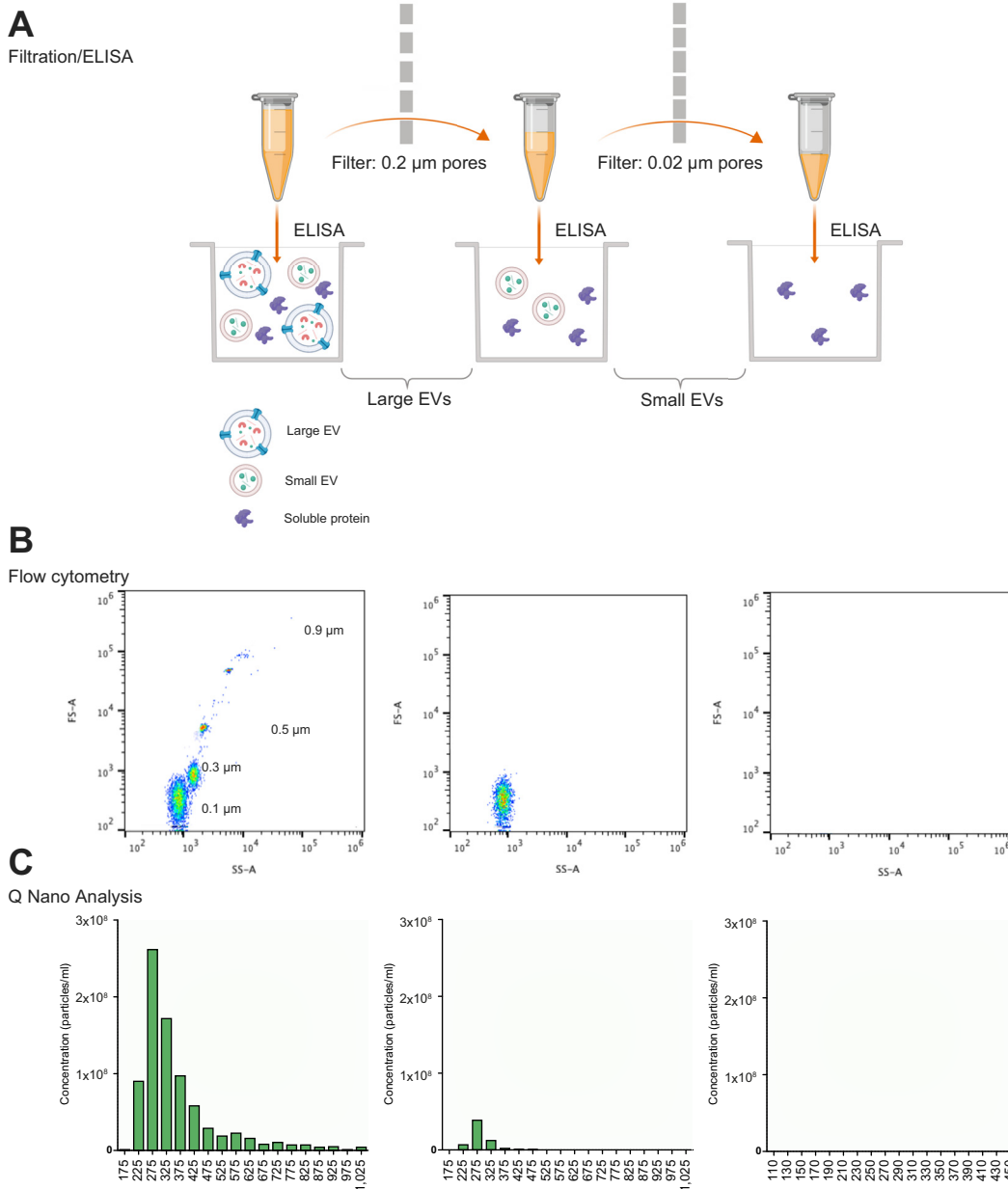


Fig. 2. Extracellular vesicle subpopulation detection method by filtration/ELISA. (A) Graphical representation of the filtration/ELISA method. ELISA is performed on platelet-free plasma before filtration, after double filtration through 0.2 μm pores, and after filtration through 0.02 μm pores. Concentration of larger extracellular vesicles is equal to the difference between protein concentration before and after double filtration through 0.2 μm pores. Concentration of small extracellular vesicles is equal to the difference between protein concentration after double filtration through 0.2 μm pores and after filtration through 0.02 μm pores. (B) Flow cytometry graphs showing calibrated Megamix-Plus FSC beads having a 0.1, 0.3, 0.5 and 0.9 μm sizes. Filtration through 0.2 μm pores removes 0.3 μm , 0.5 and 0.9 μm beads. Filtration through 0.02 μm pores removes 0.1 μm beads. (C) Tunable Resistive Pulse Sensing (TRPS, qNano) analysis of the plasma of a patient with cirrhosis before filtration, after filtration through 0.2 μm pores and after filtration through 0.02 μm pores. After filtration through 0.2 μm pores, a decrease of 92% of events between 185 and 1,000 nm was observed ($n = 2$). ELISA, enzyme-linked immunosorbent assay; EV, extracellular vesicle.

with histological grade and with NAFLD activity score and alanine aminotransferase, respectively, unlike CD4+ and CD8+ T cell subpopulations.⁸² Two lipidic extracellular vesicle markers can help to differentiate patients with non-alcoholic steatohepatitis from those with simple steatosis and from obese patients without NAFLD, but use of mass

spectrometry limits their applicability in clinical settings.⁸³

Regarding fibrosis, none of the previously cited extracellular vesicle subpopulations significantly correlated with histological fibrosis stage. On the other hand, a pilot study found that CD14+ (monocyte) and CD16+ (leukocyte)

Table 4. Summary of main clinical studies on extracellular vesicles as biomarkers in liver diseases.

| Biomarker | Var | EV size & technique | Number (patient/control) | Se/Spe (%) | AUROC PPV/NPV | Outcome | Study type | Ref. |
|--|-----|---------------------|--|------------|---------------|---------------------------------|-------------------------|---------------------|
| NAFLD | | | | | | | | |
| T Cell (CD4+ or CD8+ or iNKT) | ↑ | Larger | NAFLD: 65 | 27–59/ | 0.81–0.91 | Detection of NAFLD | Retrospective | 82 |
| Monocyte (CD14+) | | FCM | HC: 44 | 90–98 | n.a. | | | |
| Neutrophil (CD15+) | | | | | | | | |
| Platelet (CD41+) | | | | | | | | |
| Alcoholic hepatitis | | | | | | | | |
| Hepatocyte (cytokeratin-18) | ↑ | Larger | Confirmed AH: test: 46, validation: 48 | 76/81 | 0.82 | Diagnosis of AH | Prospective | 41 |
| | | Filtration/ELISA | Ruled-out AH: test: 37, validation: 20 | | 83/73 | | | |
| Haematopoietic stem cell (CD34+) & Hepatocyte (ASPGR+) | ↑ | Larger | Severe AH: non-responders: 71 | n.a. | 0.94 | Response to therapy & mortality | Retrospective | 90 |
| | | FCM | responders: 30 | | n.a. | | | |
| | | | HC: 20 | | | | | |
| Cirrhosis | | | | | | | | |
| Platelet-derived growth factor receptor β | ↑ | Small | Fibrosis 0–1: 51 | 82/41 | 0.64 | Detection of fibrosis F3-F4 | Retrospective | 134 |
| | | ELISA | Fibrosis 2–4: 97 | | n.a. | | | |
| | | | HC: 14 | | | | | |
| | | | Validation cohort: 57 | | | | | |
| Leuko-endothelial (CD31+/41–) | ↑ | Larger | Cirrhosis: 91 | n.a. | n.a. | Survival | Prospective | 39 |
| | | FCM | | | | | | |
| Hepatocyte (cytokeratin-18) | ↑ | Larger | Cirrhosis: Test cohort: 139 | n.a. | n.a. | 6-month mortality | Prospective | 40 |
| | | Filtration-ELISA | Validation cohort: 103 | | | | Competing risk analysis | |
| Hepatocellular carcinoma | | | | | | | | |
| Tumoural (AnnexinV+ EpCAM+ASPGR1+ +/- CD133+) | ↑ | Larger | HCC: 86 | 80–81/ | 0.73–0.74 | Detection of HCC | Retrospective | 143 |
| | | FCM | Cirrhosis: 49 | 47–50 | n.a. | | | |
| | | | HC: 58 | | | | | |
| miRNA-519d & -595 & -939 | ↑ | Small | Advanced HCC: 45 | n.a. | 0.82–0.84 | Detection of HCC | Retrospective | 151 |
| | | qRT-PCR | Unifocal/small: 40 | | n.a. | | | |
| | | | Cirrhosis no HCC: 30 | | | | | |
| miRNA-125 | ↓ | Small | HCC: 128 | 83/68 | 0.74 | Recurrence & survival | Prospective | 150 |
| | | qRT-PCR | | | n.a. | | | |
| Signature of miRNA-122, -148a, -1246 | ↑ | Small | HCC: 50 | 86/88 | 0.93 | Detection of HCC | Retrospective | 149 |
| | | qRT-PCR | Cirrhosis: 40 | | n.a. | | | |
| miRNA-638 | ↓ | Small | HCC: 126 | n.a. | n.a. | Overall survival | Retrospective | 171 |
| | | qRT-PCR | | | | | | |
| miRNA-21 & miRNA-10b | ↑ | Small | HCC: 124 | n.a. | n.a. | Prediction of recurrence | Retrospective | 156 |
| | | qRT-PCR | | | | | | |
| lncRNA-RP11-513115.6 & miRNA 1262 & RAB11A | ↑ | Small | HCC: 54 | 78–98/ | n.a. | Detection of early HCC | Retrospective | 152 |
| | | qRT-PCR | HCV: 42; HC: 18 | 73–95 | 72–95/79–97 | | | |
| lncRNA ENSG00000258332.1 & LINC00635 | ↑ | Small | HCC: 60 | 72–76 | 0.72–0.75 | Detection of HCC & survival | Retrospective | 147 |
| | | qRT-PCR | HBV: 96 | /78–83 | n.a. | | | |
| miRNA-92b | ↑ | Small | After LT: No recurrence: 28 | 71/63 | 0.70 | Early recurrence after LT | Retrospective | 194 |
| | | qRT-PCR | Early recurrence: 43 | | n.a. | | | |
| miRNA-21 & lncRNA-ATB | ↑ | Small | HCC: 79 | n.a. | n.a. | Overall survival | Prospective | 191 |
| | | qRT-PCR | | | | | Multivariate | |

(continued on next page)

Table 4. (continued)

| Biomarker | Var EV size & technique | Number (patient/control) | Se/Spe (%) | AUROC PPV/NPV | Outcome | Study type | Ref. |
|-----------------------------------|-------------------------|---|-----------------|-----------------|---------------------------------|--------------------------|------|
| Cholangiocarcinoma | | | | | | | |
| Total bile EVs & Total plasma EVs | ↑ Small & larger NTA | Malignant stenosis: 15 (5 CCA) Benign stenosis: 15 | 47–100 / 80–100 | 0.81–1 70/60 | Detection of malignant stenosis | Prospective Multivariate | 52 |
| Acute liver failure | | | | | | | |
| Total plasma EV | ↑ Larger FCM | Acute liver injury/failure: 50 | n.a. | n.a. | Prediction of mortality/LT | Prospective Multivariate | 206 |

AH, alcoholic hepatitis; AUROC, area under the receiver-operating characteristic; CCA, cholangiocarcinoma; EV, extracellular vesicle; FCM, flow cytometry; HC, healthy control; HCC, hepatocellular carcinoma; IncRNA, long non-coding RNA; LT, liver transplantation; qRT-PCR, quantitative reverse transcription PCR; miRNA, micro-RNA; n.a., not available; ELISA, enzyme-linked immunosorbent assay; NAFLD, non-alcoholic fatty liver disease; NPV, negative predictive value; NTA, nanoparticle tracking analysis; PPV, positive predictive value; Ref, reference; Se, sensitivity; Spe, specificity; Var, variation.

extracellular vesicles decreased with fibrosis stage.⁸⁸ These different results can be explained by the use of different extracellular vesicle separation methods, as well as the inclusion of patients with different non-alcoholic steatohepatitis severity.

Two miRNAs contained in serum extracellular vesicles, miRNA-122, a major hepatic miRNA, and miRNA-192, were found to increase with activity and fibrosis stage in several studies.^{85–87} However, these findings should be interpreted with caution since fewer than 10 patients were included in each study, and since, in 1 study, the extracellular vesicle fraction of miRNA-122 was not specifically studied.

To conclude, leukocyte extracellular vesicles have potential as biomarkers for NAFLD diagnosis, activity and fibrosis, providing that large prospective studies validate these findings. Further studies are required to confirm the potential utility of extracellular vesicles carrying miRNA-122 and -129.

Alcoholic hepatitis

The 2 main clinical challenges in alcoholic hepatitis are the non-invasive diagnosis of the disease and prediction of treatment response.

Diagnosis of alcoholic hepatitis

Our group tested whether plasma extracellular vesicle levels could be used to diagnose alcoholic hepatitis non-invasively. In 2 prospective cohorts of 83 and 68 patients with clinical suspicion of alcoholic hepatitis, we observed that plasma hepatocyte (cytokeratin-18+) extracellular vesicle levels were able to diagnose alcoholic hepatitis with good sensitivity (76%) and specificity (81%). Yet, total soluble cytokeratin-18 performed even better and can be measured more easily.⁴¹ Higher levels of hepatocyte-derived (ASPGR+) extracellular vesicles were also observed in 101 patients with severe alcoholic hepatitis compared to healthy controls.⁹⁰

Plasma concentrations of total extracellular vesicles, as well as of extracellular vesicles derived from T cells (plasma and serum), macrophages, neutrophils, haematopoietic stem cells and endothelial cells are found to be higher among patients with severe alcoholic hepatitis than among healthy controls.^{90,91} However, these changes might simply reflect the consequences of alcohol intake and not alcoholic hepatitis. Indeed, our group measured plasma extracellular vesicles derived from endothelial cells (CD62e+; CD41-/31+), platelets (CD41+), and leukocytes (CD11a+) in patients with clinical suspicion of alcoholic hepatitis and did not observe any difference between patients with and without histologically proven alcoholic hepatitis.⁴¹

Along the same lines, plasma and serum concentrations of several subpopulations of extracellular vesicles (carrying mitochondrial DNA, miRNA

or hepatocyte proteins) are higher in patients after binge drinking and/or chronic excessive alcohol consumption, as summarised in [Table S2](#).^{41,90–103} Their potential utility for diagnosis of alcoholic hepatitis should be tested in patients with a clinical suspicion of alcoholic hepatitis.

Prediction of outcome in alcoholic hepatitis

In a cohort of 101 patients with histologically proven alcoholic steatohepatitis, concentrations of circulating CD34+ (a haematopoietic stem cell marker) and ASGPR+ (a hepatocyte marker) extracellular vesicles were higher among non-responders to steroid therapy than among responders, and could predict 7-day and 1-month mortality.⁹⁰ The combination of the 2 markers could predict 7-day non-response to steroid therapy with an area under the receiver-operating characteristic (AUROC) of 0.94. Yet, no threshold that can be used in clinical practice has been established. Other extracellular vesicle subpopulations (T cell, macrophage and endothelial) evaluated in this study were higher among non-responders to steroid therapy, but were less accurate in predicting mortality.⁹⁰

To conclude, circulating hepatocyte-derived extracellular vesicles seem to be a promising biomarker for diagnosis and outcome prediction in patients with alcoholic hepatitis. Data on other subpopulations are less homogenous and need to be confirmed in future studies.

Hepatitis B and C virus infections

Extracellular vesicles could be useful markers to predict the risk of hepatitis B virus reactivation and detection of early fibrosis for hepatitis B and C. Results of studies on that topic are summarised in [Table S3](#)^{104–108} and the [supplementary text](#)^{104–110} for hepatitis B, and [Table S4](#)^{82,84,105,111–121} and the [supplementary text](#)^{82,84,105,111–122} for hepatitis C.

Cirrhosis

Different extracellular vesicle subpopulations increase in patients with cirrhosis, and are predictive of disease progression, complications and mortality ([Table S5](#)).^{39,40,47,50,100,123–135} However, whether this increase is due to excess production, decreased clearance, or (as is most likely) both, remains a source of doubt.¹⁰

Estimation of cirrhosis severity

Hepatocyte extracellular vesicle concentrations, characterised by cytokeratin-18 expression, significantly increased with Child-Pugh score in 3 independent prospective cohorts of 40, 139 and 103 patients.^{39,40} Their concentrations also correlated with model for end-stage liver disease (MELD) score and its components.⁴⁰ In the same study, concentrations of hepatocyte-derived larger

extracellular vesicles increased alongside hepatic necro-inflammatory activity, suggesting that hepatocyte injury is the driver of extracellular vesicle release.⁴⁰

Cirrhosis is known to be responsible for vascular changes including systemic vasodilation, increased cardiac output and increased intrahepatic vascular resistance. For this reason, several studies have analysed endothelial-derived extracellular vesicles.^{39,40,125,126} They all concluded that patients with cirrhosis have more endothelial-derived extracellular vesicles (CD31+/41–, CD31+/42b– or CD62e) than healthy individuals. A link between cirrhosis severity (Child-Pugh or MELD score) and plasma levels of larger leuko-endothelial (CD31+/41–) extracellular vesicles was observed in an initial cohort of 91 patients,³⁹ but this was subsequently not confirmed using the same markers (CD31+/41–),⁴⁰ and no link was observed with different markers (CD31+/42b–).¹²⁵

With systemic inflammation being a key feature of cirrhosis, several subpopulations of leukocyte-derived extracellular vesicles (CD45+, CD11a+ and CD4+) have been analysed in different studies and found to be more abundant than in healthy individuals.^{39,40,126} However, no link with cirrhosis severity was observed.

Data on platelet-derived extracellular vesicles are controversial. Three studies have found a significant increase in platelet-derived extracellular vesicles in patients with cirrhosis, compared with healthy controls, using CD62P+ as a marker.^{126–128} Four other studies have found no significant difference using the CD41+ marker.^{39,40,129,130} CD41+ extracellular vesicle levels did not change with cirrhosis severity.^{40,129} Other platelet-derived extracellular vesicles were studied and their levels were found to overlap with healthy controls.¹³⁶ These contradictory results could be due to the use of different platelet markers, as well as numerous factors influencing platelet-derived extracellular vesicle release during plasma sample preparation, making these vesicles an unreliable marker.¹³⁷

Pilot studies, described in [Table S5](#), have identified several plasma and serum miRNAs that are up- or downregulated during advanced cirrhosis, but the association with cirrhosis severity has not been investigated.^{39,40,47,50,100,123–135} Furthermore, validation is needed, given the small sample sizes and other methodological limitations.

Estimation of portal hypertension

For the time being, no extracellular vesicle subpopulation has been found to reliably estimate portal hypertension. Hepatocyte cytokeratin-18+ extracellular vesicles weakly correlated with hepatic venous pressure gradient (HVPG), but could not discriminate patients with an HVPG ≥ 10 mmHg.⁴⁰

Key point

Some extracellular vesicle subpopulations have already been evaluated in prospective studies in cirrhosis, alcoholic liver disease and hepatocellular carcinoma. In other liver diseases, extracellular vesicles still await validation.

Prediction of complications of cirrhosis

The procoagulant activity of some extracellular vesicles could play a role in portal vein thrombosis. Some studies have evaluated the ability of extracellular vesicles to predict portal vein thrombosis or other complications of cirrhosis, including ascites, hepatic encephalopathy, hepatopulmonary and hepatorenal syndromes. These studies are discussed in the [supplementary text](#).^{39,47,125,126,135,138–142}

Prediction of mortality

In a prospective collaborative study, including a validation cohort, we demonstrated that plasma levels of hepatocyte-derived extracellular vesicles can predict 6-month mortality, independently of Child-Pugh and MELD scores, using a threshold of 65 IU/L.⁴⁰ In this study, other subpopulations of plasma extracellular vesicles, including pan-leukocyte (CD11+) and endothelial (CD144+, CD62E+), were not useful for predicting mortality. However, small leuko-endothelial (CD31+/41-) extracellular vesicles also predicted 6-month mortality independently of Child-Pugh score in 2 studies.^{39,40}

The potential application of extracellular vesicles in ascites was recently investigated in 163 patients with cirrhosis.⁵⁰ The study found that low total extracellular vesicle levels in ascites predicted 30-day mortality independently of MELD score and antibiotic treatment. High percentages of neutrophil-derived (CD66b+) or lymphocyte-derived (CD3) extracellular vesicles in ascites were more commonly observed in patients with poor outcomes, but survival analyses were not performed for these subpopulations.⁵⁰

In conclusion, several subpopulations of extracellular vesicles seem to be promising candidate biomarkers for the evaluation of cirrhosis severity or prediction of mortality. Data are missing on the prediction of more specific complications and this data gap will need to be filled if personalised monitoring strategies are to be achieved.

Hepatocellular carcinoma

An unmet need is the development of tools to predict the risk of hepatocellular carcinoma (HCC) occurrence among patients with chronic liver diseases, in order to identify those requiring closer disease monitoring. However, no study has evaluated the potential of extracellular vesicles to meet this need. Conversely, abundant data suggest that extracellular vesicles could help detect HCC, predict HCC recurrence, survival and might also be associated with tumour aggressiveness.

Detection of hepatocellular carcinoma

A major challenge is to find markers facilitating early detection of HCC among patients with chronic liver diseases. Ideally, studies should include patients with early HCC and use patients with similar chronic liver diseases without HCC as

controls, but this study design has rarely been applied.^{106,123,124,126,143–154}

Total extracellular vesicles, as well as some subpopulations, increase in the plasma¹²⁴ and sera¹²³ of patients with HCC, and their concentrations normalise after liver transplantation or R0 hepatectomy, which suggests a direct relationship with tumour presence. In a pilot study, 8 patients with HCC induced by hepatitis C-related cirrhosis had higher total, hepatic (HepPar+) and endothelial (CD144+) extracellular vesicles than 5 patients without liver disease.¹²⁴ The finding for total serum extracellular vesicle concentrations was confirmed in a larger study, and a threshold was established to discriminate patients with early HCC from patients with cirrhosis alone.¹²³ A large study assessed concentrations of serum extracellular vesicles derived from tumour cells (AnnexinV+EpCAM+ASPGR1+) and showed that this subpopulation could discriminate 86 patients with HCC from 49 patients with tumour-free cirrhotic liver (3-fold increase, AUROC 0.73).¹⁴³

Expressions of several non-coding RNAs contained in extracellular vesicles are modified in patients with HCC. A meta-analysis calculated that when pooling 30 small extracellular vesicle miRNA assays, diagnosis of HCC reached a sensitivity of 80% and a specificity of 81%.¹⁵⁵ Some miRNAs are of particular interest. miRNA-21 had a higher expression in circulating serum extracellular vesicles of patients with HCC (n = 30), than in patients with chronic hepatitis B virus infection without cirrhosis and healthy controls (n = 30 per group).¹⁴⁶ These results have been confirmed by another study.¹⁵⁶ Yet, it is unclear whether HCC was the driver for circulating miRNA-21 expression, or whether cirrhosis was more prevalent among the HCC group and was responsible for the higher circulating miRNA-21 expression levels in this group. miRNA-122 expression levels in circulating serum extracellular vesicles were even shown to differentiate early HCC from cirrhosis in 2 studies.^{145,157} miRNA-122 expression increased after transarterial chemoembolisation.¹⁵⁸ Using quantification of 2 serum long non-coding RNAs (lncRNAs) carried in extracellular vesicles, 60 patients with HCC could be discriminated from 60 patients with chronic hepatitis B in 2 cohorts.¹⁴⁷ In this study, a combination of the 2 RNAs and alpha-fetoprotein yielded a sensitivity of 84% and specificity of 88%, better than alpha-fetoprotein alone.¹⁴⁷ Yet, tumour stages were advanced in this study, thus limiting immediate clinical applications.

Other circulating extracellular vesicle subpopulations have been tested, but use of low-throughput measurement techniques, absence of precise description of measurement methods or patient characteristics, small sample sizes, use of healthy controls or advanced HCC, or absence of confirmation on whether the marker truly circulates in the extracellular vesicle fraction limit interpretation ([Table S6](#)).^{123,124,126,131,133,143–154,156–190}

Predictor of recurrence and survival

Extracellular vesicles could help predict HCC recurrence after liver resection. High levels of pre-operative small extracellular vesicle miRNA-155 were predictive of lower relapse-free survival in a retrospective study of 40 patients with mainly unifocal HCC treated with first-line curative hepatectomy, although no differences in 3- and 5-year overall survival were found.¹²⁰ In another study, recurrence was higher when serum extracellular vesicle miRNA-125 expression was low among 128 patients with HCC treated by surgical resection, in multivariate analysis.¹⁵⁰

Key point

High-throughput and reproducible methods to measure extracellular vesicles are now becoming available.

Circulating extracellular vesicle RNA expression can also predict overall survival. For example, higher levels of *RAB11A* RNA in small extracellular vesicles in the serum were associated with lower relapse-free survival among 60 patients with mainly early HCC in multivariate analysis.¹⁵² Several studies have found serum small extracellular vesicle miRNA-21 to be a potentially interesting marker of outcome.^{156,191,192} Notably, a prospective study of 79 patients with HCC observed that small extracellular vesicle miRNA-21 expression was independently associated with overall survival and disease progression.¹⁹¹

To conclude, many proteins and RNAs contained in extracellular vesicles have been identified as predictors of outcome in patients with HCC (Tables S7 and S8).^{119,120,144,147,148,150,152,156–159,167,169,171,179,183,184,186,191–199} These findings should nevertheless be confirmed in patients with all stages of HCC progression, taking into account confounding factors such as comorbidities and underlying liver disease.

Primary sclerosing cholangitis & cholangiocarcinoma

Major challenges in the clinical management of patients with primary sclerosing cholangitis include the unpredictable outcome of this liver disease in terms of progression and evolution towards cholangiocarcinoma.²⁰⁰ Data on serum extracellular vesicles as markers in this setting are limited to a proteomic analysis of serum extracellular vesicles: 10 proteins were found to be differentially expressed in patients with cholangiocarcinoma (n = 43), compared with patients with primary sclerosing cholangitis (n = 30), as well as with HCC (n = 29).¹⁷⁶ Western blot analyses confirmed these results for 6 proteins, but clinical validation is needed. One team has also analysed both serum and urine extracellular vesicles, and found different RNA transcriptomic profiles between patients with cholangiocarcinoma (n = 12) and those with primary sclerosing cholangitis (n = 6), although, again, clinical confirmation is needed.²⁰¹

One of the main challenges in biliary diseases is the difficult differentiation of benign strictures from malignant ones.

Two independent groups demonstrated that bile or serum extracellular vesicles could efficiently differentiate cholangiocarcinoma from benign biliary stenosis. One group reported that total bile and serum extracellular vesicles, measured using nanoparticle tracking analysis, could distinguish malignant biliary stenosis (pancreatic cancer and cholangiocarcinoma) from benign stenosis (chronic pancreatitis), extracellular vesicles in bile were shown to have a significantly better discriminating ability than serum CA19-9.⁵² Others observed the overexpression of a small extracellular vesicle miRNA panel in bile from 26 patients with cholangiocarcinoma compared to 50 controls with benign stenosis (including 13 with primary sclerosing cholangitis).⁵¹

Beyond the setting of biliary strictures, other studies have analysed extracellular vesicles in patients with cholangiocarcinoma. One study observed that circulating tumour cell-derived (AnnexinV+EpCAM+ASGPR1+) extracellular vesicles were 3-fold more frequent in the serum of patients with cholangiocarcinoma than those with cirrhosis.¹⁴³ These tumour cell-derived extracellular vesicles were, however, unable to differentiate HCC from cholangiocarcinoma.¹⁴³ A second study identified 2 small extracellular vesicle lncRNAs that were significantly overexpressed in the bile of 35 patients with cholangiocarcinoma compared to 56 healthy controls.⁵⁴ Their combination yielded higher sensitivity and specificity than CA19-9, and was associated with lower overall survival. A third study compared the expression of small extracellular vesicle miRNA-200c-3p between 36 patients with cholangiocarcinoma and 12 healthy controls.²⁰² Expression of small extracellular vesicle miRNA-200c-3p was higher among patients with cholangiocarcinoma, and increased with tumour stage and the presence of lymph node metastases. Patients with higher levels of miRNA-200c-3p had lower disease-free and overall survival.²⁰² Two other preclinical studies found differentially expressed extracellular vesicle RNAs between patients with cholangiocarcinoma and healthy controls.^{203,204}

To conclude, several subpopulations of extracellular vesicles have the potential to become very useful tools to differentiate cholangiocarcinoma from primary sclerosing cholangitis and other tumours. The use of demanding techniques to measure extracellular vesicles would likely not be a limitation in this setting since these situations are not common and patients are usually referred to expert centres.

Acute liver failure*Detection of acute liver failure*

Diagnosis of acute liver injury/failure is usually easy, as it is based on readily available clinical and laboratory data.²⁰⁵ In contrast, prediction or early identification of drug-induced liver injury remains

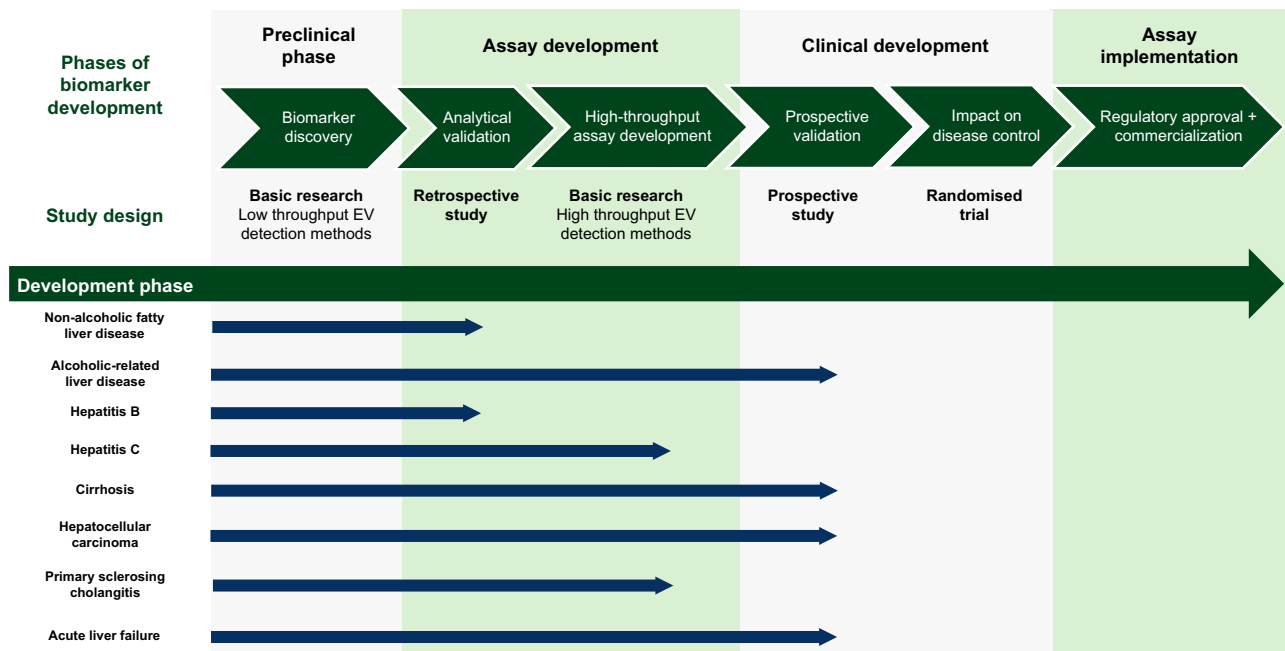


Fig. 3. Phases of development of extracellular vesicles as biomarkers for diagnosis, severity and prognosis in liver diseases. EV, extracellular vesicle.

an unmet need. Since extracellular vesicles are sensitive markers of cell injury, we can hypothesise that their circulating levels could be an early marker of drug-induced liver injury, even before liver blood test abnormalities appear. So far, data on this hypothesis are very limited. Most studies compared patients with acute liver injury to healthy controls, thereby limiting their clinical relevance (Table S9).^{206–209}

Predictor of outcome

The potential of extracellular vesicles in the acute liver injury/acute liver failure setting has been analysed by the “Acute Liver Failure Study Group” in a group of 50 patients. Plasma concentrations of total larger extracellular vesicles significantly increased in parallel with hepatic encephalopathy grade, and systemic inflammatory response syndrome.²⁰⁶ Moreover, concentrations of these extracellular vesicles were associated with death or liver transplantation at day 21 after admission, independently of other predictors (age, gender, and aetiology).²⁰⁶ Extracellular vesicles were mainly of platelet (CD41+) origin, but also of hepatocyte (ASPGR+), monocyte (CD18+) and endothelial cell (CD144+) origin and were tissue-factor positive, although each specific subpopulation was not significantly associated with severity.²⁰⁶

Conclusion

Extracellular vesicles hold promise in liver diseases as biomarkers for diagnosis and prediction of disease progression, complications, response to treatment and mortality. Although data so far are

derived from pilot studies, some subpopulations of extracellular vesicles have been robustly evaluated in independent large multicentric prospective cohorts, as summarised in Fig. 3.

Guidelines on extracellular vesicle research stress the importance of parameters such as purity, extracellular vesicle structure, physical characteristics and marker localisation, while their importance in clinical research is less explicitly covered.⁸ Other parameters which are less often considered in basic research, such as cost, assay time, labour-intensiveness, accessibility of measuring devices, inter-laboratory variability, characteristics of study population and control group, are more relevant for application in clinical settings. Several challenges must be overcome prior to considering the use of extracellular vesicles in clinical routines.²¹⁰ First, the reliability and reproducibility of the quantification methods have to be strengthened. Currently, results are likely to reflect the settings of several pre-analytical variables. For example, measured vesicle concentrations can be influenced by the minimum vesicle size that the technique can detect.²¹¹ The variation coefficient is often not mentioned in studies or is high and variable. There is no international reference preparation (gold standard) to assess the accuracy of an assay. Second, elaboration of high-throughput detection methods, not affecting sample stability and diversity, would facilitate the development of extracellular vesicle measurement. Currently, many characterisation techniques are prohibitively expensive and/or labour intensive. Third, determination of cut-off values is needed to help physicians make decisions. Fourth, results must be

validated in studies involving large cohorts of patients with comorbidities.

These limitations are currently being progressively overcome, thanks to standardisation guidelines and recommendations. Moreover, the number of studies on extracellular vesicles as biomarkers in liver diseases is growing exponentially. In the near future, these studies will provide convincing levels of evidence for both physicians and scientists.

Abbreviations

AHA, American Heart Association; AUROC, area under the receiver-operating characteristic curve; ISEV, International Society for Extracellular Vesicles; ELISA, enzyme-linked immunosorbent assay; HCC, hepatocellular carcinoma; HVPG, hepatic venous pressure gradient; lncRNA, long non-coding RNA; MELD, model for end-stage liver disease; miRNA, microRNA; MISEV, Minimal information for studies of extracellular vesicles; NAFLD, non-alcoholic fatty liver disease; qRT-PCR, quantitative reverse transcription PCR.

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Conflict of interest

Neither of the authors has any conflicts of interest to disclose.

Please refer to the accompanying [ICMJE disclosure](#) forms for further details.

Authors' contributions

Sara Thietart and Pierre-Emmanuel Rautou wrote the manuscript.

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Supplementary data

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References

- [1] van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol* 2018;19:213–228.
- [2] 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;126:859–864.
- [3] Puhm F, Afonyushkin T, Resch U, Obermayer G, Rohde M, Penz T, et al. Mitochondria are a subset of extracellular vesicles released by activated monocytes and induce type I IFN and TNF responses in endothelial cells. *Circ Res* 2019;125:43–52.
- [4] Ridger VC, Boulanger CM, Angelillo-Scherrer A, Badimon L, Blanc-Brude O, Bochaton-Piallat M-L, et al. Microvesicles in vascular homeostasis and diseases. *Thromb Haemost* 2017;117:1296–1316.
- [5] Doyle LM, Wang MZ. Overview of extracellular vesicles, their origin, composition, purpose, and methods for exosome isolation and analysis. *Cells* 2019;8:727.
- [6] van der Pol E, Böing AN, Gool EL, Nieuwland R. Recent developments in the nomenclature, presence, isolation, detection and clinical impact of extracellular vesicles. *J Thromb Haemost* 2016;14:48–56.
- [7] Mateescu B, Kowal EJK, van Balkom BWM, Bartel S, Bhattacharyya SN, Buzás EI, et al. Obstacles and opportunities in the functional analysis of extracellular vesicle RNA – an ISEV position paper. *J Extracell Vesicles* 2017;6:1286095.
- [8] Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* 2018;7:1535750.
- [9] Szabo G, Momen-Heravi F. Extracellular vesicles in liver disease and potential as biomarkers and therapeutic targets. *Nat Rev Gastroenterol Hepatol* 2017;14:455–466.
- [10] Lemoine S, Thabut D, Housset C, Moreau R, Valla D, Boulanger CM, et al. The emerging roles of microvesicles in liver diseases. *Nat Rev Gastroenterol Hepatol* 2014;11:350–361.
- [11] Coumans FAW, Brisson AR, Buzas EI, Dignat-George F, Drees EEE, El-Andaloussi S, et al. Methodological guidelines to study extracellular vesicles. *Circ Res* 2017;120:1632–1648.
- [12] Witwer KW, Buzás EI, Bemis LT, Bora A, Lässer C, Lötvall J, et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J Extracell Vesicles* 2013;2:20360.
- [13] Clayton A, Buschmann D, Byrd JB, Carter DRF, Cheng L, Compton C, et al. Summary of the ISEV workshop on extracellular vesicles as disease biomarkers, held in Birmingham, UK, during December 2017. *J Extracell Vesicles* 2018;7:1473707.
- [14] Lippi G, Fontana R, Avanzini P, Aloe R, Ippolito L, Sandei F, et al. Influence of mechanical trauma of blood and hemolysis on PFA-100 testing. *Blood Coagul Fibrinolysis* 2012;23:82–86.
- [15] Cheng HH, Yi HS, Kim Y, Kroh EM, Chien JW, Eaton KD, et al. Plasma processing conditions substantially influence circulating microRNA biomarker levels. *PLoS One* 2013;8:e64795.
- [16] Mani H, Kirchmayr K, Kläffling C, Schindewolf M, Luxembourg B, Linnemann B, et al. Influence of blood collection techniques on platelet function. *Platelets* 2004;15:315–318.
- [17] Yuana Y, Böing AN, Grootemaat AE, van der Pol E, Hau CM, Cizmar P, et al. Handling and storage of human body fluids for analysis of extracellular vesicles. *J Extracell Vesicles* 2015;4:29260.
- [18] Bæk R, Søndergaard EKL, Varming K, Jørgensen MM. The impact of various preanalytical treatments on the phenotype of small extracellular vesicles in blood analyzed by protein microarray. *J Immunol Methods* 2016;438:11–20.
- [19] Van Deun J, Mestdagh P, Agostinis P, Akay Ö, Anand S, Anckaert J, et al. EV-TRACK Consortium. EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research. *Nat Methods* 2017;14:228–232.
- [20] Wisgrill L, Lamm C, Hartmann J, Preißing F, Dragosits K, Bee A, et al. Peripheral blood microvesicles secretion is influenced by storage time, temperature, and anticoagulants. *Cytometry A* 2016;89:663–672.
- [21] Lacroix R, Judicone C, Mooberry M, Boucekine M, Key NS, Dignat-George F, et al. Standardization of pre-analytical variables in plasma microparticle determination: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. *J Thromb Haemost* 2013;11:1190–1193.
- [22] Jayachandran M, Miller VM, Heit JA, Owen WG. Methodology for isolation, identification and characterization of microvesicles in peripheral blood. *J Immunol Methods* 2012;375:207–214.

- [23] György B, Pálóczi K, Kovács A, Barabás E, Bekő G, Várnai K, et al. Improved circulating microparticle analysis in acid-citrate dextrose (ACD) anticoagulant tube. *Thromb Res* 2014;133:285–292.
- [24] Andreu Z, Rivas E, Sanguino-Pascual A, Lamana A, Marazuela M, González-Alvaro I, et al. Comparative analysis of EV isolation procedures for miRNAs detection in serum samples. *J Extracell Vesicles* 2016;5: 31655.
- [25] Gardiner C, Di Vizio D, Sahoo S, Théry C, Witwer KW, Wauben M, et al. Techniques used for the isolation and characterization of extracellular vesicles: results of a worldwide survey. *J Extracell Vesicles* 2016;5:32945.
- [26] Mol EA, Goumans M-J, Doevendans PA, Sluijter JPG, Vader P. Higher functionality of extracellular vesicles isolated using size-exclusion chromatography compared to ultracentrifugation. *Nanomedicine* 2017;13:2061–2065.
- [27] Cheng L, Sharples RA, Scicluna BJ, Hill AF. Exosomes provide a protective and enriched source of miRNA for biomarker profiling compared to intracellular and cell-free blood. *J Extracell Vesicles* 2014;3:23743.
- [28] Jeppesen DK, Fenix AM, Franklin JL, Higginbotham JN, Zhang Q, Zimmerman LJ, et al. Reassessment of exosome composition. *Cell* 2019;177:428–445.e18.
- [29] Van Deun J, Mestdagh P, Sormunen R, Cocquyt V, Vermaelen K, Vandesompele J, et al. The impact of disparate isolation methods for extracellular vesicles on downstream RNA profiling. *J Extracell Vesicles* 2014;3:24858.
- [30] Hill AF, Pegtel DM, Lambertz U, Leonardi T, O'Driscoll L, Pluchino S, et al. ISEV position paper: extracellular vesicle RNA analysis and bioinformatics. *J Extracell Vesicles* 2013;2:22859.
- [31] Takov K, Yellon DM, Davidson SM. Comparison of small extracellular vesicles isolated from plasma by ultracentrifugation or size-exclusion chromatography: yield, purity and functional potential. *J Extracell Vesicles* 2019;8:1560809.
- [32] Webber J, Clayton A. How pure are your vesicles? *J Extracell Vesicles* 2013;2:19861.
- [33] Momen-Heravi F, Balaj L, Alian S, Mantel P-Y, Halleck AE, Trachtenberg AJ, et al. Current methods for the isolation of extracellular vesicles. *Biol Chem* 2013;394:1253–1262.
- [34] Taylor DD, Shah S. Methods of isolating extracellular vesicles impact down-stream analyses of their cargoes. *Methods* 2015;87:3–10.
- [35] Stranska R, Gysbrechts L, Wouters J, Vermeersch P, Bloch K, Dierickx D, et al. Comparison of membrane affinity-based method with size-exclusion chromatography for isolation of exosome-like vesicles from human plasma. *J Transl Med* 2018;16:1.
- [36] Momen-Heravi F, Balaj L, Alian S, Trachtenberg AJ, Hochberg FH, Skog J, et al. Impact of biofluid viscosity on size and sedimentation efficiency of the isolated microvesicles. *Front Physiol* 2012;3:162.
- [37] Baranyai T, Herczeg K, Onódi Z, Voszka I, Módos K, Marton N, et al. Isolation of exosomes from blood plasma: qualitative and quantitative comparison of ultracentrifugation and size exclusion chromatography methods. *PLoS One* 2015;10:e0145686.
- [38] Vergauwen G, Dhondt B, Van Deun J, De Smedt E, Berx G, Timmerman E, et al. Confounding factors of ultrafiltration and protein analysis in extracellular vesicle research. *Sci Rep* 2017;7:2704.
- [39] Rautou P-E, Bresson J, Sainte-Marie Y, Vion A-C, Paradis V, Renard J-M, et al. Abnormal plasma microparticles impair vasoconstrictor responses in patients with cirrhosis. *Gastroenterology* 2012;143:166–176.e6.
- [40] Payancé A, Silva-Junior G, Bissonnette J, Tanguy M, Pasquet B, Levi C, et al. Hepatocyte microvesicle levels improve prediction of mortality in patients with cirrhosis. *Hepatology* 2018;68:1508–1518.
- [41] Bissonnette J, Altamirano J, Devue C, Roux O, Payancé A, Lebre D, et al. A prospective study of the utility of plasma biomarkers to diagnose alcoholic hepatitis. *Hepatology* 2017;66:555–563.
- [42] Enderle D, Spiel A, Coticchia CM, Berghoff E, Mueller R, Schlumpberger M, et al. Characterization of RNA from exosomes and other extracellular vesicles isolated by a novel Spin column-based method. *PLoS One* 2015;10:e0136133.
- [43] Böing AN, van der Pol E, Grootemaat AE, Coumans FAW, Sturk A, Nieuwland R. Single-step isolation of extracellular vesicles by size-exclusion chromatography. *J Extracell Vesicles* 2014;3:23430.
- [44] Hong C-S, Funk S, Muller L, Boyiadzis M, Whiteside TL. Isolation of biologically active and morphologically intact exosomes from plasma of patients with cancer. *J Extracell Vesicles* 2016;5:29289.
- [45] George JN, Thoi LL, McManus LM, Reimann TA. Isolation of human platelet membrane microparticles from plasma and serum. *Blood* 1982;60:834–840.
- [46] Pisitkun T, Shen R-F, Knepper MA. Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci U S A* 2004;101:13368–13373.
- [47] Awdishu L, Tsunoda S, Pearlman M, Kokoy-Mondragon C, Ghassemian M, Naviaux RK, et al. Identification of maltase glucoamylase as a biomarker of acute kidney injury in patients with cirrhosis. *Crit Care Res Pract* 2019;2019:5912804.
- [48] Merchant ML, Rood IM, Deegens JKJ, Klein JB. Isolation and characterization of urinary extracellular vesicles: implications for biomarker discovery. *Nat Rev Nephrol* 2017;13:731–749.
- [49] Sirica AE, Gores GJ, Groopman JD, Selaru FM, Strazzabosco M, Wei Wang X, et al. Intrahepatic cholangiocarcinoma: continuing challenges and translational advances. *Hepatology* 2019;69:1803–1815.
- [50] Engelmann C, Splith K, Krohn S, Herber A, Boehlig A, Boehm S, et al. Absolute quantification of microparticles by flow cytometry in ascites of patients with decompensated cirrhosis: a cohort study. *J Transl Med* 2017;15:188.
- [51] Li L, Masica D, Ishida M, Tomuleasa C, Umegaki S, Kalloo AN, et al. Human bile contains microRNA-laden extracellular vesicles that can be used for cholangiocarcinoma diagnosis. *Hepatology* 2014;60:896–907.
- [52] Severino V, Dumonceau J-M, Delhay M, Moll S, Annessi-Ramseyer I, Robin X, et al. Extracellular vesicles in bile as markers of malignant biliary stenoses. *Gastroenterology* 2017;153:495–504.e8.
- [53] Li L, Piontek KB, Kumbhari V, Ishida M, Selaru FM. Isolation and profiling of MicroRNA-containing exosomes from human bile. *J Vis Exp* 2016;2016:54036.
- [54] Ge X, Wang Y, Nie J, Li Q, Tang L, Deng X, et al. The diagnostic/prognostic potential and molecular functions of long non-coding RNAs in the exosomes derived from the bile of human cholangiocarcinoma. *Oncotarget* 2017;8:69995–70005.
- [55] Hogan MC, Lieske JC, Lienczewski CC, Nesbitt LL, Wickman LT, Heyer CM, et al. Strategy and rationale for urine collection protocols employed in the NEPTUNE study. *BMC Nephrol* 2015;16:190.
- [56] Arraud N, Linares R, Tan S, Gounou C, Pasquet J-M, Mornet S, et al. Extracellular vesicles from blood plasma: determination of their morphology, size, phenotype and concentration. *J Thromb Haemost* 2014;12:614–627.
- [57] György B, Módos K, Pállinger E, Pálóczi K, Pásztoi M, Misják P, et al. Detection and isolation of cell-derived microparticles are compromised by protein complexes resulting from shared biophysical parameters. *Blood* 2011;117:e39–e48.
- [58] Takov K, Yellon DM, Davidson SM. Confounding factors in vesicle uptake studies using fluorescent lipophilic membrane dyes. *J Extracell Vesicles* 2017;6:1388731.
- [59] Sódar BW, Kittel Á, Pálóczi K, Vukman KV, Osteikoetxea X, Szabó-Taylor K, et al. Low-density lipoprotein mimics blood plasma-derived exosomes and microvesicles during isolation and detection. *Sci Rep* 2016;6:24316.
- [60] Larson MC, Luthi MR, Hogg N, Hillery CA. Calcium-phosphate micro-precipitates mimic microparticles when examined with flow cytometry. *Cytometry A* 2013;83:242–250.
- [61] van der Pol E, Hoekstra AG, Sturk A, Otto C, van Leeuwen TG, Nieuwland R. Optical and non-optical methods for detection and characterization of microparticles and exosomes. *J Thromb Haemost* 2010;8:2596–2607.
- [62] Obeid S, Ceroi A, Mourey G, Saas P, Elie-Caille C, Boireau W. Development of a NanoBioAnalytical platform for “on-chip” qualification and quantification of platelet-derived microparticles. *Biosens Bioelectron* 2017;93:250–259.
- [63] Liang K, Liu F, Fan J, Sun D, Liu C, Lyon CJ, et al. Nanoplasmonic quantification of tumor-derived extracellular vesicles in plasma micro-samples for diagnosis and treatment monitoring. *Nat Biomed Eng* 2017;1:0021.
- [64] Koliha N, Wienczek Y, Heider U, Jüngst C, Kladt N, Krauthäuser S, et al. A novel multiplex bead-based platform highlights the diversity of extracellular vesicles. *J Extracell Vesicles* 2016;5:29975.
- [65] Corso G, Mäger I, Lee Y, Görgens A, Bultema J, Giebel B, et al. Reproducible and scalable purification of extracellular vesicles using combined bind-elute and size exclusion chromatography. *Sci Rep* 2017;7:11561.

- [66] Gool EL, Stojanovic I, Schasfoort RBM, Sturk A, van Leeuwen TG, Nieuwland R, et al. Surface plasmon resonance is an analytically sensitive method for antigen profiling of extracellular vesicles. *Clin Chem* 2017;63:1633–1641.
- [67] Jørgensen MM, Bæk R, Varming K. Potentials and capabilities of the extracellular vesicle (EV) array. *J Extracell Vesicles* 2015;4:26048.
- [68] Szatanek R, Baj-Krzyworzeka M, Zimoch J, Lekka M, Siedlar M, Baran J. The methods of choice for extracellular vesicles (EVs) characterization. *Int J Mol Sci* 2017;18:1153.
- [69] Tatischeff I, Larquet E, Falcón-Pérez JM, Turpin P-Y, Kruglik SG. Fast characterisation of cell-derived extracellular vesicles by nanoparticles tracking analysis, cryo-electron microscopy, and Raman tweezers microspectroscopy. *J Extracell Vesicles* 2012;1:19179.
- [70] Wyss R, Grasso L, Wolf C, Grosse W, Demurtas D, Vogel H. Molecular and dimensional profiling of highly purified extracellular vesicles by fluorescence fluctuation spectroscopy. *Anal Chem* 2014;86:7229–7233.
- [71] Coumans FAW, Gool EL, Nieuwland R. Bulk immunoassays for analysis of extracellular vesicles. *Platelets* 2017;28:242–248.
- [72] Ramirez MI, Amorim MG, Gadelha C, Milic I, Welsh JA, Freitas VM, et al. Technical challenges of working with extracellular vesicles. *Nanoscale* 2018;10:881–906.
- [73] van der Pol E, Sturk A, van Leeuwen T, Nieuwland R, Coumans F, ISTH-SSC-VB Working group. Standardization of extracellular vesicle measurements by flow cytometry through vesicle diameter approximation. *J Thromb Haemost* 2018;16:1236–1245.
- [74] van der Pol E, van Gemert MJC, Sturk A, Nieuwland R, van Leeuwen TG. Single vs. swarm detection of microparticles and exosomes by flow cytometry. *J Thromb Haemost* 2012;10:919–930.
- [75] Groot Kormelink T, Arksteijn GJA, Nauwelaers FA, van den Engh G, Nolte-t Hoen ENM, Wauben MHM. Prerequisites for the analysis and sorting of extracellular vesicle subpopulations by high-resolution flow cytometry. *Cytometry A* 2016;89:135–147.
- [76] Duijvesz D, Versluis CYL, van der Fels CAM, Vredenburg-van den Berg MS, Leivo J, Peltola MT, et al. Immuno-based detection of extracellular vesicles in urine as diagnostic marker for prostate cancer. *Int J Cancer* 2015;137:2869–2878.
- [77] Cointe S, Judicone C, Robert S, Mooberry MJ, Poncelet P, Wauben M, et al. Standardization of microparticle enumeration across different flow cytometry platforms: results of a multicenter collaborative workshop. *J Thromb Haemost* 2017;15:187–193.
- [78] Lacroix R, Robert S, Poncelet P, Kasthuri RS, Key NS, Dignat-George F, et al. Standardization of platelet-derived microparticle enumeration by flow cytometry with calibrated beads: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. *J Thromb Haemost* 2010;8:2571–2574.
- [79] Carnell-Morris P, Tannetta D, Siupa A, Hole P, Dragovic R. Analysis of extracellular vesicles using fluorescence nanoparticle tracking analysis. *Methods Mol Biol* 2017;1660:153–173.
- [80] Desgeorges A, Hollerweger J, Lassacher T, Rohde E, Helmbrecht C, Gimona M. Differential fluorescence nanoparticle tracking analysis for enumeration of the extracellular vesicle content in mixed particulate solutions. *Methods* 2020;177:67–73.
- [81] Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, et al. The diagnosis and management of nonalcoholic fatty liver disease: practice guidance from the American Association for the Study of Liver Diseases. *Hepatology* 2018;67:328–357.
- [82] 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;143:448–458.
- [83] 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;57:233–245.
- [84] 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.
- [85] Lee Y-S, Kim SY, Ko E, Lee J-H, Yi H-S, Yoo YJ, et al. Exosomes derived from palmitic acid-treated hepatocytes induce fibrotic activation of hepatic stellate cells. *Sci Rep* 2017;7:3710.
- [86] Pirola CJ, Fernández Gianotti T, Castaño GO, Mallardi P, San Martino J, Mora Gonzalez Lopez Ledesma M, et al. Circulating microRNA signature in non-alcoholic fatty liver disease: from serum non-coding RNAs to liver histology and disease pathogenesis. *Gut* 2015;64:800–812.
- [87] Akuta N, Kawamura Y, Watanabe C, Nishimura A, Okubo M, Mori Y, et al. Impact of sodium glucose cotransporter 2 inhibitor on histological features and glucose metabolism of non-alcoholic fatty liver disease complicated by diabetes mellitus. *Hepatol Res* 2019;49:531–539.
- [88] Welsh JA, Scorletti E, Clough GF, Englyst NA, Byrne CD. Leukocyte extracellular vesicle concentration is inversely associated with liver fibrosis severity in NAFLD. *J Leukoc Biol* 2018;104:631–639.
- [89] 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;71:1193–1205.
- [90] Sukriti S, Maras JS, Bihari C, Das S, Vyas AK, Sharma S, et al. Microvesicles in hepatic and peripheral vein can predict nonresponse to corticosteroid therapy in severe alcoholic hepatitis. *Aliment Pharmacol Ther* 2018;47:1151–1161.
- [91] Verma VK, Li H, Wang R, Hirsova P, Mushref M, Liu Y, et al. Alcohol stimulates macrophage activation through caspase-dependent hepatocyte derived release of CD40L containing extracellular vesicles. *J Hepatol* 2016;64:651–660.
- [92] Eguchi A, Franz N, Kobayashi Y, Iwasa M, Wagner N, Hildebrand F, et al. Circulating extracellular vesicles and their miR “barcode” differentiate alcohol drinkers with liver injury and those without liver injury in severe trauma patients. *Front Med (Lausanne)* 2019;6:30.
- [93] Cho Y-E, Im E-J, Moon P-G, Mezey E, Song B-J, Baek M-C. Increased liver-specific proteins in circulating extracellular vesicles as potential biomarkers for drug- and alcohol-induced liver injury. *PLoS One* 2017;12:e0172463.
- [94] Cai Y, Xu M-J, Koritzinsky EH, Zhou Z, Wang W, Cao H, et al. Mitochondrial DNA-enriched microparticles promote acute-on-chronic alcoholic neutrophilia and hepatotoxicity. *JCI Insight* 2017;2:e92634.
- [95] Momen-Heravi F, Bala S, Kodys K, Szabo G. Exosomes derived from alcohol-treated hepatocytes horizontally transfer liver specific miRNA-122 and sensitize monocytes to LPS. *Sci Rep* 2015;5:9991.
- [96] Eguchi A, Lazaro RG, Wang J, Kim J, Povero D, Williams B, et al. Extracellular vesicles released by hepatocytes from gastric infusion model of alcoholic liver disease contain a MicroRNA barcode that can be detected in blood. *Hepatology* 2017;65:475–490.
- [97] Saha B, Momen-Heravi F, Kodys K, Szabo G. MicroRNA cargo of extracellular vesicles from alcohol-exposed monocytes signals naive monocytes to differentiate into M2 macrophages. *J Biol Chem* 2016;291:149–159.
- [98] 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;290:30684–30696.
- [99] Sehrawat TS, Arab JP, Liu M, Amrollahi P, Wan M, Fan J, et al. Circulating extracellular vesicles carrying sphingolipid cargo for the diagnosis and dynamic risk profiling of alcoholic hepatitis. *Hepatology* 2020. <https://doi.org/10.1002/hep.31256>.
- [100] Nielsen MC, Andersen MN, Grønbaek H, Damgaard Sandahl T, Møller HJ. Extracellular vesicle-associated soluble CD163 and CD206 in patients with acute and chronic inflammatory liver disease. *Scand J Gastroenterol* 2020;55:588–596.
- [101] Babuta M, Furi I, Bala S, Bukong TN, Lowe P, Catalano D, et al. Dysregulated autophagy and lysosome function are linked to exosome production by micro-RNA 155 in alcoholic liver disease. *Hepatology* 2019;70:2123–2141.
- [102] Arab JP, Sehrawat TS, Simonetto DA, Verma VK, Feng D, Tang T, et al. An open-label, dose-escalation study to assess the safety and efficacy of IL-22 agonist F-652 in patients with alcohol-associated hepatitis. *Hepatology* 2019;72:441–453.
- [103] Cho Y-E, Mezey E, Hardwick JP, Salem N, Clemens DL, Song B-J. Increased ethanol-inducible cytochrome P450-2E1 and cytochrome P450 isoforms in exosomes of alcohol-exposed rodents and patients with alcoholism through oxidative and endoplasmic reticulum stress. *Hepatol Commun* 2017;1:675–690.
- [104] Sukriti S, Choudhary MC, Maras JS, Sharma S, Thangariyal S, Singh A, et al. Extracellular vesicles from hepatitis B patients serve as reservoir of hepatitis B virus DNA. *J Viral Hepat* 2019;26:211–214.
- [105] Lambrecht J, Jan Poortmans P, Verhulst S, Reynaert H, Mannaerts I, van Grunsven LA. Circulating ECV-associated miRNAs as potential clinical biomarkers in early stage HBV and HCV induced liver fibrosis. *Front Pharmacol* 2017;8:56.

- [106] Pu C, Huang H, Wang Z, Zou W, Lv Y, Zhou Z, et al. Extracellular vesicle-associated mir-21 and mir-144 are markedly elevated in serum of patients with hepatocellular carcinoma. *Front Physiol* 2018;9:930.
- [107] Yang X, Li H, Sun H, Fan H, Hu Y, Liu M, et al. Hepatitis B virus-encoded microRNA controls viral replication. *J Virol* 2017;91: e01919-16.
- [108] van der Ree MH, Jansen L, Kruize Z, van Nuenen AC, van Dort KA, Takkenberg RB, et al. Plasma microRNA levels are associated with hepatitis B e antigen status and treatment response in chronic hepatitis B patients. *J Infect Dis* 2017;215:1421-1429.
- [109] Yang Y, Han Q, Hou Z, Zhang C, Tian Z, Zhang J. Exosomes mediate hepatitis B virus (HBV) transmission and NK-cell dysfunction. *Cell Mol Immunol* 2017;14:465-475.
- [110] European Association for the Study of the Liver. Electronic address: easloffice@easloffice.eu, European Association for the Study of the Liver. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J Hepatol* 2017;67:370-398.
- [111] Santangelo L, Bordoni V, Montaldo C, Cimini E, Zingoni A, Battistelli C, et al. Hepatitis C virus direct-acting antivirals therapy impacts on extracellular vesicles microRNAs content and on their immunomodulating properties. *Liver Int* 2018;38:1741-1750.
- [112] Liu Z, Zhang X, Yu Q, He JJ. Exosome-associated hepatitis C virus in cell cultures and patient plasma. *Biochem Biophys Res Commun* 2014;455:218-222.
- [113] Bukong TN, Momen-Heravi F, Kodys K, Bala S, Szabo G. Exosomes from hepatitis C infected patients transmit HCV infection and contain replication competent viral RNA in complex with Ago2-miR122-HSP90. *Plos Pathog* 2014;10:e1004424.
- [114] Jiao X, Fan Z, Chen H, He P, Li Y, Zhang Q, et al. Serum and exosomal miR-122 and miR-199a as a biomarker to predict therapeutic efficacy of hepatitis C patients. *J Med Virol* 2017;89:1597-1605.
- [115] Fan Z, Zhang Q, Chen H, He P, Li Y, Si M, et al. Circulating microRNAs as a biomarker to predict therapy efficacy in hepatitis C patients with different genotypes. *Microb Pathog* 2017;112:320-326.
- [116] Devhare PB, Sasaki R, Shrivastava S, Di Bisceglie AM, Ray R, Ray RB. Exosome-mediated intercellular communication between hepatitis C virus-infected hepatocytes and hepatic stellate cells. *J Virol* 2017;91: e02225-16.
- [117] Welker M-W, Reichert D, Susser S, Sarrazin C, Martinez Y, Herrmann E, et al. Soluble serum CD81 is elevated in patients with chronic hepatitis C and correlates with alanine aminotransferase serum activity. *PLoS One* 2012;7:e30796.
- [118] 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;53:230-242.
- [119] Itami-Matsumoto S, Hayakawa M, Uchida-Kobayashi S, Enomoto M, Tamori A, Mizuno K, et al. Circulating exosomal miRNA profiles predict the occurrence and recurrence of hepatocellular carcinoma in patients with direct-acting antiviral-induced sustained viral response. *Bio-medicines* 2019;7:87.
- [120] Matsuura Y, Wada H, Eguchi H, Gotoh K, Kobayashi S, Kinoshita M, et al. Exosomal miR-155 derived from hepatocellular carcinoma cells under hypoxia promotes angiogenesis in endothelial cells. *Dig Dis Sci* 2019;64:792-802.
- [121] Matsuura K, De Giorgi V, Schechterly C, Wang RY, Farci P, Tanaka Y, et al. Circulating let-7 levels in plasma and extracellular vesicles correlate with hepatic fibrosis progression in chronic hepatitis C. *Hepatology* 2016;64:732-745.
- [122] AASLD-IDS A HCV Guidance Panel. Hepatitis C guidance 2018 update: AASLD-IDS recommendations for testing, managing, and treating hepatitis C virus infection. *Clin Infect Dis* 2018;67:1477-1492.
- [123] Wang W, Li H, Zhou Y, Jie S. Peripheral blood microvesicles are potential biomarkers for hepatocellular carcinoma. *Cancer Biomark* 2013;13:351-357.
- [124] Brodsky SV, Facciuto ME, Heydt D, Chen J, Islam HK, Kajstura M, et al. Dynamics of circulating microparticles in liver transplant patients. *J Gastrointest Liver Dis* 2008;17:261-268.
- [125] Zuwała-Jagiello J, Simon KA, Pazgan-Simon M. Elevated circulating endothelial cell-derived microparticle levels in patients with liver cirrhosis: a preliminary report. *Clin Exp Hepatol* 2015;1:105-111.
- [126] Campello E, Zanetto A, Spiezia L, Radu CM, Gavasso S, Ferrarese A, et al. Hypercoagulability detected by circulating microparticles in patients with hepatocellular carcinoma and cirrhosis. *Thromb Res* 2016;143:118-121.
- [127] Ogasawara F, Fusegawa H, Haruki Y, Shiraishi K, Watanabe N, Matsuzaki S. Platelet activation in patients with alcoholic liver disease. *Tokai J Exp Clin Med* 2005;30:41-48.
- [128] Fusegawa H, Shiraishi K, Ogasawara F, Shimizu M, Haruki Y, Miyachi H, et al. Platelet activation in patients with chronic hepatitis C. *Tokai J Exp Clin Med* 2002;27:101-106.
- [129] Sayed D, Amin NF, Galal GM. Monocyte-platelet aggregates and platelet micro-particles in patients with post-hepatitic liver cirrhosis. *Thromb Res* 2010;125:e228-e233.
- [130] Eyraud D, Suner L, Dupont A, Bachelot-Loza C, Smadja DM, Helley D, et al. Evolution of platelet functions in cirrhotic patients undergoing liver transplantation: a prospective exploration over a month. *PLoS One* 2018;13:e0200364.
- [131] Taleb RSZ, Moez P, Younan D, Eisenacher M, Tenbusch M, Sitek B, et al. Quantitative proteome analysis of plasma microparticles for the characterization of HCV-induced hepatic cirrhosis and hepatocellular carcinoma. *Proteomics Clin Appl* 2017;11:1700014.
- [132] Kostallari E, Hirsova P, Prasnicka A, Verma VK, Yaqoob U, Wongjarupong N, et al. Hepatic stellate cell-derived platelet-derived growth factor receptor-alpha-enriched extracellular vesicles promote liver fibrosis in mice through SHP2. *Hepatology* 2018;68:333-348.
- [133] Brandon-Warner E, Feilen NA, Culbertson CR, Field CO, deLemos AS, Russo MW, et al. Processing of miR17-92 cluster in hepatic stellate cells promotes hepatic fibrogenesis during alcohol-induced injury. *Alcohol Clin Exp Res* 2016;40:1430-1442.
- [134] Lambrecht J, Verhulst S, Mannaerts I, Sowa J-P, Best J, Canbay A, et al. A PDGFR β -based score predicts significant liver fibrosis in patients with chronic alcohol abuse, NAFLD and viral liver disease. *EBioMedicine* 2019;43:501-512.
- [135] Rautou P-E, Vion A-C, Luyendyk JP, Mackman N. Circulating microparticle tissue factor activity is increased in patients with cirrhosis. *Hepatology* 2014;60:1793-1795.
- [136] Chandler WL, Dawson KL, Ruegger MC, Teruya M, Liebl PHN, Monsour HP. Patients with cirrhosis show a relative increase in thrombin generation that is correlated with lower antithrombin levels. *Blood Coagul Fibrinolysis* 2014. <https://doi.org/10.1097/MBC.0000000000000231>.
- [137] Berckmans RJ, Nieuwland R, Böing AN, Romijn FP, Hack CE, Sturk A. Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation. *Thromb Haemost* 2001;85:639-646.
- [138] Rautou P-E, Mackman N. Microvesicles as risk markers for venous thrombosis. *Expert Rev Hematol* 2013;6:91-101.
- [139] Zanetto A, Campello E, Spiezia L, Burra P, Simioni P, Russo FP. Cancer-associated thrombosis in cirrhotic patients with hepatocellular carcinoma. *Cancers (Basel)* 2018;10:450.
- [140] Rautou P-E, Tatsumi K, Antoniaki S, Owens AP, Sparkenbaugh E, Holle LA, et al. Hepatocyte tissue factor contributes to the hypercoagulable state in a mouse model of chronic liver injury. *J Hepatol* 2016;64:53-59.
- [141] Mooberry MJ, Key NS. Microparticle analysis in disorders of hemostasis and thrombosis. *Cytometry A* 2016;89:111-122.
- [142] Campello E, Radu CM, Zanetto A, Bulato C, Shalaby S, Spiezia L, et al. Changes in plasma circulating microvesicles in patients with HCV-related cirrhosis after treatment with direct-acting antivirals. *Liver Int* 2019;40:913-920.
- [143] Julich-Haertel H, Urban SK, Krawczyk M, Willms A, Jankowski K, Patkowski W, et al. Cancer-associated circulating large extracellular vesicles in cholangiocarcinoma and hepatocellular carcinoma. *J Hepatol* 2017;67:282-292.
- [144] Abbate V, Marcantoni M, Giuliani F, Vecchio FM, Gatto I, Mele C, et al. HepPar1-positive circulating microparticles are increased in subjects with hepatocellular carcinoma and predict early recurrence after liver resection. *Int J Mol Sci* 2017;18:1043.
- [145] Sohn W, Kim J, Kang SH, Yang SR, Cho J-Y, Cho HC, et al. Serum exosomal microRNAs as novel biomarkers for hepatocellular carcinoma. *Exp Mol Med* 2015;47:e184.
- [146] Wang H, Hou L, Li A, Duan Y, Gao H, Song X. Expression of serum exosomal microRNA-21 in human hepatocellular carcinoma. *Biomed Res Int* 2014;2014:864894.
- [147] Xu H, Chen Y, Dong X, Wang X. Serum exosomal long noncoding RNAs ENSG00000258332.1 and LINC00635 for the diagnosis and prognosis of hepatocellular carcinoma. *Cancer Epidemiol Biomarkers Prev* 2018;27:710-716.

- [148] Xu H, Dong X, Chen Y, Wang X. Serum exosomal hnRNPH1 mRNA as a novel marker for hepatocellular carcinoma. *Clin Chem Lab Med* 2018;56:479–484.
- [149] Wang Y, Zhang C, Zhang P, Guo G, Jiang T, Zhao X, et al. Serum exosomal microRNAs combined with alpha-fetoprotein as diagnostic markers of hepatocellular carcinoma. *Cancer Med* 2018;7:1670–1679.
- [150] Liu W, Hu J, Zhou K, Chen F, Wang Z, Liao B, et al. Serum exosomal miR-125b is a novel prognostic marker for hepatocellular carcinoma. *Oncotargets Ther* 2017;10:3843–3851.
- [151] Fornari F, Ferracin M, Trerè D, Milazzo M, Marinelli S, Galassi M, et al. Circulating microRNAs, miR-939, miR-595, miR-519d and miR-494, identify cirrhotic patients with HCC. *PLoS One* 2015;10:e0141448.
- [152] Abd El Gwad A, Matboli M, El-Tawdi A, Habib EK, Shehata H, Ibrahim D, et al. Role of exosomal competing endogenous RNA in patients with hepatocellular carcinoma. *J Cell Biochem* 2018;119:8600–8610.
- [153] Ma X, Yuan T, Yang C, Wang Z, Zang Y, Wu L, et al. X-inactive-specific transcript of peripheral blood cells is regulated by exosomal jpx and acts as a biomarker for female patients with hepatocellular carcinoma. *Ther Adv Med Oncol* 2017;9:665–677.
- [154] Li H, Sun L, Chen X, Xiong W, Hu D, Jie S. Microvesicle microRNA profiles and functional roles between chronic hepatitis B and hepatocellular carcinoma. *Clin Transl Oncol* 2014;16:315–321.
- [155] Yang Y, Zhu R. Diagnostic value of circulating microRNAs for hepatocellular carcinoma. *Mol Biol Rep* 2014;41:6919–6929.
- [156] Tian X-P, Wang C-Y, Jin X-H, Li M, Wang F-W, Huang W-J, et al. Acidic microenvironment up-regulates exosomal miR-21 and miR-10b in early-stage hepatocellular carcinoma to promote cancer cell proliferation and metastasis. *Theranostics* 2019;9:1965–1979.
- [157] Xue X, Zhao Y, Wang X, Qin L, Hu R. Development and validation of serum exosomal microRNAs as diagnostic and prognostic biomarkers for hepatocellular carcinoma. *J Cell Biochem* 2019;120:135–142.
- [158] Suehiro T, Miyaaki H, Kanda Y, Shibata H, Honda T, Ozawa E, et al. Serum exosomal microRNA-122 and microRNA-21 as predictive biomarkers in transarterial chemoembolization-treated hepatocellular carcinoma patients. *Oncol Lett* 2018;16:3267–3273.
- [159] Fu Q, Zhang Q, Lou Y, Yang J, Nie G, Chen Q, et al. Primary tumor-derived exosomes facilitate metastasis by regulating adhesion of circulating tumor cells via SMAD3 in liver cancer. *Oncogene* 2018;37:6105–6118.
- [160] Tang J, Li Y, Liu K, Zhu Q, Yang W-H, Xiong L-K, et al. Exosomal miR-9-3p suppresses HBGF-5 expression and is a functional biomarker in hepatocellular carcinoma. *Minerva Med* 2018;109:15–23.
- [161] Li Y, Zhao J, Yu S, Wang Z, He X, Su Y, et al. Extracellular vesicles long RNA sequencing reveals abundant mRNA, circRNA, and lncRNA in human blood as potential biomarkers for cancer diagnosis. *Clin Chem* 2019;65:798–808.
- [162] Liu Y, Tan J, Ou S, Chen J, Chen L. Adipose-derived exosomes deliver miR-23a/b to regulate tumor growth in hepatocellular cancer by targeting the VHL/HIF axis. *J Physiol Biochem* 2019;75:391–401.
- [163] Zheng Q, Zhao J, Yu H, Zong H, He X, Zhao Y, et al. Tumor-specific transcripts are frequently expressed in hepatocellular carcinoma with clinical implication and potential function. *Hepatology* 2020;71:259–274.
- [164] Zhao S, Li J, Zhang G, Wang Q, Wu C, Zhang Q, et al. Exosomal miR-451a functions as a tumor suppressor in hepatocellular carcinoma by targeting LPIN1. *Cell Physiol Biochem* 2019;53:19–35.
- [165] Han Q, Lv L, Wei J, Lei X, Lin H, Li G, et al. Vps4A mediates the localization and exosome release of β -catenin to inhibit epithelial-mesenchymal transition in hepatocellular carcinoma. *Cancer Lett* 2019;457:47–59.
- [166] Cheng Z, Lei Z, Yang P, Si A, Xiang D, Tang X, et al. Exosome-transmitted p120-catenin suppresses hepatocellular carcinoma progression via STAT3 pathways. *Mol Carcinog* 2019;58:1389–1399.
- [167] Cui Y, Xu H-F, Liu M-Y, Xu Y-J, He J-C, Zhou Y, et al. Mechanism of exosomal microRNA-224 in development of hepatocellular carcinoma and its diagnostic and prognostic value. *World J Gastroenterol* 2019;25:1890–1898.
- [168] Sun L, Su Y, Liu X, Xu M, Chen X, Zhu Y, et al. Serum and exosome long non coding RNAs as potential biomarkers for hepatocellular carcinoma. *J Cancer* 2018;9:2631–2639.
- [169] Xue X, Wang X, Zhao Y, Hu R, Qin L. Exosomal miR-93 promotes proliferation and invasion in hepatocellular carcinoma by directly inhibiting TIMP2/TP53INP1/CDKN1A. *Biochem Biophys Res Commun* 2018;502:515–521.
- [170] Lin X-J, Fang J-H, Yang X-J, Zhang C, Yuan Y, Zheng L, et al. Hepatocellular carcinoma cell-secreted exosomal microRNA-210 promotes angiogenesis in vitro and in vivo. *Mol Ther Nucleic Acids* 2018;11:243–252.
- [171] Shi M, Jiang Y, Yang L, Yan S, Wang Y-G, Lu X-J. Decreased levels of serum exosomal miR-638 predict poor prognosis in hepatocellular carcinoma. *J Cell Biochem* 2018;119:4711–4716.
- [172] Wang X, Shen H, Zhangyuan G, Huang R, Zhang W, He Q, et al. 14-3-3 ζ delivered by hepatocellular carcinoma-derived exosomes impaired anti-tumor function of tumor-infiltrating T lymphocytes. *Cell Death Dis* 2018;9:159.
- [173] Li B, Mao R, Liu C, Zhang W, Tang Y, Guo Z. LncRNA FAL1 promotes cell proliferation and migration by acting as a CeRNA of miR-1236 in hepatocellular carcinoma cells. *Life Sci* 2018;197:122–129.
- [174] Zhang C, Yang X, Qi Q, Gao Y, Wei Q, Han S. lncRNA-HEIH in serum and exosomes as a potential biomarker in the HCV-related hepatocellular carcinoma. *Cancer Biomark* 2018;21:651–659.
- [175] Wang X, Kwak KJ, Yang Z, Zhang A, Zhang X, Sullivan R, et al. Extracellular mRNA detected by molecular beacons in tethered lipoplex nanoparticles for diagnosis of human hepatocellular carcinoma. *PLoS One* 2018;13:e0198552.
- [176] Arbelaez A, Azkargorta M, Krawczyk M, Santos-Laso A, Lapitz A, Perugorria MJ, et al. Serum extracellular vesicles contain protein biomarkers for primary sclerosing cholangitis and cholangiocarcinoma. *Hepatology* 2017;66:1125–1143.
- [177] Lu L, Guo D, Chen X, Xiong W, Jie S, Li H. Abnormal miRNAs targeting chromosome open reading frame genes were enriched in microvesicles derived from the circulation of HCC. *Biochem Genet* 2016;54:120–133.
- [178] Zhu L, Li J, Gong Y, Wu Q, Tan S, Sun D, et al. Exosomal tRNA-derived small RNA as a promising biomarker for cancer diagnosis. *Mol Cancer* 2019;18:74.
- [179] Wang G, Liu W, Zou Y, Wang G, Deng Y, Luo J, et al. Three isoforms of exosomal circPTGR1 promote hepatocellular carcinoma metastasis via the miR449a-MET pathway. *EBioMedicine* 2019;40:432–445.
- [180] Wu D, Yu Y, Jin D, Xiao M-M, Zhang Z-Y, Zhang G-J. Dual-aptamer modified graphene field-effect transistor nanosensor for label-free and specific detection of hepatocellular carcinoma-derived microvesicles. *Anal Chem* 2020;92:4006–4015.
- [181] Xie J-Y, Wei J-X, Lv L-H, Han Q-F, Yang W-B, Li G-L, et al. Angiopoietin-2 induces angiogenesis via exosomes in human hepatocellular carcinoma. *Cell Commun Signal* 2020;18:46.
- [182] Mjelle R, Dima SO, Bacalbasa N, Chawla K, Sorop A, Cucu D, et al. Comprehensive transcriptomic analyses of tissue, serum, and serum exosomes from hepatocellular carcinoma patients. *BMC Cancer* 2019;19:1007.
- [183] Wang S, Yang Y, Sun L, Qiao G, Song Y, Liu B. Exosomal microRNAs as liquid biopsy biomarkers in hepatocellular carcinoma. *Oncotargets Ther* 2020;13:2021–2030.
- [184] Cho HJ, Eun JW, Baek GO, Seo CW, Ahn HR, Kim SS, et al. Serum exosomal microRNA, miR-10b-5p, as a potential diagnostic biomarker for early-stage hepatocellular carcinoma. *J Clin Med* 2020;9:281.
- [185] Wang G, Zhao W, Wang H, Qiu G, Jiang Z, Wei G, et al. Exosomal MiR-744 inhibits proliferation and sorafenib chemoresistance in hepatocellular carcinoma by targeting PAX2. *Med Sci Monit* 2019;25:7209–7217.
- [186] Li W, Ding X, Wang S, Xu L, Yin T, Han S, et al. Downregulation of serum exosomal miR-320d predicts poor prognosis in hepatocellular carcinoma. *J Clin Lab Anal* 2020:e23239.
- [187] Lin H, Zhang Z. Diagnostic value of a microRNA signature panel in exosomes for patients with hepatocellular carcinoma. *Int J Clin Exp Pathol* 2019;12:1478–1487.
- [188] Cao S-Q, Zheng H, Sun B-C, Wang Z-L, Liu T, Guo D-H, et al. Long non-coding RNA highly up-regulated in liver cancer promotes exosome secretion. *World J Gastroenterol* 2019;25:5283–5299.
- [189] Lu Y, Duan Y, Xu Q, Zhang L, Chen W, Qu Z, et al. Circulating exosome-derived bona fide long non-coding RNAs predicting the occurrence and metastasis of hepatocellular carcinoma. *J Cell Mol Med* 2020;24:1311–1318.
- [190] Chen W, Quan Y, Fan S, Wang H, Liang J, Huang L, et al. Exosome-transmitted circular RNA hsa_circ_0051443 suppresses hepatocellular carcinoma progression. *Cancer Lett* 2020;475:119–128.
- [191] Lee YR, Kim G, Tak WY, Jang SY, Kwon YO, Park JG, et al. Circulating exosomal noncoding RNAs as prognostic biomarkers in human hepatocellular carcinoma. *Int J Cancer* 2019;144:1444–1452.

- [192] Zhou Y, Ren H, Dai B, Li J, Shang L, Huang J, et al. Hepatocellular carcinoma-derived exosomal miRNA-21 contributes to tumor progression by converting hepatocyte stellate cells to cancer-associated fibroblasts. *J Exp Clin Cancer Res* 2018;37:324.
- [193] Gramantieri L, Baglioni M, Fornari F, Laginestra MA, Ferracin M, Indio V, et al. LncRNAs as novel players in hepatocellular carcinoma recurrence. *Oncotarget* 2018;9:35085–35099.
- [194] Nakano T, Chen I-H, Wang C-C, Chen P-J, Tseng H-P, Huang K-T, et al. Circulating exosomal miR-92b: its role for cancer immunoediting and clinical value for prediction of posttransplant hepatocellular carcinoma recurrence. *Am J Transpl* 2019;19:3250–3262.
- [195] Fang J-H, Zhang Z-J, Shang L-R, Luo Y-W, Lin Y-F, Yuan Y, et al. Hepatoma cell-secreted exosomal microRNA-103 increases vascular permeability and promotes metastasis by targeting junction proteins. *Hepatology* 2018;68:1459–1475.
- [196] Fang T, Lv H, Lv G, Li T, Wang C, Han Q, et al. Tumor-derived exosomal miR-1247-3p induces cancer-associated fibroblast activation to foster lung metastasis of liver cancer. *Nat Commun* 2018;9:191.
- [197] Sugimachi K, Matsumura T, Hirata H, Uchi R, Ueda M, Ueo H, et al. Identification of a bona fide microRNA biomarker in serum exosomes that predicts hepatocellular carcinoma recurrence after liver transplantation. *Br J Cancer* 2015;112:532–538.
- [198] Yu L-X, Zhang B-L, Yang Y, Wang M-C, Lei G-L, Gao Y, et al. Exosomal microRNAs as potential biomarkers for cancer cell migration and prognosis in hepatocellular carcinoma patient-derived cell models. *Oncol Rep* 2019;41:257–269.
- [199] Huang X-Y, Huang Z-L, Huang J, Xu B, Huang X-Y, Xu Y-H, et al. Exosomal circRNA-100338 promotes hepatocellular carcinoma metastasis via enhancing invasiveness and angiogenesis. *J Exp Clin Cancer Res* 2020;39:20.
- [200] Lazaridis KN, LaRusso NF. Primary sclerosing cholangitis. *N Engl J Med* 2016;375:1161–1170.
- [201] Lapitz A, Arbelaiz A, O'Rourke CJ, Lavin JL, Casta AL, Ibarra C, et al. Patients with cholangiocarcinoma present specific RNA profiles in serum and urine extracellular vesicles mirroring the tumor expression: novel liquid biopsy biomarkers for disease diagnosis. *Cells* 2020;9:721.
- [202] Shen L, Chen G, Xia Q, Shao S, Fang H. Exosomal miR-200 family as serum biomarkers for early detection and prognostic prediction of cholangiocarcinoma. *Int J Clin Exp Pathol* 2019;12:3870–3876.
- [203] Wang S, Hu Y, Lv X, Li B, Gu D, Li Y, et al. Circ-0000284 arouses malignant phenotype of cholangiocarcinoma cells and regulates the biological functions of peripheral cells through cellular communication. *Clin Sci* 2019;133:1935–1953.
- [204] Gu X, Wang C, Deng H, Qing C, Liu R, Liu S, et al. Exosomal piRNA profiling revealed unique circulating piRNA signatures of cholangiocarcinoma and gallbladder carcinoma. *Acta Biochim Biophys Sin* 2020;52:475–484.
- [205] Flamm SL, Yang Y-X, Singh S, Falck-Ytter YT, Flamm SL, Lim JK, et al. American Gastroenterological Association Institute guidelines for the diagnosis and management of acute liver failure. *Gastroenterology* 2017;152:644–647.
- [206] Stravitz RT, Bowling R, Bradford RL, Key NS, Glover S, Thacker LR, et al. Role of procoagulant microparticles in mediating complications and outcome of acute liver injury/acute liver failure. *Hepatology* 2013;58:304–313.
- [207] Duan L, Ramachandran A, Akakpo JY, Weemhoff JL, Curry SC, Jaeschke H. Role of extracellular vesicles in release of protein adducts after acetaminophen-induced liver injury in mice and humans. *Toxicol Lett* 2019;301:125–132.
- [208] Schmelzle M, Splith K, Andersen LW, Kornek M, Schuppan D, Jones-Bamman C, et al. Increased plasma levels of microparticles expressing CD39 and CD133 in acute liver injury. *Transplantation* 2013;95:63–69.
- [209] Zhang Y, Wang D, Shen D, Luo Y, Che Y-Q. Identification of exosomal miRNAs associated with the anthracycline-induced liver injury in post-operative breast cancer patients by small RNA sequencing. *PeerJ* 2020;8:e9021.
- [210] Ayers L, Pink R, Carter DRF, Nieuwland R. Clinical requirements for extracellular vesicle assays. *J Extracell Vesicles* 2019;8:1593755.
- [211] van der Pol E, Coumans FaW, Grootemaat AE, Gardiner C, Sargent IL, Harrison P, et al. Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing. *J Thromb Haemost* 2014;12:1182–1192.