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Non-Alcoholic Fatty Liver Disease: Biomarkers as diagnostic tools for liver damage assessment in adult patients from Argentina

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Corresponding Author:	Pamela Valva, PhD Ricardo Gutierrez Children Hospital Buenos Aires, ARGENTINA
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Ricardo Gutierrez Children Hospital
Corresponding Author's Secondary Institution:	
First Author:	Pamela Valva, PhD
First Author Secondary Information:	
Order of Authors:	Pamela Valva, PhD
	Daniela Rios
	Paola Casciato
	Adrían Gadano
	Omar Galdame
	Eduardo Mullen
	Gustavo Bertot
	Elena De Matteo
	María Victoria Preciado
Order of Authors Secondary Information:	
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Abstract:	Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease which prevalence has been constantly increasing linked to the obesity global epidemic. 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 way 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 [hyaluronic acid (HA) and tissue inhibitor of matrix metalloprotein inhibitor-1 (TIMP-1)] and cell-death markers [cytokeratin-18 (M65) and caspase-cleaved cytokeratin-18 (M30)], which correlate with liver injury in a NAFLD patients cohort. Liver biopsies and serum from 34 NAFLD adult patients were analyzed. Histological parameters were evaluated. HA, TIMP-1, M65 and M30 were measured in serum samples. HA showed 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 diagnostic accuracy evaluation demonstrated a good performance as less invasive

	markers of significant fibrosis of both HA (AUROC 0.928) and M30 (AUROC 0.848). In conclusion, biomarkers are essential tools that may provide a quick and accurate diagnosis to patients with life-threatening NAFLD and NASH. HA and M30, together or sequentially determined, demonstrated to be straightforward tests that may be enough to predict significant fibrosis even in a primary care centre of an underdeveloped country.
Additional Information:	
Question	**Response**
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Editorial office

September, 2017

Please find attached the manuscript entitled **Non-Alcoholic Fatty Liver Disease: Biomarkers as diagnostic tools for liver damage assessment in adult patients from Argentina,** along with six tables and one illustration to be considered for publication in *European Journal of Gastroenterology & Hepatology*. Additionally you will find two supplemental tables. Here after I include the responses to the reviewer which I will also attach under the "Response to Reviewers" in the manuscript submission system. I submit the revised version of the manuscript with active change track to make more clear the grammatical corrections made by the scientific English translator. In addition, the modified parts of the manuscript according the reviewer suggestions are in italic.

This manuscript has not been submitted or accepted for publication elsewhere. The present paper no concerns patients that were studied in other published work. All authors have contributed to, seen, and approved the final submitted version of the manuscript.

This study has the approval of the Ethics Board of Ricardo Gutierrez Children Hospital and is in accordance with the 1964 Declaration of Helsinki and its later amendments. A written informed consent was obtained from all the included patients after the nature of the procedure had been fully explained.

This work was funded by grants from the National Agency for Scientific and Technology Promotion (ANPCyT) (PICT2012Nº804, PICT2014Nº1144, PICT2014Nº1553) and H.A. Barceló Foundation-Medicine University (BA-MED 005). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. No additional external funding received for this study. Therefore, the authors disclose no financial conflicts of interest.

Sincerely yours,
Dra. Pamela Valva, PhD

Gallo 1330 (1425) Bs As, TE: 4962-9138, Fax (54 11) 4963-4122

RESPONSE TO REVIEWER

REVIEWER COMMENTS:

Reviewer: The manuscript, "Non-Alcoholic Fatty Liver Disease: Biomarkers as diagnostic tools of liver damage in adult patients from Argentina" by Valva et al. searchs for correlation between serum Noninvasive markers and liver injury in NAFLD patients. Markers selected by the authors have been studied by others groups being associated with disease progression and the results found corroborate previous data from literature. My comments are as follows:

1- The text should be revised for grammatical errors.

Response: This new version of the manuscript has been reviewed by a scientific English translator who lives in Argentina. I submit the revised version of the manuscript with active change track to make more clear the grammatical corrections made by the scientific English translator.

2- Is there any information about associated morbidities of patients enrolled? i.e. patients with type 2 diabetes or metabolic syndrome?

Response: we got additional data from the clinical records of the patients enrolled in our study. Briefly, HOMA-IR median was 4.89 (range: 1.7-10.10) for NAFLD patients, while it was 3.56 (range: 1.97-7.87), 4.95 (range: 2.77-10.10) and 4.70 (range: 1.70-8.64) for not NASH, Borderline NASH and Definitive NASH, respectively. Type II Diabetes was present in 55.88% of NAFLD patients (25% not NASH, 75% Borderline NASH and 50% Definitive NASH). On the other hand, 26.47% of NAFLD patients have Hypertension (25% not NASH, 75% Borderline NASH and 27.78% Definitive NASH). Finally, according to the criteria established by Alberti KG, et al. (*Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity*. Circulation 2009;120:1640–5), Metabolic Syndrome affected 47.06% of NAFLD cases (25% not NASH, 58.33% Borderline NASH and 80% Definitive NASH). In the submitted revised version of the manuscript, we included in Table 1 the records about HOMA-IR, Type II Diabetes, Hypertension and Metabolic Syndrome.

3- The potential use of serological markers as prediction tools for the outcome of NAFLD has been performed previously in prospective cohort studies, and longitudinal observational studies; in this scenario, it is recommended to discuss the main limitations of this study.

Response: As Lykiardopoulos B et al mentioned in their article: *Development of Serum Marker Models to Increase Diagnostic Accuracy of Advanced Fibrosis in Nonalcoholic Fatty Liver Disease: The New LINKI Algorithm Compared with Established Algorithms* (Plos One, DOI:10.1371/journal.pone.0167776), a limitation of studies assessing serum fibrosis markers is that liver biopsy is used as reference standard for evaluation of

hepatic fibrosis. As we have also stated in the introduction and discussion sections of our manuscript, important limitations of liver biopsy are namely, its known sampling variability, the subjective nature of fibrosis staging and the high inter-observer variability. The limitations of liver biopsy probably impair the potential of fibrosis algorithms to reach the state of perfect surrogate fibrosis markers.

Particularly, our present study has some extra limitations. First, it was in fact a pilot study with a limited case number that makes it difficult to validate serum markers utility. However, the obtained results were similar to the ones reported in other larger adult cohorts. Second, only a few patients displayed severe fibrosis which could have been a limiting factor for the ability of the markers to distinguish between mild and moderate/severe fibrosis. Third, since we did not take into account biopsy length and fragmentation, the potential for sampling error and understaging of fibrosis remains possible. Anyway, if it is assumed that ideally, a noninvasive liver fibrosis marker should be liver-specific, easy to perform, reliable, reproducible, and inexpensive; the molecules here proposed possess these characteristics. The noninvasive biomarkers proposed here to follow up NAFLD fibrosis progression display some advantages such as lower cost than physical or patented methods, simply performed and interpreted and feasible to carry out in a facility of any primary care center of an underdeveloped country.

According with the reviewer suggestion, the limitations of our work were included in the Discussion Section of the submitted revised version of the manuscript (pages 18-19 in revised version of the manuscript but pages 19-20 in the version of active change track).

4- It has been recently reported by Lykiardopoulos et. al. the LINKI algorithm, which combines indirect fibrosis markers as: age, glucose, and AST and the direct fibrosis marker HA demonstrating to have the best diagnostic accuracy. It is recommended to be included in the discussion section, as it corroborates the author´s findings.

Response: As the reviewer mentioned, Lykiardopoulos et al developed a new noninvasive model (Linköping University-Karolinska Institute; 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 demonstrated higher AUROCs compared to other previously published serum fibrosis algorithms (FIB-4, ELF, APRI, NAFLD fibrosis score, APRI), particularly to predict advanced fibrosis. In line with this, as the reviewer suggested, we calculate LINKI-1, LINKI-2a, LINKI-2b and LINKI-2c in our cohort and the AUROCs for significant fibrosis were compared. Although all of them demonstrated 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. Interestingly, when applying the LINKI algorithms in our cohort the AUROCs obtained were better than the AUROC described by Lykiardopoulos et al for significant fibrosis. On the other hand, in contrast to the reported AUROC performance for LINKI-2a, LINKI-2b, and LINKI-2c in our cohort they showed a better diagnostic performance than LINKI-1. Therefore, as Lykiardopoulos et al mentioned in their article future studies will determine if they are more stable than LINKI-1 and which one has the best diagnostic performance.

Although, the LINKI results in our cohort did not exactly reflect Lykiardopoulos et al findings, it demonstrated promising results. According with the reviewer suggesting, we included this observation in the Discussion Section of the submitted revised version of the manuscript (page 17 in revised version of the manuscript but page 18 in the version of active change track) and the LINKI AUROC results of our cohort are presented in S2 Table.

5- Lines 173-174. Authors must mention the supporting criteria for grouped the cases for analysis in the set "healthy control+not NASH".

Response: As we describe in the result section, the four markers were evaluated in serum samples of NAFLD patients as well as in healthy donors. So, when we compared the serum levels of the evaluated markers between NAFLD patients and healthy donors significant differences were observed. However, in order to deeply describe the NAFLD population characteristics, each marker value was also compared through the 3 histological subgroups of NAFLD (not NASH, borderline, definitive NASH). Interestingly, similar results were observed when compared not NASH and healthy donor [except for M65 “not NASH” vs healthy donors (p=0.002)] as well as when comparing borderline and definitive NASH. So, this observation prompted us to group the cases in two sets “healthy control+not NASH” and “borderline+definitive NASH”. When analyzing TIMP-1, HA, M30 and M65 levels significant differences between groups for all the studied markers were observed. It is important to highlight that this arrangement was only performed in order to deeply describe the study populations and the levels of the four markers in each NAFLD subgroup, further on the analysis of the serum biomarkers as possible diagnostic tools was performed related to liver damage (analyses that did not include healthy controls).

From the point of view of the biological concerns, to group the cases in the sets "healthy control+not NASH" and “borderline+definitive NASH” makes sense considering that liver damage in terms of inflammation and fibrosis is the major parameter that differentiates borderline and definitive NASH from not NASH and controls . To clarify this point, the submitted revised version of the manuscript was rewritten in the Result Section (page 11 in revised version of the manuscript but page 12 in the version of active change track).

6- Lines 239-254 Discussion. It is preferable to include this text in "introduction section" as it seems to be more suitable.

Response: The reviewer observation is appropriate. Then, this paragraph was moved to the introduction section (pages 5-6 in both revised version of the manuscript and the version of active change track).

7- Formatting of Table 1 is confusing and does not allow a fluid interpretation of the results. Authors mention in values of Transaminases ALT (IU/l), median (range), but they do not include median. For lipid profile in Cholesterol they include range and only make mention to mg/dl.

Response: We agree with the reviewer that the Table 1 format is confuse. To clarify it and to allow an easy interpretation of the studied group characteristics we modified the Table 1. Lines and shadows were added to

separate information. Moreover, related to transaminases and lipid profile, we modified the way of expressing the results to avoid confusions. The ALT and AST data are expressed as median IU/l (range) while cholesterol and triglycerides as median mg/dl (range). Moreover, in the last version of the manuscript the AST/ALT ratio included the information of "median (range)" which was omitted in the previous one. Finally, according to reviewer suggestion we included the information about associated morbidities of the studied patients in Table 1.

8- For healthy subjects authors only mentioned that they were "without known systemic or liver disease and with normal biological and virological liver test", it is important to mention which test were performed in order to discard any injury associated with NAFLD.

Response: Healthy subject were examined by the same hepatologist team that follows the patients of the study. No clinical or biochemical evidence of liver disease or known medical illness at recruitment was observed in healthy subjects. All of them have normal abdominal ultrasonography. The same parameters which were evaluated in patients were taken into account in the healthy group. Healthy subjects turn out to have no causes of liver disease, autoimmune, genetic or endocrinologic diseases as well as hepatocellularcarcinoma (HCC). Furthermore, they were all negative for HBV, HCV and HIV as it was evidenced by negative serological markers. Routine clinical biochemical analyses included complete blood count and analysis of prothrombintime, transferrin, iron, transferrin saturation, ferritin, ALT, AST, ALP, GGT, bilirubin, fasting plasma glucose, total cholesterol, high-density lipoprotein, low density lipoprotein, and triglycerides. Blood pressure, waist circumference, bodyweight, and height were measured. Therefore, the clinical and biochemical parameters evaluated were under normal values. Finally, the alcohol consumption of the healthy group was low (men <30 g/day; women <20 g/day). According to reviewer suggestion, this point was clarified in the Material and Methods Section of submitted revised version of the manuscript (page 8 in revised version of the manuscript but page 9 in the version of active change track).

Non-Alcoholic Fatty Liver Disease: Biomarkers as diagnostic tools for liver damage assessment in adult patients from Argentina

Pamela Valva[1], Daniela Rios[1], Paola Casciato[2], Adrián Gadano[2], Omar Galdame[2], Eduardo Mullen[3], Gustavo Bertot[4], Elena De Matteo[1], María Victoria Preciado[1].

[1] Laboratory of Molecular Biology, Pathology Division, Ricardo Gutiérrez Children's Hospital; Buenos Aires, Argentina.

[2] Liver Unit, Hospital Italiano de Buenos Aires; Buenos Aires, Argentina.

[3] Pathology Division, Hospital Italiano de Buenos Aires; Buenos Aires, Argentina.

[4] H.A. Barceló Foundation-Medicine University, Buenos Aires, Argentina.

Short title: Non-Alcoholic Fatty Liver Disease Biomarkers

Corresponding author:

Valva Pamela; Instituto Multidisciplinario de Investigaciones en Patologías Pediátricas (IMIPP- CONICET-GCBA) Laboratorio de Biología Molecular, División Patología, Hospital de Niños Ricardo Gutiérrez, Buenos Aires, Argentina. Gallo 1330, C1425EFD, Buenos Aires, Argentina. Fax: +54-11-4962-4122. E-mail: valvapamela@yahoo.com

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Funder's had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

The authors disclose no financial conflicts of interest.

Abstract

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease which prevalence has been constantly increasing linked to the obesity global epidemic. 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 way 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 [hyaluronic acid (HA) and tissue inhibitor of matrix metalloprotein inhibitor-1 (TIMP-1)] and cell-death markers [cytokeratin-18 (M65) and caspase-cleaved cytokeratin-18 (M30)], which correlate with liver injury in a NAFLD patients cohort.

Liver biopsies and serum from 34 NAFLD adult patients were analyzed. Histological parameters were evaluated. HA, TIMP-1, M65 and M30 were measured in serum samples.

HA showed 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 diagnostic accuracy evaluation demonstrated a good performance as less invasive markers of significant fibrosis of both HA (AUROC 0.928) and M30 (AUROC 0.848).

In conclusion, biomarkers are essential tools that may provide a quick and accurate diagnosis to patients with life-threatening NAFLD and NASH. HA and M30, together or sequentially determined, demonstrated to be straightforward tests that may be enough

to predict significant fibrosis even in a primary care centre of an underdeveloped country.

Key Word: NAFLD, HA, TIMP-1, M30, M65

Introduction

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

Obesity generates a comprehensive proinflammatory state with high risk for metabolic comorbidities which contributes to progressively enlarge the series of patients that will develop NASH, NASH-related cirrhosis, decompensated liver disease and hepatocellular carcinoma (HCC) [3]. The emergence of this cohort is on the horizon and will introduce a significant disease burden in the field of liver transplantation. At the present time, NASH is the third most common indication for liver transplantation and it is expected to climb till to become the leading one over the next decades [4]. Strikingly, current practice guidelines do not support NAFLD screening in patients at risk in spite of its high prevalence and implicit progression to end-stage liver disease [5]. In addition, due to the elevate costs of the available tests, the liver biopsy risks 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]. So, a present

challenge is to distinguish between simple steatosis vs. NASH, since the latter increases the chances of liver disease progression [7].

The histological characterization of NAFLD ranges from simple steatosis to steatosis accompanied by inflammation and other evidences of cellular injury (NASH). NAFL encompasses: a) steatosis, b) steatosis with lobular or portal inflammation, without ballooning, or c) steatosis with ballooning but without inflammation [8]. NASH diagnosis requires histopathological evaluation to assess joint steatosis presence, ballooning and lobular inflammation [8]. Perisinusoidal fibrosis is also frequent, but it is not a diagnostic criteria. Fibrosis progression is the most significant prognostic factor that correlates with liver-related outcomes and death [9]. In this regard, 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 applied 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 inter-observer differences are frequently encountered [10]. Therefore, a growing medical need is the development of noninvasive tests that can predict initial stage and progression of liver disease over time in an accurate way [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 (ECM), 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), follow by their degradation by the matrix metalloproteins (MMPs). Moreover, MMP are also inhibited by tissue inhibitors of metalloproteins (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 be developed 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 CK-18, 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 study purposes were to evaluate the presence of matrix deposition markers [hyaluronic acid (HA) and tissue inhibitor of matrix metalloprotein inhibitor-1 (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 analyzed their diagnostic accuracy to be used as possible markers of liver damage in primary care centers in an underdeveloped country.

Material and Methods

Patients and samples

Thirty-four NAFLD Caucasian 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 (HCC), HCV, HBV and/or HIV infection. Routine clinical biochemical analyses included complete blood count and analysis of prothrombin time, transferrin, iron, transferrin saturation, ferritin, ALT, AST, ALP, GGT, 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 time of biopsy were tested.

A group of 20 adult healthy subjects with no clinical or biochemical evidence of liver disease or known medical illness at recruitment were included as controls. The same parameters which were evaluated in patients were taken into account in the healthy group. The clinical and biochemical parameters evaluated were under normal values. All healthy subjects were negative for HBV, 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 subject was included.

This study has the approval of the Ethics Board of Ricardo Gutierrez Children Hospital and is in accordance with the 1964 Declaration of Helsinki and its later amendments. A written informed consent was obtained from all patients prior to their inclusion in the study.

Histological Analysis

Two independent pathologists evaluated the histological sections in a blind manner according to the NAFLD scoring system proposed by the National Institute of Diabetes and Digestive and Kidney Disease NASH Clinical Research Network (12): a NAFLD activity score of ≥5 corresponds to a diagnosis of "definitive NASH", a score of 3-4 to "borderline NASH", and a score of ≤2 to "not NASH or simple steatosis". Fibrosis stage was also measured. Fibrosis stages ≥ 2 were considered as significant fibrosis.

Quantitative measurement of TIMP-1 and HA

Serum TIMP-1 and HA were determined by ELISA (Quantikine, R&D System Inc) according to the manufacturer's instructions.

Quantitative measurement of M30 and M65

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

Statistical analysis

GraphPad InStat software, version 3.05 was used. The Mann–Whitney U-test and unpaired t-test, ANOVA or Kruskal Wallis test were used to compare sets of data. P values <0.05 were considered significant.

The diagnostic value was assessed by the area under the receiver operating characteristic curves (AUROC). Cut-off value for the diagnosis was determined as the

maximal value at the sum of the sensitivity (Se) and specificity (Sp). AUROC, cut-off values, positive predictive values (PPV) and negative predictive values (NPV) were determined using the MedCalc demo statistical software.

The number of correctly classified cases by means of serum markers and the percentage of cases that could have not avoided the biopsy procedure were assessed.

Results

Clinical and liver biopsy findings

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 as “definitive NASH”, 35.29% as “borderline NASH” and 11.77% as “not NASH”.

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

The four markers displayed higher levels in NAFLD patients than in healthy subjects (Table 2). However, in order to deeply describe the NAFLD population characteristics, each marker value was also compared through the 3 histological subgroups of NAFLD (“not NASH”, “borderline NASH”, “definitive NASH”). Interestingly, but in agreement with inflammation and fibrosis components, similar results were observed when compared not NASH and healthy subjects [except for M65 “not NASH” vs healthy subjects (p=0.002)] as well as when comparing borderline and definitive NASH. So, this observation prompted us to group the cases in two sets “healthy subjects+not NASH” and “borderline+definitive NASH”. When analyzing TIMP-1, HA, M30 and M65 levels significant differences between groups for all the studied markers were observed (Table 2).

Regarding serum biomarkers role 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, since 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 by 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 displayed association with steatosis, inflammation and fibrosis severity. That is to say, M30 level was elevated in 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 conserved when analyzing "borderline+definitive NASH" (steatosis p=0.04; inflammation p=0.01; and fibrosis p=0.04), while in the subgroup of "definitive NASH" M30 only displayed 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 demonstrated significant association with histological injury variables. Tables 3 and 4 show the diagnostic accuracy of each marker.

It is assumed 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 demonstrated a 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 with "definitive NASH" (AUROC: 0.929, NPV: 100) (Table 3).

On the other hand, despite the M30 association with both steatosis and inflammation severity, the AUROC values were very low, but it demonstrated a good performance to predict significant fibrosis in NAFLD (AUROC: 0.848, NPV: 91.3) (Table 4). The

performance of M30 was extended to the subgroups "borderline+definitive NASH" (AUROC: 0.852) and "definitive NASH" (AUROC: 0.844) (Table 4).

The whole series of NAFLD cases with F≥2 were correctly categorized according to the HA cut-off values for significant fibrosis, while 7 out 28 (25%) patients with F<2 were misclassified as False Positive (FP). In the "borderline+definitive NASH" subgroup, 25 patients were correctly classified (6 patients were TP and 19 patients were TN), but 5 was classified in the wrong group (FP). While in the "definitive NASH" subgroup, 15 patients were correctly identified (4 TP, 11 TN), but 3 cases were FP. In accordance with the high NPV and considering that the misclassified cases were FP, only those patients with HA levels under the cut-off value could be diagnosed without significant fibrosis (61.76% NAFLD, 63.33% "borderline+definitive NASH" and 61.11% "definitive NASH" patients). In consequence, those cases with HA values higher than the cut-off cannot avoid liver biopsy (Table 5 and 6).

According to the M30 cut-off value for significant fibrosis, 30 NAFLD patients were correctly identified (4 patients were TP and 26 patients were TN) but 4 patients failed [2 FP, 2 False Negative (FN)]. In the "borderline+definitive NASH" subgroup 27 cases were accurately categorized (4 TP, 23 TN) while 3 were wrongly classified (1 FP, 2 FN). Finally, in the "definitive NASH" subgroup, 17 cases were correctly identified (3 TP, 14 TN), and 1 resulted a FN. Although more patients were correctly classified with M30 than with HA (Table 5), the FN and NPV were lower with HA; so M30 came off a good choice to be used as a single marker when HA is not available.

Conclusively, HA and M30 were evaluated either together or sequentially. When both marker cut-offs were considered jointly, only those patients with concordant results

(negative or positive for both markers) were assumed as well assigned (71% NAFLD, 79% “borderline+definitive NASH”, 77% “definitive NASH”) (Table 5). On the other hand, the sequential analysis considered HA as the first line due to its high NPV, so only those cases with HA level higher than the cut-off would proceed to M30 evaluation. With this algorithm, those cases correctly sorted were: 1) the negative ones for HA and 2) the positive ones for HA followed by 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 (Table 6).

Discussion

It has been proposed that a liver biopsy is needed to arrive to a conclusive diagnosis of NASH [23], but it is well known that besides the risks related to an invasive procedure, it has been linked with sampling error and patient care costs which could be onerous in underdeveloped countries [24]. Thus, the emergence of trustworthy noninvasive markers and tests that can accurately foretell the presence of advanced disease is an imperious need to fulfil. Among other strategies, serum aminotransferases, AST-to-platelet ratio (APRI) and AST- ALT ratio (AAR) have been proposed, but liver aminotransferases are not appropriate to be applied in a single test way [25]. In line with this, in our cohort, APRI and AAR were calculated as alternative hallmarks of liver fibrosis; however, these approaches did not improve the diagnostic accuracy performance of the other markers (S1 Table). Other authors have combined both biochemical and clinical issues (i.e. Fib-4, BARD, NFS, Fibrotest) to predict fibrosis severity; while others have brought these together with specific serum fibrosis markers (i.e. NASH Test, Fibrometer, LINKI) to do so. However, these calculation systems are difficult and burdensome to be routinely performed [2, 25, 26]. On the other hand, noninvasive techniques such as ultrasound, computed tomography, magnetic resonance imaging, and proton magnetic resonance spectroscopy can detect hepatic steatosis, but cannot consistently discriminate simple steatosis from NASH [25]. Moreover, these techniques are expensive and restricted to research centers since special equipment and trained staff are needed to perform these techniques [2, 25]. In summary, when trying to avoid liver biopsy there are no consensus on strategies for noninvasive biomarkers, therefore validated studies, especially in underdeveloped countries, are expected in prospective observational studies as well as in populations of different ethnicity and

294 geographical locations [2], since the obesity prevalence in addition to the progression of
295 histological liver damage associated with NASH display significant ethnic disparities
296 [27].
297 Many authors explored TIMP-1 and HA as potential noninvasive tools to predict
298 fibrosis in many liver diseases [5, 28-31]. Most of them considered the biomarkers as a
299 combined panel named ELF test, which involved TIMP-1, HA, and amino-terminal
300 peptide of procollagen III [28, 32, 33]. This test demonstrated good diagnostic
301 performance to predict advance stages of fibrosis; however, its availability worldwide is
302 limited, which represents a pitfall for undeveloped countries [11]. Notably, HA levels
303 seemed to be related to liver fibrosis progression as a single marker, not as a panel
304 component. Of note, in contrast to the recent results of Mizuno et al [31] who proposed
305 that HA depicted no evidence of predictive value in early fibrosis, in our adult NAFLD
306 cohort HA was strongly associated with significant fibrosis stages with a good
307 diagnostic accuracy, even when grouping the cases in either “borderline+definitive
308 NASH” or “definitive NASH”. In accordance, Suzuki et al [34] have previously
309 determined the reliability of HA to predict the severity of hepatic fibrosis in NAFLD
310 patients. They described that HA was useful for predicting severe fibrosis (≥3)
311 (AUROC:0.9, 95% CI:0.83, 0.97), but its efficacy for significant fibrosis could not be
312 evaluated due to the limited number of patients with this stage of fibrosis [34].
313 Therefore, the results obtained in our study complemented Suzuki el at. observations
314 since in our cohort significant fibrosis are represented. Kaneda et al [35] also
315 demonstrated HA to have an AUROC, NPV, Se and Sp of 0.97%, 100%, 100%, and
316 89%, respectively, for detecting severe fibrosis, and Lesmana et al and Yoneda et al [36,
317 37] also proved HA ability to differentiate between mild (F1-2) and advanced fibrosis

(F3-4).

Recently, Lykiardopoulos et al [26] developed a new noninvasive model (Linköping University-Karolinska Institute; 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 demonstrated higher AUROCs compared to other previously published serum fibrosis algorithms (FIB-4, ELF, 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 demonstrated 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 (S2 Table). Interestingly, when applying the LINKI algorithms in our cohort the AUROCs obtained were better than the AUROC described by Lykiardopoulos et al for significant fibrosis [26]. On the other hand, in contrast to the reported AUROC performance for LINKI-2a, LINKI-2b, and LINKI-2c in our cohort they showed a better diagnostic performance than LINKI-1. Therefore, as Lykiardopoulos et al mentioned in their article future studies will determine if they are more stable than LINKI-1 and which one has the best diagnostic performance.

Concerning TIMP-1, other groups reported similar observations about the higher levels of TIMP-1 in serum samples from NAFLD patients compared with those of healthy subjects [38]. Nevertheless, TIMP-1 usefulness as a marker of fibrosis severity was

dismissed in accordance with our previous study in a cohort of HCV chronically infected adult patients [39].

Finally, serum M30 was extensively validated 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 Caucasians), that M30 value as a single marker might be of lesser utility than it has been previously assumed. In our study, M30 was significantly elevated in NAFLD Caucasian patients and displayed 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 pioneering work by Feldstein et al in Caucasian population [41, 45]. However, M30 performance improved when it was combined in an algorithm with HA. These divergences reinforced the importance to perform studies which validate the M30 diagnostic accuracy in different ethnicities, regions, and age groups since it may be useful for monitoring liver damage and disease progression.

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

Finally, it is worthwhile to mention that, the present study has some limitations. First, it was in fact a pilot study with a limited case number that makes it difficult to validate serum markers utility. However, the obtained results were similar to the ones reported in

other larger adult cohorts. Second, only a few patients displayed severe fibrosis which could have been a limiting factor for the ability of the markers to distinguish between mild and moderate/severe fibrosis. Third, since we did not take into account biopsy length and fragmentation, the potential for sampling error and understaging of fibrosis remains possible. Anyway, if it is assumed that ideally, a noninvasive liver fibrosis marker should be liver-specific, easy to perform, reliable, reproducible, and inexpensive; the molecules here proposed possess these characteristics. The noninvasive biomarkers proposed here to follow up NAFLD fibrosis progression display some advantages such as lower cost than physical or patented (Fibrotest™, Fibromax™) methods, simply performed and interpreted and feasible to carry out 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 solely evaluation of HA and M30 may be enough to predict significant fibrosis as well as to evaluate fibrosis progression in NAFLD cases previously classified, according to liver biopsy, as borderline or definitive NASH. Moreover, if these markers were applied sequentially, a better sorting of cases could be achieved (Table 5 and 6). HA would be chosen as the first line assay according to its diagnostic accuracy, and then those HA values over the cut-off could be re-evaluated according to M30 cut-off. Consequently, only those cases rendering discordant results with values

over each marker cut-off 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 enough 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 so easy to assess and interpret, as are AST and ALT nowadays, so this approach would be potentially translatable to the bedside.

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534

Fig 1: Panel A: Serum HA levels related to fibrosis stages. Panel B: Serum M30 levels related to a) steatosis; b) inflammation and c) fibrosis severity. Horizontal lines inside each box represent the median, and the lower and upper borders of the box encompass the interquartile range. The vertical lines from the ends of each box encompass the extreme data points. Significant fibrosis: fibrosis stages ≥ 2. Steatosis: Grade 0, 1 and 2 (<66% of cells) *versus* score 3 (>66%). Lobular inflammation: score 0 (0 foci), 1 (<2 foci), and 2 (2-4 foci).

Table 1: Clinical and histological features of patients.

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

Table 3: Diagnostic accuracy of HA for significant fibrosis.

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

Table 5: Cases correctly classified using HA and M30.

Table 6: Percentage of patients that could not avoid the biopsy after serum marker assessment.

Supporting information

S1 Table: AAR and APRI related to significant fibrosis.

S2 Table: LINKI algorithms related to significant fibrosis.

Figure

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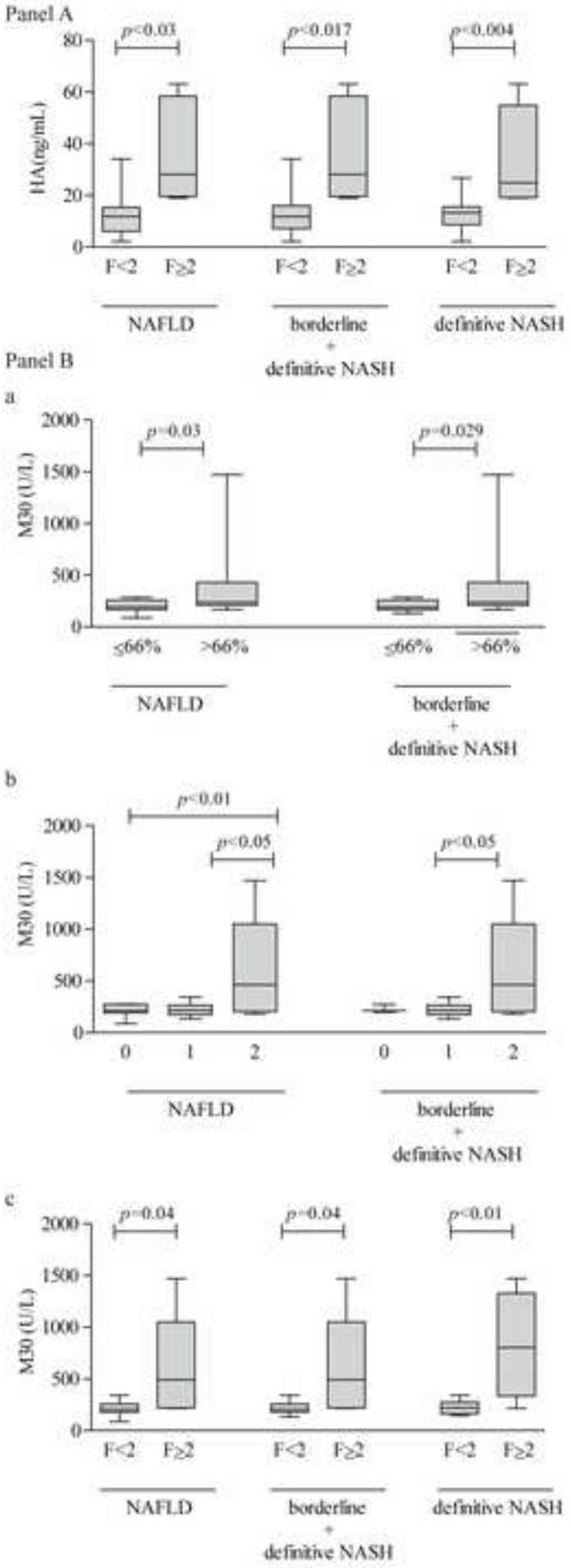

1 **Table 1: Clinical and histological features of patients.**

Factor	All patients	Not NASH	Borderline NASH	Definitive NASH
Age median ys (range)	49.5 (28-72)	37.5 (30-47)	55.5 (28-72)	45.5 (30-72)
Gender (male %)	55.88	100	41.67	55.55
Clinical and serological characteristics				
- BMI				
• Overweighed %	25	50	36.36	7.69
• Obese %	75	50	63.64	92.31
- Transaminases				
• ALT median IU/l (range)	81.5 (31-279)	76.5 (60-204)	73 (31-254)	94 (43-279)
% elevate	96	100	90.90	100
• AST median IU/l (range)	52.5 (22-208)	59.5 (29-86)	50 (22-184)	60 (35-208)
% elevated	53.57	50	54.54	53.85
• AST/ALT ratio median (range)	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 mg/dl (range)	207 (126-327)	231.5 (207-285)	206 (145-246)	200 (126-327)
• Triglycerides median mg/dl (range)	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 * (%)				
• 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

BMI: Body Mass Index; ALT: alanine aminotransferase; AST: aspartate aminotransferase. Normal ALT and AST levels were ≤32 and ≤48 IU/L, respectively when testing was done at 37ºC. The normal ranges for total cholesterol and triglyceride were 120-219 mg/dl and <150 mg/dl, respectively. *Steatosis Grade: score 0 (<5%cells), 1 (5-33%), 2 (33-66%) and 3 (>66%); lobular inflammation: score 0 (0 foci), 1 (<2 foci), 2 (2-4 foci) and 3 (>4 foci); ballooning grade: score 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 subjects.

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

Results are expressed as median (min-max). *P value of "Healthy subjects+not NASH" vs "Borderline+Definitive NASH"

Table 3: Diagnostic accuracy of HA for significant fibrosis.

	SIGNIFICANT FIBROSIS (F≥2)						
	AUROC	**95% CI**	**Cut-off** *	**Se%**	**Sp%**	**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

* ng/ml.

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

	AUROC	95% CI	Cut-off *	Se%	Sp%	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

*U/L

Table 5: Cases correctly classified using HA and M30.

	HA*	**M30***	**HA+M30†**	**HA-M30‡**
NAFLD PATIENTS	79%	88%	71%	78%
BORDERLINE +DEFINITIVE NASH PATIENTS	83%	90%	79%	85%
DEFINITIVE NASH PATIENTS	83%	94%	77%	82%

*true positive (TP) + true negative (TN), † cases with concordant results considering both markers cut-off values, ‡applying HA and M30 in a sequential form. Cases considered as positive according to HA cut-off were evaluated by M30.

Table 6: Percentage of patients that could not avoid the biopsy after serum marker assessment.

	HA*	M30*	HA+M30†	HA-M30‡
NAFLD PATIENTS	38%	-	29%	22%
BORDERLINE +DEFINITIVE NASH PATIENTS	37%	-	21%	15%
DEFINITIVE NASH PATIENTS	39%	-	23%	18%

*cases with serum HA levels higher than the cut-off, †cases with discordant results considering both serum markers, ‡applying HA and M30 in a sequential form. Cases considered as positive according to HA cut-off were evaluated by M30.

CONICET

Instituto Multidisciplinario de Investigaciones en Patologías Pediátricas (IMIPP) CONICET-División Patología, Hospital de Niños R. Gutiérrez

H N R G

RESPONSE TO REVIEWER

REVIEWER COMMENTS:

Reviewer: The manuscript, "Non-Alcoholic Fatty Liver Disease: Biomarkers as diagnostic tools of liver damage in adult patients from Argentina" by Valva et al. searchs for correlation between serum Noninvasive markers and liver injury in NAFLD patients. Markers selected by the authors have been studied by others groups being associated with disease progression and the results found corroborate previous data from literature. My comments are as follows:

1- The text should be revised for grammatical errors.

Response: This new version of the manuscript has been reviewed by a scientific English translator who lives in Argentina. I submit the revised version of the manuscript with active change track to make more clear the grammatical corrections made by the scientific English translator.

2- Is there any information about associated morbidities of patients enrolled? i.e. patients with type 2 diabetes or metabolic syndrome?

Response: we got additional data from the clinical records of the patients enrolled in our study. Briefly, HOMA-IR median was 4.89 (range: 1.7-10.10) for NAFLD patients, while it was 3.56 (range: 1.97-7.87), 4.95 (range: 2.77-10.10) and 4.70 (range: 1.70-8.64) for not NASH, Borderline NASH and Definitive NASH, respectively. Type II Diabetes was present in 55.88% of NAFLD patients (25% not NASH, 75% Borderline NASH and 50% Definitive NASH). On the other hand, 26.47% of NAFLD patients have Hypertension (25% not NASH, 75% Borderline NASH and 27.78% Definitive NASH). Finally, according to the criteria established by Alberti KG, et al. (*Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity*. Circulation 2009;120:1640–5), Metabolic Syndrome affected 47.06% of NAFLD cases (25% not NASH, 58.33% Borderline NASH and 80% Definitive NASH). In the submitted revised version of the manuscript, we included in Table 1 the records about HOMA-IR, Type II Diabetes, Hypertension and Metabolic Syndrome.

3- The potential use of serological markers as prediction tools for the outcome of NAFLD has been performed previously in prospective cohort studies, and longitudinal observational studies; in this scenario, it is recommended to discuss the main limitations of this study.

Response: As Lykiardopoulos B et al mentioned in their article: *Development of Serum Marker Models to Increase Diagnostic Accuracy of Advanced Fibrosis in Nonalcoholic Fatty Liver Disease: The New LINKI Algorithm Compared with Established Algorithms* (Plos One, DOI:10.1371/journal.pone.0167776), a limitation of studies assessing serum fibrosis markers is that liver biopsy is used as reference standard for evaluation of

hepatic fibrosis. As we have also stated in the introduction and discussion sections of our manuscript, important limitations of liver biopsy are namely, its known sampling variability, the subjective nature of fibrosis staging and the high inter-observer variability. The limitations of liver biopsy probably impair the potential of fibrosis algorithms to reach the state of perfect surrogate fibrosis markers.

Particularly, our present study has some extra limitations. First, it was in fact a pilot study with a limited case number that makes it difficult to validate serum markers utility. However, the obtained results were similar to the ones reported in other larger adult cohorts. Second, only a few patients displayed severe fibrosis which could have been a limiting factor for the ability of the markers to distinguish between mild and moderate/severe fibrosis. Third, since we did not take into account biopsy length and fragmentation, the potential for sampling error and understaging of fibrosis remains possible. Anyway, if it is assumed that ideally, a noninvasive liver fibrosis marker should be liver-specific, easy to perform, reliable, reproducible, and inexpensive; the molecules here proposed possess these characteristics. The noninvasive biomarkers proposed here to follow up NAFLD fibrosis progression display some advantages such as lower cost than physical or patented methods, simply performed and interpreted and feasible to carry out in a facility of any primary care center of an underdeveloped country.

According with the reviewer suggestion, the limitations of our work were included in the Discussion Section of the submitted revised version of the manuscript (pages 18-19 in revised version of the manuscript but pages 19-20 in the version of active change track).

4- It has been recently reported by Lykiardopoulos et. al. the LINKI algorithm, which combines indirect fibrosis markers as: age, glucose, and AST and the direct fibrosis marker HA demonstrating to have the best diagnostic accuracy. It is recommended to be included in the discussion section, as it corroborates the author´s findings.

Response: As the reviewer mentioned, Lykiardopoulos et al developed a new noninvasive model (Linköping University-Karolinska Institute; 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 demonstrated higher AUROCs compared to other previously published serum fibrosis algorithms (FIB-4, ELF, APRI, NAFLD fibrosis score, APRI), particularly to predict advanced fibrosis. In line with this, as the reviewer suggested, we calculate LINKI-1, LINKI-2a, LINKI-2b and LINKI-2c in our cohort and the AUROCs for significant fibrosis were compared. Although all of them demonstrated 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. Interestingly, when applying the LINKI algorithms in our cohort the AUROCs obtained were better than the AUROC described by Lykiardopoulos et al for significant fibrosis. On the other hand, in contrast to the reported AUROC performance for LINKI-2a, LINKI-2b, and LINKI-2c in our cohort they showed a better diagnostic performance than LINKI-1. Therefore, as Lykiardopoulos et al mentioned in their article future studies will determine if they are more stable than LINKI-1 and which one has the best diagnostic performance.

Although, the LINKI results in our cohort did not exactly reflect Lykiardopoulos et al findings, it demonstrated promising results. According with the reviewer suggesting, we included this observation in the Discussion Section of the submitted revised version of the manuscript (page 17 in revised version of the manuscript but page 18 in the version of active change track) and the LINKI AUROC results of our cohort are presented in S2 Table.

5- Lines 173-174. Authors must mention the supporting criteria for grouped the cases for analysis in the set "healthy control+not NASH".

Response: As we describe in the result section, the four markers were evaluated in serum samples of NAFLD patients as well as in healthy donors. So, when we compared the serum levels of the evaluated markers between NAFLD patients and healthy donors significant differences were observed. However, in order to deeply describe the NAFLD population characteristics, each marker value was also compared through the 3 histological subgroups of NAFLD (not NASH, borderline, definitive NASH). Interestingly, similar results were observed when compared not NASH and healthy donor [except for M65 “not NASH” vs healthy donors (p=0.002)] as well as when comparing borderline and definitive NASH. So, this observation prompted us to group the cases in two sets “healthy control+not NASH” and “borderline+definitive NASH”. When analyzing TIMP-1, HA, M30 and M65 levels significant differences between groups for all the studied markers were observed. It is important to highlight that this arrangement was only performed in order to deeply describe the study populations and the levels of the four markers in each NAFLD subgroup, further on the analysis of the serum biomarkers as possible diagnostic tools was performed related to liver damage (analyses that did not include healthy controls).

From the point of view of the biological concerns, to group the cases in the sets "healthy control+not NASH" and “borderline+definitive NASH” makes sense considering that liver damage in terms of inflammation and fibrosis is the major parameter that differentiates borderline and definitive NASH from not NASH and controls . To clarify this point, the submitted revised version of the manuscript was rewritten in the Result Section (page 11 in revised version of the manuscript but page 12 in the version of active change track).

6- Lines 239-254 Discussion. It is preferable to include this text in "introduction section" as it seems to be more suitable.

Response: The reviewer observation is appropriate. Then, this paragraph was moved to the introduction section (pages 5-6 in both revised version of the manuscript and the version of active change track).

7- Formatting of Table 1 is confusing and does not allow a fluid interpretation of the results. Authors mention in values of Transaminases ALT (IU/l), median (range), but they do not include median. For lipid profile in Cholesterol they include range and only make mention to mg/dl.

Response: We agree with the reviewer that the Table 1 format is confuse. To clarify it and to allow an easy interpretation of the studied group characteristics we modified the Table 1. Lines and shadows were added to

separate information. Moreover, related to transaminases and lipid profile, we modified the way of expressing the results to avoid confusions. The ALT and AST data are expressed as median IU/l (range) while cholesterol and triglycerides as median mg/dl (range). Moreover, in the last version of the manuscript the AST/ALT ratio included the information of "median (range)" which was omitted in the previous one. Finally, according to reviewer suggestion we included the information about associated morbidities of the studied patients in Table 1.

8- For healthy subjects authors only mentioned that they were "without known systemic or liver disease and with normal biological and virological liver test", it is important to mention which test were performed in order to discard any injury associated with NAFLD.

Response: Healthy subject were examined by the same hepatologist team that follows the patients of the study. No clinical or biochemical evidence of liver disease or known medical illness at recruitment was observed in healthy subjects. All of them have normal abdominal ultrasonography. The same parameters which were evaluated in patients were taken into account in the healthy group. Healthy subjects turn out to have no causes of liver disease, autoimmune, genetic or endocrinologic diseases as well as hepatocellularcarcinoma (HCC). Furthermore, they were all negative for HBV, HCV and HIV as it was evidenced by negative serological markers. Routine clinical biochemical analyses included complete blood count and analysis of prothrombintime, transferrin, iron, transferrin saturation, ferritin, ALT, AST, ALP, GGT, bilirubin, fasting plasma glucose, total cholesterol, high-density lipoprotein, low density lipoprotein, and triglycerides. Blood pressure, waist circumference, bodyweight, and height were measured. Therefore, the clinical and biochemical parameters evaluated were under normal values. Finally, the alcohol consumption of the healthy group was low (men <30 g/day; women <20 g/day). According to reviewer suggestion, this point was clarified in the Material and Methods Section of submitted revised version of the manuscript (page 8 in revised version of the manuscript but page 9 in the version of active change track).

Non-Alcoholic Fatty Liver Disease: Biomarkers as diagnostic tools ~~of~~ for liver damage assessment in adult patients from Argentina

Pamela Valva[1], Daniela Rios[1], Paola Casciato[2], Adrián Gadano[2], Omar Galdame[2], Eduardo Mullen[3], Gustavo Bertot[4], Elena De Matteo[1], María Victoria Preciado[1].

[1] Laboratory of Molecular Biology, Pathology Division, Ricardo Gutiérrez Children's Hospital; Buenos Aires, Argentina.

[2] Liver Unit, Hospital Italiano de Buenos Aires; Buenos Aires, Argentina.

[3] Pathology Division, Hospital Italiano de Buenos Aires; Buenos Aires, Argentina.

[4] H.A. Barceló Foundation-Medicine University, Buenos Aires, Argentina.

Short title: Non-Alcoholic Fatty Liver Disease Biomarkers

Corresponding author:

Valva Pamela; Instituto Multidisciplinario de Investigaciones en Patologías Pediátricas (IMIPP- CONICET-GCBA) Laboratorio de Biología Molecular, División Patología, Hospital de Niños Ricardo Gutiérrez, Buenos Aires, Argentina. Gallo 1330, C1425EFD, Buenos Aires, Argentina. Fax: +54-11-4962-4122. E-mail: valvapamela@yahoo.com

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Funder's had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

The authors disclose no financial conflicts of interest.

Abstract

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease which prevalence has been constantly increasing linked to the obesity global epidemic. 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 way 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 [hyaluronic acid (HA) and tissue inhibitor of matrix metalloprotein inhibitor-1 (TIMP-1)] and cell ~~-~~death markers [cytokeratin-18 (M65) and caspase-cleaved cytokeratin-18 (M30)], which correlate with liver injury in a NAFLD patients cohort.

Liver biopsies and serum from 34 NAFLD adult patients were analyzed. Histological parameters were evaluated. ~~In serum~~ HA, TIMP-1, M65 and M30 were measured in serum samples.

HA showed 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 diagnostic accuracy evaluation demonstrated a good performance as less invasive markers of significant fibrosis of both HA (AUROC 0.928) and M30 (AUROC 0.848) ~~as less invasive markers of significant fibrosis~~.

In ~~conclusion~~conclusion, ~~B~~biomarkers are essential ~~tools which~~tools that may provide a quick and accurate diagnosis to patients with life-threatening NAFLD and NASH. HA and M30, together or sequentially determined, demonstrated to be straightforward tests

that may be enough to predict significant fibrosis even in a primary care ~~centre~~centre of an underdeveloped country.

Key Word: NAFLD, HA, TIMP-1, M30, M65

Introduction

Global population health is currently threatened by the obesity epidemic that promotes premature development of the metabolic syndrome, which significantly increases the risk for liver disease early in life. Non-alcoholic fatty liver disease (NAFLD) is the most common form of chronic liver ~~disease~~ illness in all age groups, representing a serious nutritional concern due to the high prevalence of overweight and obesity [1]. NAFLD is ~~characterised~~characterized by an excessive hepatic fat accumulation and includes two conditions with different prognoses: non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) [2]. Notably, NASH is not by itself a severe hepatic lesion but it can progress towards end-stage liver diseases [2], so the identification of NASH patients is crucial to *early prevent liver damage and to improve clinical outcome.*

Obesity generates a comprehensive proinflammatory state with high risk for metabolic comorbidities which contributes to progressively enlarge the series of patients that will develop NASH, NASH-related cirrhosis, decompensated liver disease and hepatocellular carcinoma (HCC) [3]. The emergence of this cohort is on the horizon and will introduce a significant disease burden in the field of liver transplantation. At the present time, NASH is the third most common indication for liver transplantation and it is expected to climb till to become the leading one over the next decades [4]. Strikingly, current practice guidelines do not support NAFLD screening in patients at risk in spite of its high prevalence and implicit progression to end-stage liver disease [5]. In addition, due to the elevate costs of the available tests, the liver biopsy risks 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]. So, a

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present challenge is to distinguish between simple steatosis vs. NASH, since the latter increases the chances of liver disease progression [7].

The histological characterization of NAFLD ranges from simple steatosis to steatosis accompanied by inflammation and other evidences of cellular injury (NASH). NAFL encompasses: a) steatosis ~~alone~~, b) steatosis with lobular or portal inflammation, without ballooning, or c) steatosis with ballooning but without inflammation [8]. NASH diagnosis requires histopathological evaluation to assess joint steatosis presence, ballooning and lobular inflammation [8]. Perisinusoidal fibrosis is also frequent, but it is not a diagnostic criteria. Fibrosis progression is the most significant prognostic factor that correlates with liver-related outcomes and death [9]. In this regard, 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 applied as a tool to periodically monitor ~~the~~ disease outcome [10]. In addition, the amount of retrieved tissue ~~retrieved~~ can influence the diagnosis because of fat deposition, hepatocyte injury, ~~and~~ or fibrosis that can vary between lobules; moreover ~~and~~ inter-observer differences are frequently encountered [10]. Therefore, a growing medical need is the development of noninvasive tests that can predict initial stage and progression of liver disease over time in an accurate way [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, ~~for determining~~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

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extracellular matrix (ECM), ~~is~~ the key in the development of liver fibrosis [14], ~~which~~ comprises the activation of hepatic stellate cells (HSCs) with the consequent secretion of excess matrix proteins (hyaluronan, laminin, collagen, etc), follow by their degradation by the matrix metalloproteins (MMPs). Moreover, MMP are also inhibited by tissue inhibitors of metalloproteins (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 ~~the~~ apoptosis [17].

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The pathophysiological pathways involved in the development of liver damage and its progression ~~of liver damage~~ 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 be ~~induced~~ developed~~by~~ 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 CK-18, 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 study purposes ~~was~~ were to evaluate the presence of matrix deposition markers [hyaluronic acid (HA) and tissue inhibitor of matrix metalloprotein inhibitor-1 (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 analyzed their diagnostic accuracy to be used as possible markers of liver damage in primary care ~~centres~~centers in an underdeveloped country.

Material and Methods

Patients and samples

Thirty-four NAFLD Caucasian 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 (HCC), HCV, HBV and/or HIV infection. *Routine clinical biochemical analyses included complete blood count and analysis of prothrombin time, transferrin, iron, transferrin saturation, ferritin, ALT, AST, ALP, GGT, 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 time of biopsy were tested.

A group of 20 adult healthy subjects with no clinical or biochemical evidence of liver disease or known medical illness at recruitment were included as controls. The same parameters which were evaluated in patients were taken into account in the healthy group. The clinical and biochemical parameters evaluated were under normal values. All healthy subjects were negative for HBV, 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 subject was included.

This study has the approval of the Ethics Board of Ricardo Gutierrez Children Hospital and is in accordance with the 1964 Declaration of Helsinki and its later amendments. A written informed consent was obtained from all patients prior to their inclusion in the

study.

Histological Analysis

Two independent pathologists evaluated the histological sections in a blind manner according to the NAFLD scoring system proposed by the National Institute of Diabetes and Digestive and Kidney Disease NASH Clinical Research Network (12): a NAFLD activity score of ≥5 corresponds to a diagnosis of "definitive NASH", a score of 3-4 to "borderline NASH", and a score of ≤2 to "not NASH or simple steatosis". Fibrosis stage was also measured. Fibrosis stages ≥ 2 were considered as significant fibrosis.

Quantitative measurement of TIMP-1 and HA

Serum TIMP-1 and HA were determined by ELISA (Quantikine, R&D System Inc) ~~both~~ according to the manufacturer's instructions.

Quantitative measurement of M30 and M65

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

Statistical analysis

GraphPad InStat software, version 3.05 was used. The Mann–Whitney U-test and unpaired t-test, ANOVA or Kruskal Wallis test were used to compare sets of data. P values <0.05 were considered significant.

The diagnostic value was assessed by the area under the receiver operating

characteristic curves (AUROC). Cut-off value for the diagnosis was determined as the maximal value at the sum of the sensitivity (Se) and specificity (Sp). AUROC, cut-off values, positive predictive values (PPV) and negative predictive values (NPV) were determined using the MedCalc demo statistical software.

The number of correctly classified cases by means of serum markers and the percentage of cases that could have not avoided the biopsy procedure were assessed.

Results

Clinical and liver biopsy findings

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 as "definitive NASH", 35.29% as "borderline NASH" and 11.77% as "not NASH".

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

The four markers displayed higher levels in NAFLD patients than in healthy *subjects (Table 2). However, in order to deeply describe the NAFLD population characteristics, each marker value was also compared through the 3 histological subgroups of NAFLD ("not NASH", "borderline NASH", "definitive NASH"). Interestingly, but in agreement with inflammation and fibrosis components, similar results were observed when compared not NASH and healthy subjects [except for M65 "not NASH" vs healthy subjects (p=0.002)] as well as when comparing borderline and definitive NASH. So, this observation prompted us to group the cases in two sets "healthy subjects+not NASH" and "borderline+definitive NASH". When analyzing TIMP-1, HA, M30 and M65 levels significant differences between groups for all the studied markers were observed (Table 2).*

Regarding serum biomarkers role 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, since 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 ~~analysed~~analyzed by 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 displayed association with steatosis, inflammation and fibrosis severity. That is to say, M30 level was elevated in 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 conserved when ~~analysing~~analyzing ~~the~~ "borderline+definitive NASH" (steatosis p=0.04; inflammation p=0.01; and fibrosis p=0.04), while in the subgroup of "definitive NASH" M30 only displayed association ~~only~~ with fibrosis (p=0.01) (Fig 1). In contrast, M65 was not associated with any histological parameter.

Diagnostic performance of serum markers

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

It is assumed the AUROC of ~~the~~ 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 demonstrated a 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 with "definitive NASH" (AUROC: 0.929, NPV: 100) (Table 3).

On the other hand, despite the M30 association with both steatosis and inflammation severity, the AUROC values were very low, but it demonstrated a good performance to predict significant fibrosis in NAFLD (AUROC: 0.848, NPV: 91.3) (Table 4). The

performance of M30 was extended to the subgroups "borderline+definitive NASH" (AUROC: 0.852) and "definitive NASH" (AUROC: 0.844) (Table 4).

The whole series of NAFLD cases with F≥2 were correctly categorized according to the HA cut-off values for significant fibrosis, while 7 out 28 (25%) patients with F<2 were misclassified as False Positive (FP). In the "borderline+definitive NASH" subgroup, 25 patients were correctly classified (6 patients were TP and 19 patients were TN), but 5 was classified in the wrong group (FP). While in the "definitive NASH" subgroup, 15 patients were correctly identified (4 TP, 11 TN), but 3 cases were FP. In accordance with the high NPV and considering that the misclassified cases were FP, only those patients with HA levels under the cut-off value could be diagnosed without significant fibrosis (61.76% NAFLD, 63.33% "borderline+definitive NASH" and 61.11% "definitive NASH" patients). In consequence, those cases with HA values higher than the cut-off cannot avoid liver biopsy (Table 5 and 6).

According to the M30 cut-off value for significant fibrosis, 30 NAFLD patients were correctly identified (4 patients were TP and 26 patients were TN) but 4 patients failed [2 FP, 2 False Negative (FN)]. In the "borderline+definitive NASH" subgroup 27 cases were accurately categorized (4 TP, 23 TN) while 3 were wrongly classified (1 FP, 2 FN). Finally, in the "definitive NASH" subgroup, 17 cases were correctly identified (3 TP, 14 TN), and 1 resulted a FN. Although more patients were correctly classified with M30 than with HA (Table 5), the FN and NPV were lower with HA; so M30 came off a good choice to be used as a single marker when HA is not available.

Conclusively, HA and M30 were evaluated either together or sequentially. When both marker cut-offs were considered jointly, only those patients with concordant results

(negative or positive for both markers) were assumed as well assigned (71% NAFLD, 79% "borderline+definitive NASH", 77% "definitive NASH") (Table 5). On the other hand, the sequential analysis considered HA as the first line due to its high NPV, so only those cases with HA level higher than the cut-off would proceed to M30 evaluation. With this algorithm, those cases correctly sorted were: 1) the negative ones for HA and 2) the positive ones for HA followed by positive for M30 (78% NAFLD, 85% "borderline+definitive NASH", 82% "definitive NASH" of cases) (Table 5). Finally, ~~by either of the chosen approaches~~ only those patients with discordant results by either of the chosen approaches would not avoid liver biopsy (Table 6).

Discussion

It has been proposed that a liver biopsy is needed to arrive to a conclusive diagnosis of NASH [23], but it is well known that besides the risks related to an invasive procedure, it has been linked with sampling error and patient care costs which could be onerous in underdeveloped countries [24]. Thus, the emergence of trustworthy noninvasive markers and tests that can accurately foretell the presence of advanced disease is an imperious need to fulfil. Among other ~~Several~~ strategies, ~~have been proposed such as~~ serum aminotransferases, AST-to-platelet ratio (APRI) and AST- ALT ratio (AAR)~~,~~ have been proposed, but liver aminotransferases are not appropriate to be applied in a single test way [25]. In line with this, in our cohort, APRI and AAR were calculated as alternative hallmarks of liver fibrosis; however, these approaches did not improve the diagnostic accuracy performance of the other markers (S1 Table). Other authors have combined both biochemical and clinical issues (i.e. Fib-4, BARD, NFS, Fibrotest) to predict fibrosis severity; while others have brought these together with specific serum fibrosis markers (i.e. NASH Test, Fibrometer, LINKI) to do so. However, ~~this~~ these calculation ~~system~~systems ~~is~~ are difficult and burdensome to be routinely performed [2, 25, 26]. On the other hand, noninvasive techniques such as ultrasound, computed tomography, magnetic resonance imaging, and proton magnetic resonance spectroscopy can detect hepatic steatosis, but cannot consistently discriminate simple steatosis from NASH [25]. Moreover, these techniques are expensive and restricted to research ~~centres~~centers since special equipment and trained staff are needed to perform these techniques [2, 25]. In summary, when trying to avoid liver biopsy there are no consensus on strategies for noninvasive biomarkers, therefore validated studies, especially in underdeveloped countries, are expected in prospective observational

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studies as well as in populations of different ethnicity and geographical locations [2], since the obesity prevalence in addition to the progression of histological liver damage associated with NASH display significant ethnic disparities [27].

Many authors 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 named ELF test, which involved TIMP-1, HA, and amino-terminal peptide of procollagen III [28, 32, 33]. This test demonstrated good diagnostic performance to predict advance 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. Of note, in contrast to the recent results of Mizuno et al [31] who proposed that HA depicted no evidence ~~for~~ of predictive value in early fibrosis, in our adult NAFLD cohort HA was strongly associated with significant fibrosis stages with a good diagnostic accuracy, even when grouping the cases in either "borderline+definitive NASH" or "definitive NASH". In accordance, Suzuki et al [34] have previously determined the reliability of HA to predict the severity of hepatic fibrosis in NAFLD patients. They described that HA was useful for predicting severe fibrosis (≥3) (AUROC:0.9, 95% CI:0.83, 0.97), but its efficacy for significant fibrosis could not be evaluated due to the limited number of patients with this stage of fibrosis [34]. Therefore, the results obtained in our study complemented Suzuki el at. ~~work~~ observations since in our cohort significant fibrosis are represented. Kaneda et al [35] also demonstrated HA to have an AUROC, NPV, Se and Sp of 0.97%, 100%, 100%, and 89%, respectively, for detecting severe fibrosis, and Lesmana et al and Yoneda et al [36, 37] also proved HA ability to differentiate between mild (F1-2) and advanced

fibrosis (F3-4).

Recently, Lykiardopoulos et al [26] developed a new noninvasive model (Linköping University-Karolinska Institute; 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 demonstrated higher AUROCs compared to other previously published serum fibrosis algorithms (FIB-4, ELF, 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 demonstrated 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 (S2 Table). Interestingly, when applying the LINKI algorithms in our cohort the AUROCs obtained were better than the AUROC described by Lykiardopoulos et al for significant fibrosis [26]. On the other hand, in contrast to the reported AUROC performance for LINKI-2a, LINKI-2b, and LINKI-2c in our cohort they showed a better diagnostic performance than LINKI-1. Therefore, as Lykiardopoulos et al mentioned in their article future studies will determine if they are more stable than LINKI-1 and which one has the best diagnostic performance.

Concerning TIMP-1, other groups reported similar observations about the higher levels of TIMP-1 in serum samples from NAFLD patients compared with those of healthy ~~controls~~ subjects [38]. Nevertheless, TIMP-1 usefulness as a marker of fibrosis severity

was dismissed ~~again,~~ in accordance with our previous study in a cohort of HCV chronically infected adult patients [39].

Finally, serum M30 was extensively validated 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 Caucasians), that M30 value as a single marker might be of lesser utility than it has been previously assumed. In our study, M30 was significantly elevated in NAFLD Caucasian patients and displayed 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 pioneering work by Feldstein et al in Caucasian population [41, 45]. However, M30 performance improved when it was combined in an algorithm with HA. These divergences reinforced the importance to perform studies ~~to~~ which validate the M30 diagnostic accuracy in different ethnicities, regions, and age groups since it may be useful for monitoring liver damage and disease progression.

Concerning M65, the available data are limited and require further validation before integration into clinical practice [5, 45, 46]. Many authors described that the M65 level correlated with fibrosis progression in NAFLD [45, 47-49], which was not reproduced in our study. However, in accordance with Joka et al [47] M65 could differentiate simple steatosis from healthy ~~controls~~subjects, so it may be a possible marker of early stages in NAFLD.

Finally, it is worthwhile to mention that, the present study has some limitations. First, it was in fact a pilot study with a limited case number that makes it difficult to validate serum markers utility. However, the obtained results were similar to the ones reported

in other larger adult cohorts. Second, only a few patients displayed severe fibrosis which could have been a limiting factor for the ability of the markers to distinguish between mild and moderate/severe fibrosis. Third, since we did not take into account biopsy length and fragmentation, the potential for sampling error and understaging of fibrosis remains possible. Anyway, if it is assumed that ideally, a noninvasive liver fibrosis marker should be liver-specific, easy to perform, reliable, reproducible, and inexpensive; the molecules here proposed possess these characteristics. The noninvasive biomarkers proposed here to follow up NAFLD fibrosis progression display some advantages such as lower cost than physical or patented (Fibrotest™, Fibromax™) methods, simply performed and interpreted and ~~possible~~ feasible to carry out in a facility of any primary care ~~centre~~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 ~~a~~ 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 ~~as a biomarker~~, thus avoiding a liver biopsy [50].

Finally, the solely evaluation of HA and M30 may be enough to predict significant fibrosis as well as to evaluate fibrosis progression in NAFLD cases previously classified, according to liver biopsy, as borderline or definitive NASH. Moreover, if these markers were applied sequentially, a better sorting of cases could be achieved (Table 5 and 6). HA would be chosen as the first line assay according to its diagnostic accuracy, and then those HA values over the cut-off could be re-evaluated according to M30 cut-off. Consequently, only those cases rendering discordant results with values

over each marker cut-off 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 enough 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 multicentre study project, to validate and confirm our findings. If these parameters are validated in the near future, they would be so easy to assess and interpret, as are AST and ALT nowadays, so this approach would be potentially translatable to the bedside.

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Fig 1: Panel A: Serum HA levels related to fibrosis stages. Panel B: Serum M30 levels related to a) steatosis; b) inflammation and c) fibrosis severity. Horizontal lines inside each box represent the median, and the lower and upper borders of the box encompass the interquartile range. The vertical lines from the ends of each box encompass the extreme data points. Significant fibrosis: fibrosis stages ≥ 2. Steatosis: Grade 0, 1 and 2 (<66% of cells) *versus* score 3 (>66%). Lobular inflammation: score 0 (0 foci), 1 (<2 foci), and 2 (2-4 foci).

548 **Table 1: Clinical and histological features of patients**

Factor	All patients	Not NASH	Borderline NASH	Definitive NAS
Age ~~(ys)~~ median ys (range)	49.5 (28-72)	37.5 (30-47)	55.5 (28-72)	45.5 (30-72)
Gender (male %)	55.88	100	41.67	55.55
Clinical and serological characteristics				
- **BMI**				
• Overweighed %	25	50	36.36	7.69
• Obese %	75	50	63.64	92.31
- **Transaminases**				
• ALT ~~(IU/l)~~ median IU/l (range)	81.5 (31-279)	76.5 (60-204)	73 (31-254)	94 (43-279)
• % elevated	96	100	90.90	100
• AST median IU/l ~~(IU/l) median~~ (range)	52.5 (22-208)	59.5 (29-86)	50 (22-184)	60 (35-208)
• % elevated	53.57	50	54.54	53.85
• AST/ALT ratio median (range)	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 mg/dl (range)	207 (126-327)	231.5 (207-285)	206 (145-246)	200 (126-327)
• Triglycerides median mg/dl (range)	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** * (%)				
• 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	-

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• 4	-			-
n	34	4	12	18

BMI: Body Mass Index; ALT: alanine aminotransferase; AST: aspartate aminotransferase. Normal ALT and AST levels were ≤32 and ≤48 IU/L, respectively when testing was done at 37ºC. The normal ranges for total cholesterol and triglyceride were 120-219 mg/dl and <150 mg/dl, respectively. *Steatosis Grade: score 0 (<5%cells), 1 (5-33%), 2 (33-66%) and 3 (>66%); lobular inflammation: score 0 (0 foci), 1 (<2 foci), 2 (2-4 foci) and 3 (>4 foci); ballooning grade: score 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 subjects~~controlsubjetcs~~.

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

Results are expressed as median (min-max). *P value of "Healthy ~~controls~~subjects+not NASH" vs "Borderline+Definitive NASH"

562 **Table 3: Diagnostic accuracy of HA for significant fibrosis**

	SIGNIFICANT FIBROSIS (F≥2)						
	AUROC	**95% CI**	**Cut-off** *	**Se%**	**Sp%**	**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

563 * ng/ml.
564

565 **Table 4: Diagnostic accuracy of M30 for steatosis, inflammation and significant**
566 **fibrosis.**

STEATOSIS							
	AUROC	**95% CI**	**Cut-off** *	**Se%**	**Sp%**	**PPV**	**NPV**
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							
	AUROC	**95% CI**	**Cut-off** *	**Se%**	**Sp%**	**PPV**	**NPV**
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)							
	AUROC	**95% CI**	**Cut-off** *	**Se%**	**Sp%**	**PPV**	**NPV**
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

567 *U/L

568

Table 5: Cases correctly classified using HA and M30

	HA*	M30*	HA+M30†	HA-M30‡
NAFLD PATIENTS	79%	88%	71%	78%
BORDERLINE +DEFINITIVE NASH PATIENTS	83%	90%	79%	85%
DEFINITIVE NASH PATIENTS	83%	94%	77%	82%

*true positive (TP) + true negative (TN), † cases with concordant results considering both markers cut-off values, ‡applying HA and M30 in a sequential form. Cases considered as positive according to HA cut-off were evaluated by M30.

574 **Table 6: Percentage of patients that could not avoid the biopsy after serum marker**
575 **assessment**

	HA*	M30*	HA+M30†	HA-M30‡
NAFLD PATIENTS	38%	-	29%	22%
BORDERLINE +DEFINITIVE NASH PATIENTS	37%	-	21%	15%
DEFINITIVE NASH PATIENTS	39%	-	23%	18%

576 *cases with serum HA levels higher than the cut-off, †cases with discordant results considering both serum
577 markers, ‡applying HA and M30 in a sequential form. Cases considered as positive according to HA cut-off
578 were evaluated by M30.

579

Supporting information

S1 Table: AAR and APRI related to significant fibrosis

	AAR				
	F<2†	**F≥2†**	***P* value**	**AUROC**	**95% CI**
NAFLD PATIENTS	0.63 (0.368-1.07)	0.749 (0.735-0.882)	0.0388	0.7124	0.563-0.922
BORDERLINE +DEFINITIVE NASH PATIENTS	0.63 (0.368-1.07)	0.749 (0.735-0.882)	0.0448	0.7407	0.542-0.939
DEFINITIVE NASH PATIENTS	0.65 (0.394-1.07)	0.745 (0.735-0.882)	0.16	-	-
	APRI				
	F<2†	**F≥2†**	***P* value**	**AUROC**	**95% CI**
NAFLD PATIENTS	0.00047 (0.00022-0.0015)	0.00076 (0.0004-0.0016)	0.06	-	-
BORDERLINE +DEFINITIVE NASH PATIENTS	0.00046 (0.00022-0.0015)	0.0004 (0.0007659-0.001567)	0.038	0.7412	0.498-0.984
DEFINITIVE NASH PATIENTS	0.00046 (0.00025-0.00086)	0.001 (0.0004-0.0016)	0.14	-	-

AAR: aspartate aminotranferase-to-alanine aminotranferase, APRI: aspartate aminotranferase-to-platelet ratio. † Results are expressed as median (min-max).

S2 Table: LINKI algorithms related to significant fibrosis

Commented [PV1]: New table

LINKI-1		
	AUROC	**95% CI**
NAFLD PATIENTS	0.815	0.583-1.046
BORDERLINE +DEFINITIVE NASH PATIENTS	0.806	0.572-1.039
DEFINITIVE NASH PATIENTS	0.786	0.449-1.122
LINKI-2a		
	AUROC	**95% CI**
NAFLD PATIENTS	0.901	0.750-1.052
BORDERLINE +DEFINITIVE NASH PATIENTS	0.897	0.746-1.051
DEFINITIVE NASH PATIENTS	0.884	0.669-1.100
LINKI-2b		
	AUROC	**95% CI**
NAFLD PATIENTS	0.907	0.768-1.047
BORDERLINE +DEFINITIVE NASH PATIENTS	0.899	0.747-1.050
DEFINITIVE NASH PATIENTS	0.885	0.696-1.073
LINKI-2c		
	AUROC	**95% CI**
NAFLD PATIENTS	0.907	0.768-1.047
BORDERLINE +DEFINITIVE NASH PATIENTS	0.899	0.7447-1.050
DEFINITIVE NASH PATIENTS	0.885	0.662-1.100

LINKI-1: (age x 0.066) + (AST x 0.0888) + (glucosex0.34) + (HA x 0.019) - 24.136. LINKI-2a: HA x AST^2x age x (glucose) / (platelet count). LINKI-2b: HA x AST x age x $(glucose)^2$ / (platelet count). LINKI-2c: HA x AST x age x (glucose)/(√ platelet count). HA (μg/L), AST (U/L), glucose (mmol/L), age (yrs), Platelet count ($x10^9$/L).