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(54) Titre : PROCEDE SERVANT A DIAGNOSTIQUER UNE FIBROSE DU FOIE
(54) Title: METHOD FOR DIAGNOSING LIVER FIBROSIS

(57) Abrégé/Abstract:
The invention concerns a method for the detection of the presence and/or the severity of a liver disease in a patient comprising measuring in an isolated sample TIMP-1 (tissue inhibitor of metalloproteinase I), ferritin, at least one additional parameter selected from the group consisting of A2M (alpha-2-macroglobulin) and PI (prothrombin index) and optionally measuring at least one additional biochemical or clinical parameter and diagnosing the presence and/or severity of a liver disease based on the presence or measured levels of these parameters. The method can be used for monitoring therapeutic treatment of liver fibrosis and staging of liver fibrosis.

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(54) Title: METHOD FOR DIAGNOSING LIVER FIBROSIS

(57) Abstract: The invention concerns a method for the detection of the presence and/or the severity of a liver disease in a patient comprising measuring in an isolated sample TIMP-1 (tissue inhibitor of metalloproteinase I), ferritin, at least one additional parameter selected from the group consisting of A2M (alpha-2-macroglobulin) and PI (prothrombin index) and optionally measuring at least one additional biochemical or clinical parameter and diagnosing the presence and/or severity of a liver disease based on the presence or measured levels of these parameters. The method can be used for monitoring therapeutic treatment of liver fibrosis and staging of liver fibrosis.

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Method for diagnosing liver fibrosis

Field of the invention

The present invention relates to the fields of hepatology and liver fibrosis. In particular it relates to a panel of serological markers that can be used for diagnosing liver fibrosis, in particular used for diagnosing liver fibrosis due to chronic HCV infection. These markers can be used for monitoring therapeutic treatment of liver fibrosis.

Background of the invention

Fibrotic liver disease ranks as the eighth most common cause of mortality worldwide, accounting for 1.3 million deaths annually (Murray and Lopez, 1997, Lancet 349,1269-1276). The cellular mechanisms of fibrosis are complex. In response to liver injury, for example caused by chronic hepatitis C virus (HCV) infection, hepatitis B virus (HBV) infection, alcoholic or fatty liver disease, drug-induced liver disease or primary biliary cirrhosis, normally quiescent hepatic stellate cells are activated into proliferating myofibroblasts. These cells produce extracellular matrix proteins and release tissue inhibitors of metalloproteinases which bind and inactivate metalloproteinases responsible for scar degradation. As a result, fibrosis and scar may accumulate through increased production of tissue and proteins like collagen and decreased degradation of these compounds so that the function of liver is impaired (McHutchinson 2004, CME Newsletter Tx Reporter Gastroenterology, 2-4).

While hepatic fibrosis is a reversible process resulting in the accumulation of extracellular matrix, liver cirrhosis is an irreversible process which is characterized by thick bands of matrix which completely encircle the parenchyma to form nodules. If left untreated, liver fibrosis may lead to cirrhosis, maybe cancer. For these reasons timely and accurate diagnosis of liver fibrosis is essential to effective medical treatment.

Currently liver biopsy is still considered as the so-called gold standard for assessment of fibrosis and inflammation. Liver biopsy is recommended to grade and stage the

disease, confirm the diagnosis and establish a baseline against which to document improvement or disease progression, aid in determining prognosis and need for therapy (McHutchinson, see above; for review see Gebo et al. 2002 Hepatology 36, 161-172).

There exist numerous histologic grading systems that have been used to semiquantify the degree of hepatic fibrosis and inflammation in patients with chronic hepatitis C. One of the mostly used grading systems is the METAVIR system (Bedossa et al., 1994, Hepatology, 20, 15-20). METAVIR classifies hepatic fibrosis in 5 stages ranging from F0 to F4. F0 means no fibrosis, F1 corresponds to mild fibrosis (portal fibrosis without septa). The moderate to severe fibrosis classifies as F2 to F4 (F2: few septa, F3: numerous septa without cirrhosis), stage F4 corresponding to the ultimate stage of cirrhosis. Fibrosis is regarded as clinically significant starting from $F \geq 2$.

But there are several disadvantages in applying liver biopsy for diagnosing and staging fibrosis. Liver fibrosis is subject to sampling error so that the small portion of sample might not reflect the real situation in the whole liver. As such it is not an accurate marker of the dynamic process of constant degradation. Further pathologists often do not agree in their readings of histologic samples where inter- and intra-observer variability occurs in 10 to 20 % of biopsies (Cadranel et al 2000, Hepatology 32, 477-481).

Liver biopsy is an invasive and painful procedure for the patient. It is also associated with a risk of hemorrhage and other complications after the sampling. Moreover and partly due to expected complications followed by hospitalization of the patient it is a costly procedure.

Hepatic fibrosis is the principal complication of chronic HCV infection leading to the development of cirrhosis and decompensated liver disease. Directed investigation examining the development and progression of fibrosis is, therefore, essential for effective management of these patients. Evaluation of progressive fibrosis will best be accomplished with noninvasive tests capable of discriminating intermediate stages of fibrosis. A variety of single markers and marker panel algorithms have been published, but no powerful single biomarker or biomarker score is currently available that allows a

reliable prediction of fibrosis (Diagnostic Accuracy > 80 %). Further research into the development of noninvasive dynamic measures of hepatic fibrosis is strongly encouraged by the National Institutes of Health Consensus Development Conference in 2002. In particular the studies on alternatives to liver biopsy should provide enough details about the biopsy methods (average size of biopsy samples; histologically well characterized qualifying panel) to convince readers of the adequacy of reference standard. Liver biopsy is strongly dependent on optimized performance criteria and may lead to misclassification of histological stages due to interobserver variability and too small sample sizes (< 10 mm).

There has been a wide search for biochemical or serological markers which reflect fibrotic processes in liver disease and which can serve as a surrogate for liver biopsy. In the last years a couple of non-invasive or minimally invasive biochemical and serological markers have been investigated to assist in diagnosing liver diseases. In particular combinations of markers have been used to categorize patients according to their stage or degree of fibrosis.

US 6,631,330 discloses the use of a combination of at least 4 biochemical markers selected from the group consisting of α-2-macroglobulin, aspartate aminotransferase, γ-glutamyl transpeptidase, γ-globulin, total bilirubin, albumin, α1-globulin, α2-globulin, haptoglobin, β-globulin, apoA1, IL-10, TGF- β1, apoA2 and ApoB. The obtained values of 4 of these markers are mathematically combined to determine the presence of liver fibrosis. With this marker panel a diagnostic accuracy of about 80 per cent can be obtained.

The international patent application WO 2003/073822 describes a method for diagnosing the presence or severity of liver fibrosis in a patient. This method uses the combination of at least three markers which are α-2-macroglobulin, hyaluronic acid and tissue inhibitor of metalloproteinase 1 (TIMP-1). With this method a diagnostic accuracy of about 80 per cent (McHutchinson, 2004, see above) can be obtained.

There is a need to develop a non- or minimally invasive method to reach a higher diagnostic accuracy in determining liver fibrosis and classify and discriminate between different stages of fibrosis in a more reliable way than so far known in the state of the

art so that monitoring of clinical development of fibrosis during therapeutic treatment is possible. Moreover such a method should be suitable for serial testing on automatic analyzers.

Description of the invention

The problem is solved by a method according to the current invention. This method for the detection of the presence and/or the severity of a liver disease in a patient comprises the steps as follows:

a) obtaining a sample from said patient

b) measuring TIMP-1 (Tissue Inhibitor of Metalloproteinase I) in said sample

c) measuring ferritin in said sample

d) measuring at least one additional parameter selected from the group consisting of A2M (alpha-2-macroglobulin), PI (prothrombin index) in said sample

e) optionally measuring at least one additional biochemical or clinical parameter in said sample

f) diagnosing the presence and/or severity of a liver disease based on the presence or measured levels of TIMP-1, ferritin and the parameter measured according to steps d) and e)

The present invention permits a reliable differentiation between F0/F1 fibrosis from F2/F3/F4 stages. Moreover therapeutic monitoring as a control of medical treatment of liver diseases can be carried out by the method of the invention.

The method of the current invention is highly correlating with well characterized Metavir stages of hepatic fibrosis. A special advantage of the method of the current invention in comparison to state of the art methods is the usage of a qualifying panel to minimize errors of misclassification of pathological observation and of statistical models.

The method of the invention comprises a noninvasive method correlating very closely with the severity of fibrosis as determined by several methods: liver biopsy and further methods such as the determination of the area of fibrosis.

The method of the current invention is based on a statistically relevant cohort of specimens of patients with well characterized liver fibrosis, covering the total range of Metavir stages and of specimens of subjects without hepatic fibrosis due to histological findings (Metavir score: 0). The initial selection criteria of specimens is the Metavir score. This reference is confirmed in a double evaluation and in an optimized way using specimens with sizes larger than 15 mm.

The method of the current invention allows a reliable prediction of fibrosis with a diagnostic accuracy (DA) of at least 82 %, preferably at least 84 %. Since even the reference standard is no gold standard of hepatic fibrosis with respect to optional misclassification of fibrosis stages and further leads to pain and health risk to the patient the method of the present invention represents an alternative to biopsy.

The method allows the investigation of the development and progression of fibrosis providing an effective management of patients with chronic HCV. Disease monitoring of patients with chronic HCV may be performed in a short time interval in comparison to biopsy. The method allows monitoring the success of antifibrotic therapy.

The method also allows the investigation of the development and progression of fibrosis in subjects with chronic hepatic injury. This is a relatively common disorder with minimal symptoms, yet with long term risk of significant morbidity and mortality, which is defined pathologically by ongoing hepatic necrosis and inflammation in the liver, often accompanied by fibrosis. HCV is the most common form of chronic hepatic injury. The method can be applied to further forms of chronic hepatic injury: alcoholic steatohepatitis (ASH), alcoholic fatty liver disease (AFLD), non-alcoholic steatohepatitis (NASH) or non-alcoholic fatty liver disease (NAFLD).The methods of the invention can be used to monitor the severity of NASH and NAFLD. They can be used to diagnose liver fibrosis in an individual with viral hepatitis such as hepatitis A, B, C or D virus or a human immunodeficiency virus (HIV), chronic persistent or chronic active hepatitis, autoimmune liver disease, such as autoimmune hepatitis und drug-induced liver disease; primary biliary cirrhosis, primary sclerosing cholangitis, biliary atresia, liver disease resulting from medical treatment or a

congenital liver disease. The invention can be used for monitoring of treatment with a drug with the risk of liver disease. The methods can be used for diagnosing the presence or severity of fibrosis and for monitoring fibrosis, wherein fibrosis is associated with a variety of fibrotic disorders not limited to the liver: pulmonary fibrosis, kidney fibrosis, prostate fibrosis and breast fibrosis ad further fibrosis in another disorder.

According to the current invention preferred combinations of parameters are TIMP-1, ferritin and A2M (also named SNIFF 3a, SNIFF being the French abbreviation for score non-invasif de fibrose du foie; in English: non-invasive score of liver fibrosis) with a diagnostic accuracy of 82.6 %; TIMP-1, ferritin and PI (SNIFF 3) with a diagnostic accuracy of 84 %; and TIMP-1, ferritin, PI, PLT, urea, age with a diagnostic accuracy of 84.7 %. These preferred combinations can also be seen on table 3.

In the sense of the present invention the specific terms and expressions should be understood as follows:

Diagnostic accuracy (DA) is the accuracy of the test itself. This means the percentage of all tests that are truly positive or truly negative. The higher the diagnostic accuracy the more reliable are the results of the test. DA is calculated as the sum of true positives and true negatives divided by the total number of sample results and is affected by the prevalence of fibrosis in the population analyzed.

Cut-off value is the arithmetic calculated concentration of a single biomarker or of a combination of several biomarkers for the discrimination of healthy and disease state. In the understanding of the invention cut-off means a score of 0.5. If this value is above or equal to 0.5 ($\geq$0.5) this means that the Metavir stage F2 is reached for the distinction between no or mild fibrosis (Metavir stages F0 or F1) and clinically significant fibrosis CSF (Metavir stages F2, F3, F4).

Positive predictive value (PPV) is the percentage of positive tests that are truly positive.

Negative predictive value (NPV) means the percentage of negative tests that are truly negative.

Score means an arithmetic combination of several biomarkers associated with fibrosis. In particular, the score used herein has a range between 0 (minimal fibrosis) and 1 (CSF: clinically significant fibrosis).

AUROC means area under the receiver operator characteristics curve. In these curves, sensitivity is plotted against the reciprocal of specificity. An area under the ROC curve of 1.00 would indicate an ideal of 100 per cent sensitivity and 100 per cent specificity. The larger the slope at the beginning of the curve the better is the relation between sensitivity and specificity of a test.
Sensitivity is the probability of a positive test result in a patient with a disease or risk factor or other health condition.

Specificity is the probability of a negative test result in a patient who does not have the disease.

TIMP-1 (Tissue Inhibitor of Metalloproteinase I) is a 184 amino acid sialoglycoprotein with a molecular weight of 28.5 kDa (see e.g. Murphy et al Biochem J. 1981, 195,167-170) which inhibits metalloproteinases like interstitial collagenase MMP-1 or stromelysin or gelatinase B. In the understanding of the current invention the term TIMP-1 encompasses a protein with significant structural homology to human TIMP-1 inhibiting the proteolytic activity of metalloproteinases. The presence of human TIMP-1 can be detected by using antibodies that specifically detect epitopes of TIMP-1. TIMP-1 may also be determined by detection of related nucleic acids such as the corresponding mRNA.

Ferritin is a macromolecule with a molecular weight of at least 440 kD, depending on the iron content, and consists of a protein shell (apoferritin) of 24 subunits and an iron core containing an average of approximately 2500 Fe^{3+} ions (in liver and spleen ferritin). Ferritin tends to form oligomers. At least 20 isoferritins can be distinguished

with the aid of ioselectric focusing. This microheterogeneity is due to differences in the contents of the acidic H and weakly basic L subunits. The basic isoferritins are responsible for the long-term iron storage function, and are mainly found in liver, spleen and bone marrow.

The determination of ferritin is a suitable method for ascertaining the iron metabolism situation. Determination of ferritin at the beginning of therapy provides a representative measure of the body's reserves. Clinically, a threshold value of about 20 ng/ml has proved useful in the detection of prelatent iron deficiency. This value provides a reliable indication of exhaustion of the iron reserves that can be mobilized for hemoglobin synthesis. Latent iron deficiency is defined as a fall below the 12 ng/ml threshold. For manifestation of iron overloading in the body a threshold value above 400 ng/ml is regarded as useful.

For the detection of ferritin a classical sandwich immunoassay may be used in which two antibodies specific for ferritin are used to form a sandwich complex in the assay. One of the antibodies binds to a solid phase and the other antibody carries a label the signal of which is used a detection means for the presence of ferritin.

PI (prothrombin index) is useful to detect interferences in the coagulation system and can be determined by adding thromboplastine to the plasma sample and measuring the time of coagulation in seconds (so-called Quick-time).This value is correlated to an international normalized ratio that contains a correction factor that takes into account the sensitivity of the thromboplastine used.

A2M (α-2-Macroglobulin) is a conserved protein, highly abundant in plasma that serves as a protease binding protein to clear active proteases from tissue fluids. A2M does not inactivate the catalytic activity of a protease but acts by physical entrapment of the target protease by folding around the protease. A protease entrapped by A2M is thus sterically prevented from cleaving its substrate proteins. In the sense of the invention A2M may be detected by an immunological assay using specific antibodies according to test formats known to a person skilled in the art. A2M may also be determined by detection of related nucleic acids such as the corresponding mRNA

According to the invention additional biochemical or clinical parameter may be determined. Additional biochemical parameter may be any parameter directly or indirectly associated with metabolism or structure of the liver as for example urea, GGT (gamma-glutamyltransferase), hyaluronate, AST (aspartate amino transferase), MMP-2 (matrix metalloproteinase-2), ALT (alanine aminotransferase), PIIINP (N-terminal propeptide of type III procollagen), bilirubin, haptoglobin, ApoA1, PLT (number of platelets). Also hepcidin or adiponectin may be determined.

Hepcidin is a hepatic protein, originally identified as a circulating antimicrobial peptide. It is central player in the communication of body iron stores to the intestinal absorptive cells. Adiponectin is secreted by the adipocytes and circulated at relatively high systemic concentrations to influence metabolic function. Reduced serum adiponectin levels indicate an increased risk of diseases for example severity of nonalcoholic fatty liver disease (NAFLD) or nonalcoholic steatohepatitis (NASH).

Additional clinical parameters may be determined such as age, sex, weight, nutritional habits of the patient.

Urea, GGT (gamma-glutamyltransferase), hyaluronate, AST (aspartate amino transferase) and ALT (alanine amino transferase), MMP-2, PIIINP bilirubin, haptoglobin , ApoA1, hepcidin and adiponectin are determined by commercially available test kits by immunological or photometrical methods known to a person skilled in the art. Where applicable also hybridization techniques for the detection of nucleic acids that are specific for an analyte or parameter (such as the corresponding mRNA) may be used for determination of a parameter.

PLT (number of blood platelets) is the number of blood platelets and is determined by counting the platelets using a commercially available counter.
The invention makes use of the determination of a plurality of parameters. Therefore the detection of those biochemical and serological parameters of the invention that may be carried out in test formats using a solid phase is preferably carried out on miniaturized array-based test systems as described in US 2003/0017616 or WO

99/67643. These test systems have multiple spatially defined test areas each of which can be used to detect a single specific analyte or parameter. Thus a plurality of analytes can be detected in a single test run.

The term defined test areas on a solid phase is understood to mean that the test areas comprise defined regions of the solid phase which are preferably spatially separated from other test areas by inert regions. The defined test areas preferably have a diameter of 10 µm to 1 cm and particularly preferably 10 µm to 5 mm. Miniaturized test areas with a diameter of 10 µm to 2 mm are most preferred. Solid phases with several test areas are preferred which are also referred to as array systems. Such array systems are for example described in Ekins and Chu (Clin. Chem. 37, 1995, 1955-1967) and in U.S. patents nos. 5,432,099, 5,516,635 and 5,126,276. As mentioned before, an advantage of array systems is that several analyte and control determinations can be carried out simultaneously on one sample. The use of control areas to detect unspecific binding and/or interfering samples can considerably improve the reliability of the results especially with miniaturized array test systems.

In the current invention the detection of TIMP-1, A2M and Ferritin and possibly additional other biochemical parameters could for example be performed simultaneously by using such an array-based test system.

According to the invention the solid phase is any conventional support for detection methods, preferably a non-porous support, e.g. a support with a plastic, glass, metal or metal oxide surface. Porous supports such as test strips are also suitable. Spatially discrete regions (test areas) are located on this support. Immobilized solid phase receptors are applied to these test areas. The solid phase receptors are immobilized by known methods, e.g. by direct adsorptive binding, by covalent coupling or by coupling via high affinity binding pairs, e.g. streptavidin(or avidin)/biotin, antigen/antibody or sugar/lectin. The presence or/and the amount of the analyte in a sample can be determined by specific binding of components from the detection medium, e.g. of the analyte to be determined or of an analyte analogue to the solid phase receptor.

The detection of the analyte and – where appropriate – the detection of the presence of interfering reactions is achieved in the method according to the invention in a known manner by using suitable marker groups, e.g. fluorescent marker groups. Alternatively with suitable solid phases it is possible to also detect the interaction of components of the detection medium with the test and optionally control areas by determining the layer thickness of the respective area, e.g. by plasmon resonance spectroscopy.

With array systems in which several analytes from a sample are detected simultaneously, it is preferable to use a universal marker group which enables a simultaneous detection of several different analytes to different test areas. An example of such universal marker groups are marker groups which carry a receptor that can specifically interact with a complementary receptor on a test reagent, e.g. a soluble receptor for an analyte to be determined or for an analyte analogue (like antibody/antigen or streptavidin/biotin etc.).

The term sample means a biological specimen that contains or allegedly contains at least one of the markers according to the invention. For example as a sample blood, serum, plasma, urine, saliva, synovial fluid or liver tissue may be used. Fluid samples may be diluted prior to analysis if required.

To obtain a result assisting in diagnosing the disease mathematical algorithms are used known to a person skilled in the art. The obtained data is combined and evaluated by statistical methods like logistic binary regression, resulting in scores.

Figure 1 shows raw data as measured on 120 patient suffering from infection with HCV.

The invention is further illustrated by the following example:

Example

Commercially available test kits were used and all tests were performed according to the instructions given by the manufacturers as listed below.

Table 1

Biomarker	Method	Provider
AST, ALT	Clinical Blood Chemistry	Roche Diagnostics GmbH Mannheim, Germany
GGT	Clinical Blood Chemistry	Roche Diagnostics GmbH Mannheim, Germany
Bilirubin	Clinical Blood Chemistry	Roche Diagnostics GmbH Mannheim, Germany
Urea	Clinical Blood Chemistry	Roche Diagnostics GmbH Mannheim, Germany
A2M	Nephelometry	Dade Behring Marburg GmbH
Apo A1	Nephelometry	Dade Behring Marburg GmbH
Platelets	Platelet count	Bayer Diagnostics
PI	Coagulation Time	Diagnostica Stago
Hyaluronate	Elisa	Corgenix Inc.
PIIINP	RIA	Cis Bio International
YKL-40	Elisa	Quidel Corporation
TIMP1	Elisa	Amersham Pharmacia
MMP2	Elisa	Amersham Pharmacia

Figure 1 shows raw data as measured on samples of 120 patient suffering from infection with HCV. To obtain the data the test kits listed above were used.

In table 2 the diagnostic accuracy and the AUROC values are listed. It can be seen each single marker has got a DA below 80 %.

Table 2

Biomarker	Diagnostic Accuracy		Correlation		AUROC	
	%	p	r	p	c	p
A2M	76.7	$< 10^{-4}$	0.523	$< 10^{-4}$	0.800	$< 10^{-4}$
TIMP 1	72.3	$< 10^{-4}$	0.663	$< 10^{-4}$	0.813	$< 10^{-4}$
Ferritin	71.7	$< 10^{-4}$	0.433	$< 10^{-4}$	0.771	$< 10^{-4}$
HA	71.7	0.002	0.561	$< 10^{-4}$	0.762	$< 10^{-4}$
Platelets	70.8	$< 10^{-4}$	-0.523	$< 10^{-4}$	0.259	$< 10^{-4}$
AST	69.2	$< 10^{-4}$	0.444	$< 10^{-4}$	0.782	$< 10^{-4}$
Prothrombin index	69.2	$< 10^{-4}$	-0.444	$< 10^{-4}$	0.265	$< 10^{-4}$
GGT	67.5	0.002	0.229	0.012	0.721	$< 10^{-4}$
MMP 2	67.2	$< 10^{-4}$	0.451	$< 10^{-4}$	0.711	$< 10^{-4}$
ALT	66.7	0.002	0.311	0.001	0.696	$< 10^{-4}$
YKL 40	65.3	0.001	0.480	$< 10^{-4}$	0.661	0.002
Age	62.5	0.001	0.345	$< 10^{-4}$	0.706	$< 10^{-4}$
P3P	62.5	0.02	0.337	$< 10^{-4}$	0.626	0.019
Bilirubin	61.7	0.02	0.107	0.247	0.628	0.017
Haptoglobin	61.7	0.01	-0.285	0.002	0.356	0.007
ApoA1	60.0	0.03	-0.229	0.012	0.361	0.009
Sex	53.3	0.37	-	-	-	-
Urea	51.7	0.28	-0.058	0.527	0.470	0.572

Table 3 shows a comparison of DA /AUROC with state of the art methods. The methods of the current invention were shown to have superior diagnostic accuracy of clinically significant fibrosis by binary logistic regression in comparison with the methods of US 6,631,330 and WO 2003/073822.

Table 3

Method	Selected Markers	n var	n Pts	R^2	DA	AUROC
	PLT, PI, Urea, Fer, age, TIMP	6	118	0.689	84.7	
	TIMP, Fer, PI	3	119	0.629	84.0	0.904
	TIMP, Fer, A2M	3	118		82.6	0.886
WO 2003/073822	TIMP, HA, A2M	3	118		80.7	0.898
US 6,631,330 (Fibrotest)	A2M, age, Hapto, Apo, Bili, GGT, sex	7	120	0.518	80.8	0.857
US 6,631,330	A2M, age, Apo, GGT	4	120	0.487	77.5	0.859

CLAIMS:

1. A method for detecting the presence and/or severity of a liver disease in a sample from a patient comprising:

(a) measuring TIMP-1 (Tissue Inhibitor of Metalloproteinase I) in said sample;

(b) measuring Ferritin in said sample;

(c) measuring one additional parameter selected from the group consisting of A2M (alpha-2-macroglobulin) and PI (prothrombin index) in said sample; and

(d) diagnosing the presence and/or severity of liver disease based on the presence or measured levels of TIMP-1, Ferritin and the parameter measured according to step (c).

2. The method of claim 1, wherein the additional parameter is A2M.

3. The method of claim 1, wherein the additional parameter is PI.

4. Use of the method of any one of claims 1 to 3 for monitoring therapeutic treatment of liver fibrosis.

5. Use of the method of any one of claims 1 to 3 for staging liver fibrosis.

Figure 1/1
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Sample No.	Biopsy Sample (mm)	Metavir Stage	Age (years)	Sex (1:M; 2:F)	Platelets (Giga / l)	PI (%)	Urea (mmol / l)	Bilirubin (µmol / l)	GGT (UI / l)	AST (UI / l)	ALT (UI / l)	Albumin (g / l)	Gglob (g / l)	Ferritin (ng / ml)	ApoA1 (g / l)	Hyaluronate (µg / l)	A2M (mg / dl)	PIIINP (U / ml)	Haptoglobin (mg / ml)	YKL40 (ng / ml)	TIMP1 (ng / ml)	MMP2 (ng / ml)
1	25	3	63	1	123	80	7,8	11	93	66	114	41	18	627	1,30	273	374	1,20	0,41	284	2.501	2.801
2	14	0	40	2	171	96	4,5	11	11	68	157	40	10	12	1,75	55	334	0,83	0,83	43	898	1.377
3	9	0	30	1	288	89	8,6	22	27	27	59	44	9	136	1,37	25	157	1,32	1,57		912	1.252
4	24	4	45	2	81	71	4,1	11	90	118	121	35		356	1,09	497	314	1,68	0,43	316	1.556	2.265
5	20	1	51	2	217	90	6,4	6	43	28	81	44	9	64	2,53	32	190	0,74	2,47	115	913	1.243
6	23	1	35	2	165	112	15,4	5	104	104	209	37	16	141	1,76	111	239	2,32	0,38	351	2.011	1.586
7	30	1	36	1	179	97	3,2	12	17	43	76	57	15	151	1,60	22	275	0,62	1,89	193	723	1.223
8	26	3	46	1	147	90	4,3	16	148	113	120	37	18	739	1,41	69	441	0,61	1,89	312	1.474	1.128
9	11	0	58	2	238	100	7,2	8	37	34	51	44	13	55	1,89	25	177	0,92	1,26	193	1.089	1.412
10	10	0	37	1	231	86	5,0	11	66	41	79	49	12	424	1,48	25	148	0,76	1,58	60	827	1.373
11	17	3	55	2	132	80	3,9	16	23	24	42	47	18	289	1,10	40	353	0,60	1,04	312	1.367	1.345
12	22	0	31	2	250	90	4,0	3	21	19	13	43	10	44	1,32	25	236	0,94	1,26	39	669	1.291
13	16	1	32	1	276	89	3,6	10	32	45	66	45	16	162	1,83	25	158	0,74	0,91	62	774	1.463
14	15	2	38	1	178	91	5,0	9	33	34	44	50	12	208	1,97	25	389	0,73	1,07	84	975	1.448
15	21	2	69	2	236	95	8,1	7	90	55	68	44	19	179	2,06	221	290	1,19	1,97	278	1.809	2.063
16	19	4	62	1	91	74	5,4	12	28	63	133	44	20	710	1,12	126	332	1,12	0,30	158	1.848	1.759
17	26	1	34	1	246	84	5,6	11	16	46	89	49	14	174	1,25	29	221	0,70	1,02	70	973	1.430
18	17	1	58	1	196	98	11,4	15	118	18	35	42	12	261	1,51	14	174	0,97	0,30	213	1.150	1.600
19	20	1	31	2	284	120	4,8	8	83	23	61	48	10	166	1,49	25	221	0,55	1,52	66	1.009	1.098
20	20	1	69	1	138	87	12,4	13	27	30	67	50	11	120	1,40	40	555	0,40	0,30	134	1.070	1.219
21	18	1	36	2	350	101	6,6	3	23	17	40	41	14	33	1,27	25	189	1,03	0,94	108	1.308	978
22	18	0	36	1	597	88	4,6	2	37	58	76	43	17	15	1,30	52	233	0,70	2,31	90	1.340	1.745
23	16	1	33	1	535	80	8,1	4	35	38	87	41	18	6	1,10	74	340	0,40	1,69	206	925	1.273
24	20	1	33	1	178	113	4,1	5	27	33	56	43	12	138	1,33	25	204	0,64	1,70	110	807	1.242
25	20	1	68	1	260	88	5,5	6	17	40	52	41	8	98	1,30	29	327	1,00	1,06	179	998	2.229
26	19	2	53	1	183	96	8,6	9	19	52	84	39	9	502	2,14	133	114	0,71	1,16	61	1.110	1.891
27	23	1	45	1	179	103	3,9	7	38	67	120	41	11	118	0,80	25	245	0,71	1,18	44	1.300	1.214
28	16	4	44	1	117	66	4,4	8	151	89	108	46	13	352	1,09	287	314	1,35	0,91	351	1.652	1.831
29	18	2	27	1	287	95	4,0	49	65	46	130	46	8	313	0,83	20	199	0,74	1,19	87	1.674	1.081
30	18	3	29	1	146	88	7,3	6	40	25	37	48	13	107	1,71	144	299	0,87	0,30	159	1.469	1.796
31	16	4	49	1	135	83	5,6	13	173	183	242	62	8	184	1,12	292	564	1,30	0,60	282	2.200	1.865
32	15	1	23	1	211	100	3,6	10	20	26	97	45	13	107	1,40	20	247	0,70	1,26	26	884	1.256
33	20	2	37	1	260	101	4,5	5	119	21	49	42	10	297	1,31	15	178	0,65	2,71	59	833	896
34	13	0	28	1	236	103	6,4	11	87	30	60	48	8	134	1,24	14	119	0,56	0,60	48	773	1.371
35	21	1	29	2	293	108	3,7	5	59	36	44	45	8	58	1,80	25	216	0,59	1,96	40	1.173	991
36	24	1	29	1	222	98	2,1	17	386	135	234	46	15	156	1,60	20	169	0,50	1,35	106	902	1.467
37	17	4	51	2	102	70	3,1	10	392	147	111	35	40	751	1,30	216	287	1,84	0,30	351	1.926	2.695
38	19	2	62	2	189	86	5,9	17	41	46	68	47	11	198	1,21	38	231	0,87	1,21	86	1.293	1.265
39	19	4	64	2	85	116	11,8	15	134	100	162	44	10	98	1,70	354	321	1,13	1,11	309	2.501	1.556
40	15	4	56	1	104	78	4,6	14	215	28	42	39	32	528	1,60	122	235	1,20	0,70	300	1.879	2.209
41	18	2	40	1	194	100	3,7	8	547	150	364	48	17	1.114	1,19	47	359	0,75	1,60	50	1.495	1.398
42	20	0	30	1	216	97	5,5	29	16	29	84			164	1,45	12	254	0,74	1,68	69	1.024	1.198
43	24	2	43	1	150	83	5,3	10	97	58	140			365	1,60	28	277	0,50	0,88	64	1.316	1.485
44	20	1	62	2	242	100	4,0	14	67	40	47	52	9	233	2,30	45	463	0,37	0,83	33	1.216	1.125
45	15	3	48	1	161	93	2,9	10	303	28	50	46	10	489	1,37	25	320	0,67	1,07	105	1.170	1.239
46	22	0	32	1	326	93	3,5	4	27	13	14	51	10	80	1,77	25	185	0,81	1,32	35	838	1.249
47	20	1	37	1	234	86	5,4	11	100	90	203	48	15	382	1,66	25	516	1,05	0,80	97	1.483	1.434
48	24	1	34	1	222	87	4,1	12	20	22	56	53	13	113	1,10	9	275	0,81	1,45	41	989	889
49	13	0	34	1	219	103	3,7	9	105	67	113	48	6	485	1,89	28	125	0,64	0,68	76	983	1.078
50	22	4	40	1	227	83	6,2	8	153	156	392	47	13	364	0,93	33	465	1,34	0,91	73	1.896	1.499
51	24	1	43	1	175	86	3,8	10	17	43	64	44	9	116	1,25	25	222	0,70	1,13	86	985	1.043
52	24	2	38	1	182	78	5,4	6	35	124	226	45	13	311	1,22	34	319	0,99	0,52	315	1.489	1.301
53	20	1	32	1	243	97	5,0	11	11	14	33	41	10	207	1,60	33	212	0,89	1,39	71	827	1.197
54	15	2	50	2	292	96	4,4	16	20	27	56	46	12	60	1,68	56	394	0,84	1,22	40	1.234	1.463
55	17	1	32	1	202	100	5,8	12	87	18	54	51	7	299	1,70	30	252	0,62	1,35	62	593	964
56	22	4	72	1	154	85	4,7	17	190	156	159	44	12	102	1,60	156	325	0,52	0,72	320	2.138	1.789

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