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Hepcidin is a useful biomarker to evaluate hyperferritinemia associated with metabolic syndrome

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Abstract: Investigation of hyperferritinemia in metabolic syndrome patients represents a diagnostic challenge, but it is essential for the identification of individuals with iron overload. Hepcidin negatively regulates iron absorption and release. An increase in hepcidin occurs when iron levels are sufficient or in inflammatory states, conditions often associated with hyperferritinemia. Hemochromatosis causes hyperferritinemia due to iron overload, but frequently has low hepcidin levels. Our aim was to evaluate biochemical and molecular parameters related to iron metabolism in patients with metabolic syndrome. We evaluated 94 patients with metabolic syndrome according to the International Diabetes Federation criteria in a cross-sectional study. Anthropometric data and diagnostic criteria for metabolic syndrome, iron dosage, ferritin, transferrin saturation, hepcidin, and the C282Y and H63D mutations in the HFE hemochromatosis gene were evaluated. Prevalence of hyperferritinemia in the study population was 27.7% and was higher in males (46.2%) than in females (14.5%). Increase in transferrin saturation correlated with mutations in the hemochromatosis gene. Hyperferritinemia was associated to transferrin saturation and hepcidin after logistic regression analysis. In conclusion, hyperferritinemia is a frequent finding in metabolic syndrome patients, most frequently in men; and hepcidin assessment can be useful for the investigation of ferritin increase in those subjects.

Key words: diagnostic screening, ferritin, hepcidin, iron, metabolic syndrome.

INTRODUCTION

Metabolic syndrome (MS) affects approximately 20 to 25% of the adult population and is characterized by factors that increase the risk for cardiovascular disease and diabetes, such as abdominal obesity, hyperglycemia associated with insulin resistance, dyslipidemia and hypertension (Zimmet et al. 2005, Expert Panel on Detection, Evaluation,

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and Treatment of High Blood Cholesterol in Adults 2001, World Health Organization 1999, de Carvalho Vidigal et al. 2013). Among laboratorial findings, hyperferritinemia is present in roughly 15% of cases (Chen et al. 2011).

Hyperferritinemia should be carefully assessed in patients with MS, since only a minority of cases is due to true iron overload (Lorcerie et al. 2017, Goot et al. 2012). Other conditions can cause an increase of this protein, such as inflammatory processes, cancer, infections, alcoholism, nonalcoholic steatohepatitis (NASH), and neurodegenerative and chronic liver diseases (Lorcerie et al. 2017, Goot et al. 2012, Chang et al. 2013, Vaisman et al. 2000, Colli et al. 2011).

Hepcidin acts as a negative regulator of iron absorption and release, it binds to the iron transporter ferroportin causing its internalization and proteolysis (Prentice 2017). Erythropoiesis promotes a decrease in hepcidin levels, while hepcidin circulating levels can be increased in response to plasma and liver iron, and also by an inflammatory state. Consequently, several clinical conditions can influence hepcidin regulation, such as anemia, iron intake, liver disease, hemochromatosis-related mutations, inflammatory stimuli and infections, among others (Girelli et al. 2016, Prentice 2017).

Hepcidin could be an important marker to differentiate hyperferritinemia associated with MS, and hyperferritinemia caused by hereditary hemochromatosis, since hemochromatosis causes hyperferritinemia due to iron overload, but frequently has low hepcidin levels (Hare 2017, Dongiovanni et al. 2011, Trombini et al. 2011, Cardoso et al. 1998). The aim of this study was to evaluate biochemical and molecular parameters related to iron metabolism in patients with MS, using hepcidin as a potential biomarker to help identifying patients with hyperferritinemia and iron accumulation secondary to hemochromatosis.

MATERIALS AND METHODS

STUDY DESIGN

This was a cross-sectional study conducted from May, 2013, to November, 2013. A total of 94 patients from the Internal Medicine outpatient program of the Hospital de Clínicas de Porto Alegre were enrolled.

ETHICAL ASPECTS

This study was conducted after approval of the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre (CAAE 11796912.1.0000.5327 and 05770612.5.0000.5327) and participants were included after written informed consent. This study was conducted in accordance with the Declaration of Helsinki and current laws in Brazil.

PATIENT POPULATION

All patients were ≥18 years old, with MS according to criteria of the International Diabetes Federation (IDF) (Zimmet et al. 2005). The exclusion criteria were daily intake of ethanol ≥60 g for men and ≥30 g for women, diagnosis of chronic viral hepatitis, previous diagnosis of hereditary hemochromatosis or more than two blood donations in the preceding year. Patients had weight, height and waist circumference measured and a peripheral blood sample was harvested.

BIOCHEMICAL ANALYSIS

Serum concentrations of iron, ferritin and transferrin saturation were measured by routine methods with Advia 1800® and Advia Centaur (Siemens Diagnostics, Deerfield, USA). The reference values for ferritin were 10 to 291 ng/mL for women and 22 to 322 ng/mL for men, values above the upper limit were considered as hyperferritinemia. Serum glucose, cholesterol, HDL cholesterol and triglycerides values were obtained from medical records.

DETERMINATION OF HEPCIDIN

All serum samples to determine hepcidin levels were stored at -80°C, until measurements in the same assay. Hepcidin was assessed with enzyme-linked immunosorbent assay (ELISA) method through Hepcidin 25 (bioactive) ELISA kit® (DRG Diagnostics, Marburg, Germany), according to the manufacturer instructions (sensitivity of 0.35 ng/mL and linearity of 0.35 to 80 ng/mL).

MOLECULAR ANALYSIS FOR THE PRESENCE OF C282Y AND H63D MUTATIONS IN THE HFE GENE

Immediately after blood collection with EDTA anticoagulant, its DNA was extracted through commercial kit PureLink Genomic DNA Mini Kit® (Invitrogen, Carlsbad, USA) and subsequently the nucleic acid was stored at -80°C. To evaluate the presence of C282Y and H63D mutations in the HFE gene, it was amplified as described by Cardoso et al. (1998). The amplifications were performed on a Veriti thermocycler (Applied Biosystems, Foster City, USA) with annealing temperature of 63°C for C282Y and 50°C for H63D. The products were analyzed by electrophoresis in 1.5% agarose gel impregnated with GelRed®. Then, the amplified products were digested using restriction enzymes (C282Y by SnaBI at 37°C for 1h30min and H63D by BclI for 1h30min at 50°C), in a protocol adapted from Cardoso et al. (1998). Fragment analysis was performed by electrophoresis in 3.0% agarose gel impregnated with GelRed®. Heterozygote and homozygote samples identified by direct DNA sequencing were used as positive controls.

STATISTICAL ANALYSIS

Results were expressed as mean and standard deviation when parametric or medians and quartiles intervals when nonparametric. Spearman and Pearson correlations were done among all quantitative variables. Patients with normal ferritin and hyperferritinemia were compared by Student's T test or Mann-Whitney test, when applicable. Data analysis was carried out with SPSS 18.0® (Chicago, EUA). The statistical significance level was set at $P<0.05$.

RESULTS

Most patients consisted of women with a mean age of 62.2 years and BMI of 34.7 and were classified on average as obese. The other parameters were in accordance with the criteria for diagnosis of MS, according to the IDF (Zimmet et al. 2005). Table I summarizes patient's demographics, clinical and biochemical parameters. None of the included patients were receiving iron supplementation.

There was no patient with homozygosis for C282Y, while 14.9% were heterozygous and 85.1% did not have the mutation. Only 2 (2.1%) patients were homozygous for H63D mutation, whereas 24.5% were heterozygous and 73.4% did not have the mutation. One patient (1.6%) was heterozygous for both analyzed mutations. Transferrin saturation was correlated with the presence of heterozygosity in H63D ($P=0.031/\rho=0.225$).

Hyperferritinemia prevalence in the study population was 27.7%. Table II shows the correlations between ferritin levels and clinical aspects and laboratory data, there was a significant positive correlation of ferritin with cholesterol levels.

Subsequently, patients were divided into two distinct groups: patients with normal ferritin levels and patients with hyperferritinemia (Table III). Among the analyzed diagnostic criteria for MS, only the dosage of triglycerides and HDL cholesterol showed differences between the groups in univariate analysis. Male gender, cholesterol levels, transferrin saturation and hepcidin levels also differed between patients with normal ferritin levels and hyperferritinemia. Presence of high blood glucose levels, BMI and waist circumference values were similar among groups, as well as

TABLE I
Patients demographics (n=94).

Gender	
Male (%)	39 (41.5)
Female (%)	55 (58.5)
Age (years)	62.2±8.6
BMI	34.7±5.9
Abdominal circumference (cm)	114.6±11.7
SBP (mmHg)	137.3±18
DBP (mmHg)	82.0±13.2
Glucose (mg/dL)	143.1±56.2
Cholesterol (mg/dL)	187.3±46.1
HDL cholesterol (mg/dL)	41.7±8.1
Triglycerides (mg/dL)	181 (133/182)
Iron (mg/dL)	79.0±24.6
Transferrin saturation (%)	25.7±8.9
Ferritin (mg/dL)	165.8 (73.9/355.9)
Hepcidin (ng/dL)	34 (7/480)

BMI, body means index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; SBP, systolic blood pressure.
Data are shown as mean ± SD, or median (percentile 25/75), or number (n).

TABLE II
Correlation between ferritin levels and clinical aspects and laboratory data.

Variable	ρ	*P*	*P**
Dyslipidemia	-0.136	0.190	0.495
Cholesterol levels	0.216	**0.037**	**0.005**
HDL cholesterol	-0.135	0.185	0.146
LDL cholesterol	0.577	0.058	0.647
Diabetes	-0.201	0.052	0.138
Glucose levels	0.100	0.306	0.380
Hypertension	0.083	0.426	0.366

HDL, high-density lipoprotein; LDL, low-density lipoprotein.
*Adjusted for age and body weight.

the presence or absence of C282Y and H63D mutations. After logistic regression analysis, only transferrin saturation and hepcidin levels remained significantly different between the analyzed groups.

DISCUSSION

Approximately a quarter of the world population has MS, and although only 15% of patients with MS show hyperferritinemia, a large number of patients have this condition (Zimmet et al. 2005, Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults 2001, World Health Organization 1999, Chen et al. 2011). Despite the large number of female included, our results indicate the presence of hyperferritinemia in 27.7% of the study population, which are superior to those already described by Chen et al. (2011).

Hyperferritinemia was more common among men, which could be related to a greater prevalence of NASH among this gender (Williams et al. 2011, Barros et al. 2017). Women also have lower iron storage due to menstrual loss, which may reflect a lower serum ferritin. This is in agreement with other studies that considered male gender as a risk factor for iron accumulation (Dongiovanni et al. 2011).

Hepcidin was present in higher levels in patients with hyperferritinemia, indicating that synthesis of this hormone is not impaired and is responsive to iron storage (Daher and Karim 2017). Some studies showed the same hepcidin increase pattern in patients with or without MS associated hyperferritinemia when compared with patients with hereditary hemochromatosis and insulin resistance (Santos et al. 2009, Adams et al. 2013, Datz et al. 2013, Martinelli et al. 2012). Hepatic production of hepcidin is directly related to the amount of liver iron, but the increased production of hepcidin in patients with MS is not yet fully understood (Barisani et al. 2008).

Increased transferrin saturation suggests iron overload due to hemochromatosis, becoming an important tool for the diagnosis (Cardoso et al. 1998). However, in cases of hyperferritinemia associated with MS, transferrin saturation is usually within normal levels, being less useful for investigation (Chen et al. 2011, Avila et al. 2015). We observed a significant difference in transferrin saturation between patients with and without hyperferritinemia, but both groups had transferrin

TABLE III
Hyperferritinemia and associated factors.

	Patients with normal ferritin level (n=68)	Patients with hyperferritinemia (n=26)	*P* univariate	*P* multivariate*
Gender				
Male	21/39 (53.8)	18/39 (46.2)	**<0.001**	0.148
Female	47/55 (85.4)	8/55 (14.5)		
Age (years)	62.1±8.6	62.2±8.9	0.957	
BMI	34.0±4.4	35.0±6.4	0.481	
Abdominal circumference (cm)	114.5±12.2	114.8±10.4	0.919	
Glucose (mg/dL)	139.9±56.9	151.1±54.8	0.392	
Cholesterol (mg/dL)	179.7±43.1	206.8±48.7	**0.010**	0.771
Triglycerides (mg/dL)	202.0±152.1	339.8±215.4	**<0.001**	0.395
HDL cholesterol (mg/dL)	42.9±8.4	38.6±6.4	**0.020**	0.376
Transferrin saturation (%)	23.3±7.1	31.9±10.2	**<0.001**	**<0.001**
Hepcidin (ng/dL)	28.0±17.6	49.8±22.0	**<0.001**	**0.008**
Diabetes	59/77 (76.6)	18/77 (23.3)	0.070	
No-diabetes	9/17 (52.9)	8/17 (47.0)	0.070	
Normal C282Y	58/80 (72.5)	22/80 (27.5)	1.0	
Mutated C282Y	10/14 (71.4)	4/14 (28.5)	1.0	
Normal H63D	52/69 (75.3)	17/69 (24.6)	0.490	
Mutated H63D	16/25 (64)	9/25 (36)	0.490	

BMI, body means index; HDL, high-density lipoprotein.
Data are shown as mean ± standard deviation, or number/total (%).
*Logistic regression.

saturation levels within the normal range, indicating a small increase in patients with higher amount of available iron. Study of hemochromatosis associated mutations in patients with MS and hyperferritinemia should be performed only on the subset of patients that also have low hepcidin levels and/or increased transferrin saturation levels.

Our study has some limitations. The major one was that patients were not tested for the mutation that affects ferroportin, which presence has similar clinical presentation (hyperferritinemia and hyperhepcidinemia). Besides, we did not assessed the burden of liver iron using magnetic resonance imaging or liver biopsy (Cardoso et al. 1998).

In summary, in our study patients with MS and hyperferritinemia had higher hepcidin and transferrin saturation levels when compared to those with normal ferritin levels. Hyperferritinemia is a common finding in patients with MS and when associated with increased transferrin saturation, one can direct research for mutations in the HFE gene. However, when transferrin saturation levels are within normal ranges additional tests are needed, and hepcidin proved to be a promising tool in the differential diagnosis of hyperferritinemia related to MS.

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AUTHOR CONTRIBUTIONS

MRR performed the experiments, wrote the manuscript and was involved in data interpretation. DKC and FSF collected the data and participated in the experiments. DAP and GAMF designed the project, analyzed the data and contributed to the writing of the manuscript. NAM performed a critical review of the manuscript. All authors approved the final version of the manuscript.

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