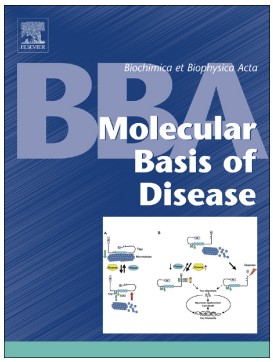

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PII: S0925-4439(18)30010-3
DOI: https://doi.org/10.1016/j.bbadis.2018.01.009
Reference: BBADIS 65025

To appear in:

Received date: 20 November 2017
Revised date: 4 January 2018
Accepted date: 7 January 2018

Please cite this article as: Joeri Lambrecht, Stefaan Verhulst, Inge Mannaerts, Hendrik Reynaert, Leo A. van Grunsven , Prospects in non-invasive assessment of liver fibrosis: Liquid biopsy as the future gold standard?. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Bbadis(2018), https://doi.org/10.1016/j.bbadis.2018.01.009

Prospects in non-invasive assessment of liver fibrosis: liquid biopsy as the future gold standard?

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Abstract

Liver fibrosis is the result of persistent liver injury, and is characterized by sustained scar formation and disruption of the normal liver architecture. The extent of fibrosis is considered as an important prognostic factor for the patient outcome, as an absence of (early) treatment can lead to the development of liver cirrhosis and hepatocellular carcinoma. Till date, the most sensitive and specific way for the diagnosis and staging of liver fibrosis remains liver biopsy, an invasive diagnostic tool, which is associated with high costs and discomfort for the patient. Over time, non-invasive scoring systems have been developed, of which the measurements of serum markers and liver stiffness are validated for use in the clinic. These tools lack however the sensitivity and specificity to detect small changes in the progression or regression of both early and late stages of fibrosis. Novel non-invasive diagnostic markers with the potential to overcome these limitations have been developed, but often lack validation in large patient cohorts. In this review, we will summarize novel trends in non-invasive markers of liver fibrosis development and will discuss their (dis-)advantages for use in the clinic.

Keywords: Chronic liver disease, Hepatic Stellate Cell, Biomarker, Diagnosis, Extracellular Vesicle, miRNA.

1. Introduction

In the 1950s, liver biopsy was introduced into the clinic [1]. In those days, this invasive procedure was considered as revolutionary, due to the possibility to visualize the presence of necrosis, inflammation, and fibrotic distortion in the affected liver. Due to the absence of other techniques with the same level of diagnostic potential, liver biopsy quickly obtained the status of being the gold standard for the diagnosis of liver fibrosis and injury. Although liver biopsy maintained this status for many years, clinicians started to doubt its efficacy in the active diagnosis and monitoring of liver disease due to some shortcomings of the technique. First, only a small tissue sample is obtained during biopsy, which represents in average $1/50.000^{th}$ of the total liver mass, making its representation for the total liver questionable [2]. Second, although clinicians and pathologists strive for standardization of the liver biopsy procedure and interpretation, inter-and intra-observer variation can never be excluded, due to variation in expertise and training [3]. Third, the invasive nature of this technique may lead to discomfort of the patient, such as pain and post-procedure complications [4]. Finally, dependent on the type of liver biopsy (with or without ultrasound, in or out-patient procedure), and possible post-procedure complications, the cost of liver biopsy can become significant (Table 1), leading to a doubtable cost-effectiveness ratio [5].

Since the implementation of liver biopsy half a century ago, intensive research partly elucidated the pathogenesis of liver fibrosis and its progression towards cirrhosis, both at the cellular and molecular level. Persistent presence of a liver injury-causing agent (viral hepatitis, drug-induced, auto-immune, alcohol-related etc.), results in epithelial cell death (hepatocyte and/or cholangiocytes) and the recruitment of inflammatory cells. This creates a hostile micro-environment to which local stromal cells such as hepatic stellate cells (HSCs) and portal fibroblasts respond by an activation process towards a myofibroblastic phenotype [6]. Of these two cell types, the HSCs are the most studied and are considered as the major source of myofibroblasts in hepatotoxic liver fibrosis [7]. Activated myofibroblasts are highly contractile cells characterized by an extensive production of extracellular matrix (ECM) proteins, resulting in scar tissue formation and increased liver stiffness, the two hallmarks of a fibrotic liver [8].

One of the major breakthroughs in Hepatology, is the description of the dynamic character of liver fibrosis. Removal or treatment of the damage-causing agent in the various types of liver disease may result in senescence [9] or apoptosis [10] of the activated HSCs, and may lead to a restoration of the functional capacity of the liver and thus improved clinical

outcome [11]. Additionally, it is found that the pace in which these dynamics occur, differs between patients, dependent on various factors such as the etiology of liver disease and environmental and host factors [12]. A static tool of fibrosis assessment such as the liver biopsy technique, which cannot be performed frequently, does not take these additional factors into consideration and may thus lead to inaccurate prognostic evaluation.

Due to the drawbacks associated with liver biopsy, and the inability of this technique to assess the dynamic character of liver fibrosis, several non-invasive diagnostic tools have been developed (Figure 1). Non-invasive tools are surrogate techniques for liver biopsy that aim to stage the degree of fibrosis without puncturing the liver. A major advantage of these methods, apart from being non-invasive, is the fact that they can be repeated frequently, which enables assessment of the evolution of fibrosis. Additionally, a larger amount of liver tissue can be evaluated. In case of sufficient sensitivity and specificity, this type of assessment of liver fibrosis could be important for patient follow-up and prognosis determination. Moreover, it could lead to major changes in drug development, as anti-fibrotic effects can be monitored frequently, and ideally, small improvements in fibrosis could be detected early, which could significantly reduce the duration of clinical trials.

2. Current clinical non-invasive techniques to assess hepatic fibrosis

Non-invasive techniques have gained popularity in current clinical settings, leading to a reduction of liver biopsies to stage the degree of liver fibrosis. Of these technologies, two different approaches have been validated in large patient cohorts with various etiologies: the detection and quantification of serum markers, and elastographic techniques measuring liver stiffness (Figure 1). While the first approach is used to obtain information about the presence of liver injury and related disruption of normal cellular homeostasis, the latter methods determine the extent of ECM deposition in the liver parenchyma.

2.1 **Serum markers**

Two large groups of serum biomarkers for liver fibrosis can be identified: direct and indirect markers. The group of direct markers covers products derived from ECM production and degradation. Examples are hyaluronic acid, matrix metalloproteases (MMPs), and tissue inhibitors of matrix metalloproteases (TIMPs). Indirect markers do not directly represent liver

injury, but are markers of liver inflammation and impaired liver function. This group includes platelet counts, alanine aminotransferase (ALT) levels, etc. [13].

The analysis of circulating factors has many advantages: ease of sampling, representation of the whole liver and thus small sampling error, good cost-effectiveness since assays can be executed in a routine laboratory setting, limited observer-related variability, and the possibility of automatization and to execute repeated measures [14]. The screening for individual factors proved to be insufficient for the detection of liver fibrosis. Therefore, combinations of multiple factors are used, leading to the development of scores and algorithms, which are shown to be a more reliable method for fibrosis detection [15]. Various risk factors, such as age, gender, waist circumference, and alcohol consumption, are found to influence the prevalence and progression of liver fibrosis, and thus the accuracy of these algorithms [16]. To improve the predictive value of these algorithms, specific risk factors are included, leading to the development of multiple clinical validated non-invasive scoring algorithms (Table 2). Some of these scoring algorithms, such as the NAFLD fibrosis score (NFS), tend to be validated for only one specific etiology of liver fibrosis. Other algorithms like the enhanced liver fibrosis (ELF) test, the fibrosis 4 (Fib-4) test, FibroMeter, FibroTest, and the Hepascore have been validated for multiple etiologies of liver fibrosis, leading to their widespread use in the clinic. Current serum-based scoring systems tend to be reliable for the detection of late-stage liver fibrosis and cirrhosis, but often lack the sensitivity to distinguish the different early stages (≤F2) of liver fibrosis [17].

2.2 Elastographic methods

Chronic activation of HSCs to a myofibroblastic phenotype is associated with an excessive production and deposition of ECM, leading to distortion of the liver microstructure and blood flow, and an increase in liver stiffness [6]. Elastographic modalities focus on the visualization and quantification of changing liver elasticity during fibrosis progression, and can be divided into two groups; ultrasound (US)-based and magnetic resonance imaging (MRI)-based elastographic methods. This latter group consists of acoustic radiation force impulse (ARFI), 2D-shear wave elastography (2D-SWE), and transient elastography (TE) [18].

FibroScan® (Echosens, Paris, France) is the most validated TE method used in the clinic, it determines the stiffness of the liver by sending out shear waves, and measuring their speed of transmission through the liver. It was first validated for the detection of late-stage liver

fibrosis and cirrhosis in patients with chronic HCV infection [18]. Since then, its high discriminative diagnostic value for severe fibrosis and cirrhosis is proven in all major etiologies of liver disease including chronic HCV infection [19], chronic HBV infection [20], NASH/NAFLD [21], alcoholic liver disease [22], and combinations of these etiologies. Limited to no inter-observer and intra-observer variability is associated with this technique [23]. On the other hand, the diagnostic performance of TE is less precise in patients with obesity or with large amounts of chest wall fat [24], and cirrhotic patients with ascites [25]. Other limitations include the inability to distinguish between the different early stages of liver fibrosis [26], the etiology-dependence of liver stiffness cut-off values [27], and the possibility to obtain false-positive results in patients with acute hepatitis, due to presence of edema and inflammation [28]. ARFI and 2D-SWE have comparable limitations and advantages as TE, with as main additional advantage, its easy implementation into a conventional ultrasound machine, and better performance in obese patients.

The most important example of an MRI-based elastographic methods is magnetic resonance elastography (MRE), which combines the MRI technique with the utility of acoustic shear waves for liver visualization [29]. This technique is more time-consuming and more expensive than other imaging techniques, and is unsuitable for use in patients with hemochromatosis or hemosiderosis, as moderate to severe iron overload in the liver parenchyma significantly decreases hepatic MRE signal intensity [30]. On the other hand, MRE does offer a whole liver examination and is feasible for obese patients [31].

Although current imaging techniques have the potential to diagnose with high accuracy significant fibrosis and cirrhosis in the affected liver, the lack of a sensitive imaging modality for early-stage liver fibrosis remains. Diagnostic panels combining such imaging techniques with values of standardized circulating markers are proposed to enhance the diagnostic potential of current tests, but have not yet found their way into the clinic. Therefore, we will next discuss some novel approaches to liver fibrosis diagnosis which could lead to the resolution of this current limitation.

3. Promising circulating markers of liver disease

To overcome the shortcomings of current non-invasive diagnostic methods, many researchers have tried to identify non-invasive scoring systems with the aim to establish a new ‘Gold standard’ for the diagnosis and staging of liver fibrosis. Focus is put on the use of

circulating nucleic acids, proteins, and lipids, with or without packaging into EVs, as novel biomarkers (Figure 2). To give an impression of potential future liver fibrosis diagnostics, we present an overview of the most recent approaches.

3.1 Circulating cell-free nucleic acids

The use of total circulating nucleic acids as diagnostic tool of liver disease is widely discussed. Circulating nucleic acids comprise DNA, mRNA, and non-coding RNAs such as microRNA (miRNA) and long non-coding RNA (lncRNA). They obtain their stability by binding to proteins or lipids, or by packaging into extracellular vesicles (EVs). The analysis of circulating nucleic acids is done by various techniques such as micro-array, next generation sequencing, and quantitative real-time polymerase chain reaction (PCR). More recent techniques such as Nanostring and droplet digital PCR, have not yet been used in biomarker discovery, but harbor great sensitivity. The Nanostring technology allows the detection and quantitation of hundreds of transcripts in one reaction by hybridization of fluorescent barcodes directly to specific nucleic acid sequences. This technique does not require amplification of the target molecule, and is known to be cost-and time-effective, easy to use, and robust [32]. Droplet digital PCR, a technology which is based on sub-partitioning of PCR samples into nanoliter droplets, provides absolute quantification of nucleic acids without the need for a standard curve [33].

3.1.1 *Cell-free DNA*

When the complete group of circulating nucleic acids is analyzed, the term cell-free nucleic acid is used [34]. Cell-free DNA (cfDNA) is released in the circulation by cells undergoing apoptosis, necrosis, or active secretion [35, 36]. Initially, while elevated levels of cfDNA were linked to the initiation and progression of the cancer pathology, an enhanced presence of cfDNA is also shown in various other pathological [37] and physiological [38] conditions. Therefore, the current focus is on specific characteristics of this cfDNA, rather than its elevated presence. One such characteristic is the methylation pattern of cfDNA. Methylation patterns are unique to each cell type, and are consistent in the various cells of a specific cell type between individuals [39]. Release of cfDNA with cell-specific methylation patterns could thus be an indicator of the prevalence of a pathological condition, with or without enhanced apoptosis and necrosis. Proof of concept is delivered for various diseases such as HCC and type-1 diabetes [40].

In the process of liver fibrosis, the mechanism behind the initiation of the myofibroblastic transdifferentiation process of HSCs involves a change in such DNA methylation patterns [41]. For example; an important requirement for the activation of HSCs, is the methylation of peroxisome proliferator-activated receptor gamma (PPARγ) by methyl-CpG-binding protein MeCP2 [42]. Enhanced methylation of PPARγ is correlated with fibrosis progression, in patients with NAFLD [43] and chronic HBV infection [44]. Analysis of circulating cfDNA shows an enhanced methylation of PPARy CpG1 and CpG2 promotor regions, in correlation with the fibrosis stage in NAFLD patients. Such hypermethylation is also found in circulating cfDNA of cirrhotic patients with alcoholic liver disease, suggesting that this phenomenon is not etiology-specific [45].

3.1.2 Cell-free non-coding RNA

Cell-free non-coding RNAs (ncRNAs) structurally resemble mRNA, but do not code for proteins. These ncRNAs can be, based on their size, divided into various subcategories such as miRNAs (which have a length of 21 to 25 nucleotides) and lncRNAs (which are longer than 200 nucleotides) [46]. This latter subtype of ncRNAs has the potential to be cell-type specific, as RNA sequencing of human HSCs identified a spectrum of over 3600 lncRNA, of which 400 are uniquely expressed in HSCs compared to +/- 40 tested normal human tissues and cell types. One such example is lncRNA-001762, which shows an enhanced expression during TGF-β stimulation of human HSCs, and of which its expression levels are the highest in HSCs compared to the other analyzed cells and tissues. LncRNA patterns are also found to be different between liver myofibroblasts, and myofibroblasts from other origins [47]. These differential expression patterns highlight the potential of circulating lncRNAs as marker for the presence of myofibroblasts in the liver. Although the field of lncRNA-analysis is relatively new, multiple lncRNAs are identified which show a dysregulation during HSC activation, making them potential candidates for biomarker discovery: lincRNA-p21 [48], growth arrest-specific 5 (GAS5) [49], maternally expressed gene 3 (MEG3) [50], H19 [51], plasmacytoma variant translocation 1 (PVT1) [52], liver fibrosis-associated lncRNA1 (lnc-LFAR1) [53], homeobox transcript antisense RNA (HOTAIR) [54], alu-mediated p21 transcriptional regulator (APTR) [55], and metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) [56]. The heterogeneity in starting material of these studies, being primary cells or cell lines of various species, could however mean only limited representation for the human *in vivo* HSC activation process. From the mentioned dysregulated lncRNAs, circulating LincRNA-p21 is known to be

down-regulated in fibrotic patients with chronic HBV infection [57] and APTR to be up-regulated in the plasma of cirrhotic patients with undisclosed etiology [55]. Due to limited knowledge concerning circulating lncRNAs during fibrosis progression, it remains however unknown if a dysregulation of such circulating lncRNAs is etiology-specific, species-specific, and truly represents the activation process of HSCs.

MiRNAs are the most abundant and most studied ncRNAs in the circulation [58]. Excellent overviews of circulating miRNAs associated with liver disease have been made by Roderburg *et al* [59], Arrese *et al* [60], and Falcon-Perez *et al* [61]. Since publication of these reviews, several new miRNAs are identified as potential biomarkers (Table 3). A decline in circulating levels of let-7a/7c/7d-5p (let-7s) is found to correlate with the progression of liver fibrosis in patients with chronic HCV infection [62]. In HBV infected patients with no/mild (F0 to F2) and severe (F3-4) fibrosis, plasma-analysis identified significant changing levels of miRNA-29a, -92a, -122, -146a, and -222. The authors propose an algorithm considering the levels of circulating miRNA-122, miRNA-222, platelet count, and alkaline phosphatase to discriminate the F3-4 group from the F0-2 group [63]. The progression of fibrosis in chronic HBV patients is also associated with significant enhanced presence of circulating miRNA-125b [64] and miRNA-185 [65]. *In situ* hybridization assay of miRNA-185 reveals an up-regulation and association of hepatic miRNA-185 to sites of myofibroblast proliferation and collagen deposition, suggesting its HSC-origin [65]. Last, in chronic HCV-infected patients, a discrimination of severe (F3-4) fibrosis from no/mild (F0-2) fibrosis is made based on the enhanced presence of circulating miR-126, miR-129, miR-203a, and miR-223 [66].

A total overview of dysregulated miRNAs during liver disease is given in table 3. As can be concluded from this overview, the identification of circulating miRNAs as biomarkers is especially focused on fibrosis in chronic HBV- and HCV-infected patients, with the uncertainty if these results are also applicable in other etiologies of liver disease. Although miRNA function and dysregulation during liver disease has been the subject of many research groups, analysis of circulating miRNA pattern has not yet found its way into the clinic due to limited patient cohorts, lack of validation of current studies, and some disadvantages associated with analysis of nucleic acids.

3.2 Extracellular Vesicles

Extracellular vesicles (EVs) are membrane-derived structures, released by cells into their microenvironment. EV is the overarching terminology for a heterogeneous group of three major subtypes of vesicles; exosomes, microvesicles and apoptotic bodies. These subtypes are divided in a subjected manner based on size, biogenesis, and some (overlapping) membrane markers, but a clear differential identification is still missing. Currently, the EV subtypes are described as follows; exosomes are the smallest subtype of EVs, with a diameter typically below 100 to 150nm, which are formed into multivesicular bodies (MVBs) and are released by fusion of the MVBs with the plasma membrane [67]; microvesicles or microparticles are bigger in size, ranging from 100nm to 1µm, and are derived from budding off the membrane [68]; apoptotic bodies have the largest diameter, ranging from 1µm to 5µm, and are formed by compartmentalization of apoptotic cells [69].

Secretion of EVs is a general characteristic of cellular homeostasis and physiology, and their presence is demonstrated in almost all body fluids. Functionality of EVs depends on their cargo which can consist of mRNA, miRNA, lncRNA, viral particles, lipids, and functional proteins. This cargo is assigned to the vesicle through specialized processes or by random events, as the vesicles contain cytoplasmic matter and its constitutes [70].

Use of blood-circulating EVs has the potential to represent disease-associated cellular changes. To obtain purified circulating EVs, various protocols can be used, which vary in yield and purity, labor-intensity, and cost of the procedure. Most common used EV isolation strategies include density centrifugation (regular ultracentrifugation, UC), density gradient centrifugation, affinity capture, membrane filtration, size exclusion chromatography, and synthetic polymer-based precipitation (Figure 2).

3.2.1 Quantification of circulating EVs

In various pathological circumstances, cells enhance their production and secretion of EVs, what causes an elevation of their total number in the circulation. This phenomenon is seen in patients with various types of liver disease, such as alcoholic liver disease [71] and early stage fibrotic patients with chronic HBV or HCV infection [72]. Quantification of the number of circulating cell-type specific EVs could be a novel tool to represent cell activation or injury. As chronic liver disease is associated with systemic inflammation, the number of circulating EVs derived from inflammatory cells is elevated, e.g. a progressive increase of CD4+ and CD8+ microvesicles (thus from CD4+ and CD8+ T cells) in patients with chronic HCV infection is

found [73]. In a similar way, in cirrhotic patients with alcohol abuse or chronic HCV infection, elevated levels of leuko-endothelial (CD31+/41-), lymphocyte (CD4+), pan-leukocyte (CD11a+), and erythrocyte (CD235a+) microvesicles are detected. Furthermore, microvesicles positive for cytokeratin-18 (CK18), a marker of epithelial cells such as hepatocytes and cholangiocytes, is detected in these patients, but not in healthy individuals [74]. Since multiple diseases are associated with an activation of inflammatory cells, the quantification of inflammatory cell-derived EVs is not specific to the liver fibrosis pathology. Changing amounts of circulating EVs derived from liver cells would therefore have a better potential to become a liver-disease specific biomarker. Cirrhotic patients with chronic HBV or HCV infection are found to have an increased presence of AnnexinV+ EpCAM+ ASGPR1+ CD133+ microvesicles [75], in which asialoglycoprotein receptor 1 (ASGPR1) is a hepatocyte-specific receptor, and EpCAM/CD133 are markers of liver progenitor cells (LPCs) [76]. Such microvesicles could thus indicate the presence of damaged hepatocytes and an enhanced ductular reaction, as caused by chronic liver injury, rather than a higher level of fibrosis.

3.2.2 EV-content as biomarker

The general heterogeneity in EV-content is shown by proteomic [77, 78] and lipidomic analysis [79], for EVs derived from *in vitro* cultures and from the plasma of healthy individuals, revealing the presence of a large multitude of proteins and lipids. Additionally, deep sequencing of plasma EVs from healthy individuals, identified an abundance of miRNAs (76,20% of all mappable reads) and significant fractions of other RNA species such as ribosomal RNA, long non-coding RNA, piwi-interacting RNA, transfer RNA, small nuclear RNA, and small nucleolar RNA [80]. Researchers teamed up for the creation of databases such as ExoCarta [81] and Vesiclepedia [82], which map this EV content, helping in the search for novel biomarkers.

— *EV protein content*. Analysis of the protein content of UC-isolated circulating EVs identifies Arginase 1 (Arg1) as biomarker candidate for hepatic injury. Its enhanced presence in EVs is observed both *in vitro* and *in vivo* by treating respectively hepatocyte cell lines and rats with hepatotoxic compounds [83]. A comparable experimental setup identifies the increasing presence of EV-associated CD40 ligand (CD40L) after treatment of a hepatocyte cell line with ethanol, and in patients with alcoholic hepatitis [84]. Although the use of EV protein content could harbor an important tool for disease identification, results of current studies are difficult to interpret, as it is shown that different EV isolation methods on plasma lead to different outcomes on proteomic analysis [85]. For example, synthetic polymer-based

precipitation is known to have a high contamination of highly abundant plasma proteins such as albumin, complement 3, and apolipoprotein E, while this contamination is only moderate in UC, and very low when using density gradient centrifugation [85, 86].

— *EV miRNA content*. Deep sequencing of circulating EV RNA content derived from healthy individuals identifies the presence of a wide spectrum of miRNAs, with an abundance of the following five; miR-99a-5p, miR-128, miR-124-3p, miR-22-3p, and miR-99b-5p [80]. It would be of great interest to identify the origin of these five EV-associated miRNAs, for use as healthy fingerprint of the cell(s) of origin. EV-associated miRNA fingerprints are still lacking in the pathology of liver fibrosis and cirrhosis. During disease progression in patients with alcoholic liver disease an enhanced presence of EV-associated miR-30a and miR-192 [71], and let-7f-5p, miR-29a-3p, and miR-340-5p [87] is detected. Cirrhotic patients with chronic HBV infection can be identified by a decrease in EV-associated miR-224, miR-122, miR-195, and miR-101 levels, as compared to patients with no or early-stage liver fibrosis [88]. MiR-192, which is down-regulated during HSC activation [89], shows decreasing levels in EVs extracted from *in vitro* activating HSCs, and in the circulating EVs of early-fibrotic patients with chronic HBV and HCV infection [72]. It is tempting to speculate that some of the EV-associated miRNAs in fibrotic patients might represent the presence of activated HSCs, and thus initiation and progression of liver fibrosis. The origin of these EVs from liver cells/HSCs *in vivo* remains to be proven before their use as biomarker can be suggested.

— *EV lncRNA content*. Although EV-associated lncRNAs have not yet been linked to fibrosis initiation and progression, their use as a diagnostic and prognostic tool in HCC is suggested, as discussed by Mohankumar and Patel [90]. The drawback of EV-associated lncRNAs is the relatively low amount of copies in the circulating EVs, when compared with for example miRNAs [80]. The discovery and use of EV-associated lncRNAs in a clinical setting will thus require highly sensitive methods of RNA analysis such as droplet digital PCR or next generation sequencing.

3.2.3 Implementation of EV-based biomarkers into the clinic

While EV content shows great promise as a fibrosis biomarker, the vesicle field has still some major issues that need to be solved before vesicle-based biomarkers can be integrated into a clinical setting (Table 4). First, a consensus should be made concerning the most optimal way for plasma collection and storage. The use of an appropriate anticoagulant should be agreed

upon, as these can influence EV yield, stability and downstream RNA analysis [91]. Secondly, a consensus concerning EV isolation should be made. Current publications are still a mixture of isolation methods, known to generate EV populations with different yield, purity, and characteristics. These issues have been addressed by the International Society for Extracellular Vesicles (ISEV) [92] and the International Society for Thrombosis and Hemostasis (ISTH) [93], leading to the proposition of specific standards and recommendations (Table 4). However, the implementation of these standards is not straightforward. Additionally, current EV isolation techniques are often time consuming and low-throughput, and thus less suitable for routine analysis in the clinic. Various high-throughput EV analysis techniques have been developed [94], but validation of these techniques, and proof of cost-effectiveness is still lacking.

3.3 Circulating lipids and proteins

Analysis of the bulk protein and lipid content of blood lead to the identification of several liver fibrosis-associated markers which are validated for clinical use, such as AST, ALT, gamma-glutamyl transferase, etc. Their informative character is considered as superior over nucleic acids, as they have a higher diversity thanks to the spectrum of post-translational modifications. However, their analysis is associated with more drawbacks [95]. Techniques to determine the circulating protein content include western blotting, enzyme-linked immunosorbent assay (ELISA), mass spectrometry, and high-performance liquid chromatography (HPLC). CyTOF mass cytometry, a more recent technique which uses transition element isotope-labeled antibodies in combination with mass spectrometry, may be important in protein-related biomarker discovery, considering this technique has high sensitivity, can analyze a great number of parameters per event, and requires little to no compensation. Its expensive reagents and slow sample acquisition, however constrains its use for everyday research [96]. Recently, several protein and lipid biomarkers in relation to progression of liver fibrosis and cirrhosis have been identified.

One such biomarker is sphingosine-1-phosphate (S1P). Cirrhotic patients with various etiologies have significant lower levels of S1P, in correlation with their MELD score, and show a prognostic meaning for one-year mortality when combined with platelet counts, hemoglobin concentrations and MELD value [97]. Complement 5 a (C5a), a member of the complement activation cascade, displays a stage-specific decrease in the plasma of fibrotic/cirrhotic patients with chronic HBV infection [98]. In patients with decompensated liver cirrhosis, various forms of fatty acid binding proteins (FABP), being L-FABP1, I-FABP2, and A-FABP4, show

enhanced presence in the circulation. Of these three analyzed FABPs, the levels of A-FABP4, which is mainly expressed in liver macrophages, are associated with 3-month mortality [99]. As the initiation and progression of liver fibrosis is marked by high turnover of fibrotic matrix, it is hypothesized that derivatives of this process could be used as diagnostic markers. One such side-product of ECM production is N-terminal pro-peptide of type III procollagen (PIIINP), which can discriminate chronic HCV-infected patients with significant and advanced fibrosis, from those with no or mild fibrosis [100, 101]. In addition, PIIINP is suggested as prognostic marker for therapy response, as treatment of HCV- and NAFLD fibrosis patients with an antifibrotic agent leads to improvement of disease outcome in those patients who have initial high PIIINP values, but not in in the patients with initial low PIIINP values [102]. Other collagen turnover derivatives such as products of MMP-degraded collagen type 3, 4, and 6 (C3M, C4M, C6M), and type 4 collagen production (P4NP7S) can identify the presence of advanced fibrosis in chronic HCV infected patients [100].

Besides the identification of changing protein levels, a change in the glycosylation, a post-translational process in which carbohydrates are attached, of many serum proteins is seen during the progression of liver disease. As most of the glycosylated proteins are derived from the liver, circulating glycosylation patterns during liver fibrosis can be observed, and be used as diagnostic test. The best known glyco-structure with changing presence during fibrosis progression is *Wisteria floribunda agglutinin*-positive Mac-2 binding protein (WFA(+)-M2BP). This isoform has an enhanced presence in the circulation during fibrosis progression in patients with chronic HBV and HCV infection [103, 104], and NAFLD [105]. Various other circulating markers are proposed, such as the enhanced presence of fucosylated haptoglobin during fibrosis progression in patients with chronic HCV infection [106], but lack validation in other etiologies of liver disease.

The mapping of total pathological glyco-alterations in a specific cell, tissue or organ leads to the identification of disease-associated glycopatterns. For the identification of such glycopatterns, lectin microarrays can be used. This high-throughput technique uses various sorts of immobilized lectins, with each their own binding specificity towards specific carbohydrate residues [107]. Such lectin microarrays lead to the identification of important lectins that can detect glyco-alterations of the alpha-1-acid glycoprotein (AGP), being *Aspergillus oryzae* (AOL) and *Maackia amurensis* (MAL), with the use of *Dature stramonium* (DSA) as normalizing signal. Alterations in AOL/DSA and MAL/DSA signal are associated

with fibrosis progression in chronic hepatitis patients [108], and their combination (named the LecT-Hepa scoring) can be used to identify significant fibrosis and cirrhosis [109].

4. Patient follow-up

Removal of the etiological source of chronic liver injury, as by weight loss, antiviral therapy, diminution of alcohol intake, etc., may lead to reversal of liver fibrosis and cirrhosis. Several anti-fibrotic therapeutics have made their way into the drug development pipeline, aiming for liver fibrosis regression when causal therapy is not applicable or insufficient. A major issue in the development of anti-fibrotic strategies is the lack of non-invasive tools to indicate early (small) changes in fibrosis dynamics. Therefore, current clinical trials remain long in time, until significant changes in fibrogenesis can be observed through biopsy, and thus require an extensive budget. In such settings of patient follow up, liquid biopsy holds great promise as a non-invasive approach to frequently measure a potential improvement of liver fibrosis. The relevance of such non-invasive, sensitive and specific biomarkers has been mainly demonstrated in antiviral therapies for liver disease. For example, in patients with HCV infection who undergo PEG-IFN treatment with or without silymarin [110] or PEG-IFN plus ribavirin treatment [111], the clinical-available ELF score was identified as a possible manner of follow up. However, in a recent phase 2 trial, in which the ASK1 inhibitor Selonsertib was tested in patients with NASH, fibrosis regression was not detected by ELF score changes [112], underlining the need for more experimental biomarkers. One such approach could be circulating miRNAs as shown in HCV patients treated with PEG-IFN complemented with ribavirin, in which miRNA-122 levels are found to be significantly higher in patients with sustained viral response compared to the non-response group [113]. Future integration of the experimental non-invasive biomarkers (discussed in this review) into ongoing clinical trials with fibrosis regression as clinical endpoint is desirable to show their efficacy and potential use in the clinic.

5. Prediction of disease outcome

Besides the real-time follow up of fibrosis dynamics, the ideal biomarker panel should be able to predict the clinical outcome of patients with chronic liver disease, identifying those that will regress towards an earlier stage of fibrosis, or progress towards cirrhosis and HCC [114]. In the search for such predictive factor, some population-based and environmental factors are identified which can influence the progression of liver disease, and should thus be taken into consideration. For example, the importance of first-degree relationship is shown by a

twelve-fold increase in the probability of advanced fibrosis development in case of first-degree relationship to a cirrhotic NAFLD patient, compared to first-degree relatives of non-NAFLD controls [115]. Such influencing factors are however often only suggestive, and lack validation in large patient cohorts.

Predictive models are generally gene-based, taking several (patho-)physiological parameters into consideration. One such predictive test is the seven-gene cirrhosis risk score (CRS), which evaluates the potential of fibrosis progression in chronic HCV infected patients, based on the presence of seven types of single nucleotide polymorphisms (SNPs) combined with the gender of the patient [116]. Another predictive SNP is rs12979860 CC, an IFNL4 SNP, which is associated with greater necro-inflammation, more rapid fibrosis progression, and eventually worse disease outcome. The predictive value of this marker is proven for chronic HCV-, chronic HBV-, and NAFLD-related fibrosis, and increases in combination with the parameters age and gender [117]. An online fibrosis-predicting algorithm, FibroGENE, evaluates the probability of significant fibrosis and cirrhosis by combining values of various blood parameters (AST, platelet count, GGT), the presence of rs12979860 CC, and the age of the patient [118]. In patients with chronic HCV infection, a strong predictive value for fibrosis progression is found for SNP rs9976971 AA of the interferon g receptor 2 gene (IFNGR2) [119], and for SNP rs2596542 TT of the major histocompatibility complex class I-related gene A (MICA) [120]. As last example, the presence of rs738409 GG SNP in patatin like phospholipase domain containing 3 (PNPLA3), also known as adiponutrin, is associated with an increased risk for enhanced fibrosis progression, cirrhosis development, and progression towards HCC in multiple etiologies of liver disease [121, 122].

6. Conclusions and outlook

The discovery and validation of imaging modalities and serological tests has led to significant reduction in use of the invasive liver biopsy. However, liver biopsy is not abandoned, due to the current inability of the non-invasive tests to provide step-wise follow up, meaning a sensitive and specific manner for the detection and differentiation between the various stages of liver fibrosis, and the possibility to detect modest progression or regression of fibrosis. In the search for the ideal biomarker which would bridge these current limitations, the current focus is on commonly used serological markers, as these are associated with a lower cost (Table 1) [123] and are thought to better inform about cellular status, compared to imaging

modalities. An additional advantage includes easy screening of the general population or of at-risk populations such as patients with diabetes and metabolic syndrome. A combination of physical examination with such sensitive serological test can be executed by a general practitioner, identifying affected individuals who should be treated or followed up in a specialized center. Screening of these individuals would help in early detection and subsequent treatment of the disease, leading to a reduction in liver disease-associated complications and mortality, and progression towards cirrhosis and HCC.

In this review, we discussed several novel tools in serological assessment, which have the possibility to complement current serological tests, or to be used as proper multifaceted diagnostic panels. While results of these novel studies are promising, some major drawbacks remain. First, to prove accurate staging of liver fibrosis by a novel serological marker, liver biopsies are still needed to identify the specific stage of fibrosis in each patient. Lack of such material, results in the inability of most studies to report other facets of their biomarker besides the efficiency to identify late stage fibrosis or cirrhosis, as compared to TE or validated serological scoring algorithms. Second, large-scale multi-etiology validation of such novel serological markers is still needed. The efficiency of the biomarker should be tested on large patient cohorts with differences in age, gender, etiology of liver disease, etc. Thirdly, inter-study differences concerning starting material, extraction procedures, analysis, normalizing factor, and more, make it hard to compare studies, and to estimate the true value of a biomarker. Finally, evaluation strategies for determining which candidate marker deserves the investment of time and money for its implementation into the clinic, are missing. The machinery and standardized procedures for analysis of circulating nucleic acids, proteins and lipids are often already implemented in the clinical setting, creating a lower cost if novel markers need to be included in this system. However, EV-analysis is completely new for the clinic, and thus requires novel procedures and equipment, associated with additional labor and costs. It should be investigated if their effectiveness as biomarker justifies this big investment.

To conclude, we believe that we are currently on a break-through in biomarker-research, thanks to the increasing knowledge in the mechanisms of liver disease, generation and management of big data, and the development of novel laboratory procedures and equipment. The outcome of these efforts will eventually lead to significant improvements in patient care.

Acknowledgments:

The Liver Cell Biology lab is supported by the Vrije Universiteit Brussel and the Fund of Scientific Research Flanders (HILIM-3D; SBO140045).

Declaration of interest:

The authors have no conflict of interest to disclose.

Figure Legends

Figure 1. Diagnostic modalities for liver fibrosis and cirrhosis. The current gold standard for detection of liver fibrosis and cirrhosis remains the liver biopsy, an invasive procedure associated with multiple drawbacks. Non-invasive diagnostic modalities have been discovered and validated for use in the clinic, being serological markers and elastographic approaches. This latter group can be divided into Ultrasound-based-techniques, such as Acoustic Radiation Force Impulse (ARFI), 2D-shear wave elastography (2D-SWE), and transient elastography (FibroScan), and magnetic resonance imaging (MRI)-based techniques, of which magnetic resonance elastography (MRE) is the most important example. Liquid biopsy has multiple advantages over elastographic modalities, such as its good cost-effectiveness, its easiness, its potential to represent the total liver, etc. Therefore, the analysis of circulating proteins, lipids, and nucleic acids (DNA, lncRNA, miRNA, …), all with or without packaging in extracellular vesicles, has been the focus of many novel research.

Figure 2. The process for identifying novel serological markers. Due to the possibility to reflect cellular homeostasis, serological markers are considered to hold a major potential to reflect progression of liver fibrosis and cirrhosis. Focus has been put on circulating nucleic acids and proteins, with or without packaging into exosomes, as novel biomarkers for liver fibrosis. Isolation of exosomes from plasma is obtained by use of various methods: differential centrifugation, density gradient centrifugation, ultrafiltration, affinity capture, size exclusion chromatography, and synthetic polymer-based precipitation. Dependent on the isolation technique used, various yield and purity of exosomes is obtained. When the total plasma content of nucleic acids or proteins is wanted, the plasma sample is treated with lysis buffers in combination with various commercial isolation kits, to obtain pure protein or nucleic acid fractions. Analysis of the exosomal/total nucleic acid content (consisting of DNA, and both coding and non-coding RNAs) can be obtained by quantitative real-time polymerase chain reaction (PCR), micro-array, next generation sequencing, and some more recent techniques such as droplet digital PCR and Nanostring. The analysis of proteins and their post-translational modifications is performed by various techniques such as western blotting, enzyme-linked immunosorbent assay (ELISA), mass spectrometry, high-performance liquid chromatography (HPLC), lectin array and CyTOF mass cytometry.

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Table 1. Average unit costs of current diagnostic modalities for liver fibrosis and cirrhosis.

	Test	Unit cost (US Dollar, $)
Invasive assessment	Liver biopsy	~ 1235.9
Elastographic methods	ARFI	~ 66.3
	CT	~ 137
	Fibroscan	~ 66.3
	MRE	~ 259
Serum markers	AAR	~ 1.2
	APRI	~ 5.3
	BARD score	~ 1.2
	ELF	~ 140
	Fib-4	~ 5.8
	FIBROspect II	~ 46
	FibroTest/FibroSure	~ 57
	Hepascore	~ 21.1
	PGAA index	~ 11.8

List adjusted form Crossan *et al.* [123]. Cost were converted from UK Pounds to US dollars by use of Organization for Economic Co-operation and Development (OECD) indices and exchange rates. AAR, AST to ALT ratio; APRI, AST-to-platelet ratio index; ARFI, Acoustic Radiation Force Impulse; CT, computerized tomography; ELF, Enhanced liver fibrosis; Fib-4, Fibrosis 4 index; MRE, Magnetic Resonance Elastography.

Table 2. Current available serological tests and scoring systems for the detection/evaluation of liver fibrosis and cirrhosis.

Serological tests and scoring systems			
Name	**Parameters**	**Discussed etiologies**	**First described in**
AST to ALT ratio (AAR)	AST, ALT	-HCV	[124]
AST-to-platelet ratio index (APRI)	AST, platelet count	-HCV -HBV	[125]
BARD score	BMI, AAR, presence of type 2 diabetes	-NAFLD	[126]
Enhanced liver fibrosis (ELF)	Age, hyaluronic acid, PIIINP, TIMP-1	-Alcoholic liver disease -NAFLD -HCV -HBV	[127]
FibroIndex	Platelet count, γ-GT, AST	-HCV	[128]
FibroMeter	Platelet count, PT-INR, AST, hyaluronic acid, α2M, gender, age	-Alcoholic liver disease -HBV -HCV -HCV with HIV coinfection	[129]
Fibrosis 4 (Fib-4) index	Age, platelet count, AST, ALT	-HCV -HBV -HCV with HIV coinfection	[130]
Fibrosis probability index (FPI)	Age, AST, total cholesterol level, insulin resistance, past alcohol intake	-HCV	[131]
FIBROSpect II	Hyaluronic acid, TIMP-1, α_2-macroglobulin	-HCV	[132]
FibroTest/FibroSure (FT)	Age, gender, α2M, haptoglobin, γ-globulin, Apo-A1, γ-GT, total bilirubin	-HCV -HBV -NAFLD -Alcoholic liver disease -HCV with HIV coinfection	[133]
FibroTest-ActiTest	Age, gender, α2M, haptoglobin, γ-globulin, Apo-A1, γ-GT, total bilirubin, ALT	-HCV	[133]
Forns index	Age, platelet count, cholesterol levels, γ-GT	-HCV	[134]
Göteborg University Cirrhosis Index (GUCI)	AST, PT-INR, platelet count	-HCV	[135]
Hepascore (HS)	Age, gender, bilirubin, γ-GT, hyaluronic acid, α2M	-HCV -HBV -Alcoholic liver disease	[136]

		-HCV with HIV coinfection	
Hui Score	BMI, platelet count, albumin, total bilirubin	-HBV	[137]
King's score	Age, AST, PT-INR, platelet count	-HCV	[138]
Lok index / HALT-C	PT-INR, AAR, platelet count	-HCV	[139]
MP3 model / Leroy score	PIIINP, MMP-1	-HCV	[140]
NAFLD Fibrosis score (NFS)	Age, hyperglycemia, BMI, platelet count, albumin, AAR	-NAFLD	[141]
PGA index	PT-INR, γ-GT, Apo-A1	-Alcoholic liver disease	[142]
PGAA index	PT-INR, γ-GT, Apo-A1, α2M	-Alcoholic liver disease	[143]
SHASTA	Hyaluronic acid, AST, albumin	-HCV with HIV coinfection	[144]
ViraHep-C model	Age, race, AST, alkaline phosphatase, platelet count	-HCV	[145]
Zeng score	Age, hyaluronic acid, α2M, γ-GT	-HBV -HBV with HIV coinfection	[146]

ALT, alanine aminotransferase; Apo-A1, apolipoprotein A_1; AST, Aspartate aminotransferase; BMI, body mass index; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; INR, International normalized ratio; MMP-1, matrix metalloproteinases-1; NAFLD, Non-alcoholic fatty liver disease; PIIINP, Procollagen III amino terminal peptide; PT, prothrombin time; TIMP-1, tissue inhibitor of metalloproteinases 1; α2M, α_2-macroglobulin; γ-GT, gamma-glutamyl transferase.

Table 3. Circulating cell-free miRNAs as biomarker for liver disease.

Circulating cell-free miRNAs	Chronic HBV infection				Chronic HCV infection			NAFLD NASH		Alcohol abuse	
	↑			↓	↑		↓	↑	↓	↑	↓
	-10a	-122-5p	-378a-3p	-26a	-16	-483-5p	-29a	-16	-630	-122	-29a
	-16	-125-5p	-423	-27a	-20a	-571	-197-3p	-19a	-744	-133a	-652
	-19b	-125b-5p	-451	-122	-21	-618	-451	-19b	-762	-155	
	-20a	-146a	-455-3p	-181b	-22	-1207-5p	-505-3p	-21		-571	
	-21	-181b	-455-5p	-197-3p	-34	-1225-5p	-650	-34			
	-22	-185	-483-3p	-223	-34a	-1275	-652	-34a			
	-23a	-192	-572	-223-3p	-92a		-762	-122			
	-23b	-192-3p	-574	-486	-122		-1974	-125b			
	-25	-193b	-575	-505-3p	-125b		Let-7a	-155-5p			
	-28	-194	-638	-654-3p	-126		Let-7c	-181a			
	-29a	-215	-801	-744	-129		Let-7d	-192			
	-30a	-222	-855-5p	-762	-133a			-200b			
	-30e-3p	-223	-1247		-134			-221			
	-92a	-342	-1974		-146a			-201b			
	-96	-342-3p	-2861		-155			-375			
	-99	-345-3p	Let-7b		-203a			-432-5p			
	-99a	-365	Let-7c		-221			-451			
	-100	-365a	Let-7f		-222			-572			
	-106a	-371a-5p			-223			-575			
	-106b	-375			-320c			-578			
	-122-3p	-378a			-423			-638			

Summary of current knowledge concerning miRNAs that show up-regulated (↑) or down-regulated (↓) levels in the plasma of patients with liver disease. List based on Roderburg *et al* [59], Arrese *et al* [60], and Falcon-Perez *et al* [61], and complemented with novel findings. HCV, hepatitis C virus; HBV, hepatitis B virus; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.

Table 4. Standardization of EV-research.

Proposed standards and recommendations for EV-research	
Subject of discussion	**Propositions**
Choice of starting material	- The use of plasma is preferred, as EV release is observed during cloth formation
Choice of coagulant	- Additional research is necessary - EDTA/ NaF-KOx / sodium citrate / citrate dextrose may be used - The use of heparin is discouraged due to its binding to EVs, and its interference with PCR analysis
Specifics of blood sampling	- Always at the same time of the circadian day - Always at the same time after food intake
Handling of the blood sample	- A 21-gauge (or larger) needle should be used - Butterfly systems should be avoided - First milliliters of samples blood should be discarded - The blood sample should be inverted 8-10 times to ensure optimal mixture with the anticoagulant - The time between blood sampling and preparation of plasma should be limited
Plasma preparation	- Consisting of 2 centrifugation steps of 15min at 2500g at room temperature - The plasma sample should be purified from any contaminating cells - The presence of hemolysis should be assessed - Platelets may be removed to obtain PFP
Plasma storage	- Consequences of plasma storage on EV-outcome remain unknown - Storage temperature remains debatable (4°C / -20°C / -80°C / -160°C)
EV isolation	- The choice of EV isolation method dependents on the required EV purity and concentration - The use of synthetic polymer-based precipitation is discouraged for EV isolation, but can be used to concentrate EV suspensions
EV storage	- EV pellets should be resuspended in PBS - The EV suspension should be stored at -80°C - Freeze-thaw cycles should be avoided
Verification EV isolation	- Various techniques can be used such as; dynamic light scattering (NanoSight), Western Blot, electron microscopy, ...

Current points of discussion concerning EV-research, and a summary of the standards and recommendations suggested in position papers by the Society of Extracellular Vesicles [92] and the International Society on Thrombosis and Haemostasis [93]. EDTA, Ethylenediaminetetraacetic acid; EV, extracellular vesicle; NAF-KOx, sodium fluoride/potassium oxalate; PBS, phosphate buffered saline; PCR, Polymerase chain reaction; PFP, platelet free plasma

Highlights

- Early diagnosis and step-wise follow up of liver fibrosis is needed
- Current non-invasive diagnostic tools lack sufficient sensitivity and specificity
- Liquid biopsy might overcome current diagnostic limitations
- Circulating vesicles and free nucleic acids, proteins, and lipids as biomarkers
- Limitations and possibilities of liquid biopsy in liver fibrosis

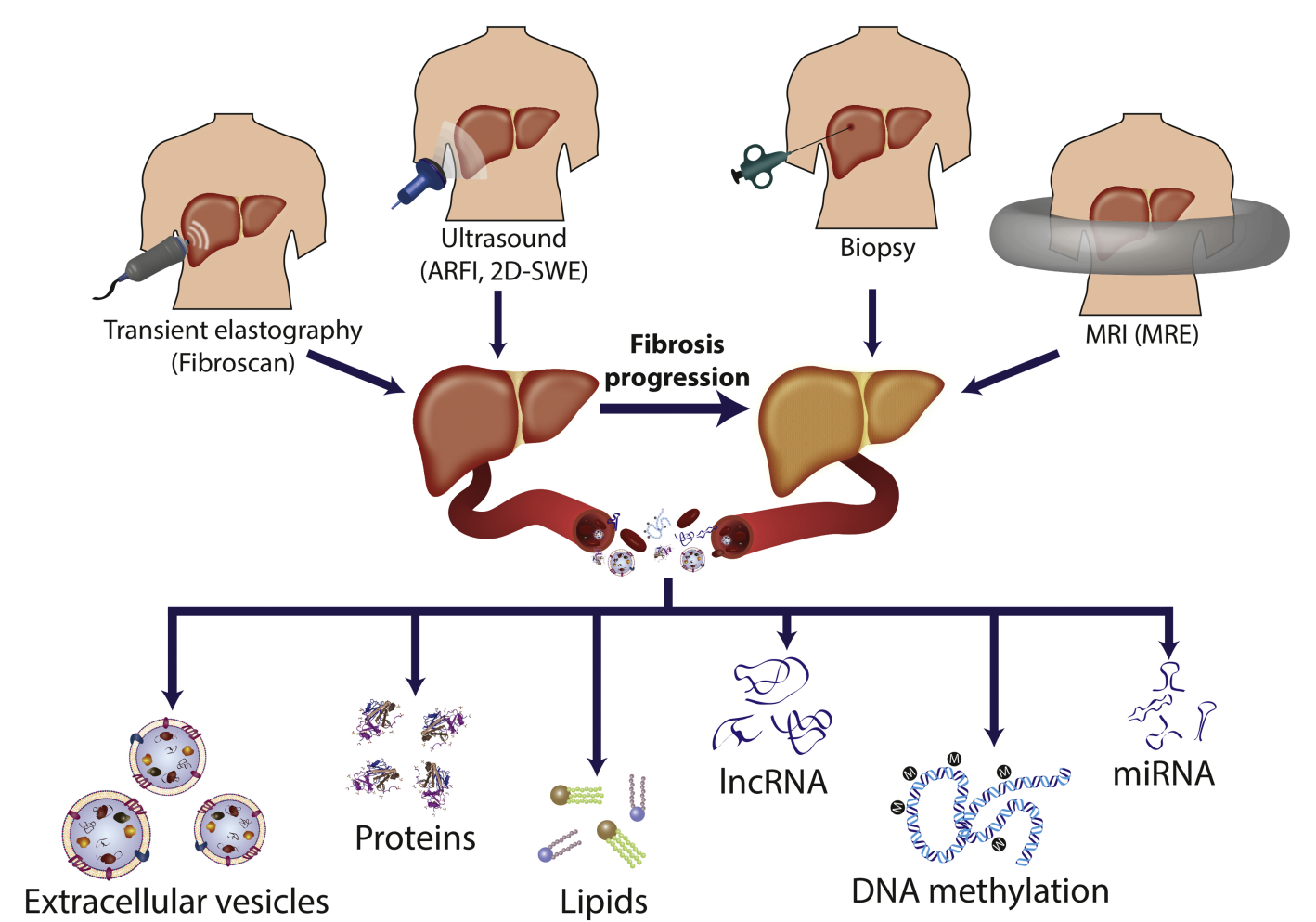
Transient elastography
(Fibroscan)
Ultrasound
(ARFI, 2D-SWE)
Biopsy
MRI (MRE)
Fibrosis
progression
Extracellular vesicles
Proteins
Lipids
lncRNA
DNA methylation
miRNA

Figure 1

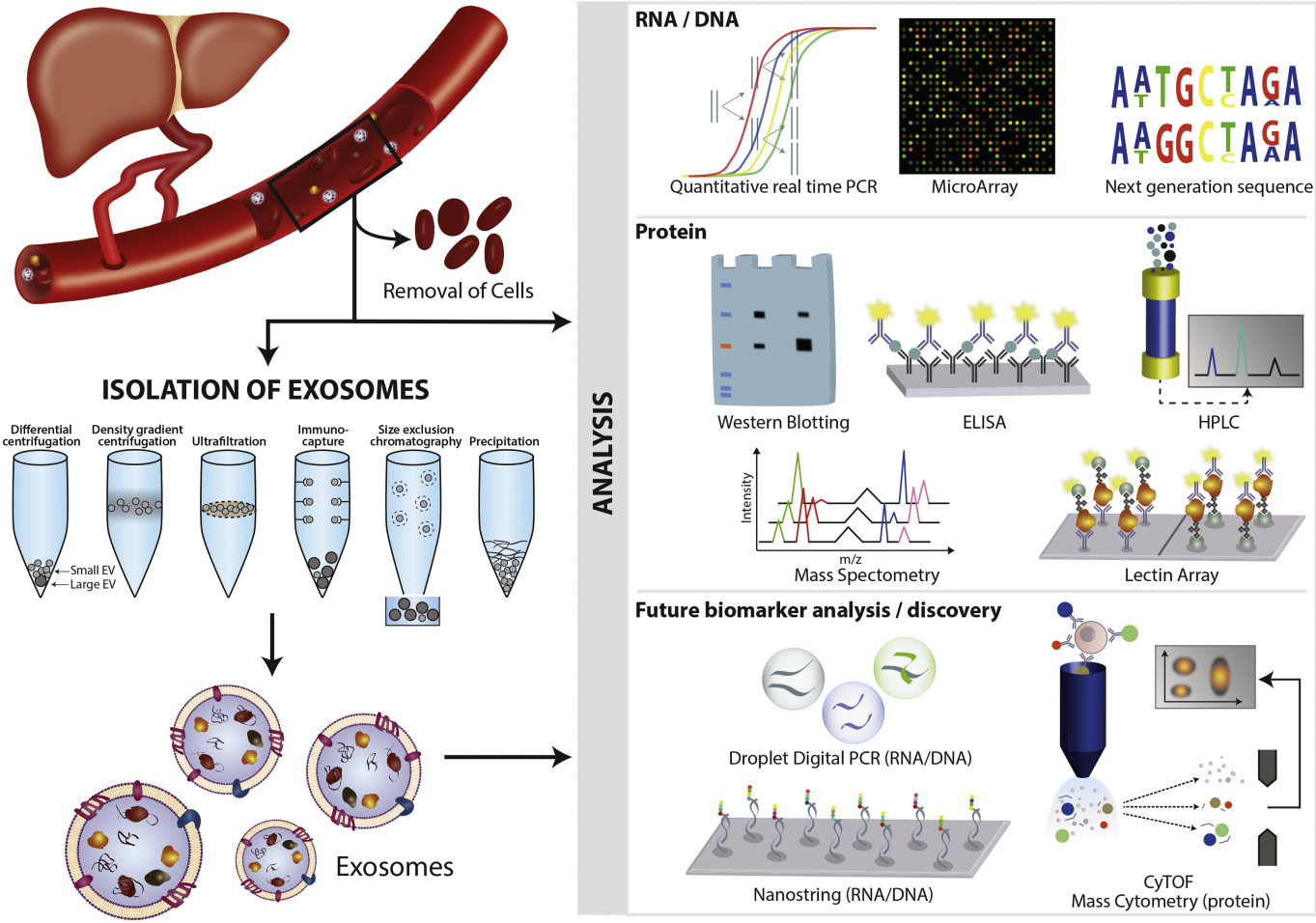
Removal of Cells
ISOLATION OF EXOSOMES
Differential centrifugation
Density gradient centrifugation
Ultrafiltration
Immuno-capture
Size exclusion chromatography
Precipitation
Small EV
Large EV
Exosomes
ANALYSIS
RNA / DNA
Quantitative real time PCR
MicroArray
AATGCTAGA
AAGGCTAGA
Next generation sequence
Protein
Western Blotting
ELISA
HPLC
Intensity
m/z
Mass Spectometry
Lectin Array
Future biomarker analysis / discovery
Droplet Digital PCR (RNA/DNA)
Nanostring (RNA/DNA)
CyTOF
Mass Cytometry (protein)

Figure 2