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(54) Title: ACCURATE BLOOD TEST FOR THE NON-INVASIVE DIAGNOSIS OF NON-ALCOHOLIC STEATOHEPATITIS

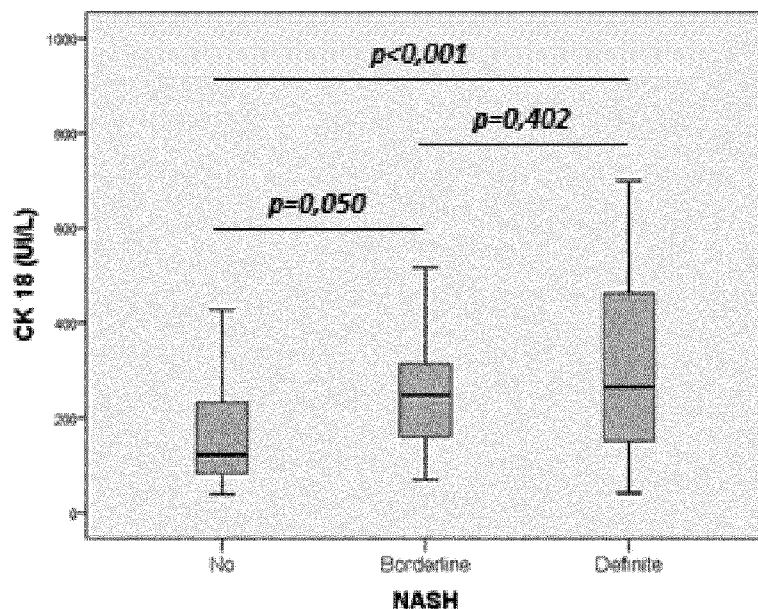


FIG. 1

(57) Abstract: The present invention relates to an in-vitro non-invasive method for assessing the presence and/or severity of NASH lesions in the liver of a subject, wherein said method comprises the steps of: a. measuring in a sample of said subject at least one biomarker, preferably 1, 2, 3 or 4 biomarkers, selected from at least one of: i. at least one biomarker reflecting apoptosis, and/or ii. at least one biomarker reflecting anthropometry, and/or iii. at least one biomarker reflecting metabolic activity, and/or iv. at least one biomarker reflecting liver status; and b. combining said at least one biomarker measure in a mathematical function.

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ACCURATE BLOOD TEST FOR THE NON-INVASIVE DIAGNOSIS OF NON-ALCOHOLIC STEATOHEPATITIS

FIELD OF INVENTION

- 5 The present invention relates to the diagnosis of non-alcoholic steatohepatitis (NASH). The present invention more specifically relates to a new and accurate blood test for the non-invasive diagnosis of non-alcoholic steatohepatitis, preferably in NAFLD patients.

BACKGROUND OF INVENTION

- 10 FLD (Fatty Liver Disease) describes a wide range of potentially reversible conditions involving the liver, wherein large vacuoles of triglyceride fat accumulate in hepatocytes via the process of steatosis (i.e. the abnormal retention of lipids within a cell).

- FLD is commonly associated with alcohol or metabolic syndromes (such as, for example, diabetes, hypertension, dyslipidemia, abetalipoproteinemia, glycogen storage
15 diseases, Weber-Christian disease, Wolman disease, acute fatty liver of pregnancy and lipodystrophy). However, it can also be due to nutritional causes (such as, for example, malnutrition, total parenteral nutrition, severe weight loss, refeeding syndrome, jejuno-ileal bypass, gastric bypass or jejunal diverticulosis with bacterial overgrowth), as well as various drugs and toxins (such as, for example, amiodarone, methotrexate, diltiazem,
20 highly active antiretroviral therapy, glucocorticoids, tamoxifen, environmental hepatotoxins) and other diseases such as inflammatory bowel disease or HIV.

- Whether it is AFLD (Alcoholic Fatty Liver Disease) or NAFLD (Non-Alcoholic Fatty Liver Disease), FLD encompasses a morphological spectrum consisting from the mildest type “liver steatosis” (fatty liver), called NAFL, to the potentially more serious
25 type “steatohepatitis”, called NASH, which is associated with liver-damaging inflammation and, sometimes, the formation of fibrous tissue. In fact, steatohepatitis has the inherent propensity to progress towards the development of fibrosis then cirrhosis

which can produce progressive, irreversible liver scarring or towards hepatocellular carcinoma (liver cancer).

Because these diseases can be potentially reversed if diagnosed early enough, or at least their consequences limited, it is crucial to be able to provide the medical field with tools
5 allowing such an early, rapid and precise diagnosis.

For a long time, the diagnosis of liver steatosis has usually been accomplished by performing a liver biopsy in order to confirm FLD and determine the grading and staging of the disease, especially NASH. Although biopsies can provide important information regarding the degree of liver damage, the procedure presents several
10 limitations, such as sampling error, invasiveness, cost, pain for patients which in turn brings forth a certain reluctance to undergo such a procedure; and finally complications may arise from such procedure, which in some cases can even lead to mortality.

Ultrasonography is also used to diagnose liver steatosis. However, this method is subjective as it is based on echo intensity (echogenicity) and special patterns of echoes
15 (texture). As a result, it is not sensitive enough and often inaccurate, especially in patients with advanced fibrosis.

In recent years, the use of non-invasive biomarkers has gained importance in the field of hepatic diagnosis. Indeed, non-invasive methods for detecting the extent of alcoholic or non-alcoholic steatohepatitis in a patient have been described. For example,
20 US2006/0172286 describes a non-invasive method for diagnosing alcoholic or non-alcoholic steatohepatitis in a patient comprising measuring the 3 biochemical markers ApoA1, ALT and AST in a sample from the patient.

Moreover, the AASLD Practice Guideline (Chalasani et al, Hepatology, 55(6):2005-2023, 2012) discloses the serum/plasma cytokeratine (CK)18 as a promising biomarker
25 for identifying steatohepatitis. However, all the studies realized on CK18 as a biomarker utilized a study-specific cut-off value. Consequently, there is not an established cut-off value for identifying steatohepatitis using this biomarker. The AASLD Practice Guideline thus concludes that although serum/plasma CK18 is a promising biomarker

for identifying steatohepatitis, it may be premature to recommend in routine clinical practice, unless a solution is found.

This invention aims at providing the solution expected by the AASLD Practice Guideline, and provides tests that may use the serum/plasma CK18 biomarker.

5 Also, this invention aims at overcoming the limitations and drawbacks of the prior art methods, that are resumed hereafter. First, prior art methods have usually been developed in cohorts of patients undergoing bariatric surgery. These morbidly obese patients represent a very particular subgroup of NAFLD patients, and thus the external validation of blood tests calibrated in such cohorts is debatable. Second, pathological
10 definition of NASH, i.e. the main study endpoint, was heterogeneous among studies and not in accordance with the latest admitted definition (Sanyal et al, Hepatology, 54(1):344-353, 2011). Third, these tests have limited accuracy for the diagnosis of NASH. Finally, none of these tests has been externally and independently validated.

The Inventors thus aimed at developing a new blood test for the non-invasive diagnosis
15 of NASH, as defined by the latest admitted pathological definition, in a well-representative cohort of NAFLD patients, wherein said blood test is more accurate than the tests of the prior art, such as, for example, more accurate than the test of US2006/0172286.

20 SUMMARY

The present invention thus relates to an in-vitro non-invasive method for assessing the presence and/or severity of NASH lesions in the liver of a subject, wherein said method comprises the steps of:

- a. measuring in a sample of said subject at least one biomarker, preferably 1, 2, 3
25 or 4 biomarkers, selected from at least one of:
 - i. at least one biomarker reflecting apoptosis, and/or
 - ii. at least one biomarker reflecting anthropometry, and/or
 - iii. at least one biomarker reflecting metabolic activity, and/or

- iv. at least one biomarker reflecting liver status; and
- b. combining said at least one biomarker measure in a mathematical function.

In one embodiment, at step (a) three of the following biomarkers are measured:

- i. at least one biomarker reflecting apoptosis, and/or
- 5 ii. at least one biomarker reflecting anthropometry, and/or
- iii. at least one biomarker reflecting metabolic activity, and/or
- iv. at least one biomarker reflecting liver status.

In one embodiment, at step (a) at least one biomarker reflecting apoptosis and at least one of the following biomarkers are measured:

- 10 i. at least one biomarker reflecting anthropometry, and/or
- ii. at least one biomarker reflecting metabolic activity, and/or
- iii. at least one biomarker reflecting liver status.

In one embodiment, at step (a) at least one biomarker reflecting apoptosis and at least one biomarker reflecting anthropometry, and at least one biomarker reflecting metabolic
15 activity are measured.

In one embodiment, the method of the invention comprises the steps of:

- a. measuring in a sample of said subject:
 - i. at least one biomarker reflecting apoptosis, wherein said biomarker reflecting apoptosis is selected from the group comprising cytokeratin 18
20 (CK18), CK18 M30, CK18 M65, AST, ALT and any combination thereof and
 - ii. at least one biomarker reflecting anthropometry, wherein said biomarker reflecting anthropometry is selected from the group comprising body mass index (BMI), body weight, age, sex, waist circumference, abdominal height,
25 hip circumference, waist/hip ratio and any combination thereof and
 - iii. at least one biomarker reflecting metabolic activity, wherein said biomarker reflecting metabolic activity is a biomarker reflecting glucose

metabolism, a biomarker reflecting lipid metabolism, or any combination thereof, preferably a biomarker reflecting glucose metabolism and
iv. optionally at least one biomarker reflecting liver status; and

b. combining said biomarkers in a mathematical function.

5 In one embodiment, said biomarker reflecting apoptosis is CK18.

In one embodiment, said biomarker reflecting anthropometry is body mass index (BMI) or body weight, preferably body mass index (BMI).

In one embodiment, said biomarker reflecting glucose activity is selected from the group comprising glycaemia, hyperglycemia, HbA1c, insulin, C peptide, HOMA,
10 QUICKI antidiabetic treatment, diabetes, type 2 diabetes and impaired fasting glucose tolerance, preferably said biomarker reflecting glucose activity is glycaemia or hyperglycemia, and more preferably said biomarker reflecting glucose activity is hyperglycemia.

In one embodiment, at step (a) four of the following biomarkers are measured:

- 15
- i. at least one biomarker reflecting apoptosis, and
 - ii. at least one biomarker reflecting anthropometry, and
 - iii. at least one biomarker reflecting metabolic activity, and
 - iv. at least one biomarker reflecting liver status.

In one embodiment, the biomarker reflecting apoptosis is selected from the group
20 comprising cytokeratin 18 (CK18), CK18 M30, CK18 M65, AST, ALT and any combination thereof.

In one embodiment, the biomarker reflecting anthropometry is selected from the group comprising body mass index (BMI), body weight, age, sex, waist circumference, abdominal height, hip circumference, waist/hip ratio and any combination thereof.

25 In one embodiment, the biomarker reflecting metabolic activity is a biomarker reflecting glucose metabolism, a biomarker reflecting lipid metabolism, or any combination thereof.

In one embodiment, the biomarker reflecting liver status is a biomarker reflecting liver fibrosis, preferably selected from the list comprising score of FIBROMETER™, BARD score, NFSA score, APRI score, AST (aspartate aminotransferase), ALT (alanine aminotransferase), AST/ALT, AST.ALT, ferritin, platelets (PLT), AST/PLT, 5 prothrombin time (PT) or prothrombin index (PI), INR (international normalized ratio), hyaluronic acid (HA or hyaluronate), haemoglobin, triglycerides, alpha-2 macroglobulin (A2M), gamma-glutamyl transpeptidase (GGT), urea, bilirubin, apolipoprotein A1 (ApoA1), type III procollagen N-terminal propeptide (P3NP), Type IV collagen, gamma-globulins (GBL), haptoglobulin, osteopontin, sodium (Na), albumin (ALB), 10 ferritin (Fer), Glucose (Glu), alkaline phosphatases (ALP), YKL-40 (human cartilage glycoprotein 39), tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), TGF, and matrix metalloproteinase 1 (MMP-1) to 9 (MMP-9), ratios and mathematical combinations thereof.

In one embodiment, wherein the biomarker reflecting liver status is a biomarker reflecting liver function, preferably selected from the list comprising AST (aspartate 15 aminotransferase), ALT (alanine aminotransferase), AST/ALT, AST.ALT, gamma-glutamyltranspeptidase (GGT), bilirubin, gamma-globulins (GLB), urea, albumin (ALB), alkaline phosphatases (ALP), alpha GST, 5'nucleotidase, ratios and mathematical combinations thereof.

20 In one embodiment, the biomarker reflecting liver status is a data resulting from a physical method for assessing liver status, preferably issued from elastometry such as, for example, a Fibroscan™.

In one embodiment, the biomarker reflecting liver status is a score resulting from a test selected from the group comprising FIBROMETER™, CIRRHOMETER™, BARD, 25 NFSA, APRI, FIB-4, Forns, Hepascore, Fibrotest™, Fibrosure, QuantiMeter, CombiMeter, E-FibroMeter, NAFLD fibrosis score, ELF, Fibrospect™.

In one embodiment, the following biomarkers are measured in step (a):

- CK18, hyperglycemia and BMI,
- BMI, AST and glycemia,

- BMI, CK18 and BARD score,
 - BMI, CK18 and glycemia,
 - Body weight, CK18 and glycemia,
 - CK18, BMI, hyperglycemia and insulin,
 - 5 - CK18 and BMI,
 - CK18, body weight, insulin, and haptoglobin,
 - CK18, body weight, NFSA score, APRI score and insulin,
 - CK18, body weight and insulin,
 - CK18, body weight and NFSA score,
 - 10 - CK18 and body weight,
 - CK18, BMI and body weight,
 - CK18 and albumin,
 - BMI and HDL cholesterol,
 - AST, HDL cholesterol and BMI,
 - 15 - BMI,
 - CK18 and HDL cholesterol,
 - CK18,
 - HDL cholesterol,
 - CK18, body weight, NFSA score, and insulin,
 - 20 - CK18, body weight, APRI score and insulin,
 - CK18, body weight, score of FibroMeter and insulin,
 - CK18, body weight, hyaluronate and insulin,
 - CK18, body weight, alpha-2-macroglobulin and insulin, or
 - CK18 body weight, ApoA1 and insulin.
- 25 In one embodiment, the mathematical function is a binary logistic regression, a multiple linear regression or any multivariate analysis.

In one embodiment, the NASH lesion is borderline NASH or definite NASH, preferably definite NASH or borderline and definite NASH.

In one embodiment, the subject is an animal, preferably a human.

In one embodiment, the NASH lesions are associated to or caused by a liver disease, preferably selected from the list comprising metabolic syndrome, non-alcoholic fatty liver disease and alcoholic chronic liver disease.

In one embodiment, the sample is a blood sample.

5

DEFINITIONS

In the present invention, the following terms have the following meanings:

- “**Nonalcoholic Fatty Liver Disease (NAFLD)**” encompasses the entire spectrum of fatty liver disease in individuals without significant alcohol consumption, ranging from fatty liver to steatohepatitis and cirrhosis (definition of the AASLD Practice Guideline, Chalasani et al, Hepatology, 55(6):2005-2023, 2012).
- “**Nonalcoholic Steatohepatitis (NASH)**” refers to the presence of hepatic steatosis and inflammation with hepatocyte injury (ballooning) with or without fibrosis (definition of the AASLD Practice Guideline, Chalasani et al, Hepatology, 55(6):2005-2023, 2012).
- “**Accuracy**” of a diagnostic test refers to the proportion of correctly classified patients by a diagnostic test.
- “**About**” preceding a figure means plus or less 10% of the value of said figure.

20 DETAILED DESCRIPTION

An object of the present invention is thus a method for measuring by non-invasive means (such as for example biomarkers) NASH lesions in the liver, and thus for diagnosing NASH in a subject. In one embodiment of the invention, the method of the invention is more accurate than the methods of the prior art.

- 25 The present invention thus relates to an in-vitro non-invasive method for assessing the presence and/or severity of NASH lesions in the liver of a subject, wherein said method comprises the steps of:

- a. measuring in a sample of said subject at least one biomarker, preferably 1, 2, 3 or 4 biomarkers, selected from at least one of:
 - i. at least one biomarker reflecting apoptosis, and/or
 - ii. at least one biomarker reflecting anthropometry, and/or
 - 5 iii. at least one biomarker reflecting metabolic activity, preferably at least one biomarker reflecting glucose metabolism, and/or
 - iv. at least one biomarker reflecting liver status; and
- b. combining said at least one biomarker measure in a mathematical function.

In one embodiment, in step (a) of the method of the invention, at least one biomarker reflecting apoptosis is measured, and optionally at least one biomarker selected from at least one of:

- i. at least one biomarker reflecting anthropometry, and/or
- ii. at least one biomarker reflecting metabolic activity, preferably at least one biomarker reflecting glucose metabolism, and/or
- 15 iii. at least one biomarker reflecting liver status.

In one embodiment of the invention, the method of the invention is for assessing the presence or the absence of NASH lesions in the liver of a subject. In another embodiment, the method of the invention is for diagnosing NASH in a subject.

In another embodiment of the invention, the method of the invention is for assessing the severity of NASH lesions in the liver of a subject.

In one embodiment of the invention, the mathematical combination of step (b) leads to a diagnostic score. According to this embodiment, the diagnostic score may correspond to a probability for the subject to present NASH lesions.

In another embodiment, the method of the invention leads to the classification of the patient in a class of a classification according to the value obtained at step (b), preferably according to the latest recommendations (Sanyal et al, 2011), i.e. the subject is diagnosed as having steatohepatitis corresponding to either definite steatohepatitis and/or borderline steatohepatitis; or no steatohepatitis.

According to the classification described by Sanyal et al (2011), the minimal criteria for the diagnosis of steatohepatitis, such as, for example, of NASH, include the presence of more than 5% macrovesicular steatosis, inflammation and liver cell ballooning, typically with a predominantly centrilobular (acinar zone 3) distribution. Moreover,
5 “definite steatohepatitis”, such as, for example, “definite NASH”, is defined by zone 3 accentuation of macrovesicular steatosis of any grade, hepatocellular ballooning of any degree, and lobular inflammatory infiltrates of any amount. “Borderline steatohepatitis” does not meet classical criteria for steatohepatitis, because the lesions may be predominantly in acinar zone 3, but liver cell ballooning is not classic or is absent.

10 In one embodiment of the invention, the NASH lesion is borderline NASH or definite NASH, preferably definite NASH.

In another embodiment, the method of the invention is for classifying the subject in a class of another known or new classification of NASH.

In a first embodiment of the invention, at least one biomarker reflecting apoptosis, and
15 at least one biomarker reflecting anthropometry are measured.

In a second embodiment of the invention, at least one biomarker reflecting apoptosis, and at least one biomarker reflecting metabolic activity, preferably at least one biomarker reflecting glucose metabolism, are measured.

In a third embodiment of the invention, at least one biomarker reflecting apoptosis, and
20 at least one biomarker reflecting liver status are measured.

In a fourth embodiment of the invention, at least one biomarker reflecting anthropometry and at least one biomarker reflecting metabolic activity, preferably at least one biomarker reflecting glucose metabolism, are measured.

In a fifth embodiment of the invention, at least one biomarker reflecting anthropometry
25 and at least one biomarker reflecting liver status are measured.

In a sixth embodiment of the invention, at least one biomarker reflecting metabolic activity, preferably at least one biomarker reflecting glucose metabolism, and at least one biomarker reflecting liver status are measured.

In one embodiment of the invention, three of the following biomarkers are measured in step (a):

- iv. at least one biomarker reflecting apoptosis, and/or
- v. at least one biomarker reflecting anthropometry, and/or
- 5 vi. at least one biomarker reflecting metabolic activity, preferably at least one biomarker reflecting glucose metabolism, and/or
- vii. at least one biomarker reflecting liver status.

In a first embodiment, at least one biomarker reflecting apoptosis, and at least one biomarker reflecting anthropometry and at least one biomarker reflecting metabolic
10 activity, preferably at least one biomarker reflecting glucose metabolism, are measured.

In a second embodiment, at least one biomarker reflecting apoptosis, and at least one biomarker reflecting anthropometry and at least one biomarker reflecting liver status are measured.

In a third embodiment, at least one biomarker reflecting apoptosis and at least one
15 biomarker reflecting metabolic activity, preferably at least one biomarker reflecting glucose metabolism, and at least one biomarker reflecting liver status are measured.

In a fourth embodiment, at least one biomarker reflecting anthropometry and at least one biomarker reflecting metabolic activity, preferably at least one biomarker reflecting glucose metabolism, and at least one biomarker reflecting liver status are measured.

20 In another embodiment of the invention, four of the following biomarkers are measured in step (a):

- i. at least one biomarker reflecting apoptosis, and
- ii. at least one biomarker reflecting anthropometry, and
- iii. at least one biomarker reflecting metabolic activity, preferably at least
25 one biomarker reflecting glucose metabolism, and
- iv. at least one biomarker reflecting liver status.

Examples of biomarkers reflecting apoptosis include, but are not limited to, cytokeratin 18 (CK18), CK18 M30, CK18 M65, aspartate aminotransferase (AST), alanine

aminotransferase (ALT) and any combination thereof. Preferably, said biomarker reflecting apoptosis is CK18 or AST, more preferably CK18.

Examples of biomarkers reflecting anthropometry include, but are not limited to, body mass index (BMI), body weight, age, sex, waist circumference, abdominal height, hip
5 circumference, waist/hip ratio and any combination thereof. Preferably, said biomarker reflecting anthropometry is selected from the group comprising BMI and body weight.

In one embodiment of the invention, the biomarker reflecting metabolic activity is a biomarker reflecting glucose metabolism, or lipid metabolism, or any combination thereof. Preferably, the biomarker reflecting metabolic activity is a biomarker reflecting
10 glucose metabolism.

Examples of biomarkers reflecting glucose metabolism include, but are not limited to glycaemia or hyperglycemia, HbA1c, insulin, C peptide, HOMA, QUICKI antidiabetic treatment, diabetes, type 2 diabetes and impaired fasting glucose tolerance. Preferably, said biomarker reflecting glucose metabolism is hyperglycemia, glycemia or insulin.
15 More preferably, said biomarker reflecting glucose metabolism is hyperglycemia or glycemia, and even more preferably hyperglycemia.

Hyperglycemia corresponds to a binary marker: the value of said marker is 0 if the subject does not present hyperglycemia, and 1 if the subject presents hyperglycemia. The skilled artisan knows how to determine if a subject presents or not hyperglycemia.
20 In one embodiment, hyperglycemia corresponds to any abnormal blood glucose level, such as, for example, higher than about 7 mmol.L (i.e. about 126 mg/dL) or anti-diabetic treatment required.

Examples of biomarkers reflecting lipid metabolism include, but are not limited to triglycerides, HDL cholesterol, LDL cholesterol, total cholesterol, adiponectin, leptin,
25 resistin, lipid-lowering drugs and dyslipidemia. Preferably, biomarkers reflecting lipid metabolism is HDL cholesterol.

Preferably, said biomarker reflecting metabolic activity is selected from the group comprising hyperglycemia, glycemia, and insulin.

HOMA or Homeostatic Model Assessment; is a test based on a ratio of glycemia and insulin.

QUICKI or quantitative assessment check index is a test equivalent to homeostasis model assessment (HOMA).

- 5 In one embodiment of the invention, the biomarker reflecting liver status is a biomarker reflecting liver fibrosis. Examples of biomarkers reflecting liver fibrosis include, but are not limited to, score of FIBROMETER™, BARD score, NFS score, APRI score, AST (aspartate aminotransferase), ALT (alanine aminotransferase), AST/ALT, AST.ALT, ferritin, platelets (PLT), AST/PLT, prothrombin time (PT) or prothrombin index (PI),
10 INR (international normalized ratio), hyaluronic acid (HA or hyaluronate), haemoglobin, triglycerides, alpha-2 macroglobulin (A2M), gamma-glutamyl transpeptidase (GGT), urea, bilirubin, apolipoprotein A1 (ApoA1), type III procollagen N-terminal propeptide (P3NP), Type IV collagen, gamma-globulins (GBL), haptoglobin, osteopontin, sodium (Na), albumin (ALB), ferritin (Fer), Glucose (Glu),
15 alkaline phosphatases (ALP), YKL-40 (human cartilage glycoprotein 39), tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), TGF, and matrix metalloproteinase 1 (MMP-1) to 9 (MMP-9), ratios and mathematical combinations thereof.

- In one embodiment of the invention, the biomarker reflecting liver status is a biomarker reflecting liver function. Examples of biomarkers reflecting liver function include, but
20 are not limited to, AST (aspartate aminotransferase), ALT (alanine aminotransferase), AST/ALT, AST.ALT, gamma-glutamyltranspeptidase (GGT), bilirubin, gamma-globulins (GLB), urea, albumin (ALB), alkaline phosphatases (ALP), alpha GST, 5' nucleotidase, ratios and mathematical combinations thereof.

- In one embodiment of the invention, the biomarker reflecting liver status is a data
25 resulting from a physical method for assessing liver status. Examples of physical methods for assessing liver status include, but are not limited to, medical imaging data and clinical measurements, such as, for example, measurement of spleen, especially spleen length. According to an embodiment, the physical method is selected from the group comprising ultrasonography, especially Doppler-ultrasonography and elastometry
30 ultrasonography and velocimetry ultrasonography (preferred tests using said data are

Fibroscan™, ARFI, VTE, supersonic imaging), MRI (Magnetic Resonance Imaging), and MNR (Magnetic Nuclear Resonance) as used in spectroscopy, especially MRI elastometry or velocimetry. Preferably, the data are Liver Stiffness Evaluation (LSE) data or spleen stiffness evaluation. According to a preferred embodiment of the invention, the data resulting from a physical method are issued from a Fibroscan™.

In one embodiment of the invention, the biomarker reflecting liver status is a score resulting from a test selected from the list comprising FIBROMETER™, CIRRHOMETER™, BARD, NFSA, APRI, FIB-4, Forns, Hepascore, Fibrotest™, Fibrosure, QuantiMeter (Calès, Hepatology 2005;42:1373-1381), CombiMeter, NAFLD fibrosis score, ELF, Fibrospect™.

BARD is a blood test based on three variables combined in a weighted sum of BMI, AST/ALT ratio and Diabetes Melitus.

NFSA : NAFLD fibrosis score (NFS) of Angulo is a blood test based on age, hyperglycemia, body mass index, platelet count, albumin, and AST/ALT ratio.

Forns is a blood test based on age, GGT, cholesterol, and platelet count.

APRI is a blood test based on platelet and AST.

ELF or European liver fibrosis is a blood test based on hyaluronic acid, P3P, TIMP-1 and age.

FibroSpect™ is a blood test based on hyaluronic acid, TIMP-1 and A2M.

FIB-4 is a blood test based on platelet, ASAT, ALT and age.

HEPAScore is a blood test based on hyaluronic acid, bilirubin, alpha2-macroglobulin, GGT, age and sex.

FIBROTEST™ is a blood test based on alpha2-macroglobulin, haptoglobin, apolipoprotein A1, total bilirubin, GGT, age and sex.

FIBROSURE™ is the American name of FIBROTEST™.

FIBROMETER™ and CIRRHOMETER™ together form to a family of blood tests, the content of which depends on the cause of chronic liver disease and the diagnostic target, and this blood test family is called FM family and detailed in the Table below:

Cause	Variables															N ^a
	Age	Sex	Weigth	A2M	HA	PI	PLT	AST	Urea	GGT	Bili	ALT	Fer	Glu	FS	
Virus																
FM V 1G	x			x	x	x	x	x	x							7
FM V 2G	x	x		x	x	x	x	x	x							8
CM V 2G	x	x		x	x	x	x	x	x							8
FM V 3G ^b	x	x		x	x		x	x	x	x						8
CM V 3G ^b	x	x		x	x		x	x	x	x						8
Alcohol																
FM A 1G	x			x	x	x										4
FM A 2G				x	x	x										3
NAFLD (steatosis)																
FM S	x		x				x	x				x	x	x		7

FM: FibroMeter, CM: CirrhoMeter,

- 5 A2M: alpha-2 macroglobulin, HA: hyaluronic acid, PI: prothrombin index, PLT: platelets, Bili: bilirubin, Fer: ferritin, Glu: glucose, FS: Fibroscan

^a Number of variables

^b HA may be replaced by GGT

- COMBIMETER™ or E-FibroMeter™ is a family of tests based on the mathematical combination of variables of the FM family (as detailed in the Table above) or of the result of a test of the FM family with elastometry, such as, for example, FIBROSCAN™ result. In one embodiment, said mathematical combination is a binary logistic regression.

- QUANTIMETER™ is a family of tests based on the mathematical combination of variables of the FM family targeted for the area of fibrosis.

Preferably, the biomarker reflecting liver status is selected from the group comprising or consisting of BARD score, haptoglobin, NFSA score, APRI score, albumin, FibroMeter™ score, hyaluronate, alpha-2-macroglobulin and ApoA1.

- In one embodiment, in the method of the invention, 1, 2, 3 4 or more of the following biomarkers are measured in step (a):

- CK18, hyperglycemia and BMI,
- BMI, AST and glycemia,
- BMI, CK18 and BARD score,
- BMI, CK18 and glycemia,
- 5 - Body weight, CK18 and glycemia,
- CK18, BMI, hyperglycemia and insulin,
- CK18 and BMI,
- CK18, body weight, insulin, and haptoglobin,
- CK18, body weight, NFSA score, APRI score and insulin,
- 10 - CK18, body weight and insulin,
- CK18, body weight and NFSA score,
- CK18 and body weight,
- CK18, BMI and body weight,
- CK18 and albumin,
- 15 - BMI and HDL cholesterol,
- AST, HDL cholesterol and BMI,
- BMI,
- CK18 and HDL cholesterol,
- CK18,
- 20 - HDL cholesterol,
- CK18, body weight, NFSA score, and insulin,
- CK18, body weight, APRI score and insulin,
- CK18, body weight, score of FibroMeter and insulin,
- CK18, body weight, hyaluronate and insulin,
- 25 - CK18, body weight, alpha-2-macroglobulin and insulin, or
- CK18 body weight, ApoA1 and insulin.

In one embodiment, the method of the invention does not comprise measuring in step (a) a biomarker reflecting lipid metabolism.

- In one embodiment, the method of the invention does not comprise measuring in step
- 30 (a) CK-18 M30, CK18 M65, resistin and adiponectin.

In one embodiment, the method of the invention does not comprise measuring in step (a) at least four biomarkers selected from the group consisting of adiponectin, CK-18, CK-18 M30, C-reactive protein, insulin-like growth factor binding protein 1 and alanine aminotransferase.

- 5 In one embodiment, the method of the invention does not comprise measuring in step (a) adiponectin, CK-18, CK-18 M30, insulin-like growth factor binding protein 1 and body mass index (BMI).

In one embodiment, the method of the invention does not comprise measuring in step (a) diabetes, sex, body mass index, triglycerides, CK-18 and CK-18 M30.

- 10 In one embodiment, the method of the invention does not comprise measuring in step (a) type 2 diabetes, triglycerides, TIMP-1 and aspartate aminotransferase.

In one embodiment of the invention, the mathematical function is a binary logistic regression, a multiple linear regression or any multivariate analysis. One skilled in the art may find in the prior art all information related to the mathematical function.

- 15 In one embodiment of the invention, the subject is an animal, preferably a human. In one embodiment, the subject is a male. In another embodiment, the subject is a female.

In one embodiment, the subject presents a BMI superior to 25 (i.e. the subject is overweight), preferably superior to 30 (i.e. the subject is obese).

In one embodiment of the invention, the subject is diagnosed with NAFLD.

- 20 In one embodiment of the invention, the subject is at risk of developing NASH lesions. Said risk may for example correspond to a genetic predisposition to NASH lesions, a familial history of NASH lesions or to overweight or obesity.

- In one embodiment of the invention, the NASH lesions are associated to or caused by a liver disease, preferably selected from the list comprising alcohol syndromes, metabolic syndromes (such as, for example, diabetes, hypertension, dyslipidemia, abetalipoproteinemia, glycogen storage diseases, Weber-Christian disease, Wolman
- 25

disease, acute fatty liver of pregnancy and lipodystrophy), non-alcoholic fatty liver disease and alcoholic chronic liver disease.

In another embodiment of the invention, the NASH lesions are associated to or caused by nutritional causes (such as, for example, malnutrition, total parenteral nutrition,
5 severe weight loss, refeeding syndrome, jejuno-ileal bypass, gastric bypass or jejunal diverticulosis with bacterial overgrowth).

In another embodiment of the invention, the NASH lesions are associated to or caused by various drugs and toxins (such as, for example, amiodarone, methotrexate, diltiazem, highly active antiretroviral therapy, glucocorticoids, tamoxifen, environmental
10 hepatotoxins).

In another embodiment of the invention, the NASH lesions are associated to or caused by inflammatory bowel disease or HIV.

According to the invention, biomarkers are measured in a sample obtained, preferably previously obtained, from the subject.

15 In one embodiment of the invention, the sample is a bodily fluid sample, such as, for example, a blood or urine sample.

Methods for measuring biomarkers in a sample are well-known from the skilled artisan. Examples of such methods include, but are not limited to, biochemistry, immunology, molecular biology or omic technologies, and physical techniques, such as, for example,
20 infrared spectroscopies.

In one embodiment of the invention, the method of the present invention is computerized. Another object of the present invention is thus a computer software for implementing the method of the invention.

The present invention also relates to an in-vitro non-invasive diagnostic test for
25 implementing the in-vitro non-invasive method as hereinabove described.

The present invention also relates to a kit for implementing the in-vitro non-invasive method as hereinabove described. According to the invention, the kit comprises means

for measuring biomarkers in a sample obtained, preferably previously obtained, from a subject.

In one embodiment, said means are PCR primers, buffers and reagents for measuring the expression of biomarkers in the sample from the subject.

- 5 In one embodiment, said means are antibodies, such as, for example, monoclonal antibodies, for detecting proteins in the sample from the subject. For example, antibodies may be used for measuring CK-18, and are commercially available, such as for example, from Santa Cruz Biotech or Abcam.

- 10 In one embodiment, said means are standard reagents, such as, for example, reagents for measuring glucose concentration in the sample from the subject, preferably in a blood sample from the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

- 15 **Figure 1** is a graph showing CK18 concentration as a function of NASH subgroups (no, borderline, definite).

Figure 2 is a graph showing the sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV), diagnostic accuracy (DA) and Youden index (sensitivity + specificity -1) as a function of NASH-score results.

20 EXAMPLES

The present invention is further illustrated by the following example.

PATIENTS AND METHODS

Patients

- 25 Patients with biopsy-proven NAFLD were consecutively included from January 2002 to March 2012 at the Angers University Hospital. NAFLD was defined as liver steatosis

on liver biopsy after exclusion of concomitant steatosis-inducing drugs (such as corticosteroids, tamoxifene, amiodarone, or methotrexate), excessive alcohol consumption (>30 g/day in men or >20 g/day in women), chronic hepatitis B or C infection, and histological evidence of other concomitant chronic liver disease. Patients
5 were excluded if they had cirrhosis complication (ascites, variceal bleeding, systemic infection, or hepatocellular carcinoma). The study protocol was conformed to the ethical guidelines of the current Declaration of Helsinki and all patients gave informed written consent.

Methods

10 ***Clinical assessment*** – Regular treatment, weight, height, and blood arterial pressure were recorded the day of the liver biopsy. Arterial hypertension, hyperglycaemia, hypertriglyceridemia, and low HDL cholesterol were defined according to the joint statement of the International Diabetes Federation, National Heart, Lung, and Blood
15 Institute, American Heart Association, World Heart Federation, International Atherosclerosis Society, and International Association for the Study of Obesity (Alberti, Circulation 2009). Arterial hypertension corresponded to systolic arterial pressure ≥ 130 mm Hg and/or diastolic arterial pressure ≥ 85 mm Hg or antihypertensive drug; hyperglycaemia to fasting serum glucose ≥ 5.55 mmol/L or anti-diabetic drug; hypertriglyceridemia to serum triglycerides ≥ 1.7 mmol/L or lipid-lowering drug; and
20 low HDL cholesterol to serum HDL-cholesterol < 1.1 mmol/L in male or < 1.3 mmol/L in female, or lipid-lowering drug.

Blood samples – Fasting blood samples were taken the day or within the week preceding liver biopsy. All blood assays were performed in the laboratory of Angers Hospital and included: AST, ALT, gamma GT, alkaline phosphatases, total bilirubin,
25 glucose, insulin, HbA1c, triglycerides, total cholesterol, HDL cholesterol, LDL cholesterol, ferritin, urea, sodium, creatinine, apolipoprotein A1, albumin, haptoglobin, alpha2 macroglobulin, hyaluronate, prothrombin index, haemoglobin, leucocytes, neutrophils, monocytes, and platelets. Serum level of apoptotic caspase-3 generated CK-18 fragments was measured on frozen samples stored at -80°C , using the M30-
30 Apoptosense enzyme-linked immunosorbent assay kit (PEVIVA, Bromma, Sweden). 5

blood tests for the diagnosis of NASH were calculated according to published formula: HAIR (Dixon Gy 2001), Palekar score (Palekar LI 2006), Gholam score (Gholam AJG 2007), Hossain score (Hosain CGH 2009), and Nice Model (Anty APT 2010).

Pathological diagnosis of NASH – Pathological examination of liver biopsies was performed by a senior expert specialized in hepatology, blinded for patient data. NASH was diagnosed as “no”, “borderline”, or “definite” according to the latest recommended definition (Sanyal et al, 2011). Histological grading and staging of NAFLD were scored according to the NASH Clinical Research Network system (Kleiner 2005). NAFLD activity score (NAS), ranging from 0 to 8, corresponded to the sum of scores for steatosis, lobular inflammation and hepatocellular ballooning.

Statistical analysis

Quantitative variables were expressed as mean $\pm$ standard deviation. Correlations between quantitative variables were determined by using the Spearman correlation coefficient (Rs). To identify the best combination of variables for the diagnosis of NASH, we performed a stepwise forward binary logistic regression including clinical data and blood parameters as independent variables. The regression score of this multivariate analysis was used to construct a new blood test ranging from 0 to 1. Diagnostic accuracy of this blood test was evaluated by the Area Under the Receiver Operating Characteristics (AUROC) and the rate of patients included in the intervals of $\geq 90\%$ negative or positive predictive values. Statistical analyses were performed using SPSS version 18.0 software (IBM, Armonk, NY, USA).

RESULTS

Patients

Characteristics of the 159 patients included are detailed in **Table 1**. 59.7% were male and mean age was 56.4 ± 11.6 years. Mean biopsy length was 30 ± 14 mm. 64 patients (40%) had no NASH, 19 (12%) had borderline NASH, and 76 (48%) had definite NASH. Mean NAS and rate of patients with $F \geq 2$ stages between no, borderline, and

definite NASH was, respectively: 2.0 ± 1.1 , 3.4 ± 0.8 , 4.3 ± 1.1 ($p < 0.001$) and 37.5%, 73.7%, 84.2 ($p < 0.001$).

Table 1: Patients characteristics at inclusion

	All	NASH			
		No	Borderline	Definite	p
Patients (n)	159	64	19	76	-
Age (years)	56.4 ± 11.6	56.4 ± 11.3	51.1 ± 16.2	57.8 ± 10.1	0.325
Male sex (%)	59.7	62.5	68.4	55.3	0.489
BMI (kg/m^2)	30.9 ± 5.4	28.7 ± 4.7	31.0 ± 7.3	32.8 ± 4.7	<0.001
Hypertension (%) ^a	75.9	69.8	57.9	85.5	0.014
Hyperglycaemia (%) ^b	67.3	50.0	57.9	84.2	<0.001
Hypertriglyceridemia (%) ^c	58.3	48.4	42.1	70.7	0.010
Low HDL cholesterol (%) ^d	60.1	56.4	66.7	61.6	0.718
Biopsy length (mm)	30.0 ± 13.7	29.3 ± 13.8	26.6 ± 13.7	31.3 ± 13.7	0.354
NAS	3.3 ± 1.5	2.0 ± 1.1	3.4 ± 0.8	4.3 ± 1.1	<0.001
Kleiner F ≥ 2 (%)	64.2	37.5	73.7	84.2	<0.001
ALT (UI/L)	65 ± 36	56 ± 32	82 ± 34	68 ± 38	0.002
AST (UI/L)	46 ± 22	39 ± 19	47 ± 15	51 ± 25	<0.001
Gamma GT (UI/L)	148 ± 165	146 ± 171	122 ± 152	156 ± 164	0.280
Alkaline phosphatases (UI/L)	79 ± 39	84 ± 45	72 ± 21	78 ± 38	0.722
Total bilirubin ($\mu\text{mol/L}$)	10.0 ± 5.6	11.0 ± 6.4	10.0 ± 7.2	9.1 ± 4.3	0.211
Albumin (g/L)	44.0 ± 3.7	44.8 ± 3.7	45.1 ± 3.2	43.1 ± 3.5	0.006
Haptoglobin (g/L)	1.24 ± 0.60	1.07 ± 0.55	1.34 ± 0.52	1.34 ± 0.64	0.040
Alpha2macroglobulin (mg/dl)	207 ± 86	190 ± 81	202 ± 89	220 ± 88	0.119
Hyaluronate ($\mu\text{g/L}$)	76 ± 98	68 ± 115	65 ± 61	86 ± 91	0.174
Apolipoprotéine A1 (g/L)	1.48 ± 0.35	1.54 ± 0.37	1.39 ± 0.36	1.45 ± 0.33	0.339
Insuline ($\mu\text{UI/ml}$)	23.2 ± 28.1	14.5 ± 12.8	16.0 ± 8.5	31.0 ± 35.8	<0.001
HbA1c (%)	6.4 ± 1.3	6.0 ± 1.5	5.9 ± 0.9	6.8 ± 1.2	<0.001
Ferritin ($\mu\text{g/L}$)	412 ± 417	380 ± 345	377 ± 215	447 ± 502	0.762
Leucocytes (G/L)	6.4 ± 1.8	5.9 ± 1.7	6.7 ± 2.1	6.6 ± 1.7	0.022
CK18 (UI/L)	303 ± 371	168 ± 123	249 ± 131	397 ± 463	<0.001

^a Systolic arterial pressure ≥ 130 mm Hg and/or diastolic arterial pressure ≥ 85 mm Hg or antihypertensive drug

^b Fasting serum glucose ≥ 5.55 mmol/L or anti-diabetic drug

^c Serum triglycerides ≥ 1.7 mmol/L or lipid-lowering drug

^d Low serum HDL-cholesterol (<1.1 mmol/L in male or <1.3 mmol/L in female) or lipid-lowering drug

Accuracy of CK18 and published blood tests for the diagnosis of NASH

CK18 level was significantly different among the 3 NASH subgroups ($p < 0.001$) but post hoc analysis showed the difference was significant only between no NASH and borderline or definite NASH (**Figure 1**). AUROC of CK18 for the diagnosis of
 5 borderline/definite NASH was 0.743 ± 0.048 , and 0.716 ± 0.048 for the diagnosis of definite NASH (**Table 2**). AUROC of HAIR, Palekar score, Gholam score, Hossain score, and Nice Model for the diagnosis of borderline/definite or definite NASH are depicted in **Table 2**.

Table 2: AUROC of CK18 and blood tests for the diagnostic of NASH

Blood test	Diagnostic target	
	Borderline/definite NASH	Definite NASH
CK18	0.743 ± 0.048	0.716 ± 0.048
HAIR	0.622 ± 0.051	0.645 ± 0.050
Palekar score	0.651 ± 0.044	0.677 ± 0.043
Gholam score	0.789 ± 0.036	0.779 ± 0.037
Hossain score	0.613 ± 0.046	0.532 ± 0.046
Nice Model	0.733 ± 0.052	0.670 ± 0.055
NASH Score	0.846 ± 0.039	0.804 ± 0.043

10 Development of the new NASH-score

We used a stepwise forward binary logistic regression to identify the independent predictors of NASH and to develop the new blood tests for NASH. Because binary logistic regression implies a binary diagnostic target, we chose to pool borderline with definite NASH versus no NASH. Indeed, the rate of $F \geq 2$ patients was not significantly
 15 different between borderline and definite NASH subgroups (73.7% vs 84.2%, $p = 0.320$), but significantly higher than in the subgroup having no NASH ($p \leq 0.005$). Moreover, a recent systematic review has shown that liver inflammation is a strong predictor of progression to advanced fibrosis in patients with NASH (Argo, JH 2009).

By multivariate analysis, the main independent predictors of borderline/definite NASH
 20 were BMI, CK18, and hyperglycaemia (**Table 3**). The regression formula of the

multivariate analysis was: $R = (0.185 \cdot \text{BMI}) + (0.006 \cdot \text{CK18}) + (1.234 \cdot \text{hyperglycaemia [0: no, 1: yes]}) - 7.277$. The new NASH-score was calculated as follow: $1 / 1 + (\exp [-R])$, and ranged from 0 to 1.

Table 3: Main independent predictors of borderline/definite NASH by stepwise forward
5 binary logistic regression.

Step	Variable	p	OR (95% CI)	β
1 st	BMI	0.003	1.203 (1.066-1.358)	0.185
2 nd	CK18	0.001	1.006 (1.002-1.010)	0.006
3 rd	Hyperglycaemia	0.034	3.436 (1.095-10.782)	1.234
-	Constant	-	-	-7.277

We also performed other multivariate analyses by successively excluding one of the three main independent predictors of borderline/definite NASH. We found 26 other scores including independent predictor(s) (**Table 4**). This table shows that the common core of all these scores is the combination of three types of markers: one reflecting
10 apoptosis, one reflecting anthropometry and another one reflecting metabolic activity. A fourth optional type is marker or test reflecting liver fibrosis.

Table 4: Summary of combinations of independent predictors of borderline/definite NASH by stepwise forward binary logistic regression.

Model	Markers or variables
1	CK18, hyperglycemia, BMI ^a
2	BMI, AST, glycemia
3	BMI, CK18, Bard score ^b
4	BMI, CK18, glycemia
5	Poids, CK18, glycemia
6	CK 18, BMI, glycemia
7	CK 18, BMI, hyperglycemia, insulin
8	CK 18, BMI
9	CK18, poids, insulin, haptoglobin ^c
10	CK 18, poids, NFSA ^b , APRI ^b , insulin
11	CK18, poids, insulin
12	CK18, poids, NFSA ^b
13	Weight, CK18
14	CK18, BMI, weight
15	CK 18, albumin
16	BMI, HDL cholesterol
17	AST, HDL cholesterol, BMI
18	BMI
19	CK18, HDL cholesterol
20	CK18
21	HDL cholesterol
22	CK 18, weight, NFSA ^b , insulin
23	CK 18, weight, APRI ^b , insulin
24	CK 18, weight, FibroMeter ^b , insulin
25	CK 18, weight, hyaluronate ^c , insulin
26	CK 18, weight, A2M ^c , insulin
27	CK 18, weight, ApoA1 ^c , insulin

^a main score

^b Bard, NFSA, APRI and FibroMeter are fibrosis tests: they can be replaced by other known non-invasive fibrosis tests.

^c Haptoglobin , hyaluronate, A2M, ApoaA1 are single fibrosis markers: they can be replaced by other known non-invasive single fibrosis markers.

Diagnostic accuracy of the NASH score

AUROC of the NASH-score for the diagnosis of borderline/definite NASH was
 5 0.846±0.039 vs 0.743±0.048 for CK18 (p=0.0098) and 0.774±0.047 for BMI
 (p=0.0287). AUROC of the NASH-score for the diagnosis of definite NASH was
 0.804±0.043 vs 0.716±0.048 for CK18 (p=0.0347) and 0.755±0.048 for BMI
 (p=0.1413). AUROC of the NASH score for the diagnosis of borderline/definite NASH
 was significantly higher than the AUROCs of the previously published blood tests for
 10 NASH ($p \leq 0.017$) excepted versus the Gholam score (p=0.170). AUROC of the NASH
 score for the diagnosis of definite NASH was significantly higher than the AUROCs of
 the previously published blood tests for NASH (p<0.057) excepted versus the Gholam
 score (p=0.436).

Negative and positive predictive values as a function of the NASH-Score results are
 15 depicted in **Figure 2**. The NASH-score included 16.5% of the patients in the interval of
 $\geq 90\%$ negative predictive value for the diagnosis of borderline/definite NASH, 53.0%
 in the interval of $\geq 90\%$ positive predictive value, and 30.4% in the remained grey zone.
 The NASH score was well-correlated with the NAS: $R_s=0.578$ (p<0.001), versus
 $R_s=0.292$ (p=0.001) for the HAIR, $R_s=0.184$ (p=0.022) for the Palekar score, $R_s=0.451$
 20 (p<0.001) for the Gholam score, $R_s=0.312$ (p<0.001) for the Hossain score, $R_s=0.529$
 (p<0.001) for the Nice Model, and $R_s=0.579$ (p<0.001) for CK18.

CLAIMS

1. An in-vitro non-invasive method for assessing the presence and/or severity of NASH lesions in the liver of a subject, wherein said method comprises the steps of:
- 5 of:
- a. measuring in a sample of said subject:
- i. at least one biomarker reflecting apoptosis, wherein said biomarker reflecting apoptosis is selected from the group comprising cytokeratin 18 (CK18), CK18 M30, CK18 M65, AST, ALT and any combination thereof
- 10 and
- ii. at least one biomarker reflecting anthropometry, wherein said biomarker reflecting anthropometry is selected from the group comprising body mass index (BMI), body weight, age, sex, waist circumference, abdominal height, hip circumference, waist/hip ratio and any combination thereof and
- 15 iii. at least one biomarker reflecting metabolic activity, wherein said biomarker reflecting metabolic activity is a biomarker reflecting glucose metabolism, a biomarker reflecting lipid metabolism, or any combination thereof, preferably a biomarker reflecting glucose metabolism and
- iv. optionally at least one biomarker reflecting liver status; and
- 20 b. combining said biomarkers in a mathematical function.
2. The method according to claim 1, wherein said biomarker reflecting apoptosis is CK18.
3. The method according to claim 1 or 2, wherein said biomarker reflecting anthropometry is body mass index (BMI) or body weight, preferably body mass
- 25 index (BMI).
4. The method according to anyone of claims 1 to 3, wherein said biomarker reflecting glucose activity is selected from the group comprising glycaemia, hyperglycemia, HbA1c, insulin, C peptide, HOMA, QUICKI antidiabetic treatment, diabetes, type 2 diabetes and impaired fasting glucose tolerance,

preferably said biomarker reflecting glucose activity is glycaemia or hyperglycemia, and more preferably said biomarker reflecting glucose activity is hyperglycemia.

- 5 **5.** The method according to anyone of claims **1** to **4**, wherein at step (a) four of the following biomarkers are measured:
- i. at least one biomarker reflecting apoptosis, and
 - ii. at least one biomarker reflecting anthropometry, and
 - iii. at least one biomarker reflecting metabolic activity, and
 - iv. at least one biomarker reflecting liver status.
- 10 **6.** The method according to anyone of claims **1** to **5**, wherein the biomarker reflecting liver status is a biomarker reflecting liver fibrosis, preferably selected from the list comprising score of FIBROMETER™, BARD score, NFSa score, APRI score, AST (aspartate aminotransferase), ALT (alanine aminotransferase), AST/ALT, AST.ALT, ferritin, platelets (PLT), AST/PLT, prothrombin time (PT)
- 15 or prothrombin index (PI), INR (international normalized ratio), hyaluronic acid (HA or hyaluronate), haemoglobin, triglycerides, alpha-2 macroglobulin (A2M), gamma-glutamyl transpeptidase (GGT), urea, bilirubin, apolipoprotein A1 (ApoA1), type III procollagen N-terminal propeptide (P3NP), Type IV collagen, gamma-globulins (GBL), haptoglobin, osteopontin, sodium (Na), albumin
- 20 (ALB), ferritin (Fer), Glucose (Glu), alkaline phosphatases (ALP), YKL-40 (human cartilage glycoprotein 39), tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), TGF, and matrix metalloproteinase 1 (MMP-1) to 9 (MMP-9), ratios and mathematical combinations thereof.
- 25 **7.** The method according to anyone of claims **1** to **5**, wherein the biomarker reflecting liver status is a biomarker reflecting liver function, preferably selected from the list comprising AST (aspartate aminotransferase), ALT (alanine aminotransferase), AST/ALT, AST.ALT, gamma-glutamyltranspeptidase (GGT), bilirubin, gamma-globulins (GLB), urea, albumin (ALB), alkaline phosphatases (ALP), alpha GST, 5' nucleotidase, ratios and mathematical combinations thereof

8. The method according to anyone of claims 1 to 5, wherein the biomarker reflecting liver status is a data resulting from a physical method for assessing liver status, preferably issued from a Fibroscan™.
9. The method according to anyone of claims 1 to 5, wherein the biomarker reflecting liver status is a score resulting from a test selected from the group comprising FIBROMETER™, CIRRHOMETER™, BARD, NFSa, APRI, FIB-4, Forns, Hepascore, Fibrotest™, Fibrosure, QuantiMeter, CombiMeter, NAFLD fibrosis score, ELF, Fibrospect™.
10. The method according to anyone of claims 1 to 9, wherein the following biomarkers are measured in step (a):
- CK18, hyperglycemia and BMI,
 - BMI, AST and glycemia,
 - BMI, CK18 and BARD score,
 - BMI, CK18 and glycemia,
 - Body weight, CK18 and glycemia,
 - CK18, BMI, hyperglycemia and insulin,
 - CK18 and BMI,
 - CK18, body weight, insulin, and haptoglobin,
 - CK18, body weight, NFSa score, APRI score and insulin,
 - CK18, body weight and insulin,
 - CK18, body weight and NFSa score,
 - CK18 and body weight,
 - CK18, BMI and body weight,
 - CK18 and albumin,
 - BMI and HDL cholesterol,
 - AST, HDL cholesterol and BMI,
 - BMI,
 - CK18 and HDL cholesterol,
 - CK18,
 - HDL cholesterol,

- CK18, body weight, NFSA score, and insulin,
 - CK18, body weight, APRI score and insulin,
 - CK18, body weight, score of FibroMeter and insulin,
 - CK18, body weight, hyaluronate and insulin,
 - 5 - CK18, body weight, alpha-2-macroglobulin and insulin, or
 - CK18 body weight, ApoA1 and insulin.
- 11.** The method according to anyone of claims **1** to **10** wherein the mathematical function is a binary logistic regression, a multiple linear regression or any multivariate analysis.
- 10 **12.** The method according to anyone of claims **1** to **11**, wherein the NASH lesion is borderline NASH or definite NASH, preferably definite NASH or borderline and definite NASH.
- 13.** The method according to anyone of claims **1** to **12**, wherein the subject is an animal, preferably a human.
- 15 **14.** The method according to anyone of claims **1** to **13**, wherein the NASH lesions are associated to or caused by a liver disease, preferably selected from the list comprising metabolic syndrome, non-alcoholic fatty liver disease and alcoholic chronic liver disease.
- 15.** The method according to anyone of claims **1** to **14**, wherein the sample is a blood
- 20 sample.

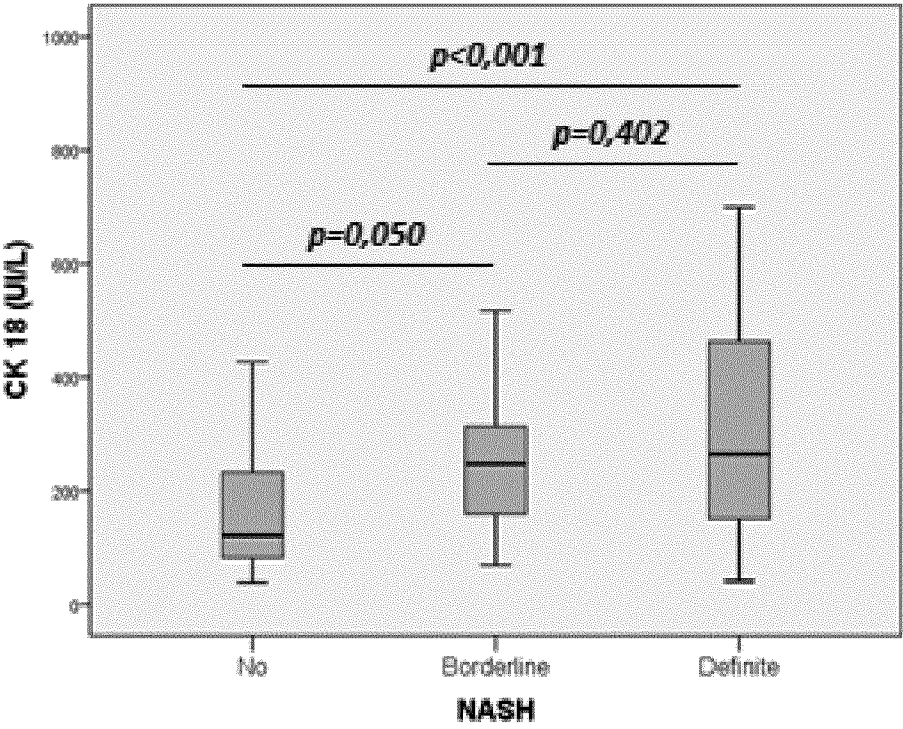


FIG. 1

2/2

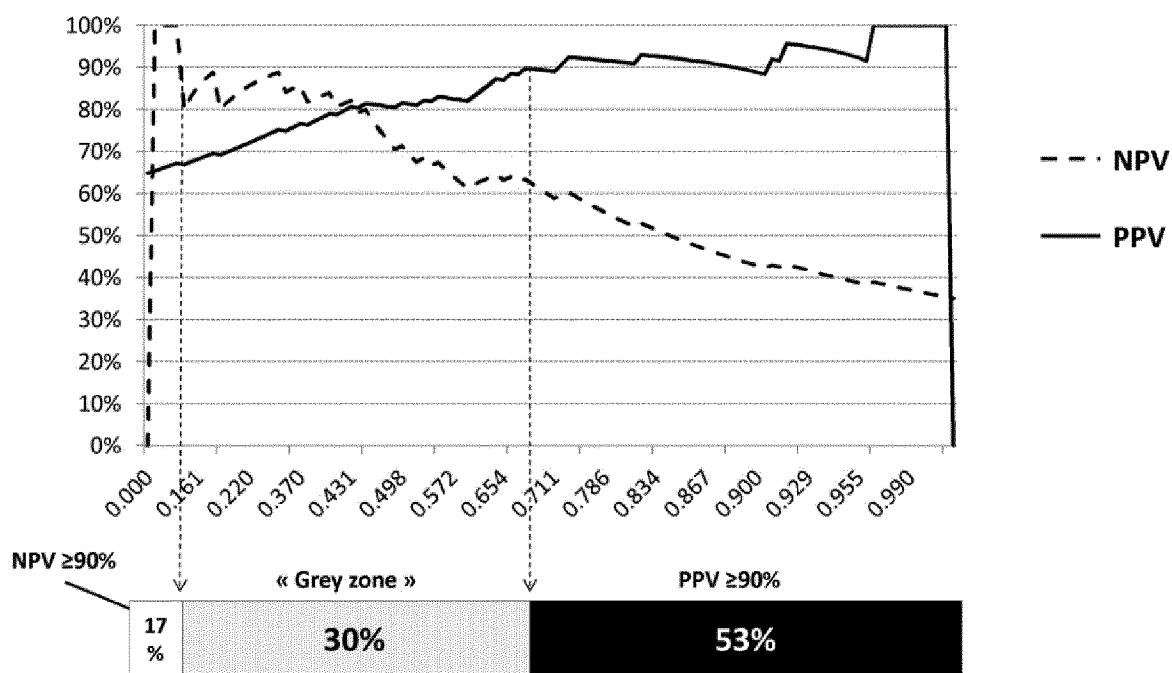


FIG. 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/070223

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/576
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2006/173629 A1 (POYNARD THIERRY [FR]) 3 August 2006 (2006-08-03) claims 1-14	1,3-8, 10-15
X	WO 2010/058295 A2 (UNIV ANGERS [FR]; CT HOSPITALIER UNIVERSITAIRE D [FR]; CALES PAUL [FR]) 27 May 2010 (2010-05-27) Table 2 and Table 2bis ; pg 24, para bridging pg 25 ; pg 25 para bridging pg 26; claims ----- -/--	1,3-7, 10-15



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

12 December 2013

Date of mailing of the international search report

03/01/2014

Name and mailing address of the ISA/

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Vadot-Van Geldre, E

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2013/070223

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M Grigorescu ET AL: "A novel pathophysiological-based panel of biomarkers for the diagnosis of nonalcoholic steatohepatitis.", Journal of physiology and pharmacology, 1 August 2012 (2012-08-01), pages 347-353, XP055092925, Retrieved from the Internet: URL: http://www.jpp.krakow.pl/journal/archiw/08_12/pdf/347_08_12_article.pdf [retrieved on 2013-12-11] table 3 -----	1,2,4-7, 11-15
X	US 2006/172286 A1 (POYNARD THIERRY [FR]) 3 August 2006 (2006-08-03) paragraph [0132]; claims 1-4,14-15 -----	1,5-9, 11-15
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