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EXTRACELLULAR VESICLES AS BIOMARKERS IN LIVER DISEASES: A CLINICIAN'S POINT OF VIEW

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Key points:

- Extracellular vesicles are membrane-bound vesicles, containing cell-derived biomolecules, such as proteins, lipids and RNAs.
- 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.
- 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.
- High-throughput and reproducible measurement methods of extracellular vesicles are now becoming available.

Abbreviations:

ABIC: age-bilirubin-INR-creatinine; ACD: acid citrate dextrose; ALT: alanine aminotransferase; AHA: American Heart Association; AUROC: Area Under the Receiver Operating Characteristics; DNA: deoxyribose nucleic acid; DNase: deoxyribonuclease; EV: extracellular vesicle; EDTA: ethylene diamine tetraacetic acid; ELISA: sandwich enzyme-linked immunosorbent assay; ISAC: International Society for Advancement of Cytometry; ISEV: International Society for Extracellular Vesicles; ISTH: International Society on Thrombosis and Haemostasis; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; HVPG: hepatic venous pressure gradient; lncRNA: long non-coding RNA; PSC: primary sclerosing cholangitis; MELD: Model for End-Stage Liver Disease; miRNA: micro-RNA; MISEV: Minimal information for studies of extracellular vesicles; NAFLD: non-alcoholic fatty liver disease; NAS score: NAFLD Activity Score; NK T cell: Natural killer T cell; RNA: ribonucleic acid; RNase: ribonuclease; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; TNM: Tumor Node Metastasis

Abstract

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 interest 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. 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, the review shows that 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 and await clinical validation, some extracellular vesicles sub-populations have already been evaluated in solid prospective studies. The challenges raised by the use of extracellular vesicles as biomarkers are also discussed here.

What are extracellular vesicles?

Extracellular vesicles (EVs) form a heterogeneous group of membrane-bound vesicles that contain cell-derived biomolecules, such as proteins, lipids, RNAs and microRNAs (miRNAs).[1–3] They sometimes also 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 biogenesis mechanisms, 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 μ m) are generated by plasma membrane budding induced by a transient phospholipid imbalance caused by externalization of phosphatidylserine (a negatively charged phospholipid), calcium dependent proteolysis of cytoskeleton and local potassium efflux responsible for cellular contraction.[4] Apoptotic bodies (size ranging from \approx 1 to 4 μ m), which are released by dying cells, are formed by a separation of 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. Communication methods of extracellular vesicles are varied and include activation of surface receptors, transfer of vesicle content into the target cell by vesicle internalization (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 detrimental process. These characteristics make extracellular vesicles useful tools for personalized medicine to diagnose disorders, evaluate prognosis, disease progression and response to treatment. Circulating extracellular vesicles have therefore emerged as attractive biomarkers in liver diseases, as shown 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 extensively reviewed elsewhere [8,11]).

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, standardization of plasma sample collection and processing is necessary to ensure comparability among studies and, ultimately, to permit 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 standardized 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 extracellular vesicle experiment protocols from a crowd-sourcing knowledge base called EV-TRACK (<http://evtrack.org>).[19]

Recommendations on blood sample collection are summarized in Box 2.[6,8,11,12,20] The International Society on Thrombosis and Haemostasis (ISTH)[21] and the International Society for Advancement of Cytometry (ISAC)[20] recommend the use of citrate tubes (as opposed to ethylene diamine tetraacetic acid (EDTA) and heparin), as their use better prevents platelet activation and formation of extracellular vesicles.[8,22] The American Heart Association's (AHA) 2017 guidelines[11] and the International Society for Extracellular Vesicles' (ISEV) 2017 position paper[7] recommend that the choice of anticoagulants be adapted to downstream analysis. For example, acid citrate dextrose (ACD) 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 is able to entirely separate extracellular vesicles from the remaining plasma. A balance must be found between maximizing recovery of extracellular vesicles (to avoid loss of information) and minimizing 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 labor 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 RNase treatment. For small cohorts, it is useful to verify reliability of separation methods, by quantifying contaminants (measuring albumin, ApoB100, ApoA1 and ApoB48) and/or by characterizing 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 summarized 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 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 depending on their size.[43] It uses a column containing porous beads: proteins are small enough to go through the pores, but not extracellular vesicles. Extracellular vesicles therefore migrate at a higher speed than soluble proteins (Fig. 1). However, if the size of a molecule (such as lipoproteins) is the same size as the size of extracellular vesicles, both will migrate at same speed and could 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 its purity is not optimal.[7,26,31,35,37,44] Automated acquisition methods have recently been developed, which makes this technique less labor

intensive and more useful in clinical settings. (b) Filtration is a technique separating smaller-sized soluble components (which are able to go through the pores) from extracellular vesicles which are retained on the filter. It is necessary to check the filter reference number which has been used, 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, ExoQuick™, miRCURY™ Exosome Isolation Kit or Invitrogen™ Total Exosome Isolation Kit) have a high extracellular vesicle recovery, with poor purity due to co-recovery of 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 be used as a concentration method after using another separation method.[12,24,34] Thus, as a general rule, the kits' applicability in a biomarker setting should be considered with due caution.[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 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 characterization 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 characterization

Several steps are proposed in the MISEV 2018 guidelines[8] in order to establish an extracellular vesicle separation method. These steps include quantification, general characterization, single vesicle characterization and are described in 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 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 has been validated.

Routine measurement of extracellular vesicles

Measurement methods for extracellular vesicles as biomarkers in routine clinical settings should exhibit the following characteristics: they should be able to detect small events as well as specific sub-populations; 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 as biomarkers are summarized 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 sub-population) 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 sub-populations 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 in order to detect small extracellular vesicles.[75,77] Yet, a major limitation resides in the variability among instruments of the smallest detectable size, which

affects the measured concentrations, and is responsible for differing sensitivities between instruments.[6,73] In response to this high interlaboratory variability, efforts to standardize 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, giving 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] Filtration/ELISA technique is described in Fig. 2.

Quantitative reverse transcription polymerase chain reaction (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 previously described.[30] Other RNA quantification methods validated for extracellular vesicles are enumerated in Table 2.[7,8,11,56,61–72] Northern blot is a robust and sensitive method to measure specific RNA transcripts, but 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 summarized 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 sub-populations; 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 in the future allow measurement of specific extracellular vesicle subpopulations.[79,80]

Extracellular vesicles in liver diseases

Extracellular vesicles appear to be attractive biomarkers for diagnosis, 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 sub-populations as biomarkers in liver diseases are summarized in Table 4 (review criteria are summarized in Supplementary Text). Unless otherwise mentioned, the studied extracellular vesicles were all derived 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. Supplementary Table 1 summarizes extracellular sub-populations as potential biomarkers in NAFLD.[2,82–89]

Diagnosis of non-alcoholic fatty liver disease

Few studies investigated extracellular vesicles for diagnosis of NAFLD. The largest included 65 patients with NAFLD and observed higher concentrations of extracellular vesicles derived from T cells and monocytes, compared with healthy controls.[82] A pilot study identified increased mitochondria-rich extracellular vesicles in obese patients with

increased serum transaminases levels, but sample sizes were small and no histology or imaging were used to characterize NAFLD.[2]

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 routines limits its applicability.[84]

Marker of activity and fibrosis

Regarding liver NAFLD activity, extracellular vesicles originating from NK T cells and monocytes (CD14+) have been shown to correlate with histological grade and with NAS score and ALT, respectively, unlike CD4+ and CD8+ T cell sub-populations.[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 sub-populations significantly correlated with histological fibrosis stage. On the other hand, a pilot study found that CD14+ (monocyte) and CD16+ (leukocyte) 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 NASH 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 one study, the extracellular vesicle fraction of miRNA-122 was not specifically studied.

To conclude, leukocyte extracellular vesicles have potential for being biomarkers of NAFLD diagnosis, activity and fibrosis, providing that large prospective studies validate these findings. Extracellular vesicles carrying miRNA-122 and -129 need further investigation.

Alcoholic hepatitis

The two main clinical challenges in alcoholic hepatitis are the elaboration of tests for non-invasive diagnosis of the disease and prediction of response to treatment.

Diagnosis of alcoholic hepatitis

Our group tested whether plasma extracellular vesicle levels could diagnose alcoholic hepatitis non-invasively. In two 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 have also been found in 101 patients with severe alcoholic hepatitis, in comparison with healthy controls.[90]

Plasma concentrations of total extracellular vesicles, as well as of extracellular vesicles derived from T cells (plasma and serum), macrophages, neutrophils, hematopoietic 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 sub-populations of extracellular vesicles (carrying mitochondrial DNA, miRNA or hepatocyte proteins) are higher in patients with binge drinking and/or chronic excessive alcohol consumption, as summarized in Supplementary Table 2.[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 hematopoietic 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 one-month mortality.[90] The combination of the two 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 sub-populations (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 of patients with alcoholic hepatitis. Data on other sub-populations are less homogenous and need to be confirmed in future studies.

Hepatitis B and C virus infections

Extracellular vesicles could be potential markers for prediction risk of hepatitis B virus reactivation and detection of early fibrosis for hepatitis B and C. Results of studies on that topic are summarized in Supplementary Table 3[104–108] and Supplementary Text[104–110] for hepatitis B, and Supplementary Table 4[82,84,105,111–121] and Supplementary Text[82,84,105,111–122] for hepatitis C.

Cirrhosis

Different extracellular vesicle sub-populations increase in patients with cirrhosis, and are predictive of disease progression, complications and mortality (Supplementary Table 5).[39,40,47,50,100,123–135] Doubt remains, however, on whether this increase is due to excess production, decreased clearance, or, most likely, both.[10]

Estimation of cirrhosis severity

Hepatocyte extracellular vesicle concentrations, characterized 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) and its components.[40] In the same study, hepatocyte larger extracellular vesicle concentrations increased with histological liver necro-inflammatory activity, suggesting that hepatocyte injury is the driver for extracellular vesicle release.[40]

Cirrhosis is known to be responsible for vascular changes including systemic vasodilation, increased cardiac output and increased intra-hepatic vascular resistance. For this reason, several studies have analyzed endothelial-derived extracellular vesicles.[39,40,125,126] They all concluded that patients with cirrhosis have higher

endothelial-derived extracellular vesicles (CD31+/41-, CD31+/42b- or CD62e) than healthy individuals. The link with cirrhosis severity (Child-Pugh score or MELD) has been observed with plasma leuko-endothelial (CD31+/41-) larger extracellular vesicles in an initial cohort of 91 patients[39], but was subsequently not confirmed using the same markers (CD31+/41-)[40] or other markers (CD31+/42b-).[125]

With systemic inflammation being a key feature of cirrhosis, several sub-populations of leukocyte-derived extracellular vesicles (CD45+, CD11a+ and CD4+) have been analyzed in different studies and found to be higher than in healthy individuals.[39,40,126] However, no link with cirrhosis severity was observed.

Data on platelet extracellular vesicles are controversial. Three studies have found a significant increase in platelet 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 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 extracellular vesicle release during plasma sample preparation, therefore making it an unreliable marker.[137]

Pilot studies, described in Supplementary Table 5, have identified several plasma and serum miRNAs that are up- or down-regulated 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 sub-population has been found to reliably estimate portal hypertension. Hepatocyte cytokeratin-18 positive extracellular vesicles weakly correlated with hepatic venous pressure gradients (HVPG), but could not discriminate patients having a HVPG ≥ 10 mmHg.[40]

Prediction of complications of cirrhosis

The procoagulant activity of some extracellular vesicles could play a role in portal vein thrombosis. Few studies have evaluated the capacity of extracellular vesicles for predicting portal vein thrombosis as well as 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 hepatocyte-derived extracellular vesicle levels can predict 6-month mortality, independently of Child-Pugh and MELD scores, using a threshold of 65 UI/L.[40] In this study, other sub-populations of plasma extracellular vesicles, including pan-leukocyte (CD11+) and endothelial (CD144+, CD62E+), were not useful for predicting mortality. Only small leuko-endothelial (CD31+/41-) extracellular vesicles also predicted 6-month mortality independently of Child-Pugh score, in two studies.[39,40] The potential interest of ascites extracellular vesicles has been investigated in 163 patients with cirrhosis.[50] The study found that low total ascites extracellular vesicle levels predicted 30-day mortality independently of MELD and antibiotic treatment. High percentages of ascites extracellular vesicles derived from neutrophil (CD66b+) or lymphocyte (CD3) were more commonly

observed in patients with poor outcomes, but survival analyses were not performed with these sub-populations.[50]

In conclusion, several sub-populations of extracellular vesicles seem to be promising candidate biomarkers to evaluate cirrhosis severity or predict mortality. Data are missing on the prediction of more specific complications and this data gap will need to be filled if personalized monitoring strategies are to be achieved.

Hepatocellular carcinoma

An unmet need is the development of tools to predict the risk of occurrence of hepatocellular carcinoma (HCC) among patients with chronic liver diseases, in order to identify those requiring closer disease monitoring. However, no study has evaluated the potential interest of extracellular vesicles in meeting this need. Conversely, abundant data suggest that extracellular vesicles could help detect HCC, predict HCC recurrence, survival and might also be associated with tumor 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 as controls patients with chronic liver diseases similar to those with HCC, but this study design has rarely been applied.[106,123,124,126,143–154]

Total extracellular vesicles, as well as some sub-populations, increase in the plasma[124] and sera[123] of patients with HCC, and their concentrations normalize after liver transplantation or R0 hepatectomy, which suggests a direct relationship with tumor 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] This result on total serum extracellular vesicles 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 tumor cells (AnnexinV+EpCAM+ASPGR1+) and showed that this sub-population could discriminate 86 patients with HCC from 49 patients with tumor-free cirrhotic liver (3-fold increase, AUROC 0.73).[143]

Expressions of several non-coding RNA 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 *per se*, was more prevalent among the HCC group and was responsible for higher circulating miRNA-21 expression levels in this group. miRNA-122 contained in circulating serum extracellular vesicles had decreased expression levels in 2 studies, and could even differentiate early HCC from cirrhosis.[145,157] Its expression increased after transarterial chemoembolization.[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 two cohorts.[147] In this study, a combination of the two RNAs and alpha-fetoprotein yielded a sensitivity of 84% and specificity of 88%, better than

alpha-fetoprotein alone.[147] Yet, tumor stages were advanced in this study, thus limiting immediate clinical applications.

Other circulating extracellular vesicle sub-populations 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 limits interpretation of the results (Supplementary Table 6).[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 plasma 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 1st line curative hepatectomy, although no difference in 3- and 5-year overall survival was 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]

Circulating extracellular vesicle RNA expression can also predict overall survival. For example, higher levels of serum small extracellular vesicle RNA RAB11A was associated with lower relapse-free survival in multivariate analysis among 60 patients with mainly early HCC.[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 serum 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 among patients with HCC (Supplementary Tables 7 and 8).[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 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), as 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 analyzed 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, here 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), bile extracellular vesicles having a significantly better discriminating ability than serum CA19-9.[52] Others have observed an overexpression of an small extracellular vesicle miRNA panel in bile extracellular vesicles from 26 patients with cholangiocarcinoma, as compared with 50 controls with benign stenosis (including 13 primary sclerosing cholangitis).[51]

Beyond the setting of biliary strictures, other studies have analyzed extracellular vesicles in patients with cholangiocarcinoma. One study observed that circulating tumor cell-derived (AnnexinV+EpCAM+ASGPR1+) extracellular vesicles were 3-fold higher in the serum of 38 patients with cholangiocarcinoma than in 49 patients with cirrhosis.[143] These tumor 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, as compared with 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 tumor stage and the presence of lymph node metastasis. Patients with higher levels of miRNA-200c-3p had lower disease-free and overall-free survivals.[202] Two other preclinical studies found differentially expressed extracellular vesicle RNAs between patients with cholangiocarcinoma and healthy controls.[203,204]

To conclude, several sub-populations of extracellular vesicles have the potential to become very useful tools to differentiate cholangiocarcinoma from PSC and other tumors. 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 centers.

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 an unmet need. Since extracellular vesicles are sensitive markers of cell injury, we can hypothesize 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 with healthy controls, thereby limiting their clinical relevance (Supplementary Table 9).[206–209]

Predictor of outcome

The interest of extracellular vesicles in the acute liver injury/acute liver failure setting has been analyzed by the “Acute Liver Failure Study Group” in a group of 50 patients. Plasma concentrations of total larger-size extracellular vesicles significantly increased with hepatic encephalopathy grade, as well as with 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 etiology).[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 sub-population was not significantly associated with severity.[206]

598

599 **Conclusion**

600 Extracellular vesicles hold promise in liver diseases as biomarkers for diagnosis and
601 prediction of disease progression, complications, response to treatment and mortality.
602 Although data so far are derived from pilot studies, some sub-populations of extracellular
603 vesicles have been robustly evaluated in independent large multicentric prospective cohorts,
604 as summarized in Fig. 3.

605 Guidelines on extracellular vesicle research stress the importance of parameters such
606 as purity, extracellular vesicle structure, physical characteristics and marker localization,
607 while their importance in clinical research is less explicitly covered.[8] Other parameters
608 which are less often considered in basic research, such as cost, assay time, labor-
609 intensiveness, accessibility of measuring devices, inter-laboratory variability, characteristics
610 of study population and control group, are more relevant for application in clinical settings.
611 Several challenges must be overcome prior to considering the use of extracellular vesicles in
612 clinical routines.[210] First, the reliability and reproducibility of the quantification methods
613 have to be strengthened. In the current state of affairs, results are likely to reflect the settings
614 of several preanalytical variables. For example, measured vesicle concentrations can be
615 influenced by the minimum vesicle size that the technique can detect.[211] The variation
616 coefficient is often not mentioned in studies or is high and variable. There is no international
617 reference preparation (gold standard) to assess trueness of assay. Second, elaboration of high-
618 throughput detection methods, not affecting sample stability and diversity, would facilitate the
619 development of extracellular vesicle measurement. Currently, many characterization
620 techniques are prohibitively expensive and/or labor-intensive. Third, determination of cut-off
621 values is needed to help physicians make decisions. Forth, validation of results is needed
622 using studies involving large cohorts of patients with comorbidities.

623 These limitations are currently being progressively overcome, thanks to
624 standardization guidelines and recommendations. Moreover, the number of studies on
625 extracellular vesicles as biomarkers of liver diseases is growing exponentially. In the near
626 future, these studies will make convincing levels of evidence available to both physicians and
627 scientists.

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1363

1364 **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.e. EV cargo)	Intermediate: co-recovery of protein aggregates and viruses	High variability (depending on rotors, dilution method, sample viscosity) Risk of EV aggregation or damage	No: Time consuming (2-9h), 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	High variability. Risk of damage and loss of biological activity.	No: time consuming (6-48h), 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 labor-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 sample volumes	[11,24]

1365 Abbreviations: EV: extracellular vesicle; Ref.: references; RNA: ribonucleic acid

1366 **Table 2: Summary of available technologies to perform extracellular vesicle**
 1367 **characterization in research settings.**

	Quantification	General characterization	Single vesicle characterization
Aim	Evaluation of EV recovery Evaluation of purity Quantification of EV subtype	Confirmation of EV presence (protein markers)	Evaluation of EV integrity Confirmation of EV presence Evaluation of EV subtype (size)
Technology	Particle number:[8] Nanoparticle tracking analysis[61] High-resolution bead-based flow cytometry Resistive pulse sensing Cryo-electron microscopy[56] Surface plasmon resonance coupled to atomic force microscopy[62] RNA quantification:[7] Bioanalyzer pico chip Quant-iT RiboGreen RNA Assay Quantitative reverse transcription polymerase chain reaction (qRT-PCR) Total protein count :[8] Colorimetric assays Fluoremetric assays Protein stain on SDS-PAGE Specific protein count:[8,11] ELISA Bead-based flow cytometry Aptamer- carbon nanotubes colorimetric assays Nanoplasmon-enhanced scattering assay[63]	Bead-based flow cytometry[8] Western blot (mainly for cell culture media)[8] Multiplex bead-based platforms [64,65] Surface plasmon resonance[66] Fluorescence scanning[67] More assays are described in [71]	High-resolution imaging technique:[8,68] Transmission electron microscopy Scanning electron microscopy Cryo-electron microscopy Atomic force microscopy Super-resolution microscopy Estimation of biophysical features:[8,69,70,72] Resistive pulse sensing Nanoparticle tracking analysis High resolution flow cytometry Asymmetric flow field fractionation Raman spectroscopy

1368 Abbreviations: EV: extracellular vesicle; ELISA: sandwich enzyme-linked immunosorbent

1369 assay; RNA: ribonucleic acid; qRT-PCR: quantitative reverse transcription polymerase chain

1370 reaction; SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis

1371 **Table 3. Detection methods of extracellular vesicles having potential for clinical use as**
 1372 **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 standardization	Only detects larger extracellular vesicles Interlaboratory variability Dedicated cytometers Labor 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]

1373 Abbreviations: EV: extracellular vesicle; Ref.: references; RNA: ribonucleic acid; qRT-PCR:

1374 quantitative reverse transcription polymerase chain reaction

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.
Nonalcoholic fatty liver disease								
T Cell (CD4+ or CD8+ or iNKT) Monocyte (CD14+) Neutrophil (CD15+) Platelet (CD41+)	↑	Larger FCM	NAFLD: 65 HC: 44	27-59 / 90-98	0.81-0.91 NA	Detection of NAFLD	Retrospective	[82]
Alcoholic hepatitis								
Hepatocyte (cytokeratin-18)	↑	Larger Filtration/ELISA	Confirmed AH: test: 46, validation: 48 Ruled-out AH: test: 37, validation: 20	76/81	0.82 83/73	Diagnosis of AH	Prospective	[41]
Hematopoietic stem cell (CD34+) & Hepatocyte (ASPGR+)	↑	Larger FCM	Severe AH: non-responders: 71 responders: 30 HC: 20	NA	0.94 NA	Response to therapy & mortality	Retrospective	[90]
Cirrhosis								
Platelet-derived growth factor receptor β	↑	Small ELISA	Fibrosis 0-1: 51 Fibrosis 2-4: 97 HC: 14 Validation cohort: 57	82/41	0.64 NA	Detection of fibrosis F3-F4	Retrospective	[134]
Leuko-endothelial (CD31+/41-)	↑	Larger FCM	Cirrhosis: 91	NA	NA	Survival	Prospective	[39]
Hepatocyte (cytokeratin-18)	↑	Larger Filtration-ELISA	Cirrhosis: Test cohort: 139 Validation cohort: 103	NA	NA	6-month mortality	Prospective Competing risk analysis	[40]
Hepatocellular carcinoma								
Tumoral (AnnexinV+ EpCAM+ASPGR1+ +/- CD133+)	↑	Larger FCM	HCC: 86 Cirrhosis: 49 HC: 58	80-81 / 47-50	0.73-0.74 NA	Detection of HCC	Retrospective	[143]
miRNA-519d & -595 & -939	↑	Small qRT-PCR	Advanced HCC: 45 Unifocal/small: 40 Cirrhosis no HCC: 30	NA	0.82-0.84 NA	Detection of HCC	Retrospective	[151]
miRNA-125	↓	Small qRT-PCR	HCC: 128	83/68	0.74 NA	Recurrence & survival	Prospective	[150]
Signature of miRNA-122, -148a, -1246	↑	Small qRT-PCR	HCC: 50 Cirrhosis: 40	86/88	0.93 NA	Detection of HCC	Retrospective	[149]
miRNA-638	↓	Small qRT-PCR	HCC: 126	NA	NA	Overall survival	Retrospective	[171]
miRNA-21 & miRNA-10b	↑	Small qRT-PCR	HCC: 124	NA	NA	Prediction of recurrence	Retrospective	[156]
lncRNA-RP11-513I15.6 & miRNA 1262 & RAB11A	↑	Small qRT-PCR	HCC: 54 HCV: 42; HC: 18	78-98 / 73-95	NA 72-95/79-97	Detection of early HCC	Retrospective	[152]
lncRNA ENSG00000258332.1	↑	Small qRT-PCR	HCC: 60 HBV: 96	72-76 / 78-83	0.72-0.75 NA	Detection of HCC	Retrospective	[147]

& LINC00635						& survival		
miRNA-92b	↑	Small qRT-PCR	After LT: No recurrence: 28 Early recurrence: 43	71/63	0.70 NA	Early recurrence after LT	Retrospective	[194]
miRNA-21 & lncRNA-ATB	↑	Small qRT-PCR	HCC: 79	NA	NA	Overall survival	Prospective Multivariate	[191]
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	NA	NA	Prediction of mortality/LT	Prospective Multivariate	[206]

1377 Abbreviations: AH: alcoholic hepatitis; AUROC: area under the receiver operating
 1378 characteristic; CCA: cholangiocarcinoma; ELISA: enzyme-linked immunosorbent assay; EV:
 1379 extracellular vesicle; FCM: flow cytometry; HC: healthy control; HCC: hepatocellular
 1380 carcinoma; lncRNA: long non-coding RNA; LT: liver transplantation; qRT-PCR: quantitative
 1381 reverse transcription polymerase chain reaction; miRNA: micro-RNA; NA: not available;
 1382 NAFLD: non-alcoholic fatty liver disease; NPV: negative predictive value; NTA:
 1383 nanoparticle tracking analysis; PPV: positive predictive value; Ref: reference; RNA:
 1384 ribonucleic acid; Se: sensitivity; Spe: specificity; Var: variation

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, separating progressively 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.

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 1000 nm was observed (n=2). Abbreviations: ELISA: sandwich enzyme-linked immunosorbent assay; EV: extracellular vesicle.

1409 **Fig. 3: Phases of development of extracellular vesicles as biomarkers for diagnosis,**
1410 **severity and prognosis in liver diseases.** Abbreviations: extracellular vesicle.

1411 Box 1. How to name extracellular vesicles?

1412 Over the last 20 years, terms used for naming different types of extracellular vesicles varied.
1413 Extracellular vesicle subtypes were previously defined by their biogenesis pathway,
1414 “exosomes” having an endosomal origin, “microvesicles” deriving from plasma membrane
1415 and “apoptotic bodies” being released by dying cells. However, no specific marker of
1416 biogenesis pathway has been found, and extracellular vesicle size and protein composition
1417 overlap between “exosomes” and “microvesicles”. Accordingly, both “Minimal information
1418 for studies of extracellular vesicles” (MISEV) guidelines 2018 and American Heart
1419 Association guidelines 2017 recommend using the term “extracellular vesicles” to avoid
1420 confusion.[6,8] Sub-classification of these extracellular vesicles can be based on operational
1421 aspects including size (small, *i.e.* inferior to 100 or 200 nm vs. medium/large, *i.e.* superior to
1422 100 or 200 nm), density, biochemical composition, and cell condition or origin (*e.g.*
1423 hepatocyte extracellular vesicle, apoptotic body, hypoxic extracellular vesicle).

1424 **Box 2. Blood sample collection, platelet free plasma preparation and storage, adapted**
1425 **from ISEV 2013, AHA 2017, ISAC 2016 and ISTH 2015 guidelines.**[6,11,12,20] ISEV:
1426 International Society of Extracellular Vesicles; AHA: American Heart Society; ISAC:
1427 International Society for Advancement of Cytometry; ISTH: International Society of
1428 Thrombosis and Hemostasis; EDTA: ethylene diamine tetraacetic acid
1429
1430 Prefer fasting blood samples.
1431 If possible, collect samples during same time period.
1432 Adapt anticoagulant to downstream analysis: citrate, EDTA or sodium fluoride/potassium.
1433 Avoid heparin-based anticoagulants.
1434 For venipuncture, use 21-gauge needles or larger. Avoid butterfly systems.
1435 Remove cuff rapidly after venipuncture and discard first 2-3 mL.
1436 Properly fill tubes to obtain correct blood/anticoagulant ratio. Invert tubes 8-10 times without
1437 shaking.
1438 Stabilize tubes and store vertically. Minimize bench-top storage time < 30-60 minutes.
1439 Avoid analyzing hemolyzed samples.
1440 Double centrifugate at 2500 g for 15 minutes at room temperature, set the lowest deceleration
1441 of centrifuge.
1442 Use a clean plastic tube for second centrifugation. Do not collect the last 0.5 cm of plasma
1443 above the buffy coat.
1444 Freeze and store at -80 °C.

Fig. 1

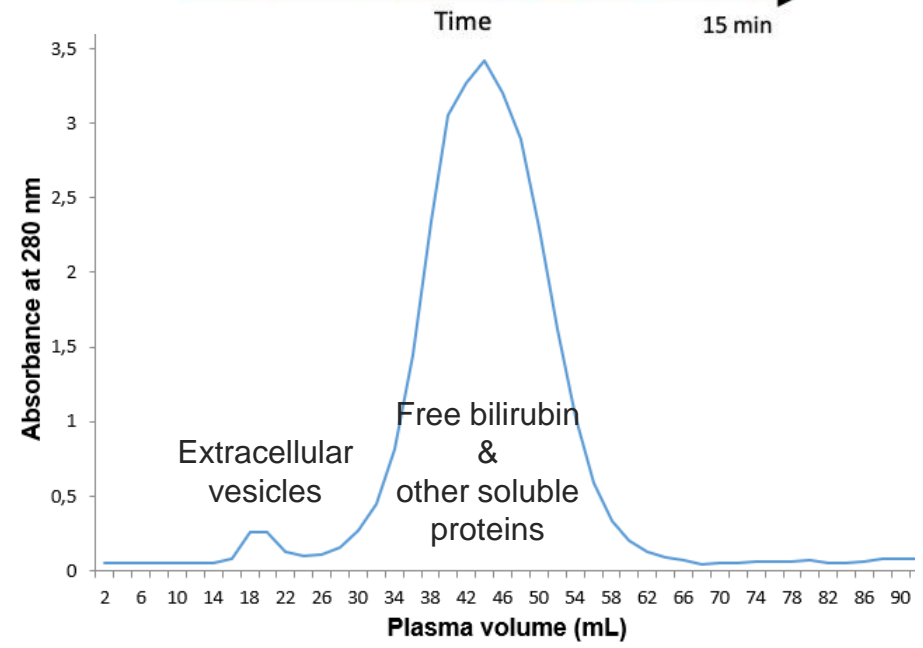
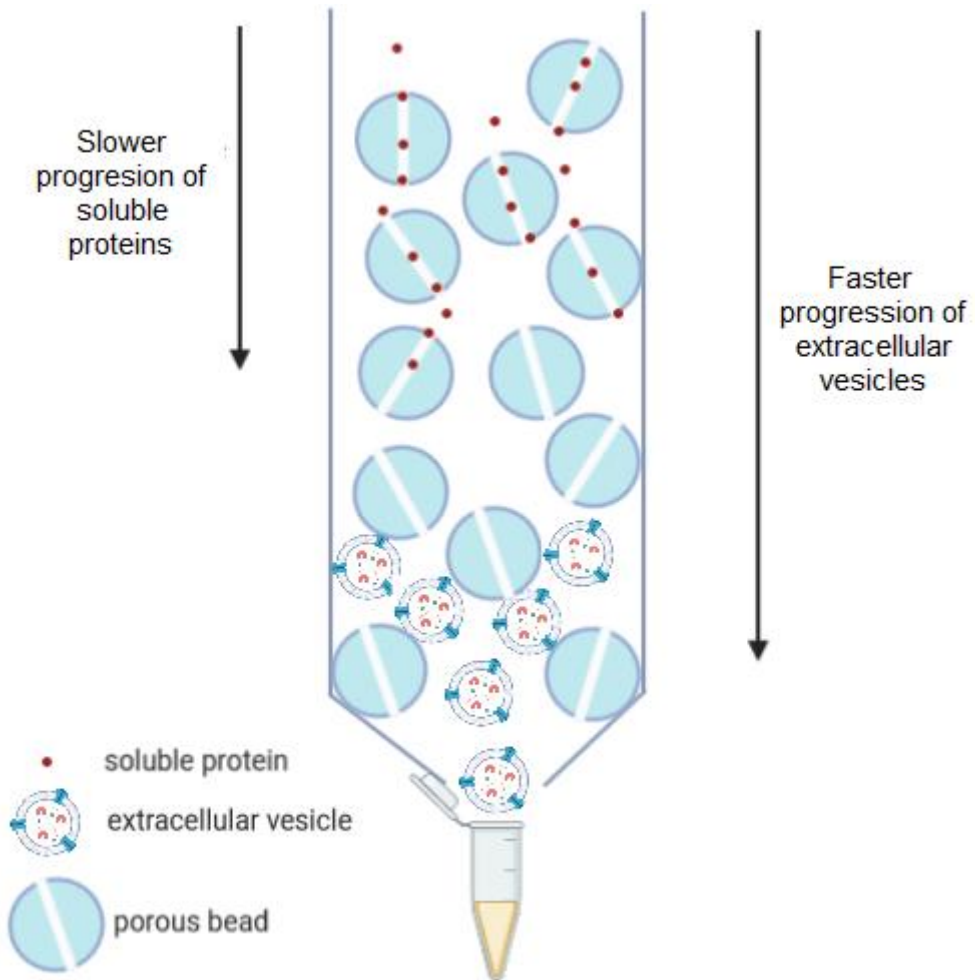
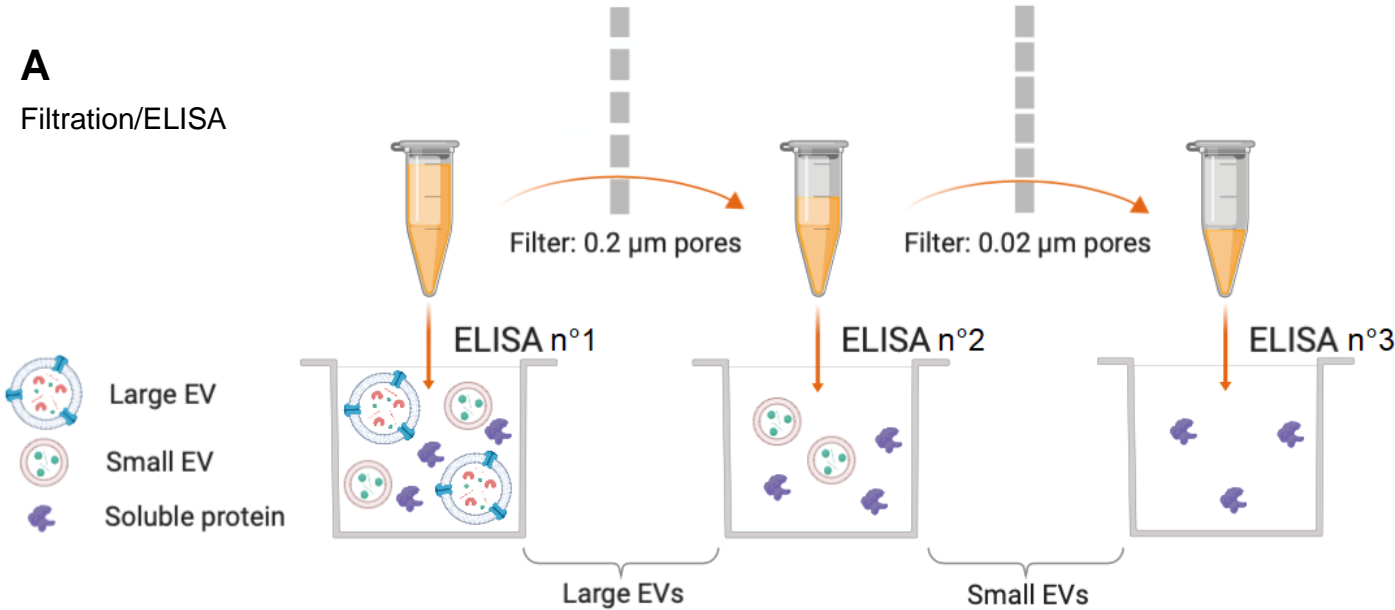


Fig. 2

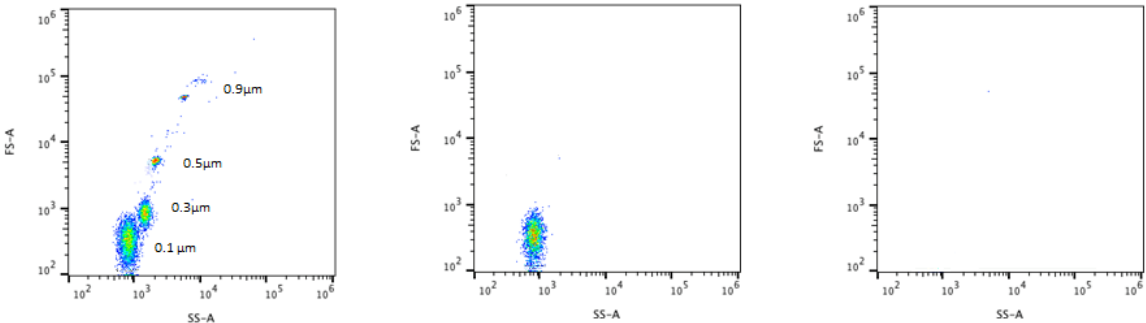
A

Filtration/ELISA



B

Flow cytometry



C

Q Nano Analysis

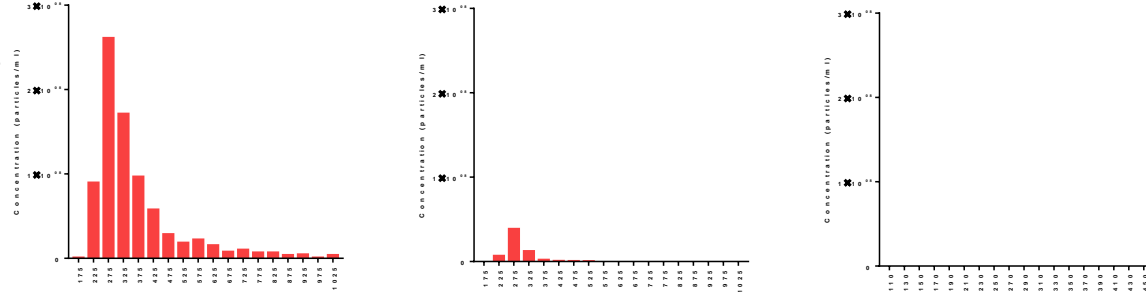
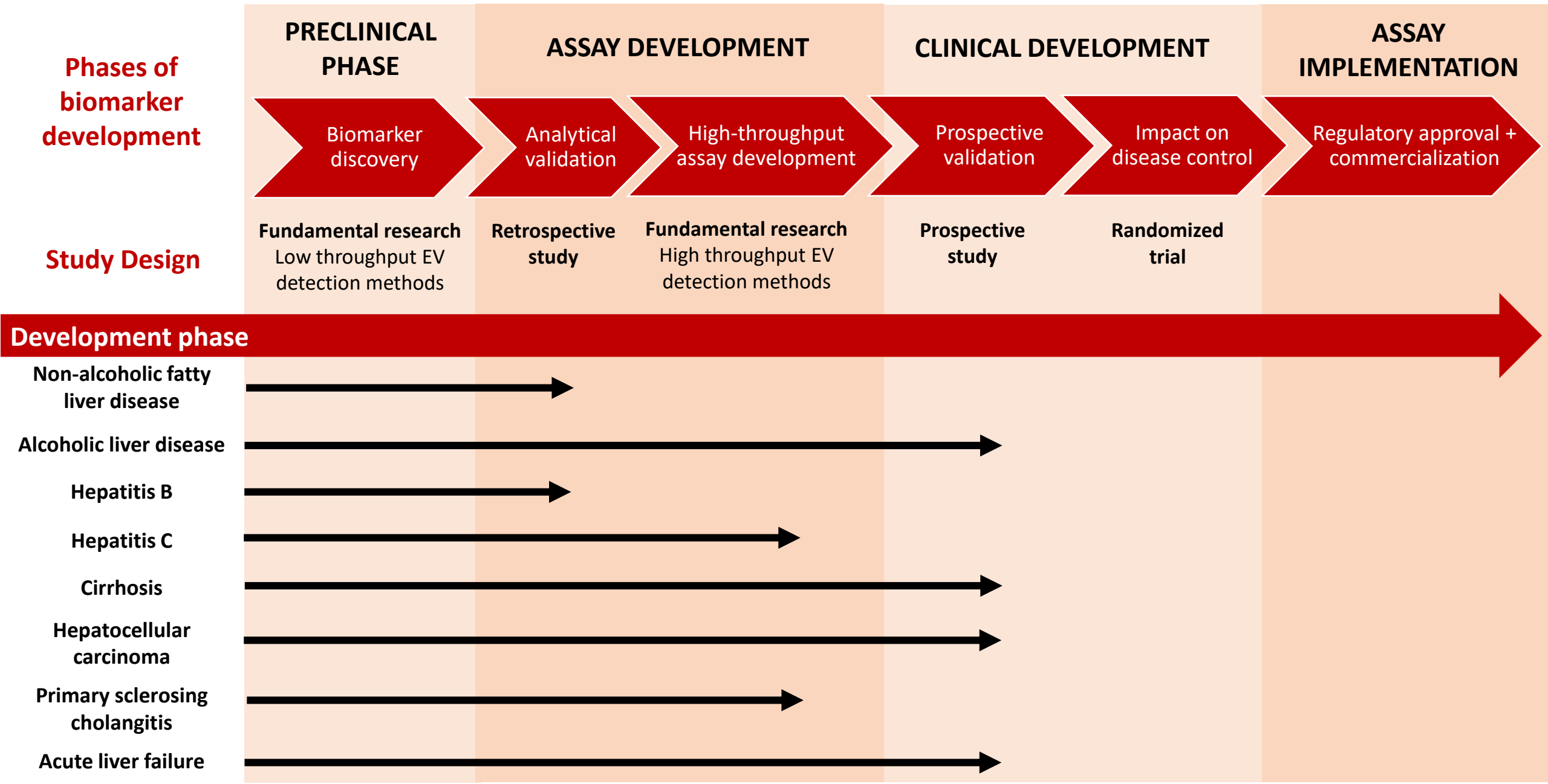
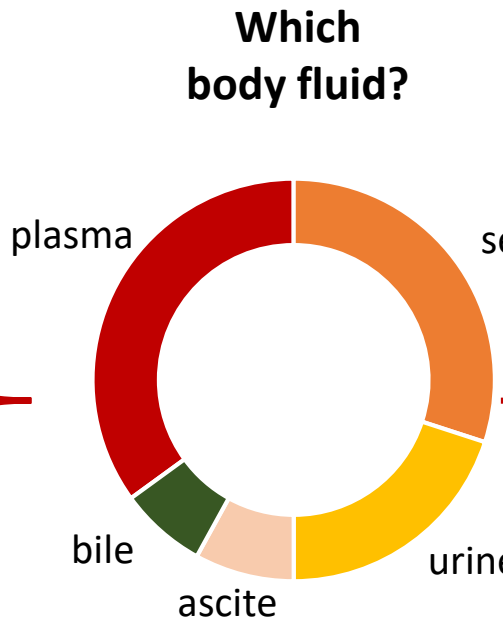
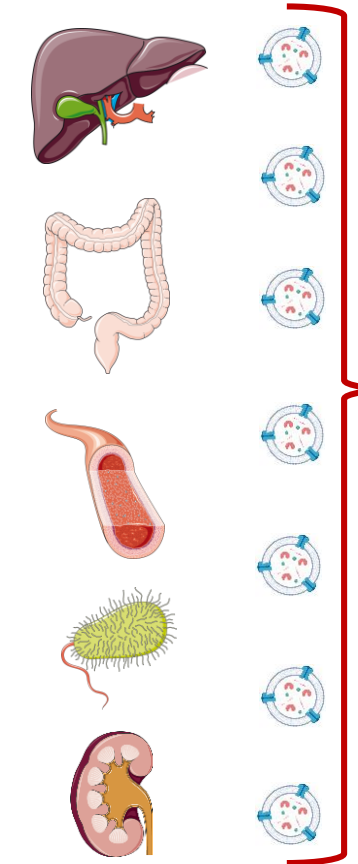


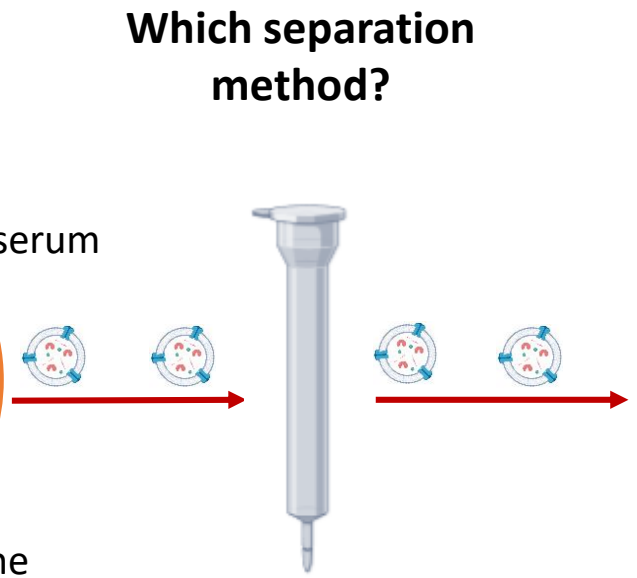
Fig. 3



Which extracellular vesicle subpopulation?

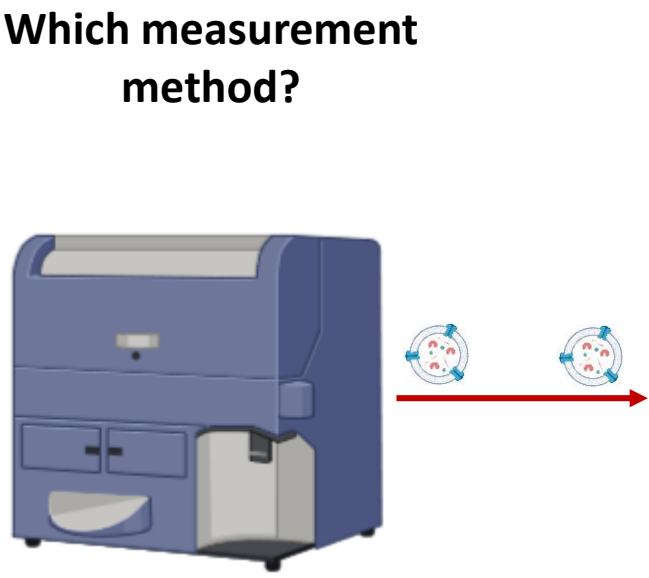


Which body fluid?



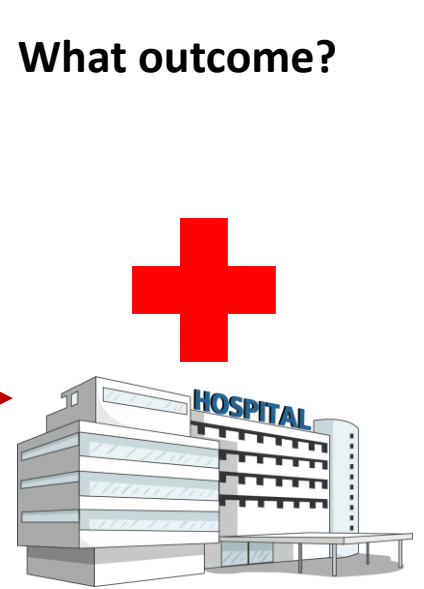
Which separation method?

Size exclusion chromatography
Filtration
Precipitation kits



Which measurement method?

Flow cytometry
qRT-PCR
Filtration/ELISA



What outcome?

Diagnosis
Severity
Prognosis