IMPROVED DIAGNOSIS OF METABOLIC DYSFUNCTION-ASSOCIATED STEATOHEPATITIS

BACKGROUND OF THE INVENTION

Metabolic dysfunction-associated steatohepatitis (MASH) is a chronic liver disease characterized histologically by the accumulation of fat, hepatocyte damage and inflammation resembling alcoholic hepatitis. MASH can lead to liver fibrosis, cirrhosis, liver failure and/or hepatocellular carcinoma (HCC).

Until recently, MASH was largely underdiagnosed, as it carries no obvious symptoms in its early stages, and also because of the lack of widely available non-invasive tests specifically developed to diagnose the disease. At-risk MASH status, which is defined as having MASH, MAS score ≥4 and fibrosis stage F≥2, represents an important MASH sub-population to identify. Indeed, these patients are associated to elevated risks of disease worsening, notably cirrhosis, and higher risk of liver-related and all-cause mortality. Due to the technical limitations and also risks of biopsies, which are the clinical reference standard for the diagnosis of MASH and fibrosis, the development of blood-based non-invasive tests (NITs) is of major importance. Different NITs have been developed, mainly to fit with fibrosis stages. However, there is still unmet medical need to provide more efficient and robust blood-based NIT for addressing a composite Fibrosis x NAS endpoint when detecting at-risk MASH patients. In this context, we evaluated whether it would be possible to provide a NIT improved over the NITs of the prior art.

SUMMARY OF THE INVENTION

The present invention is based on the detailed analysis of a significant dataset of clinical trial. It is herein provided a non-invasive method for the identification of at-risk MASH subjects.

Accordingly, the present invention relates to a method for the diagnosis of at-risk metabolic dysfunction-associated steatohepatitis (MASH) in a subject, wherein said method comprises quantifying the levels of miR-34a-5p, in particular hsa-miR-34a-5p, and Thrombospondin 2 (TSP2 or TSP-2) in a biological fluid sample of said subject.

Particularly, the invention relates to a method for the diagnosis, screening, monitoring or prognosis of at-risk MASH in a subject, said method comprising the step of quantifying the levels of miR-34a-5p and Thrombospondin 2 (TSP2) in a biological fluid sample of said subject; combining the quantified levels in a mathematical function to assign a score; and comparing said score with a cutoff value to determine whether said subject is of risk of having at-risk MASH.

In a particular embodiment, the score is compared with several cutoff values to determine whether said subject is of high, low or indeterminate risk of having at-risk MASH.

More specifically, the invention relates to a method for the diagnosis, screening, monitoring or prognosing of at-risk MASH in a subject, said method comprising:
- quantifying the levels of miR-34a-5p and TSP-2 in a biological fluid sample of said subject;
- combining the quantified levels in a mathematical function to assign a score; and
- comparing said score with cutoff values to determine whether said subject is of risk of having at-risk MASH.

In a particular embodiment, the method of the invention is for the diagnosis of at-risk MASH in a subject.

In a particular embodiment, the mathematical function includes a logistic regression equation.

In another embodiment, the biological fluid sample is a blood, serum or plasma sample, preferably a serum sample.

In yet another embodiment, the subject suffers from obesity, insulin resistance, glucose intolerance, type 2 diabetes mellitus (T2DM), prediabetes, dyslipidaemia, hypertriglyceridaemia, or high blood pressure.

According to another aspect, the invention relates to a computer program comprising instructions that, when executed by a processor/processing means, cause the processor/processing means to:
- receive quantified levels of miR34a-5p and TSP-2;
- calculate a score from these quantified levels of the subject, from a mathematical function as described herein; and
- assign the subject into the group of at-risk subjects or not at-risk subjects based upon the calculated score compared to predetermined cutoff values.

In yet another aspect, the invention relates to a computer-readable medium comprising the computer program disclosed herein. In a particular embodiment, the computer-readable medium is a non-transitory medium or a storage medium.

In addition, the present invention relates to specific anti-MASH or anti-fibrotic agents for use in the treatment of at-risk MASH in a subject in need thereof, wherein the subject has been classified as having at-risk MASH thanks to the method disclosed herein.

In still another aspect, the invention relates to a kit for diagnosing, screening, monitoring or prognosis of at-risk MASH in a subject, said kit comprising means for determining the levels of TSP-2 and miR-34a-5p.

In a particular embodiment, the kit comprises an antibody or an aptamer or a peptide directed against TSP-2 and primers useful for quantifying miR-34a-5p.

FIGURES

Figure 1 represents boxplots of a modelization combining TSP-2 and miR-34a-5p (TMIR) and the single biomarkers (TSP-2 and miR-34a-5p) in matched subpopulations by gender. Y axis represents the quantity of TSP-2 expressed as $\log_{10}$(TSP-2 (ng/mL)) (Fig. 1A), of miR-34a-5p expressed as $\log_{10}$(miR-34a-5p (Fold)) (Fig. 1B) and a score calculated by the modelization TMIR (for Fig. 1C). Boxplots are presented with associated Student t-tests for mean comparisons, *** $p < 0.001$, * $p < 0.05$ and NS if $p \geq 0.05$.

Figure 2 represents boxplots of the modelization TMIR and the single biomarkers (TSP-2 and miR-34a-5p) in matched subpopulations by age. * $p < 0.05$ and NS: $p \geq 0.05$. Y axis represents the quantity of TSP-2 expressed as $\log_{10}$(TSP-2 (ng/mL)) (Fig. 2A), of miR-34a-5p expressed as $\log_{10}$(miR-34a-5p (Fold)) (Fig. 2B) and a score calculated by the modelization TMIR (for Fig. 2C).

Figure 3 represents boxplots of the modelization TMIR and the single biomarkers (TSP-2 and miR-34a-5p) in matched subpopulations by T2D status. NS: $p \geq 0.05$. Y axis represents the quantity of TSP-2 expressed as $\log_{10}$(TSP-2 (ng/mL)) (Fig. 3A), of miR-34a-5p expressed as $\log_{10}$(miR-34a-5p (Fold)) (Fig. 3B) and a score calculated by the modelization TMIR (for Fig. 3C).

Figure 4 represents Roc curves for at-risk MASH endpoint obtained in validation cohort with different NITs.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a non-invasive method that can be used to aid discrimination between at-risk MASH and not at-risk MASH in a subject, from a biological fluid sample of the subject.

Histological scoring/staging systems have been developed to assess Metabolic dysfunction-associated steatotic liver disease (MASLD) activity level and fibrosis stage and to estimate the risk of evolution to clinical liver outcomes. The MASLD-Activity-Score (MAS) has been developed to assess the severity of MASLD. MAS is the sum of three histological scores determined from liver biopsy slices:

- S: Steatosis score: 0: <5%; 1: 5-33%; 2: 34-66% and 3: >66%;
- LI: Lobular Inflammation score (foci per 20x field): 0: none; 1: <2 foci; 2: 2-4 foci and 3: >4 foci; and
- HB: Ballooning degeneration score: 0: none; 1: few; 2: many cells/prominent ballooning.

Using this scoring system, a "patient with MASH" has MAS≥3, with at least 1 point in steatosis, at least 1 point in lobular inflammation and at least 1 point in hepatocyte ballooning. A "non-MASH" patient is a patient having either (i) a MAS≥3 with at least one of steatosis, lobular inflammation and hepatocyte ballooning scores equal to 0; or (ii) a MAS<3. In addition, in the context of the present invention, a patient is excluded as being a MASH patient if said patient has viral hepatitis, autoimmune liver disease, alcohol-related liver disease, drug-induced liver disease or congenital causes of chronic liver disease such as hereditary hemochromatosis, Wilson's disease, alpha-1-antitrypsin deficiency and polycystic ovary syndrome.

Localization and extent of fibrosis (F) at histological exam signs the severity (advancement) of MASH. The NASH-CRN (Nonalcoholic SteatoHepatitis Clinical Research Network) has developed a dedicated fibrosis staging system (Kleiner, D.E *et al*, Hepatology, 2005 Jun; 41(6):1313-21).

MASH Clinical Research Network Scoring System Definitions	**F Score**
Perisinusoidal or periportal fibrosis	1
Mild perisinusoidal fibrosis (zone 3)	1a
Moderate perisinusoidal fibrosis (zone 3)	1b
Portal/periportal fibrosis	1c
Perisinusoidal and portal/periportal fibrosis	2

Bridging fibrosis	3
Cirrhosis	4

Using this fibrosis staging system, patients with no or minimal fibrosis (F=0-1) are generally not considered at risk of cirrhosis, liver failure, HCC (hepatocellular carcinoma) or liver-related death. Patients with significant (F=2) and advanced fibrosis (F=3) are at increasing risk of developing cirrhosis, liver failure, HCC and liver-related death. Patients with compensated cirrhosis have severe fibrosis (F=4) and are at high risk of liver failure (decompensated cirrhosis), HCC and liver-related deaths. Identifying patients who are at risk of developing HCC, cirrhotic complications and liver-related deaths is the ultimate reason for liver assessment. As defined by the FDA and EMA, patients at risk of liver outcomes who should be pharmacologically treated are those with MAS≥4 (with score ≥ 1 for each of steatosis, lobular inflammation and ballooning) and NASH-CRN fibrosis score (F) ≥ 2.

Accordingly, in the context of the present invention, a patient with "at-risk MASH", otherwise referred to as an "at-risk patient" or as a "patient at risk of hepatic outcome", is a patient with a MAS higher or equal to 4, a S score higher or equal to 1, a LI score higher or equal to 1, a HB score higher or equal to 1 and a F score of higher or equal to 2. It defines a subgroup of MASH patients having a high risk of developing at least one life-threatening liver outcome such as cirrhosis, liver failure, HCC and liver-related death.

The terms "subject" and "patient" may be used interchangeably herein and refer to a human subject. As mentioned above, MASH occurs more commonly in patients suffering from metabolic disorders. In addition, MASH is known to be associated to comorbidities such as metabolic disorders. Therefore, the method of the present invention can more particularly benefit to those patients presenting such comorbidities. Common comorbidities of MASH include obesity, insulin resistance, glucose intolerance, T2DM, prediabetes, dyslipidaemia, hypertriglyceridaemia, hypertension (high blood pressure) and cardiovascular disease. Older age may also predispose to HCC in MASH patients. Therefore, in a particular embodiment, the patient suffers from a metabolic disorder, such as obesity, insulin resistance, glucose intolerance, T2DM, prediabetes, dyslipidaemia, hypertriglyceridaemia, and high blood pressure.

In a particular embodiment, as used herein the expression “screening” refers to the selection of a patient to be treated or not to be treated in a cohort or in a clinical study. The screening of patients may imply a diagnostic step and then the distribution of the patient in a group: to be treated and not to be treated.

As used herein, the term "monitoring" refers to a comparison of the score calculated at two or more time points. Monitoring is the ongoing, systematic collection and analysis of data as a project or condition progresses, like a clinical study or a treatment protocol. According to the invention, if the score increases during time, the pathology progresses whereas if the score decreases during time, the pathology regresses. The monitoring is possible due to the noninvasiveness of the method according to the present invention. Indeed, the ease of use of this assay allows repetitive measures and the follow-up of a patient in the time course of a pathology. In conclusion, the monitoring is the application of the method of diagnosis along time for a patient.

As used herein, the expression "biological fluid sample" refers to a blood, serum or plasma sample, preferably a serum sample.

Analysis of a biological fluid sample can be performed using several analytical methods, depending on the type of biomarkers to be quantified. Such analytical methods include quantitative RT-PCR, mass spectrometry, immunoPCR and immunodetection. One can also cite the use of a biochip to implement the simultaneous analysis of multiple biomarkers.

In a particular embodiment, miR34a-5p is has-miR34a-5p.

In a particular embodiment, the level of miR-34a-5p is measured as Cq (amplification cycle) or fold change, preferably as fold. Suitable methods are readily available to those skilled in the art. Specific RNAs can be quantified after extraction of total RNA from the biological fluid sample, using commercial kits for example. Extraction efficiency can be monitored to minimize sample-to-sample variation. Such monitoring can be conducted by addition of an internal process control (IPC) to the sample before total RNA extraction. Such IPC can correspond to a miRNA molecule of known sequence which is heterologous to the sample. For example, one can use a miRNA molecule found in a different species than human, such as a miRNA molecule from *Caenorhabditis elegans*, in particular Cel-miR-40-3p (SEQ ID NO:1: UCACCGGGUGUACAUCAGCUAA). Such IPC can be contained in synthetic vesicles. To quantify the level of miR-34a-5p, positive controls with a known miR-34a-5p Cq value can be used, such as three positive controls with a known miR-34a-5p Cq value. These positive controls can cover the range of miR-34a-5p level in MASH population. In case three positive controls are used, one corresponds to a low miR-34a-5p level, one to a medium level and another to a high level. The medium level can also be referred to as the calibrator, serving in the assay to calculate the fold change value. The positive controls are processed at the same

time as the tested sample of the subject. Reverse transcription is carried out simultaneously on i) extracted total RNA from samples spiked with IPC and ii) on total RNA from positive controls spiked with the IPC as well, for example using a commercial reverse transcription kit. Quantification can then be implemented on cDNA using, for example, a commercial quantitative PCR kit. Cq determination mode can be regression. Moreover, for each patient sample, transcript abundance can be expressed as fold change using both IPC and calibrators Cqs according to equations used to normalize the calibrator against the IPC, to normalize the sample against the IPC, and to quantify miR-34a-5p level as fold change.

In the following paragraph, we describe a specific, but non-limiting method to achieve miR34a-5p quantification. Briefly, total RNA is extracted from a patient serum samples, using Promega magnetic bead-based extraction Maxwell® Plasma and Serum Kit (AS1680, Promega) and RCS48 Instrument (AS8500, Promega) according to the manufacturer's instructions. To monitor extraction efficiency and to minimise sample-to-sample variation, synthetic vesicles containing *Caenorhabditis elegans* Cel-miR-40-3p (Mature miRNA sequence UCACCGGGUGUACAUCAGCUAA-3', Integrated DNA Technologies, purification RNAse free HPLC) are used as IPC and are added to each sample prior to RNA extraction. Three positive controls with a known miR-34a Cq values (low [C1=32 Cq], medium also called calibrator [C2=30.7 Cq], and high [C3=28Cq] has-miR34a-5p levels) covering the range of miR-34a expression in MASH population are processed at the same time of tested samples with the medium standard serving also as calibrator to the assay to calculate the fold change value. Total RNA from serum samples, containing IPC and total RNA from positive controls containing IPC as well are concomitantly reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit (4366597, Applied Biosystems, Thermo Fisher Scientific). Reverse transcription reaction is carried out in a final mixture of 24 µL containing 3µL of TaqMan MicroRNA Assay 5X and incubated in a Thermal Cycler T100 (Biorad). cDNAs are stored in low binding tubes at -20°C until further use. Expression of mature miRNAs is quantified according to the manufacturer's instructions using the TaqMan miRNA RT-qPCR Assay 20X and TaqMan Universal Master Mix II, no Uracil-N-Glycosilase (UNG) (4440040, Applied Biosystems, ThermoFisher Scientific). A fixed volume of 5 µL of total cDNA is used as a template for the qPCR assay using a CFX96 Real-Time PCR detection System. The miR-34a TaqMan assay (sequence of mature hsa-miR-34a-5p = UGGCAGUGUCUUAGCUGGUUGU (SEQ ID NO:2), miR-base number=MIMAT0000255; and sequence of mature Cel-miR-40-3p= UCACCGGGUGUACAUCAGCUAA (SEQ ID NO:1), miR-base number= MIMAT0000011) is used. The Cq determination mode is regression. For each patient sample, miRNA level is expressed as fold change using both IPC and calibrators Cqs according to the following equations:

- Step 1: Normalization of calibrator C2 against Internal Process Control

$\Delta Cq_{\text{C2 miR-34}} = Cq_{\text{C2 miR-34a}} - Cq_{\text{C2 miR-40 in C2}}$

- Step 2: Normalization of sample against Internal Process Control

$\Delta Cq_{\text{Sample miR-34a}} = Cq_{\text{sample miR-34a}} - Cq_{\text{sample miR-40}}$

- Step 3: Sample miR-34a delta expression calculation in Cq

$\Delta\Delta Cq_{\text{sample miR-34}} = \Delta Cq_{\text{Sample}} - \Delta Cq_{\text{C2}}$

- Step 4: Sample delta expression of miR-34a expressed as fold

hsa-miR-34a Fold Change (FC) = $2^{-\Delta\Delta Cq}$

The circulating levels of TSP-2 may be measured by any conventional methodology well known in the art, such as immunoassays (e.g. ELISA (enzyme-linked immunosorbent assay), immunoturbidimetry, immuno-nephelometry, immune cytometry, protein array). For example, the levels of TSP-2 can be determined by antibodies, aptamers or peptides directed against said marker.

In a particular embodiment, the level of TSP-2 is measured as ng/mL. Specific TSP-2 can be quantified by the Quantikine® ELISA for Human Thrombospondin-2 Immunoassay from R&D Systems (Minneapolis, USA). The assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human TSP-2 has been pre-coated onto a microplate. Standards (from 0.313 ng/mL to 20 ng/mL) and serum (diluted 15-fold) are pipetted into the wells and any TSP-2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody (human TSP-2 conjugated to horseradish peroxidase with preservatives) is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of TSP-2 bound in the initial step. The color development is stopped, and the intensity of the color is measured. The standard curve is created by reducing the data using computer software capable of generating a four-parameter logistic (4-PL) curve-fit.

In a particular embodiment, the circulating levels of miR-34a-5p and TSP-2 are measured from one or more blood-derived sample(s) from the subject. In that case, the same kind of sample is used each time a measure has to be done. For the sake of clarity, this means that if a previous measure was done from a serum sample, the subsequent measures are done from serum samples of the same subject. Likewise, if the previous measure was done from a blood or plasma sample, the subsequent measures are done from blood or plasma samples, respectively, of the same subject. In a particular embodiment, the circulating levels of the markers are measured from one or more serum sample(s) from the subject.

According to another embodiment, the selection of at-risk MASH patients among MASLD patients depends on a score calculated with the levels of miR-34a-5p and TSP-2 of the patient. The further comparison of the score with cutoff values can be used to assign the patient in a group of patients who will receive a treatment or in a group of patients who will not receive a treatment.

According to the invention, each said cutoff value may be a specific value or a range of values.

Thus, preferably, each of the biomarker level of the subject can be introduced into a mathematical function to produce an output value that correlates with at-risk MASH status. The method thus can be used to discriminate subjects as having at-risk MASH or not having at-risk MASH. One skilled in the art is aware of numerous suitable methods for developing mathematical function, and all of these are within the scope of the present invention. In a particular embodiment, the mathematical function includes a logistic regression equation.

In a further embodiment, the method of the present invention implements the following formula:

$$S = \frac{1}{1 + \exp(-y)}$$

wherein

$y = \beta_0 + \beta_1 * \log_{10}(\text{TSP-2 (ng/mL)}) + \beta_2 * \log_{10}(\text{miR-34a-5p (Fold)})$.

In a particular embodiment, β_0 is comprised between -11.3 and 2.5, in particular between -9.859 and 1.124, more particularly equal to -5.2897.

In a particular embodiment, β_1 is comprised between 1.5 and 6.5, in particular between 1.824 and 6.1574, more particularly equal to 3.8547.

In a particular embodiment, β_2 is comprised between 0.7 and 3.4, in particular between 0.985, 2.9841, more particularly equal to 1.8733.

By way of example, the following equation can be used for the diagnosis of at-risk MASH.

y= -5.2897+ 3.8547 * log10(TSP2 (ng/mL)) + 1.8733 * log10(miR-34a-5p (Fold)).

The score calculated from the mathematical function can then be compared to a predetermined cutoff value. According to an embodiment, the cutoff is comprised between 0.13 and 0.92. In a particular embodiment, the cutoff is comprised between 0.2806 and 0.7337. In a more particular embodiment, the cutoff is equal to 0.5526.

According to another embodiment, the score calculated from the mathematical function can be compared to predetermined cutoff values, such as low and high cutoff values. In this context,

a calculated S value lower that the low cutoff is indicative of a subject not having at-risk MASH, and a calculated S value greater or equal to the high cutoff value is indicative of a subject having at-risk MASH.

In a particular embodiment, the low cutoff is comprised between 0.28 and 0.48, in particular between 0.37 and 0.39. In a further particular embodiment, the low cutoff is equal to 0.3806. In another particular embodiment, the high cutoff is comprised between 0.59 and 0.89, in particular between 0.62 and 0.65. In a further particular embodiment, the high cutoff is equal to 0.6337.

The present invention also relates to a computer program comprising instructions that, when executed by a processor/processing means, cause the processor/processing means to:
- receive quantified levels of miR-34a-5p and TSP-2;
- calculate a score from these quantified levels of the subject, from a mathematical function as described above; and
- assign the subject into the group of at-risk subjects or not at-risk subjects based upon the calculated score compared to predetermined cutoff values.

The present invention further provides a computer readable medium comprising the computer program described therein. According to a particular embodiment, the computer-readable medium is non-transitory medium or a storage medium.

In some embodiments, thanks to the method of the invention, a decision may be taken to give life-style recommendations to a subject (such as a food regimen or providing physical activity recommendations), to medically take care of a subject (e.g. by setting regular visits to a physician or regular examinations, for example for regularly monitoring markers of liver damage), or to administer at least one MASH or liver fibrosis therapy to the patient, to treat or prevent at-risk MASH. In a particular embodiment, a decision may be taken to give life-style recommendations to a subject or to administer at least one MASH or liver fibrosis therapy. The invention thus further relates to an anti-MASH or anti-fibrotic compound for use in a method for treating MASH in a subject in need thereof, wherein the subject has been identified thanks to a method according to the invention.

The term "treatment", as used herein, relates to both therapeutic measures and prophylactic or preventive measures, wherein the goal is to prevent or slow down (lessen) an undesired physiological change or disorder. Beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, stabilizing pathological state (specifically not worsening),

slowing down or stopping the progression of the disease, improving or mitigating the pathological condition. Particularly, for the purpose of the present invention, treatment is directed to slow the progression of MASH and/or fibrosis and reduce the risk of further complications. It can also involve prolonging survival in comparison with the expected survival if the treatment is not received.

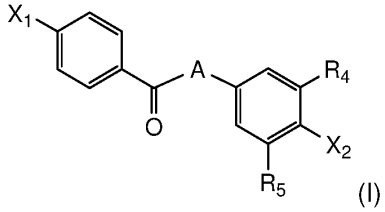
In the context of the present invention, a patient to be treated is a patient with at-risk MASH and a patient not to be treated is a patient not at-risk MASH.

The anti-MASH or anti-fibrotic agent is administered in a therapeutically effective amount. As used herein, the expression "therapeutically effective amount" refers to an amount of the drug effective to achieve a desired therapeutic result. A therapeutically effective amount of a drug may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of drug to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of agent are outweighed by the therapeutically beneficial effects. The effective dosages and dosage regimens for drug depend on the disease or condition to be treated and may be determined by the persons skilled in the art. A physician having ordinary skill in the art may readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician could start doses of drug employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. In general, a suitable dose of a composition of the present invention will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect according to a particular dosage regimen. Such an effective dose will generally depend upon the factors described above.

In a particular embodiment, the invention relates to an anti-MASH compound for use in a method for treating MASH in a subject suffering from at-risk MASH, wherein the subject has been classified as having at-risk MASH thanks to the method according to the invention.

Illustrative anti-MASH and anti-fibrotic compounds are listed below:

- a compound of formula (I) or a pharmaceutically acceptable salt thereof:



(I)

wherein:

X1 represents a halogen atom, a R1 group or G1-R1 group;

A represents a CH=CH or CH2-CH2 group;

X2 represents a G2-R2 group;

G1 represents an atom of oxygen;

G2 represents an atom of oxygen or sulfur;

R1 represents a hydrogen atom, an unsubstituted alkyl group, an aryl group or an alkyl group that is substituted by one or more substituents selected from halogen atoms, alkoxy groups, alkylthio groups, cycloalkyl groups, cycloalkylthio groups and heterocyclic groups;

R2 represents an alkyl group substituted by a –COOR3 group, wherein R3 represents a hydrogen atom or an alkyl group that is substituted or not by one or more substituents selected from halogen atoms, cycloalkyl groups and heterocyclic groups; and

R4 and R5, identical or different, represent an alkyl group that is substituted or not by one or more substituent selected from halogen atoms, cycloalkyl groups and heterocyclic groups;

- AMP activated protein kinase stimulators such as PXL-770, MB-11055, Debio-0930B, metformin, CNX-012, O-304, mangiferin calcium salt, eltrombopag, carotuximab, and imeglimin;

- Bile acids such as obeticholic acid (OCA), ursodeoxycholic acid (UDCA), norursodeoxycholic acid, and ursodiol;

- CCR antagonists such as cenicriviroc (CCR2/5 antagonist), PG-092, RAP-310, INCB-10820, RAP-103, PF-04634817, and CCX-872;

- Dipeptidyl peptidase IV (DPP4) inhibitors such as evogliptin, vidagliptin, fotagliptin, alogliptin, saxagliptin, tilogliptin, anagliptin, sitagliptin, retagliptin, melogliptin, gosogliptin, trelagliptin, teneligliptin, dutogliptin, linagliptin, gemigliptin, yogliptin, betagliptin, imigliptin, omarigliptin, vidagliptin, and denagliptin;

- Farnesoid X receptor (FXR) agonists such as obeticholic acid (OCA), tropifexor (LJN452), cilofexor (GS9674), Nidufexor (LMB763), EDP-305, AKN-083, INT-767, GNF-5120, LY2562175, INV-33, NTX-023-1, EP-024297, Px-103, SR-45023, TERN-101 (6-{4-[5-Cyclopropyl-3-(2,6-dichloro-phenyl)-isoxazol-4-ylmethoxy]-piperidin-1-yl}-1-methyl-1H-indole-3 carboxylic acid), TERN-201, TERN-501 and TERN-301;

- Fibroblast Growth Factor 19 (FGF-19) receptor ligand or functional engineered variant of FGF-19;

- Fibroblast Growth Factor 21 (FGF-21) agonists such as PEG-FGF21 (pegbelfermin, formely BMS-986036), YH-25348, BMS-986171, YH-25723, LY-3025876, and NNC-0194-0499;

- engineered Fibroblast Growth Factor 19 (FGF-19) analogues such as NGM-282 (aldafermin);

- Glucagon-like peptide-1 (GLP-1) analogs such as semaglutide, liraglutide, exenatide, albiglutide, dulaglutide, lixisenatide, loxenatide, efpeglenatide, taspoglutide, MKC-253, DLP-205, and ORMD-0901;
- Nicotinic acid such as Niacin and Vitamin B3;
- nitazoxanide (NTZ), its active metabolite tizoxanide (TZ) or other prodrugs of TZ such as RM-5061;
- PPAR alpha agonists such as fenofibrate, ciprofibrate, pemafibrate, gemfibrozil, clofibrate, binifibrate, clinofibrate, clofibric acid, nicofibrate, pirifibrate, plafibride, ronifibrate, theofibrate, tocofibrate, and SR10171;
- PPAR gamma agonists such as pioglitazone, deuterated pioglitazone, rosiglitazone, efatutazone, ATx08-001, OMS-405, CHS-131, THR-0921, SER-150-DN, KDT-501, GED-0507-34-Levo, CLC-3001, and ALL-4;
- PPAR delta agonists such as GW501516 (Endurabol or ({4-[({4-methyl-2-[4-(trifluoromethyl)phenyl]-1,3-thiazol-5-yl}methyl)sulfanyl]-2-methylphenoxy}acetic acid)), MBX8025 (Seladelpar or {2-methyl-4-[5-methyl-2-(4-trifluoromethyl- phenyl)-2H-[l,2,3]triazol-4-ylmethylsylfanyl]-phenoxy}-acetic acid), GW0742 ([4-[[[2-[3-fluoro-4-(trifluoromethyl)phenyl]-4-methyl-5-thiazolyl]methyl]thio]-2-methyl phenoxy]acetic acid), L165041, HPP-593, and NCP-1046;
- PPAR alpha/gamma dual agonists (also named glitazars) such as saroglitazar, aleglitazar, muraglitazar, tesaglitazar, and DSP-8658;
- PPAR gamma/delta dual agonists such as conjugated linoleic acid (CLA), and T3D-959;
- PPAR alpha/gamma/delta pan agonists or PPARpan agonists such as IVA337, TTA (tetradecylthioacetic acid), bavachinin, GW4148, GW9135, bezafibrate, lanifibranor, lobeglitazone, and CS038;
- Sodium-glucose transport (SGLT) 2 inhibitors such as licoglifozin, remogliflozin, dapagliflozin, empagliflozin, ertugliflozin, sotagliflozin, ipragliflozin, tianagliflozin, canagliflozin, tofogliflozin, janagliflozin, bexagliflozin, luseogliflozin, sergliflozin, HEC-44616, AST-1935, and PLD-101.
- stearoyl CoA desaturase-1 inhibitors/fatty acid bile acid conjugates such as aramchol, GRC-9332, steamchol, TSN-2998, GSK-1940029, and XEN-801;
- thyroid receptor β (THR β) agonists such as VK-2809, resmetirom (MGL-3196), MGL-3745, SKL-14763, sobetirome, BCT-304, ZYT-1, MB-07811 and eprotirome;
- Vitamin E and isoforms; vitamin E combined with vitamin C and atorvastatin.

In a particular embodiment, the anti-MASH agent is selected from pegbelfermin, cenicriviroc, dapagliflozin, dulaglutide, empagliflozin, fenofibrate, lanifibranor, liraglutide, obeticholic acid, pioglitazone, resmetirom, saroglitazar magnesium, seladelpar, semaglutide, sitagliptin, TERN-101, TERN-201 and tropifexor.

The invention also relates to a kit for diagnosing, screening, monitoring or prognosing of at-risk MASH in a subject. Said kit comprises means for determining the levels of TSP-2 and miR-34a-5p. In a particular embodiment, said kit comprises specific antibodies, aptamers or peptide to measure TSP-2 and primers useful for quantifying miR-34a-5p.

Antibodies directed against TSP-2 may be any monoclonal, polyclonal and/or conjugated antibodies directed against TSP-2 known in the art. For instance, said antibody directed against TSP-2 is Monoclonal antibody Thrombospondin 2 (4) (Ref: sc-136238, Santa Cruz Biotechnology, Texas, USA).

Primers useful for quantifying miR-34a-5p may be designed according to any conventional methods. For instance, these primers may be TaqMan™ probes.

The kit of the invention may further comprise immunoassay standards and reagents. In a particular embodiment, the kit comprises at least one specific positive control for TSP-2 and/or at least one specific positive control for miR-34a-5p.

Particularly, said positive control for TSP-2 comprises from 11 to 300 ng/mL of TSP-2. The positive control for miR-34a-5p comprises from 32Cq to 28 Cq of miR-34a-5p.

More particularly, the kit of the invention further comprises 3 positive controls for TSP-2 and 3 positive controls for miR-34a-5p, wherein said positive controls for TSP-2 respectively comprise 30, 50 and 100 ng/mL of TSP-2; wherein said positive controls for miR-34a-5p comprises low [C1=32 Cq], medium also called calibrator [C2=30.7 Cq], and high [C3=28Cq] miR34a-5p levels, respectively.

The invention is further described with reference to the following, non-limiting, examples.

EXAMPLES

1. **Datasets**

The study population consists of patients that were screened for potential inclusion in the Resolve-It phase 3 clinical trial. All patients with biopsies results available, and blood samples used for biomarkers measurements with less than 90 days between biopsies and blood collections dates were selected as of potential utility for this study.

We then selected all patients with full data on a selected set of biomarkers, as well as on the usual demographical and clinical parameters (age, sex, Type-2 Diab, Dyslipidemia, Arterial Hypertension [HT], BMI).

This selection led to a total of 2036 patients. Among those patients, we randomly selected 500 (~25%) patients, keeping the rest of the population for being potentially included in the validation dataset. To control for potential confounding factors when processing the modelization and the selection of relevant biomarkers, we applied a propensity score matching algorithm to homogenize the following parameters among patients with and without at-risk MASH : Age, sex, Type-2 Diab, Dyslipidemia, Arterial Hypertension, BMI.

This process led to a selection of 360 patients, 180 with and 180 without at-risk MASH, well-balanced for the parameters indicated in Table 1. In this training cohort, the mean age of patients was 54 years of age, 64% of them were male, 35% had T2D and 75% were obese. Regarding histology, 49% of patients without at-risk MASH had fibrosis stage F1, while at-risk MASH patients were equally distributed between F2 and F3 (44% in each).

Table 1:

	All Data		Training cohort					Validation cohort			
			Non at-risk MASH		At-risk MASH			Non at-risk MASH		At-risk MASH	
	n	value	n	value	n	value	p value	n	value	n	value	p value
Gender, Male	2206	61.24% (1351)	180	63.89% (115)	180	63.33% (114)	1	953	64.95% (619)	893	56.33% (503)	0,0002
Age (years)	2206	53.91±11.73	180	53.49±11.36	180	53.54±11.43	0.9668	953	53.27±11.83	893	54.75±11.72	0.0072
Age by class,<45/46-55/56-64/>65	2206	23.6%/ 27.2%/ 29.3%/ 19.9%	180	21.7%/ 28.9%/ 32.2%/ 17.2%	180	21.1%/ 28.9%/ 32.2%/ 17.8%	0.9964	953	26%/ 27.9%/ 26.5%/ 19.5%	893	21.8%/ 25.8%/ 31%/ 21.4%	0,0007
Type 2 Diabetes	2206	42.16% (930)	180	35% (63)	180	35% (63)	1	953	33.58% (320)	893	54.2% (484)	<0.0001
Dylsipidemia	2206	48.19% (1063)	180	38.89% (70)	180	39.44% (71)	1	953	46.48% (443)	893	53.64% (479)	0.0025
Arterial hypertension	2206	56.39% (1244)	180	55.56% (100)	180	55% (99)	1	953	50.47% (481)	893	63.16% (564)	<0.0001
Metabolic Syndrom	1944	83.95% (1632)	143	86.71% (124)	177	79.1% (140)	0.1021	760	81.45% (619)	864	86.69% (749)	0.0047
BMI (kg/m^2)	2203	33.54±6.07	180	33.67±5.4	180	33.72±5.48	0.9333	950	33.06±6.18	893	33.98±6.16	0.0015
Obese	2203	70.4% (1551)	180	76.11% (137)	180	73.33% (132)	0.6276	950	66.53% (632)	893	72.79% (650)	0.0041
Ast (IU/L)	2205	41.81±30.06	180	33.36±21.72	180	51.23±34.15	<0.0001	953	32.41±23.27	892	51.64±33.23	<0.0001
Alt (IU/L)	2205	57.37±43.51	180	45.78±29.89	180	70.04±41.79	<0.0001	953	45.38±35.16	892	69.97±49.6	<0.0001
Ast/Alt ratio	2205	0.81±0.32	180	0.81±0.31	180	0.78±0.29	0.4474	953	0.82±0.33	892	0.82±0.31	0.928
GGT (IU/L)	2205	74.47±94.18	180	64.71±79.18	180	88.54±105.31	0.0158	953	61.64±80.68	892	87.31±105.34	<0.0001
ALP (IU/L)	2204	84.88±31.12	180	86.16±32.72	180	85.75±28.5	0.9003	953	83.16±30.53	891	86.3±31.86	0.0308
FPG (mmol/L)	2197	6.02±1.82	180	5.89±1.66	180	6.26±2.2	0.0735	949	5.61±1.5	888	6.44±1.97	<0.0001
HbA1c (%)	2206	6.18±1.01	180	6.07±1	180	6.15±0.97	0.4066	953	5.99±0.94	893	6.41±1.04	<0.0001
Triglycerides (mmol/L)	2205	1.96±1.19	180	1.73±0.9	180	1.95±1.14	0.0466	953	1.88±1.08	892	2.1±1.35	0,0001
Platelet (10e9/L)	2197	237.53±67.92	180	238.92±69.44	180	232.76±62.65	0.3774	949	244.07±69	888	231.22±66.92	0,0001
MASLD	2206	92.38% (2038)	180	85% (153)	180	100% (180)	<0.0001	953	85.2% (812)	893	100% (893)	<0.0001
MASH	2206	69.31% (1529)	180	36.11% (65)	180	100% (180)	<0.0001	953	41.03% (391)	893	100% (893)	<0.0001
At-risk MASH	2206	48.64% (1073)	180	0% (0)	180	100% (180)	<0.0001	953	0% (0)	893	100% (893)	<0.0001
Fibrosis	2206	1.9±1.15	180	1.24±1.15	180	2.68±0.68	<0.0001	953	1.18±1.06	893	2.64±0.62	<0.0001
Fibrosis by stage, 0/1/2/3/4	2206	13.2%/ 25.6%/ 25.3%/ 29.7%/ 6.1%	180	26.1%/ 48.9%/ 5.6%/ 13.9%/ 5.6%	180	0%/ 0%/ 43.9%/ 43.9%/ 12.2%	NA	953	25.6%/ 50.1%/ 8.2%/ 12.8%/ 3.4%	893	0%/ 0%/ 43.9%/ 48.2%/ 8%	NA
MAS	2206	4.24±1.98	180	2.83±1.72	180	5.45±1.01	<0.0001	953	2.9±1.68	893	5.7±1.08	<0.0001
MAS by category, 0-1, 2-3, 4-5, 6-8	2206	12.2%/ 20.3%/ 37.5%/ 30%	180	26.7%/ 40%/ 25.6%/ 7.8%	180	0%/ 0%/ 52.2%/ 47.8%	NA	953	23.2%/ 39.3%/ 31.2%/ 6.3%	893	0%/ 0%/ 43.8%/ 56.2%	NA

The training process led to the selection of a biomarker panel comprising two biomarkers: TSP-2 and miR-34a-5p. Based on this result, we selected all patients with available measures of these two biomarkers, fulfilling the condition regarding biopsies results availability and ≤ 90 days between blood and biopsy dates, and who were not part of the training dataset. This led to an independent validation dataset comprising 1846 patients. Principal characteristics are reported in Table 1 above.

In this validation dataset, and as usually observed in MASH databases, population with at-risk MASH had significantly higher percentages of T2D, dyslipidemia, HT and obesity. Concerning histology, 85% of patients without at-risk MASH had MASLD, and 41% were affected with MASH.

2. Statistical analyses

Descriptive statistics were generated for baseline characteristics and reported as means±sd or %(n), and patients without and with at-risk MASH were compared using appropriate 2-sample tests (Student t test for comparison of means and $\chi 2$ test for comparison of percentages).

The modelization process was performed using a Stepwise algorithm, comparing sequentially binomial logistic regression models for the detection of at-risk MASH based on their associated BIC (Bayesian Information Criterion), starting with an empty model and sequentially inserting the variables that minimize this criterion. The process stopped when none of the remaining variables led to a decrease of the BIC value. For this, the stat R package (*step* function) was used. The regression coefficients of the selected model were extracted and associated 95% CI were reported.

Specific cutoffs values were derived using the training dataset. A low cutoff to achieve 85% sensitivity (Sen) and a high cutoff to achieve 90% specificity (Spe) were extracted, and diagnostic performances (Sen, Spe, PPV, NPV, Acc) associated to these cutoffs were computed in both the training and validation datasets. Also, a Youden cutoff that maximize Sen + Spe is reported.

The overall diagnostic performance of non-invasive tests and biomarkers are estimated using AUROC values, and these statistics are tested for significant differences using Delong tests. AUROC values are reported with 95%CI estimated using 1000 bootstrap samples. Associated ROC curves are graphically reported.

Boxplots are presented with associated Student t-tests for mean comparisons, and p values are graphically reported as follow : **** if $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ and NS if $p \geq 0.05$.

All propensity score matching algorithms were executed using the matchIt function from the MatchIt R package, and all statistical analyses were performed using R programming language version 4.3.0.

When executing the stepwise algorithm, the combination of TSP-2 and miR-34a-5p was found as the best combination, as it returned the lowest BIC before any further biomarker added led to an increase in BIC. Based on this selection of biomarkers, the following equation was obtained using a binomial logistic regression model:

$$S = \frac{1}{1 + \exp(-y)}$$

wherein

$y = \beta_0 + \beta_1 * \log_{10}(\text{TSP2 (ng/mL)}) + \beta_2 * \log_{10}(\text{miR-34a-5p (Fold)})$.

Coefficients of this model are reported in Table 2 with associated 95%CI, all of them reaching high significance with associated p values <0.0001.

Table 2:

	β_0	β_1	β_2
Coefficient	-5.2897	3.8547	1.8733
CI (95%)	(-8.3366, -2.6685)	(2.0904, 5.8884)	(1.0201, 2.8044)

Based on this modelization and the training dataset, cutoff values to achieve a 85% sensitivity (Low cutoff) and 90% specificity (high cutoff) are fixed at 0.3806 et 0.6337, respectively. The Youden cutoff, which maximizes the sum of sensitivity and specificity of the present diagnostic method, is fixed at 0.5526.

3. Results

3.1 Performance of the combination TSP-2 and miR-34a-5p vs single biomarkers

The performance of the modelization described above (named TMIR) is then tested in the independent validation dataset, in comparison with that of TSP-2 alone or miR-34a-5p alone. To measure this, we computed the AUROC values for TSP2 alone and miR34-a alone and compare those AUROCs with the one achieved by modelization TMIR. Results obtained in the validation dataset, comprising 1846 patients, are reported in Table 3.

Table 3: AUROC values for at-risk MASH detection

	N	n at-risk	AUC (95% CI)	*p* value
TSP-2+miR34a	1846	893	0.84 (0.82, 0.86)	-
TSP-2	1846	893	0.8 (0.79, 0.82)	<0.0001
miR34a	1846	893	0.79 (0.77, 0.81)	<0.0001

The modelization TMIR achieved a significantly higher AUROC than both biomarkers when used alone, with p values<0.0001, confirming the better performance the combination of TSP-2 and miR-34a-5p in one model.

3.2 Robustness of the combination TSP-2 and miR-34a-5p

We further analyzed the potential robustness of the combination of TSP-2 and miR-34a-5p against three different parameters of interest in the diagnostic and MASH fields. These parameters were the age, sex and T2D status of patients. Indeed, it's important to guarantee that any diagnostic test could be interpreted irrespective of these patients' parameters.

To do this, we used the validation dataset and applied a propensity score matching algorithm to extract two subpopulations in each case, while balancing for the following list of potential confounding factors: age, sex, prevalence of T2D, dyslipidemia and HT, BMI, prevalence of at-risk MASH, fibrosis, steatosis, ballooning and lobular inflammation by class.

This process led to the creation of 2 subpopulations for each parameter of interest (Age, sex, T2D), each time these subpopulations being well-balanced for the other parameters listed above. For the 2 subpopulations of age, we create one subpopulation comprising patients ages ≤50 and the second with patients ages ≥60 to ensure a clear separation of age between both subpopulations. For gender, age and T2D, we extracted a total of 1242 (621 in each category), 676 (338 in each category) and 1170 (585 in each category) patients respectively.

The distributions of the modelization TMIR and its associated biomarkers in each associated categories (gender, age and T2D status) are reported as boxplots in Fig. 1, 2 and 3.

As we can observed in Fig. 1, TSP-2 expression was reduced in male vs female, while the expression of miR-34a was higher in Male vs Female, as previously observed in past studies. The combination of both biomarkers in a modelization, led this test to be associated with distributions that were not significantly impacted by the gender of patients.

Regarding age, while TSP-2 was not impacted by this parameter, miR-34a-5p was significantly reduced in older patients. However, the magnitude of this decrease did not have a sufficient

impact once combined with TSP-2 to lead to a significant age impact on the modelization TMIR as reported in Fig. 2.

Finaly, when focusing and comparing patients with and without T2D, we observed that none of the biomarkers were significantly impacted by this parameter, both returning similar distributions. This led the modelization TMIR to be associated with similar distribution across these two subpopulations as reported in Fig. 3.

These results show that the diagnostic method of the invention is not significantly impacted by the gender, the age and T2D status of a patient.

Overall, the combination of TSP-2 with miR-34a-5p shows high robustness and could be used with fixed cutoff irrespective of the patients' parameters, e.g. the gender, the age and T2D status.

3.3 Performance of the combination TSP-2 and miR-34a-5p vs other NITs

Finally, the overall performances of the combination of TSP-2 and miR-34a-5p in the modelization TMIR for the detection of at-risk MASH were compared with other usual non-invasive tests: FIB-4, ELF, NFS and ALT.

To do so, among all patients included in the validation cohort, we selected those with full data on these NITs, leading to a dataset of 1671 patients. We derived the AUROC values and associated Delong tests for AUROC difference between the combination and other NITs. Results are summarized in Table 4. The associated ROC curves are also graphically reported in Fig. 4.

Table 4: AUROC values for at-risk MASH detection

	N	n at-risk	AUC (95% CI)	*p* value
TSP-2+miR-34a-5p	1671	770	0.85 (0.83, 0.87)	-
FIB4	1671	770	0.66 (0.64, 0.69)	<0.0001
ELF	1671	770	0.71 (0.69, 0.73)	<0.0001
NFS	1671	770	0.62 (0.59, 0.65)	<0.0001
ALT	1671	770	0.7 (0.68, 0.73)	<0.0001

In this population, the modelization TMIR achieved an AUROC of 0.85, significantly higher than those achieved by the other NITs, all p values <0.0001. This result confirms the high performance of this combination of biomarkers and notably the gain of using this new diagnostic test over the usual ones.

Therefore, we have identified an efficient and robust NIT. This new diagnostic method needs a limited number of biomarkers (TSP-2 and miR-34a-5p), the quantification of both of them being performed in serum.

Most interestingly, this new model provides a high diagnostic performance. Moreover, we have demonstrated that this new NIT is not impacted by gender, age and T2DM status. This provides a new valuable tool for diagnosing at-risk MASH subjects.

CLAIMS

1. A method for the diagnosis, screening, monitoring or prognosis of at-risk metabolic dysfunction-associated steatohepatitis (MASH) in a subject, said method comprising the steps of:
- quantifying the levels of miR-34a-5p and TSP-2 in a biological fluid sample of said subject;
- combining the quantified levels in a mathematical function to assign a score; and
- comparing said score with a cutoff value to determine whether said subject is an at-risk MASH subject.

2. The method according to claim 1, wherein the comparison of said score is made with cutoff values.

3. The method according to claim 1 or 2, wherein the mathematical function includes a logistic regression function.

4. The method according to any one of claims 1 to 3, wherein the biological fluid sample is a blood, serum or plasma sample.

5. The method according to any one of claims 1 to 4, wherein the biological fluid sample is a serum sample.

6. The method according to any one of claims 1 to 5, wherein the subject suffers from obesity, insulin resistance, glucose intolerance, T2DM, prediabetes, dyslipidaemia, hypertriglyceridaemia, or high blood pressure.

7. A computer program comprising instructions that, when executed by a processor/processing means, cause the processor/processing means to:
- receive quantified levels of miR-34a-5p and TSP-2;
- calculate a score from these quantified levels of the subject, from a mathematical function; and
- assign the subject into the group of at-risk subjects or not at-risk subjects based upon the calculated score compared to predetermined cutoff values.

8. A computer readable medium comprising the computer program according to claim 7.

9. The computer readable medium according to claim 8, which is a non-transitory medium or a storage medium.

10. An anti-MASH or anti-fibrotic agent for use in the treatment of at-risk MASH in a subject in need thereof,
wherein the anti-MASH agent is selected from pegbelfermin, cenicriviroc, dapagliflozin, dulaglutide, empagliflozin, fenofibrate, lanifibranor, liraglutide, obeticholic acid, pioglitazone, resmetirom, saroglitazar magnesium, seladelpar, semaglutide, sitagliptin, TERN-101, TERN-201 and tropifexor, and
wherein the subject has been classified as having at-risk MASH thanks to the method according to any one of claims 1 to 6.

11. A kit for diagnosing, screening, monitoring or prognosis of at-risk MASH in a subject, said kit comprising means for determining the levels of TSP-2 and miR-34a-5p.

12. The kit according to claim 11, comprising an antibody or an aptamer or a peptide directed against TSP2 and primers useful for quantifying miR-34a-5p.