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Prevalence of C282Y, H63D and S65C gene mutations in patients with hyperferritinemia undergoing therapeutic phlebotomy suspected of having hereditary hemochromatosis

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ARTIGO ORIGINAL

RESUMO

A hemocromatose é uma doença autossômica recessiva causada pelo excesso de ferro depositado nos órgãos. Sua prevalência varia de acordo com as diferenças étnicas da região estudada, sendo menor em Brasileiros do que em Caucasianos ou Norte-europeus. Este estudo transversal retrospectivo analisou 222 pacientes submetidos a flebotomia terapêutica entre março de 2011 e julho de 2014, em um banco de sangue de um hospital terciário com objetivo de avaliar a prevalência da mutação genética C282Y em indivíduos avaliados com hiperferritinemia e comparar com características geográficas, clínicas, ferritina sérica e saturação de transferrina. Concluiu-se que a prevalência da mutação C282Y era maior nesta população da região sul comparada a relatos anteriores da população brasileira, ainda inferior aos de norte-europeus

Palavras-chave: hemocromatose, polimorfismo genético, ferritina sérica, flebotomia.

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ABSTRACT

Background: Hemochromatosis is an autosomal recessive disorder caused by excessive iron overload in parenchymal organs. In Brazil, the prevalence of hereditary hemochromatosis (HH) may vary compared to other countries due to different ethnical backgrounds according to the geographic region studied. The prevalence of C282Y mutation of the HFE gene ranges from three to eight times lower in Brazilians than in Caucasians of Northern European origin. Methods: A retrospective cross-sectional study was carried out in 222 patients submitted to therapeutic phlebotomy between March 2011 and July 2014 in a tertiary hospital blood center. The prevalence of HH was determined in 89 (47.3%) out of 188 (84.6%) patients presenting hyperferritinemia, and divided in two genotype groups: C282Y mutation and other mutations. The HH patients' geographical and clinical characteristics, serum ferritin rate (SF) and transferrin saturation (TS) were analyzed. Results: The HH group consisted of 71 males (79.7%) and 18 (20.3%) females, with a median age of 51.5 ± 10.6 years. There were 36 (40.0%) symptomatic patients and 65 (73.0%) tested positive for the HFE mutation. The initial baseline TS values were significantly higher in primary HH patients (48.6%, IC 95% 43.2% - 55.7%) compared to those with secondary HH (35.0%, IC 95% 27.5% 41.8%, p=0.001). Conclusion: The prevalence of C282Y mutation was higher in this population from the South region compared to previous reports in the Brazilian population, yet inferior to that of Northern Europeans.

Keywords: hemochromatosis; genetic polymorphisms; serum ferritin; phlebotomy

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INTRODUCTION

Initially, hereditary hemochromatosis (HH) was considered an idiopathic disease and believed to be a rare syndrome, being identified only after patients have become symptomatic in the late phase of the disease. Since the important discovery of the specific HFE genes in 1996, main advances have been made as for the physiopathology understanding, added to early disease diagnosis and identification of new mutations with specific disease subtype characteristics. Treatment protocols and family screening could, then, be established and more recently disease testing has become more available, so that HH prevalence has increased over time. [1]

Hereditary hemochromatosis is an autosomal recessive disease characterized by a genetic predisposition to excessive iron absorption from the diet. At the disease end-stage, structural and functional damage to the affected organs are observed. [2] The liver is the most affected by iron accumulation, with the consequent development of fibrosis leading to cirrhosis, which is a risk factor to develop hepatocellular carcinoma. [3] The pancreas and the heart are also affected organs, leading to diabetes mellitus and heart failure.

Patients with established HH diagnosis and iron overload must be included in a regular therapeutic phlebotomy program to avoid progression and late complications of the disease, and may have a normal life survival rate [4, 5]. The prevalence of C282Y mutation in Brazil is reported to be lower if compared to the Northern Europeans. However, due to the extensive ethnical diversity in different geographic regions, the prevalence of HFE mutations is not homogenous in the Brazilian territory. In this scenario, the main aim of the study was to evaluate the prevalence of HH in patients who underwent therapeutic phlebotomy in a hemotherapy service in the South of Brazil of Northern European descent. The objective is to describe the sociodemographic, clinical, genetic and laboratory characteristics associated with HH in this specific population.

METERIALS AND METHODS

Survey design and participants:

A cross-sectional study was carried out among 222 patients undergoing therapeutic phlebotomy between March 2011 and July 2014 in a hemotherapy service of a tertiary hospital in Passo Fundo, Brazil. The prevalence of HH was determined in 188/222 (84.7%) patients referred to the blood center with hyperferritinemy, of both primary and secondary etiology. This HH classification was based on [6] socio-demographic and clinical characteristics, as well as serum ferritin (SF) and transferrin saturation (TS) measurements. Patient selection was performed by reviewing the database during the period when patients were followed at the hemotherapy service.

Laboratory assessment:

The initial HH diagnosis was carried out by the patients' primary physicians, who were led to the hemotherapy service for phlebotomy. If the patient had no blood tests, the blood bank hematologist would request it. Among the 99 patients investigated for secondary hemochromatosis (hyperferritinemia), only 18 patients were tested for genetic mutations, and all resulted negative for C282Y, H63D and S65C genes. The test was not performed in the remaining patients, as there were secondary causes that justified the high ferritin levels, so they were excluded from this study.

In our study, the inclusion criteria were age equal or greater than 18 years, TS values equal or superior to 45% for both genders, in accordance with international reports, whose concept of elevated transferrin saturation ranges from 45 to 60% [7, 8], FS values equal or superior to 300 µg/L for males and 200µg/L for females. Hemoglobin (Hb) levels above 11g/dL and hematocrit (Ht) above 33% were required for patients to undergo therapeutic phlebotomy.

The exclusion criteria were patients with primary polycythemia (polycythemia vera), secondary polycythemia and secondary hemochromatosis.

Inclusion criteria for therapeutic phlebotomy:

Phlebotomy was prescribed by the primary physicians, and indications were reassessed by the hematologist from the hemotherapy service. In total, there were 89 patients with suspected diagnosis of HH who underwent the procedure. When the patient had high ferritin and transferrin saturation index, but no genetic testing

Statistical analysis:

The statistical analyses were carried out with the help of IBM SPSS Statistics 23.0. The categorical data were expressed as absolute and relative frequency and the numerical as mean ± standard deviation. The therapeutic phlebotomy indication was performed using variance analysis with adjustment according to gender and age. The symptom proportions were compared using Pearson chi-square. The TS measures, basal ferritin and hematocrit comparisons, among the subjects with primary and secondary hemochromatosis, were carried out using t Student test. The estimated marginal means were expressed with the respective confidence interval of 95%. Tests with probability value of 0.05 were considered statistically significant.

RESULTS

Patient demographics:

Two hundred and twenty-two patients were included in the study, with a median age of 54.9 ± 12.3 years, being 187 (84.2%) males and 35 (15.8%) females. In this study group, 219 (98.6%) patients were self-reportedly classified as European Caucasians. Most patients came from 22 cities in the south of Brazil. Table 1 presents the socio-demographic characteristics of the population studied.

Table 1: Socio-demographic characteristics of patients referred for therapeutic phlebotomy (n=222)

Socio-demographic variables	Therapeutic phlebotomy indication				p
	Primary polycythemia (n = 11)	Secondary polycythemia (n = 23)	Primary hemochromatosis (n = 89)	Secondary hemochromatosis (n = 99)	
Age (years)	62.6[b] ± 13.2	56.6[ab] ± 16.9	51.5[a] ± 10.6	56.8[ab] ± 11.8	0.003
Age					
< 60 years	4 (2.8%)	12 (8.4%)	68 (47.6%)	59 (41.3%)	0.008
60 years or more	7 (8.9%)	11 (13.9%)	21 (26.6%)	40 (50.6%)	
Sex					

Male	5 (2.7%)	20 (10.7%)	72 (38.5%)	90 (48.1%)	0.001
Female	6 (17.1%)	3 (8.6%)	17 (48.6%)	9 (25.7%)	

Results from the one-way ANOVA and Tukey post hoc test. Values express absolute, relative or mean frequency ± standard deviation; Rio Grande do Sul State.

Laboratory measurements and clinical data:

Among the 222 patients eligible for therapeutic phlebotomy, 188 (84.6%) had hemochromatosis and 34 (15.3%) had polycythemia. Of those, 11 (32.3%) patients had primary polycythemia and 23 (67.6%) secondary polycythemia. Among the 188 patients with hemochromatosis, 89 (47.3%) were suggested to be hereditary and 99 (52.6%) had the secondary form of the disease, as summarized in Table 2.

Table 2: Clinical and laboratory characteristics of patients submitted to phlebotomy for hyperferritinemy (n= 222)

Previous history and comorbidities	Global (n = 222)	Therapeutic phlebotomy indication			
		Primary polycythemia (n = 11)	Secondary polycythemia (n = 23)	Primary hemochromatosis (n = 89)	Secondary hemochromatosis (n =99)
Previous donation	24 (10.8%)	—	2 (8.6%)	11 (12.3%)	11 (11.1%)
Previous transfusion	9 (4.0%)	—	1 (4.3%)	1 (1.1%)	7 (7.0%)
Family history	29 (13.0%)	—	—	25 (28.0%)	4 (4.0%)
Symptoms	69 (31.0%)	7 (63.6%)	6 (26.0%)	36 (40.4%)	20 (20.2%)
Hepatic	62 (27.9%)	—	—	31 (34.8%)	31 (31.3%)
Endocrine	28 (12.6%)	—	3 (13.0%)	10 (11.2%)	15 (15.1%)
Dyslipidemia	28 (12.6%)	—	—	17 (19.1%)	11 (11.1%)
Hematology/Oncology	28 (12.6%)	—	1 (4.3%)	10 (11.2%)	6 (6.0%)
Cardiovascular	36 (16.2%)	1 (9.0 %)	8 (34.7%)	13 (14.6%)	14 (14.1%)
Renal	3 (1.3%)	—	3 (13.0%)	—	—
Pulmonary	5 (2.2%)	—	4 (17.3%)	—	1 (1.0%)
TGI	2 (0.9%)	—	—	—	2 (2.0%)
Others	8 (3.6%)	—	2 (8.6%)	4 (4.4%)	2 (2.0%)
Baseline hematocrit (Ht)	43.9 ± 5.2	50.9 ± 4.5	52.2 ± 7.4	42.7 ± 3.7	42.4 ± 3.1

Values express absolute and relative frequency or mean ± standard deviation.

Among the 188 with hyperferritinemia, 89 (47.3%) patients were referred, with HH diagnosis for phlebotomy, to the blood center. However, only 65 (73%) presented an HFE genetic test result (C282Y, H63D and S65C) on their first consultation with the hematologist.

Only 11/89 (12.3%) patients had previously been blood donors. One particular (1.1%) patient was diagnosed with HH, associated with spherocytosis, and had previously received multiple blood transfusions (twelve units of red blood cells) during pregnancy. Familiar investigation for HH diagnosis or any related pathology showed that 25 (28.1%) patients mentioned having at least one family member with HH. These patients comprised 18 (72.0%) males and 7 (28.0%) females. Other 19 patients (29.2%) had a positive genetic study. None of the 4 (4.0%) patients with secondary hemochromatosis that reported a family history of HH carried out specific genetic evaluation.

From the 188 patients with high ferritin levels, including both primary and secondary hemochromatosis, 83 (44.1%) patients presented their genetic tests during the phlebotomy procedure, of which 18 (21.7%) were tested negative for genetic mutations, and were, thus, considered to have secondary hemochromatosis. Likewise, only 66 out of 89 (74.1 %) patients referred to the blood bank for phlebotomy, which performed genetic HFE testing. Of those, 65 (73.0 %) were positive for an HFE mutation. One patient (1.2%), was considered a possible case of HH even in the absence of mutation for the subtype 1 of HH and probably due to a different undetected mutation.

Considering the identified mutations, the H63D gene was the most prevalent, found in 24/65 (36.9%) affected patients in heterozygosity and in 13 (20.0%) patients in homozygosity. The C282Y mutation occurred in heterozygosity in 12 (18.5%) patients and in homozygosity in 8 (12.3%). The S65C mutation in homozygosity was present in 2 (3.1%) patients. Heterozygous C282Y/H63D occurred in 5 (7.7%) patients, while heterozygous H63D/S65C in only 1 (1.5%). The overall prevalence of the affected HFE gene included 43 (65.1%) cases of H63D, followed by 25 (37.8%) cases of C282Y and 3 (4.5%) cases of S65C. Of note, 2/89 patients with hemochromatosis who tested negative for genetic mutations descended from Caucasians and Africans, while another patient who descended from Caucasians and native south Americans had heterozygous C282Y

genotype.

Among the 89 patients with HH, 31 (86.0%) were male and 5 (14.0%) were female, and 36 (40.4%) patients were symptomatic. A significantly higher frequency of symptomatic disease was observed in patients with the C282Y mutation, as compared to the other mutations of the HFE gene, namely 15/25 (60.0%) vs 14/39 (35.0%), p = 0.059, as shown in Table 3. In this HH population, 15 patients showed evidence of iron overload, 8 diagnosed by liver biopsy and 7 by Magnetic Resonance Imaging T2* (MRI). The main signs and symptoms referred by patients with HH stratified by gender are summarized in Table 3.

Table 3: Frequency of symptoms in patients with hemochromatosis submitted to phlebotomy for hyperferritinemia according to demographic, clinical, genetic and laboratory variables

Symptoms and signs	Symptoms and signs		p
	Yes	No	
Sex			
Male (n = 71)	50 (30.9%)	112 (69.1%)	0.420
Female (n = 18)	6 (23.1%)	20 (76.9%)	
Therapeutic phlebotomy indication			
Primary hemochromatosis	36(40.4%)	53 (59.6%)	0.002
Secondary hemochromatosis	20 (20.2%)	79 (79.8%)	
Gene mutation			
Mutation C282Y	15 (60.0%)	10 (40.0%)	0.059
Other genetic mutations	14 (35.9%)	25 (64.1%)	
Hereditary Hemochromatosis			
Yes	36(40.4%)	53 (59.6%)	0.002
No	20 (20.2%)	79 (79.8%)	
Tiredness			
Yes	5 (100.0%)	0 (0.0%)	0.005
No	31 (36.9%)	53 (63.1%)	
Fatigue			
Yes	12 (92.3%)	1 (7.7%)	< 0.001
No	24 (31.6%)	52 (68.4%)	
Arthralgia			
Yes	14 (87.5%)	2 (12.5%)	< 0.001
No	22 (30.1%)	51 (69.9%)	
Abdominal pain			
Yes	6 (75.0%)	2 (25.0%)	0.037
No	30 (37.0%)	51 (63.0%)	
Organ iron overload			
Yes	10 (66.7%)	5 (33.3%)	0.023
No	26 (35.1%)	48 (64.9%)	

Liver biopsy			
Yes	8(80.0%)	2 (20.0%)	0.007
No	28 (35.4%)	51 (64.6%)	
Transferrin saturation rate			
Yes	17 (51.5%)	16 (48.5%)	0.126
No	12 (33.3%)	24 (66.7%)	

Baseline TS values were found to be significantly higher among patients with HH (48.6% [43.2% – 55.7%]) as compared with those with secondary hemochromatosis (35.0%, [27.5%-41.8%]), p=0.001. After adjustment for gender and presence of symptoms, neither showed statistical significance. Likewise, no significant statistical difference was observed comparing baseline serum ferritin levels in patients with HH (966.6 ng/mL, [735.9-10029.1]) and secondary hemochromatosis (1023.8ng mL, [439.1 – 1025.7], p=0.703) after adjustment for gender and presence of symptoms.

No significant statistical difference was observed between baseline hematocrit in patients with HH (42.7%, [40.8 – 42.7]) and secondary hemochromatosis (42.4%, [40.3 – 43.0]), p=0.601, after adjustment for gender and presence of symptoms. Male patients presented with significantly higher baseline hematocrit levels than females, 42.7 (42.1 – 43.3) vs 40.7 (39.2- 42.3), respectively (p= 0.021).

Taking into account patients with HH undergoing therapeutic phlebotomy, hematocrit varied from 34% to 50%, mean 42.7%. In male patients, the hematocrit varied from 34% to 48%, mean 40.7%. Table 4 describes hematimetric parameters of patients with primary and secondary hemochromatosis.

Table 4: Hematimetric parameters of patients with primary and secondary hemochromatosis

Baseline	Therapeutic phlebotomy indication		
	Primary (Hereditary) hemochromatosis	Secondary hemochromatosis	p
Baseline TSI (%)	48.6 ± 18.3 (n = 42)	35.0 ± 12.8 (n = 26)	0.001

Baseline ferritin (ng/mL)	966.6 ± 646.8 (n = 78)	1023.8 ± 1147.5 (n = 75)	0.703
Baseline hematocrit (%)	42.7 ± 3.7 (n = 89)	42.4 ± 3.1 (n = 99)	0.601

Mean ± standard deviation.

DISCUSSION

In the present study, 34 patients (15.3%) had polycithaemia and 188 (84.6%) hemochromatosis. Among the ones with polycithaemia, 11 (32.3%) were primary and 23 (67.6%) secondary. From the 188 cases of patients with hemochromatosis, 89 (47.3%) were hereditary and 99 (52.6%) secondary.

Among the 188 patients with hyperferritinemy, 83(44.1%) were genetically investigated toward HFE gene, 18(21.7%) had a negative result for genetic mutation and were considered to have secondary hemochromatosis. The remaining 65(73.0%) had a positive HFE mutation, and 1 (1.1%), even in the absence of mutation for HH 1 subtype, was considered as carrier of hereditary hemochromatosis.

In a 14-year-retrospective work, from 1998 to 2012, among causes of phlebotomy indication, there were 11 (11%) which presented for therapeutic phlebotomy caused by secondary hyperferritinemy.[9]

In the Brazilian literature, just one study was carried out in Ribeirão Preto, São Paulo, from November 1997 to August 1998, whose result found, in a total of 105 phlebotomy performed, 76 cases of (72%) secondary polycythemia, 16 (15%) polycythemia vera, 4 (4%) porphyria catenae tarda, with or without C hepatitis, and 9 (9%) hereditary hemochromatosis. [10]

In our study HH patients had a male predominance of (71/89, 79.7%) with a mean age 51.5 ± 10.6, and most of them (87/89, 97.8%) were self-reportedly Caucasians of European descent.

Testing for the HFE gene was performed in 83 (44.1%) patients, 18 of which (21.7%) had negative results and were, thus, considered to have secondary hemochromatosis. Among the 89 patients with HH, 65 (73.0%) tested positive for the HFE mutation. One case (1.1%) that was tested negative for HH 1 subtype was considered a possible HH subject with a non HFE mutation. No results were obtained in 23 (25.8%) patients. There were 11 (11%) patients referred for therapeutic phlebotomy

caused by secondary hyperferritinemia.

Our results contrast with a previous study from Ribeirão Preto, São Paulo, which analyzed 105 phlebotomies performed from November 1997 to August 1998 and found a prevalence of only 9 (9%) cases of hereditary hemochromatosis. [10]A cohort of patients with hyperferritinemia from Rio Grande do Norte with 183 patients from both genders, aged 15 to 70 years showed evidence of C282Y and H63D mutations for the HFE gene in 89 patients (48%), being 9 (5%) C282Y in heterozygosis, 2 (1.10%) C282Y in homozygosis, 56 (31%) H63D in heterozygosis, 16 (8.70%), H63D in homozygosis and 6 (3.30%) in hybrid heterozygosis (H63D/C282Y). Male patients presented with a higher concentration of serum ferritin. [11]

Another study of patients with HH undergoing therapeutic phlebotomy was done between January 2002 and May 2012, and showed a possibility of missed diagnosis or error in diagnosis of the disease, emphasizing that all suspect cases of hereditary hemochromatosis should have genetic testing performed before starting phlebotomy. [12] However, several studies report that other genetic and environmental factors can alter phenotypic penetrance by clinical and bio clinical manifestation of the expression of gene C282Y in homozygosis, like the haplotype disparity A*01-B*08 or A*03-B*07 in male and female patients, as a determinant sign in the regulation of gene expression. [13, 14]

In our study, the analysis of the family history of patients with diagnosed or suspected HH revealed that 25/89 patients (28.1%) mentioned to have at least one family member with HH, which is rarely diagnosed before the second decade of life, most of the patients become symptomatic between 40 and 50 years old [15, 16, 17]. Thus, the current guideline for first-degree relatives of patients with HH is to undergo genetic testing and TS measurement after 20 years of age. [18,19] Factors that may increase the risk of HH include a positive family history of HH, the presence of liver disease or type I diabetes mellitus and northern or western European descent.[20] Among the comorbidities, the presence of hepatitis C, alcohol abuse or hereditary anemia are risk factors for iron overload, which are also important prognostic factors.[21]

In this study, only 11 patients (12.3%) had previously been blood donors. Donors, who are suspected of having iron overload, either with a family history or, suggestive clinical workup, must be evaluated or followed by a hematologist. [22] Previous reports

found that [23], more than 30% of blood donors have HFE mutations.

Most of the patients in this study (73, 82.9%) come from the 6th CRS (Passo Fundo), in the north of Rio Grande do Sul, Brazil. Depending on the population studied, genetic testing for the HFE mutations shows that homozygosity for the C282Y mutation prevails and varies between 60% and 100%, followed by a prevalence of 0 to 7% for C282Y/H63D genotype, 0 to 4% for H63D/H63D genotype, 0 to 15% to H63D in heterozygosis and 0 to 21% with normal genotype.[21] Mutations C282Y and H63D account for two thirds of the Brazilian patients with HH, making the likelihood of other mutations of HFE gene, like S65C, or other genes related to iron metabolism less probable to be found. [24]

Among the genes involved in HFE gene, 43 cases (65.15%) for H63D followed by 25 (37.87%) for gene C282Y and 3 (4.54%) for S65C were found in this work. The genotype C282Y is found in more than 90% of the patients with HH in the north of Europe and in more than 80% of Americans [25].

In the present work, values of baseline TS were significantly higher among subjects with HH, compared to those with secondary hemochromatosis. However, there was no statistical difference, as for the baseline FS, between patients with primary and secondary hemochromatosis. Independent of the confirmation of the genotype diagnosis, the presence of iron overload already indicates the start of phlebotomy treatment to remove the excess of iron.[26]

In Brazil, hereditary hemochromatosis frequency may have variations compared to other countries because of its continental dimension and differences in the ethnic background of each geographic region, which may result in different regional prevalence of the disease. The prevalence of the C282Y mutation of the HFE gene is from three to eight times lower in Brazilian people than in Caucasian from Northern Europe.

Nowadays it is routine practice to investigate FS level in regular checkups. However, in several cases TS is not requested, which may limit the suspected diagnosis of HH and underestimate the disease prevalence in the region, which may be actually higher. Among the limitations for precise diagnosis are the lack of genetic testing for the HFE gene or other subtypes of HH, and also T2*magnetic resonance image, which is not available in the health public service.

The conclusion is that C282Y mutation in the population studied was slightly

higher than the prevalence described in the Brazilian population in previous reports, yet inferior to that of northern Europe. Other genes, besides C282Y, were associated with target organ lesion. Transferrin saturation remains a fundamental tool for HH diagnosis. The mean age, at the beginning of the treatment, in our study population portrays late diagnosis, reinforcing the importance of multidisciplinary actions *to* improve diagnosis and early treatment to prevent the irreversible complications of this disease.

Our study has some limitations due to: TS is not required in many cases of high ferritin level by the assistant physicians. Magnetic Resonance Imaging T2* (MRI) testing was limited due to cost and lack of availability in the public health service, as well the genetic study for HFE gene, only the main mutations were analyzed in the HFE gene, the other subtype mutations, non HFE gene, are not affordable, and are mainly performed in research centers.

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