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A diagnostic model to differentiate simple steatosis from nonalcoholic steatohepatitis based on the likelihood ratio form of Bayes theorem

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Abstract

Objective: To evaluate the performance of a diagnostic model based on a composite index using clinical and laboratory data, including cardiovascular biomarkers, to help practitioners to differentiate patients with simple steatosis from those with nonalcoholic steatohepatitis (NASH).

Design and methods: 101 patients with biopsy proven features of nonalcoholic fatty liver disease were included. We investigated the usefulness of 9 biomarkers in predicting the histological disease severity, including routine biochemical tests, C-reactive protein, soluble intercellular adhesion molecule-1 (sICAM-1) and anthropometric evaluation. Receiver operating characteristic (ROC) curves and likelihood ratios (LRs) were used to evaluate the fit of each test. A composite index was calculated as the product of each individual test LR.

Results: In a model patient who has all positive tests, the post-test probability for NASH would be 99.5%.

Conclusion: The capacity of each individual biomarker to independently predict the disease outcome was lower than a composite index constructed after multiplying the LR for each individual test combined into a "multimarker" score.

Keywords: Biomarkers; Diagnostic model; Fatty liver NASH; Nonalcoholic fatty liver disease; sICAM-1

Introduction

Nonalcoholic fatty liver disease (NAFLD) is increasingly prevalent worldwide. The disease affects 10 to 24% of the general population in various countries [1], and parallels the frequency of the obesity and type 2 diabetes epidemic [2].

NAFLD refers to a wide spectrum of liver diseases, ranging from fatty liver alone to nonalcoholic steatohepatitis (NASH) with evidence of liver cell injury, a mixed inflammatory lobular infiltrate, and variable fibrosis [3]. Patients with simple steatosis usually have a benign prognosis for liver disease. In contrast, up to 20% of patients with NASH can progress to cirrhosis [4]. Moreover, survival of patients with NASH is reduced as these subjects more often die from cardiovascular and liver-related causes [5]. In addition, results from cross-sectional studies showed that the presence of cardiovascular complications in patients with NAFLD increases with the histological severity of the disease [6].

These findings may be related to that plasma homocysteine concentrations are significantly higher in patients with NASH, suggesting that plasma homocysteine can be considered as a good predictive point for discrimination of NASH from simple steatosis [7].

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Nevertheless, NAFLD has no definitive biochemical markers. In fact, despite that in most of the patients the disease can be associated with mild elevation of serum aminotransaminase levels [3], advanced stages of liver disease can be observed in some patients with normal results of this liver test [8].

Liver ultrasound scan is the most common screening approach for NAFLD in asymptomatic patients. However, it is not useful to distinguish between the two main histological and prognostic patterns of NAFLD: simple steatosis and NASH.

Liver biopsy (LB), therefore, has been recommended for both confirming diagnosis and providing prognostic information, particularly when assessing the presence of liver inflammation and fibrosis [3]. Unfortunately, LB–for many reasons–does not meet the challenge to massive screening for significant liver injury in patients with NAFLD. The global trend of an increasing prevalence of fatty liver, not only in adult but also in children, and the increasingly high prevalence of co-morbidities associated with NAFLD, such as obesity and type 2 diabetes, all together depict a scenario in which performing a LB in about 30% of symptomatic individuals results impractical and not cost-effective. Moreover, LB is an invasive and costly procedure, and even in the most experienced hands is prone to complications, showing a mortality rate between 0.1 and 0.01% [9]. Hence, the indication of LB in patients with NAFLD is becoming controversial, at least for the routine recognition and management of fatty liver disease in clinical practice.

An area of particular interest is the use of noninvasive biomarkers as surrogate and endpoints of disease. Based on the knowledge of the pathogenesis of NAFLD, we hypothesized that clinical data and anthropometric measurements in addition to laboratory tests may be combined to establish a simple and accurate diagnostic test to distinguish between the major clinical forms of NAFLD. In this study, we propose a diagnostic model based on a composite index using both clinical and routine laboratory data, including cardiovascular biomarkers to help, especially but not only, general practitioners, to differentiate those patients with simple steatosis from those with NASH.

Materials and methods

Patients selection

We performed a cross sectional study on NAFLD in a county Hospital of the city of Buenos Aires. This study involved 101 unrelated patients (32 males and 69 females) with proven through biopsy features of NAFLD, including ultrasonographic examinations (US) suggestive of fatty infiltration performed by the same operator.

Patients were considered for inclusion if they had histopathologic evidence of fatty liver disease, either simple steatosis or NASH, on liver biopsy performed within the study period. Based on the histological findings, 41 patients were assigned to the simple steatosis group and 60 to the NASH group.

Secondary causes of steatosis, including alcohol abuse ($\geq$30 g alcohol daily for men and $\geq$20 g for women), total parenteral nutrition, hepatitis B and hepatitis C virus infection, and the use of drugs known to precipitate steatosis were always excluded. By using standard clinical and laboratory evaluation as well as liver biopsy features when applicable, autoimmune liver disease, metabolic liver disease, Wilson's disease, and α-1-antitrypsin deficiency were likewise ruled out in all patients.

Physical, anthropometric and biochemical evaluation

Health examinations included anthropometric measurements, a questionnaire on health-related behaviors, and biochemical determinations.

Body mass index (BMI) was calculated as weight/height2 (kg/m2) and used as an index for relative weight. Additionally, waist and hip circumference were also assessed.

Blood was drawn from 12-hour-fasting subjects who had lain in a supine resting position for at least 30 min. Serum insulin, total cholesterol, HDL and LDL-cholesterol, triglycerides, plasma glucose and liver function tests were measured by standard clinical laboratory techniques.

Initially, abnormal liver function test were defined according to the following criteria: 1) elevated serum alanine (ALT) and/or aspartate aminotransferase (AST), defined as >41 U/L, 2) gamma-glutamyl-transferase (GGT) >50 U/L, and 3) alkaline phosphatase (AP) >250 UI/L. All biochemical determinations were measured using a Hitachi-912 Autoanalyzer (Roche, Diagnostic, Buenos Aires, Argentina).

Homeostasis Model Assessment (HOMA) was used to evaluate an insulin resistance index and was calculated as fasting serum insulin (μU/mL) × fasting plasma glucose (mmol/L)/22.5. Elevated blood pressure was defined as systolic arterial blood pressure (SABP) $\geq$130 mm Hg and/or DABP$\geq$85 mm Hg or receipt of anti-hypertensive medications.

Cardiovascular biomarkers

Serum C-reactive protein (CRP) was measured in duplicate to evaluate low-grade inflammation by an agglutination of the latex particles coated with anti-human C-reactive protein assay (CRP-Latex, BioSystems S.A., Barcelona, Spain) with a detection limit of 1.0 mg/L.

A quantitative determination of soluble intercellular adhesion molecule-1 (sICAM-1) was performed in duplicate by a solid phase sandwich enzyme linked immuno sorbent assay by using a monoclonal specific antibody for ICAM-1 (Diaclone, France). Serum concentrations are expressed in ng/mL.

Body fat content and abdominal subcutaneous fat thickness

Measurement of body fat content was performed by using a bioelectrical impedance method at 50 kHz and 500 μA (OMRON Body Fat Analyzer, model HBF-306, OMRON Healthcare, INC Illinois, U.S.A.). The body fat content is calculated by a formula that includes five factors: electric resistance, height, weight, age and gender. Body fat percentage (%) was calculated as Body fat mass (lbs.)/Body weight (lbs.) × 100.

We also included a measurement of the thickness of the subcutaneous fat of the anterior abdominal wall by ultrasonographic evaluation using a 3.5 MHz linear type B-mode probe.

The transducer was transversely placed perpendicular to the skin in the midline of abdomen, between the xiphoid process and umbilicus, and the maximum thickness of the subcutaneous fat was measured three times and the mean value was taken.

Liver biopsies and histological evaluation

A percutaneous liver biopsy (LB) was performed with ultrasound guidance and modified 1.4 mm diameter Menghini needles (Hepafix, Braun, Germany) on an outpatient basis. Liver biopsy specimens were routinely fixed in 40 g/L formaldehyde (pH 7.4) embedded in paraffin and stained with hematoxylin and eosin, Masson trichrome and silver impregnation for reticular fibers. The same liver pathologist, who was blinded to patient details, read all biopsies. All biopsies were at least 2 cm in length and contained a minimum of 8 portal tracts. The degree of steatosis was assessed according to the system developed by Brunt et al. [10] based on the percentage of hepatocytes containing macrovesicular fat droplets, as follows: grade 0, no steatosis; grade 1, <33% of hepatocytes containing macrovesicular fat droplets; grade 2, 33%–66% of hepatocytes containing macrovesicular fat droplets; and grade 3, >66% of hepatocytes containing macrovesicular fat droplets.

NASH was defined as steatosis plus mixed inflammatory-cell infiltration, hepatocyte ballooning and necrosis, glycogen nuclei, Mallory's hyaline, and any stage of fibrosis, including absent fibrosis [3], and was also scored as described by Brunt et al. [10]. The severity of necroinflammatory activity was expressed on a 3-point scale, as follows: grade 1 (mild), grade 2 (moderate) and grade 3 (severe) as described by Brunt et al. [10]. The severity of fibrosis was expressed on a 4-point scale, as follows: 0=none, 1=perivenular and/or perisinusoidal fibrosis in zone 3, 2=combined pericellular portal fibrosis, 3=septal/bridging fibrosis, and 4=cirrhosis. Histological features of patients with simple steatosis and NASH are shown in Table 1.

All the investigations performed in this study were conducted in accordance with the guidelines of The Declaration of Helsinki. Written consent from individuals was obtained in accordance with the procedures approved by the Ethical Committee of our institution.

Statistical analysis

For univariate analysis, differences between groups were assessed by the non-parametric Mann–Whitney Test to avoid any assumption about variable distribution and homoscedasticity. For testing the association between markers and disease severity, we used logistic regression analysis with disease severity as the dependent (response) variable, and HOMA, AP, γ-GT, ALT, AST, C-RP and sICAM-1 as continuous predictor variables.

To evaluate the diagnostic performance of each independent variable (clinical or biochemical marker) to discriminate about histological diagnosis, we performed a receiver operating characteristic (ROC) curve analysis. Positive (PPV) and negative (NPV) predictive value of different tests were calculated.

Table 1
Histological features of patients with simple steatosis and NASH

Histological features	Simple steatosis patients	NASH patients
Degree of steatosis		
1	11	6
2	19	19
3	11	35
Necroinflammatory activity		
1	NA	26
2		32
3		2
Fibrosis stage		
0	NA	29
1		19
2		2
3		9
4		1

NASH: nonalcoholic steatohepatitis. NA: not applicable.

Additionally, we used likelihood ratios (LRs) as an alternative method of judging the accuracy of each test.

For the evaluation of each test performance, we used the post-test probability compared to the pretest probability, the probability of disease presence as estimated before diagnostic testing (usually the prevalence of the disease that in our case was around 55–60%). Post-test probability is calculated as post odds/(1+post-test odds). To obtain the post-test odds, we multiplied the pretest odds by the LR. The pre-test odds (the odds of disease before getting the test) were computed as pretest probability/(1−pretest probability).

Besides, LRs were used to combine the results of multiple diagnostic tests and to calculate the post-test probability for the target disorder. A composite index was then calculated in which, the LR of the whole set of findings is the product of the LR of each individual test (LR1×LR2×LR3×…×LRn) [11].

We used the CSS/Statistica program package, StatSoft V 6.0 (Tulsa, USA) to perform these analyses.

Results

Clinical features and laboratory findings of the patients according to NAFLD status are shown in Table 2.

Significant differences were observed between simple steatosis and NASH about fasting plasma glucose and insulin, HOMA index, AP and sICAM-1. However, logistic regression indicated that only HOMA index (OR: 1.26, 95% CI: 1.02–1.56, $p<0.03$) and AP (OR: 1.007, 95% CI: 1.001–1.012, $p<0.01$) are independent predictors of NASH.

ROC area under the curve (AUROC) results for anthropometric measurements, liver tests, insulin resistance and inflammation and endothelial damage biomarkers to discriminate histological diagnosis are shown in Table 3.

As a single marker, AP had the greatest AUROC (0.69) compared with other clinical or biochemical parameter. The optimal diagnostic accuracy of liver function tests to discri-

Table 2
Clinical features and laboratory findings of patients according to NAFLD status

Variables	Simple steatosis patients	NASH patients	*P* value
Number of subjects	41	60	
Female/male	26/15	43/17	NS
Age; years	52.3	54.6	NS
BMI; kg/m2	32.1±5.3	33.7±6.6	NS
Waist circumference, cm	100.6±19.8	105.0±13.9	NS
Waist/hip ratio	0.9±0.05	0.9±0.07	NS
SABP; mmHg	126.5±15.9	127.1±16.1	NS
DABP; mmHg	79.2±9.7	77.6±13.7	NS
Fasting plasma glucose; mmol/L	5.37±1.17	6.45±2.41	<0.04
Fasting plasma insulin; pmol/L	80.6±46.5	120.1±91.6	<0.02
HOMA index	2.9±2.1	4.8±4.1	<0.007
Total cholesterol; mmol/L	5.55±1.49	5.67±1.06	NS
HDL cholesterol; mmol/L	1.28±0.65	1.28±0.41	NS
LDL-cholesterol; mmol/L	3.24±1.53	3.15±1.31	NS
Triglycerides; mmol/L	1.94±1.14	2.26±1.44	NS
Uric acid; mmol/L	375±750	286±369	NS
ALT, U/L	60.4±79.0	64.37±46.85	NS
AST, U/L	40.1±17.4	49.4±34.3	NS
GGT, U/L	63.7±47.4	75.6±68.2	NS
AP, U/L	214.8±97.7	286.2±112.2	<0.002
C-reactive protein; mg/L	7.3±2.5	8.8±4.8	NS
sICAM-1; ng/mL	496.1±235.1	650.1±327.4	0.05
Body fat content (%)	36.9±8.8	38.5±8.1	NS
Subcutaneous fat thickness (cm)	45.8±10.9	53.8±15.0	NS

NAFLD: nonalcoholic fatty liver disease. Nonalcoholic steatohepatitis: NASH. SABP and DABP: systolic and diastolic arterial blood pressure, HOMA: homeostatic model assessment. ALT and AST: serum alanine and aspartate aminotransferase, GGT: gamma-glutamyl-transferase, AP: alkaline phosphatase. sICAM-1: soluble intercellular adhesion molecule-1. Results are expressed as mean±SD. *P* value stands for statistical significance using Mann–Whitney Test. NS: non-significant. All measurements are in SI units.

minate between the 2 histological outcomes was observed for ALT (the better cutoff value for ALT was 22). At this cutoff, which is almost reduced by half the upper cutoff value of the reference range, the sensitivity of ALT was 96.63%. However, the ability of ALT to correctly identify absence of disease was very low, showing a specificity of 24.4%.

In addition, test LRs were used to evaluate the fit of each test. We multiplied 9 LRs attributable to 9 items in the medical history of the patients (ALT, AST, AP, γGG, HOMA, CRP, sICAM-1, BMI and waist circumference) to construct a clinical model. This approach showed an incremental ability to predict the histological outcome. For instance, considering a model patient with all the positive tests (all positive LRs), the post-test probability for NASH would be 99.5%. In the same way, considering a model with all negative tests (all the negative LRs), the post-test probability for NASH would be negligible (0.3%).

The AUROC for the composite index computing the products of all LRs was 0.795 (SE 0.044), 95% CI 0.703–0.896), see Fig. 1. The sensitivity and specificity with a cutoff of 1.31 were 68.3% and 82.9%, respectively. The PPV and NPV were 85.4 and 64.2, respectively.

Discussion

We investigated the usefulness of 9 biomarkers for predicting the histological severity of NAFLD (simple steatosis and NASH) in 101 patients with proven through biopsy features of the disease.

The 9 biomarkers were selected based on both the previous knowledge about the pathogenesis of NAFLD disease (insulin resistance is a major contributor to the pathogenesis and disease progression of NAFLD) and biologic plausibility (the most common cause of a mild elevation of serum ALT is NAFLD [3]). We also measured two cardiovascular biomarkers: C-reactive protein (a marker of inflammation) and sICAM-1 (a marker of endothelial dysfunction), as NAFLD is linked to increased cardiovascular risk, endothelial dysfunction and carotid atherosclerosis [6,12,13].

We observed that the most informative biomarkers for predicting NASH were AP and HOMA index. However, we observed that the capacity of each individual biomarker to independently predict the disease outcome was lower than a composite index constructed after computing the LR of each individual test combined into a “multimarker” score.

Even though after subtracting some biomarkers, such as BMI that showed a high cutpoint, CRP or sICAM-1 that is particularly orientated for either inflammation or cardiovascular complications, we observed a still high post-test probability for

Table 3
Receiver operating characteristic (ROC) area under the curve results to discriminate about histological diagnosis (simple steatosis and NASH)

Variable	Area (SE)	95% CI	Cutoff value	Sensitivity (95% CI)	Specificity (95% CI)	P-LR	N-LR	PPV	NPV
BMI	0.563 (0.059)	0.460–0.663	37	30.0 (18.9–43.2)	87.2 (72.6–95.7)	2.34	0.80	78.3	44.7
Waist circumference	0.576 (0.058)	0.474–0.675	105	51.7 (38.4–64.8)	67.5 (50.9–81.4)	1.59	0.72	70.5	48.2
ALT	0.582 (0.057)	0.479–0.680	22	96.6 (88.3–99.5)	24.4 (12.4–40.3)	1.28	0.14	64.8	83.3
AST	0.597 (0.057)	0.494–0.694	29	84.6 (75.0–93.9)	36.6 (22.1–53.1)	1.36	0.37	66.2	65.2
AP	0.690 (0.053)	0.588–0.780	239	69.0 (55.5–80.5)	64.1 (47.2–78.8)	1.92	0.48	74.1	58.1
GGT	0.553 (0.059)	0.449–0.653	71	40.7 (28.2–54.3)	76.9 (60.7–88.8)	1.76	0.77	72.7	46.2
HOMA	0.664 (0.055)	0.559–0.757	3.13	64.3 (50.4–76.6)	66.7 (49.8–80.9)	1.93	0.54	73.5	56.5
CRP	0.589 (0.060)	0.483–0.689	6.5	63.3 (49.9–75.4)	58.8 (40.7–75.3)	1.54	0.62	73.1	47.6
sICAM-1	0.639 (0.064)	0.523–0.744	556.9	56.6 (42.3–70.2)	73.1 (52.2–88.4)	2.10	0.59	81.1	45.2

NASH: nonalcoholic steatohepatitis. HOMA: homeostatic model assessment. ALT and AST: serum alanine and aspartate aminotransferase, GGT: gamma-glutamyl-transferase, AP: alkaline phosphatase. CRP: C-reactive protein. sICAM-1: soluble intercellular adhesion molecule-1.
SE: standard error. CI: confidence interval. P-LR: positive likelihood ratio. N-LR: negative likelihood ratio. PPV: positive predictive value. NPV: negative predictive value.

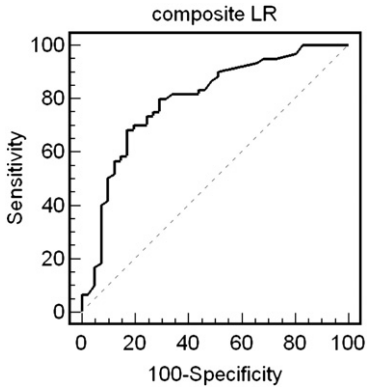

Fig. 1. ROC curve for the composite index computing the products of all likelihood ratios (LRs) to discriminate non-alcoholic steatohepatitis (NASH) from simple steatosis.

NASH (95.7%) with the composite index that incorporates liver tests (ALT, AST, AP and γGT), HOMA and waist circumference for a model patient with these 6 positive tests. For instance, patients who show ALT ≥ 22, AST ≥ 29, AP ≥ 239, γGT ≥ 71, HOMA ≥ 3.13 and a waist circumference ≥ 105 are more likely to have NASH than simple steatosis in a 95.7% of the cases. Otherwise, patients who show ALT < 22, AST < 29, AP < 239, γGT < 71, HOMA < 3.13 and a waist circumference < 105 are very unlikely to have NASH, as the probability of this to happen is less than 1%. These estimations sharply contrast with a priori probability of having NASH of 55–60% — the prevalence of NASH in our population of patients with ultrasonographic findings indicative of fatty liver.

The main strength of our study is the application of a simple and efficient composite index for predicting NAFLD outcomes (simple steatosis or NASH) by computing the LRs with information about the pretest probability of disease to determine the post-test odds and then the posttest probability of having NASH.

We chose this approach because computing LRs and posttest odds after a series of diagnostic tests is much easier than using the sensitivity/specificity method, and clinicians can determine the probability of a disorder (also called "posttest probability"), given the result of each test for each individual patient. More important, because LRs do not vary when disorder prevalence varies, it can be generalized to other patients based on Bayes' theorem [14]. Additionally, LRs are ratios of probabilities, and can be treated in the same way as risk ratios for the purposes of calculating confidence intervals [14].

Some limitations of our analysis deserve comment. First, we selected biomarkers based on the knowledge about NAFLD pathogenesis and results from previous clinical studies. We acknowledge that other not tested biomarkers, particularly concerning to liver fibrosis, might have provided additional information. In the same way, specific adipokines–such as TNF-alpha, leptin, and adiponectin–are also interesting biomarkers to test. However, we rather preferred to evaluate markers related with underlying risk factors for cardiovascular disease, such as proinflammatory state and endothelial dysfunction.

Second, inter-subject biological variability in tested biomarkers needs further validation not only in a different setting but also in a different population. In addition, even though we included in the study a sample of patients with a well-characterized phenotype, it would be important to validate these results in large-scale longitudinal studies increasing the sample size.

Finally, despite we computed laboratory results obtained by autoanalyzers using standardized methods–mostly universally accepted–, it may be useful to further evaluate the variability in the analytical technique and transferability between laboratories before the proposed cutoffs are accepted as universally valid.

Our findings regarding the association of single biomarkers with the risk of NASH are consistent with the results of previous studies. For instance, Shimada et al. evaluated the performance of several test for diagnosing early-stage NASH, and reported an AUROC of 0.757 for HOMA with a cutoff level over 3, among other markers [15]. In addition, Gholam et al. reported that statistical models incorporating markers of liver injury (AST) and hyperglycemia may be useful in predicting the presence of liver pathology in a population of severely obese patients [16].

A remarkable study encompassing a large sample of NAFLD patients from different centers around the world recently described the "NAFLD fibrosis score". Despite that the main endpoint of this study was to predict the presence or absence of advanced fibrosis, it is worth mentioning that in agreement with us, BMI and hyperglycemia, among other variables, were independent indicators of advanced liver disease—fibrosis [17].

Few studies compare biomarkers from different pathways or assess the incremental performance of a multimarker panel for risk prediction of NAFLD. Palekar et al. evaluated a diagnostic model for differentiating steatosis from steatohepatitis utilizing both clinical characteristics and a panel of biochemical markers of lipid peroxidation and fibrosis [18]. The authors calculated a composite index by summing several risk factors (age, gender, AST, BMI, hyaluronic and ALT/AST ratio), and observed that the presence of three or more risk factors had a sensitivity, specificity, PPV, and NPV of 73.7%, 65.7%, 68.2%, and 71.4%, respectively. One of the limitations of this study is that there were significant differences in age and gender between simple steatosis and NASH patients, and these 2 risk factors are included in the clinical model. In addition, the sum of risk factors does not give the appropriate weight to the most sensitive and specific markers.

Another approach for non-invasive quantitative estimate of liver steatosis is the SteatoTest that is calculated by combining 9 biochemical markers [19]. Despite the reasonable performance of this test, the main limitation is the use of an algorithm that is undisclosed by the trade company that commercialize the product (its cost is around 100€), and cost-effectiveness and accessibility also influences the clinical decision to measure when evaluating new biomarkers.

As a final point we wish to mention that along with BMI, AP and HOMA, one of the tests showing the higher positive LR for NASH was sICAM-1 (2.10). This result is consistent with our

previous observation that sICAM-1 levels were significantly associated with NAFLD severity [20]. This finding may explain the link between NAFLD and increased cardiovascular risk and endothelial dysfunction.

In summary, our study show that the clinicians can combine the LRs with information about the pretest probability of disease to determine the post-test probability of disease and can predict NAFLD severity to minimize the need of liver biopsy for distinguishing between simple steatosis disease and NASH, at least in routine clinical practice, until therapeutic options become available. Experts can perform LB to quantify hepatic steatosis, inflammation, necrosis, and fibrosis when eventually deciding therapeutic choices such as bariatric surgery or experimental drug treatment for NASH.

Our proposal to combine non-invasive, simple to perform and cheap diagnostic tests may help to distinguish the two main above-described histological patterns of NAFLD and can aid clinical assessment of disease probability so that therapeutic decisions can be made in patients' best interest.

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