ORIGINAL ARTICLES

Hyaluronic acid predicts hepatic fibrosis in children with nonalcoholic fatty liver disease

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Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in children and adolescents, and it may progress to liver fibrosis and cirrhosis. Liver biopsy, which is the recognized gold standard for the diagnosis of hepatic fibrosis, is invasive. Thus, there has been increasing interest in the development of noninvasive markers. Hyaluronic acid (HA) has been shown to be a good marker of liver fibrosis in adults. In the current study, we evaluated the association of HA with liver fibrosis in 100 consecutive children with biopsy-proven NAFLD. In all, 65% of the children had liver fibrosis. Using proportional-odds ordinal logistic regression, we found that values of HA ≥ 1200 ng/mL made the absence of fibrosis (F0) unlikely (7%, 95% confidence interval (CI): 1% to 14%), whereas values of HA ≥ 2100 ng/mL made F2, F3, or F4 fibrosis likely (89%, 95% CI: 75% to 100%). Our study shows that HA is a predictor of fibrosis in children with NAFLD followed at a tertiary care center. Additional studies are needed to test whether HA can be employed to predict liver fibrosis in pediatric populations with similar and lower prevalence of liver fibrosis. (Translational Research 2010;156:229–234)

Abbreviations: ALT = aspartate aminotransferase; AST = aspartate aminotransferase; BMI = body mass index; CI = confidence interval; ECM = extracellular matrix; GGT = gamma glutamyl transferase; HA = hyaluronic acid; HOMA-R = homeostasis model assessment of insulin resistance; NAFLD = nonalcoholic fatty liver disease; NASH = nonalcoholic steatohepatitis; NPV = negative predictive value; OR = odds ratio; PPV = positive predictive value; ROC = receiver operating characteristic curve; SDS = standard deviation score

Parallel to the current pandemic of overweight and obesity, nonalcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease in children and adolescents in Western countries.[1,2] The prevalence of pediatric NAFLD is 3% to 10% in normal-weight subjects and reaches a value of 80% in obese individuals.[3,4] These data are alarming because, even if the long-term course of pediatric NAFLD is not yet known, some evidence exists of a possible course toward cirrhosis and liver failure, resulting in an increased need for liver transplantation.[5,6]

Therefore, the early detection of NAFLD in children and adolescents is necessary to prevent the development

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Submitted for publication March 29, 2010; revision submitted May 14, 2010; accepted for publication May 27, 2010.

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1931-5244/$ - see front matter

doi:10.1016/j.trsl.2010.05.008

AT A GLANCE COMMENTARY

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Background

Recently, hyaluronic acid (HA) has been shown to be one of the best performing direct markers of liver fibrosis in adults.

Translational Significance

Here, we demonstrated the association of serum HA levels with liver fibrosis in children with biopsy-proven NAFLD, suggesting that it may allow a simple and efficient screening of patients at risk of progressive liver disease needing subsequent investigation. The translational impact of our research is that HA assessment might come into clinical practice for the management of children with suspected NASH, contributing to simplify and improve the management of untreated patients, as well as those included in a therapeutic plan.

of advanced liver disease, both in pediatric age as well as later in life.[7-9] The progression of NAFLD toward liver fibrosis and cirrhosis strongly depends on the presence of a necroinflammatory and fibrogenic milieu defined as nonalcoholic steatohepatitis (NASH).[10,11] The distinction between simple fatty liver and NASH, and the exclusion of competing causes of chronic liver disease, is based on the histopathologic evaluation of liver tissue. However, because liver biopsy is invasive, painful, and expensive, there has been increasing interest in the development of noninvasive markers for the diagnosis of NASH and liver fibrosis.[12]

Many noninvasive markers of liver fibrosis have been proposed so far.[13] Although all available markers have suboptimal diagnostic accuracy, they may reduce the need for liver biopsy when used alone or in combination.[14] These findings are more relevant for the pediatric setting, in which liver biopsy is perceived as more risky than in adults.[15] Among the proposed markers, some reflect alterations of hepatic function but not of extracellular matrix (ECM) metabolism and, therefore, are labeled ''indirect markers.'' Those markers directly linked to modifications in ECM turnover during fibrogenesis are instead defined ''direct markers.''[16-19]

Among direct markers, hyaluronic acid (HA) is one of the best predictors of liver fibrosis in adults.[20] HA is a glycosaminoglycan synthesized by ECM-producing cells, including activated hepatic stellate cells. The circulating levels of HA might reflect not only the stage of disease but also ECM metabolism and, to some extent, inflammatory activity within the liver.[20] Recent studies performed in patients with chronic viral hepatitis C either before[21,22] or after[23] liver transplantation have reinforced the notion that HA is a low-cost and accurate marker for the staging of liver fibrosis. A recent study showed that serum HA is a marker of fibrosis in unselected children undergoing liver biopsy,[24] but the possibility of employing HA for the prediction of liver fibrosis in children with NAFLD has not been tested so far.

In the current study, we evaluated the association of serum HA levels with the degree of liver fibrosis in children with NAFLD, aiming to determine the diagnostic performance of HA as a single, low-cost, and easily available marker of hepatic fibrosis suitable for everyday clinical practice.

METHODS

Patients. This cross-sectional study involved 100 consecutive children and adolescents (68 males and 32 females) with biopsy-proven NAFLD referred to the Liver Unit of the ''Bambino Gesù'' Children's Hospital and Research Institute between May 2006 and November 2009. Exclusion criteria were (1) excessive alcohol intake ($\geq$20 g/day), (2) hepatitis A, B, C, D, E, or G or cytomegalovirus or Epstein-Barr virus infection, (3) autoimmune liver disease, (4) metabolic liver disease, (5) celiac disease, (6) Wilson's disease, (7) alpha-1-antitrypsin deficiency, (8) total parenteral nutrition, and (9) use of steatogenic drugs. The study protocol was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the ''Bambino Gesù'' Children's Hospital. Informed consent was obtained from each patient and/or at least 1 legal guardian.

Anthropometric and laboratory measurements. Weight and height were measured and body mass index (BMI) was calculated and converted to standard deviation scores (SDSs) using the CDC 2000 reference data.[25] Alanine transaminase (ALT), aspartate transaminase (AST), and gamma-glutamyl-transferase (GGT) were measured using standard methods as described elsewhere.[26] Glucose was measured using standard laboratory methods and insulin by radioimmunoassay (Myria Technogenetics, Milan, Italy). The homeostasis model assessment index of insulin resistance (HOMA-R) was calculated as (fasting insulin [μU/mL] $\times$ fasting glucose [mmol/L]/22.5). Serum HA was collected at the time of liver biopsy and immediately stored at –80 °C. HA was then measured using an enzyme-linked binding protein assay (Hyaluronan; R&D Systems, Minneapolis, Minn) and is reported as ng/mL.

Table I. Anthropometric and laboratory measurements of the 100 study subjects stratified by stage of liver fibrosis

	F0 (n = 35)				F1 (n = 50)				F2+ (n = 15)				JT test
	Median	IQR	Min	Max	Median	IQR	Min	Max	Median	IQR	Min	Max	*P* value
Age (years)	12	3	5	17	11	3	5	17	11	4	8	18	0.839
Weight (kg)	58.7	23.6	19.4	100.0	58.2	25.0	19.4	98.0	62.0	18.0	34.8	84	0.914
Weight (SDS)	1.92	1.21	0.31	3.70	2.20	1.13	0.04	4.20	1.90	0.84	0.17	2.83	0.929
Height (m)	1.54	0.20	1.11	1.78	1.52	0.14	1.09	1.80	1.57	0.29	1.35	1.84	0.753
Height (SDS)	0.95	1.91	−1.08	3.39	0.64	1.73	−1.05	3.54	1.05	0.98	−0.24	2.97	0.373
BMI (kg/m^2)	25.3	6.0	15.9	38.1	26.0	6.9	16.5	38.3	24.5	4.7	19.2	33.3	0.663
BMI (SDS)	1.96	0.78	0.28	2.86	2.06	0.76	0.77	3.33	1.68	0.77	−0.58	2.55	0.623
ALT (U/L)	59	26	28	126	59	24	14	192	54	25	14	93	0.283
AST (U/L)	45	26	21	71	48	34	21	151	32	26	20	61	0.487
GGT (U/L)	22	5	11	71	22	11	11	83	19	5	11	33	0.194
HOMA-R	2.1	2.0	0.7	8.3	2.1	1.4	0.8	9.5	2.2	2.7	1.1	6.0	0.635
HA (ng/mL)	351	430	74	1600	1170	260	230	1560	2070	960	1340	3000	<0.001

Abbreviations: JT, Jonckheere-Terpstra test for ordered alternatives (both ascending and descending); SDS, standard deviation score; HOMA-R, homeostasis model assessment of insulin resistance.

Liver histopathology. Liver biopsies were performed using an automatic core biopsy device (Biopince, Amedic, Sweden) with a 18-G needle, 150 mm long, and the ability to cut tissues with lengths up to 33 mm with great precision.[27] Liver biopsies, which were at least 15 mm, were read by a single pathologist who was unaware of the patient's clinical and laboratory data. The biopsies were processed routinely (formalin fixed and paraffin embedded) and analyzed by different staining. Hematoxylin and eosin staining was used to evaluate the architecture of liver parenchyma, hepatocyte abnormalities, and inflammatory infiltrates. Van Gieson staining was used for the assessment of fibrosis and architectural changes. NALFD and NASH were diagnosed using the criteria developed by the NASH Clinical Research Network.[28] Briefly, steatosis was graded on a 4-point scale: grade 0 = steatosis involving <5% of hepatocytes; grade 1 = steatosis involving up to 33%; grade 2 = steatosis involving 33% to 66%; and grade 3 = steatosis involving >66%. Lobular inflammation was graded on a 4-point scale: grade 0 = no foci; grade 1 = less than 2 foci per 200× field; grade 2 = 2–4 foci per 200× field; and grade 3 = >4 foci per 200× field. Hepatocyte ballooning was graded from 0 to 2: 0 = none, 1 = few balloon cells, and 2 = many balloon cells. Fibrosis was quantified using a 5-level scale: 0 = no fibrosis; 1 = peri-sinusoidal or periportal fibrosis (1a = mild, zone 3 and perisinusoidal; 1b = moderate, zone 3 and perisinu-soidal; 1c = portal/periportal); 2 = perisinusoidal and portal/periportal; 3 = bridging fibrosis; and 4 = cirrhosis.

Statistical analysis. Descriptive statistics are reported as median, interquartile range (IQR), and minimum and maximum values because of skewed distributions. IQR was calculated as the difference between the 75th and 25th percentile. The Jonckheere-Terpstra test for ordered alternatives (both ascending and descending) was used to test the existence of a trend between ordinally coded liver fibrosis (F0 = stage 0; F1 = stage 1; and F2+ = stages 2, 3, and 4) and HA and the other variables of interest.[29] The Fisher exact test was used to evaluate the association between categorical variables and liver fibrosis.[29] Spearman's rho was used to evaluate the association between continuous variables.[29] We assessed the ability of HA to predict liver fibrosis using proportional-odds logistic regression.[30,31] Ordinally coded liver fibrosis was the response variable and HA was the predictor. Odds ratios (ORs) and 95% bootstrapped confidence intervals (95% CIs) were calculated, with bias-corrected accelerated bootstrap performed on 1000 random samples of 100 subjects. The OR obtained from this model is a measure of the change in the odds from less severe to more severe liver fibrosis.[31] The equality of slopes among the levels of liver fibrosis was checked using the Brant test.[32] The model fit was evaluated using m-asymptotic diagnostic plots and the receiver operating characteristic (ROC) curves for the 2 binary models underlying the proportional-odds model (F0 vs F1 and F2+, and F0 + F1 vs F2+).[32] Positive predictive values (PPVs) and negative predictive values (NPVs) were also calculated for cut points selected on the basis of the previous analysis.[33] All statistical tests were 2-tailed, and statistical significance was assigned to a P-value < 0.05. A statistical analysis was performed using Stata version 11 (StataCorp, College Station, Tex).

RESULTS

Table I reports the anthropometric and clinical measurements of the 100 subjects (68 male and 32 female

Table II. Distribution of histopathologic lesions in the 100 study subjects stratified by stage of liver fibrosis

	F0 (n = 35)				F1 (n = 50)				F2+ (n = 15)				FE test
Degree or stage	0	1	2	3	0	1	2	3	0	1	2	3	P value
Steatosis	0	5	20	10	0	9	34	7	0	3	6	6	0.179
Inflammation	2	23	10	0	2	36	12	—	0	11	4	—	0.966
Ballooning	17	18	0	—	26	24	0	—	10	5	0	—	0.481

Abbreviations: F0, fibrosis stage 0; F1, fibrosis stage 1; F2+, fibrosis stages 2, 3 and 4; FE, Fisher's exact test.

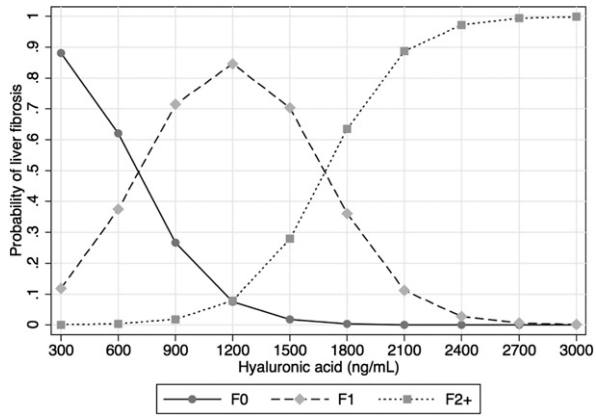

Fig 1. Probability of liver fibrosis as detected by plasma levels of HA (proportional-odds logistic regression).

subjects) stratified by degree of liver fibrosis; 35% of the subjects had no liver fibrosis (F0), whereas 50% had F1 and 15% had F2+ liver fibrosis. Among the subjects with F2+ fibrosis, 11 had F2, 2 had F3, and 2 had F4 fibrosis. The distribution of liver fibrosis in male and female subjects was as follows: F0 38% versus 28%, F1 48% versus 53%, and F2+ 13% versus 18%. The subjects with F0, F1, and F2+ fibrosis had similar values of anthropometry, liver enzymes, and HOMA-R, whereas HA showed an increasing trend ($P < 0.001$) for increasing values of liver fibrosis. We found no association between HA and ALT, AST, GGT and HOMA-R ($P \geq 0.106$). Table II reports the distribution of histopathologic lesions in the study subjects. No association was found between the stage of liver fibrosis and the degree of steatosis, inflammation, or ballooning ($P \geq 0.371$).

At proportional-odds ordinal logistic regression, an increase of 300 ng/mL unit of HA was associated with an OR of 4.51 (bootstrapped 95% CI: 2.50–8.13, $P < 0.001$) for more severe versus less severe liver fibrosis. Figure 1 depicts the probability of liver steatosis for increments of 300 ng/mL of hyaluronic acid, and Table III gives the associated 95% CI for this probability. The ROC area associated to the use of HA to diagnose any degree of liver fibrosis (ie, F1 and F2+ versus F0 was 0.88 [95% CI: 0.81–0.96]). Using the ≥1200 ng/mL cut point, the PPV of HA was 0.90 (exact 95% CI: 0.77–0.97), and the NPV was 0.53 (exact 95% CI: 0.39–0.66). The ROC area associated with the use of HA to diagnose significant liver fibrosis (ie, F2+ versus F0 and F1) was 0.95 (95% CI: 0.91–0.99). Using the 2100 ng/mL cut point, the PPV of HA was 0.40 (exact 95% CI: 0.05–0.85), and the NPV was 0.91 (exact 95% CI: 0.83–0.96). Both PPVs and NPVs, and their confidence intervals, are influenced clearly by the high prevalence (65%) of liver fibrosis is our series and by the fact that just 15% of patients had F2+ fibrosis.[15,33] It is important to note, however, that the main aim of this study was not to develop binary cut points but to model the HA–fibrosis relationship as ordinal not only giving a better possibility to cross test our findings in external populations but also providing the potential user of HA with a larger and less arbitrary spectrum of probabilities than allowed by dichotomization.

DISCUSSION

Because of the current epidemic of obesity, NAFLD is a booming health care problem that should not be underestimated, especially in children.[2] Although liver biopsy is the recognized gold standard for the diagnosis of NASH and liver fibrosis,[28] it is an invasive technique that is perceived as more risky in children than in adults.[27] Even more importantly, liver biopsy is not suitable for repeated short-term assessments during follow-up.[34] Because of this evidence, many efforts have been directed at developing noninvasive methods to separate simple steatosis from NASH and to detect liver fibrosis in patients with fatty liver.[35]

The detection of liver fibrosis in a patient with NAFLD would imply the presence of progressive liver disease and make the differentiation between simple fatty liver and NASH less important.[35] The noninvasive methods evaluated so far for the assessment of fibrosis in pediatric NASH include a proprietary algorithm based on a panel of serum markers that was shown to be accurate at detecting F2+ fibrosis in a selected series of children with NAFLD followed at a tertiary care center.[15,36] Although the available methods for the detection of fibrosis are being validated, it is important to test noninvasive and low-cost methods not needing

Table III. Probability of liver fibrosis as detected by serum levels of hyaluronic acid (proportional-odds logistic regression)

	F0			F1			F2+		
HA (ng/mL)	Prob	Lower	Upper	Prob	Lower	Upper	Prob	Lower	Upper
300	0.88	0.79	0.98	0.12	0.02	0.21	0.00	0.00	0.00
600	0.62	0.46	0.78	0.37	0.22	0.53	0.00	0.00	0.01
900	0.27	0.14	0.40	0.71	0.58	0.85	0.02	0.00	0.04
1200	0.07	0.01	0.14	0.85	0.75	0.95	0.08	0.01	0.14
1500	0.02	0.00	0.04	0.70	0.54	0.87	0.28	0.11	0.45
1800	0.00	0.00	0.01	0.36	0.13	0.59	0.64	0.40	0.87
2100	0.00	0.00	0.00	0.11	0.02	0.24	0.89	0.75	1.00
2400	0.00	0.00	0.00	0.03	0.02	0.07	0.97	0.93	1.00
2700	0.00	0.00	0.00	0.01	0.01	0.02	0.99	0.98	1.00
3000	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	1.00

Abbreviations: F0, fibrosis stage 0; F1, fibrosis stage 1; F2+, fibrosis stages 2, 3 and 4; Prob, probability of liver fibrosis; Lower, lower 95% confidence interval of the probability of liver fibrosis; Upper, upper 95% confidence interval of the probability of liver fibrosis.

referral of the patient to a specialized center where transient elastography or other diagnostic methods are available.[37] The ideal method would be available at any level of health care (ie, from general practice to tertiary care), especially in view of the high prevalence of NAFLD in the general population. This finding would allow, in fact, a screening of the pediatric population with NAFLD for associated liver fibrosis. A first screening performed by the general practitioner by some easily available tests would allow the selection of patients to be referred to specialized centers for subsequent evaluation.

Two recent studies showed that HA is a good marker of severe fibrosis in adults with NAFLD,[38,39] and another study confirmed these findings in a sample of unselected children undergoing liver biopsy.[24] Our study, which was performed only in children with NAFLD, confirms the potential of HA for the prediction of liver fibrosis. We modeled fibrosis as ordinal to gain a better insight into the HA–fibrosis relationship. According to our data, a value of HA $\geq$ 1200 ng/mL makes the absence of fibrosis unlikely (7%, 95% CI: 1% to 14%) and a value of HA $\geq$ 2100 ng/mL makes significant fibrosis very likely (89%, 95% CI: 75% to 100%).

Our study shows, for the first time, that serum HA is a predictor of the degree of hepatic fibrosis in a pediatric population with NAFLD. If our data are confirmed by subsequent studies, HA may allow a simple and efficient screening of patients at risk of progressive liver disease needing additional investigation, including the execution of liver biopsy. More studies are needed to confirm our findings and to test whether HA can be employed to predict liver fibrosis in pediatric populations with similar and lower prevalence of liver fibrosis.[13]

Giorgio Bedogni and Massimo Pinzani contributed equally to this study.

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