Serum markers of liver fibrosis: Combining the BIPED classification and the neo-epitope approach in the development of new biomarkers

Sanne Skovgård Veidal, Anne-Christine Bay-Jensen, Gervais Tougas, Morten Asser Karsdal and Ben Vainer

Department of Pharmacology, Nordic Bioscience, Herlev, Denmark
Novartis Pharma AG Translational Medicine, Basel, Switzerland
Department of Pathology, Rigshospitalet, University of Copenhagen, Denmark

Abstract. Background: Fibrosis is a central histological feature of chronic liver diseases and is characterized by the accumulation and reorganization of the extracellular matrix. The gold standard for assessment of fibrosis is histological evaluation of a percutaneous liver biopsy. Albeit a considerable effort have been invested in finding alternative non-invasive approaches, these have not been sufficiently successful to replace biopsy assessment.

Aim: To identify the extracellular matrix proteins of interest, that as protein degradation fragments produced during extracellular matrix metabolism neo-epitopes, may be targeted for novel biochemical marker development in fibrosis. We used the recently proposed BIPED system (Burden of disease, Investigative, Prognostic, Efficacy and Diagnostic) to characterise present serological markers.

Methods: Pubmed was search for keywords; Liver fibrosis, neo-epitopes, biomarkers, clinical trail, extra cellular matrix, protease, degradation, fragment.

Results and Conclusion: Implementation of BIPED categorization in the development and validation of fibrosis biomarkers to simplify and standardize the use of existing and future biomarkers seems advantageous. In addition, a systematic use of the neo-epitope approach, i.e. the quantification of peptide epitopes generated from enzymatic cleavage of proteins during extracellular remodeling, may prove productive in the quest to find new markers of liver fibrosis.

Keywords: Fibrosis, biomarkers, neo-epitope, liver, diagnostics, BIPED, extracellular matrix, collagen, proteoglycan

1. Introduction

Chronic liver diseases are major global health problems with approximately 800,000 deaths annually worldwide [6,128]. Chronic liver injury, irrespective of the cause, is associated with progressive liver fibrosis, which is observed microscopically as excessive deposition and abnormal distribution of extracellular matrix (ECM) components. Progression of fibrosis eventually leads to end stage cirrhosis [29]. The sequence of events in liver fibrosis resembles that of wound healing and scar formation, including recruitment of inflammatory cells and proliferation and/or activation of matrix-producing cells, such as hepatic stellate cells (HSC), endothelial cells and hepatocytes. During chronic damage to the liver, the continuous insult leads to a constant imbalance between fibrogenesis and fibrolysis, which
Fig. 1. Sequence of fibrogenesis as a result of liver cell injury. Liver cell injury causes activation of HSC and transdifferentiation into matrix-synthesizing MFB. \( \Delta \) = TIMP; \( \mathcal{O} \) = MMP; \( \mathcal{O} \) = Proteoglycan; \( \mathcal{O} \) = Collagen. Abbreviations used: HBV/HCV, Hepatitis B/V virus; NAFLD, non-alcoholic fatty liver disease; ECM, extra cellular matrix; EMT, epithelial-to-mesenchymal transition; HSC, Hepatic stellate cell; MFB, Myofibroblast.

results in alteration of the tissue composition and accumulation of connective tissue [108].

Identification and characterization of the cell types and the different mediators involved in liver fibrogenesis have expanded significantly during recent years [10, 42,85]. HSC are identified as the driving force of liver fibrosis. When HSC are activated by inflammatory mediators [37] they differentiate into hepatic myofibroblast-like cells (hMFB) capable of expression and secretion of several connective tissue components (e.g. collagens, elastin, proteoglycans, and hyaluronan) (Fig. 1) [37,74]. HSC are believed to be the main source of ECM proteins accumulated in the liver during chronic liver disease. Besides HSC, resent research has clearly demonstrated that other cell types contribute to the hMFB-pool [30,124,126]. These cells can be from local sources such as portal myofibroblasts [61] as well as newly formed HSC that originate from a process called epithelial-to-mesenchymal transition (EMT) in which biliary epithelial cells or hepatocytes transform into fibroblasts [56]. In addition there is a contribution to the hMFB-pool from outside the liver from cells like bone marrow [27] and circulating fibrocytes [102]. If the insult to the liver does not subsides, this excessive ECM deposition eventually results in liver cirrhosis and, ultimately, in liver failure.

Studies of humans and of animal models have suggested that some elements of fibrosis are reversible and, in specific circumstances, resolution with restoration to near normal organ architecture can be achieved [23, 24,45,53,98]. Recent work on animal models continues to provide solid foundations to the essence of this concept [12]. The change of paradigm associated to these findings is set to provoke an increase in the efforts to develop new therapies that modulate or reverse the fibrogenic process.

Because of the invasive nature and the potential side-effects of liver biopsy, serological disease markers are at present important tools for patient follow-up and treatment assessment. Candidate biomarkers for liver fibrosis have previously been classified into two categories [37]: Class I fibrosis markers are hypothesis-driven and based on molecular findings made in the study of fibrogenesis. Class II fibrosis markers are
Table 1
Collagens of the hepatic extracellular matrix

<table>
<thead>
<tr>
<th>Group</th>
<th>Collagen type</th>
<th>Chains</th>
<th>Origin</th>
<th>Cellular source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fibril forming collagens</em></td>
<td>I [34,36,78]</td>
<td>α₁(1) α₂(1)</td>
<td>Widely distributed</td>
<td>HSC, MFB, sinusoidal, and portal endothelial</td>
</tr>
<tr>
<td></td>
<td>III [34,36,78]</td>
<td>α₁(III)</td>
<td>Widely distributed</td>
<td>HSC, MFB, sinusoidal, and portal endothelial</td>
</tr>
<tr>
<td><em>Basement membrane collagens</em></td>
<td>V [34,36,78]</td>
<td>α₁(V), α₂(V), α₃(V)</td>
<td>Widely distributed</td>
<td>HSC, MFB, sinusoidal, and portal endothelial</td>
</tr>
<tr>
<td></td>
<td>IV [34,36,78]</td>
<td>α₁(IV) α₂(IV)</td>
<td>Basement membranes</td>
<td>HSC, MFB, sinusoidal, and portal endothelial</td>
</tr>
<tr>
<td><em>Microfibrillar collagen</em></td>
<td>VI [122]</td>
<td>α₁(VI) α₂(VI)</td>
<td>Ubiquitously expressed in the</td>
<td>HSC, MFB, sinusoidal, portal endothelial</td>
</tr>
<tr>
<td><em>Hexagonal network-forming collagens</em></td>
<td>VIII [62]</td>
<td>α₁(VIII) α₂(VIII)</td>
<td>Subendothelial space</td>
<td>HSC, MFB, sinusoidal, portal endothelial</td>
</tr>
<tr>
<td><em>FACTIT collagens</em></td>
<td>XIV [17]</td>
<td></td>
<td>Mainly found in mesenchyme</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td><em>Multiplexins</em></td>
<td>XIX [86]</td>
<td></td>
<td>Basement membranes, particularly in the perisinusoidal space</td>
<td>Hepatocytes &gt; bile duct epithelial</td>
</tr>
<tr>
<td></td>
<td>XVIII [118]</td>
<td></td>
<td></td>
<td>&gt; entothelial cells &gt; HSC and MFB</td>
</tr>
</tbody>
</table>

Fibrogenesis during chronic liver diseases is a dynamic process involving complex cellular and molecular mechanisms [77]. Excessive fibrogenesis is the result of an imbalance between degradation and formation of ECM components. This will ultimately lead to increased liver size and density with progressively impaired liver function as the end-result. These ECM macromolecules are mainly fibrous proteins with structural and adhesive functions, such as collagens and proteoglycans.

2. Potential targets: The hepatic extracellular matrix

Fibrogenesis during chronic liver diseases is a dynamic process involving complex cellular and molecular mechanisms [77]. Excessive fibrogenesis is the result of an imbalance between degradation and formation of ECM components. This will ultimately lead to increased liver size and density with progressively impaired liver function as the end-result. These ECM macromolecules are mainly fibrous proteins with structural and adhesive functions, such as collagens and proteoglycans.

2.1. Collagens

Collagens are responsible for the structural integrity of the ECM of most connective tissues, including that of the liver. The ECM content results from a fine balance between synthesis and degradation tightly controlled through regulation of gene expression and protein secretion, but also through endogenous protease inhibition and protein degradation by metalloproteinas (MMPs) and cysteine proteases [32,68,79]. Ten collagen types have been described in the liver (Table 1) [111]. Of those, the two major collagens are the fibril-forming types I and III. Fibril-forming collagens are predominantly synthesized by HSC as precursor molecules with large propeptide extensions at both the N- and C-terminal ends [31]. These propeptides are used as markers for liver fibrogenesis under various settings [20,41]. The mature propeptides are cleaved from procollagen by N- or C-terminal proteinases, and the mature collagen is then integrated into the ECM [31, 34]. During fibrogenesis, type I and III collagen levels increase up to 8 times [36] with a significantly higher increase of type I collagen than of type III collagen, changing the I/III ratio from 1:1 in the healthy liver to 2:1 in the cirrhotic liver. As demonstrated in Table 1,
Table 2: Proteoglycans of the hepatic extracellular matrix

<table>
<thead>
<tr>
<th>Group</th>
<th>Proteoglycans</th>
<th>Other origin</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large extracellular proteoglycans</td>
<td>Aggrecan [60,67]</td>
<td>Articular cartilage chondrocytes, intervertebral disc, nasal cartilage</td>
<td>Extracellular matrix stability (hyaluronan binding)</td>
</tr>
<tr>
<td>(aggregating and hyaluronan-binding)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small leucine-rich proteoglycans (collagen-binding)</td>
<td>Decorin [9,79]</td>
<td>Connective tissue, cartilage, bone</td>
<td>Binds to and connect collagen molecules (matrix stabilization and thickness)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Organogenesis Binding of TGFβ</td>
</tr>
<tr>
<td></td>
<td>Biglycans [26,83]</td>
<td>Capillary endothelium, skin (keratinocytes), epithelium of kidney</td>
<td>Cell differentiation Binds and connect collagen fibrils</td>
</tr>
<tr>
<td></td>
<td>Fibromodulin [67,115]</td>
<td>Connective tissue, bone, cartilage, kidney, lung, intestine</td>
<td>Regulate orientation of collagen fibers</td>
</tr>
<tr>
<td></td>
<td>Lumican [9,67]</td>
<td></td>
<td>Controls spacing and thickness of collagen fibers</td>
</tr>
<tr>
<td></td>
<td>Perlecan [33,91,107]</td>
<td>All basement membranes</td>
<td>TGFβ receptor and signaling Possible reservoir of TGFβ/β2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cell-adhesion</td>
</tr>
</tbody>
</table>

3. The balance of tissue formation and degradation – proteases and their natural inhibitors

The imbalance in liver fibrosis between synthesis and degradation of ECM results in conversion of the low-density subendothelial matrix into matrix rich in interstitial collagens (Fig. 2). The increase in collagen and proteoglycan can be the results of; increased protein production, impaired protein degradation, or diminished matrix degradation or a combination of those. The process of decreased protein degradation has recently received increased attention [8,47]. In the extracellular space, matrix degradation occurs predominantly as a consequence of the action of MMPs. MMPs are secreted from cells into the extracellular space as proenzymes, which are then activated by a number of specific, usually cell surface-associated, cleavage mechanisms. The active enzymes are in turn inhibited by a family of tissue inhibitors of metalloproteinases (TIMP) [3]. By this combination of mechanisms, extracellular matrix degradation is closely regulated, which prevents inadvertent tissue damage.

As a consequence, excessive matrix deposition may be the consequence of either increased formation, decreased degradation of connective tissue components or both.

4. The gold standard for identification and follow-up of liver diseases

Histopathologic examination of percutaneous liver biopsies is the gold standard for establishing diagnosis.
Fig. 2. Tissue loss, gain, and transformation during pathogenesis rely on extracellular matrix remodeling. The left picture shows ECM in healthy conditions where the cells (●) and ECM molecules (\(\downarrow\)) are highly organized. During fibrosis development the ECM changes composition and structure resulting in accumulation and changed structure (Right picture).

and staging of liver disease and it is the primary end point when evaluating the efficacy of new antifibrotic therapies. The analysis of consecutive liver specimens further allows evaluation of the progression of fibrosis (i.e. the stage of the disease [63]) and of the disease activity. However, the histopathological examination of liver biopsy presents a series of drawbacks. Besides being invasive with a mortality rate of 1/1,000–1/10,000 and with a rate of severe complications of 1/200, it is prone to sampling error since only 1/50,000 of the liver mass is examined and is subjected to a reproducibility of only 35–45% [38]. Furthermore, histological evaluation is highly dependent on the experience of the pathologist, and even in the best of hands, the method may not unambiguously determine the stage of disease [38].

Different interpretation systems have been designed to minimize these uncertainties. The basic idea behind those systems is to standardize and integrate two or more histological features considered by medical consensus as having the highest predictive value (e.g. fibrosis, inflammatory activity and necrosis). Quantitative as well as qualitative scoring systems have been proposed. The semi-quantitative Knodell score [64] – and its modification, the Ishak score [52] – has been preferred for assessment of hepatitis in clinical trials. This evaluation system combines the assessment of periporal and/or bridging necrosis, intralobular degeneration and focal necrosis, portal inflammation, and fibrosis into an overall score.

5. Which information can be obtained from measuring serological biomarkers of liver fibrosis?

The use of liver biopsies provides static information of the disease, and some insights into the response to treatment when performed as serial biopsies. Serological biochemical markers have been used in other settings for prognostic use, especially for identification of “fast progressors” or those who possibly would benefit the most from treatment [109].

This implies that biopsies and serological markers may supply independent information, much as serological markers of bone resorption in combination with a bone mineral density testing by X-ray have provided osteoporotic patients with improved diagnostic and prognostic values [48,59]. The combination of a marker highly sensitive to changes together with a status assessment may be of use in monitoring patients with chronic liver disease.

6. Classification of biomarkers

Numerous attempts have been made to explore non-invasive markers that are capable of providing accurate information about fibrogenesis and the extent of fibrosis in the liver [117]. So far, however, the impact of biochemical markers of fibrosis in clinical practice has been very limited. Even when markers have proven useful in follow-up studies by providing complementary information to the histopathological analysis, no biomarker or combination of biomarkers has shown significant clinical or preclinical validity in replacing needle biopsy or minimizing animal experimentation [37,38].

This failure of success may have different causes, of which one may be inconsequence in the classification of potential biomarkers. The most comprehensive classification of fibrosis biomarkers was published by Dr. Gressner and colleagues [38], who separated biomarkers into Class I and Class II biomarkers. Class I biomarkers are those intended to reflect ECM turnover and/or fibrogenic cell changes. Class II are indirect serum biomarkers based on algorithmic evaluations of commonly observed functional alterations of the liver that do not necessarily reflect ECM turnover and/or fibrogenic cell changes. Generally, Class I biomarkers
derive from hypothesis-driven research based on ECM biology and cellular mechanisms of pathology, whereas Class II biomarkers are identified from retrospective studies in which combinations of mostly classical clinical biochemistry parameters associated to liver function were tested for their predictive value. A disadvantage of this classification is that it does not provide information about the potential clinical use of the biomarkers nor does it go far enough in terms of recognizing, differentiating and understanding them. Class I biomarkers, in particular, originating from different lines of basic research and their changes, might be very difficult to interpret from a clinical or pathophysiological perspective. A functional classification, establishing concise standard definitions of biomarker types, would allow for a better understanding of the relationship between individual markers and between markers and clinical features. It would also help focusing research efforts and, ultimately, the chances of finding markers with in vivo applicability.

7. The BIPED classification

The BIPED classification categorizes biomarkers according to key parameters that are needed for assessment of clinical trials in research and development. Several classes have been defined: (i) Biomarkers that describe the progression of disease and that correlate with known clinical parameters (Burden of disease – B); (ii) biomarkers that capture the effect of an intervention in both known and unknown biological mechanisms associated with clinical outcome and that can act as a surrogate marker, i.e. changes in these biomarkers can predict the clinical outcome (Efficacy – E); (iii) diagnostic biomarkers enabling identification of patients within the population and identification of subgroups within the diseased population (Diagnostic – D); (iv) biomarkers that identify subjects with high risk of progression (Prognostic – P); and (v) biomarkers not fully validated in a study population and therefore can be used solely for scientific investigations (Investigatory – I). Figure 3 illustrates the relationship between the different biomarkers in the context of a study population.
The purpose of a D-marker is to provide a clear discrimination between diseased and non-diseased subjects within the total population. A new diagnostic biomarker should be evaluated by comparison with the established gold standard with the appropriate spectrum of subjects. Initial verification would be obtained in a population from a cross-sectional dataset (optimally including patients with conditions that may clinically be confused with the target disease) or a case-control design with subjects with and without documented fibrosis. The clinical usefulness of a D-marker needs to be evaluated by a series of parameters, including high sensitivity, high specificity and high positive/negative predictive value (PPV/NPV). As additional statistical parameters used for D-markers, the positive likelihood ratio (LR), and the area under the curve (AUC), derived from receiver operator curve (ROC) analyses, are used to assess the probability that a positive or negative test indicate those with or without the disease (e.g. liver fibrosis).

A B-marker, in contrast, should be able to assess the severity and the extent of disease within the group of individuals, who are classified as having the diagnosis by using the diagnostic marker. This is evaluated typically at a single time point in either baseline assessments of subjects enrolled in a clinical trial or by examining cross-sectional data of individuals with liver disease from different cohorts. In this case, the necessary comparison with the gold standard is focused on disease severity; while the parameters used to assess B-markers are similar to those used for D-markers.

A P-marker is a marker that predicts either the future onset or the progression of the disease. Longitudinal studies (both prospective and retrospective) are required to evaluate this kind of markers, showing an association of the marker at baseline with the risk of progression or of developing the disorder. The purpose of a P-marker is to predict future episodes and not to classify individuals by a given criteria. Relative risk (RR) or odds ratio (OR), given the presence or absence of the marker, are used to describe dichotomous or categorical outcomes, while the RR or OR per SD increase or decrease in the marker, and/or the AUC estimated from ROC are used for P-markers examined as continuous variables. Thus, for an outcome such as progression of the disease assessed by a liver histopathology score, the methods of analyses are different from those of D- and B-markers.

An ideal E-marker is a serum biomarker of disease that, when measured serially, is associated with an improved or beneficial clinical or histopathological outcome among patients who are given a specific treatment, i.e. a surrogate efficacy marker that is predictive for gold standard outcome. In its most simple form it would provide a dichotomous outcome, discriminating between responders and non-responders, and in other settings it provides information about efficacy of treatment among individuals at high risk of developing a given liver disease. The serial evaluation of an E-marker should be focused on the intervention group in a randomized controlled trial. E-markers are typically continuous, and therefore regression models are used for assessment that correlate changes in biomarker levels (e.g. per unit or standard deviations) to changes in an outcome variable.

8. Classification of liver fibrosis biomarkers according to BIPED

Despite the fact that in many clinical studies on biomarkers targeting it has been suggested that the proposed biomarkers can be applied as either B- or D-markers, less than a handful of the suggested fibrosis biomarkers have reached relevant clinical application up to this point. In Table 3, liver disease biomarkers analyzed in clinical trials is organized according to the new BIPED classification of biomarkers recently introduced in the field of cartilage diseases [7].

The presented framework of assessment focuses on the BIPED categorization, with less attention drawn to sensitivity and specificity performance of the individual markers in the individual studies. In order to compare the performance of different markers with specificity and sensitivity, comparable study populations or even similar assessment techniques performed in the same study population are needed. This is most optimally compared to a gold standard following subsequent odds-ratio calculations. Unfortunately, most study populations are constructed with diseased individuals compared with selected healthy controls, and do therefore not reflect the clinical situation but a nested tailored situation. Hence, the specificity and sensitivity becomes highly dependent on the study population. Classification by the BIPED system may enable researcher from different research backgrounds to communicate in a robust assessment framework, with special focus on diagnostic, prognostic and possible burden of disease evaluations.

An additional category has recently been added to the BIPED classification, namely markers defining the stage of a disease (S-marker) [7]. It can be argued that
### Table 3

<table>
<thead>
<tr>
<th>Class</th>
<th>Biomarker</th>
<th>Parameters</th>
<th>Chronic liver disease</th>
<th>BIPED classification</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Hyaluronan</td>
<td>Hyaluronan</td>
<td>HCV</td>
<td>I, D</td>
<td>[15,80,94,127]</td>
</tr>
<tr>
<td>I</td>
<td>IGF-1</td>
<td>IGF-1</td>
<td>HCV</td>
<td>I</td>
<td>[75]</td>
</tr>
<tr>
<td>I</td>
<td>Leptin</td>
<td>Leptin</td>
<td>HCV</td>
<td>I</td>
<td>[76]</td>
</tr>
<tr>
<td>I</td>
<td>PIIIP</td>
<td>PIIIP</td>
<td>HCV</td>
<td>I</td>
<td>[72,120]</td>
</tr>
<tr>
<td>Mixed I</td>
<td>Zheng et al. index</td>
<td>HA, PIIICP, PIIINP, Laminin, C-IV</td>
<td>Chronic hepatitis</td>
<td>I</td>
<td>[131]</td>
</tr>
<tr>
<td>Mixed I</td>
<td>Lebensztjen et al. index</td>
<td>Laminin-2, C-IV, MMP2, MMP9-TIMP1</td>
<td>HBV</td>
<td>I</td>
<td>[69]</td>
</tr>
<tr>
<td>Mixed I</td>
<td>Lebensztjen et al. index</td>
<td>Tenascin, hyaluronan, Collagen VI, TIMP-1</td>
<td>HBV</td>
<td>I</td>
<td>[70]</td>
</tr>
<tr>
<td>Mixed I</td>
<td>Patel et al. index</td>
<td>Hyaluronan, TIMP-1, α₂-macroglobulin</td>
<td>HCV</td>
<td>D, I</td>
<td>[95]</td>
</tr>
<tr>
<td>Mixed I</td>
<td>Patel et al. index</td>
<td>TIMP-1, tenasin, collagen IV, PIIINP, MMP2, laminin, Hyaluronan</td>
<td>NASH</td>
<td>I</td>
<td>[73]</td>
</tr>
<tr>
<td>II</td>
<td>Forns-index</td>
<td>Age, platelet count, γGT, cholesterol</td>
<td>HCV HIV/HCV</td>
<td>D, I</td>
<td>[11,13,28,72,90]</td>
</tr>
<tr>
<td>II</td>
<td>FibroTest</td>
<td>Haptoglobin, α₂-macroglobulin, apolipoprotein A1, γGT, bilirubin</td>
<td>HCV HIV/HCV</td>
<td>I, D, E</td>
<td>[11,13,40,44,55,72, 73,88,90,91,92,99,102, 104,105,109]</td>
</tr>
<tr>
<td>II</td>
<td>Actitest</td>
<td>FibroTest + ALT</td>
<td>HCV</td>
<td>I, D, E</td>
<td>[44,87,100,101]</td>
</tr>
<tr>
<td>II</td>
<td>APRI</td>
<td>AST, platelet count</td>
<td>HIV/HCV</td>
<td>D, I</td>
<td>[2,11,13,14,17,44,51,72, 73,88,90,91,92,99,102, 104,105,109]</td>
</tr>
<tr>
<td>II</td>
<td>Hepscore</td>
<td>Bilirubin, γGT, hyaluronan, α₂-macroglobulin, age, gender</td>
<td>HCV HIV/HCV</td>
<td>D, I, B</td>
<td>[1,13,43,71,72]</td>
</tr>
<tr>
<td>II</td>
<td>FIB-4</td>
<td>Platelet count, AST, ALT, age</td>
<td>HIV/HCV</td>
<td>I, D</td>
<td>[13,119]</td>
</tr>
<tr>
<td>II</td>
<td>SHASTA</td>
<td>Hyaluronan, albumin, AST</td>
<td>HIV/HCV</td>
<td>I</td>
<td>[13]</td>
</tr>
<tr>
<td>II</td>
<td>Fibroindex</td>
<td>FORN+APRI</td>
<td>HCV</td>
<td>D, I, E</td>
<td>[65]</td>
</tr>
<tr>
<td>II</td>
<td>Fibrometer test</td>
<td>Platelet count, prothrombin index, AST, α₂-macroglobulin, hyaluronan, urea, age</td>
<td>HIV/HCV</td>
<td>D, I</td>
<td>[13,14,43,71,72]</td>
</tr>
<tr>
<td>II</td>
<td>NFSA</td>
<td>Age, hyperglycaemia, body mass index, platelets, albumin, AST/ALT</td>
<td>NAFLD</td>
<td>I</td>
<td>[14]</td>
</tr>
<tr>
<td>II</td>
<td>Ultrasound + APRI</td>
<td>AST, platelet count, Ultrasound</td>
<td>HCV</td>
<td>D, I</td>
<td>[92]</td>
</tr>
<tr>
<td>II</td>
<td>Metwally et al. index</td>
<td>Platelet count, albumin, AST, history of blood transfusion, HBV core antibody</td>
<td>HCV</td>
<td>D, I</td>
<td>[81]</td>
</tr>
<tr>
<td>II</td>
<td>Mohammadnejad et al. index</td>
<td>Age, HBV DNA levels, alkaline phosphatase, albumin, platelet counts, AST</td>
<td>HCV</td>
<td>D, I</td>
<td>[84]</td>
</tr>
<tr>
<td>II</td>
<td>FibroSpect II</td>
<td>Hyaluronan, TIMP-1, α₂-macroglobulin</td>
<td>HCV</td>
<td>D, I</td>
<td>[96,114,129]</td>
</tr>
<tr>
<td>II</td>
<td>Stepwise combination algorithms</td>
<td>Combination of APRI and Fibrotest</td>
<td>HCV</td>
<td>D, I</td>
<td>[112]</td>
</tr>
<tr>
<td>II</td>
<td>Imbret-Bisman index</td>
<td>α₂ macroglobulin, AST, ALT γGT, total bilirubin, albumin, α₁ globulin, α₂ globulin, β globulin, γ globulin, apolipoprotein A</td>
<td>HCV</td>
<td>I</td>
<td>[51]</td>
</tr>
<tr>
<td>II</td>
<td>Nunes et al.</td>
<td>Age, Platelets, INR, CD4, AST/ALT, Hyaluronan, YKL-40, PIIINP</td>
<td>HCV/HIV</td>
<td>D, I</td>
<td>[90]</td>
</tr>
<tr>
<td>II</td>
<td>Fibroscan + +</td>
<td>Fibroscan, Fibrotest, APRI</td>
<td>HCV</td>
<td>D, I</td>
<td>[18]</td>
</tr>
</tbody>
</table>

Staging markers are in fact B-markers assessing the extent of disease. In arthritis, markers of this class are capable of measuring the severity within a particular joint, and/or severity in terms of number of joints involved. We postulate that, in liver fibrosis, staging markers are a subcategory of the burden of disease-markers, assuming that fibrosis is equally distributed in the entire liver, an assumption that is also made on liver biopsy sampling. However, this is not necessarily the case as was described previously, and a new category therefore had to be established for the staging markers.

Biochemical markers measured in serum/urine are the product of systemic events, in which many local specific events contribute to that pool of biomarker epitope. Biomarkers require validation as the assessment of the assay or measurement performance characteristics including sensitivity, specificity, and reproducibility [116,123]. Even though some biochemical markers
Fig. 4. Neo-epitopes – protease degradation of proteins. A) Fragments are generated of a protein by enzymatic activity. B) The enzymatic cleavage leads to two highly specific fragments. C) After blasting and homology search antibodies are developed against the specific cleavage site. During development the antibodies are screen against different de-selection peptides (e.g. elongated peptides) to make sure the developed antibody do not react with the whole protein, but reacts only against the generated specific cleavage site- the neo-epitope.

have been shown to correlate with the number of affected sites, such as collagen type II degradation markers and number of affected joints in OA [82], tissue specificity and pathological relation must be carefully evaluated, possible by application of the BIPED criteria [7,57]. Particular emphasis should be directed to whether novel markers may be a diagnostic or prognostic marker [57]. Even though the combined approach with proteases degradation fragments may increase the accuracy of the biomarker, the exact tissue distribution and contribution deserves attention to obtain maximal disease accuracy and assay precision.

9. The neo-epitope approach

Different approaches exist to biochemical biomarker development [105,110]. The main approaches taken are an unbiased proteomic approach involving a range of powerful techniques [66,130], and a more simple hypothesis driven approach [57]. Each approach provide advantages and drawback, however a deeper discussion is out of the current scope.

Matrix remodeling is an integrated process of tissue development, maintenance and pathogenesis.

Endopeptidases such as MMPs and cysteine proteases play major roles in the degradation of extracellular macromolecules such as the collagen and proteoglycans. The proteolytic action of the MMPs results in generation of specific cleavage fragments, called neo-epitopes (Fig. 4). Even though many components of the ECM as well as enzymes responsible for remodeling may overlap between different tissues, the combination of a specific protease and a specific ECM protein component may provide a unique combination for a specific tissue or a specific disease mechanism.

The neo-epitope approach has been used extensively in bone and cartilage diseases which are diseases with extensive ECM remodeling [109]. In alignment with the BIPED criteria, serological markers of diagnosis, efficacy and prognosis have been identified and exploited during drug development for osteoarthritis and osteoporosis [109]. Imbalance between tissue formation and tissue degradation is involved in both diseases. For example, evidence points to the fact that postmenopausal bone loss is the result of both an increase in bone resorption and an increase in bone formation, where the excessive bone resorption leads to a net bone loss [58]. Furthermore, MMP generated fragments of type II collagen (CTX-II), have been demonstrated and extensively used in osteoarthritis as both a diagnostic, prognostic and efficacy marker [4,22,104], thereby assisting researchers in understanding key biological questions related to progression of the disease. These key learnings may be transferred into the area of liver fibrosis. The enzymes presently receiving the most attention in the liver fibrosis field are the MMPs in combination with collagen type I and III, and protease-generated fragments of collagen types I and III may be relevant targets for biochemical markers development for measuring high turnover during fibrogenesis.
At present there are limited ECM neo-epitope studies concerning liver fibrosis. Guañabens N et al. [40] evaluated the bone turnover markers N-telopeptide of type I collagen (NTX), C-telopeptide of type I collagen (CTX) and N-terminal pro-peptide of collagen type I (PINP) in 34 women with primary biliary cirrhosis, a disease with increased liver fibrosis. The aim was to evaluate the influence of a noninvasive disease with increased connective tissue synthesis or degradation on the levels of the bone turnover markers. The level of NTX, CTX and PINP were elevated in patients compared to controls and correlated with the histological stage of the disease. Even though the levels correlates with the disease, the bone turnover markers would give a high background in many liver fibrosis diseases due to high bone turnover, which is a typical complication in fibrotic liver diseases [19,21,93].

10. Conclusion

We have introduced the BIPED classification and classified the existing liver fibrosis biomarkers used in clinical trial according to this system. Although considerable work has been done, none of the biomarkers have yet proved itself suitable for clinical use in the evaluation of patients with chronic liver disease. We recommend that the BIPED criterion is implemented in the development and validation of biomarkers, so that clinicians and researchers are easily guided in the use of existing and future biomarkers. We would also like to draw attention to the neo-epitope approach, which has provided instrumental biochemical marker tools for researchers within the fields of osteoarthritis and osteoporosis. As many pathologies involves extensive extracellular matrix remodelling, and fibrotic diseases in particular are related to extensive ECMR with matrix deposition and protease expression/regulations, the lessons learned from pathologies may suggest optimized strategy for targeting specific tissue components with real pathophysiological significance.

The ultimate goal for the use of biomarkers is to increase the sensitivity and the specificity of the follow-up of chronic liver patients, minimizing the need for invasive liver biopsy procedures in this clinical setting. Application of biomarkers would provide the clinicians with improved tools to personalize the treatment, to follow the progression of the disease and to monitor the response to treatment.

Declaration

Sanne Skovgård-Veidal, Anne-Christine Bay-Jensen and Morten Asser Karsdal are employees of Nordic Bioscience. Gervais Tougas is an employee of Novartis. Morten Asser Karsdal owns stocks and shares in Nordic Bioscience.

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