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Distinct phenotype of non-alcoholic fatty liver disease in patients with low levels of free copper and of ceruloplasmin

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ABSTRACT – **Background** – Copper deficiency has been linked to alterations in lipid metabolism and hepatic steatosis. Oxidative stress plays a role in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). One of the enzymes that neutralize oxidative stress is Cu/Zn superoxide dismutase, which depends on the availability of adequate amounts of copper. **Objective** – Correlate the levels of ceruloplasmin and of non-ceruloplasmin-bound copper (NCBC) with clinical, biochemical and histological parameters of non-alcoholic fatty liver disease (NAFLD) patients. **Methods** – Data from 95 consecutively admitted NAFLD patients who underwent liver biopsy composed the groups based on ceruloplasmin levels lower than 25 mg/dL and on negative NCBC. The risk factors for NAFLD in each group were compared. **Results** – Body mass index was lower in patients with ceruloplasmin <25 mg/dL (29.1±3.47 vs 32.8±6.24 kg/m^2; *P*=0.005) as were the levels of LDL, HDL and total cholesterol, when compared with their counterparts with ceruloplasmin >25 mg/dL (101±38 vs 116±35 mg/dL, *P*=0.05; 43±9 vs 51±16 mg/dL, *P*=0.01; 174±43 vs 197±39 mg/dL, *P*=0.01, respectively). Mean serum ferritin levels were higher in the ceruloplasmin <25 mg/dL group (343±327 vs 197±190 ng/mL; *P*=0.02). Otherwise, patients with negative NCBC had higher HOMA-IR (8.2±14.7 vs 4.6±3.7; *P*=0.03). Age, gender, hypertension and diabetes showed no statistical difference. **Conclusion** – Patients with NAFLD had different clinical and biochemical markers according to the levels of NCBC and ceruloplasmin.

HEADINGS – Non-alcoholic fatty liver disease. Ceruloplasmin. Copper, deficiency. Oxidative stress.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) encompasses a large spectrum of disease that ranges from benign steatosis (NAFL) and non-alcoholic steatohepatitis (NASH) with hepatocellular injury (ballooning, inflammation) to fibrosis that eventually, in some cases, progresses to cirrhosis, end-stage liver disease and hepatocellular carcinoma[1,2]. Nowadays, NAFLD is the most common chronic liver disease in Western countries, present in 20% to 40% of the general population[3,4]. The prevalence of NASH in Western countries is approximately 2%–6%[5], accounting for more than 50% of cryptogenic cirrhosis[6]. The pathogenesis of NAFLD and NASH is closely related with metabolic syndrome and should be called a "hepatic feature of metabolic syndrome", although almost 10% of NAFLD patients have no comorbidities in some series[7,8].

The causes of NAFLD in patients with normal body mass index (BMI) and no metabolic risk factors have not been completely clarified. Given the multifactorial events in the pathogenesis of this disease, genetic factors and/or specific dietary habits might be responsible for the development of NAFLD in lean individuals, even in the absence of metabolic disorders[9,10].

Copper deficiency has been linked to alterations in lipid metabolism and to hepatic steatosis. Oxidative stress plays a role in the pathogenesis of NAFLD. One of the enzymes that neutralize oxidative stress is Cu/Zn superoxide dismutase, which depends on the availability of adequate amounts of copper, otherwise copper and iron homeostasis are intrinsically related to each other. For example, the ceruloplasmin (that contains most of the body's serum copper) acts as a ferroxidase and when it is devoid can lead to iron overload, which causes oxidative damage to the liver[11,12]. We aimed to correlate ceruloplasmin levels and serum copper concentration with clinical, biochemical and histological parameters in patients with NAFLD.

METHODS

The current study is a retrospective cross-sectional study of a cohort of 235 subjects with liver biopsy-proven NAFLD. All patients had been enrolled between 2009 and 2013 and the levels of ceruloplasmin and of serum copper were measured within six months before or after the biopsy date. Only patients with liver biopsy analyses available were included, following the NAFLD American Association for the Study of Liver Diseases (AASLD) guidelines. Data were collected by medical attendants from interview, laboratorial tests and liver biopsies that were analyzed by an expert pathologist. This study was reviewed and approved by the Ethics Committees of University of São Paulo School of Medicine Department of Gastroenterology and conforms to the ethical guidelines of the Declaration of Helsinki; all patients signed an informed consent.

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From 235 selected patients, only 95 had ceruloplasmin and serum copper levels measured during the six-month period before or after liver biopsy, and only these were included. All patients had alcohol intake less than 100 g/week or 140 g/week for women and men, respectively. Patients were divided into groups based on ceruloplasmin levels and on non-ceruloplasmin-bound copper (NCBC) less than zero µg/dL (no free copper available), calculated using the formula "total serum copper − (ceruloplasmin x 3.15)"[13]. The cut-off of 25 mg/dL for the ceruloplasmin variable was established with the premise to maximize the difference between homeostatic model assessment index (HOMA-IR) medians[14].

The risk factors for NAFLD (obesity, diabetes, hypertension and dyslipidemia) in each group were compared. Insulin resistance was indirectly determined by HOMA-IR as the product of fasting insulinemia (mU/mL) and glycemia (mM), which was divided by 22.5. Insulin resistance was considered when HOMA-IR was higher than 2.7. Steatohepatitis were defined by the histological NAFLD score activity (NAS) ≥5[9,15,16].

Qualitative variables were expressed as percent (frequency) while quantitative variables were expressed as mean ± standard deviation. Comparisons between study groups were performed considering t- and Mann-Whitney tests for quantitative variables. The assumptions of normality and homogeneity of variances were verified by Anderson-Darling[17] and Levene[18] tests, respectively. In addition, Fisher's test was performed to evaluate differences for qualitative variables. A *P*-value was considered statistically significant if <0.05. All calculations were performed using R program, version 3.0.2[19].

RESULTS

Clinical and biochemical characteristics from all patients are displayed in TABLE 1. From 95 patients, 68% were female, 44% had diabetes, 62% arterial hypertension and 65% had dyslipidemia.

BMI was lower in patients with ceruloplasmin levels <25 mg/dL (29.1±3.47 vs 32.8±6.24 kg/m²; *P*=0.005) as were the levels of LDL, HDL and total cholesterol, when compared with their counterparts with ceruloplasmin higher than 25 mg/dL (101±38 vs 116±35 mg/dL, *P*=0.05; 43±9 vs 51±16 mg/dL, *P*=0.01; 174±43 vs 197±39 mg/dL, *P*=0.01, respectively). The mean of serum ferritin levels was higher in the ceruloplasmin <25 mg/dL group (343±327 vs 197±190 ng/mL; *P*=0.02). Otherwise, patients with negative NCBC had (without statistical significance) higher total cholesterol, HDL and LDL levels (194±41 vs 187±42 mg/dL, *P*=0.39; 50±17 vs 47±12 mg/dL, *P*=0.89; 113±38 vs 109±35 mg/dL, *P*=0.64, respectively), more steatohepatitis (*P*=0.1) and significantly higher HOMA-IR (8.2±14.7 vs 4.6±3.7; *P*=0.03). Comparisons between these parameters are displayed in TABLE 2.

TABLE 1. Main characteristics of all patients with non alcoholic fatty liver disease.

Variable	Mean ± SD*	Median (range)
Age (years)	53.8±11.12	55 (21–71)
Body mass index	31.55±5.69	31.35 (19.6–55)
Serum copper (µg/dL)	91.02±23.56	92 (27–150)
Free serum copper	-1.03±21.32	1.75 (-72–62)
Ceruloplasmin (mg/dL)	29.22±8.73	29 (7–53)
Glicemia (mg/dL)	108.94±32.83	98 (63–241)
Insulin (mIU/L)	18.19±12.48	15 (3.7–70.5)
HOMA-IR	5.95±9.4	4.03 (0.7–63.3)
AST (U/L)	39.19±27.04	32.5 (10–156)
ALT (U/L)	51.14±37.8	37.5 (13–174)
GGT (U/L)	75.82±88.18	44.5 (8–476)
Total cholesterol (mg/dL)	190.13±41.9	179 (91–287)
HDL (mg/dL)	48.8±14.75	48 (24–116)
LDL (mg/dL)	111.36±36.64	104 (32–213)
Triglycerides (mg/dL)	166.13±80.1	147 (56–399)
Variable	**n**	*%*
Gender M-F	30–65	32–68
Diabetes	42	44
Hypertension	59	62
Dyslipidemia	62	65

SD: standard deviation; HOMA-IR: homeostatic model assessment index; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transpeptidase; HDL: High-density lipoprotein; LDL: low density lipoproteins.

TABLE 2. Comparison of main clinical and biochemical parameters between the groups of patients according to the levels of ceruloplasmin and serum free copper.

Parameters	Ceruloplasmin		*P* value	Negative free copper		*P* value	Free copper		*P* value
	≤25 mg/dL (35)	>25 mg/dL (64)		<0 µg/dL (40)	≥0 µg/dL (55)		<15 µg/dL (77)	≥15 µg/dL (18)	
BMI	29.1±3.47	32.8±6.24	0.005	31.9±5.2	31.2±6	0.5	31.3±4.9	32.6±8.5	0.6
Hypertension	65%	69%	0.8	70%	66%	0.8	68%	66%	1.0
DM	51%	46%	0.6	54%	43%	0.3	47%	53%	0.7
Cholesterol	174±43	197±39	0.01	194±41	187±42	0.39	186±40	208±44	0.08
LDL	101±38	116±35	0.05	113±38	109±35	0.64	106±35	131±36	0.01
HDL	43±9	51±16	0.01	50±17	47±12	0.89	50±15	43±11	0.1
HOMA-IR	4.57±2.9	6.7±11.5	0.8	8.2±14.7	4.6±3.7	0.03	5.9±10.1	5.9±3.2	0.2
NASH	80%	79%	1.0	87%	74%	0.1	77%	88%	0.3
AST	40.5±28.4	38.5±26.5	0.7	41.2±31.6	37.7±23.5	0.9	37.7±27.5	45.9±24.2	0.04
Ferritin	343	197	0.02	249	238	0.5	245	233	0.9

BMI: body mass index; DM: diabete mellitus; LDL: low density lipoproteins; HOMA-IR: homeostatic model assessment index; NASH: non-alcoholic steatohepatitis; AST: aspartate aminotransferase.

DISCUSSION

In the present study, NAFLD patients with levels of ceruloplasmin less than 25 mg/dL exhibited lower BMI, lower LDL, HDL and total cholesterol levels, and higher levels of ferritin when compared with their counterparts with ceruloplasmin higher than 25 mg/dL. This is a value close to that of 28.6 mg/dL found by Nobili et al in pediatric patients that better distinguished children with a NAS ≥5 from those with NAS less than 5 with 92% specificity, 76% specificity and 82% accuracy[20]. The authors speculated that the levels of ceruloplasmin are lower in children with more severe NAFLD secondary to liver dysfunction, which may reflect higher susceptibility to oxidative stress at the hepatocyte level.

Trying to interpret the results of the aforementioned study and of ours, we hypothesized that the primary event in NAFLD/NASH patients with lower ceruloplasmin levels could be related to copper deficiency. Indirectly, we investigated whether the levels of NCBC had any relationship with some clinical and biochemical parameters usually related to NAFLD. HOMA-IR was significantly higher in patients with NCBC less than zero. These data may suggest that NAFLD patients with lower levels of ceruloplasmin and lower levels of NCBC may have different clinical and biochemical features among all patients with NAFLD, for instance lower BMI, hyperferritinemia and higher HOMA-IR.

NAFLD patients certainly do not have the heritable Wilson's disease deficiency in *ATP7B*, but lower copper content could be a negative stimulus for ceruloplasmin synthesis, or even an acquired deficiency similar to that observed in Wilson's disease could be observed in NAFLD patients secondary to the absence of enough copper to be incorporated in the molecular structure of ceruloplasmin. This protein without the incorporation of six to seven copper ions is very labile and has a shorter life time[21]. This could even be one reason for the lower levels of Cu/Zn superoxide dismutase in NAFLD patients[22-24]. Indeed, low levels of cuproenzymes were described during the status of copper deficiency, especially those of ceruloplasmin[25,26] In copper-deficient rats, copper administration by oral gavage increased the rate of ceruloplasmin synthesis, in contrast to normal rats in which plasma ceruloplasmin levels were not elevated. It means that, at least in copper deficiency, copper availability controls the rate of synthesis, activation and plasma concentration of ceruloplasmin[27].

Our data suggest that levels of copper may represent an additional disease-modifying factor relevant to NAFLD in lean patients. In our investigation, lower NCBC concentrations were associated with higher degrees of HOMA-IR and low levels of ceruloplasmin with lower BMI. Some other human and animal studies corroborated these findings. Rats on low dietary copper develop liver steatosis, suggesting that inadequate copper availability may increase lipid accumulation in the liver[28]. Epidemiological and experimental studies have shown that copper deficiency is linked to atherogenic dyslipidemia[29,30] and investigations in rodent models found that copper deficiency induces hypertriglyceridemia and hypercholesterolemia, and also modifies LDL and VLDL composition[31]. In contrast, lower concentrations of serum and muscle cholesterol were detected after feeding supranormal copper concentrations to chickens[32]. These studies provide evidence that low copper bioavailability can profoundly alter lipid metabolism and that it may therefore be involved in the development of NAFLD. In contrast to experimental models of copper-induced deficiency and to a clinical study of hypercholesterolemic patients that demonstrated an increased amount of all lipoproteins and lower copper concentrations respectively[31,33], in this cohort of patients, ceruloplasmin lower than 25 mg/dL related significantly to lower levels of total cholesterol, HDL and LDL. Unfortunately, only incomplete information is available on drug intake to exclude that the lower levels of the total cholesterol and its fractions can be related to the drug administration at the time of measuring the level of copper and ceruloplasmin. It must be stressed that the administration of statins or estrogen consumption reduces or increases ceruloplasmin and copper levels, respectively[34,35].

There is some evidence suggesting that the primary event in NAFLD could be related to low levels of copper in the liver. Aigner et al and Yang et al demonstrated that patients with NAFLD had lower levels of liver copper, and lower values of serum ceruloplasmin when compared with other diseases and normal controls[28]. Comparing NAFLD and NASH patients, the latter showed the lowest levels of tissue copper. The reason why there is a low hepatic concentration of copper in patients with NASH could be key to understanding the starting process. A reduction in the intestinal absorption of copper could be an explanation for this phenomenon. An experimental model of fructose-induced NASH demonstrated that, although in part secondary to a marginally copper-deficiency diet, Sprague-Dawley rats had impaired duodenum Ctr1 (copper transporter 1) expression that may be the primary event responsible for decreased copper absorption and subsequent low plasma copper, low liver copper content and eventual low plasma ceruloplasmin activity[36]. In this study, a combined iron overload was also observed, based on high levels of liver iron and of plasma ferritin, a common observation in NAFLD patients[37].

Serum levels of ferritin, an iron-binding protein that can store Fe^{3+} ions, reflect the body's iron store and ferritin has two opposite functions, an anti-oxidative function by its chelating effect of free iron and a promoter function for oxidative stress by releasing free iron[38]. An inverse correlation between ceruloplasmin and ferritin was previously observed in a healthy population[39]. Hence, it is a hypothesis that ceruloplasmin may act against oxidative stress induced by ferritin, or that low levels of ceruloplasmin impair iron export from hepatocytes and the endoplasmic reticulum system and even ferroportin expression[38-40]. Spontaneous oxidation of Fe^{2+} (ferrous iron) is potentially harmful because it triggers free radical formation, and ceruloplasmin-induced iron oxidation would prevent the oxidative stress induced by Fe^{2+}. In the current study, serum ferritin levels were higher in the ceruloplasmin <25 mg/dL group corroborating the aforementioned information[41].

Regardless of dietary habits, NAFLD has been shown to be associated with insulin resistance independently of BMI. Particularly in non-obese subjects, dysglycemia was found to be an independent risk factor for NAFLD. Since the degree of insulin resistance and prevalence of metabolic syndrome may increase from lean to overweight and obese NAFLD patients, it could be assumed that NAFLD often develops in the early phases of insulin resistance before the development of clinically evident metabolic disorders. However, we found that patients with negative free serum copper had higher HOMA-IR and, although NAFLD has been shown to be closely associated with insulin resistance, this may not be the case for all NAFLD patients. A review by Tilg and Moschen[42] pointed out that inflammation may precede hepatic steatosis. In this context, the authors suggested that steatosis is a "bystander phenomenon" and not necessarily the cause of inflammation, adopting a multiple hits hypothesis in order to explain the pathogenesis of NAFLD.

These data give insight into the mechanisms involved in the development of hepatic steatosis and inflammation, which might also apply to lean individuals as our study suggests[7,8].

On the other hand, increased oxidative stress is considered a key trigger in the pathogenesis of human NAFLD and one of the enzymes counteracting oxidative stress, Cu/Zn superoxide dismutase, depends on adequate copper availability, suggesting a link between copper availability and antioxidant defenses in NAFLD. In addition, Sprague-Dawley rats exhibited increased activity of the proinflammatory protein cyclooxygenase-2 when fed a diet with a low copper content[43]. Moreover, insulin resistance and, in particular, NAFLD are frequently accompanied by disturbances of iron homeostasis that are molecularly linked to low copper bioavailability and decreased levels of the copper-containing ferroxidase ceruloplasmin and of mitochondrial dysfunction[40]. Humans express about a dozen proteins that require copper for function. Limitation in the activity of cuproenzymes, such as those related to oxidative removal or iron oxidation, can explain the pleiotropic effect of copper deficiency. Any antagonist of copper absorption could induce a copper deficiency, even with otherwise adequate copper intake and a healthy intestine, as in cases of zinc-induced copper deficiency due to high dietary zinc content[44].

One of the challenges to identify copper deficiency is the ability for its detection. Currently, the most widely used criterion is the level of plasma copper. Plasma copper is lowered only in marginal or severe copper-deficient humans. Since the major pool of plasma copper is the one bound to ceruloplasmin molecules, measurement of ceruloplasmin activity correlates highly with plasma copper and can substitute as a protocol. One limitation of the measurement of ceruloplasmin as a biomarker is the fact that it is an acute-phase protein. Thus, inflammatory stimuli to the liver, such as IL-1 or IL-6, result in higher ceruloplasmin release to plasma. If this occurs in a copper-deficient human, a false measurement could mask the deficient state[42]. Moreover, ceruloplasmin levels are higher in women than in men, and in the former when they are using oral contraceptives or if they are pregnant[35]. These variables were not controlled in this study, and maybe the differences between the groups with respect to ceruloplasmin levels could be more evident. These differences need to be checked further with all these variables under control.

Some limitations of our study were the observational and transversal nature, small sample and unevaluated diet, medical drugs, exercise, waist circumference, insulin application or smoking, factors that influence ceruloplasmin status, copper and cholesterol levels. Another disadvantage was that there was no measure of copper concentration in liver biopsy specimens to correlate with serum levels or measure of copper-dependent proteins to show the effects of relative copper deficiency. Despite of those limitations, the impact of results of the current study increase evidence that disturbances in copper metabolism may have a pivotal role in the pathogenesis in a group of NAFLD patients.

Patients with NAFLD had different clinical and biochemical markers according to the levels of NCBC and of ceruloplasmin, suggesting that alterations in the metabolism of copper could have some role in NAFLD development.

Our study correlates the levels of copper and of ceruloplasmin with lipid metabolism, insulin resistance and iron metabolism with NAFLD, and finds lower levels of ceruloplasmin in non-obese NAFLD individuals. However, further studies are required to clarify the role of ceruloplasmin and copper levels in NAFLD pathogenesis.

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Authors' contribution

Nunes VS, Andrade AR, Guedes ALV: data collection, writing manuscript. Diniz MA: methodology and statistical analysis. Oliveira CP: review manuscript. Cançado ELR: project development, review and writing manuscript.

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Nunes VS, Andrade AR, Guedes ALV, Diniz MA, Oliveira CP, Cançado ELR. Fenótipo distinto da doença hepática gordurosa não alcoólica em pacientes com níveis reduzidos de cobre livre e ceruloplasmina. Arq Gastroenterol. 2020;57(3):249-53.

RESUMO – **Contexto** – A deficiência de cobre tem sido relacionada a alterações no metabolismo lipídico e esteatose hepática. O estresse oxidativo desempenha um papel fundamental na fisiopatologia da doença hepática gordurosa não alcoólica. Uma das enzimas que neutralizam o estresse oxidativo é a Cobre/Zinco superoxido dismutase, que depende da disponibilidade de quantidades adequadas de cobre. **Objetivo** – Correlacionar os níveis de ceruloplasmina e de cobre não ligado à ceruloplasmina (NCBC) com parâmetros clínicos, bioquímicos e histológicos de pacientes com doença hepática gordurosa não alcoólica (DHGNA). **Métodos** – Dados de 95 pacientes com DHGNA internados consecutivamente e submetidos à biópsia hepática compuseram os grupos com base em níveis de ceruloplasmina inferiores a 25 mg/dL e em NCBC negativo. Os fatores de risco para DHGNA em cada grupo foram comparados. **Resultados** – O índice de massa corporal foi menor nos pacientes com ceruloplasmina <25 mg/dL (29,1±3,47 vs 32,8±6,24 kg/m^2; *P*=0,005), assim como os níveis de LDL, HDL e colesterol total, quando comparados aos seus pares com ceruloplasmina >25 mg/dL (101±38 vs 116±35 mg/dL, *P*=0,05; 43±9 vs 51±16 mg/dL, *P*=0,01; 174±43 vs 197±39 mg/dL, *P*=0,01, respectivamente). Os níveis médios de ferritina sérica foram maiores no grupo ceruloplasmina <25 mg/dL (343±327 vs 197±190 mg/mL; *P*=0,02). Os pacientes com NCBC negativo apresentaram maior HOMA-IR (8,2±14,7 vs 4,6±3,7; *P*=0,03). Idade, sexo, hipertensão e diabetes não mostraram diferença estatística. **Conclusão** – Pacientes com DHGNA apresentaram diferentes marcadores clínicos e bioquímicos de acordo com os níveis de NCBC e ceruloplasmina.

DESCRITORES – Hepatopatia gordurosa não alcoólica. Ceruloplasmina. Cobre, deficiência. Estresse oxidativo.

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