Nonalcoholic fatty liver disease: biomarkers as diagnostic tools for liver damage assessment in adult patients from Argentina

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Background Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease whose prevalence has been increasing constantly and linked to the global obesity epidemic. The NAFLD histologic spectrum ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), which can progress to cirrhosis and hepatocellular carcinoma. Liver biopsy is the only reliable means to diagnose and stage NASH, but its invasive nature limits its use. Therefore, the prediction of hepatic injury by means of the development of new noninvasive tests represents a growing medical need. Our aim was to evaluate matrix deposition and cell-death markers, which correlate with liver injury in an NAFLD patient cohort.
Patients and methods Liver biopsies and serum from 34 NAFLD adult patients were analyzed. Histological parameters were evaluated. Matrix deposition [hyaluronic acid (HA) and tissue inhibitor of matrix metalloproteinase inhibitor-1 (TIMP-1)] and cell-death markers [cytokeratin-18 (M65) and caspase-cleaved cytokeratin-18 (M30)] were measured in serum samples.
Results HA showed an association with fibrosis severity (P=0.03) and M30 with steatosis (P=0.013), inflammation (P=0.004), and fibrosis severity (P=0.04). In contrast, TIMP-1 and M65 showed no association with any histological parameter of liver injury. The evaluation of diagnostic accuracy showed good performance as less invasive markers of significant fibrosis of both HA (area under the receiver operating characteristic curve: 0.928) and M30 (area under the receiver operating characteristic curve: 0.848).
Conclusion Biomarkers are essential tools that may provide a quick and accurate diagnosis for patients with life-threatening NAFLD and NASH. HA and M30, together or determined sequentially, have been found to be straightforward tests that may be sufficient to predict significant fibrosis even in a primary care center of an underdeveloped country. Eur J Gastroenterol Hepatol 30:637–644

Introduction

The health of the global population is currently threatened by the obesity epidemic, which promotes premature development of the metabolic syndrome, which in turn significantly increases the risk for liver disease early in life. Nonalcoholic fatty liver disease (NAFLD) is the most common form of chronic liver illness in all age groups, representing a major nutritional concern because of the high prevalence of overweight and obesity [1]. NAFLD is characterized by an excessive hepatic fat accumulation and includes two conditions with different prognoses: nonalcoholic fatty liver and nonalcoholic steatohepatitis (NASH) [2]. Notably, NASH is not by itself a severe hepatic lesion, but it can progress toward end-stage liver diseases [2]; thus, the identification of NASH patients is crucial to prevent liver damage early and to improve clinical outcome.

Obesity induces a comprehensive proinflammatory state with a high risk for metabolic comorbidities that contributes toward a progressive increase in patients who will develop NASH, NASH-related cirrhosis, decompensated liver disease, and hepatocellular carcinoma [3]. At present, NASH is the third most common indication for liver transplantation, and it is expected to increase and become the most common indication over the next decades [4]. Strikingly, current practice guidelines do not support NAFLD screening in patients at risk despite its high prevalence and implicit progression to end-stage liver disease [5]. In addition, because of the increased costs of the available tests, the risks of liver biopsy, and the lack of an effective treatment to offer to patients, NAFLD screening has been opposed [2]. However, the NAFLD progressive form should be identified in patients at risk (age > 50 years, type 2 diabetes mellitus, obesity, or metabolic syndrome) [6]. Therefore, the present challenge is to distinguish between simple steatosis versus NASH as the latter increases the likelihood of liver disease progression [7].

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The histological characterization of NAFLD ranges from simple steatosis to steatosis accompanied by inflammation and other evidences of cellular injury (NASH). Nonalcoholic fatty liver encompasses (a) steatosis, (b) steatosis with lobular or portal inflammation, without ballooning, or (c) steatosis with ballooning, but without inflammation [8]. The diagnosis of NASH requires a histopathological evaluation to assess the combined presence of steatosis, ballooning, and lobular inflammation [8]. Perisinusoidal fibrosis is also frequent, but it is not a diagnostic criterion. Fibrosis progression is the most significant prognostic factor that correlates with liver-related outcomes and death [9]. In this respect, liver biopsy is the gold standard providing important diagnostic and prognostic information; however, it remains a costly and invasive procedure with inherent risks. Thus, it cannot be used as a tool to periodically monitor disease outcome [10]. In addition, the amount of retrieved tissue can influence the diagnosis because of fat deposition, hepatocyte injury, or fibrosis that can vary between lobules; moreover, interobserver differences are frequently encountered [10]. Therefore, there is a growing medical need to develop noninvasive tests that can predict the initial stage and progression of liver disease over time in an accurate manner [11]. Currently, although little progress has been achieved in clinical practice, there are several noninvasive diagnostic methods that are being validated, namely, serum markers and imaging methods, to determine liver damage [12]. It is well known that abnormal liver function tests are poor indicators of NAFLD [6]; therefore, tracers of extracellular matrix remodeling represent attractive candidates because they directly evaluate the process of fibrogenesis [13]. The balance between deposition and removal of extracellular matrix, the key in the development of liver fibrosis [14], comprises the activation of hepatic stellate cells (HSCs) with the consequent secretion of excess matrix proteins (hyaluronan, laminin, collagen, etc.), followed by their degradation by the matrix metalloproteinases (MMPs). Moreover, MMP are also inhibited by tissue inhibitors of metalloproteinases (TIMPs) [15]. The serum levels of hyaluronic acid (HA) reflect the activity of HSC cells [16]; meanwhile, TIMP-1 protects collagen from MMP fibrolysis and also inhibits HSC apoptosis [17].

The pathophysiological pathways involved in the development of liver damage and its progression from simple steatosis to NASH is still uncertain; however, emerging data suggest that apoptosis of hepatocytes plays a central role in NAFLD. Particularly, NASH is considered to develop in two consecutive steps, excess fat accumulation and subsequent liver necroinflammation, the so-called 'two-hit hypothesis' [18]. Recent reports describe that the accumulation of free fatty acids in the hepatocytes leads to an increase in their cell death by apoptosis [19,20]. Engulfment of apoptotic bodies by HSC stimulates their fibrogenic activity; therefore, it could be a mechanism that leads to fibrosis through hepatocyte apoptosis [21]. The apoptotic process is mediated by activated caspases that cleave several intracellular substrates including CK18, the major intermediate filament protein in the liver. Cleaved CK18 is released through apoptosis; meanwhile, uncleaved CK18 is released during both necrosis and apoptosis.

The aims of this study were to evaluate the presence of matrix deposition markers (HA and TIMP-1) as well as cell-death markers [soluble fraction of cytokeratin-18 (M65) and caspase-generated neoepitope of the cytokeratin-18 proteolytic fragment (M30)] in a cohort of adult patients with NAFLD and to analyze their diagnostic accuracy for use as possible markers of liver damage in primary care centers in an underdeveloped country.

Patients and methods

Patients and samples

Thirty-four NAFLD White adult patients who attended the Hospital Italiano de Buenos Aires were enrolled.

Patients had no other causes of liver disease, autoimmune, genetic, or endocrinologic diseases, hepatocellular carcinoma, hepatitis C virus (HCV), hepatitis B virus, and/or HIV infection. Routine clinical biochemical analyses included complete blood count and analysis of prothrombin time, transferrin, iron, transferrin saturation, ferritin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, γ-glutamyltransferase, bilirubin, fasting plasma glucose, total cholesterol, high-density lipoprotein, low-density lipoprotein, and triglycerides. Blood pressure, waist circumference, bodyweight, and height were measured. Patients who consumed alcohol (men > 30 g/day; women > 20 g/day) were excluded.

Formalin-fixed paraffin-embedded liver biopsies and serum samples at the time of biopsy were tested.

A group of 20 healthy adults with no clinical or biochemical evidence of liver disease or known medical illness at recruitment were included as controls. The same parameters that were evaluated in patients were taken into account in the healthy group. All healthy participants were negative for hepatitis B virus, HCV, and HIV as evidenced by negative serological markers. Finally, the alcohol consumption of the healthy group was low. Only a serum sample from each healthy participant was included.

This study was approved by the Ethics Board of Ricardo Gutierrez Children Hospital and was carried out in accordance with the 1964 Declaration of Helsinki and its later amendments. A written informed consent was obtained from all patients before their inclusion in the study.

Histological analysis

Two independent pathologists evaluated the histological sections in a blinded manner according to the NAFLD scoring system proposed by the National Institute of Diabetes and Digestive and Kidney Disease NASH Clinical Research Network: a NAFLD activity score of at least 5 corresponds to a diagnosis of 'definitive NASH', a score of 3–4 corresponds to 'borderline NASH', and a score of less than or equal to 2 corresponds to 'not NASH or simple steatosis'. The stage of fibrosis was also measured. Fibrosis stages of at least 2 were considered to indicate significant fibrosis.

Quantitative measurement of TIMP-1 and HA

Serum TIMP-1 and HA were determined by enzyme-linked immunosorbent assay (ELISA) (Quantikine; R&D System Inc., Minneapolis, Minnesota, USA) according to the manufacturer's instructions.

Quantitative measurement of M30 and M65

Serum M30 and M65 were determined using the commercial quantitative sandwich enzyme immunoassay technique (M30-Apoptosense ELISA and M65-EpiDeath ELISA Kit, PEVIVA; Bromma, Sweden, respectively) according to the manufacturer's instructions.

Statistical analysis

GraphPad InStat software, version 3.05 (California Corporation, San Diego, California, USA) was used. The Mann–Whitney *U*-test and unpaired *t*-test, analysis of variance, or Kruskal–Wallis test were used to compare sets of data. *P* values less than 0.05 were considered significant.

The diagnostic value was assessed by the area under the receiver operating characteristic curves (AUROC). The cutoff value for the diagnosis was determined as the maximal value of the sum of the sensitivity and specificity. AUROC, cutoff values, positive predictive values, and negative predictive values (NPVs) were determined using the MedCalc demo statistical software; Ostend, Belgium.

The number of correctly classified cases by serum markers and the percentage of patients in whom the biopsy procedure could not have been avoided were assessed.

Results

Clinical and liver biopsy findings

The clinical and histological features of patients are described in Table 1. In accordance with the report of the NASH Clinical Research Network, 52.94% of patients were diagnosed with 'definitive NASH', 35.29% with 'borderline NASH', and 11.77% with 'no NASH'.

Quantitative assessment of TIMP-1, HA, M30, and M65

The four markers showed higher levels in NAFLD patients than in the healthy participants (Table 2). However, to describe the NAFLD population characteristics in more detail, each marker value was also compared through the three histological subgroups of NAFLD ('not NASH', 'borderline NASH', 'definitive NASH'). Interestingly, but in agreement with inflammation and fibrosis components, similar results were observed on comparing participants without NASH and healthy participants [except for M65 'not NASH' vs. healthy participants ($P = 0.002$)] as well as on comparing patients with borderline and definitive NASH. Therefore, this observation led us to group the cases into two sets: 'healthy participants + not NASH' and 'borderline + definitive NASH'. On analyzing TIMP-1, HA, M30, and M65 levels, significant differences were observed between groups for all the markers studied (Table 2).

In terms of the role of serum biomarkers as liver damage predictors, TIMP-1 showed no significant differences among fibrosis stages, hepatitis severity, or steatosis grade. Meanwhile, HA showed association with fibrosis severity as it was increased in NAFLD patients with significant fibrosis ($P = 0.03$) (Fig. 1). Moreover, this marker showed a sustained association with significant fibrosis when the cohort was analyzed on the basis of more precise groups (Fig. 1), namely, both the subgroup of patients with 'borderline + definitive NASH' ($P = 0.017$) and 'definitive NASH' ($P = 0.004$).

M30 showed an association with steatosis, inflammation, and fibrosis severity. That is, the M30 level was elevated in

Table 1. Clinical and histological features of patients

Factors	All patients	Not NASH	Borderline NASH	Definitive NASH
Age [median (range)] (years)	49.5 (28–72)	37.5 (30–47)	55.5 (28–72)	45.5 (30–72)
Sex: male (%)	55.88	100	41.67	55.55
Clinical and serological characteristics (%)				
BMI				
Overweight	25	50	36.36	7.69
Obese	75	50	63.64	92.31
Transaminases				
ALT [median (range)] (IU/l)	81.5 (31–279)	76.5 (60–204)	73 (31–254)	94 (43–279)
%Elevated	96	100	90.90	100
AST [median (range)] (IU/l)	52.5 (22–208)	59.5 (29–86)	50 (22–184)	60 (35–208)
%Elevated	53.57	50	54.54	53.85
AST/ALT [median (range)] (IU/l)	0.71 (0.368–1)	0.54 (0.41–0.95)	0.71 (0.36–0.88)	0.71 (0.36–0.89)
Lipid profile				
Cholesterol [median (range)] (mg/dl)	207 (126–327)	231.5 (207–285)	206 (145–246)	200 (126–327)
Triglycerides [median (range)] (mg/dl)	166 (60–465)	281.5 (156–465)	157 (60–391)	158 (76–375)
HOMA-IR [median (range)]	4.89 (1.7–10.10)	3.56 (1.97–7.87)	4.95 (2.77–10.10)	4.70 (1.70–8.64)
Type II diabetes (%)	55.88	25	75	50
Hypertension (%)	26.47	25	75	27.78
Metabolic syndrome (%)	47.06	25	58.33	80
Histological characteristics				
Steatosis (%)[a]				
0	–	–	–	–
1	17.65	50	33.33	–
2	26.47	50	50	5.56
3	55.88	–	16.67	94.44
Lobular inflammation				
0	20.59	100	25	–
1	61.76	–	75	66.64
2	17.65	–	–	33.33
3	–	–	–	–
Ballooning (%)				
0	14.71	100	8.33	–
1	61.76	–	83.34	61.11
2	23.53	–	8.33	38.89
NAFLD activity score (%)				
≤2	11.77			
3–4	35.29			
≥5	52.94			
Fibrosis (%)				
0	67.65	100	58.33	66.67
1	14.71	–	25	11.11
2	11.76	–	–	22.22
3	5.88	–	16.67	–
4	–			–
n	34	4	12	18

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HOMA-IR, homeostatic model assessment of insulin resistance; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.
Normal ALT and AST levels were ≤32 and ≤48 IU/l, respectively, when testing was performed at 37°C. The normal ranges for total cholesterol and triglycerides were 120–219 and <150 mg/dl, respectively.
[a]Steatosis grade: scores 0 (<5% cells), 1 (5–33%), 2 (33–66%), and 3 (>66%); lobular inflammation: scores 0 (0 foci), 1 (<2 foci), 2 (2–4 foci), and 3 (>4 foci); ballooning grade: scores 0 (none), 1 (few ballooning cells), and 2 (many cells/prominent cells); fibrosis stage: score 1 [a, b = mild (1a)/moderate (1b) zone 3 perisinusoidal fibrosis; 1c = only portal fibrosis]; 2 (zone 3 and portal/periportal fibrosis); 3 (bridging fibrosis); and 4 (cirrhosis).

Table 2. TIMP-1, HA, M30, and M65 levels in NAFLD patients and healthy participants

	Healthy participants	NAFLD	*P* value	Healthy participants + not NASH	Borderline + definitive NASH	*P* value[a]
TIMP-1 (ng/ml)	114.90 (92.58–181.11)	163.88 (89.87–557.36)	0.017	114.90 (92.58–242.39)	166.37 (89.87–557.36)	0.0046
HA (ng/ml)	6.205 (2.59–28.24)	13.69 (2.16–63.06)	0.02	6.205 (2.59–28.24)	13.70 (2.16–63.06)	0.02
M30 (U/l)	92.33 (71.29–121.61)	218.17 (87.34–1470.8)	< 0.0001	99.65 (71.29–277.43)	218.17 (133.39–1470.8)	0.0001
M65 (U/l)	72.53 (0–286.44)	460.24 (106.38–2166.2)	< 0.0001	227.56 (0–479.29)	477.69 (106.38–2166.2)	< 0.0001

Results are expressed as median (minimum–maximum).
HA, hyaluronic acid; M30, caspase-cleaved cytokeratin-18; M65, cytokeratin-18; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; TIMP-1, tissue inhibitor of matrix metalloproteinase inhibitor-1.
[a]*P* value of 'healthy participants + not NASH' versus 'borderline + definitive NASH'.

NAFLD patients with severe steatosis (grade 3) ($P = 0.013$), severe inflammation grade ($P = 0.004$), and significant fibrosis ($P = 0.04$). This association profile was maintained when analyzing 'borderline + definitive NASH' (steatosis, $P = 0.04$; inflammation, $P = 0.01$; and fibrosis, $P = 0.04$), whereas in the subgroup of 'definitive NASH', M30 only showed an association with fibrosis ($P = 0.01$) (Fig. 1). In contrast, M65 was not associated with any histological parameter.

Diagnostic performance of serum markers

The diagnostic performance was only evaluated for those serum markers that had shown a significant association with histological injury variables. Tables 3 and 4 show the diagnostic accuracy of each marker.

It is assumed that the AUROC of a marker must be equal to or greater than 0.800 to be considered a less invasive test as good as a liver biopsy to evaluate liver damage [22]. Under this assumption, HA showed good performance (AUROC: 0.928, NPV: 100) for significant fibrosis in NAFLD, both in the subgroup of patients with 'borderline + definitive NASH' (AUROC: 0.924, NPV: 100) as well as in patients with 'definitive NASH' (AUROC: 0.929, NPV: 100) (Table 3).

However, despite the association of M30 with both steatosis and inflammation severity, the AUROC values were very low, but it showed good performance in predicting significant fibrosis in NAFLD (AUROC: 0.848, NPV: 91.3) (Table 4). The good performance of M30 in predicting significant fibrosis was also observed in the subgroups 'borderline + definitive NASH' (AUROC:0.852) and 'definitive NASH' (AUROC: 0.844) (Table 4).

The whole series of NAFLD cases with $F \geq 2$ were categorized correctly according to the HA cutoff values for significant fibrosis, whereas seven (25%) out 28 patients with $F < 2$ were misclassified as false positive (FP). In the 'borderline + definitive NASH' subgroup, 25 patients were classified correctly [six patients were true positive (TP) and 19 patients were true negative (TN)], but five were classified in the wrong group (FP). However, in the 'definitive NASH' subgroup, 15 patients were identified correctly (four TP, 11 TN), but three cases were FP. In accordance with the high NPV and considering that the misclassified cases were FP, only those patients with HA levels below the cutoff value could be diagnosed without significant fibrosis (61.76% NAFLD, 63.33% 'borderline + definitive NASH', and 61.11% 'definitive NASH' patients). Therefore, those cases with HA values higher than the cutoff could not avoid liver biopsy (Tables 5 and 6).

According to the M30 cutoff value for significant fibrosis, 30 NAFLD patients were identified correctly (four patients were TP and 26 patients were TN), but four patients failed [two FP, two false negative (FN)]. In the 'borderline + definitive NASH' subgroup, 27 cases were categorized accurately (four TP, 23 TN), whereas three were classified wrongly (one FP, two FN). Finally, in the 'definitive NASH' subgroup, 17 cases were identified correctly (three TP, 14 TN) and one was an FN. Although more patients were classified correctly with M30 than with HA (Table 5), the FN and NPV were lower with HA; thus, M30 may be a good choice for use as a single marker when HA is not available.

Conclusively, HA and M30 were evaluated either together or sequentially. When both marker cutoffs were considered in combination, only those patients with concordant results (negative or positive for both markers) were correctly categorized (71% NAFLD, 79% 'borderline + definitive NASH', 77% 'definitive NASH') (Table 5). However, the sequential analysis considered HA as the first line because of its high NPV; thus, only those cases with HA levels higher than the cutoff would continue to M30 evaluation. With this algorithm, those cases that were categorized correctly (i) the negative ones for HA and (ii) the positive ones for HA, followed by those positive for M30 (78% NAFLD, 85% 'borderline + definitive NASH', 82% 'definitive NASH' of cases) (Table 5). Finally, only those patients with discordant results by either of the chosen approaches would not avoid liver biopsy and they should performed liver biopsy to know their fibrosis severity (Table 6).

Discussion

It has been proposed that a liver biopsy is needed to arrive at a conclusive diagnosis of NASH [23], but it is well known that besides the risks related to an invasive procedure, it has been linked to sampling error and patient care costs, which could be onerous in underdeveloped countries [24]. Thus, the development of reliable noninvasive markers and tests that can accurately predict the presence of advanced disease is urgently needed. Among other strategies, serum aminotransferases, aspartate aminotransferase-to-platelet ratio (APRI), and AST–ALT ratio have been proposed, but liver aminotransferases are not appropriate to be applied in a single test [25]. In line with this, in our cohort, APRI and AST–ALT ratio were calculated as alternative hallmarks of liver fibrosis; however, these approaches did not improve the diagnostic accuracy performance of the other markers (Supplementary Table 1, Supplemental digital content 1, *http://links.lww.com/EJGH/A260*). Other authors have combined both biochemical and clinical issues (i.e. FIB-4, BARD, NFS, Fibrotest) to predict fibrosis severity, whereas others have combined these with specific serum fibrosis markers [i.e. NASH Test, Fibrometer, Linköping University-Karolinska

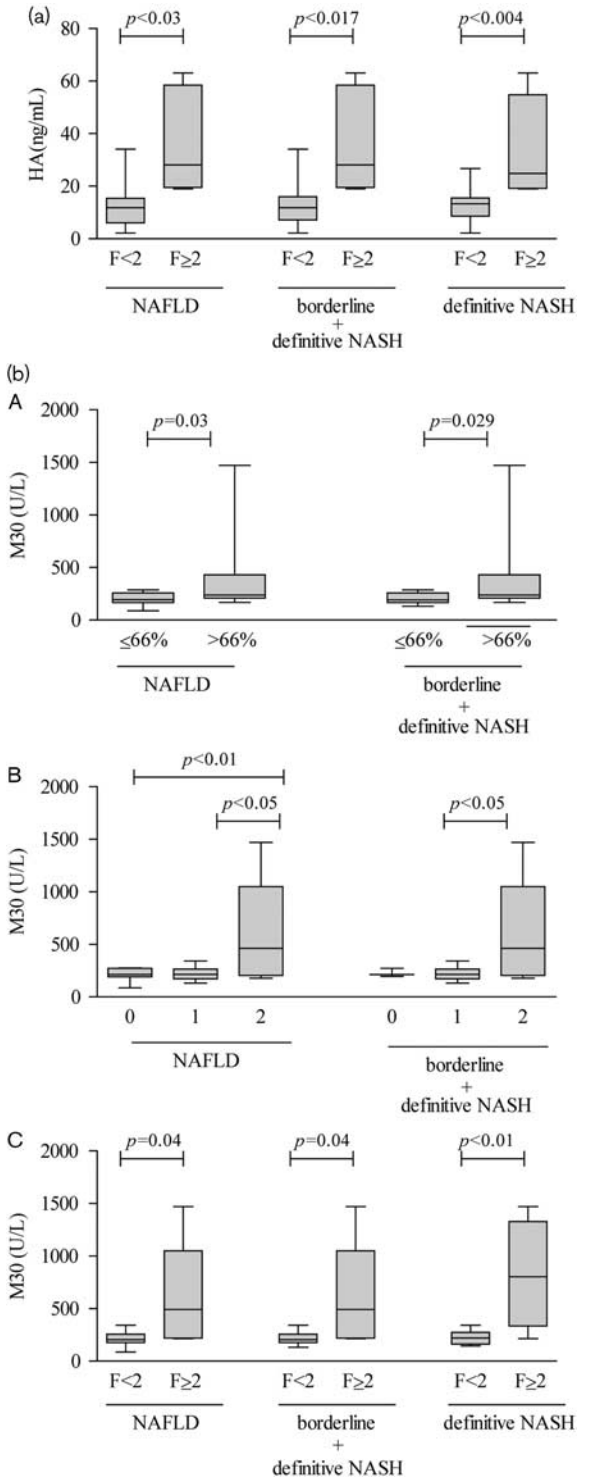

Fig. 1. (a) Serum HA levels related to fibrosis stages. (b) Serum M30 levels related to: A: steatosis; B: inflammation; and C: fibrosis severity. Horizontal lines within each box represent the median, and the lower and upper borders of the box show the interquartile range. The vertical lines from the ends of each box show the extreme data points. Significant fibrosis: F ≥ 2. Steatosis: grades 0, 1, and 2 (< 66% of cells) versus score 3 (>66%). Lobular inflammation: scores 0 (0 foci), 1 (< 2 foci), and 2 (2–4 foci). HA, hyaluronic acid; M30, caspase-cleaved cytokeratin-18; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

Institute (LINKI)]. However, these calculation systems are difficult and cumbersome for routine use [2,25,26]. However, noninvasive techniques such as ultrasound, computed tomography, MRI, and proton magnetic resonance spectroscopy can detect hepatic steatosis, but cannot consistently differentiate simple steatosis from NASH [25]. Moreover, these techniques are expensive and restricted to research centers as special equipment and trained staff are needed to perform these techniques [2,25]. In summary, when attempting to avoid liver biopsy, there is no consensus on strategies for noninvasive biomarkers; therefore, validated studies, especially in underdeveloped countries, should be carried out in prospective observational studies as well as in populations of different ethnicities and geographical locations [2] as the prevalence of obesity in addition to the progression of histological liver damage associated with NASH show significant ethnic disparities [27].

Many authors have explored TIMP-1 and HA as potential noninvasive tools to predict fibrosis in many liver diseases [5,28–31]. Most of them considered the biomarkers as a combined panel called the ELF test, which involved TIMP-1, HA, and aminoterminal peptide of procollagen III [28,32,33]. This test showed good diagnostic performance in predicting advanced stages of fibrosis; however, its availability worldwide is limited, which represents a pitfall for undeveloped countries [11]. Notably, HA levels seemed to be related to liver fibrosis progression as a single marker, not as a panel component. It is noteworthy that in contrast to the recent results of Mizuno *et al.* [31], who proposed that HA showed no evidence of predictive value in early fibrosis, in our adult NAFLD cohort, HA was associated strongly with significant fibrosis stages with good diagnostic accuracy, even when grouping the cases into either 'borderline + definitive NASH' or 'definitive NASH'. In agreement with this, Suzuki *et al.* [34] have previously determined the reliability of HA in predicting the severity of hepatic fibrosis in NAFLD patients. They described that HA was useful for predicting severe fibrosis (≥3) (AUROC: 0.9, 95% confidence interval: 0.83–0.97), but its efficacy for significant fibrosis could not be evaluated because of the limited number of patients with this stage of fibrosis [34]. Therefore, the results obtained in our study confirmed the observations of Suzuki *et al.* [34] as in our cohort, significant fibrosis are represented. Kaneda *et al.* [35] also reported HA to have an AUROC, NPV, sensitivity, and specificity of 0.97, 100, 100, and 89%, respectively, for detecting severe fibrosis, and Lesmana *et al.* [36] and Yoneda *et al.* [37] also proved the ability of HA to differentiate between mild (F1–F2) and advanced fibrosis (F3–F4).

Recently, Lykiardopoulos *et al.* [26] developed a new noninvasive model (LINKI) for predicting fibrosis in NAFLD patients. The LINKI model was designed as different mathematical combinations of certain parameters named LINKI-1 (which includes HA, AST, glucose, and age), LINKI-2a, LINKI-2b, and LINKI-2c (which include HA, AST, glucose, age, and platelet count). All these LINKI algorithms showed higher AUROCs compared with other previously published serum fibrosis algorithms (FIB-4, enhanced liver fibrosis, APRI, NAFLD fibrosis score, APRI), particularly to predict advanced fibrosis. In line with this, in our cohort, LINKI-1, LINKI-2a, LINKI-2b, and LINKI-2c were calculated and the AUROCs for significant fibrosis were compared. Although all of them

Table 3. Diagnostic accuracy of HA for significant fibrosis

	Significant fibrosis (F ≥ 2)						
	AUROC	95% CI	Cutoff (ng/ml)	Sensitivity (%)	Specificity (%)	PPV	NPV
NAFLD patients	0.928	0.768–0.990	16.38	100	82.61	60.0	100
Borderline + definitive NASH patients	0.924	0.766–0.989	17.96	100	83.33	60.0	100
Definitive NASH patients	0.929	0.705–0.996	16.17	100	85.71	66.7	100

AUROC, area under the receiver operating characteristic curve; CI, confidence interval; HA, hyaluronic acid; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NPV, negative predictive value; PPV, positive predictive value.

Table 4. Diagnostic accuracy of M30 for steatosis, inflammation, and significant fibrosis

	AUROC	95% CI	Cutoff (U/l)	Sensitivity (%)	Specificity (%)	PPV	NPV
Steatosis							
NAFLD patients	0.709	0.508–0.864	196.38	85.71	57.14	66.7	80.0
Borderline + definitive NASH patients	0.721	0.503–0.883	196.38	85.71	60.00	75.0	75.0
Inflammation							
NAFLD patients	0.553	0.355–0.740	343.13	33.33	100	100	84.6
Borderline + definitive NASH patients	0.722	0.503–0.884	343.13	50.00	100	100	85.7
Significant fibrosis (F ≥ 2)							
NAFLD patients	0.848	0.663–0.955	284.73	66.67	95.45	80.0	91.3
Borderline + definitive NASH patients	0.852	0.648–0.962	284.73	66.67	94.44	80.0	89.5
Definitive NASH patients	0.844	0.528–0.982	343.13	75.00	100	100	88.9

AUROC, area under the receiver operating characteristic curve; CI, confidence interval; M30, caspase-cleaved cytokeratin-18; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NPV, negative predictive value; PPV, positive predictive value.

Table 5. Cases classified correctly using HA and M30

	HA (%)[a]	M30 (%)[a]	HA + M30 (%)[b]	HA − M30 (%)[c]
NAFLD patients	79	88	71	78
Borderline + definitive NASH patients	83	90	79	85
Definitive NASH patients	83	94	77	82

HA, hyaluronic acid; M30, caspase-cleaved cytokeratin-18; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.
[a]True positive + true negative.
[b]Cases with concordant results considering the cutoff values of both markers.
[c]Applying HA and M30 in a sequential form. The result of HA were analyzed in the first time, then cases considered positive according to the HA cutoff were evaluated by M30. Therefore, those cases correctly categorized were (i) the negative ones for HA and (ii) the positive ones for HA and positive for M30.

Table 6. Percentage of patients in whom the biopsy could not be avoided after serum marker assessment

	HA (%)[a]	M30 (%)[a]	HA + M30 (%)[b]	HA − M30 (%)[c]
NAFLD patients	38	–	29	22
Borderline + definitive NASH patients	37	–	21	15
Definitive NASH patients	39	–	23	18

HA, hyaluronic acid; M30, caspase-cleaved cytokeratin-18; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.
[a]Cases with serum HA levels higher than the cutoff.
[b]Cases with discordant results considering both serum markers.
[c]Applying HA and M30 in a sequential form. Cases considered positive according to the HA cutoff were evaluated by M30.

showed good performance (AUROC > 0.80) for predicting significant fibrosis in NAFLD and also in 'borderline- + definitive NASH' and 'definitive NASH', these approaches did not improve the diagnostic accuracy performance of HA alone (Supplementary Table 2, Supplemental digital content 1, *http://links.lww.com/EJGH/A260*). Interestingly, on applying the LINKI algorithms in our cohort, the AUROCs obtained were better than the AUROC reported by Lykiardopoulos *et al.* [26] for significant fibrosis. However, in contrast to the AUROC reported for LINKI-2a, LINKI-2b, and LINKI-2c, in our cohort, a better diagnostic performance than LINKI-1 was found. Therefore, as Lykiardopoulos *et al.* [26] reported in their article, future studies should determine whether LINKI-2a, LINKI-2b and LINKI-2c are more reliable than LINKI-1 and which one shows the best diagnostic performance.

For TIMP-1, other groups reported similar observations of higher levels of TIMP-1 in serum samples from NAFLD patients compared with those of healthy participants [38]. Nevertheless, the usefulness of TIMP-1 as a marker of fibrosis severity was rejected in agreement with our previous study in a cohort of HCV chronically infected adult patients [39].

Finally, serum M30 was validated extensively as a single marker of NASH and was recognized as the most promising noninvasive test [7,28,40–44]. However, Cusi *et al.* [7] recently reported in a NAFLD cohort with an ethnic mix proper from Texas, USA (few African-Americans, most Mexican-Hispanics, a third of Whites), that the M30 value as a single marker might be less valuable than it has been assumed previously. In our study, M30 was significantly increased in NAFLD White patients and showed an association with liver damage. Indeed, the most relevant result was that it turned out to be a fibrosis biomarker with a high diagnostic accuracy, which was in agreement with the pioneering work carried out by Feldstein *et al.* in a White population [41,44]. However, the performance of M30 improved when it was combined in an algorithm with HA. These divergences reinforce the importance of carrying out studies that validate the diagnostic accuracy of M30 in different ethnicities, regions, and age groups as it may be useful for monitoring liver damage and disease progression.

For M65, the available data are limited and require further validation before integration into clinical practice [5,45,46]. Many authors reported that the M65 level correlated with fibrosis progression in NAFLD [45,47–49], which was not reproduced in our study. However, in agreement with Joka *et al.* [47], M65 could differentiate simple steatosis from healthy participants; thus, it may be a possible marker of early stages in NAFLD.

Finally, it worth mentioning that the present study has some limitations. First, this was in fact a pilot study with a limited number of participants, which makes it difficult to validate the utility of serum markers. However, the results obtained were similar to those reported in other larger adult cohorts. Second, only a few patients had severe fibrosis, which could have been a limiting factor for the ability of the markers to distinguish between mild and moderate/severe fibrosis. Third, as we did not take into account biopsy length and fragmentation, the potential for sampling error and understaging of fibrosis remains possible. Nonetheless, if it is assumed that ideally, a noninvasive liver fibrosis marker should be liver specific, easy to perform, reliable, reproducible, and inexpensive, the target proposed here possess these characteristics. The noninvasive biomarkers proposed here for follow-up of NAFLD fibrosis progression have some advantages such as lower cost than physical or patented (Fibrotest, Fibromax) methods, easy to use and interpret, and feasible in a facility of any primary care center of an underdeveloped country. The key to a robust prevention program will depend on the early individualization, treatment, and monitoring of high-risk patients by detecting disease-specific biomarkers [50]. They are essential for screening strategies applied to patients with fatty liver disease and for diagnosing patients with life-threatening NAFLD and NASH more quickly. This would enable classification and staging of disease using a simple blood test, thus avoiding a liver biopsy [50].

Finally, the evaluation of only HA and M30 may be enough to predict significant fibrosis as well as to evaluate fibrosis progression in NAFLD cases classified previously, according to liver biopsy, as borderline or definitive NASH. Moreover, if these markers were applied sequentially, better categorization of cases could be achieved (Tables 5 and 6). HA could be chosen as the first-line assay on the basis of its diagnostic accuracy, and then HA values above the cutoff could be re-evaluated according to the cutoff of M30. Consequently, only those cases rendering discordant results with values over each marker cutoff should not avoid liver biopsy.

Noninvasive markers are reliable tools for screening patients with fatty liver disease. They allow a quick and accurate diagnosis of patients with life-threatening NAFLD and NASH. Serum HA and M30 are straightforward tests that may be sufficient to predict significant fibrosis as well as to evaluate fibrosis progression even in a primary care center of an underdeveloped country. It would be useful to study larger cohorts in our region, perhaps in a multicenter project, to validate and confirm our findings. If these parameters are validated in the near future, they would be very easy to assess and interpret, as are AST and ALT nowadays; thus, this approach would be potentially translatable to the bedside.

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Conflicts of interest

There are no conflicts of interest.

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