Review Article

Liver-derived Extracellular Vesicles: a Cell by Cell Overview to Isolation and Characterization Practices

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Abstract

BACKGROUND: Extracellular vesicles (EVs) are a diverse group of membrane-bound nanovesicles potentially released by every cell. With the liver's unique ensemble of cells and its fundamental physiological tasks, elucidating the role of EV-mediated hepatic cellular crosstalk and their role in different pathologies has been gaining the attention of many scientists.
SCOPE OF REVIEW: The present review shifts the perspective into practice: we aim to critically discuss the methods used to purify and to biochemically analyse EVs from specific liver resident cells, including hepatocytes, hepatic stellate cells, cholangiocytes, liver sinusoidal endothelial cells, Kupffer cells, liver stem cells. The review offers a reference guide to current approaches.
MAJOR CONCLUSIONS: Strategies for EV isolation and characterization are as varied as the research groups performing them. We present main advantages and disadvantages for the methods, highlighting common causes for concern, such as FBS handling, reporting of cell viability, EV yield and storage, differences in differential centrifugations, suboptimal method descriptions, and method transferability. We both looked at how adaptable the research between human and rodent cells *in vitro* is, and also assessed how well either of them translates to *ex vivo* settings.
GENERAL SIGNIFICANCE: We reviewed methodological practices for the isolation and analysis of liver-derived EVs, making a cell type specific user guide that shows where to start, what has worked so far and to what extent. We critically discussed room for improvement, placing a particular focus on working towards a potential standardization of methods.

1. Introduction

Extracellular vesicles (EVs) is a collective term referring to a diverse group of small membrane vesicles virtually released by all cell types, and which are generally being categorized according to their biogenesis:[1,2] apoptotic bodies are blebs of the dying cell membrane and have the broadest size range; microvesicles, sometimes referred to as microparticles or ectosomes, stem from the outward budding of the cellular membrane. Exosomes, which tend to be the smallest subpopulation, are released into the extracellular space after multivesicular bodies (MVB) fuse with the cell membrane (Figure 1). It is still not really possible to isolate one subpopulation from the others, and while it is believed that they may display biomolecules that are enriched to different extents, their overlapping composition, density and size, as well as the absence of subtype-specific markers still make for a considerable challenge.[3,4] The recent discovery of their role in intercellular communication captivated the attention of a growing number of scientists anticipating the enormous potential of EVs in the fields of diagnostics and drug delivery.[5,6] For some pathological dispositions EVs can be applied as liquid biopsies, and that has sparked a lot of interest from a diagnostic perspective. EVs are enriched in selected biomolecules, they are intrinsically equipped to protect their cargo from degradation, and while their complexity offers many characterization opportunities (see Figure 1), they are still simpler to analyse than total blood or serum samples. The interest in EVs as drug delivery systems stems from their potential advantages over synthetic carriers: they are bioavailable, biocompatible, resistant to RNAases and proteases (high physicochemical stability), capable of long-distance communication and they are intrinsically able to interact with cells even across species.[7–9]

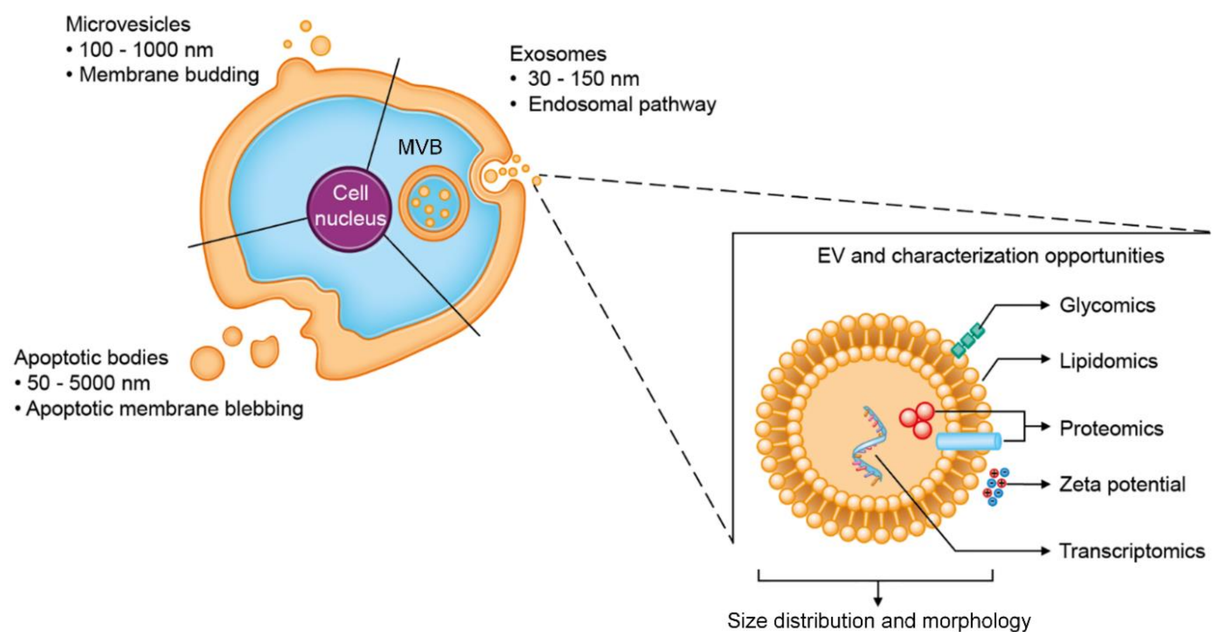

Figure 1: EV nomenclature according to biogenesis; zoomed insert shows a schematic representation of a single EV with characterization opportunities.

The increasing scientific interest in EVs[10] has led to the establishment of dedicated, open access databases such as Vesiclepedia,[11] EVpedia[12] and EV-TRACK,[13] which are being regularly updated. The majority of the information is concerning proteins, whereas EV-TRACK sets itself apart by focusing on method transparency.
As reviewed elsewhere,[14,15] there are significant challenges (small yields, co-purification of contaminants, etc.) in finding the most efficient protocols for the isolation and sufficient characterization of EVs. We will discuss them in an effort to encourage sharing the current knowledge of the more practical scientific trends in those areas, starting with the present review on liver-derived EVs.

The liver is a large and complex organ responsible for a variety of essential physiological tasks including protein synthesis, lipid storage regulation, xenobiotic detoxification, and offering support to both immunological activity and food digestion.[16] It is difficult to understate its importance. When organ function is compromised, hepatic diseases are directly responsible for as many as 2 million deaths per year: liver cirrhosis alone kills 1.16 million people every year, and hepatocellular carcinoma accounts for the death of 788’000 more, meaning that combined they cause 3.5% of all yearly deaths in the world.[17,18] The global health burden of liver associated conditions is not sufficiently addressed as of yet.[19] With its unique ensemble of diverse cells (see Figure 2), the liver offers the opportunity to study intra- and inter-cellular communication. Elucidating the role of EV-mediated hepatic cellular crosstalk has gained the attention of many researchers, who have been able to review its critical role in both health and disease, pointing to differences in the set of EVs that are released, especially in the case of tumors.[20–25]

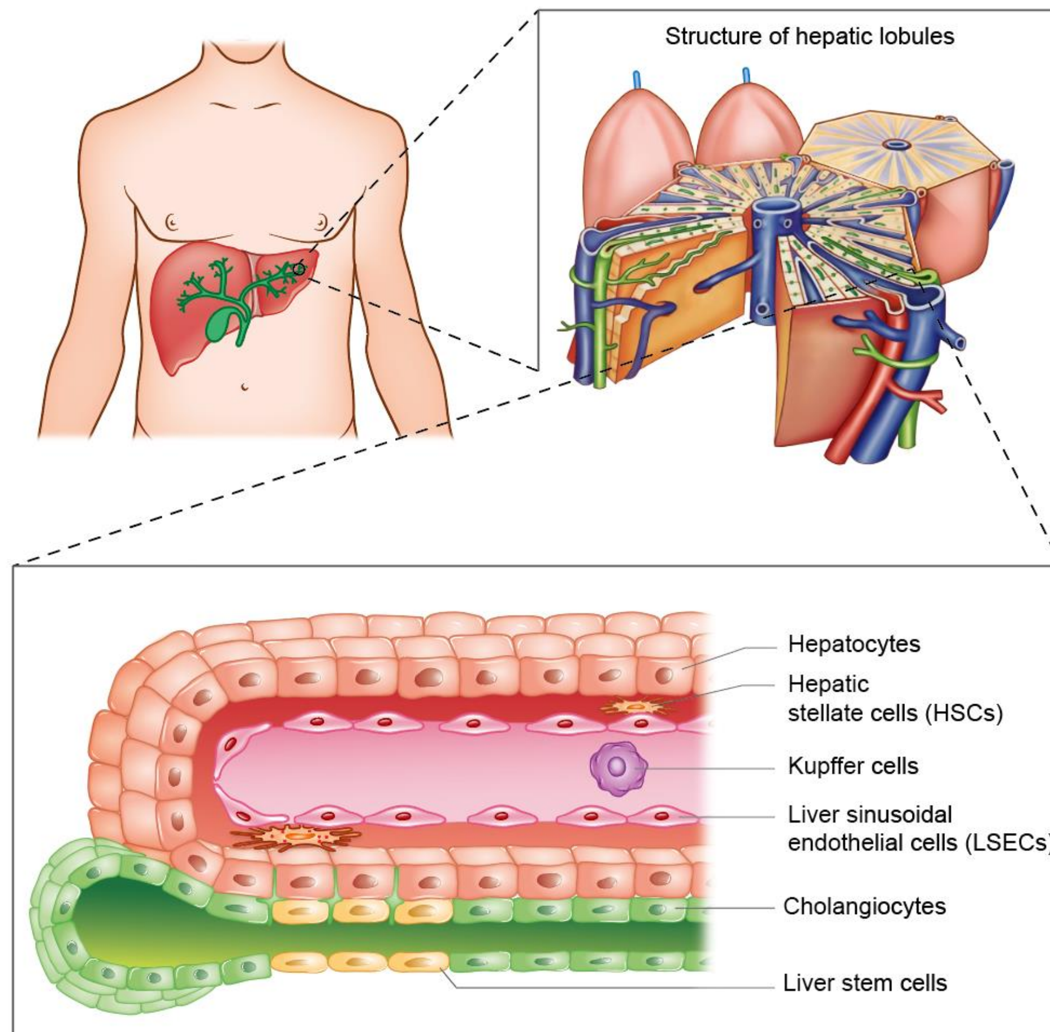

Figure 2: Liver location, structure of hepatic lobules (adapted with permission[26]**) and their anatomy at the cellular level: hepatocytes (the most abundant cells in the liver and responsible for the most tasks, see chapter 2), hepatic stellate cells (vitamin A storing cells, chapter 3), cholangiocytes (modifying the bile along the bile ducts, chapter 4), liver sinusoidal endothelial cells (lining the fenestrated layer of blood vessels, chapter 5), Kupffer cells (liver resident macrophages, chapter 6), liver stem cells (potentially playing a role in liver regeneration, chapter 7).**

The present review shifts the perspective into practice: we aim to critically consider the methods used to purify and to biochemically analyse EVs from specific cells, offering a user/reference guide to current practices (see Table 1). The most common and (when applicable) the most original/promising protocols will be examined in order to provide a discussion frame. Diving into the different methodological approaches, we will also highlight limitations and possible inconsistencies. Finally, the (interspecies) method transferability and the translational applicability of these practices will be examined. We will look within the individual cell specific chapters if the same or similar methods could be transferred to different *in vitro* systems (rodent cells, co-cultures), but we will also explore the practical strategies for EV-isolation and characterization that were applied *ex vivo* and in the clinic from patients with liver-related conditions (chapter 8). It is worth noting, that we are only covering method employment and it is not our role to comment on the quality of the results gained from them. As to the nomenclature, we strived to keep it as it was applied in the referenced manuscripts, only changing it to “EVs” if we needed to paraphrase, since it is the preferred generic term.[1,2]

Table 1: Overview of the methods for liver-derived EVs.

Related Chapter		EV origin	FBS handling	Isolation techniques	Yield	Storage	Analysis/ Characterization
[*In vitro*/Cell culture]							
2	**h-Hepatocytes**	Hep3B,[27–35] HepG2,[27–33,36–50] PLC/PRF/5,[28,29,40,51] 97H,[35,52] LM3,[35,38,52,53] Huh6,[27] Huh7,[a] Huh-7.5,[54,55] Bel-7402,[50] Q64-7703,[37] MH CC92-H,[37] LO2,[37,52] LSQT-2,[38] MHCC97L,[38] SMMO7721,[38] HKCI-C3,[56] HKCI-B [56]	UC-dep,[b] ExoFree-FBS,[36,57] SSt (12, 24, 72 h)[c]	dUc,[d] filtration,[e] ExoQuick™,[f] Total Exosome Isolation™ (TEI),[43,53] density gradient UC,[g] sucrose cushion UC[30,58]	protein content,[28,43,59,60] relative particle number,[h] particle number per number of cells,[35,56] molar concentration[27]	at 4 °C for no longer than 48 h,[30] at -70 °C,[39] at -80 °C,[i] dried EV-pellet at -80 °C[27]	TEM,[j] immunogold TEM,[k] flow cytometry,[l] western blot,[m] NTA,[n] AFM,[27] DLS,[o] BCA assay,[p] Bradford assay,[33,43,56–58] RNA,[q] proteomics,[32,35] custom colorimetric nanoplasmonic assay (molar concentration)[27]
	r-Hepatocytes	primary rat hepatocytes,[32,41,61] primary mouse hepatocytes,[62–66] Hca-F,[67] Hca-P,[67] H22,[68] IMH[69]	n/f	dUc, density gradient UC filtration	protein content, relative particle number, particle number per number of cells	n/f	TEM, DLS, zeta potential, western blot, fluorescence microscopy, qRT-PCR, proteomics, flow cytometry NTA
3	**h-HSCs**	LX-2[70–73]	SSt (16, 48 h),[71–73] ExoFree-FBS[70]	dUc,[72,73] ExoQuick™[70]	relative particle number[73]	n/f	NTA,[73] DLS,[72] zeta potential, [72] RNA, [70] TEM,[71–73] western blot[72,73]
	r-HSCs	primary rat HSCs,[74] primary mouse HSCs,[72,75–77]	UC-dep,[76] SSt (16, 48 h)[74]	dUc,[r] density gradient UC,[20,74] TEI[77]	relative particle number[75,76]	at 4 °C and used within 72 h[74]	TEM,[20,72,74–76] DLS,[20,72] zeta potential,[72]

		rat HSC-derived PMF[20]					western blot,[20,72,75,77] fluorescence microscopy[76] qRT-PCR,[77] proteomics,[20,74,78] flow cytometry,[20] RNA[74]
4	**h-Cholangiocytes**	EGI1,[79] TFK-1,[79] H69[80]	UC-dep,[80] SSt (48 h)[79]	dUc, [79][80] filtration[79]	relative particle number[79][80]	at -80°C[79]	NTA, [79]·[80] TEM, [79]·[80] proteomics, [79] western blot[79]
	r-Cholangiocytes	603B,[74] primary mice cholangiocytes[81]	UC-dep,[81] SSt (16 h)[74]	dUc[74] density gradient UC[74]	n/f	n/f	TEM, [74][81] RNA[74] Protein analysis[74] DLS,[81] RNA[81]
5	**h-LSECs**	TMNK-1[82]	n/f	ExoQuick, [82] dUc[83]	protein content [82],[83]	n/f	flow cytometry, [82] TEM, [83] immunoblot, [83] Bradford assay, [83] BCA assay, [82] RNA[83]
	r-LSECs	TSEC[84]	UC-dep then 20% FBS medium sterile filtration[84]	n/f	relative particle number, [84] protein content[84]	n/f	NTA, [84] western blot, [84] TEM,[84] Bradford assay[84]
6	**h-Kupffer Cells**	THP-1,[83,85,86] monocyte-derived macrophages[87]	ExoFree-FBS[87]	dUc, [84,85] filtration,[85] ExoQuick, TEI[87]	relative particle number,[85] protein content[84]	n/f	NTA,[85] EM, [84,[87] western blot, [84,[87] Bradford assay,[84] RNA[84]
	r-Kupffer Cells	mice hepatic macrophages[36,65,66]	UC-dep[35]	dUc, [35,64,65] sucrose gradient[64,65]	relative particle number[64,65]	n/f	NTA[64,65] flow cytometry,[35] DLS,[35] TEM,[35] RNA[35]
7	**h-Liver Stem Cells**	HLSCs[88–97][3]	SSt (24 h)[163–173]	dUc,[163–173] custom charge-based precipitation method, [98] size exclusion chromatography[97]	relative particle number[163–173]	at -80°C with 5% (v/v) DMSO[88–97]	NTA, [88–97] TEM, [88–97] western blot, [88–97] RNA, [88–97] Raman spectroscopy,[97]

							flow cytometry[88–97]
	r-Liver Stem Cells	Thp1+ cells[99]	n/f	dUc[99]	Spectrophotometry[99]	n/f	Spectrophotometry[99]
[*ex vivo*/Clinic]							
8	**human**	blood (circulation), [100–159] blood (liver), [160] bile[161] intraoperatively[162]	n/f	dUc[s)] ExoQuick or TEI[t)]	protein content, relative particle number	at -80°C	NTA, TEM, MS, western blot
	rodent	blood (circulation) [215–251]	n/f	ExoQuick	relative particle number	at -80°C	TEM, RNA Protein analysis, DLS

Footnotes

a) [27,34,47,53,57,63,69,180,188,205–207]
b) [27–29,32,37,38,40,52,53,56,57,63,69,206,208]
c) [45,48,49,51,64,205,209,210]
d) [27–33,35–38,40–42,45,46,48,51,56–58,63,64,69,167,188,205,206,211]
e) [30–33,51,54,56,167]
f) [39,44,45,50,54,55,209,210,212]
g) [41,42,45,56,188]
h) [38,45,46,63,64,69]
i) [28,29,31,37,40,50,51,53,63,69,206,213]
j) [28,29,33,35,37–40,47–50,53,60,69,188,208]
k) [30,31,41,46,52,56,58,180,214]
l) [28,40,41,47–49,54,213,215]
m) [27,33,39,40,45,46,53,56–59,69,216,217]
n) [32,35,37,38,45,46,51,60,63,64,69,167,179,207,218]
o) [36,41,43,48,53,57]
p) [27,30,31,39–41,48,50,53,54,59,60]
q) [28,34,36,51,57,179,207,212,216,218]
r) [20,72,74,76,78,219]
s) [32,36–38,62,63,83,220]
t) [70,77,86,209,221]

Abbreviations

h: human
r: rodent (either mouse or rat)
dUc: differential centrifugation including UC
SSt: serum starvation
UC-dep: depleted of EVs by UC
n/f: not found (either non-applicable or not disclosed)

AFM: atomic force microscopy
BCA: bicinchoninic acid
DLS: dynamic light scattering
MS: mass spectrometry
NTA: nanoparticle tracking analysis
TEM: transmission electron microscopy
qRT-PCR: quantitative real-time polymerase chain reaction

2. EVs from Hepatocytes

Hepatocytes are the most abundant cells in the liver, comprising about 70-80% of its mass, they are dedicated to protein synthesis (serum albumin, transferrin and other glycoproteins), while also being the main site for glycolysis and only site for bile salts production in the body.[222–224] Another essential task relying on hepatocytes is detoxification: they metabolize both exogenous (e.g., drugs, toxins) and endogenous (e.g., steroids) compounds.[225,226] More notable still is their ability to regenerate the injured liver.[227] Given their prominent presence, their multifaceted roles, and their direct association to many liver diseases (fatty liver, chronic hepatitis, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, liver fibrosis, hepatocellular carcinoma),[228] it is not surprising that they have been the most frequent focus of research involving liver-derived EVs so far.

2.1 Isolation strategies

EVs have been isolated from both cryopreserved primary hepatocytes[54,60,179,212] as well as from cell lines (see Table 1).

Berardocco et al. 2017[27] isolated EVs from Hep3B, HepG2, Huh7 and HuH6 human hepatocarcinoma (HCC) cell lines by differential centrifugation: first cells were removed (300 x g, 10 min), then apoptotic bodies (2'000 x g, 30 min), and finally cell organelles (16'000 x g, 20 min). EVs were then pelleted by ultracentrifugation (UC; 120'000 x g for 70 min) and the EV-enriched pellet was resuspended in PBS and purified by repeating the UC step. Everything was performed at 4 °C, and the final EV-pellet was drained, rapidly frozen in liquid nitrogen and stored at -80 °C before analysis. Cells were grown in cell culture medium supplied with 10% FBS, but EVs were isolated after having it exchanged with medium containing 1% FBS and 0.25% human serum albumin (hBSA), both of which had been previously depleted of EVs by UC (120'000 x g for 5 h). We appreciated the care that was taken to depleting FBS and hBSA of EVs prior to use; experimental corroboration on the chosen protocol was missing, yet it has been previously reported that even 18 h long UC is not necessarily effective to remove all FBS-EVs.[229]

As to the isolation of the hepatocyte derived EVs themselves, other groups worked similarly, but preferred different centrifugation times and forces (e.g. Cannito et al. 2017:[48] 3'000 x g for 15 min, then UC at 100'000 x g for 90 minutes at 10 °C). Why some numbers were chosen over others is rarely explicitly justified: sometimes it is disclosed that previously reported methods have been adopted (e.g. Fang T. et al. 2018[38] citing Lässer et al. 2012[230]), and oftentimes published practices were adapted without explaining what prompted the tweak. A direct experimental validation could not always be found, which is a major weakness of these protocols.

He et al. 2015[56] added a filtration step (0.22 μm) that followed their differential centrifugation (500 x g for 10 min, then 16'500 x g for 30 min) and preceded the UC (110'000 x g, 70 min). After this, the EV-pellet was washed and purified in PBS by performing a UC step on a 40% (w/v) sucrose cushion, which

was followed by yet another UC wash in PBS. The protocol seemed to address the concern that UC can lead to co-isolation of other impurities (protein aggregates, lipoproteins) more carefully.
Others avoided UC by favoring commercially available precipitation kits such as ExoQuick™[50,54,216] and Total Exosome Isolation™ (TEI).[43,58] These kits have been suggested to be more easily translated into clinical settings because not every hospital has an ultracentrifuge, the required accessories, and the specifically trained personnel, and they occasionally yield more and even more pure EVs,[231] although there are still conflicting reports about it.[232,233]However, they come with their own set of perplexities: different precipitation kits are commercially available, but their composition being proprietary information makes it hard to evaluate their efficacy in excluding contaminants and in their ability to work with EVs from any cell type.
Yet another approach was to opt for membrane affinity spin columns such as ExoEasy Maxi™, opted by Cao et al. 2019.[167]
It is worth pointing out that the storage strategy is not always disclosed, let alone the validation thereof. Similar to Berardocco et al. 2017, EV pellets were stored at -80 °C by other groups too,[28,29,31,37,40,50,51,53,63,69,206,213] mostly after re-suspending them in PBS (when the final volume is shared, it tends to be between 50 and 500 µL), or at -70 °C.[39] We particularly appreciated the caution of Xiao et al. 2010,[30] who stored EVs at 4 °C for no longer than 48 h before use.

2.2 Characterization

When evaluating the quality of the isolation methods, we would like to point to the approach of Thacker et al. 2018,[45] who tested different protocols, directly comparing them to UC: ExoQuick™ was shown to lead to the highest co-purification of extravesicular contaminants, while OptiPrep™ yields were deemed too low. They describe culturing HepG2 in T75 flasks, 2.1 million cells/mL in 10 mL at seeding, and harvesting EVs after 2-3 days, starting from 4 mL of cell culture medium and calculating back the number of particles/mL for yield comparisons (10^{11} p/mL by ExoQuick™, 10^{10} p/mL by UC). It would have been beneficial to report the number of cells and their viability at the time of medium collection to use the method performances for future comparisons of protocol efficiency, not only efficacy. This would allow to find whether EVs can be isolated by a certain approach, but also to evaluate how many EVs can be retrieved from a specific number of cells, and how viable the cells were at the time of EV collection to minimize concerns about isolation of apoptotic bodies. Tentative quantification by other groups, when reported, was sometimes shown as relative release after measuring concentration by nanoparticle tracking analysis (NTA),[46,63,234] better still by normalizing it to cell number.[56,210] Others favoured protein quantification[31,37,43] (even normalizing it to a number of cells[60]), or counting EVs as seen by EM.[52] Little attention was given to explicitly stating the cell viability; exceptions include Eguchi et al. 2017,[36] reporting on 90.5 - 94.5% cell viability, and Xiao et al. 2010[30] documenting 95%.
The intrinsic uncertainty associated with isolation and purification techniques makes the characterization steps all the more important. We found Berardocco et al. 2017,[27] again to be thorough in their approach using gel electrophoresis for protein profiling, atomic force microscopy (AFM) for morphology and size, and a previously developed colorimetric nanoplasmonic assays (see Maiolo et al. 2015[235]) to evaluate EV-purity and concentration. Protein content determination was performed by bicinchoninic

acid (BCA) assay and western blot analysis was done for EV and non-EV markers (GM130, calnexin, Hsp70, CD63, CD9, TGM2, EpCAM, E-cadherin, LGR5 and β-actin). Total RNA from EVs was purified using the Fatty Tissue RNA Purification kit, SOLiD Library preparation, sequencing and bioinformatic analysis was done by GENOMNIA.

Other groups have favoured dynamic light scattering (DLS) for EV-size determination,[36,48,57] tuneable resistive pulse sensing (TRPS, i.e. qNano™),[56] nanoparticle tracking analysis (NTA; for size and concentration),[37,38,49] transmission electron microscopy (TEM)[28,29,33,35,37–40,47–50,53,60,69,188,208] (sometimes combined to immunogold staining of EV markers),[30,31,41,46,52,56,58,180,214] or cryo-TEM (for size and morphology)[187,193,194,236] and even scanning EM (SEM).[43] Quantification and marker expression analysis of EVs by flow cytometry was also done[28,40,41,47–49,54,213,215] (e.g. Cobb et al. 2018,[54] using a kit for exosome immunocapture and colorimetric quantification). Proteomic analysis on hepatocyte-derived EVs was performed to various degrees by several groups, we will only mention a few;[35,61,174] the same applies to total and specific RNA evaluations.[36,51,52,216] Lipidomics characterization was found in the study by Kakazu et al. 2016,[69] who looked at ceramides and non-esterified Fas (cell surface death receptor) by mass spectrometry (MS). Finally, Wu et al. 2018[60] reported an EV zeta potential between -20 and -30 mV.

2.3 Interspecies method transferability

Different groups have retrieved hepatocyte-EVs originating from mice and rats and we would like to highlight those providing comparison to human cells.

Povero et al. 2013[41] compared HepG2 to rat-derived hepatocyte-EV, isolating them by differential centrifugation (including UC) and, for selected experiment, further purified them by 10 to 70% sucrose gradient UC (150’000 x g, 18 h, 10 °C). Eguchi et al. 2017[36] had isolated by UC EVs from HepG2 and from primary mice hepatocytes, as well as blood-EVs from mice plasma. A few researchers used the Huh7 cell line, again looking at EVs released from mice hepatocytes after isolating them by UC.[63,69,180] When looking at increasing the complexity of the analyzed system without going *in vivo* still, we would like to single out Dioufa et al. 2017,[237] for using their own *ex vivo* human liver microphysiological system (MPS), developed with primary hepatocytes and non-parenchymal cells (NPCs) from the liver. EVs from the liver MPS were isolated by polymeric precipitation with the TEI kit, and they were then analyzed by TEM and for total RNA, as well as for cDNA and miRNA; exosomes markers were evaluated by western blot using the Exo-Check™ exosome antibody array). More interesting still, some managed to compare human hepatocytes cell lines to primary rodents cells as well as samples obtained from human donors within the same study (see chapter 8).[32,62,63]

3. <u>EVs from Hepatic Stellate Cells</u>

Hepatic stellate cells reside in the space of Disse (Figure 2), between hepatocytes and endothelial cells, and are mainly responsible for storing vitamin A in cytoplasmic lipid droplets.[238] Upon liver injury, however, they undergo transdifferentiation into a myofibroblast-like state, i.e., they become activated, progressively lose their lipid droplets and start promoting fibrogenesis, most notably by deposition of excessive and collagen rich extracellular matrix.[239,240] When the injuries are repeated during chronic diseases and the fibrosis is not resolved, the excessive deposition of scar tissue eventually leads to cirrhosis and the loss of organ function. The pivotal role of HSCs in liver fibrosis makes these cells crucial therapeutic and diagnostic targets.[241]

3.1 Isolation strategies

For the *in vitro* isolation of HSC-derived EVs, a common cell line seems to be the LX-2, which has the advantage of being able to grow under serum free conditions.[242]

Brandon-Warner et al. 2016[70] isolated EVs from cell culture medium using the ExoQuick™ precipitation kit, after culturing the cells with 10% of commercially available Exo-free FBS. Information about cell number and viability at the time of EV-collection and the total yield are missing, making it hard to judge the efficiency of their method. Purification steps after this were not performed and it is not specified how EVs were stored between isolation and analysis.

Serial centrifugation was the preferred method for Chen L. et al. 2014, Charrier et al. 2014 and Wang et al. 2018.[71,72,243] Cell culture medium for EV-isolation had been prepared without FBS, and the LX-2 cells were serum-starved for 48-72 h but a measure of cell number, density and viability is unfortunately missing. The specific isolation protocols were found inconsistent, because Charrier et al. 2014 refer their readers to Chen L. et al. 2014, which in turn cite Thery et al. 2006,[244] an overview of different protocols for the isolation of EVs. While the projects described in these articles were not focusing on the EVs isolated from HSCs, more descriptive protocols would better facilitate reproducibility. Wang et al. 2018[243] adapted an "exosome" isolation procedure based on cholangiocytes and previously reported in Li L. 2016,[245]. Unfortunately, Li et al. refer to yet another isolation protocol (Li L. et al. 2014,[155] see chapter 8) which makes it challenging to compare them.

3.2 Characterization

Brandon-Warner et al. 2016[70] investigated total RNA, which was isolated using the SeraMiR™ exosome RNA purification kit, then quantified and analysed it to compare differences in the expression levels of individual miRNAs in cells and exosomes.

Charrier et al. 2014[71] characterized their isolated EVs by electron microscopy, expression of key markers and size, based on protocols from Thery et al. 2011[246] and Chen et al. 2014.[72] The latter explains their characterization steps for HSC-EVs isolated from mice, adding that "similar procedures" were used for EVs originating from LX-2 cells. They performed western blots (CD9), TEM, DLS analysis, zeta potential measurements, and analysed exosomal and cellular RNAs for the presence of miR-21, which was determined by real time polymerase chain reaction (RT-PCR).

While not as frequently explored as hepatocyte-derived EVs (see chapter 2), HSC-EVs have been analysed by a diverse array of means. Reporting of the protocols frequently relied on additional references to be looked up across different papers, which can complicate the ease with which reported methods could be reproduced.

3.3 Interspecies method transferability

There are even more examples for research involving murine and rat HSC-derived EVs than their human counterpart. In Chen L. et al. 2015,[78] Chen L. and Brigstock 2016[76] and Chen L. et al. 2016[75] EVs were purified as described in Chen L. et al. 2014[72] (differential centrifugation and UC, referring the reader to Thery et al. 2006[244]) from primary mice cells. EVs were analysed by NTA, and imaged by cryo-TEM. Exosomal mRNA was determined by quantitative RT-PCR, while cellular EV-uptake was observed by confocal microscopy (EVs were stained with PKH26). Exosomal proteins were evaluated by western blot. Within this study (Chen et al. 2014), they also looked at circulating EVs but changed their isolation method. EVs were harvested from murine sera using PureExo Exosome Isolation Kits. Total exosomal RNA from sera was prepared using miRNeasy mini kits.
Povero et al. 2014[20] and Witek et al. 2009[74] used primary rat HSCs: the former opted for differential centrifugation (including UC, as applied to HepG2, see chapter 2), while the latter preferred polymeric precipitation (TEI). Lambrecht et al. 2017[77] evaluated EVs from mice HSCs and those found in human plasma (see chapter 8).
A rigorous parallelization of isolation and characterization practices is missing, but research with HSC-derived EVs has been steadily gaining attraction, and their biochemical properties as analysed from rodent models are being increasingly explored in more complex settings.

4. <u>EVs from Cholangiocytes</u>

Cholangiocytes are epithelial cells lining the bile ducts (Figure 2), which can differ in size and morphology just as the bile duct tree itself does. Their main physiological role is the modification of the bile coming from the liver while it is being transported along the biliary ducts into the intestine.[247–250] Pathologies directly associated with cholangiocytes include primary biliary cholangitis, and primary sclerosing cholangitis, for which liver transplantation is the only available cure. Primary sclerosing cholangitis also leads to different cancers collectively known as cholangiocarcinoma.[250–252]

4.1 Isolation strategies

Cholangiocyte-EVs were preferentially isolated by serial centrifugation followed by UC. Sato et al. 2017[80] used the H69 cell line, cultured with FBS that had been depleted of EVs by UC (120‘000 x g, 18 h). For EV isolation, they first removed cell debris (300 x g for 10 min, then 3’000 x g for 30 min), after which they filtered the supernatants (0.22 µm) before UC (120’000 x g, 3 h). EV-pellets were then

washed with 30 mL PBS, pelleted again by UC (120’000 x g, 2 h) and re-suspended in 0.5 mL PBS. The adopted strategy of performing a filtration followed by PBS washes indicates a concern EV-purification. The description of the methods shows an attention to details that enable reproducibility, such as sharing the volume of PBS used to re-suspend the final pellet, which also helps put the yield as shown in the NTA distribution profiles into perspective.

A slightly different approach was found in Arbelaiz et al. 2017,[79] which used the H69, EGI1 and TFK-1 cell lines as well as patient blood (see chapter 8). Cell culture medium was centrifuged (1’500 x g, 15 min, 4 °C), the supernatant filtered (0.22 µm), and centrifuged again (10’000 x g, 30 min, 4 °C) before two UC steps (100’000 x g, 75 min, 4 °C). Samples were store at -80 °C. It was not possible to extrapolate an absolute yield making comparison of EV-retrieval difficult.

4.2 Characterization

To determine the size and concentration of the isolated EVs, NTA and TEM were performed both by Sato et al. 2017 and by Arbelaiz et al. 2017; however, the latter additionally analyzed the EVs’ protein profile by mass spectrometry, and evaluated the expression of different EV-markers (CD9, CD63, CD81) by western blot. Research articles about cholangiocyte-derived EVs are more limited in numbers rather than in quality, already providing fruitful isolation protocols and an ample baseline of analytical characterizations to build upon.

4.3 Interspecies method transferability

Centrifugation and UC proved to be a viable strategy for the isolation of EVs from mice cholangiocytes too. Witek et al. 2009[74] performed it when working with the 603B cells, after 16 h of serum starvation by first removing cells and debris (2’000 x g, 15 min, twice) and then pelleting of EVs by UC (50’000 x g, 45 min, twice). For selected experiments they would add a purification step by sucrose gradient centrifugation (100’000 x g, 15 h). Fresh samples were analyzed for proteomics or they were stored at 4 °C for no longer than 72 h before performing TEM and RNA analysis.

Li X et al. 2018[81] worked with murine large and small cholangiocytes (MLEs, MSEs) and they cleared conditioned cell culture medium from cell debris by centrifugation (2’000 x g for 15 minutes followed by 16’000 x g for 20 min, both at 4 °C), and pelleted EVs by UC (100’000 x g, 70 min, at 4 °C). EVs were resuspended in sterile PBS and stored at -80 °C for further analysis. EV-size was determined by DLS and confirmed by TEM, and levels of mRNA H19 were assessed. Interestingly, they also isolated EVs from mice (and human) sera too, as further described in chapter 8.

5. <u>EVs from Liver Sinusoidal Endothelial Cells</u>

Liver sinusoidal endothelial cells (LSECs) form the fenestrated endothelial layer at the interface between cells in the blood and the HSCs in the space of Disse. LSECs act as efficient pinocytotic scavengers for

particles that are smaller than 0.2 µm, making them the central players in clearing the body of blood-borne viruses.[253,254] When working with EVs from LSECs, researchers were interested infections caused by hepatitis B and C viruses (HBV and HCV).

5.1 Isolation strategies

EVs were isolated from primary LSECs and from the immortalized TMNK-1 cell line by Giugliano et al. 2015,[82] using the ExoQuick™ precipitation kit.

Li J et al. 2013[83] preferred serial centrifugations: 300 x g for 10 min, 2'000 x g for 10 min, 10' 000 x g for 30 min; EVs were then pelleted by UC (100'000 x g for 70 min), washed in PBS and pelleted by UC again (100'000 x g, 70 min). For selected experiments sucrose density-gradient centrifugation was performed.

5.2 Characterization

The characterization of LSEC-EVs in Giugliano 2015 was limited to the determination of total protein content by BCA and to detection using CD63-labeled Dynabeads. Beadbound EVs were labeled with anti-CD63-PE and anti-CD81-PerCP-eFluor 710 and finally quantified by flow cytometry.

Li J et al. 2013 quantified the protein content by Bradford assay, and characterized the EVs by electron microscopy, by immunoblot for exosomal (CD63, TSG101, Alix, LAMP2, β-actin, Hsp90) and non-exosomal markers (GRP94, EEA1, Cytochrome C), and by microarray analysis of exosomal mRNA and miRNA.

While restricted to a few research articles, EVs from LSECs have been successfully purified and characterized. Follow up research could expand the field by providing more insights into optimized storage strategies or yield determination practices that would allow for comparisons across studies.

5.3 Interspecies method transferability

When looking at the protocols applied to studying LSEC-derived EVs from rodents, Wang et al. 2015[255] compared EVs isolated from a mice immortalized LSEC cell line (TSEC[256]) to murine serum-derived exosomes. The description of isolation and purification methods referred the reader to Huebert et al. 2010,[256] Thery et al. 2006,[244] Tu et al. 2015,[257] whose protocols were not related to EVs from LSECs.

LSECs were cultured in medium with 10% FBS, which was prepared with 20% FBS first and then depleted of FBS-EVs by UC and sterile filtration. EVs were characterized by NTA, immunogold-EM (CD81, TfR, CD63) and western blot. They also mention using 50 µg of exosomes per experiment, as determined by Bradford assay, although an exact number of cells required to achieve such a yield was missing, making it challenging to analytically compare method efficiency across studies.

6. EVs from Kupffer Cells

The resident macrophages in the liver, found in the hepatic sinusoid, are known as Kupffer cells.[258] Since the liver is frequently in contact with exogenous material, suppressing “unwanted” immune responses is essential. Kupffer cells have been shown to provide anti-inflammatory signals that allow homeostatic immunological tolerance under healthy conditions. As part of the innate immune system, these macrophages phagocyte invading pathogens and play a critical role for the initiation of immunological responses when inflammation and recruitment of other cells becomes required by the triggered defence mechanism.[259–261] Dysregulation of these processes is directly connected to pathologies discussed in previous chapters, because Kupffer cells communicate through EVs with hepatocytes, HSCs, Cholangiocytes and LSECs.

6.1 Isolation strategies

Aucher et al. 2013[85] chose the THP-1 cells as their model for liver macrophages, collecting their apoptotic bodies and exosomes. The isolation methods was adapted from Hristov et al. 2004[262] with 800 x g for 10 minutes to remove (endothelial) cells and then pelleting of apoptotic bodies at 16’500 x g for 20 min. For the isolation of the exosomes it cites the work of Mittelbrunn et al. 2011.[263] The latter stated that donor cells (T cells) were cultured with 10% FBS (depleted of bovine EVs by UC at 100’000 x g overnight). Still Mittelbrunn et al. 2011 explain how the conditioned cell culture medium was cleared by centrifugation (320 x g, 5 min); the resulting supernatant was filtered through 0.22 µm membranes, and EVs were finally pelleted by UC (100’000 x g, 60 min, 4 °C). While the methods could be extrapolated, the reliance on references for method description sometimes risks to undercut all the work that was done. Ideally, the methods for EV isolation should also be experimentally validated for every new cell type regardless.

Li J et al. 2013[83] and Saha et al. 2016[86] also used THP-1 cells to model Kupffer cells, as well as primary human monocytes. Saha et al. 2016 (who cultured cells using commercially available ExoFree-FBS™) opted for EV-precipitation with ExoQuick™, which was performed after two centrifugations (1’500 x g for 10 min, then 10’000 x g for 20 min) followed by one filtration (0.8 µm) step.

Polymer precipitation was the strategy for Zhou et al. 2016,[87] although TEI kit was preferred. The cells that were used were purified human monocytes were obtained grown in 10% exosome-free FBS.

6.2 Characterization

Saha et al. 2016 analyzed THP-1-derived EVs by NTA for size and concentration, and determined the protein content by Bradford assay. Li J et al. 2013[83] analyzed protein content by Bradford as well, but they additionally performed EM, immunoblot analysis of exosomal markers, and microarray analysis of exosomal mRNA and miRNA.

Zhou et al. 2016 characterized their isolated EVs by EM, western blot for both EV and non-EV markers (Alix, LAMP2, cytochrome c, HSP70, CD63, GAPDH) and immunofluorescence; macrophage-EVs were labelled with the PKH67 dye, then given to Huh-7 cells and observed by fluorescence microscopy.
Future reports about EVs from Kupffer cells can expand the existing literature by providing additional insights into storage and yield optimization practices.

6.3 Interspecies method transferability

While Eguchi et al. 2017[36] had been very thorough in their analysis of hepatocyte-EVs (FACS, DLS, TEM, miRNA, see chapter 2), they also took a look at the EVs shed by hepatic macrophages they isolated from mice, and compared the two in terms of morphology (TEM) and concentration (FACS).
Nojima et al. 2016[65,66] reported working with EVs originating from mice Kupffer cells. The EVs were isolated by differential centrifugation (300 x g for 10 min, then 16’000 x g for 30 min), after which the supernatant was filtred through a 0.22 µm membrane. EVs were subsequently collected after two UC steps (120’000 x g for 70 min). The EV-pellet was re-suspended in PBS and purified on a Tris/sucrose/D2O density cushion UC (100’000 x g for 90 min). EVs were then transferred into a new tube, and collected after one final UC step (120’000 x g for 70 min). EV yields under different conditions were precisely reported, as well as the activity of neutral ceramidase and sphingosine kinase.

7. EVs from liver stem cells

Several studies have suggested the presence of liver-resident stem cells, which along with hepatocytes contribute to liver regeneration, but it remains a controversial topic, as reviewed elsewhere.[264] The identification of oval cells and their role in liver regeneration contributes to our current understanding of the process,[265] more confidently so after establishing their precursor role to hepatocytes,[266] and their localization in the canals of Hering[267] between bile capillaries and interlobular bile ducts (Figure 2). A population of more committed hepatic stem/progenitor cells known as small hepatocytes had been first observed in rat cells in 1992,[268] and subsequently isolated from both adult rats[269] and humans.[270]
Herrera et al. 2006[271], were able to isolate a human liver stem-like cell (HLSC) population expressing markers of mesenchymal (but not hematopoietic) stem cells from the tissues of adult human livers and from cryopreserved primary human hepatocytes. HLSCs showed the ability to self-renew and to differentiate into osteogenic, endothelial and insulin-producing cells, while starting with a partial commitment to the hepatocyte lineage. Since 2006, it has been regularly reported on HSLC and HLSC-derived EVs.[88–97]

7.1 Isolation strategies

For the isolation of EVs described in the following paragraph, HLSCs were cultured in the absence of FBS prior to cell culture medium collection, and their documented viability was between 97 and 99%, obviating concerns about co-isolation of FBS-EVs and limiting the presence of apoptotic bodies.

The article by Herrera et al. from 2014[88–97] exemplifies the group's general preference of differential centrifugation (including UC)[88–97] for EV isolation: after performing two centrifugations at 3'000 x g for 20 min to remove cells and debris, EVs were isolated by 2 h UC (100'000 x g, 4°C). EVs were then either used fresh or after storing them at -80 °C (resuspended in RPMI with 5% (v/v) dimethyl sulfoxide). They provided precise storing conditions and reported no differences in the biochemical activity between freshly used and stored EVs.

Variations of this protocol can be found in other articles published by this group. For example, Herrera had opted for a single centrifugation at 2'000 x g for 20 min followed by two UC steps (100'000 x g) of 1 h each in 2010,[90] and then modified the protocol again to one centrifugation at 3'000 x g for 20 min, a second one at 10'000 x g for 1 h, and a final UC (100'000 x g) for 1 h.[92] Gualerzi et al. 2019[97] added a size exclusion chromatography (SEC) purification step.

Interestingly, Deregibus et al. in 2016[98] proposed an alternative approach by developing a custom charge-based strategy for EV isolation, in which they use protamine and PEG 35 kDa.[98] A comparison with commercially available precipitation kits was not performed, but their method yielded results that were comparable to the UC approach in terms of EV-quality, and higher in terms of isolated EV-quantity (not only in cell cultures but also in serum and saliva samples, see chapter 8).

7.2 Characterization

Herrera et. al 2014 offers many of the characterization steps of HLSC-EV. Size distribution was determined by NTA. To trace EVs by fluorescent microscopy, EVs were labeled with 1 µM Dil dye. Cytofluorimetric analysis was done using fluorescein isothiocyanate, phycoerythrin or allophycocyanin conjugated antibodies (CD73, CD44, CD105, CD90, CD107, CD63, CD29, CD81, CD146, HLA-class I). They also performed FACS after absorption on beads by incubating 10 µg EVs with latex beads then with the aforementioned antibodies. EV protein content was quantified by Bradford assay; immunoblotting was also performed (CD63, CD81, Alix and Hsp9).

Additional characterization for HLSC-EVs include zeta potential, TEM and analysis of RNA (Deregibus et al. 2016), as well as a novel method based on Raman spectroscopy for purity determination established by Gualerzi et al. 2019.[97] The method used Raman spectra to measure the protein-to-lipid and nucleic acid-to-lipid ratios.

7.3 Interspecies method transferability

Liver stem cell EVs originating from rats have been described by Ichinohe et al. in 2017.[99] The liver resident stem/progenitor cells used were isolated by their research group before[272] (Thy1-positive cells[273]), but it is difficult to directly compare them to the HLSCs because there are no studies comparing their EVs. EVs from Thy+ cells were isolated using the commercially available precipitation kit ExoQuick™, and the described characterization was limited to EV quantification by NanoDrop 1000

spectrometer, since they administered 1.6 µg of EVs to different liver cells and look at their effect on cell morphology, proliferation and IL17rb receptor expression.

8. EVs from clinical settings

There has been extensive research delving into the diagnostic potential of EVs in the context of liver-associated conditions, albeit mostly looking into circulating vesicles, both in human patients[100–159] and in rodents.[215–251] The increased complexity of the system makes it difficult to trace their origin back to a specific cell type, but first efforts have been made to make that connection. We will highlight here research that endeavored to connect previously discussed *in vitro* settings to *ex vivo* findings.

8.1 Isolation strategies

Liver-derived EVs have been recovered *in* and *ex vivo* from liver blood directly,[160] bile[161] and even from cancer cells that were extracted intraoperatively.[162] The isolation of EVs from human patients mirrored protocols developed for cell culture systems, with differential centrifugation (including UC)[32,36–38,62,63,83,220] and polymer precipitation (ExoQuick™ and TEI)[70,77,86,209,221] being the most prevalent approaches. Samples collected from blood circulation are consistently stored at -80 °C, mostly after depleting them of cells and platelets.

8.2 Characterization

Typically, the analysis of circulating EVs is focused on the biomarkers of interest. For example, Sohn et al. 2015[136] analysed the expression levels of serum exosomal microRNAs (miR-18a, -21, -93, -106b, -221, -222 and -224, -101, -122 and -195) of patients suffering from different liver conditions including chronic hepatitis B, liver cirrhosis and hepatocellular carcinoma. This also tends to happen when looking at specific cells. For example, Brandon-Warner et al. 2016,[70] who collected EVs from HSCs *in vitro* as well as from human plasma, performed RNA isolation using exoEasy™ serum and plasma kit, followed by QiaZol total RNA purification; for exomes they only show miR data.

8.3 Interspecies method transferability

Nojima et al. 2016[66],[65] isolated EVs from primary mice hepatocytes and Kupffer cells *in vitro* by differential UC and sucrose gradient, while in the same studies describe also mice serum-derived EVs (even from cardiac puncture[65]) being isolated by ExoQuick™. The isolation strategies were not transferred from *in vitro* to *ex vivo* in these cases, but the characterization practices (DLS for size, CD81-antigen-ELISA for quantification) were.
Lambrecht et al. 2017[77] evaluated the levels of miRNA-122, -150, -192, -21, -200b, and -92a by qRT-PCR from human plasma samples as well as from primary mice HSCs. In both instances, EV isolation

was performed by TEI. Duan et al. 2019[62] compared compared findings from primary rat hepatocytes and human plasma. A wider comparison was performed by Hirsova et al. 2016:[63] Huh7 cells and primary mice hepatocytes both served as *in vitro* models in addition to their research with *ex vivo* human samples. In their research with cholangiocytes, Li X. et al. 2018[81] looked at EVs isolated from primary mice cells *in vitro*, but took it a step further *ex vivo*, evaluating both murine and human sera-derived EVs. Their methods included differential centrifugation (with a UC step) for EV-isolation, DLS and TEM for size analysis and assessment of mRNA H19 levels. Cho et al. 2017[32] isolated EVs from a variety of samples as well: human cell lines (HepG2, He3B), rat primary hepatocytes, human sera, rat sera. While differential centrifugation worked *in vitro*, they noted how extra steps were required *ex vivo* to reduce contamination with plasma proteins (e.g., albumin). EV isolation from plasma samples was thus optimized by comparing three alternative methods: density gradient UC (30% Optiprep), ExoQuick™, and an optimized ExoQuick™ protocol, which included 3 washing steps. Deregibus et al. 2016[98] were perhaps more interesting from a methodological transferability perspective, since they compared the performance of a custom charge-based precipitation method to differential UC, using samples derived from HLSCs (see chapter 7), human serum and human saliva.

9. Final general remarks

Strategies for liver-derived EV isolation and characterization are as varied as the research groups tackling the challenge. While the main advantages and disadvantages for each approach have been presented, we would like to highlight some of the key aspects that emerged.

FBS handling. Cell culture approaches have become indispensable to simplify EV research before diving into significantly more complex *ex vivo* samples, but most cell lines require FBS for optimal growth. Depriving them of it might easily result in additional stress that will affect results to an unpredictable extent. Alternatives to FBS have already been proposed, such as chemically defined media or human platelet lysate[274] and these may replace FBS-supplemented cell culture models.[275] Cells can be successively deprived of FBS to limit the impact of outright elimination of it, but more frequently than that, research groups opt to depleting their FBS of EVs by ultracentrifugation prior to use or they directly use commercially available ExoFreeFBS. The experimental validation for either of these steps is rarely reported, but it would be an important addition given the questionable efficacy of some the most common methods.[229] Even under serum-replacement conditions, miRNA contaminants have reportedly been found.[276] Whenever serums starvation (i.e., culturing without serum) is feasible, it would be the preferred option.

Cell viability. Looking at the EVs collected *in vitro*, the cell number and viability at EV-harvest are seldom mentioned. Depending on the study, 90-99% vital cells is what was deemed appropriate when documented at all. As even a few dead cells can contribute to the presence of apoptotic bodies that can influence the EV-population it is important to report the number of viable cells in each study.[1]

Yield. EV yield directly impacts the characterization possibilities because it determines whether there is enough material to perform analyses such as cryo-TEM imaging. It also speaks to the efficiency of the

isolation methods., revealing how well does a specific technique perform, especially when compared to alternatives. An absolute yield would also allow to evaluate upstream applicability, i.e., whether mass production would be a feasible option or not (e.g. for the use of liver-derived EVs as drug delivery systems). Because of all of these considerations, the importance of disclosing and being able to compare this data is easy to see, yet when looking at the quantification of EV-recovery, we found the information either omitted/lacking or hard to extrapolate and ultimately to compare between studies. The particle number per million cells measure would be a convenient option to express the yield and to compare its efficiency across studies. Quantification by mass of EV-associated proteins would also be viable alternative, if other co-isolated proteins can be excluded, preferably by number of cells as well.

Storage. EV storage is a particularly relevant subject when EV isolation and characterization are not performed on the same day, which is often the case given how much time most of the described protocols require. Storage insights were not always provided, and their validation even less frequently. It has already been reported that storage modality can affect the EVs[277–279] which is why we think it is important to share this information. Trehalose, mannitol and polyethylene glycol had been evaluated as possible cryoprotectants in the aforementioned studies, although not in the context of liver-derived EVs as of yet. With the exception of dimethyl sulfoxide,[88–97] the use of cryoprotectants for storage below -20 °C is rarely considered.

Differences in differential (ultra-)centrifugation. This isolation technique has become the gold standard in EV research for the very good reason that it works. It is cited in well over half the articles referenced in this review, but the protocols are not standardized. Even when analyzing EVs originating from the same cell types, different groups tend to have different approaches with sometimes unexplained differences arise within the same group which makes comparison challenging. The number of centrifugation steps vary and, the relative centrifugal forces and centrifugal times applied at each step are rarely consistent. When there is sufficient purity validation and subsequent sample characterization, this methodological diversity might not be an insurmountable issue, but it is worth keeping it in mind when comparing results, and also when choosing which protocol to follow.

Suboptimal method description. There is a general lack of rigorous standardization of methods in EV research, that was addressed with a position paper first published in 2014,[280] then expended upon in 2018.[1] EV-TRACK is a platform aiming at method transparency. Methods descriptions could be shared on EV-TRACK when the information would otherwise be left out of a publication. Implementing this would improve reproducibility and, more nuancedly, it would allow to compare method efficiencies across studies. Experimental validation of some practices is not always shared, which may be a problem when the methods used have been reported to have weaknesses, such as depletion of FBS-EVs. The growing community working on EVs is becoming more aware of the need for standardization in this young field of research,[281] and the MISEV guidelines[1] remain undoubtedly the reference text in these regards.

10. Conclusions and perspectives

We reviewed the current methodological practices for the isolation and analysis of those liver derived EVs, making a cell type specific user guide. Liver-derived EVs are gaining attention as a research topic, and there are already a few research papers addressing EVs from every liver resident cell type. Much work has already been published, but a rigorous standardization is needed. We moreover highlighted common causes for concern and critically reviewed room for improvement: the bigger issue that emerged was suboptimal method description and transparency.

We found hepatocyte-derived EVs to be analyzed the most, perspective studies might tackle EVs originating from other cell types more frequently. EVs from human induced pluripotent stem cells (iPSC)-derived hepatocyte like cells have not been analysed in detail yet, even though these cells are being taken under consideration as cell culture models for the liver.[282–284] EVs from iPSCs have been studied in the context of liver fibrosis, but they were still not differentiated into hepatocyte like cells.[285] The method transferability and translational applicability have also been a prominent topic of our discussion because working with EVs is inherently complicated even in single cell cultures, but many researchers have endeavored to escalate the challenge to co-cultures systems, to animal models (rodents), and to human patients. The comparisons have not always been complete, but the efforts put into positively tracing EVs and their associated biomolecules back to a specific cell type in increasingly more complex settings are a first important step to better understand liver diseases and we hope to see more of that in the future.

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