



HAL
open science

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

Sara Thietart, Pierre-Emmanuel Rautou

► **To cite this version:**

Sara Thietart, Pierre-Emmanuel Rautou. Extracellular vesicles as biomarkers in liver diseases: A clinician's point of view. *Journal of Hepatology*, 2020, 73, pp.1507 - 1525. 10.1016/j.jhep.2020.07.014 . hal-03493927

HAL Id: hal-03493927

<https://hal.science/hal-03493927v1>

Submitted on 21 Nov 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

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

3
4 Sara Thietart (1) & Pierre-Emmanuel Rautou (1,2,3)

5
6 1-Université de Paris, Centre de recherche sur l'inflammation, Inserm, F-75018 Paris, France

7 2-Service d'Hépatologie, DHU Unity, Pôle des Maladies de l'Appareil Digestif, Hôpital
8 Beaujon, AP-HP, Clichy, France

9 3-Centre de Référence des Maladies Vasculaires du Foie, French Network for Rare
10 Liver Diseases (FILFOIE), European Reference Network (ERN) 'Rare-Liver'

11
12 Corresponding author:

13 Name: Prof. Pierre-Emmanuel Rautou, MD, PhD

14 Address: Service d'hépatologie, Hôpital Beaujon, 100, boulevard du Général Leclerc, 92100

15 Clichy, France

16 Tel: +33.1.40. 87.52.83

17 Fax: +33.1. 40.87.55.30

18 *E-mail address:* pierre-emmanuel.rautou@inserm.fr

19
20 Key words: microvesicle; exosome; microparticle; vesicle; liver; alcohol

21
22 Word count: Abstract (256), Key points (4), Manuscript (5496), Tables (4), Figures (3),
23 Boxes (2), Supplementary Tables (9), References (211)

24
25 Conflict of interest: Neither of the authors has any conflicts of interest to disclose.

26 Financial support: This work was supported by the “Institut National de la Santé et de la
27 Recherche Médicale” (ATIP AVENIR), the “Agence Nationale pour la Recherche” (ANR-18-
28 CE14-0006-01, RHU QUID-NASH) and by « Émergence, Ville de Paris ».

29

30 Authors’ contributions: Sara Thietart and Pierre-Emmanuel Rautou wrote the manuscript.

31

32 **Key points:**

- 33 • Extracellular vesicles are membrane-bound vesicles, containing cell-derived
34 biomolecules, such as proteins, lipids and RNAs.
- 35 • Extracellular vesicles are promising biomarkers for diagnosis of liver diseases and
36 prediction of disease progression, complications, response to treatment and mortality.
37 Different extracellular vesicle subpopulations have been studied, using total
38 extracellular vesicle count, proteins, lipids, RNAs and microRNAs.
- 39 • Some extracellular vesicle subpopulations have already been evaluated in prospective
40 studies in cirrhosis, alcoholic liver disease and hepatocellular carcinoma. In other liver
41 diseases, extracellular vesicles still await validation.
- 42 • High-throughput and reproducible measurement methods of extracellular vesicles are
43 now becoming available.

44 **Abbreviations:**

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

58 **Abstract**

59 Extracellular vesicles are membrane-bound vesicles containing proteins, lipids, RNAs and
60 microRNAs. They can originate from both healthy and stressed cells, and provide a snapshot
61 of the cell of origin in physiological and pathological circumstances. Various processes that
62 may give rise to the release of extracellular vesicles occur in liver diseases, including
63 hepatocyte apoptosis, hepatic stellate cell activation, liver innate immune system activation,
64 systemic inflammation, and organelle dysfunction (mitochondrial dysfunction and
65 endoplasmic reticulum stress). Numerous studies have therefore investigated the potential
66 interest of extracellular vesicles as biomarkers in liver diseases. This review provides an
67 overview of the methods that can be used to measure extracellular vesicle concentrations in
68 clinical settings, ranging from plasma preparation to extracellular vesicle measurement
69 techniques. We also provide a comprehensive review of studies that test extracellular vesicles
70 as diagnostic, severity and prognostic biomarkers in various liver diseases, including non-
71 alcoholic and alcoholic steatohepatitis, viral hepatitis B and C infections, cirrhosis, primary
72 liver cancers, primary sclerosing cholangitis and acute liver failure. In particular, the review
73 shows that extracellular vesicles could be useful tools to evaluate activity and fibrosis in non-
74 alcoholic fatty liver disease, predict risk of hepatitis B virus reactivation, predict
75 complications and mortality in cirrhosis, detect early hepatocellular carcinoma, detect
76 malignant transformation in primary sclerosing cholangitis and predict outcomes in acute
77 liver failure. While most studies draw on data derived from pilot studies and await clinical
78 validation, some extracellular vesicles sub-populations have already been evaluated in solid
79 prospective studies. The challenges raised by the use of extracellular vesicles as biomarkers
80 are also discussed here.

81 **What are extracellular vesicles?**

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

88 Extracellular vesicles are classified into several subtypes, each with different
89 biogenesis mechanisms, which are thoroughly reviewed elsewhere.[1] Briefly, exosomes (size
90 ranging from 40 to 100-200 nm) are generated in intraluminal vesicles by inward budding of
91 the limiting membrane of early endosomes during the maturation process of multivesicular
92 endosomes. Exosomes are then released into the extracellular milieu by fusion of
93 multivesicular bodies with the plasma membrane.[1] “Microvesicles” (previously called
94 “microparticles”, with sizes ranging from 100-200 nm to 1 μ m) are generated by plasma
95 membrane budding induced by a transient phospholipid imbalance caused by externalization
96 of phosphatidylserine (a negatively charged phospholipid), calcium dependent proteolysis of
97 cytoskeleton and local potassium efflux responsible for cellular contraction.[4] Apoptotic
98 bodies (size ranging from \approx 1 to 4 μ m), which are released by dying cells, are formed by a
99 separation of plasma membrane from the cytoskeleton.[5] Apoptotic bodies will not be
100 discussed in this review, as they are the same size as platelets, and are therefore eliminated
101 during the first steps of platelet-free plasma processing.[6,7]

102 Due to overlapping size, density and composition, a precise definition of these
103 subgroups of extracellular vesicles has not yet been firmly established (Box 1).[8] It is now
104 recommended that extracellular vesicles be classified by size, density, biochemical
105 composition or cellular origin and not by biogenesis.[8] In this review, we therefore use the

106 terms “small”, and “larger” extracellular vesicles, using a size cut-off of 100 to 200 nm (the
107 smallest size of extracellular vesicle that can be detected using flow cytometry).

108

109 Once released into the extracellular compartment, extracellular vesicles can reach
110 target cells for intercellular communication. Communication methods of extracellular vesicles
111 are varied and include activation of surface receptors, transfer of vesicle content into the
112 target cell by vesicle internalization (phagocytosis, endocytosis) or membrane fusion.[1] The
113 role of extracellular vesicles in cell-cell communication in liver diseases has been thoroughly
114 reviewed elsewhere.[9,10]

115 Extracellular vesicles are released following cell activation or apoptosis. Their
116 composition depends on their cellular origin and on the stimulus responsible for their
117 formation.[9] Circulating extracellular vesicle concentrations and composition thus vary with
118 disease conditions with some specificity for the ongoing detrimental process. These
119 characteristics make extracellular vesicles useful tools for personalized medicine to diagnose
120 disorders, evaluate prognosis, disease progression and response to treatment. Circulating
121 extracellular vesicles have therefore emerged as attractive biomarkers in liver diseases, as
122 shown in this review.

123

124 **Measurement methods in clinical use**

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

129

130 ***Patient requirements***

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

136

137 *Pre-analytical requirements*

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

142

143 *Plasma sample collection, preparation and storage*

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

154

155 Recommendations on blood sample collection are summarized in Box 2.[6,8,11,12,20]
156 The International Society on Thrombosis and Haemostasis (ISTH)[21] and the International
157 Society for Advancement of Cytometry (ISAC)[20] recommend the use of citrate tubes (as
158 opposed to ethylene diamine tetraacetic acid (EDTA) and heparin), as their use better prevents
159 platelet activation and formation of extracellular vesicles.[8,22] The American Heart
160 Association's (AHA) 2017 guidelines[11] and the International Society for Extracellular
161 Vesicles' (ISEV) 2017 position paper[7] recommend that the choice of anticoagulants be
162 adapted to downstream analysis. For example, acid citrate dextrose (ACD) is considered to be
163 more suitable for RNA analysis.[7,11,23]

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

168

169 *Separation methods*

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

172 Currently, no extracellular vesicle separation method is able to entirely separate
173 extracellular vesicles from the remaining plasma. A balance must be found between
174 maximizing recovery of extracellular vesicles (to avoid loss of information) and minimizing
175 contaminants (soluble proteins, protein aggregates, lipoproteins, nucleic acids and viruses).
176 Some authors use combinations of techniques such as ultrafiltration, density gradients or
177 washing with extracellular vesicle-free buffer. This increases purity, but is more labor
178 intensive.[8,25] Consideration should also be given to the fact that separation methods can
179 influence important extracellular vesicle characteristics, such as size, integrity, composition

180 and functional properties.[18,26,27]. Separation methods can isolate non-vesicular circulating
181 miRNAs or RNAs together with extracellular vesicles.[28,29] Since treatment of extracellular
182 vesicles with deoxyribonuclease/ribonuclease (DNase/RNase) does not interfere with RNA
183 analysis and degrades externally bound RNA, its use is recommended by the ISEV and
184 AHA.[7,11,30] Interpretation of data should take into account isolation techniques and use of
185 RNase treatment. For small cohorts, it is useful to verify reliability of separation methods, by
186 quantifying contaminants (measuring albumin, ApoB100, ApoA1 and ApoB48) and/or by
187 characterizing the aspect of separate extracellular vesicles (for example, by using transmission
188 electron microscopy).[11,31] A ratio of at least 3×10^{10} vesicles per μg of protein is proposed
189 to define high purity.[32]

190

191 The main extracellular vesicle separation methods applicable for biomarker use are
192 summarized in Table 1.[6,7,11,12,22,24,29,33–42] Some separation methods are unlikely to
193 be suitable for use in clinical routines, including differential centrifugation, density gradient
194 centrifugation and immunoaffinity capture. These methods are described in Supplementary
195 Text.[6,11,12,19,25,33–35,42] Other separation methods seem to be better adapted to clinical
196 settings: (a) Size exclusion chromatography separates extracellular vesicles from other
197 components depending on their size.[43] It uses a column containing porous beads: proteins
198 are small enough to go through the pores, but not extracellular vesicles. Extracellular vesicles
199 therefore migrate at a higher speed than soluble proteins (Fig. 1). However, if the size of a
200 molecule (such as lipoproteins) is the same size as the size of extracellular vesicles, both will
201 migrate at same speed and could therefore be co-isolated.[31] This technique has been shown
202 to give intact and functional vesicles, as well as fewer protein and lipoprotein contaminants
203 than other methods, although its purity is not optimal.[7,26,31,35,37,44] Automated
204 acquisition methods have recently been developed, which makes this technique less labor

205 intensive and more useful in clinical settings. (b) Filtration is a technique separating smaller-
206 sized soluble components (which are able to go through the pores) from extracellular vesicles
207 which are retained on the filter. It is necessary to check the filter reference number which has
208 been used, as filter type and pore size have been shown to heavily influence recovery.[8,38]
209 The effect on vesicle disruption of the forces applied to push samples through the filters is
210 unknown.[34] (c) Precipitation kits (such as, ExoQuick™, miRCURY™ Exosome Isolation
211 Kit or Invitrogen™ Total Exosome Isolation Kit) have a high extracellular vesicle recovery,
212 with poor purity due to co-recovery of protein complexes and extracellular vesicle
213 aggregates.[8,29] Their application should be limited to samples known to be rich in small
214 extracellular vesicles, for the study of RNA, or be used as a concentration method after using
215 another separation method.[12,24,34] Thus, as a general rule, the kits' applicability in a
216 biomarker setting should be considered with due caution.[11]

217

218 *Other biofluid collection and separation methods*

219 In addition to plasma, extracellular vesicles have been detected in other biological
220 fluids, including serum, urine, ascites and bile. Methods to collect these biofluids and separate
221 extracellular vesicles are described in Supplementary Text.[6,11,12,20,24,45–55]

222

223 *Analytical techniques*

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

228

229 *Accurate characterization*

230 Several steps are proposed in the MISEV 2018 guidelines[8] in order to establish an
231 extracellular vesicle separation method. These steps include quantification, general
232 characterization, single vesicle characterization and are described in Supplementary
233 Text.[8,11,56–60] Available technologies that may be used to perform all these steps are
234 listed in Table 2.[7,8,11,56,61–72] These steps should be considered when setting up the
235 detection of an extracellular vesicle marker, to assess the results of separation methods and to
236 establish the likelihood that the biomarker is associated with extracellular vesicles and not
237 with other co-separated materials.[8] These steps do not need to be repeated, once the
238 separation and routine measurement method has been validated.

239

240 *Routine measurement of extracellular vesicles*

241 Measurement methods for extracellular vesicles as biomarkers in routine clinical
242 settings should exhibit the following characteristics: they should be able to detect small events
243 as well as specific sub-populations; to have low inter-laboratory variability; and to use widely
244 available devices, with quick acquisition time and limited costs. The 3 main detection
245 methods commonly used for extracellular vesicles as biomarkers are summarized in Table
246 3.[7,11,39,71–76]

247 High sensitivity-flow cytometry on a dedicated device can simultaneously detect light
248 scatter (*i.e.* diameter) and fluorescence signal (*i.e.* an extracellular vesicle sub-population)
249 from extracellular vesicles passing one by one in front of a laser beam.[6] It is the most
250 commonly used technique for measuring concentrations of total extracellular vesicles as well
251 as sub-populations of extracellular vesicles, and can also determine cellular origin. Dedicated
252 high-resolution flow cytometers are required, using bead-based calibrations or a scatter-
253 diameter relationship model in order to detect small extracellular vesicles.[75,77] Yet, a major
254 limitation resides in the variability among instruments of the smallest detectable size, which

255 affects the measured concentrations, and is responsible for differing sensitivities between
256 instruments.[6,73] In response to this high interlaboratory variability, efforts to standardize
257 the technique are currently underway.[73,77,78]

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

265 Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a method
266 that quantifies a specific RNA transcript.[7] It is the most widely used method in biomarker
267 identification as it is robust, low-cost, and has high analytical sensitivity.[72] The check list of
268 experimental details for RNA analysis has been previously described.[30] Other RNA
269 quantification methods validated for extracellular vesicles are enumerated in Table
270 2.[7,8,11,56,61–72] Northern blot is a robust and sensitive method to measure specific RNA
271 transcripts, but is time consuming, and thus not adapted to clinical laboratories.

272 Other detection methods that are often used in research settings and are less applicable
273 in clinical settings are summarized in Table 2. Nanoparticle tracking analysis, which has
274 outperformed dynamic light scattering, is a method that determines size and concentration of
275 extracellular vesicles by measuring their Brownian motion. However, these techniques are
276 limited by the fact that: they are unable to detect specific extracellular vesicle sub-
277 populations; exact concentrations can only be estimated with a risk of high inter-laboratory
278 variability; and they require special equipment.[11] Current development of fluorescent

279 detection using nanoparticle tracking analysis devices may in the future allow measurement of
280 specific extracellular vesicle subpopulations.[79,80]

281

282 **Extracellular vesicles in liver diseases**

283 Extracellular vesicles appear to be attractive biomarkers for diagnosis, estimating
284 severity and prognosis in liver diseases, including non-alcoholic and alcoholic steatohepatitis,
285 chronic viral hepatitis B and C infections, cirrhosis, primary liver cancers, primary sclerosing
286 cholangitis and acute liver failure. The main clinical studies evaluating extracellular vesicle
287 sub-populations as biomarkers in liver diseases are summarized in Table 4 (review criteria are
288 summarized in Supplementary Text). Unless otherwise mentioned, the studied extracellular
289 vesicles were all derived from plasma.

290

291 ***Non-alcoholic fatty liver disease***

292 Non-invasive tools to assess steatohepatitis and fibrosis in non-alcoholic fatty liver
293 disease (NAFLD) do not entirely reflect the variety of liver histological changes in these
294 patients.[81] Extracellular vesicles that reflect ongoing cell damage might be able to fill this
295 gap and better capture the disease's complexity. Supplementary Table 1 summarizes
296 extracellular sub-populations as potential biomarkers in NAFLD.[2,82–89]

297

298 ***Diagnosis of non-alcoholic fatty liver disease***

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

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

305 A pattern of 12 miRNAs carried by serum extracellular vesicles could discriminate 12
306 patients with non-alcoholic steatohepatitis from patients with chronic hepatitis B and C
307 infections and from healthy controls, although limited access to microarrays in clinical
308 routines limits its applicability.[84]

309

310 *Marker of activity and fibrosis*

311 Regarding liver NAFLD activity, extracellular vesicles originating from NK T cells
312 and monocytes (CD14+) have been shown to correlate with histological grade and with NAS
313 score and ALT, respectively, unlike CD4+ and CD8+ T cell sub-populations.[82] Two lipidic
314 extracellular vesicle markers can help to differentiate patients with non-alcoholic
315 steatohepatitis from those with simple steatosis and from obese patients without NAFLD, but
316 use of mass spectrometry limits their applicability in clinical settings.[83]

317 Regarding fibrosis, none of the previously cited extracellular vesicle sub-populations
318 significantly correlated with histological fibrosis stage. On the other hand, a pilot study found
319 that CD14+ (monocyte) and CD16+ (leukocyte) extracellular vesicles decreased with fibrosis
320 stage.[88] These different results can be explained by the use of different extracellular vesicle
321 separation methods, as well as the inclusion of patients with different NASH severity.

322 Two miRNAs contained in serum extracellular vesicles, miRNA-122, a major hepatic
323 miRNA, and miRNA-192, were found to increase with activity and fibrosis stage in several
324 studies.[85–87] However, these findings should be interpreted with caution since fewer than
325 10 patients were included in each study, and since, in one study, the extracellular vesicle
326 fraction of miRNA-122 was not specifically studied.

327

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

332

333 ***Alcoholic hepatitis***

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

336

337 *Diagnosis of alcoholic hepatitis*

338 Our group tested whether plasma extracellular vesicle levels could diagnose alcoholic
339 hepatitis non-invasively. In two prospective cohorts of 83 and 68 patients with clinical
340 suspicion of alcoholic hepatitis, we observed that plasma hepatocyte (cytokeratin-18+)
341 extracellular vesicle levels were able to diagnose alcoholic hepatitis with good sensitivity
342 (76%) and specificity (81%). Yet, total soluble cytokeratin-18 performed even better and can
343 be measured more easily.[41] Higher levels of hepatocyte-derived (ASPGR+) extracellular
344 vesicles have also been found in 101 patients with severe alcoholic hepatitis, in comparison
345 with healthy controls.[90]

346 Plasma concentrations of total extracellular vesicles, as well as of extracellular
347 vesicles derived from T cells (plasma and serum), macrophages, neutrophils, hematopoietic
348 stem cells and endothelial cells are found to be higher among patients with severe alcoholic
349 hepatitis than among healthy controls.[90,91] However, these changes might simply reflect
350 the consequences of alcohol intake and not alcoholic hepatitis. Indeed, our group measured
351 plasma extracellular vesicles derived from endothelial cells (CD62e+; CD41-/31+), platelets
352 (CD41+), and leukocytes (CD11a+) in patients with clinical suspicion of alcoholic hepatitis

353 and did not observe any difference between patients with and without histologically proven
354 alcoholic hepatitis.[41]

355 Along the same lines, plasma and serum concentrations of several sub-populations of
356 extracellular vesicles (carrying mitochondrial DNA, miRNA or hepatocyte proteins) are
357 higher in patients with binge drinking and/or chronic excessive alcohol consumption, as
358 summarized in Supplementary Table 2.[41,90–103] Their potential utility for diagnosis of
359 alcoholic hepatitis should be tested in patients with a clinical suspicion of alcoholic hepatitis.

360

361 *Prediction of outcome in alcoholic hepatitis*

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

371

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

375

376 *Hepatitis B and C virus infections*

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

382

383 *Cirrhosis*

384 Different extracellular vesicle sub-populations increase in patients with cirrhosis, and
385 are predictive of disease progression, complications and mortality (Supplementary Table
386 5).[39,40,47,50,100,123–135] Doubt remains, however, on whether this increase is due to
387 excess production, decreased clearance, or, most likely, both.[10]

388

389 *Estimation of cirrhosis severity*

390 Hepatocyte extracellular vesicle concentrations, characterized by cytokeratin-18
391 expression, significantly increased with Child-Pugh score in 3 independent prospective
392 cohorts of 40, 139 and 103 patients.[39,40] Their concentrations also correlated with Model
393 for End-Stage Liver Disease (MELD) and its components.[40] In the same study, hepatocyte
394 larger extracellular vesicle concentrations increased with histological liver necro-
395 inflammatory activity, suggesting that hepatocyte injury is the driver for extracellular vesicle
396 release.[40]

397

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

402 endothelial-derived extracellular vesicles (CD31+/41-, CD31+/42b- or CD62e) than healthy
403 individuals. The link with cirrhosis severity (Child-Pugh score or MELD) has been observed
404 with plasma leuko-endothelial (CD31+/41-) larger extracellular vesicles in an initial cohort of
405 91 patients[39], but was subsequently not confirmed using the same markers (CD31+/41-)[40]
406 or other markers (CD31+/42b-).[125]

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

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

420 Pilot studies, described in Supplementary Table 5, have identified several plasma and
421 serum miRNAs that are up- or down-regulated during advanced cirrhosis, but the association
422 with cirrhosis severity has not been investigated.[39,40,47,50,100,123–135] Furthermore,
423 validation is needed, given the small sample sizes and other methodological limitations.

424

425 *Estimation of portal hypertension:*

426 For the time being, no extracellular vesicle sub-population has been found to reliably
427 estimate portal hypertension. Hepatocyte cytokeratin-18 positive extracellular vesicles weakly
428 correlated with hepatic venous pressure gradients (HVPG), but could not discriminate patients
429 having a HVPG ≥ 10 mmHg.[40]

430

431 *Prediction of complications of cirrhosis*

432 The procoagulant activity of some extracellular vesicles could play a role in portal
433 vein thrombosis. Few studies have evaluated the capacity of extracellular vesicles for
434 predicting portal vein thrombosis as well as other complications of cirrhosis, including
435 ascites, hepatic encephalopathy, hepatopulmonary and hepatorenal syndromes. These studies
436 are discussed in the Supplementary Text.[39,47,125,126,135,138–142]

437

438 *Prediction of mortality*

439 In a prospective collaborative study, including a validation cohort, we demonstrated
440 that plasma hepatocyte-derived extracellular vesicle levels can predict 6-month mortality,
441 independently of Child-Pugh and MELD scores, using a threshold of 65 UI/L.[40] In this
442 study, other sub-populations of plasma extracellular vesicles, including pan-leukocyte
443 (CD11+) and endothelial (CD144+, CD62E+), were not useful for predicting mortality. Only
444 small leuko-endothelial (CD31+/41-) extracellular vesicles also predicted 6-month mortality
445 independently of Child-Pugh score, in two studies.[39,40] The potential interest of ascites
446 extracellular vesicles has been investigated in 163 patients with cirrhosis.[50] The study
447 found that low total ascites extracellular vesicle levels predicted 30-day mortality
448 independently of MELD and antibiotic treatment. High percentages of ascites extracellular
449 vesicles derived from neutrophil (CD66b+) or lymphocyte (CD3) were more commonly

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

452

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

457

458 ***Hepatocellular carcinoma***

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

465

466 *Detection of hepatocellular carcinoma*

467 A major challenge is to find markers facilitating early detection of HCC, among
468 patients with chronic liver diseases. Ideally, studies should include patients with early HCC
469 and use as controls patients with chronic liver diseases similar to those with HCC, but this
470 study design has rarely been applied.[106,123,124,126,143–154]

471 Total extracellular vesicles, as well as some sub-populations, increase in the
472 plasma[124] and sera[123] of patients with HCC, and their concentrations normalize after
473 liver transplantation or R0 hepatectomy, which suggests a direct relationship with tumor
474 presence. In a pilot study, 8 patients with HCC induced by hepatitis C-related cirrhosis had

475 higher total, hepatic (HepPar+) and endothelial (CD144+) extracellular vesicles than 5
476 patients without liver disease.[124] This result on total serum extracellular vesicles
477 concentrations was confirmed in a larger study, and a threshold was establish to discriminate
478 patients with early HCC from patients with cirrhosis alone.[123] A large study assessed
479 concentrations of serum extracellular vesicles derived from tumor cells
480 (AnnexinV+EpCAM+ASPGR1+) and showed that this sub-population could discriminate 86
481 patients with HCC from 49 patients with tumor-free cirrhotic liver (3-fold increase, AUROC
482 0.73).[143]

483 Expressions of several non-coding RNA contained in extracellular vesicles are
484 modified in patients with HCC. A meta-analysis calculated that when pooling 30 small
485 extracellular vesicle miRNA assays, diagnosis of HCC reached a sensitivity of 80% and a
486 specificity of 81%.[155] Some miRNAs are of particular interest. miRNA-21 had a higher
487 expression in circulating serum extracellular vesicles of patients with HCC (n = 30), than in
488 patients with chronic hepatitis B virus infection without cirrhosis and healthy controls (n = 30
489 per group).[146] These results have been confirmed by another study.[156] Yet, it is unclear
490 whether HCC was the driver for circulating miRNA-21 expression, or whether cirrhosis *per*
491 *se*, was more prevalent among the HCC group and was responsible for higher circulating
492 miRNA-21 expression levels in this group. miRNA-122 contained in circulating serum
493 extracellular vesicles had decreased expression levels in 2 studies, and could even
494 differentiate early HCC from cirrhosis.[145,157] Its expression increased after transarterial
495 chemoembolization.[158] Using quantification of 2 serum long non-coding RNAs (lncRNAs)
496 carried in extracellular vesicles, 60 patients with HCC could be discriminated from 60
497 patients with chronic hepatitis B in two cohorts.[147] In this study, a combination of the two
498 RNAs and alpha-fetoprotein yielded a sensitivity of 84% and specificity of 88%, better than

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

501 Other circulating extracellular vesicle sub-populations have been tested, but use of
502 low-throughput measurement techniques, absence of precise description of measurement
503 methods or patient characteristics, small sample sizes, use of healthy controls or advanced
504 HCC, or absence of confirmation on whether the marker truly circulates in the extracellular
505 vesicle fraction limits interpretation of the results (Supplementary Table
506 6).[123,124,126,131,133,143–154,156–190]

507

508 *Predictor of recurrence and survival*

509 Extracellular vesicles could help predict HCC recurrence after liver resection. High
510 levels of pre-operative plasma small extracellular vesicle miRNA-155 were predictive of
511 lower relapse-free survival in a retrospective study of 40 patients with mainly unifocal HCC
512 treated with 1st line curative hepatectomy, although no difference in 3- and 5-year overall
513 survival was found.[120] In another study, recurrence was higher when serum extracellular
514 vesicle miRNA-125 expression was low among 128 patients with HCC treated by surgical
515 resection, in multivariate analysis.[150]

516 Circulating extracellular vesicle RNA expression can also predict overall survival. For
517 example, higher levels of serum small extracellular vesicle RNA RAB11A was associated
518 with lower relapse-free survival in multivariate analysis among 60 patients with mainly early
519 HCC.[152] Several studies have found serum small extracellular vesicle miRNA-21 to be a
520 potentially interesting marker of outcome.[156,191,192] Notably, a prospective study of 79
521 patients with HCC observed that serum small extracellular vesicle miRNA-21 expression was
522 independently associated with overall survival and disease progression.[191]

523 To conclude, many proteins and RNAs contained in extracellular vesicles have been
524 identified as predictors of outcome among patients with HCC (Supplementary Tables 7 and
525 8).[119,120,144,147,148,150,152,156–159,167,169,171,179,183,184,186,191–199] These
526 findings should nevertheless be confirmed in patients with all stages of HCC progression,
527 taking into account confounding factors such as comorbidities and underlying liver disease.

528

529 ***Primary sclerosing cholangitis & cholangiocarcinoma***

530 Major challenges in clinical management of patients with primary sclerosing
531 cholangitis include the unpredictable outcome of this liver disease in terms of progression and
532 evolution towards cholangiocarcinoma.[200] Data on serum extracellular vesicles as markers
533 in this setting are limited to a proteomic analysis of serum extracellular vesicles: 10 proteins
534 were found to be differentially expressed in patients with cholangiocarcinoma (n = 43), as
535 compared with patients with primary sclerosing cholangitis (n = 30), as well as with HCC (n
536 = 29).[176] Western blot analyses confirmed these results for 6 proteins, but clinical
537 validation is needed. One team has also analyzed both serum and urine extracellular vesicles,
538 and found different RNA transcriptomic profiles between patients with cholangiocarcinoma
539 (n = 12) and those with primary sclerosing cholangitis (n = 6), although, here again, clinical
540 confirmation is needed.[201]

541

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

544 Two independent groups demonstrated that bile or serum extracellular vesicles could
545 efficiently differentiate cholangiocarcinoma from benign biliary stenosis. One group reported
546 that total bile and serum extracellular vesicles, measured using nanoparticle tracking analysis,
547 could distinguish malignant biliary stenosis (pancreatic cancer and cholangiocarcinoma) from

548 benign stenosis (chronic pancreatitis), bile extracellular vesicles having a significantly better
549 discriminating ability than serum CA19-9.[52] Others have observed an overexpression of an
550 small extracellular vesicle miRNA panel in bile extracellular vesicles from 26 patients with
551 cholangiocarcinoma, as compared with 50 controls with benign stenosis (including 13
552 primary sclerosing cholangitis).[51]

553

554 Beyond the setting of biliary strictures, other studies have analyzed extracellular
555 vesicles in patients with cholangiocarcinoma. One study observed that circulating tumor cell-
556 derived (AnnexinV+EpCAM+ASGPR1+) extracellular vesicles were 3-fold higher in the
557 serum of 38 patients with cholangiocarcinoma than in 49 patients with cirrhosis.[143] These
558 tumor cell-derived extracellular vesicles were, however, unable to differentiate HCC from
559 cholangiocarcinoma.[143] A second study identified 2 small extracellular vesicle lncRNAs
560 that were significantly overexpressed in the bile of 35 patients with cholangiocarcinoma, as
561 compared with 56 healthy controls.[54] Their combination yielded higher sensitivity and
562 specificity than CA19-9, and was associated with lower overall survival. A third study
563 compared the expression of small extracellular vesicle miRNA-200c-3p between 36 patients
564 with cholangiocarcinoma and 12 healthy controls.[202] Expression of small extracellular
565 vesicle miRNA-200c-3p was higher among patients with cholangiocarcinoma, and increased
566 with tumor stage and the presence of lymph node metastasis. Patients with higher levels of
567 miRNA-200c-3p had lower disease-free and overall-free survivals.[202] Two other preclinical
568 studies found differentially expressed extracellular vesicle RNAs between patients with
569 cholangiocarcinoma and healthy controls.[203,204]

570 To conclude, several sub-populations of extracellular vesicles have the potential to
571 become very useful tools to differentiate cholangiocarcinoma from PSC and other tumors.
572 The use of demanding techniques to measure extracellular vesicles would likely not be a

573 limitation in this setting since these situations are not common and patients are usually
574 referred to expert centers.

575

576 *Acute liver failure*

577 *Detection of acute liver failure*

578 Diagnosis of acute liver injury/failure is usually easy, as it is based on readily
579 available clinical and laboratory data.[205] In contrast, prediction or early identification of
580 drug-induced liver injury remains an unmet need. Since extracellular vesicles are sensitive
581 markers of cell injury, we can hypothesize that their circulating levels could be an early
582 marker of drug-induced liver injury, even before liver blood test abnormalities appear. So far,
583 data on this hypothesis are very limited. Most studies compared patients with acute liver
584 injury with healthy controls, thereby limiting their clinical relevance (Supplementary Table
585 9).[206–209]

586

587 *Predictor of outcome*

588 The interest of extracellular vesicles in the acute liver injury/acute liver failure setting
589 has been analyzed by the “Acute Liver Failure Study Group” in a group of 50 patients.
590 Plasma concentrations of total larger-size extracellular vesicles significantly increased with
591 hepatic encephalopathy grade, as well as with systemic inflammatory response
592 syndrome.[206] Moreover, concentrations of these extracellular vesicles were associated with
593 death or liver transplantation at day-21 after admission, independently of other predictors
594 (age, gender, and etiology).[206] Extracellular vesicles were mainly of platelet (CD41+)
595 origin, but also of hepatocyte (ASPGR+), monocyte (CD18+) and endothelial cell (CD144+)
596 origin and were tissue-factor positive, although each specific sub-population was not
597 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. Fourth, 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.

628

Acknowledgments.

629

We thank Marion Tanguy for providing images and for her helpful advice and Camille

630

Florimond for superb technical assistance. We thank Kathryn Gordon for her thorough

631

English re-reading of the paper.

632 **References**

- 633 [1] van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular
634 vesicles. *Nat Rev Mol Cell Biol* 2018;19:213–28.
635 <https://doi.org/10.1038/nrm.2017.125>.
- 636 [2] Garcia-Martinez I, Santoro N, Chen Y, Hoque R, Ouyang X, Caprio S, et al.
637 Hepatocyte mitochondrial DNA drives nonalcoholic steatohepatitis by activation of
638 TLR9. *J Clin Invest* 2016;126:859–64. <https://doi.org/10.1172/JCI83885>.
- 639 [3] Puhm F, Afonyushkin T, Resch U, Obermayer G, Rohde M, Penz T, et al.
640 Mitochondria Are a Subset of Extracellular Vesicles Released by Activated Monocytes
641 and Induce Type I IFN and TNF Responses in Endothelial Cells. *Circ Res*
642 2019;125:43–52. <https://doi.org/10.1161/CIRCRESAHA.118.314601>.
- 643 [4] Ridger VC, Boulanger CM, Angelillo-Scherrer A, Badimon L, Blanc-Brude O,
644 Bochaton-Piallat M-L, et al. Microvesicles in vascular homeostasis and diseases.
645 *Thromb Haemost* 2017;117:1296–316. <https://doi.org/10.1160/TH16-12-0943>.
- 646 [5] Doyle LM, Wang MZ. Overview of Extracellular Vesicles, Their Origin, Composition,
647 Purpose, and Methods for Exosome Isolation and Analysis. *Cells* 2019;8.
648 <https://doi.org/10.3390/cells8070727>.
- 649 [6] van der Pol E, Böing AN, Gool EL, Nieuwland R. Recent developments in the
650 nomenclature, presence, isolation, detection and clinical impact of extracellular
651 vesicles. *J Thromb Haemost* 2016;14:48–56. <https://doi.org/10.1111/jth.13190>.
- 652 [7] Mateescu B, Kowal EJK, van Balkom BWM, Bartel S, Bhattacharyya SN, Buzás EI, et
653 al. Obstacles and opportunities in the functional analysis of extracellular vesicle RNA -
654 an ISEV position paper. *J Extracell Vesicles* 2017;6:1286095.
655 <https://doi.org/10.1080/20013078.2017.1286095>.

- 656 [8] Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al.
657 Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a
658 position statement of the International Society for Extracellular Vesicles and update of
659 the MISEV2014 guidelines. *J Extracell Vesicles* 2018;7:1535750.
660 <https://doi.org/10.1080/20013078.2018.1535750>.
- 661 [9] Szabo G, Momen-Heravi F. Extracellular vesicles in liver disease and potential as
662 biomarkers and therapeutic targets. *Nature Reviews Gastroenterology & Hepatology*
663 2017;14:455–66. <https://doi.org/10.1038/nrgastro.2017.71>.
- 664 [10] Lemoine S, Thabut D, Housset C, Moreau R, Valla D, Boulanger CM, et al. The
665 emerging roles of microvesicles in liver diseases. *Nat Rev Gastroenterol Hepatol*
666 2014;11:350–61. <https://doi.org/10.1038/nrgastro.2014.7>.
- 667 [11] Coumans FAW, Brisson AR, Buzas EI, Dignat-George F, Drees EEE, El-Andaloussi S,
668 et al. Methodological Guidelines to Study Extracellular Vesicles. *Circ Res*
669 2017;120:1632–48. <https://doi.org/10.1161/CIRCRESAHA.117.309417>.
- 670 [12] Witwer KW, Buzás EI, Bemis LT, Bora A, Lässer C, Lötvall J, et al. Standardization of
671 sample collection, isolation and analysis methods in extracellular vesicle research. *J*
672 *Extracell Vesicles* 2013;2. <https://doi.org/10.3402/jev.v2i0.20360>.
- 673 [13] Clayton A, Buschmann D, Byrd JB, Carter DRF, Cheng L, Compton C, et al. Summary
674 of the ISEV workshop on extracellular vesicles as disease biomarkers, held in
675 Birmingham, UK, during December 2017. *J Extracell Vesicles* 2018;7:1473707.
676 <https://doi.org/10.1080/20013078.2018.1473707>.
- 677 [14] Lippi G, Fontana R, Avanzini P, Aloe R, Ippolito L, Sandei F, et al. Influence of
678 mechanical trauma of blood and hemolysis on PFA-100 testing. *Blood Coagul*
679 *Fibrinolysis* 2012;23:82–6. <https://doi.org/10.1097/MBC.0b013e32834c6cb5>.

- 680 [15] Cheng HH, Yi HS, Kim Y, Kroh EM, Chien JW, Eaton KD, et al. Plasma processing
681 conditions substantially influence circulating microRNA biomarker levels. *PLoS ONE*
682 2013;8:e64795. <https://doi.org/10.1371/journal.pone.0064795>.
- 683 [16] Mani H, Kirchmayr K, Kläffling C, Schindewolf M, Luxembourg B, Linnemann B, et
684 al. Influence of blood collection techniques on platelet function. *Platelets* 2004;15:315–
685 8. <https://doi.org/10.1080/09537100410001711505>.
- 686 [17] Yuana Y, Böing AN, Grootemaat AE, van der Pol E, Hau CM, Cizmar P, et al.
687 Handling and storage of human body fluids for analysis of extracellular vesicles. *J*
688 *Extracell Vesicles* 2015;4:29260. <https://doi.org/10.3402/jev.v4.29260>.
- 689 [18] Bæk R, Søndergaard EKL, Varming K, Jørgensen MM. The impact of various
690 preanalytical treatments on the phenotype of small extracellular vesicles in blood
691 analyzed by protein microarray. *J Immunol Methods* 2016;438:11–20.
692 <https://doi.org/10.1016/j.jim.2016.08.007>.
- 693 [19] EV-TRACK Consortium, Van Deun J, Mestdagh P, Agostinis P, Akay Ö, Anand S, et
694 al. EV-TRACK: transparent reporting and centralizing knowledge in extracellular
695 vesicle research. *Nat Methods* 2017;14:228–32. <https://doi.org/10.1038/nmeth.4185>.
- 696 [20] Wisgrill L, Lamm C, Hartmann J, Preißing F, Dragosits K, Bee A, et al. Peripheral
697 blood microvesicles secretion is influenced by storage time, temperature, and
698 anticoagulants. *Cytometry A* 2016;89:663–72. <https://doi.org/10.1002/cyto.a.22892>.
- 699 [21] Lacroix R, Judicone C, Mooberry M, Boucekine M, Key NS, Dignat-George F, et al.
700 Standardization of pre-analytical variables in plasma microparticle determination:
701 results of the International Society on Thrombosis and Haemostasis SSC Collaborative
702 workshop. *J Thromb Haemost* 2013. <https://doi.org/10.1111/jth.12207>.

- 703 [22] Jayachandran M, Miller VM, Heit JA, Owen WG. Methodology for isolation,
704 identification and characterization of microvesicles in peripheral blood. *J Immunol*
705 *Methods* 2012;375:207–14. <https://doi.org/10.1016/j.jim.2011.10.012>.
- 706 [23] György B, Pálóczi K, Kovács A, Barabás E, Bekő G, Várnai K, et al. Improved
707 circulating microparticle analysis in acid-citrate dextrose (ACD) anticoagulant tube.
708 *Thromb Res* 2014;133:285–92. <https://doi.org/10.1016/j.thromres.2013.11.010>.
- 709 [24] Andreu Z, Rivas E, Sanguino-Pascual A, Lamana A, Marazuela M, González-Alvaro I,
710 et al. Comparative analysis of EV isolation procedures for miRNAs detection in serum
711 samples. *J Extracell Vesicles* 2016;5:31655. <https://doi.org/10.3402/jev.v5.31655>.
- 712 [25] Gardiner C, Di Vizio D, Sahoo S, Théry C, Witwer KW, Wauben M, et al. Techniques
713 used for the isolation and characterization of extracellular vesicles: results of a
714 worldwide survey. *J Extracell Vesicles* 2016;5:32945.
715 <https://doi.org/10.3402/jev.v5.32945>.
- 716 [26] Mol EA, Goumans M-J, Doevendans PA, Sluijter JPG, Vader P. Higher functionality
717 of extracellular vesicles isolated using size-exclusion chromatography compared to
718 ultracentrifugation. *Nanomedicine* 2017;13:2061–5.
719 <https://doi.org/10.1016/j.nano.2017.03.011>.
- 720 [27] Cheng L, Sharples RA, Scicluna BJ, Hill AF. Exosomes provide a protective and
721 enriched source of miRNA for biomarker profiling compared to intracellular and cell-
722 free blood. *J Extracell Vesicles* 2014;3. <https://doi.org/10.3402/jev.v3.23743>.
- 723 [28] Jeppesen DK, Fenix AM, Franklin JL, Higginbotham JN, Zhang Q, Zimmerman LJ, et
724 al. Reassessment of Exosome Composition. *Cell* 2019;177:428-445.e18.
725 <https://doi.org/10.1016/j.cell.2019.02.029>.

- 726 [29] Van Deun J, Mestdagh P, Sormunen R, Cocquyt V, Vermaelen K, Vandesompele J, et
727 al. The impact of disparate isolation methods for extracellular vesicles on downstream
728 RNA profiling. *J Extracell Vesicles* 2014;3. <https://doi.org/10.3402/jev.v3.24858>.
- 729 [30] Hill AF, Pegtel DM, Lambertz U, Leonardi T, O’Driscoll L, Pluchino S, et al. ISEV
730 position paper: extracellular vesicle RNA analysis and bioinformatics. *J Extracell*
731 *Vesicles* 2013;2. <https://doi.org/10.3402/jev.v2i0.22859>.
- 732 [31] Takov K, Yellon DM, Davidson SM. Comparison of small extracellular vesicles
733 isolated from plasma by ultracentrifugation or size-exclusion chromatography: yield,
734 purity and functional potential. *J Extracell Vesicles* 2019;8:1560809.
735 <https://doi.org/10.1080/20013078.2018.1560809>.
- 736 [32] Webber J, Clayton A. How pure are your vesicles? *J Extracell Vesicles* 2013;2.
737 <https://doi.org/10.3402/jev.v2i0.19861>.
- 738 [33] Momen-Heravi F, Balaj L, Alian S, Mantel P-Y, Halleck AE, Trachtenberg AJ, et al.
739 Current methods for the isolation of extracellular vesicles. *Biol Chem* 2013;394:1253–
740 62. <https://doi.org/10.1515/hsz-2013-0141>.
- 741 [34] Taylor DD, Shah S. Methods of isolating extracellular vesicles impact down-stream
742 analyses of their cargoes. *Methods* 2015;87:3–10.
743 <https://doi.org/10.1016/j.ymeth.2015.02.019>.
- 744 [35] Stranska R, Gysbrechts L, Wouters J, Vermeersch P, Bloch K, Dierickx D, et al.
745 Comparison of membrane affinity-based method with size-exclusion chromatography
746 for isolation of exosome-like vesicles from human plasma. *J Transl Med* 2018;16:1.
747 <https://doi.org/10.1186/s12967-017-1374-6>.
- 748 [36] Momen-Heravi F, Balaj L, Alian S, Trachtenberg AJ, Hochberg FH, Skog J, et al.
749 Impact of biofluid viscosity on size and sedimentation efficiency of the isolated
750 microvesicles. *Front Physiol* 2012;3:162. <https://doi.org/10.3389/fphys.2012.00162>.

- 751 [37] Baranyai T, Herczeg K, Onódi Z, Voszka I, Módos K, Marton N, et al. Isolation of
752 Exosomes from Blood Plasma: Qualitative and Quantitative Comparison of
753 Ultracentrifugation and Size Exclusion Chromatography Methods. PLoS ONE
754 2015;10:e0145686. <https://doi.org/10.1371/journal.pone.0145686>.
- 755 [38] Vergauwen G, Dhondt B, Van Deun J, De Smedt E, Berx G, Timmerman E, et al.
756 Confounding factors of ultrafiltration and protein analysis in extracellular vesicle
757 research. Sci Rep 2017;7:2704. <https://doi.org/10.1038/s41598-017-02599-y>.
- 758 [39] Rautou P-E, Bresson J, Sainte-Marie Y, Vion A-C, Paradis V, Renard J-M, et al.
759 Abnormal plasma microparticles impair vasoconstrictor responses in patients with
760 cirrhosis. Gastroenterology 2012;143:166-176.e6.
761 <https://doi.org/10.1053/j.gastro.2012.03.040>.
- 762 [40] Payancé A, Silva-Junior G, Bissonnette J, Tanguy M, Pasquet B, Levi C, et al.
763 Hepatocyte microvesicle levels improve prediction of mortality in patients with
764 cirrhosis. Hepatology 2018;68:1508–18. <https://doi.org/10.1002/hep.29903>.
- 765 [41] Bissonnette J, Altamirano J, Devue C, Roux O, Payancé A, Lebrech D, et al. A
766 prospective study of the utility of plasma biomarkers to diagnose alcoholic hepatitis.
767 Hepatology 2017;66:555–63. <https://doi.org/10.1002/hep.29080>.
- 768 [42] Enderle D, Spiel A, Coticchia CM, Berghoff E, Mueller R, Schlumpberger M, et al.
769 Characterization of RNA from Exosomes and Other Extracellular Vesicles Isolated by
770 a Novel Spin Column-Based Method. PLoS ONE 2015;10:e0136133.
771 <https://doi.org/10.1371/journal.pone.0136133>.
- 772 [43] Böing AN, van der Pol E, Grootemaat AE, Coumans FAW, Sturk A, Nieuwland R.
773 Single-step isolation of extracellular vesicles by size-exclusion chromatography. J
774 Extracell Vesicles 2014;3. <https://doi.org/10.3402/jev.v3.23430>.

- 775 [44] Hong C-S, Funk S, Muller L, Boyiadzis M, Whiteside TL. Isolation of biologically
776 active and morphologically intact exosomes from plasma of patients with cancer. *J*
777 *Extracell Vesicles* 2016;5:29289. <https://doi.org/10.3402/jev.v5.29289>.
- 778 [45] George JN, Thoi LL, McManus LM, Reimann TA. Isolation of human platelet
779 membrane microparticles from plasma and serum. *Blood* 1982;60:834–40.
- 780 [46] Pisitkun T, Shen R-F, Knepper MA. Identification and proteomic profiling of exosomes
781 in human urine. *Proc Natl Acad Sci USA* 2004;101:13368–73.
782 <https://doi.org/10.1073/pnas.0403453101>.
- 783 [47] Awdishu L, Tsunoda S, Pearlman M, Kokoy-Mondragon C, Ghassemian M, Naviaux
784 RK, et al. Identification of Maltase Glucoamylase as a Biomarker of Acute Kidney
785 Injury in Patients with Cirrhosis. *Crit Care Res Pract* 2019;2019:5912804.
786 <https://doi.org/10.1155/2019/5912804>.
- 787 [48] Merchant ML, Rood IM, Deegens JKJ, Klein JB. Isolation and characterization of
788 urinary extracellular vesicles: implications for biomarker discovery. *Nat Rev Nephrol*
789 2017;13:731–49. <https://doi.org/10.1038/nrneph.2017.148>.
- 790 [49] Sirica AE, Gores GJ, Groopman JD, Selaru FM, Strazzabosco M, Wei Wang X, et al.
791 Intrahepatic Cholangiocarcinoma: Continuing Challenges and Translational Advances.
792 *Hepatology* 2019;69:1803–15. <https://doi.org/10.1002/hep.30289>.
- 793 [50] Engelmann C, Splith K, Krohn S, Herber A, Boehlig A, Boehm S, et al. Absolute
794 quantification of microparticles by flow cytometry in ascites of patients with
795 decompensated cirrhosis: a cohort study. *J Transl Med* 2017;15:188.
796 <https://doi.org/10.1186/s12967-017-1288-3>.
- 797 [51] Li L, Masica D, Ishida M, Tomuleasa C, Umegaki S, Kalloo AN, et al. Human bile
798 contains microRNA-laden extracellular vesicles that can be used for

- 799 cholangiocarcinoma diagnosis. *Hepatology* 2014;60:896–907.
800 <https://doi.org/10.1002/hep.27050>.
- 801 [52] Severino V, Dumonceau J-M, Delhaye M, Moll S, Annessi-Ramseyer I, Robin X, et al.
802 Extracellular Vesicles in Bile as Markers of Malignant Biliary Stenoses.
803 *Gastroenterology* 2017;153:495-504.e8. <https://doi.org/10.1053/j.gastro.2017.04.043>.
- 804 [53] Li L, Piontek KB, Kumbhari V, Ishida M, Selaru FM. Isolation and Profiling of
805 MicroRNA-containing Exosomes from Human Bile. *J Vis Exp* 2016.
806 <https://doi.org/10.3791/54036>.
- 807 [54] Ge X, Wang Y, Nie J, Li Q, Tang L, Deng X, et al. The diagnostic/prognostic potential
808 and molecular functions of long non-coding RNAs in the exosomes derived from the
809 bile of human cholangiocarcinoma. *Oncotarget* 2017;8:69995–70005.
810 <https://doi.org/10.18632/oncotarget.19547>.
- 811 [55] Hogan MC, Lieske JC, Lienczewski CC, Nesbitt LL, Wickman LT, Heyer CM, et al.
812 Strategy and rationale for urine collection protocols employed in the NEPTUNE study.
813 *BMC Nephrol* 2015;16:190. <https://doi.org/10.1186/s12882-015-0185-3>.
- 814 [56] Arraud N, Linares R, Tan S, Gounou C, Pasquet J-M, Mornet S, et al. Extracellular
815 vesicles from blood plasma: determination of their morphology, size, phenotype and
816 concentration. *J Thromb Haemost* 2014;12:614–27. <https://doi.org/10.1111/jth.12554>.
- 817 [57] György B, Módos K, Pállinger E, Pálóczi K, Pásztói M, Misják P, et al. Detection and
818 isolation of cell-derived microparticles are compromised by protein complexes
819 resulting from shared biophysical parameters. *Blood* 2011;117:e39-48.
820 <https://doi.org/10.1182/blood-2010-09-307595>.
- 821 [58] Takov K, Yellon DM, Davidson SM. Confounding factors in vesicle uptake studies
822 using fluorescent lipophilic membrane dyes. *J Extracell Vesicles* 2017;6:1388731.
823 <https://doi.org/10.1080/20013078.2017.1388731>.

- 824 [59] Sódar BW, Kittel Á, Pálóczi K, Vukman KV, Osteikoetxea X, Szabó-Taylor K, et al.
825 Low-density lipoprotein mimics blood plasma-derived exosomes and microvesicles
826 during isolation and detection. *Sci Rep* 2016;6:24316.
827 <https://doi.org/10.1038/srep24316>.
- 828 [60] Larson MC, Luthi MR, Hogg N, Hillery CA. Calcium-phosphate microprecipitates
829 mimic microparticles when examined with flow cytometry. *Cytometry A* 2013;83:242–
830 50. <https://doi.org/10.1002/cyto.a.22222>.
- 831 [61] van der Pol E, Hoekstra AG, Sturk A, Otto C, van Leeuwen TG, Nieuwland R. Optical
832 and non-optical methods for detection and characterization of microparticles and
833 exosomes. *J Thromb Haemost* 2010;8:2596–607. [https://doi.org/10.1111/j.1538-](https://doi.org/10.1111/j.1538-7836.2010.04074.x)
834 [7836.2010.04074.x](https://doi.org/10.1111/j.1538-7836.2010.04074.x).
- 835 [62] Obeid S, Ceroi A, Mourey G, Saas P, Elie-Caille C, Boireau W. Development of a
836 NanoBioAnalytical platform for “on-chip” qualification and quantification of platelet-
837 derived microparticles. *Biosens Bioelectron* 2017;93:250–9.
838 <https://doi.org/10.1016/j.bios.2016.08.100>.
- 839 [63] Liang K, Liu F, Fan J, Sun D, Liu C, Lyon CJ, et al. Nanoplasmonic Quantification of
840 Tumor-derived Extracellular Vesicles in Plasma Microsamples for Diagnosis and
841 Treatment Monitoring. *Nat Biomed Eng* 2017;1. [https://doi.org/10.1038/s41551-016-](https://doi.org/10.1038/s41551-016-0021-0021)
842 [0021](https://doi.org/10.1038/s41551-016-0021-0021).
- 843 [64] Koliha N, Wiencek Y, Heider U, Jüngst C, Kladt N, Krauthäuser S, et al. A novel
844 multiplex bead-based platform highlights the diversity of extracellular vesicles. *J*
845 *Extracell Vesicles* 2016;5:29975. <https://doi.org/10.3402/jev.v5.29975>.
- 846 [65] Corso G, Mäger I, Lee Y, Görgens A, Bultema J, Giebel B, et al. Reproducible and
847 scalable purification of extracellular vesicles using combined bind-elute and size

- 848 exclusion chromatography. *Sci Rep* 2017;7:11561. [https://doi.org/10.1038/s41598-017-](https://doi.org/10.1038/s41598-017-10646-x)
- 849 10646-x.
- 850 [66] Gool EL, Stojanovic I, Schasfoort RBM, Sturk A, van Leeuwen TG, Nieuwland R, et
- 851 al. Surface Plasmon Resonance is an Analytically Sensitive Method for Antigen
- 852 Profiling of Extracellular Vesicles. *Clin Chem* 2017;63:1633–41.
- 853 <https://doi.org/10.1373/clinchem.2016.271049>.
- 854 [67] Jørgensen MM, Bæk R, Varming K. Potentials and capabilities of the Extracellular
- 855 Vesicle (EV) Array. *J Extracell Vesicles* 2015;4:26048.
- 856 <https://doi.org/10.3402/jev.v4.26048>.
- 857 [68] Szatanek R, Baj-Krzyworzeka M, Zimoch J, Lekka M, Siedlar M, Baran J. The
- 858 Methods of Choice for Extracellular Vesicles (EVs) Characterization. *Int J Mol Sci*
- 859 2017;18. <https://doi.org/10.3390/ijms18061153>.
- 860 [69] Tatischeff I, Larquet E, Falcón-Pérez JM, Turpin P-Y, Kruglik SG. Fast
- 861 characterisation of cell-derived extracellular vesicles by nanoparticles tracking analysis,
- 862 cryo-electron microscopy, and Raman tweezers microspectroscopy. *J Extracell*
- 863 *Vesicles* 2012;1. <https://doi.org/10.3402/jev.v1i0.19179>.
- 864 [70] Wyss R, Grasso L, Wolf C, Grosse W, Demurtas D, Vogel H. Molecular and
- 865 Dimensional Profiling of Highly Purified Extracellular Vesicles by Fluorescence
- 866 Fluctuation Spectroscopy. *Anal Chem* 2014;86:7229–33.
- 867 <https://doi.org/10.1021/ac501801m>.
- 868 [71] Coumans FAW, Gool EL, Nieuwland R. Bulk immunoassays for analysis of
- 869 extracellular vesicles. *Platelets* 2017;28:242–8.
- 870 <https://doi.org/10.1080/09537104.2016.1265926>.

- 871 [72] Ramirez MI, Amorim MG, Gadelha C, Milic I, Welsh JA, Freitas VM, et al. Technical
872 challenges of working with extracellular vesicles. *Nanoscale* 2018;10:881–906.
873 <https://doi.org/10.1039/c7nr08360b>.
- 874 [73] van der Pol E, Sturk A, van Leeuwen T, Nieuwland R, Coumans F, ISTH-SSC-VB
875 Working group. Standardization of extracellular vesicle measurements by flow
876 cytometry through vesicle diameter approximation. *J Thromb Haemost* 2018;16:1236–
877 45. <https://doi.org/10.1111/jth.14009>.
- 878 [74] van der Pol E, van Gemert MJC, Sturk A, Nieuwland R, van Leeuwen TG. Single vs.
879 swarm detection of microparticles and exosomes by flow cytometry. *J Thromb*
880 *Haemost* 2012;10:919–30. <https://doi.org/10.1111/j.1538-7836.2012.04683.x>.
- 881 [75] Groot Kormelink T, Arkesteijn GJA, Nauwelaers FA, van den Engh G, Nolte-'t Hoen
882 ENM, Wauben MHM. Prerequisites for the analysis and sorting of extracellular vesicle
883 subpopulations by high-resolution flow cytometry. *Cytometry A* 2016;89:135–47.
884 <https://doi.org/10.1002/cyto.a.22644>.
- 885 [76] Duijvesz D, Versluis CYL, van der Fels CAM, Vredenburg-van den Berg MS, Leivo J,
886 Peltola MT, et al. Immuno-based detection of extracellular vesicles in urine as
887 diagnostic marker for prostate cancer. *Int J Cancer* 2015;137:2869–78.
888 <https://doi.org/10.1002/ijc.29664>.
- 889 [77] Cointe S, Judicone C, Robert S, Mooberry MJ, Poncelet P, Wauben M, et al.
890 Standardization of microparticle enumeration across different flow cytometry
891 platforms: results of a multicenter collaborative workshop. *J Thromb Haemost*
892 2017;15:187–93. <https://doi.org/10.1111/jth.13514>.
- 893 [78] Lacroix R, Robert S, Poncelet P, Kasthuri RS, Key NS, Dignat-George F, et al.
894 Standardization of platelet-derived microparticle enumeration by flow cytometry with
895 calibrated beads: results of the International Society on Thrombosis and Haemostasis

- 896 SSC Collaborative workshop. *J Thromb Haemost* 2010;8:2571–4.
897 <https://doi.org/10.1111/j.1538-7836.2010.04047.x>.
- 898 [79] Carnell-Morris P, Tannetta D, Siupa A, Hole P, Dragovic R. Analysis of Extracellular
899 Vesicles Using Fluorescence Nanoparticle Tracking Analysis. *Methods Mol Biol*
900 2017;1660:153–73. https://doi.org/10.1007/978-1-4939-7253-1_13.
- 901 [80] Desgeorges A, Hollerweger J, Lassacher T, Rohde E, Helmbrecht C, Gimona M.
902 Differential fluorescence nanoparticle tracking analysis for enumeration of the
903 extracellular vesicle content in mixed particulate solutions. *Methods* 2020.
904 <https://doi.org/10.1016/j.ymeth.2020.02.006>.
- 905 [81] Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, et al. The
906 diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from
907 the American Association for the Study of Liver Diseases. *Hepatology* 2018;67:328–
908 57. <https://doi.org/10.1002/hep.29367>.
- 909 [82] Kornek M, Lynch M, Mehta SH, Lai M, Exley M, Afdhal NH, et al. Circulating
910 microparticles as disease-specific biomarkers of severity of inflammation in patients
911 with hepatitis C or nonalcoholic steatohepatitis. *Gastroenterology* 2012;143:448–58.
912 <https://doi.org/10.1053/j.gastro.2012.04.031>.
- 913 [83] Kakazu E, Mauer AS, Yin M, Malhi H. Hepatocytes release ceramide-enriched pro-
914 inflammatory extracellular vesicles in an IRE1 α -dependent manner. *J Lipid Res*
915 2016;57:233–45. <https://doi.org/10.1194/jlr.M063412>.
- 916 [84] Murakami Y, Toyoda H, Tanahashi T, Tanaka J, Kumada T, Yoshioka Y, et al.
917 Comprehensive miRNA expression analysis in peripheral blood can diagnose liver
918 disease. *PLoS ONE* 2012;7:e48366. <https://doi.org/10.1371/journal.pone.0048366>.

- 919 [85] Lee Y-S, Kim SY, Ko E, Lee J-H, Yi H-S, Yoo YJ, et al. Exosomes derived from
920 palmitic acid-treated hepatocytes induce fibrotic activation of hepatic stellate cells. *Sci*
921 *Rep* 2017;7. <https://doi.org/10.1038/s41598-017-03389-2>.
- 922 [86] Pirola CJ, Fernández Gianotti T, Castaño GO, Mallardi P, San Martino J, Mora
923 Gonzalez Lopez Ledesma M, et al. Circulating microRNA signature in non-alcoholic
924 fatty liver disease: from serum non-coding RNAs to liver histology and disease
925 pathogenesis. *Gut* 2015;64:800–12. <https://doi.org/10.1136/gutjnl-2014-306996>.
- 926 [87] Akuta N, Kawamura Y, Watanabe C, Nishimura A, Okubo M, Mori Y, et al. Impact of
927 sodium glucose cotransporter 2 inhibitor on histological features and glucose
928 metabolism of non-alcoholic fatty liver disease complicated by diabetes mellitus.
929 *Hepatol Res* 2019;49:531–9. <https://doi.org/10.1111/hepr.13304>.
- 930 [88] Welsh JA, Scorletti E, Clough GF, Englyst NA, Byrne CD. Leukocyte extracellular
931 vesicle concentration is inversely associated with liver fibrosis severity in NAFLD. *J*
932 *Leukoc Biol* 2018;104:631–9. <https://doi.org/10.1002/JLB.5A1217-501R>.
- 933 [89] Guo Q, Furuta K, Lucien F, Gutierrez Sanchez LH, Hirsova P, Krishnan A, et al.
934 Integrin β 1-enriched extracellular vesicles mediate monocyte adhesion and promote
935 liver inflammation in murine NASH. *J Hepatol* 2019;71:1193–205.
936 <https://doi.org/10.1016/j.jhep.2019.07.019>.
- 937 [90] Sukriti S, Maras JS, Bihari C, Das S, Vyas AK, Sharma S, et al. Microvesicles in
938 hepatic and peripheral vein can predict nonresponse to corticosteroid therapy in severe
939 alcoholic hepatitis. *Aliment Pharmacol Ther* 2018;47:1151–61.
940 <https://doi.org/10.1111/apt.14564>.
- 941 [91] Verma VK, Li H, Wang R, Hirsova P, Mushref M, Liu Y, et al. Alcohol stimulates
942 macrophage activation through caspase-dependent hepatocyte derived release of

- 943 CD40L containing extracellular vesicles. *J Hepatol* 2016;64:651–60.
944 <https://doi.org/10.1016/j.jhep.2015.11.020>.
- 945 [92] Eguchi A, Franz N, Kobayashi Y, Iwasa M, Wagner N, Hildebrand F, et al. Circulating
946 Extracellular Vesicles and Their miR “Barcode” Differentiate Alcohol Drinkers With
947 Liver Injury and Those Without Liver Injury in Severe Trauma Patients. *Front Med*
948 (Lausanne) 2019;6:30. <https://doi.org/10.3389/fmed.2019.00030>.
- 949 [93] Cho Y-E, Im E-J, Moon P-G, Mezey E, Song B-J, Baek M-C. Increased liver-specific
950 proteins in circulating extracellular vesicles as potential biomarkers for drug- and
951 alcohol-induced liver injury. *PLoS ONE* 2017;12:e0172463.
952 <https://doi.org/10.1371/journal.pone.0172463>.
- 953 [94] Cai Y, Xu M-J, Koritzinsky EH, Zhou Z, Wang W, Cao H, et al. Mitochondrial DNA-
954 enriched microparticles promote acute-on-chronic alcoholic neutrophilia and
955 hepatotoxicity. *JCI Insight* 2017;2. <https://doi.org/10.1172/jci.insight.92634>.
- 956 [95] Momen-Heravi F, Bala S, Kodys K, Szabo G. Exosomes derived from alcohol-treated
957 hepatocytes horizontally transfer liver specific miRNA-122 and sensitize monocytes to
958 LPS. *Sci Rep* 2015;5:9991. <https://doi.org/10.1038/srep09991>.
- 959 [96] Eguchi A, Lazaro RG, Wang J, Kim J, Povero D, Williams B, et al. Extracellular
960 vesicles released by hepatocytes from gastric infusion model of alcoholic liver disease
961 contain a MicroRNA barcode that can be detected in blood. *Hepatology* 2017;65:475–
962 90. <https://doi.org/10.1002/hep.28838>.
- 963 [97] Saha B, Momen-Heravi F, Kodys K, Szabo G. MicroRNA Cargo of Extracellular
964 Vesicles from Alcohol-exposed Monocytes Signals Naive Monocytes to Differentiate
965 into M2 Macrophages. *J Biol Chem* 2016;291:149–59.
966 <https://doi.org/10.1074/jbc.M115.694133>.

- 967 [98] Wang R, Ding Q, Yaqoob U, de Assuncao TM, Verma VK, Hirsova P, et al. Exosome
968 Adherence and Internalization by Hepatic Stellate Cells Triggers Sphingosine 1-
969 Phosphate-dependent Migration. *J Biol Chem* 2015;290:30684–96.
970 <https://doi.org/10.1074/jbc.M115.671735>.
- 971 [99] Sehrawat TS, Arab JP, Liu M, Amrollahi P, Wan M, Fan J, et al. Circulating
972 extracellular vesicles carrying sphingolipid cargo for the diagnosis and dynamic risk
973 profiling of alcoholic hepatitis. *Hepatology* 2020. <https://doi.org/10.1002/hep.31256>.
- 974 [100] Nielsen MC, Andersen MN, Grønbæk H, Damgaard Sandahl T, Møller HJ.
975 Extracellular vesicle-associated soluble CD163 and CD206 in patients with acute and
976 chronic inflammatory liver disease. *Scand J Gastroenterol* 2020:1–9.
977 <https://doi.org/10.1080/00365521.2020.1759140>.
- 978 [101] Babuta M, Furi I, Bala S, Bukong TN, Lowe P, Catalano D, et al. Dysregulated
979 Autophagy and Lysosome Function Are Linked to Exosome Production by Micro-RNA
980 155 in Alcoholic Liver Disease. *Hepatology* 2019;70:2123–41.
981 <https://doi.org/10.1002/hep.30766>.
- 982 [102] Arab JP, Sehrawat TS, Simonetto DA, Verma VK, Feng D, Tang T, et al. An Open-
983 Label, Dose-Escalation Study to Assess the Safety and Efficacy of IL-22 Agonist F-652
984 in Patients With Alcohol-associated Hepatitis. *Hepatology* 2019.
985 <https://doi.org/10.1002/hep.31046>.
- 986 [103] Cho Y-E, Mezey E, Hardwick JP, Salem N, Clemens DL, Song B-J. Increased ethanol-
987 inducible cytochrome P450-2E1 and cytochrome P450 isoforms in exosomes of
988 alcohol-exposed rodents and patients with alcoholism through oxidative and
989 endoplasmic reticulum stress. *Hepatol Commun* 2017;1:675–90.
990 <https://doi.org/10.1002/hep4.1066>.

- 991 [104] Sukriti S, Choudhary MC, Maras JS, Sharma S, Thangariyal S, Singh A, et al.
992 Extracellular vesicles from hepatitis B patients serve as reservoir of hepatitis B virus
993 DNA. *J Viral Hepat* 2019;26:211–4. <https://doi.org/10.1111/jvh.12995>.
- 994 [105] Lambrecht J, Jan Poortmans P, Verhulst S, Reynaert H, Mannaerts I, van Grunsven LA.
995 Circulating ECV-Associated miRNAs as Potential Clinical Biomarkers in Early Stage
996 HBV and HCV Induced Liver Fibrosis. *Front Pharmacol* 2017;8:56.
997 <https://doi.org/10.3389/fphar.2017.00056>.
- 998 [106] Pu C, Huang H, Wang Z, Zou W, Lv Y, Zhou Z, et al. Extracellular Vesicle-Associated
999 mir-21 and mir-144 Are Markedly Elevated in Serum of Patients With Hepatocellular
1000 Carcinoma. *Front Physiol* 2018;9:930. <https://doi.org/10.3389/fphys.2018.00930>.
- 1001 [107] Yang X, Li H, Sun H, Fan H, Hu Y, Liu M, et al. Hepatitis B Virus-Encoded
1002 MicroRNA Controls Viral Replication. *J Virol* 2017;91.
1003 <https://doi.org/10.1128/JVI.01919-16>.
- 1004 [108] van der Ree MH, Jansen L, Kruize Z, van Nuenen AC, van Dort KA, Takkenberg RB,
1005 et al. Plasma MicroRNA Levels Are Associated With Hepatitis B e Antigen Status and
1006 Treatment Response in Chronic Hepatitis B Patients. *J Infect Dis* 2017;215:1421–9.
1007 <https://doi.org/10.1093/infdis/jix140>.
- 1008 [109] Yang Y, Han Q, Hou Z, Zhang C, Tian Z, Zhang J. Exosomes mediate hepatitis B virus
1009 (HBV) transmission and NK-cell dysfunction. *Cell Mol Immunol* 2017;14:465–75.
1010 <https://doi.org/10.1038/cmi.2016.24>.
- 1011 [110] European Association for the Study of the Liver. Electronic address:
1012 easloffice@easloffice.eu, European Association for the Study of the Liver. EASL 2017
1013 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J*
1014 *Hepatol* 2017;67:370–98. <https://doi.org/10.1016/j.jhep.2017.03.021>.

- 1015 [111] Santangelo L, Bordoni V, Montaldo C, Cimini E, Zingoni A, Battistelli C, et al.
1016 Hepatitis C virus direct-acting antivirals therapy impacts on extracellular vesicles
1017 microRNAs content and on their immunomodulating properties. *Liver Int*
1018 2018;38:1741–50. <https://doi.org/10.1111/liv.13700>.
- 1019 [112] Liu Z, Zhang X, Yu Q, He JJ. Exosome-associated hepatitis C virus in cell cultures and
1020 patient plasma. *Biochem Biophys Res Commun* 2014;455:218–22.
1021 <https://doi.org/10.1016/j.bbrc.2014.10.146>.
- 1022 [113] Bukong TN, Momen-Heravi F, Kodys K, Bala S, Szabo G. Exosomes from hepatitis C
1023 infected patients transmit HCV infection and contain replication competent viral RNA
1024 in complex with Ago2-miR122-HSP90. *PLoS Pathog* 2014;10:e1004424.
1025 <https://doi.org/10.1371/journal.ppat.1004424>.
- 1026 [114] Jiao X, Fan Z, Chen H, He P, Li Y, Zhang Q, et al. Serum and exosomal miR-122 and
1027 miR-199a as a biomarker to predict therapeutic efficacy of hepatitis C patients. *J Med*
1028 *Virol* 2017;89:1597–605. <https://doi.org/10.1002/jmv.24829>.
- 1029 [115] Fan Z, Zhang Q, Chen H, He P, Li Y, Si M, et al. Circulating microRNAs as a
1030 biomarker to predict therapy efficacy in hepatitis C patients with different genotypes.
1031 *Microb Pathog* 2017;112:320–6. <https://doi.org/10.1016/j.micpath.2017.10.003>.
- 1032 [116] Devhare PB, Sasaki R, Shrivastava S, Di Bisceglie AM, Ray R, Ray RB. Exosome-
1033 Mediated Intercellular Communication between Hepatitis C Virus-Infected
1034 Hepatocytes and Hepatic Stellate Cells. *J Virol* 2017;91.
1035 <https://doi.org/10.1128/JVI.02225-16>.
- 1036 [117] Welker M-W, Reichert D, Susser S, Sarrazin C, Martinez Y, Herrmann E, et al. Soluble
1037 serum CD81 is elevated in patients with chronic hepatitis C and correlates with alanine
1038 aminotransferase serum activity. *PLoS ONE* 2012;7:e30796.
1039 <https://doi.org/10.1371/journal.pone.0030796>.

- 1040 [118] Kornek M, Popov Y, Libermann TA, Afdhal NH, Schuppan D. Human T cell
1041 microparticles circulate in blood of hepatitis patients and induce fibrolytic activation of
1042 hepatic stellate cells. *Hepatology* 2011;53:230–42. <https://doi.org/10.1002/hep.23999>.
- 1043 [119] Itami-Matsumoto S, Hayakawa M, Uchida-Kobayashi S, Enomoto M, Tamori A,
1044 Mizuno K, et al. Circulating Exosomal miRNA Profiles Predict the Occurrence and
1045 Recurrence of Hepatocellular Carcinoma in Patients with Direct-Acting Antiviral-
1046 Induced Sustained Viral Response. *Biomedicines* 2019;7.
1047 <https://doi.org/10.3390/biomedicines7040087>.
- 1048 [120] Matsuura Y, Wada H, Eguchi H, Gotoh K, Kobayashi S, Kinoshita M, et al. Exosomal
1049 miR-155 Derived from Hepatocellular Carcinoma Cells Under Hypoxia Promotes
1050 Angiogenesis in Endothelial Cells. *Dig Dis Sci* 2019;64:792–802.
1051 <https://doi.org/10.1007/s10620-018-5380-1>.
- 1052 [121] Matsuura K, De Giorgi V, Schechterly C, Wang RY, Farci P, Tanaka Y, et al.
1053 Circulating let-7 levels in plasma and extracellular vesicles correlate with hepatic
1054 fibrosis progression in chronic hepatitis C. *Hepatology* 2016;64:732–45.
1055 <https://doi.org/10.1002/hep.28660>.
- 1056 [122] AASLD-IDSAs HCV Guidance Panel. Hepatitis C Guidance 2018 Update: AASLD-
1057 IDSA Recommendations for Testing, Managing, and Treating Hepatitis C Virus
1058 Infection. *Clin Infect Dis* 2018;67:1477–92. <https://doi.org/10.1093/cid/ciy585>.
- 1059 [123] Wang W, Li H, Zhou Y, Jie S. Peripheral blood microvesicles are potential biomarkers
1060 for hepatocellular carcinoma. *Cancer Biomark* 2013;13:351–7.
1061 <https://doi.org/10.3233/CBM-130370>.
- 1062 [124] Brodsky SV, Facciuto ME, Heydt D, Chen J, Islam HK, Kajstura M, et al. Dynamics of
1063 circulating microparticles in liver transplant patients. *J Gastrointest Liver Dis*
1064 2008;17:261–8.

- 1065 [125] Zuwała-Jagiełło J, Simon KA, Pazgan-Simon M. Elevated circulating endothelial cell-
1066 derived microparticle levels in patients with liver cirrhosis: a preliminary report. *Clin*
1067 *Exp Hepatol* 2015;1:105–11. <https://doi.org/10.5114/ceh.2015.55567>.
- 1068 [126] Campello E, Zanetto A, Spiezia L, Radu CM, Gavasso S, Ferrarese A, et al.
1069 Hypercoagulability detected by circulating microparticles in patients with
1070 hepatocellular carcinoma and cirrhosis. *Thromb Res* 2016;143:118–21.
1071 <https://doi.org/10.1016/j.thromres.2016.05.021>.
- 1072 [127] Ogasawara F, Fusegawa H, Haruki Y, Shiraishi K, Watanabe N, Matsuzaki S. Platelet
1073 activation in patients with alcoholic liver disease. *Tokai J Exp Clin Med* 2005;30:41–8.
- 1074 [128] Fusegawa H, Shiraishi K, Ogasawara F, Shimizu M, Haruki Y, Miyachi H, et al.
1075 Platelet activation in patients with chronic hepatitis C. *Tokai J Exp Clin Med*
1076 2002;27:101–6.
- 1077 [129] Sayed D, Amin NF, Galal GM. Monocyte-platelet aggregates and platelet micro-
1078 particles in patients with post-hepatic liver cirrhosis. *Thromb Res* 2010;125:e228-233.
1079 <https://doi.org/10.1016/j.thromres.2009.12.002>.
- 1080 [130] Eyraud D, Suner L, Dupont A, Bachelot-Loza C, Smadja DM, Helley D, et al.
1081 Evolution of platelet functions in cirrhotic patients undergoing liver transplantation: A
1082 prospective exploration over a month. *PLoS ONE* 2018;13:e0200364.
1083 <https://doi.org/10.1371/journal.pone.0200364>.
- 1084 [131] Taleb RSZ, Moez P, Younan D, Eisenacher M, Tenbusch M, Sitek B, et al.
1085 Quantitative proteome analysis of plasma microparticles for the characterization of
1086 HCV-induced hepatic cirrhosis and hepatocellular carcinoma. *Proteomics Clin Appl*
1087 2017;11. <https://doi.org/10.1002/prca.201700014>.
- 1088 [132] Kostallari E, Hirsova P, Prasnicka A, Verma VK, Yaqoob U, Wongjarupong N, et al.
1089 Hepatic stellate cell-derived platelet-derived growth factor receptor-alpha-enriched

- 1090 extracellular vesicles promote liver fibrosis in mice through SHP2. *Hepatology*
1091 2018;68:333–48. <https://doi.org/10.1002/hep.29803>.
- 1092 [133] Brandon-Warner E, Feilen NA, Culberson CR, Field CO, deLemos AS, Russo MW, et
1093 al. Processing of miR17-92 Cluster in Hepatic Stellate Cells Promotes Hepatic
1094 Fibrogenesis During Alcohol-Induced Injury. *Alcohol Clin Exp Res* 2016;40:1430–42.
1095 <https://doi.org/10.1111/acer.13116>.
- 1096 [134] Lambrecht J, Verhulst S, Mannaerts I, Sowa J-P, Best J, Canbay A, et al. A PDGFR β -
1097 based score predicts significant liver fibrosis in patients with chronic alcohol abuse,
1098 NAFLD and viral liver disease. *EBioMedicine* 2019;43:501–12.
1099 <https://doi.org/10.1016/j.ebiom.2019.04.036>.
- 1100 [135] Rautou P-E, Vion A-C, Luyendyk JP, Mackman N. Circulating microparticle tissue
1101 factor activity is increased in patients with cirrhosis. *Hepatology* 2014;60:1793–5.
1102 <https://doi.org/10.1002/hep.27033>.
- 1103 [136] Chandler WL, Dawson KL, Ruegger MC, Teruya M, Liebl PHN, Monsour HP. Patients
1104 with cirrhosis show a relative increase in thrombin generation that is correlated with
1105 lower antithrombin levels. *Blood Coagul Fibrinolysis* 2014.
1106 <https://doi.org/10.1097/MBC.0000000000000231>.
- 1107 [137] Berckmans RJ, Nieuwland R, Böing AN, Romijn FP, Hack CE, Sturk A. Cell-derived
1108 microparticles circulate in healthy humans and support low grade thrombin generation.
1109 *Thromb Haemost* 2001;85:639–46.
- 1110 [138] Rautou P-E, Mackman N. Microvesicles as risk markers for venous thrombosis. *Expert*
1111 *Rev Hematol* 2013;6:91–101. <https://doi.org/10.1586/ehm.12.74>.
- 1112 [139] Zanetto A, Campello E, Spiezia L, Burra P, Simioni P, Russo FP. Cancer-Associated
1113 Thrombosis in Cirrhotic Patients with Hepatocellular Carcinoma. *Cancers (Basel)*
1114 2018;10. <https://doi.org/10.3390/cancers10110450>.

- 1115 [140] Rautou P-E, Tatsumi K, Antoniak S, Owens AP, Sparkenbaugh E, Holle LA, et al.
1116 Hepatocyte tissue factor contributes to the hypercoagulable state in a mouse model of
1117 chronic liver injury. *J Hepatol* 2016;64:53–9.
1118 <https://doi.org/10.1016/j.jhep.2015.08.017>.
- 1119 [141] Mooberry MJ, Key NS. Microparticle analysis in disorders of hemostasis and
1120 thrombosis. *Cytometry A* 2016;89:111–22. <https://doi.org/10.1002/cyto.a.22647>.
- 1121 [142] Campello E, Radu CM, Zanetto A, Bulato C, Shalaby S, Spiezia L, et al. Changes in
1122 plasma circulating microvesicles in patients with HCV-related cirrhosis after treatment
1123 with direct-acting antivirals. *Liver Int* 2019. <https://doi.org/10.1111/liv.14234>.
- 1124 [143] Julich-Haertel H, Urban SK, Krawczyk M, Willms A, Jankowski K, Patkowski W, et
1125 al. Cancer-associated circulating large extracellular vesicles in cholangiocarcinoma and
1126 hepatocellular carcinoma. *J Hepatol* 2017;67:282–92.
1127 <https://doi.org/10.1016/j.jhep.2017.02.024>.
- 1128 [144] Abbate V, Marcantoni M, Giuliante F, Vecchio FM, Gatto I, Mele C, et al. HepPar1-
1129 Positive Circulating Microparticles Are Increased in Subjects with Hepatocellular
1130 Carcinoma and Predict Early Recurrence after Liver Resection. *Int J Mol Sci* 2017;18.
1131 <https://doi.org/10.3390/ijms18051043>.
- 1132 [145] Sohn W, Kim J, Kang SH, Yang SR, Cho J-Y, Cho HC, et al. Serum exosomal
1133 microRNAs as novel biomarkers for hepatocellular carcinoma. *Exp Mol Med*
1134 2015;47:e184. <https://doi.org/10.1038/emm.2015.68>.
- 1135 [146] Wang H, Hou L, Li A, Duan Y, Gao H, Song X. Expression of serum exosomal
1136 microRNA-21 in human hepatocellular carcinoma. *Biomed Res Int* 2014;2014:864894.
1137 <https://doi.org/10.1155/2014/864894>.
- 1138 [147] Xu H, Chen Y, Dong X, Wang X. Serum Exosomal Long Noncoding RNAs
1139 ENSG00000258332.1 and LINC00635 for the Diagnosis and Prognosis of

- 1140 Hepatocellular Carcinoma. *Cancer Epidemiol Biomarkers Prev* 2018;27:710–6.
1141 <https://doi.org/10.1158/1055-9965.EPI-17-0770>.
- 1142 [148] Xu H, Dong X, Chen Y, Wang X. Serum exosomal hnRNPH1 mRNA as a novel
1143 marker for hepatocellular carcinoma. *Clin Chem Lab Med* 2018;56:479–84.
1144 <https://doi.org/10.1515/cclm-2017-0327>.
- 1145 [149] Wang Y, Zhang C, Zhang P, Guo G, Jiang T, Zhao X, et al. Serum exosomal
1146 microRNAs combined with alpha-fetoprotein as diagnostic markers of hepatocellular
1147 carcinoma. *Cancer Med* 2018;7:1670–9. <https://doi.org/10.1002/cam4.1390>.
- 1148 [150] Liu W, Hu J, Zhou K, Chen F, Wang Z, Liao B, et al. Serum exosomal miR-125b is a
1149 novel prognostic marker for hepatocellular carcinoma. *Onco Targets Ther*
1150 2017;10:3843–51. <https://doi.org/10.2147/OTT.S140062>.
- 1151 [151] Fornari F, Ferracin M, Trerè D, Milazzo M, Marinelli S, Galassi M, et al. Circulating
1152 microRNAs, miR-939, miR-595, miR-519d and miR-494, Identify Cirrhotic Patients
1153 with HCC. *PLoS One* 2015;10. <https://doi.org/10.1371/journal.pone.0141448>.
- 1154 [152] Abd El Gwad A, Matboli M, El-Tawdi A, Habib EK, Shehata H, Ibrahim D, et al. Role
1155 of exosomal competing endogenous RNA in patients with hepatocellular carcinoma. *J*
1156 *Cell Biochem* 2018;119:8600–10. <https://doi.org/10.1002/jcb.27109>.
- 1157 [153] Ma X, Yuan T, Yang C, Wang Z, Zang Y, Wu L, et al. X-inactive-specific transcript of
1158 peripheral blood cells is regulated by exosomal Jpx and acts as a biomarker for female
1159 patients with hepatocellular carcinoma. *Ther Adv Med Oncol* 2017;9:665–77.
1160 <https://doi.org/10.1177/1758834017731052>.
- 1161 [154] Li H, Sun L, Chen X, Xiong W, Hu D, Jie S. Microvesicle microRNA profiles and
1162 functional roles between chronic hepatitis B and hepatocellular carcinoma. *Clin Transl*
1163 *Oncol* 2014;16:315–21. <https://doi.org/10.1007/s12094-013-1078-1>.

- 1164 [155] Yang Y, Zhu R. Diagnostic value of circulating microRNAs for hepatocellular
1165 carcinoma. *Mol Biol Rep* 2014;41:6919–29. [https://doi.org/10.1007/s11033-014-3578-](https://doi.org/10.1007/s11033-014-3578-7)
1166 7.
- 1167 [156] Tian X-P, Wang C-Y, Jin X-H, Li M, Wang F-W, Huang W-J, et al. Acidic
1168 Microenvironment Up-Regulates Exosomal miR-21 and miR-10b in Early-Stage
1169 Hepatocellular Carcinoma to Promote Cancer Cell Proliferation and Metastasis.
1170 *Theranostics* 2019;9:1965–79. <https://doi.org/10.7150/thno.30958>.
- 1171 [157] Xue X, Zhao Y, Wang X, Qin L, Hu R. Development and validation of serum
1172 exosomal microRNAs as diagnostic and prognostic biomarkers for hepatocellular
1173 carcinoma. *J Cell Biochem* 2019;120:135–42. <https://doi.org/10.1002/jcb.27165>.
- 1174 [158] Suehiro T, Miyaaki H, Kanda Y, Shibata H, Honda T, Ozawa E, et al. Serum exosomal
1175 microRNA-122 and microRNA-21 as predictive biomarkers in transarterial
1176 chemoembolization-treated hepatocellular carcinoma patients. *Oncol Lett*
1177 2018;16:3267–73. <https://doi.org/10.3892/ol.2018.8991>.
- 1178 [159] Fu Q, Zhang Q, Lou Y, Yang J, Nie G, Chen Q, et al. Primary tumor-derived exosomes
1179 facilitate metastasis by regulating adhesion of circulating tumor cells via SMAD3 in
1180 liver cancer. *Oncogene* 2018;37:6105–18. <https://doi.org/10.1038/s41388-018-0391-0>.
- 1181 [160] Tang J, Li Y, Liu K, Zhu Q, Yang W-H, Xiong L-K, et al. Exosomal miR-9-3p
1182 suppresses HBGF-5 expression and is a functional biomarker in hepatocellular
1183 carcinoma. *Minerva Med* 2018;109:15–23. [https://doi.org/10.23736/S0026-](https://doi.org/10.23736/S0026-4806.17.05167-9)
1184 4806.17.05167-9.
- 1185 [161] Li Y, Zhao J, Yu S, Wang Z, He X, Su Y, et al. Extracellular Vesicles Long RNA
1186 Sequencing Reveals Abundant mRNA, circRNA, and lncRNA in Human Blood as
1187 Potential Biomarkers for Cancer Diagnosis. *Clin Chem* 2019.
1188 <https://doi.org/10.1373/clinchem.2018.301291>.

- 1189 [162] Liu Y, Tan J, Ou S, Chen J, Chen L. Adipose-derived exosomes deliver miR-23a/b to
1190 regulate tumor growth in hepatocellular cancer by targeting the VHL/HIF axis. *J*
1191 *Physiol Biochem* 2019. <https://doi.org/10.1007/s13105-019-00692-6>.
- 1192 [163] Zheng Q, Zhao J, Yu H, Zong H, He X, Zhao Y, et al. Tumor-Specific Transcripts Are
1193 Frequently Expressed in Hepatocellular Carcinoma With Clinical Implication and
1194 Potential Function. *Hepatology* 2019. <https://doi.org/10.1002/hep.30805>.
- 1195 [164] Zhao S, Li J, Zhang G, Wang Q, Wu C, Zhang Q, et al. Exosomal miR-451a Functions
1196 as a Tumor Suppressor in Hepatocellular Carcinoma by Targeting LPIN1. *Cell Physiol*
1197 *Biochem* 2019;53:19–35. <https://doi.org/10.33594/000000118>.
- 1198 [165] Han Q, Lv L, Wei J, Lei X, Lin H, Li G, et al. Vps4A mediates the localization and
1199 exosome release of β -catenin to inhibit epithelial-mesenchymal transition in
1200 hepatocellular carcinoma. *Cancer Lett* 2019;457:47–59.
1201 <https://doi.org/10.1016/j.canlet.2019.04.035>.
- 1202 [166] Cheng Z, Lei Z, Yang P, Si A, Xiang D, Tang X, et al. Exosome-transmitted p120-
1203 catenin suppresses hepatocellular carcinoma progression via STAT3 pathways. *Mol*
1204 *Carcinog* 2019;58:1389–99. <https://doi.org/10.1002/mc.23022>.
- 1205 [167] Cui Y, Xu H-F, Liu M-Y, Xu Y-J, He J-C, Zhou Y, et al. Mechanism of exosomal
1206 microRNA-224 in development of hepatocellular carcinoma and its diagnostic and
1207 prognostic value. *World J Gastroenterol* 2019;25:1890–8.
1208 <https://doi.org/10.3748/wjg.v25.i15.1890>.
- 1209 [168] Sun L, Su Y, Liu X, Xu M, Chen X, Zhu Y, et al. Serum and exosome long non coding
1210 RNAs as potential biomarkers for hepatocellular carcinoma. *J Cancer* 2018;9:2631–9.
1211 <https://doi.org/10.7150/jca.24978>.
- 1212 [169] Xue X, Wang X, Zhao Y, Hu R, Qin L. Exosomal miR-93 promotes proliferation and
1213 invasion in hepatocellular carcinoma by directly inhibiting

- 1214 TIMP2/TP53INP1/CDKN1A. *Biochem Biophys Res Commun* 2018;502:515–21.
1215 <https://doi.org/10.1016/j.bbrc.2018.05.208>.
- 1216 [170] Lin X-J, Fang J-H, Yang X-J, Zhang C, Yuan Y, Zheng L, et al. Hepatocellular
1217 Carcinoma Cell-Secreted Exosomal MicroRNA-210 Promotes Angiogenesis In Vitro
1218 and In Vivo. *Mol Ther Nucleic Acids* 2018;11:243–52.
1219 <https://doi.org/10.1016/j.omtn.2018.02.014>.
- 1220 [171] Shi M, Jiang Y, Yang L, Yan S, Wang Y-G, Lu X-J. Decreased levels of serum
1221 exosomal miR-638 predict poor prognosis in hepatocellular carcinoma. *J Cell Biochem*
1222 2018;119:4711–6. <https://doi.org/10.1002/jcb.26650>.
- 1223 [172] Wang X, Shen H, Zhangyuan G, Huang R, Zhang W, He Q, et al. 14-3-3 ζ delivered by
1224 hepatocellular carcinoma-derived exosomes impaired anti-tumor function of tumor-
1225 infiltrating T lymphocytes. *Cell Death Dis* 2018;9:159. [https://doi.org/10.1038/s41419-](https://doi.org/10.1038/s41419-017-0180-7)
1226 017-0180-7.
- 1227 [173] Li B, Mao R, Liu C, Zhang W, Tang Y, Guo Z. LncRNA FAL1 promotes cell
1228 proliferation and migration by acting as a CeRNA of miR-1236 in hepatocellular
1229 carcinoma cells. *Life Sci* 2018;197:122–9. <https://doi.org/10.1016/j.lfs.2018.02.006>.
- 1230 [174] Zhang C, Yang X, Qi Q, Gao Y, Wei Q, Han S. lncRNA-HEIH in serum and exosomes
1231 as a potential biomarker in the HCV-related hepatocellular carcinoma. *Cancer Biomark*
1232 2018;21:651–9. <https://doi.org/10.3233/CBM-170727>.
- 1233 [175] Wang X, Kwak KJ, Yang Z, Zhang A, Zhang X, Sullivan R, et al. Extracellular mRNA
1234 detected by molecular beacons in tethered lipoplex nanoparticles for diagnosis of
1235 human hepatocellular carcinoma. *PLoS ONE* 2018;13:e0198552.
1236 <https://doi.org/10.1371/journal.pone.0198552>.
- 1237 [176] Arbelaiz A, Azkargorta M, Krawczyk M, Santos-Laso A, Lapitz A, Perugorria MJ, et
1238 al. Serum extracellular vesicles contain protein biomarkers for primary sclerosing

- 1239 cholangitis and cholangiocarcinoma. *Hepatology* 2017;66:1125–43.
1240 <https://doi.org/10.1002/hep.29291>.
- 1241 [177] Lu L, Guo D, Chen X, Xiong W, Jie S, Li H. Abnormal miRNAs Targeting
1242 Chromosome Open Reading Frame Genes were Enriched in Microvesicles Derived
1243 from the Circulation of HCC. *Biochem Genet* 2016;54:120–33.
1244 <https://doi.org/10.1007/s10528-015-9705-x>.
- 1245 [178] Zhu L, Li J, Gong Y, Wu Q, Tan S, Sun D, et al. Exosomal tRNA-derived small RNA
1246 as a promising biomarker for cancer diagnosis. *Mol Cancer* 2019;18:74.
1247 <https://doi.org/10.1186/s12943-019-1000-8>.
- 1248 [179] Wang G, Liu W, Zou Y, Wang G, Deng Y, Luo J, et al. Three isoforms of exosomal
1249 circPTGR1 promote hepatocellular carcinoma metastasis via the miR449a-MET
1250 pathway. *EBioMedicine* 2019;40:432–45. <https://doi.org/10.1016/j.ebiom.2018.12.062>.
- 1251 [180] Wu D, Yu Y, Jin D, Xiao M-M, Zhang Z-Y, Zhang G-J. Dual-Aptamer Modified
1252 Graphene Field-Effect Transistor Nanosensor for Label-Free and Specific Detection of
1253 Hepatocellular Carcinoma-Derived Microvesicles. *Anal Chem* 2020;92:4006–15.
1254 <https://doi.org/10.1021/acs.analchem.9b05531>.
- 1255 [181] Xie J-Y, Wei J-X, Lv L-H, Han Q-F, Yang W-B, Li G-L, et al. Angiopoietin-2 induces
1256 angiogenesis via exosomes in human hepatocellular carcinoma. *Cell Commun Signal*
1257 2020;18:46. <https://doi.org/10.1186/s12964-020-00535-8>.
- 1258 [182] Mjelle R, Dima SO, Bacalbasa N, Chawla K, Sorop A, Cucu D, et al. Comprehensive
1259 transcriptomic analyses of tissue, serum, and serum exosomes from hepatocellular
1260 carcinoma patients. *BMC Cancer* 2019;19:1007. [https://doi.org/10.1186/s12885-019-](https://doi.org/10.1186/s12885-019-6249-1)
1261 6249-1.

- 1262 [183] Wang S, Yang Y, Sun L, Qiao G, Song Y, Liu B. Exosomal MicroRNAs as Liquid
1263 Biopsy Biomarkers in Hepatocellular Carcinoma. *Onco Targets Ther* 2020;13:2021–30.
1264 <https://doi.org/10.2147/OTT.S232453>.
- 1265 [184] Cho HJ, Eun JW, Baek GO, Seo CW, Ahn HR, Kim SS, et al. Serum Exosomal
1266 MicroRNA, miR-10b-5p, as a Potential Diagnostic Biomarker for Early-Stage
1267 Hepatocellular Carcinoma. *J Clin Med* 2020;9. <https://doi.org/10.3390/jcm9010281>.
- 1268 [185] Wang G, Zhao W, Wang H, Qiu G, Jiang Z, Wei G, et al. Exosomal MiR-744 Inhibits
1269 Proliferation and Sorafenib Chemoresistance in Hepatocellular Carcinoma by Targeting
1270 PAX2. *Med Sci Monit* 2019;25:7209–17. <https://doi.org/10.12659/MSM.919219>.
- 1271 [186] Li W, Ding X, Wang S, Xu L, Yin T, Han S, et al. Downregulation of serum exosomal
1272 miR-320d predicts poor prognosis in hepatocellular carcinoma. *J Clin Lab Anal*
1273 2020:e23239. <https://doi.org/10.1002/jcla.23239>.
- 1274 [187] Lin H, Zhang Z. Diagnostic value of a microRNA signature panel in exosomes for
1275 patients with hepatocellular carcinoma. *Int J Clin Exp Pathol* 2019;12:1478–87.
- 1276 [188] Cao S-Q, Zheng H, Sun B-C, Wang Z-L, Liu T, Guo D-H, et al. Long non-coding RNA
1277 highly up-regulated in liver cancer promotes exosome secretion. *World J Gastroenterol*
1278 2019;25:5283–99. <https://doi.org/10.3748/wjg.v25.i35.5283>.
- 1279 [189] Lu Y, Duan Y, Xu Q, Zhang L, Chen W, Qu Z, et al. Circulating exosome-derived
1280 bona fide long non-coding RNAs predicting the occurrence and metastasis of
1281 hepatocellular carcinoma. *J Cell Mol Med* 2020;24:1311–8.
1282 <https://doi.org/10.1111/jcmm.14783>.
- 1283 [190] Chen W, Quan Y, Fan S, Wang H, Liang J, Huang L, et al. Exosome-transmitted
1284 circular RNA hsa_circ_0051443 suppresses hepatocellular carcinoma progression.
1285 *Cancer Lett* 2020;475:119–28. <https://doi.org/10.1016/j.canlet.2020.01.022>.

- 1286 [191] Lee YR, Kim G, Tak WY, Jang SY, Kweon YO, Park JG, et al. Circulating exosomal
1287 noncoding RNAs as prognostic biomarkers in human hepatocellular carcinoma. *Int J*
1288 *Cancer* 2019;144:1444–52. <https://doi.org/10.1002/ijc.31931>.
- 1289 [192] Zhou Y, Ren H, Dai B, Li J, Shang L, Huang J, et al. Hepatocellular carcinoma-derived
1290 exosomal miRNA-21 contributes to tumor progression by converting hepatocyte
1291 stellate cells to cancer-associated fibroblasts. *J Exp Clin Cancer Res* 2018;37:324.
1292 <https://doi.org/10.1186/s13046-018-0965-2>.
- 1293 [193] Gramantieri L, Baglioni M, Fornari F, Laginestra MA, Ferracin M, Indio V, et al.
1294 LncRNAs as novel players in hepatocellular carcinoma recurrence. *Oncotarget*
1295 2018;9:35085–99. <https://doi.org/10.18632/oncotarget.26202>.
- 1296 [194] Nakano T, Chen I-H, Wang C-C, Chen P-J, Tseng H-P, Huang K-T, et al. Circulating
1297 exosomal miR-92b: Its role for cancer immunoediting and clinical value for prediction
1298 of posttransplant hepatocellular carcinoma recurrence. *Am J Transplant* 2019.
1299 <https://doi.org/10.1111/ajt.15490>.
- 1300 [195] Fang J-H, Zhang Z-J, Shang L-R, Luo Y-W, Lin Y-F, Yuan Y, et al. Hepatoma cell-
1301 secreted exosomal microRNA-103 increases vascular permeability and promotes
1302 metastasis by targeting junction proteins. *Hepatology* 2018;68:1459–75.
1303 <https://doi.org/10.1002/hep.29920>.
- 1304 [196] Fang T, Lv H, Lv G, Li T, Wang C, Han Q, et al. Tumor-derived exosomal miR-1247-
1305 3p induces cancer-associated fibroblast activation to foster lung metastasis of liver
1306 cancer. *Nat Commun* 2018;9:191. <https://doi.org/10.1038/s41467-017-02583-0>.
- 1307 [197] Sugimachi K, Matsumura T, Hirata H, Uchi R, Ueda M, Ueo H, et al. Identification of
1308 a bona fide microRNA biomarker in serum exosomes that predicts hepatocellular
1309 carcinoma recurrence after liver transplantation. *Br J Cancer* 2015;112:532–8.
1310 <https://doi.org/10.1038/bjc.2014.621>.

- 1311 [198] Yu L-X, Zhang B-L, Yang Y, Wang M-C, Lei G-L, Gao Y, et al. Exosomal
1312 microRNAs as potential biomarkers for cancer cell migration and prognosis in
1313 hepatocellular carcinoma patient-derived cell models. *Oncol Rep* 2019;41:257–69.
1314 <https://doi.org/10.3892/or.2018.6829>.
- 1315 [199] Huang X-Y, Huang Z-L, Huang J, Xu B, Huang X-Y, Xu Y-H, et al. Exosomal
1316 circRNA-100338 promotes hepatocellular carcinoma metastasis via enhancing
1317 invasiveness and angiogenesis. *J Exp Clin Cancer Res* 2020;39:20.
1318 <https://doi.org/10.1186/s13046-020-1529-9>.
- 1319 [200] Lazaridis KN, LaRusso NF. Primary Sclerosing Cholangitis. *N Engl J Med*
1320 2016;375:1161–70. <https://doi.org/10.1056/NEJMra1506330>.
- 1321 [201] Lapitz A, Arbelaiz A, O'Rourke CJ, Lavin JL, Casta AL, Ibarra C, et al. Patients with
1322 Cholangiocarcinoma Present Specific RNA Profiles in Serum and Urine Extracellular
1323 Vesicles Mirroring the Tumor Expression: Novel Liquid Biopsy Biomarkers for
1324 Disease Diagnosis. *Cells* 2020;9. <https://doi.org/10.3390/cells9030721>.
- 1325 [202] Shen L, Chen G, Xia Q, Shao S, Fang H. Exosomal miR-200 family as serum
1326 biomarkers for early detection and prognostic prediction of cholangiocarcinoma. *Int J*
1327 *Clin Exp Pathol* 2019;12:3870–6.
- 1328 [203] Wang S, Hu Y, Lv X, Li B, Gu D, Li Y, et al. Circ-0000284 arouses malignant
1329 phenotype of cholangiocarcinoma cells and regulates the biological functions of
1330 peripheral cells through cellular communication. *Clin Sci* 2019;133:1935–53.
1331 <https://doi.org/10.1042/CS20190589>.
- 1332 [204] Gu X, Wang C, Deng H, Qing C, Liu R, Liu S, et al. Exosomal piRNA profiling
1333 revealed unique circulating piRNA signatures of cholangiocarcinoma and gallbladder
1334 carcinoma. *Acta Biochim Biophys Sin (Shanghai)* n.d.
1335 <https://doi.org/10.1093/abbs/gmaa028>.

- 1336 [205] Flamm SL, Yang Y-X, Singh S, Falck-Ytter YT, Flamm SL, Lim JK, et al. American
1337 Gastroenterological Association Institute Guidelines for the Diagnosis and
1338 Management of Acute Liver Failure. *Gastroenterology* 2017;152:644–7.
1339 <https://doi.org/10.1053/j.gastro.2016.12.026>.
- 1340 [206] Stravitz RT, Bowling R, Bradford RL, Key NS, Glover S, Thacker LR, et al. Role of
1341 procoagulant microparticles in mediating complications and outcome of acute liver
1342 injury/acute liver failure. *Hepatology* 2013;58:304–13.
1343 <https://doi.org/10.1002/hep.26307>.
- 1344 [207] Duan L, Ramachandran A, Akakpo JY, Weemhoff JL, Curry SC, Jaeschke H. Role of
1345 extracellular vesicles in release of protein adducts after acetaminophen-induced liver
1346 injury in mice and humans. *Toxicol Lett* 2019;301:125–32.
1347 <https://doi.org/10.1016/j.toxlet.2018.11.005>.
- 1348 [208] Schmelzle M, Splith K, Andersen LW, Kornek M, Schuppan D, Jones-Bamman C, et
1349 al. Increased plasma levels of microparticles expressing CD39 and CD133 in acute
1350 liver injury. *Transplantation* 2013;95:63–9.
1351 <https://doi.org/10.1097/TP.0b013e318278d3cd>.
- 1352 [209] Zhang Y, Wang D, Shen D, Luo Y, Che Y-Q. Identification of exosomal miRNAs
1353 associated with the anthracycline-induced liver injury in postoperative breast cancer
1354 patients by small RNA sequencing. *PeerJ* 2020;8:e9021.
1355 <https://doi.org/10.7717/peerj.9021>.
- 1356 [210] Ayers L, Pink R, Carter DRF, Nieuwland R. Clinical requirements for extracellular
1357 vesicle assays. *J Extracell Vesicles* 2019;8:1593755.
1358 <https://doi.org/10.1080/20013078.2019.1593755>.
- 1359 [211] van der Pol E, Coumans F a. W, Grootemaat AE, Gardiner C, Sargent IL, Harrison P, et
1360 al. Particle size distribution of exosomes and microvesicles determined by transmission

1361 electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse
1362 sensing. *J Thromb Haemost* 2014;12:1182–92. <https://doi.org/10.1111/jth.12602>.
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

1375 **Table 4: Summary of main clinical studies on extracellular vesicles as biomarkers in**
 1376 **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

1385 **Fig. 1: Size exclusion chromatography. Left.** The column contains porous beads separating
1386 soluble proteins (which migrate at lower speed, as they are small enough to go through the
1387 pores) from extracellular vesicles. **Right.** As an example, plasma from a patient with Child-
1388 Pugh C cirrhosis goes through a size exclusion chromatography column, separating
1389 progressively the extracellular vesicle compartment from the bilirubin-rich protein
1390 compartment. The chromatogram shows absorbance at 280 nm of each 2 mL fraction of
1391 plasma: extracellular vesicles are detected first, followed by soluble proteins.

1392

1393 **Fig. 2: Extracellular vesicle subpopulation detection method by filtration/ELISA. A.**
1394 Graphical representation of the filtration/ELISA method. ELISA is performed on platelet free
1395 plasma before filtration, after double filtration through 0.2 μm pores, and after filtration
1396 through 0.02 μm pores. Concentration of larger extracellular vesicles is equal to the difference
1397 between protein concentration before and after double filtration through 0.2 μm pores.
1398 Concentration of small extracellular vesicles is equal to the difference between protein
1399 concentration after double filtration through 0.2 μm pores and after filtration through 0.02 μm
1400 pores. **B.** Flow cytometry graphs showing calibrated Megamix-Plus FSC beads having a 0.1,
1401 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
1402 beads. Filtration through 0.02 μm pores removes 0.1 μm beads. **C.** Tunable Resistive Pulse
1403 Sensing (TRPS, qNano) analysis of the plasma of a patient with cirrhosis before filtration,
1404 after filtration through 0.2 μm pores and after filtration through 0.02 μm pores. After filtration
1405 through 0.2 μm pores, a decrease of 92% of events between 185 and 1000 nm was observed
1406 (n=2). Abbreviations: ELISA: sandwich enzyme-linked immunosorbent assay; EV:
1407 extracellular vesicle.

1408

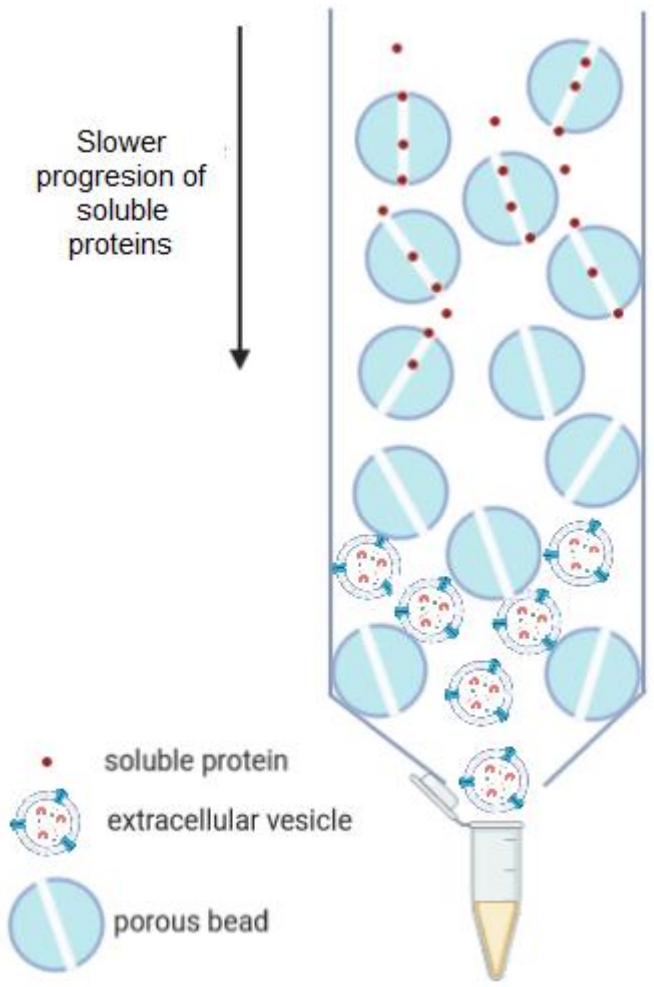
1409 **Fig. 3: Phases of development of extracellular vesicles as biomarkers for diagnosis,**
1410 **severity and prognosis in liver diseases.** Abbreviations: extracellular vesicle.

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



Free bilirubin & other soluble proteins

Extracellular vesicles

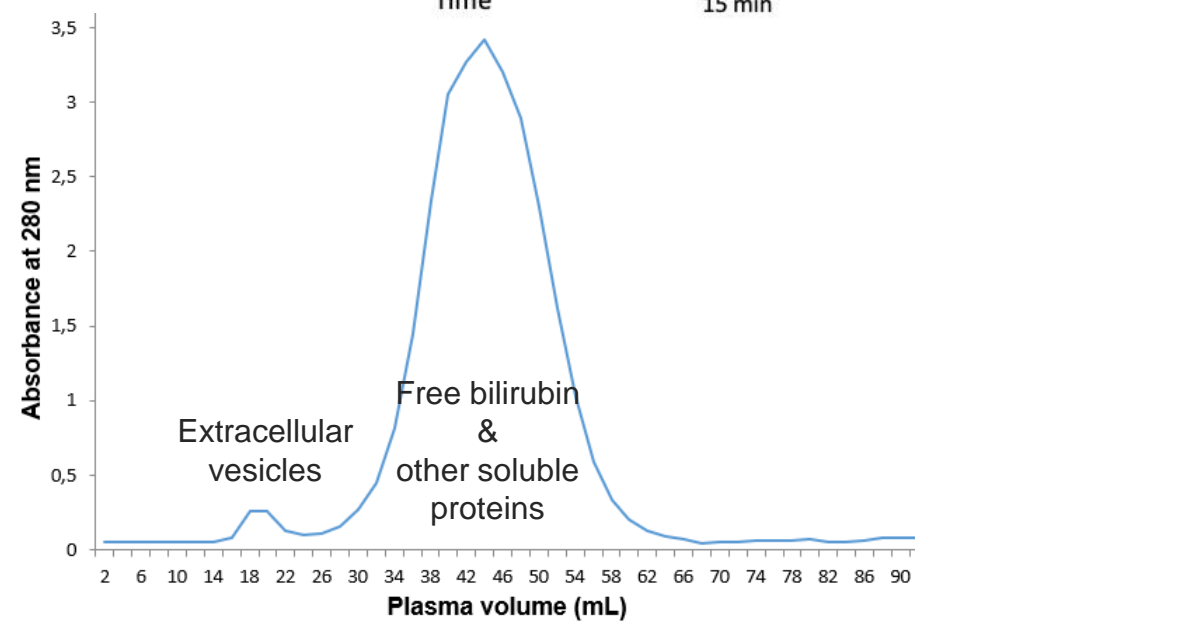


Fig. 2

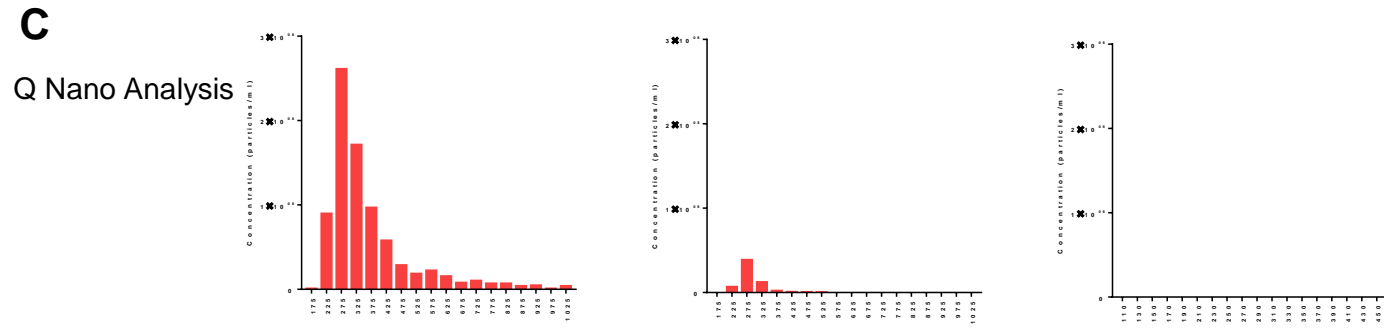
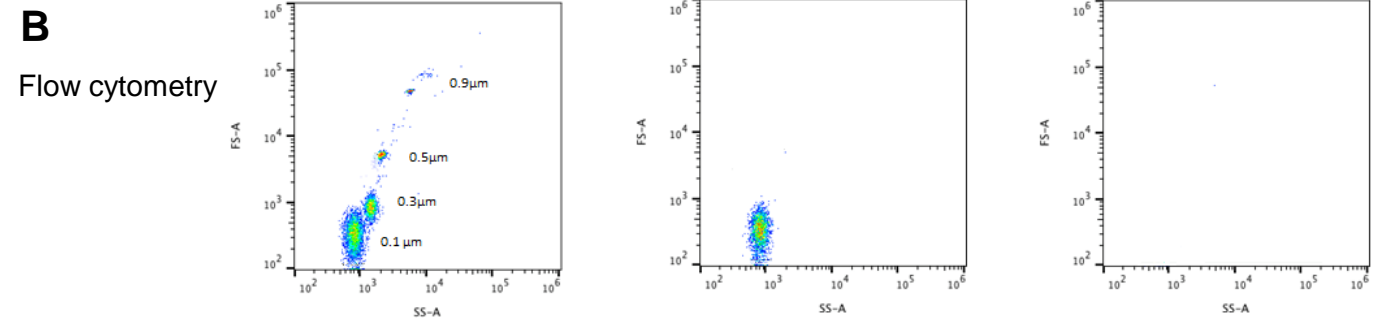
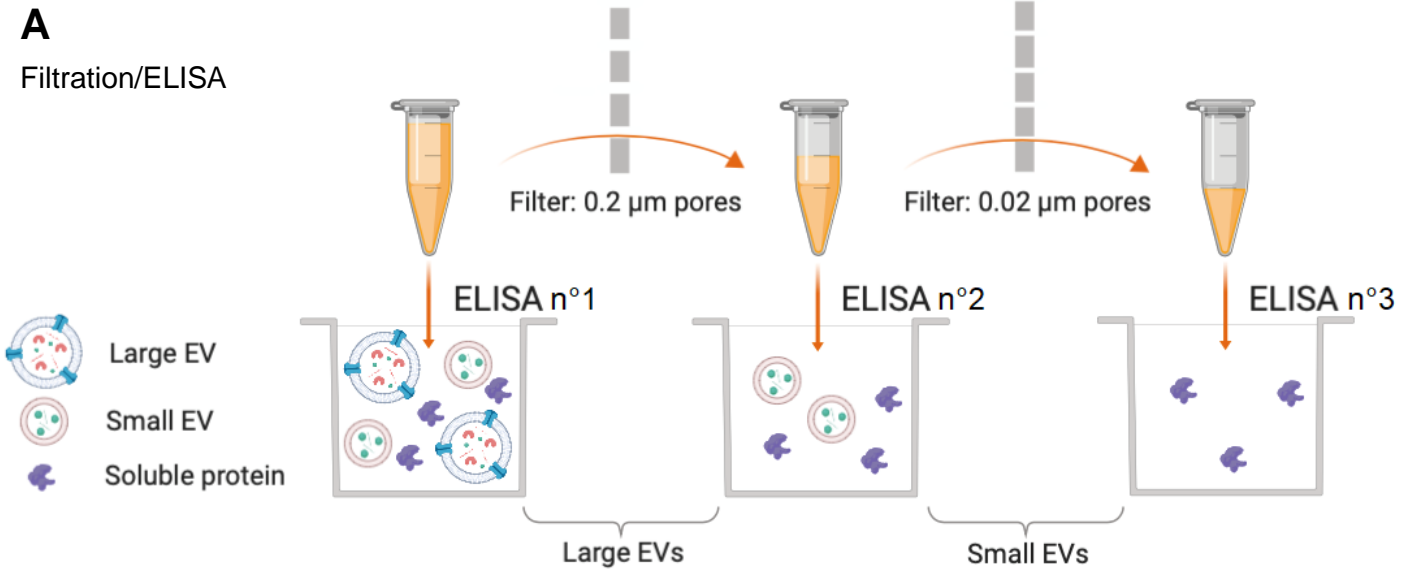
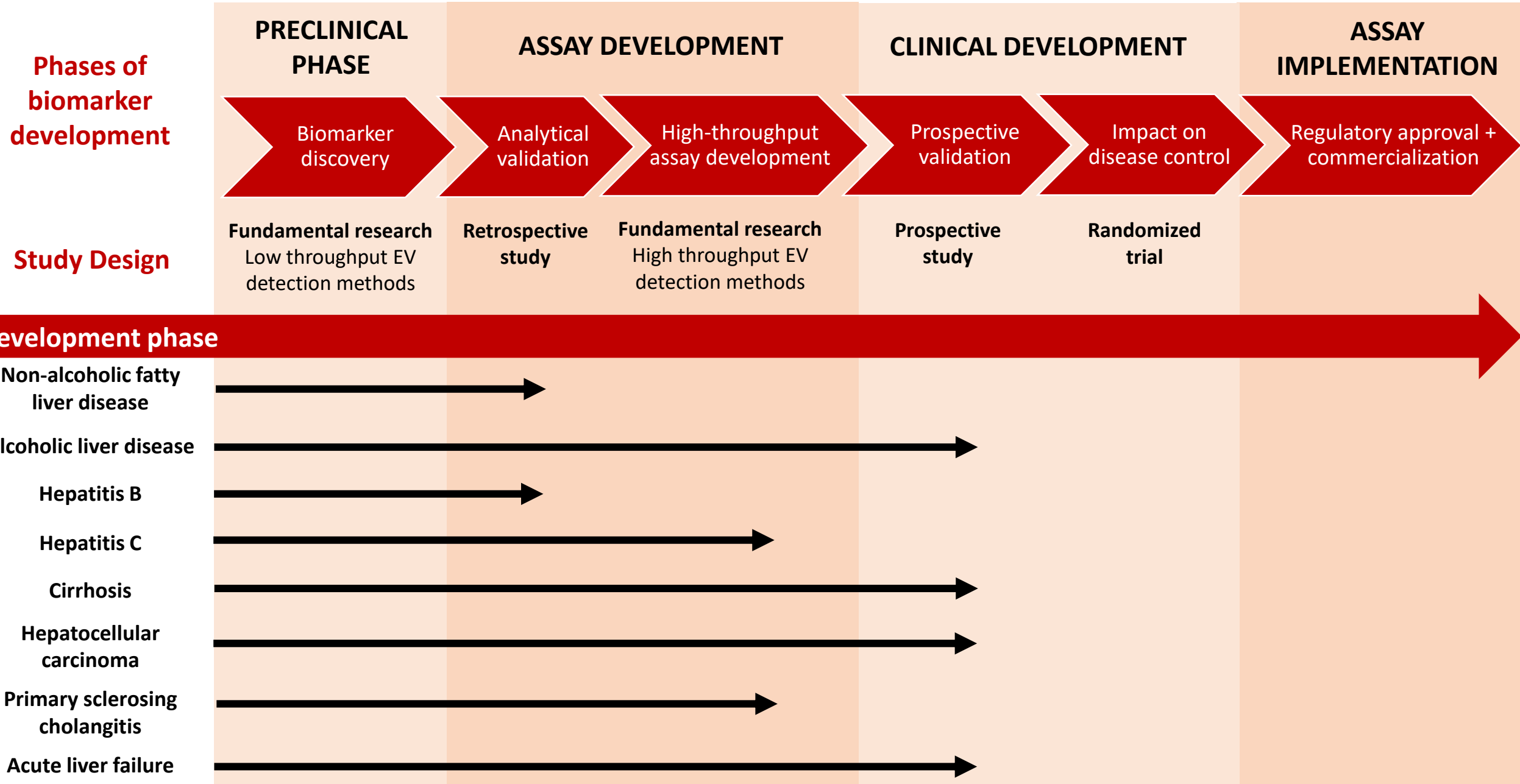
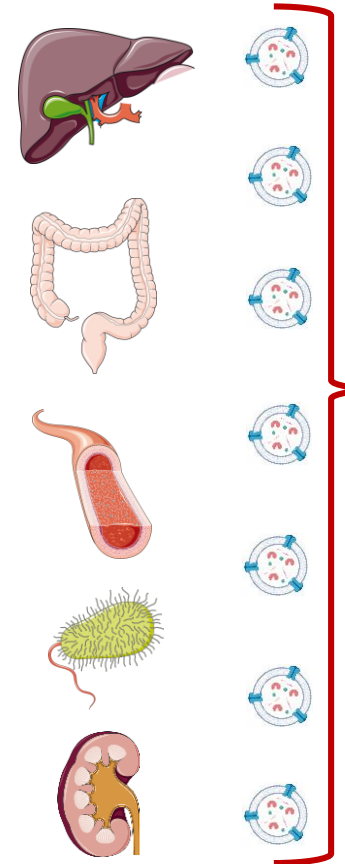


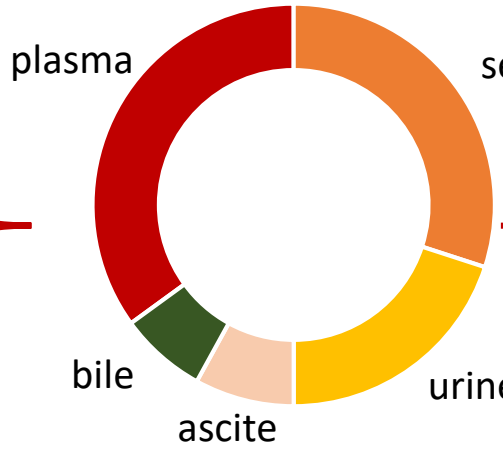
Fig. 3



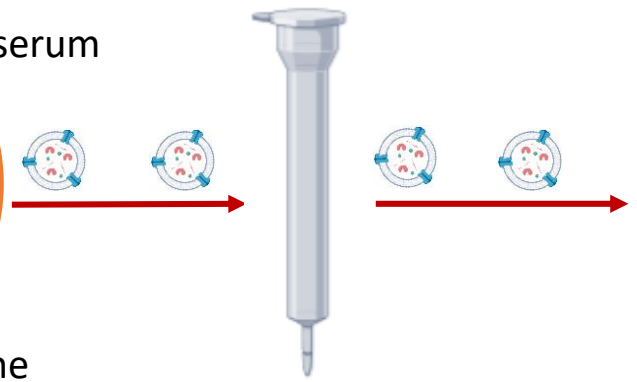
Which extracellular vesicle subpopulation?



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