METHOD FOR MONITORING PATIENTS WITH METABOLIC DYSFUNCTION-ASSOCIATED STEATOHEPATITIS

The present invention relates to a method for monitoring patients with metabolic dysfunction-associated steatohepatitis (MASH).

BACKGROUND

Initiated by presence of steatosis in the liver, metabolic dysfunction-associated steatotic liver disease (MASLD), formerly known as non-alcoholic fatty liver disease (NAFLD), is the liver disease with most cases in the world (~25%) (Cotter TG and Rinella M. *Gastroenterology*. 2020 May;158(7):1851-1864). It can progress to metabolic dysfunction-associated steatohepatitis (MASH), formerly named nonalcoholic steatohepatitis or NASH. MASH is the worst form of MASLD. MASH is a silent disease which can progress to cirrhosis and liver-related events. At-risk MASH corresponds to patients with a fibrosis stage of 2 or more, and a MASLD-Activity-Score (MAS) of 4 points or more, with at least 1 point in each MAS component (steatosis, lobular inflammation, ballooning). This population of at-risk MASH patients has an increased risk of disease progression, associated with liver-related events and mortality.

In the context of MASH, MAS usually progresses before fibrosis progresses, and it may be important to track this evolution early enough to improve handling of patients in standard of care. In addition, numerous ongoing MASH clinical trials raise the necessity to easily and efficiently track the evolution of MASH patients to evaluate the efficacy of treatments in these clinical trials.

Both U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) defined the endpoints of MASH clinical trials as the resolution of MASH and/or the improvement of liver fibrosis. It is therefore necessary to develop a tool which is capable of monitoring these endpoints during a MASH clinical trial.

The current method for diagnosing MASH or liver fibrosis is liver biopsy, which is an invasive and expensive procedure, and associated with potential complications. As an invasive procedure, it is not possible to often perform a liver biopsy on a patient and a delay between two biopsies must be respected for the safety of patients. Liver biopsy cannot be carried out at a large-scale population level and presents logistical complexity during clinical trials: a histopathologist

expert must read the liver biopsy in order to assess histology components. Furthermore, sampling variability of liver biopsy may be present (Ratziu V, et al., *Gastroenterology*. 2005 Jun;128(7):1898-906.). A biopsy may not reflect the changes in the whole liver since it is a needle biopsy and the liver tissue obtained from the subcapsular region is not representative of the whole liver. Therefore, liver biopsy cannot reasonably be used for monitoring the evolution of MASH in a patient.

Currently, no non-invasive tests (NIT) have been approved to be able to monitor MASH and/or liver fibrosis evolution in MASH clinical trials or in standard MASH patients care.

Therefore, there is still an unmet medical need to develop a NIT method capable of easily and accurately monitoring the evolution of MASH and/or liver fibrosis in a subject with MASH.

SUMMARY OF THE INVENTION

The present invention is based on the detailed analysis of dataset of clinical trial. By analyzing the score which is obtained from mathematical functions combining the circulating levels of hsa-miR-34a-5p and YKL-40 and the gender of MASH patients during patient follow-up, the inventor have shown that the evolution of said score is correlated with the evolution of MASH and/or of liver fibrosis of said patient.

More surprisingly, the inventors have shown that the slope value of these scores calculated at different timepoints during the follow-up of a MASH patient can be used as an indication of the evolution of MASH and/or liver fibrosis in said subject. More particularly, the slope value of these scores is indicative of whether said patient has MASH resolution and/or liver fibrosis improvement. It is thus herein provided a non-invasive test for monitoring the evolution of MASH and/or liver fibrosis in a subject with MASH based on said slope value. Said method can also be applied to evaluate the efficacy of a medical treatment of MASH and/or liver fibrosis in a subject with MASH.

The ease of use of this non invasive assay allows repetitive measures and regular follow-up of a patient. during a medical treatment.

The first aspect of the present invention relates to a method for monitoring the evolution of MASH and/or liver fibrosis or for evaluating the efficacy of a medical treatment of MASH and/or liver fibrosis in a subject with MASH.

In an embodiment, the method of the present invention comprises:

-providing (i) the circulating levels of hsa-miR-34a-5p and YKL-40 in biological fluid samples, which are obtained at two or more timepoints from the subject, and (ii) the gender of the subject,

- combining the provided levels and the gender in a mathematical function to assign a score at each timepoint,

-combining the scores of each timepoint to calculate a slope value of the scores of said subject, and

-comparing said slope value with a reference slope value.

In a particular embodiment, the score is a value S2 which is a logit value of S1 obtained from a logistic function:

$$S1 = \frac{1}{1 + \exp(-y)}$$

wherein

y = β0 + β1∗log10(miR-34a-5p (Fold)) + β2∗log10(YKL-40 (ng/ml)) + β3 ∗ Gender + β4 ∗log10(miR-34a-5p (Fold)) ∗ Gender

wherein

Gender is 0 if the subject is a female, or 1 if the subject is a male;

β0 is comprised between -3 and 3, in particular between -2 and 2;

β1 is comprised between 1 and 5, in particular between 2 and 4;

β2 is comprised between 0 and 4.5, in particular between 0.5 and 3;

β3 is comprised between -2 and 2, in particular between -1 and 1; and

β4 is comprised between -1 and 2, in particular between 0 and 2.

In a more particular embodiment, the reference slope value of S2 slope values is comprised between -0.00230 and 0.00000. In a more particular embodiment, the reference slope value of S2 slope values is equal to -0.00088.

In a still more particular embodiment, the slope value of a subject lower than or equal to the reference slope value is indicative of the subject having MASH resolution and/or liver fibrosis improvement; and a slope value of the subject higher than the reference slope value is indicative of the subject having neither MASH resolution nor liver fibrosis improvement.

In an embodiment, the biological fluid sample is a blood, serum or plasma sample. In a preferred embodiment, the biological fluid sample is a serum sample.

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:

a) receive levels of hsa-miR-34a-5p and YKL-40 measured at two or more timepoints of a subject with MASH and the gender of the subject,

b) combine the levels and the gender in a mathematical function to assign a score at each timepoint,

c) calculate a slope value of the subject from the scores of each timepoint,

d) compare the slope value of the subject with a reference slope value,

e) determine whether the subject has MASH resolution and/or liver fibrosis improvement, wherein if the slope value of the subject is lower than or equal to the reference slope value, which is indicative of the subject having MASH resolution and/or liver fibrosis improvement; if the slope of the subject is higher than the reference slope, which is indicative of the subject having neither MASH resolution nor liver fibrosis improvement.

In yet another aspect, the present invention relates to a computer readable recording medium storing instructions for executing the method of the present invention.

FIGURES

Figure 1: Receiver Operating Characteristics (ROC) curve analysis of slopes of S2 values.

Figure 2A:

Bottom line: evolution profile of S2 values collected at 8 timepoints in a group of MASH patients with MASH resolution and/or fibrosis improvement.

Top line: evolution profile in a group of MASH patients with neither MASH resolution nor fibrosis improvement.

Figure 2B: slope values of the S2 values of figure 2A. Column “Yes”: patients with MASH resolution and/or fibrosis improvement (Yes); column “No”: patients with neither MASH resolution nor fibrosis improvement (No). The horizontal dotted line corresponds to a value of a descriptive slope = 0, which corresponds to a stable slope.

Figure 3A: individual evolution profiles of S2 values collected at 8 timepoints in 4 patients with MASH (A, B, C, D).

Figure 3B: individual slope values of 4 patients with MASH (A, B, C, D) calculated from S2 values obtained at 8 timepoints of figure 3A. The horizontal dotted line corresponds to the reference slope value (Youden cutoff).

DETAILED DESCRIPTION OF THE INVENTION

Metabolic dysfunction-associated steatotic liver disease (MASLD) is defined as a condition characterized by liver fat accumulation in the presence of at least one of the following three metabolic conditions: overweight/obesity, type 2 diabetes mellitus (T2DM), or at least two of seven metabolic risk abnormalities in those subjects who do not have T2DM and are lean by ethnic-specific body mass index (BMI) criteria: (1) waist circumference $\geq$ 102/88 cm in Caucasian men and women (or $\geq$ 90/80 cm in Asian men and women); (2) blood pressure $\geq$ 130/85 mmHg or specific drug treatment; (3) plasma triglycerides $\geq$ 150 mg/dl ($\geq$ 1.70 mmol/L) or specific drug treatment; (4) plasma high-density lipoprotein (HDL)-cholesterol < 40 mg/dl (< 1.0 mmol/L) for men and < 50 mg/dl (< 1.3 mmol/L) for women or specific drug treatment; (5) prediabetes (i.e., fasting glucose levels 100 to 125 mg/dl [5.6 to 6.9 mmol/L], or 2-h post-load glucose levels 140 to 199 mg/dl [7.8 to 11.0 mmol] or HbA1c 5.7% to 6.4% [39 to 47 mmol/mol]); (6) Homeostasis model assessment (HOMA) of insulin resistance score $\geq$ 2.5; and (7) plasma high-sensitivity C-reactive protein (CRP) level > 2 mg/L.

Histological scoring/staging systems have been developed to assess 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.

The term “steatosis” refers to the process describing the abnormal retention of lipids or fat accumulation within the liver. The term “hepatocellular ballooning” is usually defined, at the light microscopic level, based on hematoxylin and eosin (H&E) staining, as cellular enlargement 1.5-2 times the normal hepatocyte diameter, with rarefied cytoplasm. It refers more generally to the process of hepatocyte cell death. The term “lobular inflammation” refers to the

presence of lobular inflammatory foci (grouped inflammatory cells) at microscopic examination of a hematoxylin and eosin (H&E) stained slice of a liver biopsy.

According to the present invention, the term "fibrosis" or "liver fibrosis" denotes a pathological condition of excessive deposition of fibrous connective tissue in the liver. More specifically, fibrosis is a pathological process, which includes a persistent fibrotic scar formation and overproduction of extracellular matrix by the connective tissue, as a response to tissue damage. Physiologically, the deposit of connective tissue can obliterate the architecture and function of liver.

The different stages of liver fibrosis are defined by the Kleiner scoring system (Kleiner *et al,* Hepatology 2005, Vol 41, Issue 6, 1313-1321) wherein:

F0 refers to a subject with an absence of liver fibrosis;

F1 refers to a subject with portal or perisinusoidal fibrosis;

F2 refers to a subject with portal/periportal and perisinusoidal fibrosis;

F3 refers to a subject with septal or bridging liver fibrosis;

F4 refers to a subject with liver cirrhosis.

Using this fibrosis staging system, patients with no or minimal fibrosis (F=0 or 1) are generally not considered at risk of cirrhosis, liver failure, HCC (hepatocellular carcinoma) or liver-related death. Patients with significant (F=2) or severe (F=3) liver fibrosis are at risk of developing cirrhosis, liver failure, HCC and liver-related death. Patients with compensated cirrhosis (F=4) are at high risk of liver failure (decompensated cirrhosis), HCC and liver-related deaths.

Within the context of the present invention, a "subject with MASH" or a "MASH subject" has MAS≥4, with at least 1 point in steatosis, at least 1 point in lobular inflammation, at least 1 point in hepatocyte ballooning and a liver fibrosis of stage 1, 2 or 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 or polycystic ovary syndrome.

As used herein, the term "monitoring" refers to the tracking of the evolution of a disease or condition. Monitoring is the ongoing, systematic collection and analysis of data as a protocol or condition progresses, for example during a clinical study or a treatment protocol.

Within the context of the present invention, the “evolution of MASH” is characterized by two parameters in a subject with MASH: (i) whether MASH is resolved; (ii) whether liver fibrosis is improved. In a particular embodiment, the invention relates to a method for monitoring the resolution of MASH and/or the improvement of liver fibrosis in a subject with MASH.

Within the context of the present invention, “MASH resolution” is defined as the presence, in a subject with baseline MASH, of any grade of steatosis, no ballooning, only minimal lobular inflammation (score ≤ 1) and, at the same time, no worsening of the stage of liver fibrosis compared to liver fibrosis stage at the first timepoint.

Within the context of the present invention, “fibrosis improvement” is defined as the improvement, in a subject with baseline MASH, of liver fibrosis by at least 1 stage without any worsening of MASH, i.e. no worsening of ballooning and lobular inflammation, no more than 1 score change in steatosis, compared to the stage of MASH at the first timepoint.

The stage of MASH is determined by MAS score.

The term “subject who has baseline MASH” refers to a patient with MASH at the first timepoint of the method of the invention.

Subjects who had baseline MASH and achieves MASH resolution are defined as “MASH resolvers”.

Patients who had baseline MASH and achieves fibrosis improvement are defined as “fibrosis improvers”.

In a particular embodiment, the present invention provides a method for determining ”MASH resolvers” and/or “fibrosis improvers” from MASH patients.

The terms "subject" and "patient" may be used interchangeably herein and refer to a human subject.

The implementation of the present invention is based on the providing of (i) the circulating levels of hsa-miR-34a-5p and YKL-40 in a subject with MASH and (ii) the gender of said subject.

The circulating level of YKL-40 in a biological fluid sample may be measured by any conventional method 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 YKL-40 can be determined by antibodies, aptamers

or peptides respectively directed against said markers from the serum. The level of YKL-40 can be measured using a YKL-40-specific binding agent, such as an anti- YKL-40 antibody. For example, we can cite methods based on immunodetection, such as ELISA (enzyme-linked immunosorbent assay). In this respect, commercial kits for YKL-40 level determination are readily available, such as the Quantikine® Chitinase 3-like 1 Immunoassay (ref.: DC3L10, R&D Systems, Inc.).

Circulating hsa-miR-34a-5p in a biological fluid sample may be measured by common technique used in molecular biology such as RNA extraction, reverse transcription and quantitative PCR with specific primers and/or probes from the serum.

In a particular embodiment, the circulating levels of hsa-miR-34a-5p and YKL-40 in a biological fluid sample are simultaneously measured by an analytical method, such as biochips or immunoPCR.

In particular, Immuno-PCR relies on the use of an antibody, which has been conjugated to an oligonucleotide. This oligonucleotide can be then amplified by PCR and this conjugate acts as a bridge between the immunoreaction and DNA amplification. This method combines the versatility and robustness of immunoassays with the exponential signal amplification power of the PCR. Typically, IPCR allows a 10-1,000-fold increase in sensitivity over the analogous ELISA. This method is in particular described in WO2024/251754.

The measurements of the levels of these markers are conducted in biological fluid samples of the subject, such as a sample of blood or a blood-derived sample, e.g. serum or plasma, or a sample of saliva, interstitial fluid, or urine. In a particular embodiment, the biological fluid sample is a sample of blood, serum, or plasma. Preferably, the sample is a serum sample.

According to the present invention, a biological fluid sample may be analyzed immediately after collection or be stored in cold environments, for example in freezers, for further analysis.

According to the present invention, the term “timepoint” refers to a period of time during which one or more biological fluid samples are collected for the purpose of determining the levels of hsa-miR-34a-5p and YKL-40 of a subject in said period of time. A timepoint may be a period of time from several minutes to several days. For instance, said timepoint may be a period of 3 days. According to one embodiment, only one biological fluid sample of a subject is collected at one timepoint. According to said embodiment, the levels of hsa-miR-34a-5p and YKL-40 of a subject at a timepoint are obtained from the same sample. According to another embodiment,

more than one, e.g. two or three, biological fluid samples are collected at one timepoint. According to a more particular embodiment, the respective levels of hsa-miR-34a-5p and YKL-40 of a subject at a timepoint are mean levels of those measured in all samples collected at one timepoint. According to another particular embodiment, the levels of hsa-miR-34a-5p and YKL-40 of a subject at a timepoint are obtained from different samples.

In general, the first timepoint occurs at the beginning of the follow-up of a subject, e.g. at the beginning of a treatment protocol of MASH and/or liver fibrosis or at the beginning of a life-style protocol or of a diet plan, just before the therapy has been initiated by the patient. The last timepoint may occur at the end of the follow-up of said subject, e.g. at the end of the treatment protocol of MASH and/or liver fibrosis, the end of a life-style protocol or of a diet plant, or the moment when MASH resolution or fibrosis improvement is achieved. Thanks to the non-invasive property of the present method, the measurement at each timepoint for collecting samples may be scheduled as many as needed. The number of timepoints may be determined according to the time course of a treatment protocol or of a life-style protocol. In a particular embodiment, the method of the invention comprises from 6 to 10 timepoints. In a more particular embodiment, the method of the invention comprises 8 timepoints. Two adjacent timepoints may be separated by a period of from several days to several months, for example from 2 weeks to 6 months. In a particular embodiment, two adjacent measurement timepoints are separated by a period comprised from 9 to 12 weeks.

The levels of these markers at each timepoint and the gender of the subject are combined in a mathematical function to assign a score at each timepoint. Said score correlates with the evolution of MASH. One skilled in the art is aware of numerous suitable methods for developing mathematical functions, and all of these are within the scope of the present invention.

In a particular embodiment, the score is a value S1 obtained by a logistic function.

In a more particular embodiment, the score is a value S2 which is a logit value of said S1 score obtained by a logistic function.

The value S2 is calculated according to the following mathematical function:

$$S2=\log(S1/1-S1)$$

In a further embodiment, S1 is obtained from the following logistic function:

$$S1 = \frac{1}{1 + \exp(-y)}$$

wherein

$y = \beta0 + \beta1 * \log10(\text{miR-34a-5p (Fold)}) + \beta2 * \log10(\text{YKL-40 (ng/ml)}) + \beta3 * \text{Gender} + \beta4 * \log10(\text{miR-34a-5p (Fold)}) * \text{Gender}$; and

wherein Gender is 0 if the subject is a female, or 1 if the subject is a male.

In a particular embodiment, β0 is comprised between -3 and 3, in particular between -2 and 2.

In a particular embodiment, β1 is comprised between 1 and 5, in particular between 2 and 4.

In a particular embodiment, β2 is comprised between 0 and 4.5, in particular between 0.5 and 3.

In a particular embodiment, β3 is comprised between -2 and 2, in particular between -1 and 1.

In a particular embodiment, β4 is comprised between -1 and 2, in particular between 0 and 2.

In a further particular embodiment, β0 is comprised between -3 and 3, β1 is comprised between 1 and 5, β2 is comprised between 0 and 4.5, β3 is comprised between -2 and 2 and β4 is comprised between -1 and 2. In yet another particular embodiment, β0 is comprised between -2 and 2, β1 is comprised between 2 and 4, β2 is comprised between 0.5 and 3, β3 is comprised between -1 and 1 and β4 is comprised between 0 and 2.

By way of example, the following equations can be used for calculating a S1 value.

equation 1:

$y = -1.4539 + 2.3003 * \log10(\text{miR-34a-5p (Fold)}) + 1.0598 * \log10(\text{YKL-40 (ng/ml)}) - 0.0533 * \text{Gender} + 0.4514 * \log10(\text{miR-34a-5p (Fold)}) * \text{Gender}$

equation 2:

$y = -0.8756 + 3.3957 * \log10(\text{miR-34a-5p (Fold)}) + 2.5248 * \log10(\text{YKL-40 (ng/ml)}) - 0.6496 * \text{Gender} + 0.2873 * \log10(\text{miR-34a-5p (Fold)}) * \text{Gender}$

equation 3:

$y = 1.1543 + 2.5678 * \log10(\text{miR-34a-5p (Fold)}) + 1.7859 * \log10(\text{YKL-40 (ng/ml)}) + 0.3514 * \text{Gender} + 0.7264 * \log10(\text{miR-34a(5p (Fold)}) * \text{Gender}$

According to the method of the invention, the scores calculated from a same mathematical function as described above at each timepoint for a subject with MASH are combined to calculate a slope value of these scores.

In a particular embodiment, the S2 values at each timepoint for a subject with MASH are combined to calculate a slope value of these S2 values.

A slope value of a series of scores may be obtained by using any conventional data analytic method, i.e. linear regression.

In a more particular embodiment, the slope value of the S2 values as defined above is calculated by a linear model of S2 values over time.

According to the method of the invention, said slope value is compared with a reference slope value. Said reference slope value is an optimal cutoff value allowing to classify a MASH patient as having MASH resolution and/or fibrosis improvement, or to classify a treatment of MASH and/or liver fibrosis as efficient. Said reference slope value is chosen to maximize the number of correctly classified patients.

According to an embodiment of the invention, a slope value lower than or equal to the reference slope value is indicative of a MASH subject having MASH resolution and/or liver fibrosis improvement, i.e. said MASH subject may have either MASH resolution or liver fibrosis improvement, or have both MASH resolution and liver fibrosis improvement.

According to another embodiment of the invention, a slope value higher than the reference slope value is indicative of a MASH subject having neither MASH resolution nor liver fibrosis improvement.

In a yet another embodiment, a slope value lower than or equal to the reference slope value is indicative of a treatment of MASH and/or liver fibrosis being efficient for a MASH subject; a slope value higher than the reference slope value is indicative of a treatment of MASH and/or liver fibrosis being inefficient for a MASH subject.

Said reference slope value may be determined by any conventional data analytic method. For instance, said reference slope value is determined according to a ROC curve of the slope values of the S2 values obtained from a cohort of MASH patients.

In a particular embodiment, said reference slope value is determined by a standard analytical method (Unal I. *Comput Math Methods Med.* 2017:3762651. Epub 2017 May 31.) using the

Youden index (YOUDEN WJ. *Cancer.* 1950 Jan;3(1):32-5). In a more particular embodiment, the reference slope value is a value which allows to maximize the sum of sensitivity and specificity of the method of the present invention.

Still more particularly, the reference slope value is the reference slope value of S2 slope value and comprised between -0.00230 and 0.00000. In a preferred embodiment, the reference slope value is equal to -0.00088.

Thanks to the methods of the invention, according to the monitoring outcome, a decision may be taken to give life-style recommendations to a subject with MASH (such as a food regimen or providing physical activity recommendations), to apply at least one MASH therapy and/or liver fibrosis therapy to said patient, or to adjust the MASH treatment protocol of the patient.

The easy use of the method of the invention allows to monitor without delay the achievement or not of the endpoints of the clinical trials of drugs for treating MASH, and thus monitor the efficiency of a clinical trial for treating MASH.

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:

a) receive levels of hsa-miR-34a-5p and YKL-40 measured at two or more timepoints of a subject with MASH and the gender of the subject,
b) combine the levels and the gender in a mathematical function to assign a score at each timepoint,
c) calculate a slope value of the subject from the scores of each timepoint,
d) compare the slope value of the subject with a reference slope value,
e) determine whether the subject has MASH resolution and/or liver fibrosis improvement, wherein if the slope value of the subject is lower than or equal to the reference slope value, which is indicative of the subject having MASH resolution and/or liver fibrosis improvement; if the slope of the subject is higher than the reference slope, which is indicative of the subject having neither MASH resolution nor liver fibrosis improvement.

In a particular embodiment, the score obtained in step (b) is a value S2 which is the logit value of a value S1 obtained according to the aforementioned logistic function.

In another particular embodiment, the reference slope value is a reference slope value of S2 slope value and comprised between -0.00230 and 0.00000. In a more particular embodiment, said reference slope value is equal to -0.00088.

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. Suitable media are RAM, CD-ROM, or disk drives.

The present invention also relates to a computer readable recording medium storing instructions for executing the method of the present invention.

In a particular embodiment, the computer readable recording medium comprises instructions for executing the steps a) to e) of the above-described computer program.

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

EXAMPLES

1. MATERIALS AND METHODS

1.1 Study patient cohort

Resolve-IT is the study patient cohort used. Resolve-IT is a double-blind, placebo-controlled, multicenter, international phase 3 clinical trial (NCT02704403). Resolve-IT evaluated the efficacy and safety of elafibranor in patients aged 18-75 years with NASH and fibrosis ranging from 1 to 3. Patients who were enrolled in Resolve-IT were considered for potential inclusion in this analysis.

1.2 Patients follow-up

Patients who were included and followed until Resolve-IT interim phase had performed two liver biopsies: a first one during screening visit (SV) and a second one during visit 7. Blood dosage was performed in these patients during every visit (screening visit (SV) and visits 1-2-3-4-5-6-7), allowing to measure circulating hsa-miR-34a-5p and YKL-40 levels at every timepoint.

In the protocol, a mean delay of 9 weeks was expected between screening visit and visit 1. A mean delay of 12 weeks was expected between each other pair of visits (V1-V2, V2-V3, V3-V4, V4-V5, V5-V6, V6-V7). Patients follow-up was approximately a year and a half.

For this analysis, all visits were taken into account (SV, and visits 1-2-3-4-5-6-7), resulting in 2 liver biopsies and 8 blood dosages for each patient.

1.3 Patients selection

Patients integrated in the analysis are MASH patients included in Resolve-IT phase 3 clinical trial, who had a liver-biopsy proven fibrosis stage of 1, 2, or 3; a MAS score of 4 or more points; a ballooning score of 1 point or more; a lobular inflammation score of 1 point or more; a steatosis of 1 point or more. Selected patients for the analysis had available liver biopsy at screening visit and visit 7, had less than 180 days between each liver biopsy and the corresponding blood dosage (at screening visit and visit 7), and had available blood dosage of hsa-miR-34a-5p and YKL-40 at every visit (Screening visit, and visits 1-2-3-4-5-6-7).

This selection led to 711 patients used for the analyses.

1.4 Slope value calculation

Patients are followed from screening visit to visit 7, which provides 8 timepoints for each patient. At each timepoint, a S1 value is calculated based on the measured levels of circulating hsa-miR-34a-5p and YKL-40 and the gender of a patient and according to the logistic function below:

$$S1 = \frac{1}{1 + \exp(-y)}$$

wherein

y = -0.8756 + 3.3957 ∗ log10(miR-34a-5p (Fold)) + 2.5248 ∗ log10(YKL-40 (ng/ml)) - 0.6496 ∗ Gender + 0.2873 ∗ log10(miR-34a-5p (Fold)) ∗ Gender

Each S1 value of a patient is then converted to a S2 value, which is the logit value of S1. S2 is obtain by using the following mathematical function: S2=log(S1/1-S1). A linear model of S2 values over the time can be constructed. Based on said model, we can obtain the slope value of S2 values of each patient.

A Receiver Operating Characteristic (ROC) curve can then be computed (Figure 1), corresponding to the sensitivity and specificity reached with different slope values used as classification cutoffs. The AUC value corresponds to the area under the ROC curve, the larger the better. An AUC of 0.5 means a random classification of patients. An AUC of 1 means a perfect classification of patients.

The optimal cutoff of said ROC curve is determined at -0.00088. Said value allows to maximize the sum of sensitivity and specificity and is used as Youden cutoff. A Youden cutoff is defined as the reference slope value to classify patients based on their slope values.

In order to compute confidence intervals around Youden cutoff, we used bootstrap resampling method. Bootstrap resampling consists in simulating k simulated datasets. In this case, k=1000. Each simulated dataset had a sample of n (n being the sample size of the original dataset, thus n=711). From each of the 1000 simulated datasets through bootstrap, we computed Youden cutoff. We ended up with 1000 Youden cutoffs. Based on 1000 simulated Youden cutoffs, it was possible to extract a standard error around original Youden cutoff and thus to compute confidence intervals (95%). This calculation allowed us to estimate the following results around original Youden cutoff: -0.00088 [-0.00230; 0].

2. Results

By applying Youden cutoff (-0.00088), an AUC of 0.621 is achieved. This AUC is significantly higher than a random classification of patients (i.e. AUC of 0.5). The performances of Youden cutoff are specified in table 1 below:

Table 1:

	Performances
Sample size	711
Prevalence	248 (35%)
AUC [95% CI]	0.621 [0.577 – 0.663]
Sensitivity	48%
Specificity	72%
PPV	48%
NPV	72%

PPV: positive predictive value

NPV: negative predictive value

Results of Table 1 mean that we detect 48% of the patients with MASH resolution and/or fibrosis improvement, and 72% of the patients without MASH resolution nor fibrosis improvement. Among the patients predicted positive, 48% of them are true positive. Among the patients predicted negative, 72% of them are true negative.

The S2 values were measured at 8 timepoints (SV, and visits 1-2-3-4-5-6-7) in MASH patients who were confirmed by biopsy as having MASH resolution and/or fibrosis improvement and in MASH patients who were confirmed by biopsy as having neither MASH resolution nor fibrosis improvement. The slope values of the S2 values obtained from these two groups of patients were also calculated. Figure 2A shows that, compared to patients having neither MASH resolution nor fibrosis improvement, patients having MASH resolution and/or fibrosis improvement have in general lower S2 values at the same timepoint. Most importantly, we can observe that the S2 values of patients having MASH resolution and/or fibrosis improvement show a tendency to decrease over time. This tendency is confirmed in Figure 2B, which shows that the slope value of patients having MASH resolution and/or fibrosis improvement is significantly lower than the slope value of patients having neither MASH resolution nor fibrosis improvement.

In order to verify the performance of the method of the invention, the method was applied to four patients with baseline MASH. The histology of these patients is summarized in table 2.

Table 2:

	Histology					
Patient	Baseline lobular inflammation (SV)	End of study lobular inflammation (visit 7)	Baseline ballooning (SV)	End of study ballooning (visit 7)	Baseline fibrosis (SV)	End of study fibrosis (visit 7)
A	3	3	2	2	3	3
B	2	3	1	2	2	3
C	3	1	2	0	3	1
D	2	0	1	0	2	1

Based on the histologic results, patients A and B have neither MASH resolution nor fibrosis improvement, while patients C and D have MASH resolution and fibrosis improvement at the end of follow-up.

The time (number of days) elapsed between screening visit and other visits is summarized in table 3.

Table 3:

	Time (days)							
Patient	SV	V1	V2	V3	V4	V5	V6	V7
A	0	55	140	217	309	385	476	553
B	0	63	145	225	314	392	480	560
C	0	59	137	210	302	385	475	555
D	0	70	155	230	319	391	472	552

The collected S1 values from the four patients are summarized in table 4.

Table 4:

	S1 value							
Patient	SV	V1	V2	V3	V4	V5	V6	V7
A	0.6	0.62	0.58	0.61	0.65	0.59	0.62	0.6
B	0.5	0.55	0.57	0.59	0.62	0.65	0.7	0.75
C	0.82	0.76	0.72	0.73	0.61	0.56	0.32	0.27
D	0.37	0.32	0.25	0.24	0.26	0.24	0.12	0.07

These S1 values are transformed to their logit values (S2) and illustrated in table 5.

Table 5:

	S2 value							
Patient	SV	V1	V2	V3	V4	V5	V6	V7
A	0.41	0.49	0.32	0.45	0.62	0.36	0.49	0.41
B	0.00	0.2	0.28	0.36	0.49	0.62	0.85	1.10
C	1.52	1.15	0.94	0.99	0.45	0.24	-0.75	-0.99
D	-0.53	-0.75	-1.10	-1.15	-1.05	-1.15	-1.99	-2.59

The evolution profiles of S2 values of these four patients are illustrated in figure 3A.

The slope value for each patient (S2 value against the time) is computed and compared with the Youden cutoff (figure 3B).

The slope value of each patient and the patient classification according to the present invention are also summarized in table 6.

Table 6:

Patient	Slope	Classification
A	0.00004	Neither MASH resolution nor fibrosis improvement
B	0.00176	Neither MASH resolution nor fibrosis improvement
C	-0.00441	MASH resolution and/or fibrosis improvement
D	-0.00309	MASH resolution and/or fibrosis improvement

We can observe that the classification the patients A, B, C and D according to the method of the invention is correlated with the results determined by biopsy.

These results show that the method of the invention allows to efficiently and easily monitor the evolution of MASH and/or liver fibrosis in a subject with MASH. Since this method is non-invasive and can be implemented as often as necessary, it can also be used to evaluate the efficacy of a medical treatment of MASH and/or liver fibrosis in a subject with MASH.

CLAIMS

1. A method for monitoring the evolution of metabolic dysfunction-associated steatohepatitis (MASH) and/or liver fibrosis, or for evaluating the efficacy of a medical treatment of MASH and/or liver fibrosis in a subject with MASH, wherein said method comprises:

-providing (i) the circulating levels of hsa-miR-34a-5p and YKL-40 in biological fluid samples, which are obtained at two or more timepoints from the subject, and (ii) the gender of the subject,

- combining the provided levels and the gender in a mathematical function to assign a score at each timepoint,

-combining the scores of each timepoint to calculate a slope value of the scores of said subject, and

-comparing said slope value with a reference slope value.

2. The method according to claim 1, wherein the score is a value S2 which is a logit value of S1 obtained from a logistic function:

$$S1 = \frac{1}{1 + \exp(-y)}$$

wherein

$y = \beta0 + \beta1 * \log10(\text{miR-34a-5p (Fold)}) + \beta2 * \log10(\text{YKL-40 (ng/ml)}) + \beta3 * \text{Gender} + \beta4 * \log10(\text{miR-34a-5p (Fold)}) * \text{Gender}$

wherein

Gender is 0 if the subject is a female, or 1 if the subject is a male;

β0 is comprised between -3 and 3, in particular between -2 and 2;

β1 is comprised between 1 and 5, in particular between 2 and 4;

β2 is comprised between 0 and 4.5, in particular between 0.5 and 3;

β3 is comprised between -2 and 2, in particular between -1 and 1; and

β4 is comprised between -1 and 2, in particular between 0 and 2.

3. The method according to claim 2, wherein the reference slope value of S2 slope value is comprised between -0.00230 and 0.00000.

4. The method according to claim 3, wherein the reference slope value of S2 slope value is equal to -0.00088.

5. The method according to any one of claims 1 to 4, wherein a slope value of the subject lower than or equal to the reference slope value is indicative of the subject having MASH resolution and/or liver fibrosis improvement; and wherein a slope value of the subject higher than the reference slope value is indicative of the subject having neither MASH resolution nor liver fibrosis improvement.

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

7. The method according to claim 6, wherein the biological fluid sample is a serum sample.

8. A computer program comprising instructions that, when executed by a processor/processing means, cause the processor/processing means to:

a) receive levels of hsa-miR-34a-5p and YKL-40 measured at two or more timepoints of a subject with MASH and the gender of the subject,
b) combine the levels and the gender in a mathematical function to assign a score at each timepoint,
c) calculate a slope value of the subject from the scores of each timepoint,
d) compare the slope value of the subject with a reference slope value,
e) determine whether the subject has MASH resolution and/or liver fibrosis improvement, wherein if the slope value of the subject is lower than or equal to the reference slope value, which is indicative of the subject having MASH resolution and/or liver fibrosis improvement; if the slope of the subject is higher than the reference slope, which is indicative of the subject having neither MASH resolution nor liver fibrosis improvement.

9. The computer program according to claim 8, wherein the score is a value S2 which is the logit value of a value S1 obtained according to the logistic function defined in claim 2.

10. The computer program according to claim 9, wherein the reference slope value of S2 slope value is comprised between -0.00230 and 0.00000.

11. The computer program according to claim 10, wherein the reference slope value of S2 slope value is equal to -0.00088.

12. A computer readable recording medium storing instructions for executing the method of any one of claims 1 to 7.