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ORIGINAL PAPER

Can stanniocalcin-2 be regarded as a novel non-invasive biomarker of advanced liver steatosis in obese children? A preliminary study

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ABSTRACT

Introduction: With the increasing prevalence of liver steatosis, non-invasive methods are sought to detect it and to assess the degree of lipid accumulation in the liver. The aim of this preliminary study was to assess the serum concentrations of stanniocalcin-2 (STC-2), pregnancy-associated plasma protein A (PAPP-A) and insulin-like growth factor binding protein (IGFBP-4) among children with obesity, check if they detect liver steatosis and determine whether they differentiate mild and advanced steatosis.

Material and methods: This prospective study involved 62 obese children (39 boys and 23 girls, median age 13.75 years). Infectious, autoimmune, metabolic and toxic liver conditions were excluded. All subjects underwent body composition analysis, anthropometric measurements, abdominal ultrasound and routine blood chemistry analyses. STC-2, PAPPA and IGFBP-4 were measured using ELISA kits. The control group consisted of 19 non-obese, healthy children with correct body mass index and without organic pathologies.

Results: The concentration of examined biomarkers in obese children with liver steatosis was significantly higher than in the control group and STC-2 was also significantly higher in children with advanced steatosis in comparison to children with mild steatosis. In the group of obese children with liver steatosis STC-2 positively correlated with: gamma-glutamyltransferase, cholesterol, triglycerides, low-density lipoprotein cholesterol, homeostatic model assessment-insulin resistance and waist-hip ratio. The ability of serum STC-2 to detect advanced liver steatosis was significant (AUC = 0.746, $p = 0.0022$, cut-off 135.27 pg/ml).

Conclusions: This preliminary study demonstrated that STC-2 can be regarded as a potential non-invasive marker of advanced liver steatosis in obese children. The other analyzed parameters (PAPP-A and IGFBP-4) seem not to be useful in diagnosis of liver steatosis in obese children.

KEY WORDS:

obesity, PAPP-A, liver steatosis, STC-2, IGFBP-4.

INTRODUCTION

Non-invasive diagnostic methods have become increasingly important in various diseases, especially in pediatric population. A significant role is played by biomarkers, defined by the National Health Institute as indicators of natural biological processes, pathogenic processes or responses to an exposure or intervention [1]. Biomarker research also represents an evolving area within hepatology. Advances in technology have led to a rise in the discovery of putative biomarkers of the whole spectrum of liver injury, including fibrosis and steatosis [2, 3].

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Recently, potential non-invasive biomarkers of liver steatosis have also aroused wide interest in order to achieve diagnostic and prognostic effectiveness in tackling this growing global health concern.

What is more, new, non-invasive parameters are sought not only to detect liver steatosis but also to predict the degree of lipid accumulation in the liver [4–6]. Increasing evidence suggests that insulin-like growth factor (IGF) insulin-like growth factor binding protein (IGFBP-4), pregnancy-associated plasma protein A (PAPP-A) and serum concentration of stanniocalcin-2 (STC-2) may be linked to a number of pathological conditions, including metabolic disorders, cardiovascular diseases and non-alcoholic fatty liver disease (NAFLD), and the cooperation of these three agents was named the IGFBP-4/PAPP-A/STC-2 axis [7].

Stanniocalcin, a glycosylated peptide hormone, was originally shown to play a critical role in calcium and phosphate homeostasis. Various studies have indicated the possible involvement of two mammalian stanniocalcins (STC-1 and 2) in diverse biological processes including cell proliferation and apoptosis, inflammation, oxidative stress and metabolism [8]. STC-2 is predicted to function in an autocrine and/or paracrine fashion. Abundant STC-2 protein expression was also observed in the liver and sparse studies indicate its role in obesity and liver steatosis [9]. On the other hand, PAPP-A is a metalloprotease, expressed and present in a wide range of tissues, including adipose tissue. The highest levels are found in pregnant women; therefore PAPP-A serves as a biomarker of pregnancy-associated conditions. PAPP-A is also abundantly present in unstable atherosclerotic plaques and circulating concentrations of PAPP-A are increased in cardiovascular patients [10]. It has been shown recently that STC-2 interacts with PAPP-A, inhibiting its proteolytic activity toward IGFBP-4. IGFBP-4 is a critical regulator of the activity of IGF. The liver is the main source of circulating IGFBP-4, but the protein is secreted by a number of cell types, including adipocytes. Increased levels of IGFBP-4 result in reduction in IGF signaling [10]. Experimental and clinical data about the role of IGF-1 in cardiovascular disease are ambiguous and do not provide strong evidence. Interestingly, a recent single study suggested that STC-2 mediated PAPP-A inhibition ameliorates atherosclerosis in hypercholesterolemic mice [11].

PAPP-A and STC-2 were also identified as novel modulators of IGF-1 bioavailability and therefore its role in human growth. STC-2 inhibits PAPP-A's ability to cleave IGFBPs, thereby resulting in decreased levels of free IGF-1 and consequently decreased IGF-1 signaling [12].

The most common cause of liver steatosis in adults and children is NAFLD. Moreover, NAFLD is considered a hepatic manifestation of metabolic syndrome [13]. The parameters of the STC-2/PAPP-A/IGFBP-4 axis examined in this study are connected with metabolic disorders and potentially also with liver steatosis. Therefore, the aim of the study was to investigate parameters of the IGFBP-4/PAPP-A/STC-2 axis in obese children and check if they can be helpful in detecting liver steatosis in children, with particular emphasis on differentiating between mild and advanced steatosis.

MATERIALS AND METHODS

The prospective study included 62 consecutive children (39 boys and 23 girls, age 11.5–16 years, median age 13.75) with obesity (body mass index [BMI] > 95c), admitted to the department due to suspected liver disease (hepatomegaly, elevated serum alanine aminotransferase (ALT), and/or fatty liver in ultrasound examination). The control group consisted of 19 non-obese, healthy children (11 boys and 8 girls, median age 13.75, with BMI < 95c), without organic pathologies, hospitalized in the department because of accidental swallowing of a foreign body or functional disorders of the digestive tract.

The study was approved by the local bioethics committee. Written consent was obtained from all the children's parents.

All participants underwent physical examination with anthropometric measurements. Weight measurement was performed barefoot and wearing minimal garments by the same physician in all individuals, on the same, calibrated scale. To measure height the participant was asked to step onto the scale with the back to the scale and stand up straight with heels together. Body mass index was calculated by dividing weight (kg) by height squared (m^2). Waist and hip circumference were measured according to the World Health Organization protocol [14]. Waist-hip ratio (WHR) was calculated as the waist measurement divided by the hip measurement.

Viral hepatitis (HCV, HBV), selected metabolic liver diseases (Wilson's disease, alpha-1-antitrypsin deficiency), cystic fibrosis, celiac disease, autoimmune hepatitis, and toxic conditions were excluded in the studied group. None of the children in the cohort suffered from endocrine disorders including type 2 diabetes mellitus; nor did they receive any drugs.

Blood sampling was performed in the fasting state. Alanine aminotransferase, aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), total serum bilirubin, total serum cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides (TG), uric acid, fasting glucose and insulin were assessed with validated automated methods. Homeostatic model assessment-insulin resistance (HOMA-IR) was calculated according to Matthews *et al.* [15].

Abdominal ultrasonography (USG) was performed in all participants by the same experienced radiologist using a General Electric Voluson E8 with a convex 3–5 MHz transducer. The degree of liver steatosis was assessed according to a four-grade scale (0–3) described by Savery-

TABLE 1. Baseline characteristics of examined patients ($n = 62$)

Data of patients	Median	25–75 quartile
Age (years)	13.7	11.5–16
ALT [IU/l]	30.5	16–48
AST [IU/l]	25	20–35
GGT [IU/l]	23	17–30
Bilirubin [mg/dl]	0.47	0.35–0.65
Total cholesterol [mg/dl]	167	145–178
HDL cholesterol [mg/dl]	44.5	39–53
LDL cholesterol [mg/dl]	97	75–114
Triglycerides [mg/dl]	89	61–124
Glucose [mg/dl]	89	84–93
Insulin [µIU/ml]	15.4	12–20.8
HOMA-IR	3.4	2.6–4.5
Uric acid [mg/dl]	6.2	5.2–7.3
BMI [kg/m^2]	29.2	26.2–32.3
Waist [cm]	93	88.5–101
Hip [cm]	106	97.5–113.5
WHR	0.91	0.86–0.96
Fat (%)	35.8	28.7–39.9
Fat mass [kg]	25.8	20.4–34.5
FFM [kg]	48.2	41.3–61
Muscle mass [kg]	45.9	39.65–58.45
TBW [kg]	35.3	30.2–44.7
TBW (%)	47.6	44–52.15
STC-2 [pg/ml]	109.1	91.6–148.4
PAPP-A [pg/ml]	477.1	429.3–513.2
IGFBP-4 [ng/ml]	90.0	28.6–127.9

ALT – alanine aminotransferase, AST – aspartate aminotransferase, BMI – body mass index, FFM – fat-free mass, GGT – gamma-glutamyltransferase, HDL – high-density lipoprotein, HOMA-IR – homeostatic model assessment-insulin resistance, IGFBP-4 – insulin-like growth factor binding protein, LDL – low-density lipoprotein, PAPP-A – pregnancy-associated plasma protein A, STC-2 – serum concentration of stanniocalcin-2, TBW – total body water, WHR – waist-hip ratio

TABLE 2. Comparison of serum concentration of stanniocalcin-2, pregnancy-associated plasma protein A and insulin-like growth factor binding protein, between obese children with liver steatosis (group 1) and control group (group 2)

Variable	Group 1 (obese children with liver steatosis, n = 38)	Group 2 (control, $n = 19$)	p
STC-2 [pg/ml]	120.0	76.4	0.002
PAPP-A [pg/ml]	472.9	433.2	0.04
IGFBP-4 [ng/ml]	78.3	28.1	0.002

IGFBP-4 – insulin-like growth factor binding protein, PAPP-A – pregnancy-associated plasma protein A, STC-2 – serum concentration of stanniocalcin-2

muttu *et al.* [16]. Mild liver steatosis was defined as stage 1, and advanced steatosis as stage 2 or 3.

Fatty liver index (FLI), considered as an indicator of fatty liver in adults, was calculated using an algorithm based on BMI, waist circumference, GGT and TG concentration.

A body fat analyzer (Tanita, Tokyo, Japan) was used to measure body composition by bioelectrical impedance. The following measurements were taken: body fat percentage (fat %), fat mass, fat-free mass, muscle mass and total body water mass and percentage.

Stanniocalcin-2, PAPP-A and IGFBP-4 were measured using specific enzyme-linked immunoassay (ELISA, Wuhan EIAab Science Co., Ltd, China).

STATISTICAL ANALYSES

Biochemical tests and anthropometric parameters were expressed as median and 25th–75th quartile (Q1–Q3). Statistical analyses were performed using Statistica 10.0. The Mann-Whitney *U* test was used for non-parametric data. The relationship between biochemical tests was analyzed by the Spearman rank-correlation test. Results were considered statistically significant at $p \leq 0.05$.

ETHICAL

This research was funded by the Medical University of Bialystok (SUB/1/DN/21/001/1143, N/ST/2B/18/001/1143).

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the Medical University of Bialystok (nr R-I-002/427/2015, 29.10.2015.).

Informed consent was obtained from all subjects involved in the study.

RESULTS

Table 1 presents baseline characteristics of examined children.

Liver steatosis was diagnosed in ultrasound only in 38 obese children (61.3%). The concentrations of STC-2, PAPP-A and IGFBP-4 in obese children with liver steatosis were significantly higher than in the control group (Table 2).

Among all obese patients, 18 children had mild liver steatosis on USG (stage 1), and 20 children developed advanced steatosis (stage 2 or 3) (Table 3). 18 children had mild liver steatosis on USG (stage 1, age: 11–16 years, median 13; 9 boys and 9 girls), and 20 children developed advanced steatosis (stage 2 or 3, age: 11.5– 15.3, median 12.8; 2 girls and 18 boys).

The concentration of STC-2 was significantly higher in children with advanced steatosis in comparison to children with mild steatosis. PAPP-A, IGFBP-4 and FLI did not differ between those two groups ($p = 0.82$). Moreover, children with advanced steatosis had significantly higher activity of ALT, AST, GGT and higher concentration of serum uric acid (Table 3).

In the examined group of obese children (n = 62) we found a significant positive correlation of STC-2 with:

TABLE 3. Comparative characteristics of subgroups of children with mild (grade 1) and advanced (grade 2 + 3) steatosis in ultrasound

Data of patients	Mild steatosis in USG ($n = 18$) Median; 25–75 quartile	Advanced steatosis in USG ($n = 20$) Median; 25–75 quartile	p
Age (years)	13; 11–16	12.75; 11.5–15.25	NS
ALT [IU/l]	27; 16–42	63; 41–91	< 0.001
AST [IU/l]	22; 20–28	38.5; 32–54	< 0.001
GGT [IU/l]	23.5; 20–27	31.5 ; 24–50	0.02
Bilirubin [mg/dl]	0.46; 0.36–0.64	0.51; 0.35–0.71	NS
Total cholesterol [mg/dl]	167; 142–177	168.5; 145.5–182.5	NS
HDL cholesterol [mg/dl]	49; 41–56	43.5; 41–48	NS
LDL cholesterol [mg/dl]	96.5; 81–111	98; 60–114	NS
Triglycerides [mg/dl]	71.5; 61–137	102; 71.5–139	NS
Glucose [mg/dl]	86.5; 84–89	86.5; 81.5–92.5	NS
Insulin [µIU/ml]	14.35; 12–16.3	18.4; 12.85–24.8	NS
HOMA-IR	3.04; 2.6–3.6	4.21; 2.7–5.45	NS
Uric acid [mg/dl]	6.1; 5.1–6.9	7.01; 6–7.5	0.04
BMI [kg/m2]	28.7; 27.1–32.3	29; 24.7–33.4	NS
Waist [cm]	93; 88–98	93.5; 89.5–104.5	NS
Hip [cm]	101; 99–110	101; 90.75–111	NS
WHR	0.9; 0.85–0.96	0.94; 0.9–1.02	NS
Fat (%)	37.7; 33.7–41.2	33; 27.05–38.3	NS
Fat mass [kg]	28.1; 24.1–34.9	22.1; 16.75–30.7	NS
FFM (kg)	46.4; 42.3–57.1	46; 39.8–68.95	NS
Muscle mass [kg]	44.9; 40.5–55.5	43.65; 37.7–65.55	NS
TBW [kg]	34; 31–41.8	33.7; 29.15–50.5	NS
TBW%	46.35; 43.5–49.1	49.05; 45.15–53.45	NS
STC-2 [pg/ml]	102.5; 43.6–130.9	152.7; 104.7–181.1	0.008
PAPP-A [pg/ml]	469.6; 393.6–515.8	487.8; 434.5–521	NS
IGFBP-4	91.1; 38.2–143.3	67.1; 25.6–104.9	NS
FLI	40.95; 31.5–77.8	47.57; 29–82.3	NS

ALT – alanine aminotransferase, AST – aspartate aminotransferase, BMI – body mass index, FFM – fat-free mass, FLI – fatty liver index, GGT – gamma-glutamyltransferase, HDL – high-density lipoprotein, HOMA-IR – homeostatic model assessment-insulin resistance, IGFBP-4 – insulin-like growth factor binding protein, LDL – low-density lipoprotein, PAPP-A – pregnancy-associated plasma protein A, STC-2 – serum concentration of stanniocalcin-2, TBW – total body water, USG – ultrasonography, WHR – waist-hip ratio

ALT ($r = 0.29$, $p = 0.02$), AST ($r = 0.25$, $p = 0.05$), GGT ($r = 0.26$, $p = 0.04$), TG ($r = 0.3$, $p = 0.02$), insulin ($r = 0.48$, $p < 0.001$), HOMA-IR ($r = 0.48$, $p < 0.001$), uric acid ($r = 0.28$, $p = 0.03$), waist ($r = 0.25$, $p = 0.05$), WHR ($r = 0.27$, $p = 0.036$), the intensity of liver steatosis in ultrasound ($r = 0.28$, $p = 0.03$). The majority of correlations were weak, except for the correlation of STC-2 with parameters of insulin resistance (insulin and HOMA-IR), which were moderate.

In the group of obese children with liver steatosis (n = 38) STC-2 positively correlated with: GGT ($r = 0.43$, $p = 0.008$), cholesterol ($r = 0.32$, $p = 0.05$), TG ($r = 0.32$, $p = 0.05$), LDL cholesterol ($r = 0.33$, $p = 0.05$), insulin ($r = 0.48$, $p = 0.003$), HOMA-IR ($r = 0.45$ $p = 0.004$) and WHR ($r = 0.35$, $p = 0.04$).

ROC ANALYSIS (MILD STEATOSIS VS. ADVANCED STEATOSIS)

The ability of serum STC-2 to detect advanced liver steatosis was significant (AUC = 0.746, $p = 0.0022$, sensitivity = 65%, specificity = 78%, cut-off 135.27 pg/ml). PAPP-A and IGFBP-4 did not allow a useful prediction (Figure 1).

DISCUSSION

To our knowledge, this is the first prospective study to assess the STC-2/PAPP-A/IGFBP-4 axis in obese children with liver steatosis. We found that stanniocalcin-2 levels were significantly higher in children with obesity

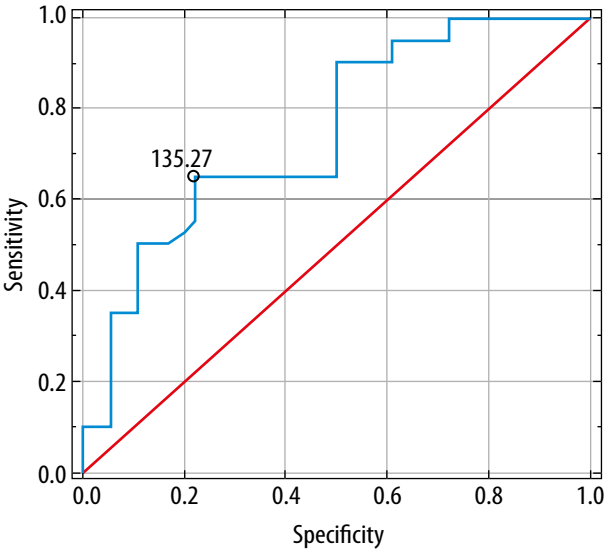

FIGURE 1. ROC curve of serum concentration of stanniocalcin-2 to detect children with advanced liver steatosis

and liver steatosis in comparison to lean, healthy children. What is more, STC-2 can differentiate children with mild and advanced liver steatosis, which was also confirmed with ROC analysis. Therefore STC-2 can be regarded as a non-invasive biomarker of liver steatosis in obese children.

The role of STC-2 has been investigated in an animal model. Zhao *et al.* [17] found that expression levels of STC-2 were significantly reduced in the livers of leptin-deficient and high-fat-diet induced obese mice. Furthermore, systemic administration of STC-2 recombinant protein or adenovirus-mediated overexpression of STC-2 ameliorated hepatosteatosis and hypertriglyceridemia in obese mice. However, the data did not confirm the signaling pathways modulated by STC-2 and described earlier at the molecular level in tumor cells and osteoblasts [18, 19]. That is why the authors speculate that the role and downstream signaling pathways of STC-2 might be tissue- or cell-specific, and the role of STC-2 in hepatic TG homeostasis needs further investigations. Moreover, the authors found that STC-2 activated mainly the signal transducer and activator of transcription (STAT3) signaling pathway to inhibit lipogenic gene expression. Previously, in another animal model, Jiao *et al.* [20] also proposed that STC-2 acts as an anorectic factor, which leads to a significant reduction in body weight in mice by activating the STAT3 signaling pathway. Meanwhile, in another study Sookoian *et al.* [21] suggested a potential role of the STAT3 polymorphisms and their haplotypes in susceptibility to NAFLD and disease severity. Genetic variants in STAT3 might therefore be connected with the discrepancies between the results of the above-mentioned studies on an animal model and our study.

Considering the conflicting outcomes of animal model studies and our results, and knowing the role of STC-2 in different processes in humans, we cannot rule out that the higher STC-2 levels found in our study in obese children and its higher concentration in advanced steatosis could be explained by upregulation of STC-2 as an adaptation to oxidative stress and a response to inflammation in the liver.

Physiological parameters regulating levels of the IGFBP-4/STC-2/PAPP-A axis in humans were presented by Panagiotou *et al.* [22]. The authors found that female gender and percentage of total body fat were positively correlated with STC-2 levels; however, the study involved healthy, young adult participants with normal BMI, and in our study we did not find a correlation between STC-2 and sex or parameters of body mass analysis in the cohort of children with obesity.

Results similar to ours were observed by Lake *et al.* [23]. The aim of their study was to determine the coordinated regulation of ER stress-associated genes in the progressive stages of human NAFLD. Human liver samples were categorized as normal, steatosis, NASH ("fatty"), and NASH ("not fatty"). The samples were analyzed by protein expression and mRNA. STC-2 mRNA and protein were significantly upregulated among NASH samples. The authors concluded that STC-2 may have an important role in the initiation of adaptive mechanisms given previous accounts of its cytoprotective properties during disease. These results are consistent with the findings of our study and may support our analysis.

Pourteymour *et al.* [24] used mRNA sequencing as an untargeted approach to identify novel myokines regulated by acute or long-term exercise in middle-aged, sedentary, overweight men. Among other numerous transcripts, the authors found discrepancies between STC-2 levels *in vitro* and *in vivo*: STC-2 was more highly expressed in differentiated human myotubes, as compared to skeletal muscle biopsies.

On the other hand, Lopez *et al.* [25] found that adult diabetic patients who presented the highest glycosylated hemoglobin values exhibited the lowest STC-2 expression. The authors hypothesized that STC-2 might be a part of adaptive signaling pathways and enhanced STC-2 expression might be a protective mechanism. This conclusion may also explain the findings of our study.

Ortega *et al.* [26], in their pioneer study, analyzed expression of the components of the IGF-1/PAPP-A/STC-2 axis in the vein wall in adult patients with chronic venous disease. The authors found an increase in genetic and protein expression of PAPP-A and a decrease in STC-2 expression in the vein wall, concluding that those parameters perform essential functions in response to oxidative stress or endoplasmic reticulum stress. In our research, we assessed PAPP-A and STC-2 in serum, not directly in the place of inflammation and tissue damage, which might explain the conflicting results.

Data concerning the IGFBP-4/PAPP-A/STC-2 axis in the pediatric population are scarce. Woelfle *et al.* reported a higher serum IGFBP-4 level in obese children, in com-

parison to lean controls, whereas PAPP-A serum levels did not differ between obese and lean children [27]. In our research, the result concerning IGFBP-4 was similar, but PAPP-A concentration in our cohort was higher compared to lean controls. In contrast to our study, the authors did not assess the serum concentration of STC-2.

The strength of our study lies in finding a potential non-invasive marker not only to detect liver steatosis in children but also to differentiate degrees of steatosis. Nevertheless, our research is the first to highlight the importance of IGFBP-4/PAPP-A/STC-2 markers in liver steatosis in children. The fatty liver index has proven invalid in children in predicting liver steatosis, which was also confirmed in our study.

However, our study also has a number of potential limitations. First, the sample size is relatively small and for that reason we consider it as a preliminary study. Additional studies are required to validate our observations. Secondly, the gold standard in NAFLD diagnosis is liver biopsy; however, due to its invasiveness it is not routinely performed in the pediatric population. Moreover, our patients did not meet the criteria to undergo liver biopsy according to the ESPGHAN Hepatology Committee.

CONCLUSIONS

In this preliminary study we found that STC-2 is elevated in obese children, correlates with biochemical markers of insulin resistance and hepatocyte injury, and differentiates patients with mild and severe liver steatosis, and therefore can be regarded as a novel non-invasive biomarker of liver steatosis in children. The other analyzed parameters (PAPP-A and IGFBP-4) seem not to be useful in diagnosis of liver steatosis in obese children.

DISCLOSURE

The authors declare no conflict of interest.

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