Levels of Serum Ceruloplasmin Associate With Pediatric Nonalcoholic Fatty Liver Disease

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ABSTRACT

Objectives: Nonalcoholic fatty liver disease (NAFLD) in adolescents and children is rapidly becoming one of the most common causes of chronic liver disease worldwide. NAFLD varies from simple fatty liver to nonalcoholic steatohepatitis (NASH) with possible fibrosis. Several studies suggest that oxidative stress plays a central role in several metabolic abnormalities and cellular damage that characterize NAFLD. We investigated whether transition metals and their related proteins were related to NAFLD symptoms and their underlying processes.

Methods: We measured copper, iron, ceruloplasmin (Cp) concentration and activity, transferrin (Tf), ferroxidase activity, and ferritin, and we calculated Tf saturation and Cp to Tf ratio (Cp/Tf) as an index of the activity of the antioxidant Cp-Tf system in 100 children with biopsy-proven NAFLD. Pediatric patients were grouped by nonalcoholic fatty liver disease score (NAS) $\geq$ 5 (30 subjects) and NAS $<$ 5 (70).

Results: Cp distinguished children with NAS $\geq$ 5 from those with NAS $<$ 5 with an accuracy of 82%. Specifically, a receiver operator characteristics curve showed that a cutoff of 28.6 mg/dL separated NAS $\geq$ 5 from NAS $<$ 5 with a specificity of 92% and a sensitivity of 76%. The Cp/Tf ratio, as well as copper concentration and Cp activity, decreased in the NAS $\geq$ 5 group, pointing out an imbalance in metal regulation. Either copper or Cp concentrations were lower in subjects having ballooning.

Conclusions: Serum antioxidant capacity owing to Cp failure is strongly associated with NAFLD-related damage. Further studies are, however, required to clarify the role of Cp in NAFLD pathogenesis and to evaluate its potential application as diagnostic marker.

Key Words: ceruloplasmin, ceruloplasmin activity, ceruloplasmin-transferrin antioxidant system, nonalcoholic fatty liver disease, nonalcoholic steatohepatitis

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Nonalcoholic fatty liver disease (NAFLD) has reached epidemic proportions and is rapidly becoming one of the most common causes of chronic liver disease worldwide (1). The estimated prevalence of the disease depends on the method used for diagnosis. It has been, however, reported that NAFLD affects approximately 3% to 10% of children in Western countries (2). Moreover, an increase of up to 70% in the rate of this disorder was observed in overweight/obese children (2).

The histological pattern of NAFLD varies from simple fatty liver to nonalcoholic steatohepatitis (NASH), the latter being often associated with fibrosis that may eventually progress to cirrhosis and hepatocellular carcinoma (3).

Pediatric NAFLD is closely associated with insulin resistance (IR) and other phenotypic manifestations of metabolic syndrome, including overweight/obesity, visceral adiposity, type 2 diabetes, hypertriglyceridemia, and arterial hypertension (2,4,5). Recent evidence also supports an association between NAFLD and atherosclerosis in children, probably because the proinflammatory state that characterizes NASH may also have proatherogenic effects (6).

The pathogenesis of NAFLD has remained poorly understood, and the mechanisms are still undergoing active investigation. The simple fatty liver results from a 2-way cause-and-effect relation between intrahepatic free fatty acid (FFA) and IR, whereas the progression to NASH has been attributed oxidative stress, imbalance of adipokines levels, proinflammatory cytokine induction, activation of endotoxin-mediated immune response, and hepatic stellate cell activation (3).

Several studies, however, suggest that oxidative stress plays a central role in several metabolic abnormalities and cellular damage that characterize NASH development. In particular, increased expression of the cytochrome P450 (CYP 2E1) and reactive oxygen species (ROS) dependents on FFA concentrations as well as mitochondrial dysfunctions have been observed in both humans livers and an animal model of NASH (7). Moreover, FFA oxidation via the peroxisomal β-oxidation and the microsomal ω-oxidation have been reported (7). Because the liver is the primary organ of both lipid metabolism and metal detoxification processes, disturbances in its function could be closely associated with NAFLD development via oxidative stress (Haber-Weiss and Fenton reactions) (8,9). Defects in protein function associated with metal homeostasis, such as ferritin, transferrin (Tf), ceruloplasmin (Cp), metallothionein, and lactoferrin, could exacerbate oxidative stress (10). We recently reported that in our pediatric NAFLD population, iron was present in just a few patients and in low deposition (11). Furthermore, diverse studies on animal models of NAFLD (12) and humans (13,14) have pointed out a condition of copper deficiency that is associated with lipid accumulation and oxidative stress. Whether copper dysfunction in NAFLD is isolated rather being associated with a systemic transition metal dysmetabolism remains an open question. To address this issue, we studied 100 children affected by NAFLD, investigating proteins related to copper and iron status.

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METHODS

Patients and Laboratory/Clinical Data

In the present study we analyzed 100 archival serum samples (stored at −80°C) derived from our cohort of well-characterized subjects with liver biopsy-proven NAFLD. Indications for liver biopsy in this series were previously reported (6). In particular, the samples came from patients with NAFLD referred from January 2008 to March 2011 to the Hepatology Unit of Bambino Gesù Children's Hospital (Rome, Italy). Exclusion criteria included intake of alcohol and drugs (eg, valproate, amiodarone, or prednisone), the presence of hepatic viral infections, and a history of parenteral nutrition at the time of biopsy. Autoimmune liver disease, metabolic liver disease, Wilson disease, and α-1-antitrypsin–associated liver disease were ruled out using standard clinical, laboratory, and histological criteria. The study was approved by the ethics committee of the Bambino Gesù Hospital, and informed consent was obtained from each patient or responsible guardian.

Weight and height were measured using standard procedures. Body mass index (BMI) was calculated and converted to standard deviation scores (SDS) using US reference data (15). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl aminotransferase (GGT), glucose, total triglycerides, total cholesterol, and low-density lipoprotein (LDL) were evaluated using standard laboratory methods. Insulin was measured by radioimmunoassay (Myria Technogenetics, Milan, Italy). Levels of glucose and insulin were measured at 0, 30, 60, 90, and 120 minutes during an oral glucose tolerance test (OGTT) performed with 1.75 g glucose per kilogram of body weight (up to 75 g). IR and sensitivity were determined, respectively, by the homeostatic model assessment of insulin resistance (HOMA-IR) using the formula: insulin resistance = (insulin × glucose)/22.5; and by the insulin sensitivity index (ISI) derived from the OGTT using the formula: ISI = (10,000/square root of [fasting glucose × fasting insulin] × [mean glucose × mean insulin during OGTT]) (16,17).

Systemic Inflammation Markers

High-sensitivity C-reactive protein (hs-CRP), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL6) serum levels were analyzed by enzyme-linked immunosorbent assay (ELISA) following the instructions of the manufacturer (supplied with kit purchased from BioVendor).

Liver Histology

Liver tissue samples were fixed in buffered formalin embedded in paraffin and sliced into 3-μm sections, and standard histological stains were performed: hematoxylin and eosin (H&E), periodic Schiff acid without and with diastase (PAS/PAS-D), and Van Gieson trichrome stain. The criteria of Kleiner et al (18) were applied based on overall impression of the pathologist, to diagnose NASH. The main histological features of NAFLD were scored according to the scoring system developed by the NASH Clinical Research Network (CRN) (18). 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; grade 3 = more than 4 foci per 200× field. Portal chronic inflammation was also evaluated (0–1). Hepatocyte ballooning was graded from 0 to 2: 0 = none, 1 = few balloon cells, 2 = many/prominent balloon cells. Stage of fibrosis was quantified on a 5-point scale: stage 0 = no fibrosis; stage 1 = perisinusoidal or periportal (1a = mild, zone 3, perisinusoidal; 1b = moderate, zone 3, perisinusoidal; 1c = portal/periportal); stage 2 = perisinusoidal and portal/periportal; stage 3 = bridging; and stage 4 = cirrhosis.

As the purpose of this study was to assess whether characterization of the inflammatory infiltrate provided additional information besides validated indices of liver damage, we subdivided cases into 2 subgroups according to NAFLD activity score (nonalcoholic fatty liver disease score [NAS] ≥ 5 or NAS <5). NAS is obtained by the sum of scores for steatosis (0–3), lobular inflammation (0–3), and ballooning (0–2), thus ranging from 0 to 8. NAS ≥ 5 were diagnosed as NASH and confirmed by a pathologist.

Biochemical Investigations

The cohort of 100 archival serum samples was analyzed for biochemical variables. Briefly, measurements of serum copper were obtained either by atomic absorption spectroscopy (An Analyst 300 Perkin Elmer atomic absorption spectrophotometer equipped with a graphite furnace platform HGA 800.) or colorimetrically, following the method of Abe et al (19) (Randox Laboratories Ltd, Crumlin, UK) automated on Cobas Mira Plus (Horiba ABX, Montpellier, France). Data produced by the latter were used in the statistical analysis. Iron was measured by photometric test using Ferene in the following way: iron bound to Tf is released in an acidic medium as ferric iron and is then reduced to ferrous iron in the presence of ascorbic acid; ferrous iron forms a blue complex with Ferene (20). Tf (21) and Cp (22) levels were measured by immunoturbidimetric assays (Horiba ABX): serum was mixed with the purified immunoglobulin fraction of, respectively, a rabbit anti-human Tf antibody solution and a rabbit anti-human Cp antiserum, containing 15 mmol/L NaN_3 as stabilizer. The resulting immune complexes are measured by turbidimetry. More details on these methods can be found in Hussain et al (23). We also computed the ratio between Cp and Tf serum concentrations (Cp/Tf), which is a statistical index conceived on the basis of experimental analysis of electron paramagnetic signals of the Cp—binding a copper in the oxidized state (Cu^{2+})—and of the apotransferrin. Tf saturation (% Tf-sat) was calculated by dividing serum iron (μg/dL) by the total iron-binding capacity (TBC = TF in mg/dL × 1.25) and multiplying by 100. Ferritin was measured by latex-enhanced turbidimetric immunoassay (24). Serum was mixed with a suspension (w/v) of 0.07% latex particles coupled to a rabbit anti-human ferritin antiserum, in the presence of a glycine buffer. The resulting immune complexes were measured by turbidimetry. All reagents were ABX Pentra from Horiba ABX.

Cp activity measurements were assayed by an automation of the *o*-dianisidine method (25–28), and data were expressed in international unit per liter (IU/L). Ferroxidase measurements were performed by applying an automation of the kinetic ferroxidase method (29) and were expressed in absorption per minute (Abs/min). Reagents employed in enzymatic assays were all from Sigma-Aldrich (St. Louis, MO). All biochemical measures were automated on a Cobas Mira Plus (Horiba ABX) and performed in duplicate.

Statistical Analyses

Children were grouped accordingly to the severity of liver disease: NAS ≥ 5 and NAS < 5. Groups were compared for demographic, clinical, and biological variables under study. Associations between variables were evaluated with chi-square or Fisher exact test and ANOVA, respectively, for categorical and continuous variables. Correlation analyses between continuous variable scores (Pearson *r*) were performed.

TABLE 1. Demographic and clinical characteristics of the child population subdivided by NAS < 5 and NAS ≥ 5

	All (100)	NAS < 5 (N = 70)	NAS ≥ 5 (N = 30)	*P*
Sex, M/F	68/32	51/19	17/13	0.16*
Age, years	11.1 (0.26)	11.4 (0.3)	10.5 (0.5)	0.13
Weight, kg	58.9 (49; 72.5)	63.8 (52.2; 74.9)	55.4 (48.3; 62)	0.06
Height, m	1.53 (1.43; 1.60)	1.54 (1.44; 1.60)	1.51 (1.38; 1.59)	0.49
BMI, kg/m^2	25 (23; 29)	26 (23; 29)	24 (23; 26)	0.05
HOMA-IR	2.1 (1.6; 3.3)	2.0 (1.6; 3.0)	2.2 (1.6; 3.5)	0.35
ISI	3.8 (2.9; 5.5)	4.0 (3.0; 5.5)	3.2 (2.8; 5.2)	0.56
Cholesterol level, mg/dL	174 (135; 190)	172 (134; 190)	176 (146; 188)	0.56
Triglyceride level, mg/dL	90 (70; 125)	89 (64; 128)	90 (78; 123)	0.68
ALT, IU/L	57 (48; 74)	60 (55; 77)	54 (45; 63)	0.16
AST, IU/L	45 (32; 59)	45 (33; 63)	45 (27; 57)	0.12
GGT, IU/L	22 (19; 24)	22 (19; 30)	21 (18; 22)	0.02
LDL, mg/dL	70 (60; 90)	70 (60; 90)	80 (60; 90)	0.43
hs-CRP, mg/dL	1.44 (0.90; 2.46)	1.47 (1.43; 2.46)	1.42 (0.90; 2.46)	0.42
TNF-α, pg/mL	6.27 (2.12; 10.64)	7.23 (2.70; 10.12)	5.54 (2.12; 10.64)	0.02
IL6, pg/mL	8.03 (3.65; 27.87)	8.03 (3.65; 25.25)	8.28 (3.91; 27.87)	0.63

Values presented are mean (standard deviation scores) for age, median (25th; 75th percentiles) otherwise. ALT = alanine aminotransferase; AST = aspartate aminotransferase; BMI = body mass index; CRP = C-reactive protein; GGT = gamma-glutamyltransferase; HOMA-IR = homeostatic model assessment of insulin resistance; IL6 = interleukin-6; ISI = insulin sensitivity index; LDL = low-density lipoprotein; NAS = nonalcoholic fatty liver disease score; TNF-α = tumor necrosis factor-α. * Fisher exact test.

Copper, iron, Cp concentration, Cp activity, Cp specific activity, ferroxidase activity, Tf, Tf-saturation, Cp/Tf, ferritin, age, BMI, HOMA-IR, ISI, AST, ALT, and LDL were investigated with univariate and multivariate logistic regression analyses to evaluate which ones were related to NAS. Variables associated with NAS with a $P < 0.2$ in univariate analysis were included in the multivariate logistic model.

Receiver operator characteristics (ROC) curves analysis on the identified biological variables was used to assess their diagnostic validity and to set the most useful cutoff value for sensitivity and specificity.

Fractional polynomials were used to account for nonlinear associations between outcomes and predictors. Odds were converted to probabilities, and nomograms depicting the probability of the outcomes as a function of Cp (transformed where needed to account for nonlinearities) were developed. For example, transformed Cp was calculated as ([Cp (mg/dL)/10] – 2). Akaike information criterion (AIC) was used to compare Cp and copper for their ability to predict the outcomes of interest (30). Statistical software package STATA 11.2 (StataCorp LP, College Station, TX) was used for all of the analyses.

RESULTS

The baseline patient demographic, clinical parameters, and markers of systemic inflammation were reported in Table 1. Specifically, 70 children were included in the NAS < 5 group and 30 in the NAS ≥ 5. Histopathological features associated with NAFLD were reported in Table 2 for all 100 patients according to NAS group. Biochemical values of variables associated with copper and iron metabolism were reported in both groups (Table 3).

We performed a logistic regression univariate analysis on our data to evaluate the effects of demographics (sex and age), clinical markers (BMI, AST, ALT, HOMA-IR, ISI, LDL), and biochemical markers of the metal panel (copper, iron, Cp concentration, Cp activity, Cp-specific activity, ferroxidase activity, Tf saturation, Cp/Tf, ferritin) on the probability of developing NASH. The analysis revealed that copper, Cp—measured as concentration or activity—and Cp/Tf differed between the 2 groups. On this basis, we created a multivariate logistic model to analyze the effects of the biometal markers, which significantly differed between the 2 groups in the probability of belonging to the more severe group (NAS ≥ 5). The biochemical variables that entered the analysis were copper, Cp (concentration and activity), Cp/Tf, ferroxidase activity, age, and sex. The model revealed that besides sex (being female increased the risk 1.8-fold, odds ratio 2.79; 95% CI [confidence interval] 0.88–8.84; $P = 0.080$), a 1-unit decrease in Cp concentration increased the probability of developing NAS ≥ 5 by 25% (odds ratio 0.80; 95% CI 0.73–0.88; $P = 0.000$).

When we studied the relation between Cp *o*-dianisidine activity and the total serum ferroxidase activity, we noted that

TABLE 2. Distribution of histopathological lesions in the 100 study subjects based by NAS

Histological features	Degree or stage	All (N = 100)	NAS < 5 (N = 70)	NAS ≥ 5 (N = 30)
Steatosis	1	17	17	0
	2	58	50	8
	3	25	3	22
Inflammation	0	4	4	0
	1	68	62	6
	2	28	4	24
Ballooning	0	52	51	1
	1	48	19	29
	2	0	0	0
Fibrosis	0	35	24	11
	1	50	38	12
	2+	15	8	7

NAS = nonalcoholic fatty liver disease score.

TABLE 3. Biochemical variables as indices of metals (copper and iron) metabolism, in child population subdivided by NAS < 5 and NAS ≥ 5

	NAS <5 (n = 70)	NAS ≥ 5 (n = 30)	Normal values	*P*
Copper, μmol/L	15.1 (2.3)	12.5 (3.6)	11–24.4	0.000
Ceruloplasmin, mg/dL	36.4 (5.3)	28.1 (7.2)	20–60	0.000
Ceruloplasmin activity, IU/L	97.1 (22.2)	76.3 (30.1)	62–140	0.001
Ferroxidase activity, Abs/min	54.4 (20.9)	65.7 (23.7)	—	0.022
Transferrin, g/L	3.1 (0.5)	2.9 (0.7)	2–3.6	0.048
Transferrin sat, %	29.4 (17.6)	30.6 (22.2)	15–50	0.765
Ferritin, ng/mL	68.1 (42.6)	57.3 (39.6)	15–120	0.241
Fe, μg/dL	112.4 (69.6)	108.6 (78.8)	22–135	0.809
Cp/Tf ratio ($\times 10^{-2}$)	12.0 (2.1)	10.1 (2.1)	—	0.001
Ceruloplasmin specific activity, IU/mg ($\times 10^{-1}$)	2.7 (0.6)	2.8 (0.9)	—	0.411

Data are presented as mean (standard deviation). NAS = nonalcoholic fatty liver disease score.

the lower the Cp (either concentration [$r = -0.5$, $P < 0.001$] or activity [$r = -0.3$, $P < 0.001$]), the higher the ferroxidase activity was.

Finally, ROC curve on concentration units was used to assess Cp validity in discriminating children affected by more severe NAFLD (NAS ≥ 5). A cutoff of 28.6 mg/dL effectively separated NAS ≥ 5 from NAS <5 (area under curve 82%) with a specificity of 92% and a sensitivity of approximately 76% (Fig. 1).

We deepened the relation between Cp and the clinical signs of NAFLD in the entire cohort. Cp was associated with the odds of NASH, ballooning, inflammation, and steatosis but not with the odds of fibrosis (logistic regression models not shown). The association with the odds of steatosis was inverse linear, whereas that with the odds of NASH, ballooning, and inflammation was inverse quadratic. Figure 2A shows a nomogram plotting the probability of steatosis obtained at logistic regression as a function of Cp (mg/dL), and Fig. 2 (B-D) shows NASH, ballooning, and inflammation as a function of transformed Cp (TCp).

Table 4 shows that values of transformed TCp < 0.06 (Cp > 40.8 mg/dL, n = 19) and TCp > 0.18 (Cp < 23.6 mg/dL, n = 9) could be used, respectively, to rule out or in NASH, ballooning, and inflammation in our patients; however, ruling in inflammation is much less accurate than ruling it out at these cutoff points.

Finally, copper had substantially the same behavior as Cp, with a minor amount of predictive power for these outcomes, as revealed by the following Akaike information criterion: 81 versus 100 for NASH, 103 versus 119 for ballooning, 94 versus 105 for inflammation and 124 versus 130 for steatosis (logistic regression models not shown).

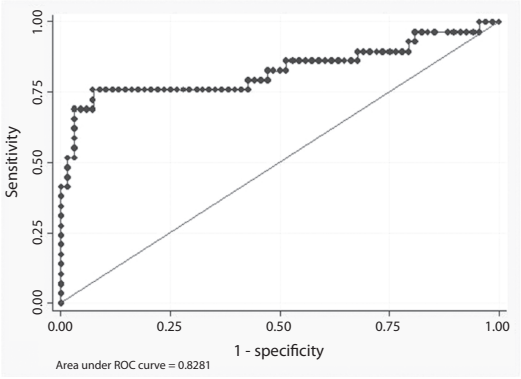

FIGURE 1. ROC curve on ceruloplasmin concentration values. A serum Cp concentration of 28.6 mg/dL distinguished effectively severe nonalcoholic fatty liver disease (NAFLD) with nonalcoholic fatty liver disease score (NAS) ≥ 5 with a specificity of 92% and a sensitivity of approximately 76% (area under curve 82%).

DISCUSSION

The main result of our study is that Cp decreases seem to discriminate children with more severe NAFLD, confirming and extending to the pediatric population previous data on adults (13). Specifically, a cutoff of 28.6 mg/dL distinguished NAS ≥ 5 from NAS < 5 with an accuracy of 82%, pointing out its potential as a noninvasive and supportive marker of the disease. In line with hyaluronic acid (31), Cp detection may serve as an additional noninvasive test for the screening of children with suspected NAFLD at liver function tests and ultrasound. Furthermore, Cp was also associated with steatosis. However, the accuracy of this prediction was much lower than that with NASH, ballooning, and inflammation and not enough to be used for practical purposes.

Another relevant biological facet that comes out of the present study is the involvement of Cp in the altered pathways resulting in ballooning, highlighting new therapeutic strategies to counteract or contain this typical sign of NAFLD. Specifically, the strong association between Cp dysfunction and ballooning hepatocytes suggests a link between Cp deficiency and the processes underlying this liver damage, as it has been already described for Mallory bodies (32). Although the potential of Cp as a noninvasive marker of NAFLD, any conclusion can be drawn about causative factors of its decreased levels observed in the bloodstream of patients with NAFLD, as well as if Cp is a cause or a consequence of liver dysfunction in this disease.

Diverse hypotheses suggest that Cp variations in NAFLD can be a reflection of liver dysfunction: For example, Wilson disease and aceruloplasminemia, both characterized by liver dysfunction, have decreased levels of Cp—even though upon diverse mechanisms—and share some signs with NAFLD, such as steatosis, ballooning, and inflammation.

One possible hypothesis to the lower serum Cp found in children with more severe NAFLD could be that the higher the NAS score, the sicker the liver, and the less capable it is of biosynthesized an active Cp. Within this line of reasoning, it could be speculated that the lower serum Cp levels may resemble a lower intrahepatic Cp content secondary to liver dysfunction, which, in turn, may reflect higher susceptibility to oxidative stress at the hepatocyte level, in terms of peroxidation or accumulation of lipids, misfolded proteins from still unidentified mechanisms, which may result in ballooning formation in NASH. This is suggested by the fact that Cp has a high antioxidant power. In fact, mainly from early studies on serum, Cp has been described to take part in one of the main antioxidant system of the body, which is mainly effective in

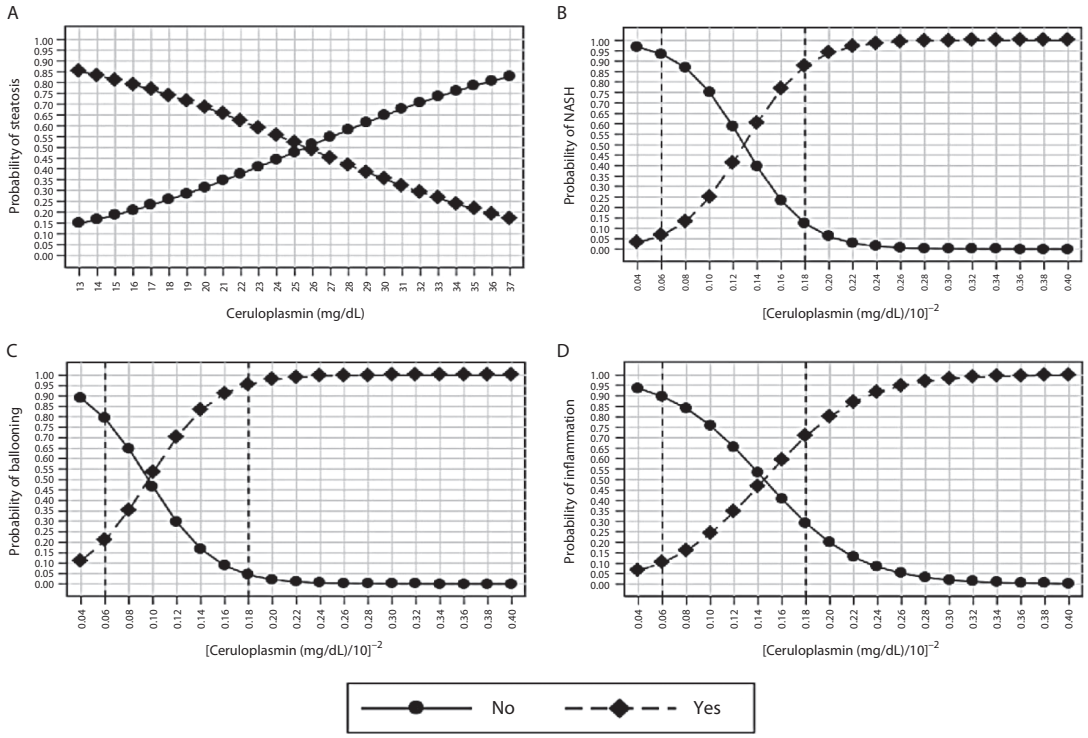

FIGURE 2. Probability of steatosis (A), nonalcoholic steatohepatitis (NASH) (B), ballooning (C), and inflammation (D) according to serum ceruloplasmin levels. The relation with steatosis is inverse linear whereas that with NASH, ballooning, and inflammation is inverse quadratic.

counteracting oxidative stress generated by transition metals in the bloodstream (33,34). Studies in the early 1980s (35) demonstrated that the Cp/Tf ratio reflects the combined antioxidant activity of Cp bonding of copper in the oxidized state (Cu^{2+}) (36) and of the apotransferrin (37), both measured through electron paramagnetic resonance spectrometry. In the present NASH study, as we previously did with patients who had strokes (38) and patients with Alzheimer disease (39), we used the Cp/Tf ratio obtained by measuring concentration units to represent the functionality of this Cp-Tf system, which is otherwise expensive to monitor by electron paramagnetic resonance spectrometry (35). Previous studies demonstrated that the activation of the Cp-Tf system is directed to counteract lipid peroxidation as, for example, in experimental hypercholesterolemia (35). If the sensitive fall in the Cp-Tf system activity that we found in the present study can reflect an impairment of the antioxidant machinery at hepatic level cannot be elucidated by present results. However, it is important to note that the anti- or prooxidant role of Cp is still debated (40–44).

TABLE 4. Probability of NASH, ballooning, and inflammation at selected cutoff points of transformed ceruloplasmin

	TCp	Rules	Point	Lower	Upper
NASH	<0.06	OUT	0.93	0.88	0.99
NASH	>0.18	IN	0.88	0.74	1.00
Ballooning	<0.06	OUT	0.79	0.68	0.90
Ballooning	>0.18	IN	0.96	0.89	1.00
Inflammation	<0.06	OUT	0.90	0.82	0.97
Inflammation	>0.18	IN	0.71	0.51	0.91

Lower = lower 95% CI of probability; NASH = nonalcoholic steatohepatitis; Point = point estimate of probability; TCp = transformed ceruloplasmin ([Cp (mg/dL)/10]$^{-2}$); Upper = upper 95% CI confidence of probability.

Another result of the present study is that, while Cp levels appear disarranged, markers of iron status, that is, iron, ferritin, Tf, and Tf saturation, were apparently normal in our children affected by NAFLD. Conversely, a recent study has shown increased iron deposits in liver and other organs of adult patients with NAFLD (14). The discordance between present results and those published on adult NAFLD (14) can find an explanation when considering our data on ferroxidase activity. When we analyzed the Cp *o*-dianisidine activity in relation to the total serum ferroxidase activity, we noted that the lower the Cp, the higher the ferroxidase activity was, suggesting a vicarious ferroxidase activity in the serum of children. This additional serum ferroxidase activity—called ferroxidase II (45)—suggests an unaffected iron metabolism in children with NAFLD, as it has been described also in a study on patients with Wilson disease, which reported that, despite lower levels of Cp, patients with Wilson disease have a ferroxidase activity less affected, and a normal iron metabolism (45).

In spite of the fact that we found that systemic inflammation is only sustained by significant increased levels of TNF-α in our patients, a role of inflammation in Cp cannot be ruled out because Cp is an acute phase reactant (33,46); however, the inverse trend of Cp with respect to systemic inflammation suggests primarily liver dysfunction.

The present study has a number of limitations, including the need for patient selection on the basis of the NAS score, the lack of data on healthy controls, and the small number of cases analyzed that possibly result in sampling bias, thus deserving further confirmatory studies. Nevertheless, here we clearly show that Cp circulating levels increase inversely to the severity of pediatric NAFLD and display a close correlation with the histological features of ballooning.

In summary, these findings suggest that Cp level detection in serum, combined with the well-defined hyaluronic acid (31), may

serve as a supportive noninvasive test for NAFLD in the pediatric population. If confirmed in large-population studies, Cp assay, combined with other noninvasive biomarkers and imaging (ie, transient elastography), could help to better discriminate those children who really need liver biopsy for NAFLD diagnosis confirmation. Furthermore, this novel marker could be useful for following up with patients during lifestyle interventions and/or pharmacological therapy.

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