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Regular paper

Retinol binding protein-4 as a serum biomarker of intrahepatic lipid content in obese children — preliminary report

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Objectives: Obesity, insulin resistance and dyslipidemia are the most significant risk factors of non-alcoholic fatty liver disease (NAFLD) but the role of adipokines in the pathogenesis of this disease is not clear. Assessment of retinol binding protein (RBP-4) seems to be promising because data from animal and human studies suggest its role in the patomechanism of insulin resistance. Therefore, the aim of the study was to evaluate the serum levels of RBP-4 in children with NAFLD. Methods: Fasting serum level of RBP-4 was determined in 42 obese children with suspected liver disease and 20 lean controls. The degree of liver steatosis was graded in ultrasound according to Saverymuttu. The intrahepatic lipid content was assessed noninvasively in a semiquantitative fashion using ^{1}HMR spectroscopy (1.5-T scanner with PRESS sequence). Results: Fatty liver was confirmed in 30 children by ultrasonography (16 of them had also increased alanine transaminase (ALT) activity). Serum concentrations of RBP-4 were significantly higher in obese children with NAFLD compared to controls. Significant correlations were found between RBP-4 level and ultrasonographic grade of liver steatosis, intrahepatic lipid content (^{1}HMRS) and triglycerides level, while the serum level of RBP-4 was not significantly higher in children with advanced liver steatosis (grade 2–3, n = 11) compared to patients with mild steatosis (grade 1, n = 19). The ability of RBP-4 to differentiate children with advanced liver steatosis from those with mild steatosis was not significant. Conclusion: RBP-4 can be considered as a convenient serum marker of intrahepatic lipid content in obese children.

Keywords: adipokines, RBP-4, children, NAFLD, obesity, biomarker

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INTRODUCTION

Recent data suggest that non-alcoholic fatty liver disease (NAFLD) is the most common liver pathology in adults and children (Argo & Caldwell, 2009; Loomba *et al*., 2009). Obesity, insulin resistance and lipid abnormalities play a crucial role in the development of this disease (Angulo, 2002; Patton *et al*., 2006). Recently the role of adipokines, peptides derived mainly from the adipose tissue, in the pathogenesis of metabolic syndrome, as well as NAFLD which is considered a hepatic manifestation of this syndrome, is widely discussed (Kershaw & Flier, 2004; Louthan *et al*., 2005; Lebensztejn *et al*., 2009). The newly identified adipokine retinol binding protein-4 (RBP-4) is of particular interest in this context because data from animal studies suggest its role in the patomechanism of insulin resistance (Yang *et al*., 2005). Data from human adults are conflicting and studies in children have special value because pediatric patients could be regarded as an ideal model for the study of pathogenesis of obesity-related liver disease. Children display early stages of the disease, an absence of major confounding factors (e.g. alcohol and other environmental influences) often seen in adults. Therefore, the aim of the study was to evaluate the RBP-4 serum level in obese children with NAFLD. We also aimed to determine for the first time the correlation of the serum RBP-4 with intrahepatic lipid content assessed noninvasively in a semiquantitative fashion using ^{1}HMR spectroscopy in this group of children.

MATERIALS

The study was carried out prospectively on 42 obese children (mean age 12.2 years, range 7–17, 27 boys and 15 girls) admitted to the Department of Pediatrics, Gastroenterology and Allergology of the Medical University of Białystok with suspected liver disease (hepatomegaly and/or ultrasonographic liver brightness and/or increased ALT activity). Informed consent was obtained from all patients' parents and the protocol was approved by the ethics committee of the Medical University of Białystok, Poland.

NAFLD was diagnosed in those children with steatosis confirmed in liver ultrasound evaluation and elevation of serum ALT activity. Viral hepatitis (HBV, HCV), toxic, autoimmune hepatitis (AIH) and metabolic liver diseases (Wilson disease, α1 antitrypsin deficiency, cystic fibrosis), as well as diabetes were excluded in all the children. Body mass index (BMI) and waist to hip ratio (WHR) were calculated for all the children. Serum level of total cholesterol, lipoproteins HDL and LDL, triglycerides as well as standard liver tests which included total bilirubin, alanine transaminase (ALT), aspartate transami-

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Abbreviations: ALT, aspartate transaminase (EC 2.6.1.2); AST, aspartate transaminase (EC 2.6.1.1); GGT, γ-glutamyltranspeptidase (EC 2.3.2.2); AUC, area under curve; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HOMA-IR, homeostasis model assessment — insulin resistance; ^{1}HMR, proton magnetic resonance; NAFLD, non-alcoholic fatty liver disease, RBP, retinol binding protein; ROC, receiver operating characteristics; WHR, waist to hip ratio

nase (AST) and γ-glutamyltranspeptidase (GGT) were measured directly by automated methods. Insulin resistance by homeostasis model (HOMA-IR) (Matthews *et al.*, 1985) was also measured.

METHODS

Serum RBP-4 level. Fasting serum level of RBP-4 was measured in all the patients and 20 non-obese controls of the same age using EIA commercial kits (DRG Diagnostics).

Ultrasound and ^{1}HMR spectroscopy. The degree of liver steatosis was graded from 0 (no steatosis) to 3, according to Saverymuttu *et al.* (1986) based on ultrasonographic examination (General Electric LOQIQ 500, convex 3–5 MHz). Steatosis grade was assessed in a blinded fashion by the same radiologist without knowledge of the patients' laboratory or clinical data. In order to determine specificity and sensitivity of the assay, we arbitrarily defined advanced liver steatosis as a score > 1 according to the semiquantitative scale used. ^{1}HMR spectroscopy was performed with 1.5-T scanner (Picker Eclipse) and with PRESS sequence; intrahepatic lipid content was assessed in relative units in comparison with unsupressed water signal. In all spectra, the peaks of highest intensity were observed in the range of 1.1–1.5 ppp, characteristic for lipid groups $(CH_2)_n$ (Lip 2). The groups CH_3 (Lip 1) and CH_2=CH–CH_2 (Lip 3) at 0.8–1.1 ppp and 1.9–2.3 ppp had signals of markedly lower intensity. Intrahepatic lipid content (IHLC) was calculated according to the formula:

IHLC = Lip 1 peak area + Lip 2 peak area + Lip 3 peak area/non-suppressed water area × 1000 (Tarasów *et al.*, 2002).

Statistics. Serum parameters were expressed as mean values ± standard deviation (S.D.). Statistical analysis was performed with the Mann-Whitney two-sample test. The relationship between results of the biochemical tests and liver ultrasound scores was analyzed by the Spearman rank-correlation test for nonparametric data and by the Pearson method for parametric data. The differences between groups 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 advanced liver steatosis. 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) (Vida, 1993).

RESULTS

Thirty children (70%) had liver steatosis in ultrasound examination (group I); 16 of them also had an increased serum ALT activity (group Ia — hepatopathic obese children — NAFLD). Thirteen children (30%) had neither liver brightness in ultrasound nor increased ALT activity (group II — non-hepatopathic obese children).

Anthropometric parameters

There were no significant differences between the groups regarding age and BMI; however, the NAFLD children displayed a significantly higher value of WHR. We also found the higher level of WHR ($P = 0.034$) as well as RBP-4 ($P = 0.0056$) and liver intrahepatic lipid content in MRS ($P = 0.009$) in boys than in girls.

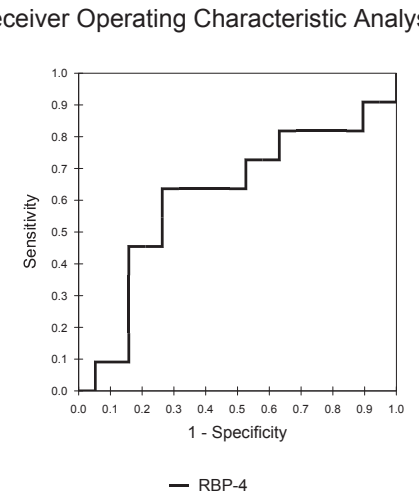

Figure 1. ROC curve of ability of RBP-4 to detect advanced liver steatosis according to Saverymuttu *et al.* (1986) in children with fatty liver.

Laboratory parameters

There were no significant differences between the groups regarding levels of bilirubin, lipid profile, glucose, insulin, HOMA-IR and RBP-4. However, the NAFLD children displayed a significantly higher activity of liver enzymes.

^{1}HMR spectroscopy assessment

The NAFLD children had significantly higher values of intrahepatic lipid content assessed by ^{1}HMR spectroscopy.

The characteristics of the examined groups of children are presented in Table 1.

Serum concentration of RBP-4

The serum RBP-4 level in children with liver steatosis in ultrasound (n = 30) and diagnosed NAFLD (n = 16) was significantly higher ($P < 0.0001$) compared to lean controls (n = 20).

There were significant positive correlations of ultrasonographic grade of liver steatosis in NAFLD children and RBP-4 ($r = 0.45$; $P = 0.05$), insulin ($r = 0.56$; $P = 0.02$), HOMA-IR ($r = 0.55$; $P = 0.03$) as well as intrahepatic lipid content ($r = 0.66$; $P = 0.005$). We also found a highly significant correlation between RBP-4 level and intrahepatic lipids and level of triglycerides ($r = 0.64$, $P = 0.007$; $r = 0.72$, $P = 0.002$, respectively).

Diagnostic value of RBP-4 for identification of patients with advanced liver steatosis

Nineteen children had mild liver steatosis and eleven children had advanced steatosis in ultrasound examination. The concentration of RBP-4 was not significantly different in children with advanced liver steatosis compared to patients with mild steatosis (33.6 ± 8.1 mg/l *vs* 30.4 ± 7.4 mg/l).

The ability of RBP-4 to differentiate the children with advanced liver steatosis from those with mild steatosis was not significant (AUC = 0.6124) (Fig. 1).

Table 1. Characteristics of examined subgroups of obese children

Characteristics of patients	Group I (n = 30) mean ± S.D.	Group Ia (n = 16) mean ± S.D.	Group II (n = 12) mean ± S.D.	*P* I *vs* II	*P* Ia *vs* II
Age (years)	12.17 ± 2.42	12.43 ± 2.12	12.33 ± 1.92	NS*	NS
BMI (kg/m^2)	29.5 ± 3.73	29.97 ± 3.36	30.86 ± 4.74	NS	NS
WHR	1.02 ± 0.07	1.02 ± 0.05	0.97 ± 0.06	0.05	0.01
ALT (IU/l)	51 ± 49	77 ± 56	24 ± 15	0.0033	0.0001
GGT (IU/l)	24 ± 11	34 ± 24	24 ± 28	NS	0.0022
Bilirubin (mg%)	0.7 ± 0.3	0.7 ± 0.2	0.7 ± 0.4	NS	NS
Cholesterol (mg/dl)	167 ± 36	165 ± 45	156 ± 21	NS	NS
Lipoprotein HDL (mg/dl)	51 ± 10	53 ± 12	53 ± 10	NS	NS
Lipoprotein LDL (mg/dl)	92 ± 30	88 ± 38	82 ± 20	NS	NS
Triglycerides (mg/dl)	120 ± 55	115 ± 64	102 ± 54	NS	NS
Glucose (mg/dl)	84 ± 7	82 ± 8	84 ± 4	NS	NS
Insulin (μIU/ml)	17.24 ± 7.7	17.4 ± 8.07	18.5 ± 14.95	NS	NS
HOMA–IR	2.14 ± 0.92	2.16 ± 0.95	1.83 ± 0.99	NS	NS
RBP-4 (mg/l)	1.3 ± 0.93	1.05 ± 0.41	1.1 ± 0.5	NS	NS
Intrahepatic lipid content (RU-relative units)	140 ± 89	170 ± 99	49 ± 29	< 0.0001	< 0.0001

*NS, not significant

DISCUSSION

A novel finding of our work is that serum concentration of RBP-4 correlates with ultrasonographic grade of liver steatosis and intrahepatic lipid content measured by ^{1}HMR spectroscopy in NAFLD children. To the best of our knowledge, this is the first pediatric report in which serum RBP-4 has been correlated with ectopic liver fat content assessed noninvasively in a semiquantitative fashion using ^{1}HMR spectoscopy. Our findings are in agreement with studies in adults (Stefan *et al*., 2007; Perseghin *et al*., 2007). The liver is the major source (80%) of RBP-4 secretion in rodents and in humans (Yang *et al*., 2005). Therefore, RBP-4 can be considered as a convenient serum marker of intrahepatic lipid content in obese children.

The data concerning serum concentration of RBP-4 in adults are conflicting. We found a significantly higher serum concentration of RBP-4 in NAFLD children compared to lean controls. Our report is consistent with the data of Seo *et al*. (2008) and Wu *et al*. (2008), who showed a higher RBP-4 level in adult NAFLD patients than in controls. However, other studies displayed no differences between controls and NAFLD adults (Milner *et al*., 2009; Cengiz *et al*., 2009), or even lower serum RBP-4 in NAFLD patients compared with a control group (Schina *et al*., 2009). These discrepancies may be explained by differences in age, anthropometric indices and liver tests among the studies and different methodology of RBP-4 examination (Graham *et al*., 2007). It should be stressed that liver function greatly impacts serum RBP-4 and its level decreases with the progression of the disease (Yagmur *et al*., 2007).

We also found that RBP-4 level was not significantly different in children with advanced liver steatosis compared to patients with mild steatosis diagnosed by ultrasound. The ability of RBP-4 to differentiate the children with advanced liver steatosis from those with mild steatosis was not significant. Therefore, RBP-4 does not predict advanced liver steatosis in this group of obese children.

Liver biopsy was not performed in our patients because of its invasiveness and some limitations such as sampling errors. Furthermore, histological diagnosis of NAFLD does not affect the treatment of obesity-related liver disease in paediatric patients (Ratziu *et al*., 2005; Patton *et al*., 2006). Moreover, the main aim of our study was not to examine the correlation between RBP-4 and the stage of liver fibrosis (it has already been assessed in children by Nobili *et al*., 2009), but rather to look for a correlation of this adipokine with ectopic intrahepatic lipid content assessed noninvasively in a semiquantitative fashion using ^{1}HMR spectroscopy. We also used ultrasound to identify and grade fatty liver due to its noninvasiveness, availability and sufficient specificity and sensitivity (Saadeh *et al*., 2002; Joy *et al*., 2003). It has to be stressed that the liver ultrasound examination was performed by the same radiologist and using the same equipment, increasing the reliability of the procedure. Recently, Nobili *et al*. (2009) tested the power of RBP-4 in predicting the degree of liver involvement in children with biopsy-proven NAFLD and found that high levels of this adipokine were associated significantly with low necroinflammatory activity, a low NAFLD score and a low fibrosis score. In adults, Alkhouri *et al*. (2009), Milner *et al*. (2009) and Schina *et al*. (2009) found no significant differences in RBP-4 level between patients with biopsy-proven simple steatosis and NASH (non-alcoholic steatohepatitis). However, Schina *et al*. (2009) confirmed that RBP-4 immunohistochemical score in the liver was positively correlated with the grade of steatosis and stage of fibrosis, and serum RBP-4 was significantly lower in NAFLD patients compared with control group.

Although findings in mice assigned RBP-4 a key role in the pathogenesis of insulin resistance (Yang *et al*., 2005), in humans the link between this adipokine and insulin resistance is not clear. We did not find a correlation between RBP-4 level and insulin resistance or anthropometric indices (BMI, WHR). In contrast with our report, Stefan *et al*. (2007) found an association of RBP-4 serum level with HOMA-IR, but others did not find

such a correlation in adults with NAFLD (Milner *et al*., 2009; Schina *et al*., 2009; Alkhouri *et al*., 2009). Our data concerning anthropometric indices are in agreement with the data presented by Perseghin *et al*. (2007). Moreover, Stefan *et al*. (2007) failed to find an association between RBP-4 and total, subcutaneal, or visceral amount of fat. Moreover, we found a positive correlation between RBP-4 and triglycerides' level, consistent with the results of others (Seo *et al*., 2008; Milner *et al*., 2009).

We conclude that although we did not confirm the relationships between RBP-4 level and major pathogenic indices of NAFLD (obesity, insulin resistance), this adipokine positively correlated with intrahepatic lipids assessed noninvasively in a semiquantitative fashion using ^{1}HMR spectroscopy. Therefore, RBP-4 can be regarded as a serum marker of intrahepatic lipid content in obese children but this finding needs to be confirmed in larger studies with biopsy-proven NAFLD.

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