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Cytokeratin-18 and hyaluronic acid levels predict liver fibrosis in children with non-alcoholic fatty liver disease*

Dariusz M. Lebensztejn[1]✉, Aldona Wierzbicka[2], Piotr Socha[2], Maciej Pronicki[3], Elżbieta Skiba[4], Irena Werpachowska[1] and Maciej Kaczmarski[1]

[1]Department of Pediatrics, Gastroenterology and Allergology, Medical University of Bialystok, Bialystok, Poland; [2]Department of Gastroenterology, Hepatology and Immunology, Children's Health Memorial Institute, Warszawa, Poland; [3]Department of Pathology, Children's Health Memorial Institute, Warszawa, Poland; and [4]Department of Pediatric Oncology and Hematology, Medical University of Bialystok, Bialystok, Poland

Objectives: There is a need to replace liver biopsy with non-invasive markers that predict the degree of liver fibrosis in fatty liver disease related to obesity. Therefore, we studied four potential serum markers of liver fibrosis and compared them with histopathological findings in liver biopsy in children with non-alcoholic fatty liver disease (NAFLD). Methods: We determined fasting serum level of hyaluronic acid (HA), laminin, YKL-40 and cytokeratin-18 M30 in 52 children (age range 4–19, mean 12 years, 80% of them were overweight or obese) with biopsy-verified NAFLD. Viral hepatitis, autoimmune and metabolic liver diseases (Wilson's disease, alpha-1-antitrypsin deficiency, cystic fibrosis) were excluded. Fibrosis stage was assessed in a blinded fashion by one pathologist according to Kleiner. Receiver operating characteristics (ROC) analysis was used to calculate the power of the assays to detect liver fibrosis (AccuROC, Canada). Results: Liver fibrosis was diagnosed in 19 children (37%). The levels of HA and CK18M30 were significantly higher in children with fibrosis compared to children without fibrosis (p=0.04 and 0.05 respectively). The ability of serum HA (cut-off 19.1 ng/ml, Se=84%, Sp=55%, PPV=52%, NPV=86%) and CK18M30 (cut-off 210 u/l, Se=79%, Sp=60%, PPV=56%, NPV=82%) to differentiate children with fibrosis from those without fibrosis was significant (AUC=0.672 and 0.666, respectively). The combination of both markers was superior (AUC=0.73, p=0.002). Laminin and YKL-40 levels did not allow a useful prediction. Conclusions: Cytokeratin-18 and hyaluronic acid are suitable serum markers predicting liver fibrosis in children with NAFLD. Studying these markers may identify patients at risk of disease progression.

Keywords: children, cytokeratin-18, hyaluronic acid, laminin, NAFLD, YKL-40

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INTRODUCTION

Obesity in childhood is an increasing health problem that has reached an alarming proportion all over the world. Non-alcoholic fatty liver disease (NAFLD), which is closely associated with obesity, is the most common cause of chronic liver disease both in adults and children (Matteoni *et al.*, 1999; Patton *et al.*, 2006). The histopathological features of this disease may take the form of simple steatosis or show progression to steatohepatitis, fibrosis and even cirrhosis, liver insufficiency and hepatocellular carcinoma (Matteoni *et al.*, 1999; Patton *et al.*, 2006; Schwimmer *et al.*, 2006; Feldstein *et al.*, 2009). Recently, liver transplantation for paediatric NAFLD cirrhosis has been reported (Feldstein *et al.*, 2009). For that reason monitoring the progression of the disease (mainly fibrosis) is urgently needed.

Unambiguous diagnosis of NAFLD and especially staging of fibrosis require liver biopsy, which due to its invasiveness and other limitations (e.g., sampling error) cannot be widely used in clinical practice in children (Ratziu *et al.*, 2005). Furthermore, histological diagnosis of NAFLD does not affect the treatment of obesity-related liver disease in paediatric patients and it only provides static information about the amount of fibrotic tissue accumulated in the liver (Patton *et al.*, 2006). Therefore, there is a need for non-invasive parameters that could predict the dynamics or at least the stage of liver fibrosis in children and thus obviate the requirement of liver biopsy.

From the broad spectrum of extracellular-matrix (ECM) components we chose hyaluronic acid (HA), laminin and YKL-40 (human cartilage glycoprotein-39). Normal serum concentration of these markers are not significantly affected by body growth and therefore appear to be useful in assessing ECM metabolism in paediatric liver disease (Trivedi *et al.*, 1993; Johansen *et al.*, 1996). In our previous study we confirmed the usefulness of hyaluronic acid and laminin levels in predicting liver fibrosis in children with chronic hepatitis B (Lebensztejn *et al.*, 2004; 2007).

We also analysed the serum concentration of cytokeratin-18 (keratin-18), which can be regarded as a marker of apoptosis. It has previously been found that apoptosis of hepatocytes may play an important role in the pathogenesis of NAFLD, mainly in fibrogenesis and progression to cirrhosis (Wieckowska *et al.*, 2006).

The aim of the study was to assess serum concentrations of the selected biomarkers in children with NAFLD in relation to liver fibrosis diagnosed by liver biopsy and to determine the diagnostic value of those serum markers for identification of patients with liver fibrosis.

✉e-mail: lebensztejn@hoga.pl

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Abbreviation: AUC, area under curve; BMI, body mass index; CK, cytokeratin; ECM, extracellular matrix; F, fibrosis; HA, hyaluronic acid; HOMAIR, homeostasis model assessment-insulin resistance; NAFLD, non-alcoholic fatty liver disease; ROC, receiver operating characteristics.

STUDY GROUP

The study was carried out on 52 children (mean age 12 years, range 4–19, 39 boys and 13 girls) with biopsy-verified NAFLD (80% of them were overweight or obese). Viral hepatitis (HBV, HCV), autoimmune hepatitis and metabolic liver diseases (Wilson's disease, alpha-1-antitrypsin deficiency, cystic fibrosis) were excluded. No subject had clinical diabetes. As a control group, 25 non-obese children (8–16 years, mean age — 12 years) were included without anamnestical, or laboratory signs of organic liver diseases or other systemic diseases.

Informed consent was obtained from parents of the patients studied and the protocol was approved by the ethics committee of the Medical University of Bialystok.

METHODS

Serum level of total cholesterol, HDL and LDL lipoproteins, triglycerides as well as standard liver tests (alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyltranspeptidase (GGT)) were measured directly by validated automated methods. Insulin resistance was assessed by homeostatis model (HOMA-IR) (Matthews *et al.*, 1985).

Measurement of serum fibrosis markers. Fibrosis markers were measured in serum samples (obtained after an overnight fast of 12 h) using commercial kits: hyaluronic acid (Corgenix Inc., USA); cytokeratin-18 (M30) (Peviva AB, Sweden), laminin (Takara Bio Inc.,); YKL-40 (METRA, Quidel Corp., USA).

Histological analysis. All children underwent liver biopsy on the day after serum sampling. Liver biopsy was performed in children with liver brightness in ultrasound examination and/or elevated serum ALT activity. Liver specimens were fixed in buffered formalin and embedded in paraffin. Fibrosis stage was assessed in a blinded fashion by a single pathologist without knowledge of the patients' laboratory or clinical data according to Kleiner *et al.* (2005) using a 5-point scale: F0 — no fibrosis, F1 — mild/moderate perisinusoidal or portal fibrosis, F2 — both perisinusoidal and portal fibrosis, F3 — bridging fibrosis, F4 — cirrhosis.

Statistics. Biochemical serum parameters were expressed as medians and interquartile (Q1-Q3) ranges. Statistical analysis was performed with the Mann-Whitney two-sample test for nonparametric data. The relationship between biochemical tests and liver fibrosis scores was analyzed by the Spearman rank-correlation test for nonparametric data and by the Pearson method for parametric data. Tests were considered statistically significant at $P<0.05$. Receiver operating characteristics (ROC) analysis (AccuROC, Montreal, Canada) was used to calculate the power of the assay to detect liver fibrosis. Sensitivity of the assay was plotted against false positivity (1-specificity). Comparison of the area under curve (AUC) was performed using a two-tailed *p*-test, which compares the AUC with the diagonal line of no information (AUC 0.5).

RESULTS

Characteristics of the patients

Selected biochemical and histological data are presented in Table 1.

Table 1. Characteristics of examined children with NAFLD.

Data of patients	Median	Q1	Q3
Age (years)	12.1	10.5	14.4
BMI (kg/m²)	15.6	23	28.5
ALT (IU/l)	70	51	114
AST (IU/l)	37	28	62
GGT (IU/l)	35	25	52
Total cholesterol (mg%)	170	148.5	202
Lipoproteins HDL (mg%)	39	32	45
Lipoproteins LDL (mg%)	101	89	128
Triglycerides (mg%)	110	72	160.5
Glucose (mg%)	97.5	82	91
Insulin (μIU/ml)	14	9	24
HOMA-IR	1.9	0	3.7
HA (ng/ml)	19.9	17.2	23.2
Laminin (ng/ml)	549.9	423.5	738.5
YKL-40 (ng/ml)	36.7	27.2	54.1
CK-18 M30 (U/l)	215	150	342

Serum concentration of fibrosis markers

Liver fibrosis was diagnosed in 19 children (37%); according to the Kleiner scale its degree was evaluated to be F1 in 14 children, F2 in one child and F3 in 3 children. Serum concentrations of HA, laminin and YKL-40 were significantly higher ($P<0.05$) in NAFLD patients with fibrosis (20.5 ng/ml; 19.5–34.8, 623 ng/ml; 357–980, 42.6 ng/ml; 27.7–61.1, respectively) than in controls (13.5 ng/ml; 12–14.2, 54 ng/ml; 47–66, 28.6 ng/ml; 24.4–32); CK-18 M30 level was also higher in children with liver fibrosis (311 U/l; 210–378) than in the control group (234 U/l; 187–270) but it did not achieve statistical significance.

There was a significant correlation of CK-18 M30 level with the stage of liver fibrosis ($r=0.32$, $P=0.023$). Hyaluronan, laminin and YKL-40 did not correlate with fibrosis stage. There were no correlations between serum fibrosis markers levels and total cholesterol, HDL,

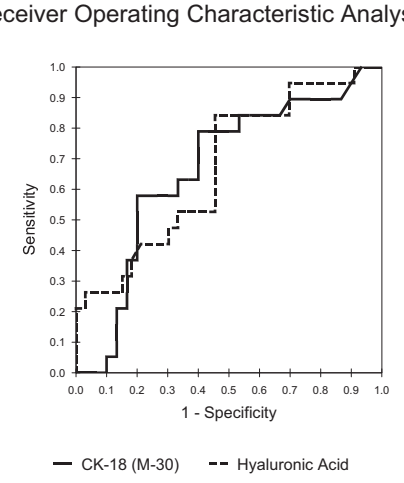

Figure 1. ROC curve of ability of HA and cytokeratin-18 M30 to predict morphological liver fibrosis in children with NAFLD.

Table 2. Characteristics of subgroup of NAFLD children without fibrosis (n = 33) and with liver fibrosis (n = 19).

Data of patients	NAFLD children without fibrosis (n=33) Median; Q1-Q3	NAFLD children with fibrosis (F1-F3) (n=19) Median; Q1-Q3	P
Age (years)	12.5; 10.7–14.6	11.8; 9.3–14	NS
BMI (kg/m^2)	25.8; 23.5–29.8	24.5; 20.3–27.8	NS
ALT (IU/l)	69.5; 53–114	75; 43–107.5	NS
AST (IU/l)	37.5; 28–52	37; 31–69	NS
GGTP (IU/l)	35; 25–48	34; 22.5–57	NS
Total cholesterol (mg/dl)	171; 150–204	169; 132–198	NS
Lipoproteins HDL (mg/dl)	37; 31–45	40; 32–47	NS
Lipoproteins LDL (mg/dl)	101; 89–127	101; 72–138	NS
Triglycerides (mg/dl)	118; 88–162	169; 132–198	NS
Glucose (mg/dl)	87; 81–90	84; 82–92	NS
Insulin (μIU/ml)	14; 9.6–23	13.5; 8.1–33	NS
HOMA-IR	1.78; 0–3.5	2.45; 0.2–4.3	NS
HA (ng/ml)	18.5; 16.2–22.2	20.5; 19.5–34.8	0.04
Laminin (ng/ml)	532; 427–644	623; 357–980	NS
YKL-40 (ng/ml)	36.7; 26.8–47.8	42.6; 27.7–61.1	NS
CK-18 M30 (U/l)	177.5; 137–306	311; 210–378	0.05

LDL, triglycerides, HOMA-IR or standard liver tests, except for YKL-40 which correlated with ALT ($r = 0.45$, $P = 0.003$) and GGT ($r = 0.49$, $P = 0.001$).

Diagnostic value of serum fibrosis markers for identification of patients with liver fibrosis

The levels of HA and CK-18 M30 were significantly higher in children with diagnosed fibrosis compared to NAFLD children without fibrosis ($P = 0.04$; 0.05, respectively) (Table 2).

The ability of serum HA (cut-off 19.1 ng/ml, Se = 84%, Sp = 55%, PPV = 52%, NPV = 86%) and CK-18 M30 (cut-off 210 u/l, Se=79%, Sp=60%, PPV = 56%, NPV = 82%) levels to differentiate NAFLD children with fibrosis from those without fibrosis was significant (AUC = 0.672, $P = 0.04$; AUC = 0.666, p = 0.05 respectively) (Fig. 1). The combination of both markers was superior to HA or CK-18 alone (AUC = 0.73, $P = 0.002$, Se = 74%, Sp = 79%, PPV = 56%, NPV = 63%). Using hyaluronic acid and cytokeratin-18 levels combined, 35 out of 52 children could be correctly allocated either to the group without or with fibrosis, potentially avoiding biopsy in 67.35% of the examined children. Laminin and YKL-40 did not allow a useful prediction.

DISCUSSION

We evaluated the serum concentrations of hyaluronic acid, laminin, YKL-40 and cytokeratin-18 in children with diagnosed NAFLD and compared those data with histological liver fibrosis. It should be stressed that we only examined well-defined, biopsy-proven NAFLD Caucasian children. It is widely accepted that studies in paediatric patients have special value because children can be regarded as an ideal model for investigation of NAFLD; they have earlier stages of the disease (cirrhosis is rare) and no major confounding factors (e.g., alcohol or other environmental influences) often seen in adults. We found that only cytokeratin-18 level correlated with fibrosis stage. Still, one should view liver biopsy results as only an approximation of true liver histology, therefore some discrepancy between serum fibrosis markers and the stage of fibrosis is to be expected. Some authors have suggested that histological evaluation of liver bioptate is not sensitive enough to detect small changes in the stage of liver fibrosis and serum markers may in fact be more accurate than biopsy in staging the disease (Afdhal, 2004; Lebensztejn *et al.,* 2005). According to Poynard *et al.* (2004) inadequate evaluation of liver biopsy rather than inaccuracy of biomarkers (FibroTest) was more commonly the cause for divergent results between biopsy and markers.

In this study we did not analyse the relationship between biomarkers and histological NAFLD activity score. NAS is a nonweighted sum of steatosis, ballooning and inflammation (Kleiner *et al.*, 2004). Observations from previous studies indicated that NAS does not correlated well with pediatric NAFLD (Carter-Kent *et al.*, 2009). Children rarely demonstrated ballooning of hepatocytes or Mallory bodies and a high proportion of children (up to 40%) could not be classified into NASH or simple steatosis group (Lavine *et al.*, 2004; Valva *et al.*, 2008; Fitzpatrick *et al.*, 2010).

For that reason the main aim of the study was to determine if chosen biochemical parameters have any

clinical usefulness as markers of liver fibrosis. We found significantly higher levels of CK-18 M30 and HA in NAFLD children with diagnosed fibrosis compared to those without morphological fibrosis. In ROC analysis we confirmed that the ability of serum HA and CK-18 M30 to differentiate children with fibrosis from those without fibrosis was significant and for the first time we found that combination of both markers was superior in identification of patients at disease progression. Laminin and YKL-40 did not allow a useful prediction.

Only Fitzpatrick *et al.* (2010) analysed both CK-18 M30 and HA levels in children with biopsy-proven NAFLD, but they did not estimate the correlation between biomarkers and fibrosis stage and they did not analyse the combination of the markers examined. Using the NAS scoring they found that CK-18 M30 is useful in stratifying disease severity in pediatric NAFLD but HA did not achieve significance in predicting steatohepatitis or significant fibrosis. Our findings concerning HA are in agreement with the data published by Nobili *et al.* (2010) who also confirmed the potential of this marker for the prediction of liver fibrosis in NAFLD children. In adults with NAFLD a correlation between CK-18 (Diab *et al.*, 2008; Feldstein *et al.*, 2009; Civera *et al.*, 2010) and HA (Suzuki *et al.,* 2005; Malik *et al.*, 2009) and the stage of liver fibrosis was also found.

The potential limitation of our study includes selection bias because our group of NAFLD children was evaluated at a tertiary-level paediatric hepatology unit. We also did not analyze the usefulness of the biomarkers examined in differentiating children with advanced liver fibrosis from those with mild fibrosis because the number of children with stage F2/F3 was too low (n = 4).

In conclusion, cytokeratin-18 M30 and hyaluronic acid seem to be suitable serum markers predicting liver fibrosis in children with NAFLD. Their application identifies with good accuracy patients at risk of disease progression.

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