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

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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 succesfull 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 (**B**urden of disease, **I**nvestigative, **P**rognostic, **E**fficacy and **D**iagnostic) 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

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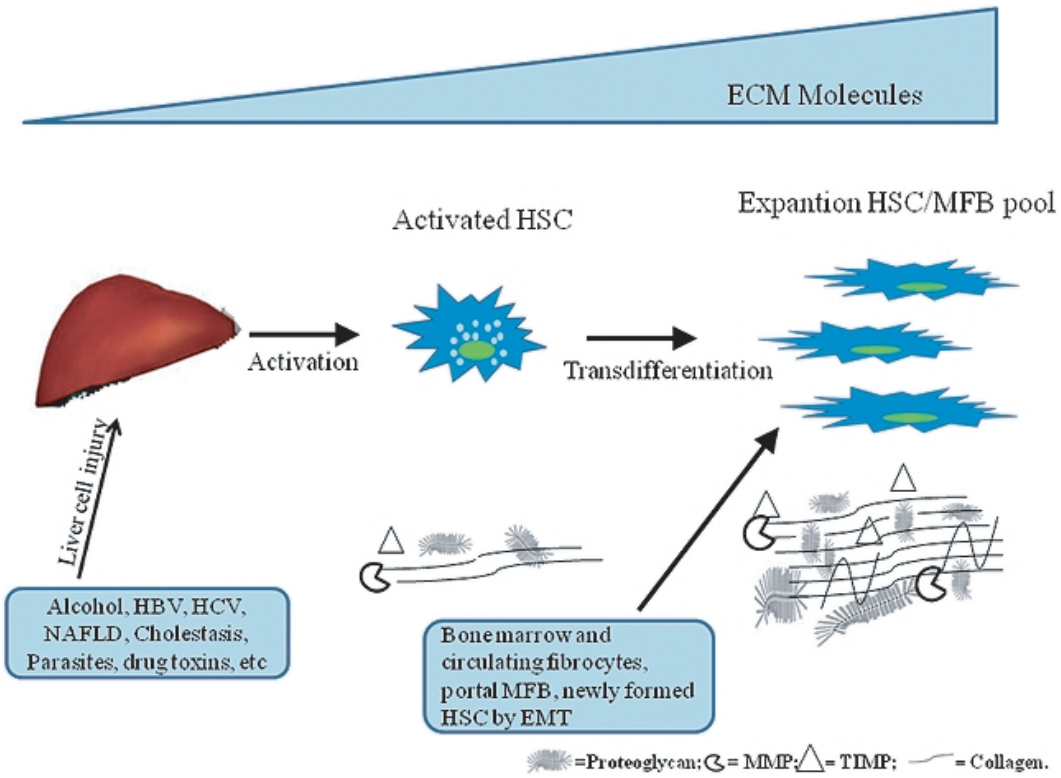

Fig. 1. Sequence of fibrogenesis as a result of liver cell injury. Liver cell injury causes activation of HSC and transdifferentiation into matrix-synthesizing MFB. △ = TIMP; ᔓ = MMP; ≈ =Proteoglycan; ⌒ = Collagen. Abbreviations used: HBV/HCV, Hepatitis B/V virus; NAFLD, non-alcoholic fatty liver disease; 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 circulationg 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

Group	Collagen type	Chains	Origin	Cellular source
Fibril forming collagens	I [34,36,78]	α_1(I) α_2(I)	Widely distributed	HSC, MFB, sinusoidal, and portal endothelial
	III [34,36,78]	α_1(III)	Widely distributed	HSC, MFB, sinusoidal, and portal endothelial
	V [34,36,78]	α_1(V), α_2(V), α_3(V)	Widely distributed	
Basement membrane collagens	IV [34,36,78]	α_1(IV) α_2(IV)	Basement membranes	HSC, MFB, sinusoidal, and portal endothelial, and bile duct epithelial (levavasseur 1995)
Microfibrillar collagen	VI [122]	α_1(VI) α_2(VI)	Ubiquitously expressed in the interstitial ECM	HSC, MFB, sinusoidal, portal endothelial, and bile duct epithelial
Hexagonal network-forming collagens	VIII [62]	α_1(VIII) α_2(VIII)	Subendothelial space	Endothelial cells
FACIT collagens	XIV [17]		Mainly found in mesenchyme	Mesenchymal cells
	XIX [86]			
Multiplexins	XVIII [118]		Basement membranes, particularly in the perisinusoidal space	Hepatocytes $>$ bile duct epithelial $>$ entothelial cells $>$ HSC and MFB

mainly clinical laboratory tests that can relate biochemical changes in serum or plasma to liver disease status. Both approaches have been employed in the quest to find alternatives to liver biopsy examination, which is the current gold standard in liver disease diagnostic and monitoring. At present, the available scientific literature on liver fibrosis biomarkers contains a sizable number of experimental studies which differ in scope, design and methodology, making comparison of markers and approaches difficult. A classification system using simple robust parameters to categorize and evaluate the application the individual fibrosis biomarker, i.e. diagnostic, prognostic or burden of disease indications, would help understand the state of the art of novel and excisting markers and may allow better implementation of biochemical markers in study design and patient monitoring.

This review describes major ECM components that may be targeted in fibrosis biomarker research as potential type I biomarkers and introduces the BIPED classification (Burden of disease, Investigative, Prognostic, Efficacy of treatment and diagnostic). Finally, based on our experience from other pathologies of extensive ECM remodeling, we suggest to implement a novel approach for the identification and development of biochemical markers, namely by use of the neo-epitopes approach.

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 metalloproteinases (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 is demonstrated in Table 1,

Table 2
Proteoglycans of the hepatic extracellular matrix

Group	Proteoglycans	Other origin	Function
Large extracellular proteoglycans (*aggregating and hyaluronan-binding*)	Aggrecan [60,67]	Articular cartilage chondrocytes, intervertebral disc, nasal cartilage	Extracellular matrix stability (hyaluronan binding)
Small leucine-rich proteoglycans (*collagen-binding*)	Decorin [9,79]	Connective tissue, cartilage, bone	Binds to and connect collagen molecules (matrix stabilization and thickness) Organogenesis Binding of TGFβ
	Biglycans [26,83]	Capillary endothelium, skin (keratinocytes), epithelium of kidney	Cell differentiation Binds and connect collagen fibrils
	Fibromodulin [67,115]	Connective tissue, bone, cartilage	Regulate orientation of collagen fibers
	Lumican [9,67]	Cornea, muscle, cartilage, kidney, lung, intestine	Controls spacing and thickness of collagen fibers
Cell-associated proteoglycans	Syndecans [5,107,125]	Widely distributed – often cell membrane bound	Binds collagens, fibronectin, thrombospondin, tenascin and bFGF TGFβ receptor and signaling Possible reservoir of TGFβ
	Perlecan [33,91,107]	All basement membranes	Selective barrier for macromolecules Cell-adhesion

the liver also contains other collagen types, all of which may be involved in the excessive fibrogenesis during fibrosis, leading to the end result of cirrhosis with a total of up to six times more collagen than in the normal liver [106].

2.2. Proteoglycans

Increasing attention is directed to the measurement and understanding of proteoglycans in the liver tissue, as these molecules in various forms and composition are associated with liver function. Proteoglycans are a diverse group of macromolecules that covalently bind a variable number of glycosaminoglycan (GAG) side chains to a core protein [46]. Table 2 lists the most studied proteoglycans found in the liver.

In the liver, proteoglycans are localized to the extracellular, pericellular spaces and on the cell surface, where they participate in cell-cell, cell-matrix and protein-protein interactions [55]. In the normal liver, the net amount of proteoglycans is low [78], whereas the level of several proteoglycans is increased during liver fibrosis [35]. Decorin is the most extensively studied proteoglycan in liver fibrosis [25,33,49,83] followed by biglycan, perlecan, aggrecan (in rodents), syndecan and lumican all showing elevated levels as a response to chronic liver injury. Animal studies have shown that chronic liver damage causes deposition of especially decorin, perlecan and biglycan in fibrotic septa [33,83], and syndecan-1 and -2 are increased on both mRNA and protein levels in the cirrhotic livers [55]. Indeed, an altered deposition and composition of proteoglycans in fibrotic tissue will lead to marked changes in the physiochemical properties of the tissue, inevitably changing the accessibility of regulatory factors and the cellular responses.

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

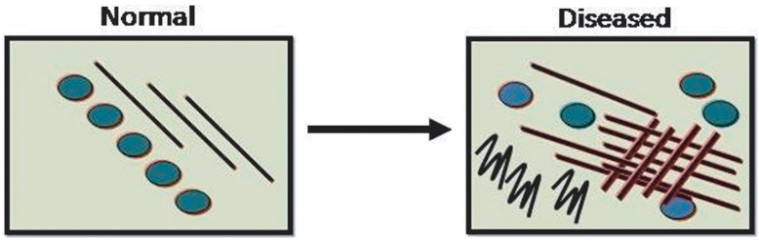

Fig. 2. Tissue loss, gain, and transformation during pathogenesis rely on extracellular matrix remodeling. The left picture shows ECM in healthy conditions where the cells (●) and ECM molecules (\) are highly organized. During fibrosis development the ECM changes composition and struction resulting in 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 periportal 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

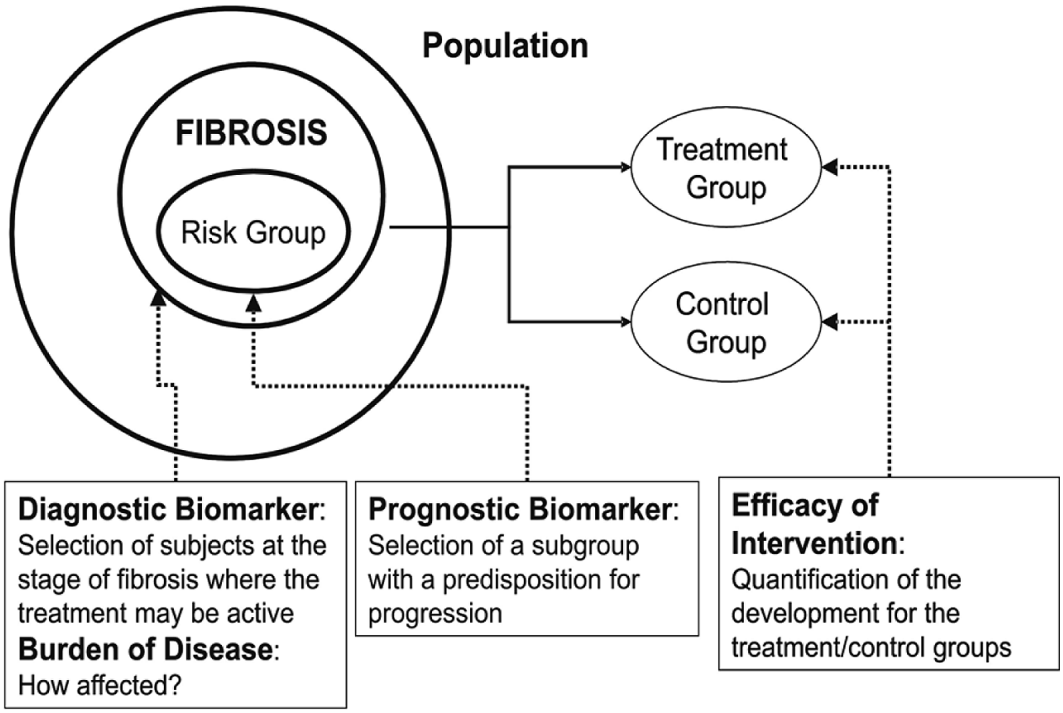

Fig. 3. Use of the BIPED classification to characterize markers of disease in a study population. The I-marker is the promising markers that need validation in the total population before application as B- or D-markers. B- and D-markers can define a study group within the total population (with or without disease) and within the study population (severity), respectively. The P-marker will give information about the progression of the disease in the study group or who are likely to develop the disease. The E-marker will give information about the efficacy of treatment received by the study group. S-marker defines the exact pathological stage of the disease.

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
Biomarkers of liver fibrosis classified into the BIPED classification

Class	Biomarker	Parameters	Chronic liver disease	BIPED classification	Reference
I	Hyaluronan	Hyaluronan	HCV	I, D	[15,80,94,127]
I	IGF-I	IGF-I	HCV	I	[75]
I	Leptin	Leptin	HCV	I	[76]
I	PIIIP	PIIIP	HCV	I	[15]
Mixed I	MP3	PIIINP, MMP1	HCV	D, I, E	[72,120]
Mixed I	Zheng et al. index	HA, PIIICP, PIIINP, Laminin, C-IV	Chronic hepatitis	I	[131]
Mixed I	Lebensztjen et al. index	Laminin-2, C-IV, MMP2, MMP9-TIMP1 index	HBV	I	[69]
Mixed I	Lebensztjen et al. index	Tenascin, hyaluronan, Collagen VI, TIMP-1	HBV	I	[70]
Mixed I	Tsochatzis et al. index	Leptin, adiponectin, resistin	HCV, HBC, NASH	I	[121]
Mixed I	Patel et al. index	Hyaluronan, TIMP-1, α_2-macroglobulin	HCV	D, I	[95]
Mixed I	Lieber et al.	TIMP-1, tenascin, collagen IV, PIIINP, MMP2, laminin, Hyaluronan	NASH	I	[73]
II	Forns-index	Age, platelet count, γGT, cholesterol	HCV HIV/HCV	D, I	[11,13,28,72,90]
II	FibroTest	Haptoglobin, α_2-macroglobulin, apolipoprotein A1, γGT, bilirubin	HCV HIV/HCV NAFLD NAFLD in diabetes patients	I, D, E	[11,13,40,44,45,55,72, 73,88,90,91,92,99,102, 104,105,109]
II	Actitest	FibroTest + ALT	HCV	I, D, E	[44,87,100,101]
II	APRI (Wai-index)	AST, platelet count	HIV/HCV HCV NAFLD	D, I	[2,11,13,14,17,44,51,72, 73,92,97,99,120,121,126]
II	Hepascore	Bilirubin, γGT, hyaluronan, α_2-macroglobulin, age, gender	HCV HIV/HCV	D, I, B	[1,13,43,71,72]
II	FIB-4	Platelet count, AST, ALT, age	HIV/HCV	I, D	[13,119]
II	SHASTA	Hyaluronan, albumin, AST	HIV/HCV	I	[13]
II	Fibroindex	FORN+APRI	HCV	D, I, E	[65]
II	Fibrometer test	Platelet count, prothrombin index, AST, α_2-macroglobulin, hyaluronan, urea, age	HIV/HCV HCV NAFLD	D, I	[13,14,43,71,72]
II	NFSA	Age, hyperglycaemia, body mass index, platelets, albumin, AST/ALT	NAFLD	I	[14]
II	Ultrasound + APRI	AST, platelet count, Ultrasound	HCV	D, I	[92]
II	Metwally et al. index	Platelet count, albumin, AST, history of blood transfusion, HBV core antibody	HCV	D, I	[81]
II	Mohamadnejad et al. index	Age, HBV DNA levels, alkaline phosphatase, albumin, platelet counts, AST	HCV	D, I	[84]
II	Fibro*Spect* II	Hyaluronan, TIMP-1, α_2-macroglobulin	HCV	D, I	[96,114,129]
II	Stepwise combination algorithms	Combination of APRI and Fibrotest	HCV	D, I	[112]
II	Imbert-Bismut index	α_2 macroglobulin, AST, ALT γGT, total bilirubin, albumin, α_1 globulin, α_2 globulin, β globulin, γ globulin, apolipoprotein A_1	HCV	I	[51]
II	Nunes et al.	Age, Platelets, INR, CD4, AST/ALT, Hyaluronan, YKL-40, PIIINP	HCV/HIV HCV	D, I	[90]
II	Fibroscan + + +	Fibroscan, Fibrotest, APRI	HCV	D, I	[18]

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

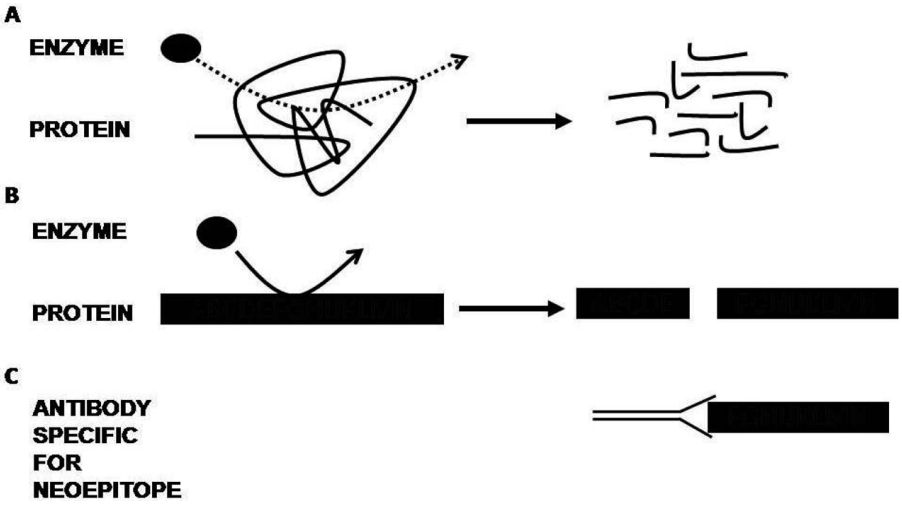

Fig. 4. Neo-epitopes – protease degradation of proteins. A) Fragments are generated of a protein by enzymatic activity. B) The enzymatic cleavage leads to two highly specific fragments. C) After blasting and homology search antibodies are developed against the specific cleavage site. During development 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 approached with proteases degradation fragments may increase the accuracy of the biomarker, the exact tissue distribution and contribustion 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 approached 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 extensibley 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 nonskeletal 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 classifiction 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 osteoporois. 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 suggestan 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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