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RDW as differential parameter between microcytic anemias in "pure" and concomitant forms

O RDW como parâmetro diferencial entre anemias microcíticas nas formas "pura" e em concomitância

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ABSTRACT

Introduction: Iron deficiency anemia and thalassemia minor are microcytic and hypochromic types of anemia commonly found in our environment. The correct differentiation between them is of great clinical importance, although it is often hampered by the coexistence of diseases that may alter the results of standard tests for their discrimination, in addition to the significant costs of such tests. **Objective:** The objective of this study was to investigate the discriminatory power of red cell distribution width (RDW) between iron deficiency anemia and thalassemia minor. **Method:** Blood parameters were compared in 227 patients with iron deficiency anemia and/or thalassemia minor after diagnosis confirmed by molecular biology and HbA2 measurement for alpha thalassemia and beta thalassemia trait respectively. The frequency of alpha thalassemia trait in a population from two public hospitals of Minas Gerais was also determined. **Result and conclusion:** RDW was able to differentiate iron deficiency anemia from thalassemia trait, what indicates that this blood count parameter is a useful tool since concomitant disorders are excluded. In addition, a high frequency of the $-\alpha^{3.7}$ mutation was observed in the study population (20.3%), justifying its investigation when another cause for microcytic anemia is absent.

Key words: iron deficiency anemia; thalassemia trait; red blood cell distribution width.

INTRODUCTION

Iron deficiency anemia and thalassemia minor are among the most frequent hypochromic and microcytic anemias in our environment.

Iron deficiency anemia is the most common nutritional disorder, affecting 30%-70% of the population in developing countries[1]. This type of anemia is the final phase of a process that begins with exhaustion of iron stores and continues with iron depletion from the other compartments that contain it, compromising normal erythropoiesis[2]. Thus, its diagnosis is given by tests assessing iron metabolism, aiming to reveal the absence of iron stores in the reticuloendothelial system[1].

Thalassemias are genetic disorders characterized by the partial decrease or total absence of globin chain production; the alpha and beta forms[3, 4] bear clinical relevance. Beta thalassemia minor presents slight or mild clinical manifestations, and its diagnosis is made by hemoglobin electrophoresis, in which increased hemoglobin A2 (HbA2) is the indicative parameter of this hemoglobinopathy[4, 5].

Each chromosome 16 presents two genes that encode the alpha chain. Consequently, there are four types of alpha thalassemia, depending on the number of mutated genes. The deficiency due to four-gene deletions, characterized by absent production of alpha chains, is incompatible with life[6], while three-gene deletions present severe anemia, and may require regular transfusions. The two-gene deletion produces mild microcytosis and hypochromia, with normal or slightly decreased hemoglobin levels. The one-gene defect is hematologically and clinically asymptomatic (silent carrier state)[3, 4, 7-10].

The presence of hemoglobin H (HbH) in hemoglobin electrophoresis, associated with marked microcytic anemia,

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is compatible with the diagnosis of HbH disease. But it is not possible to establish the diagnosis of alpha thalassemia trait by hemoglobin electrophoresis; it is given by exclusion of other microcytic anemias. That is why the correct diagnosis requires search for mutations in the alpha globin genes by molecular biology methods[11]. The prevalence of silent carriers in the Brazilian population is estimated to be 10%-20%; the prevalence of the alpha thalassemia trait, 1%-3%, with the one-gene deletion ($-\alpha^{3.7}$) being the most commonly reported[12]. Another common mutation is the 4.2 Kb deletion ($-\alpha^{4.2}$)[13, 14].

When the diagnosis of iron deficiency anemia and thalassemia minor, both alpha and beta, is based only on hematological parameters and erythrocyte morphology, these forms of thalassemia may be erroneously diagnosed as iron deficiency anemia. It is necessary to carry out the gold standard tests to differentiate between these two types of anemia, once the therapeutic conduct is distinct for each case. These tests, however, are more expensive, and, sometimes, inaccessible to a disadvantaged population. Therefore the identification of these types of anemia is very important, aimed at optimizing the differential diagnosis, using, if possible, just a complete blood count (CBC). One of the parameters of the CBC suggested for differentiation is the red blood cell distribution width (RDW). As verified by Bessman and Feinstein[15], homogeneous microcytosis is observed in patients with thalassemia minor when in comparison with that of iron deficiency anemia patients. As a result, RDW tends to be higher in iron deficiency anemia than in thalassemia minor[5].

OBJECTIVE

This study is aimed at investigating the discriminatory power of RDW between iron deficiency anemia and thalassemia minor, with the diagnosis of alpha thalassemia trait being confirmed by molecular biology. The frequency of alpha thalassemia minor was also determined in a populational sample with microcytosis and hypochromia coming from two public hospitals of the state of Minas Gerais.

METHODS

Ethical aspects

This study was approved by the ethics committees of the following institutions: Universidade Federal de Minas Gerais (Report 344/09), Hospital das Clínicas (Report 114/09) and Hospital Governador Israel Pinheiro (Report 129/09).

Sample characteristics

The first group of participants was composed of 30 adults recruited at the hematology outpatient clinic of Hospital das Clínicas in Belo Horizonte (MG). They presented either mean corpuscular volume (MCV) below 80 fl, with clinical and laboratorial characteristics suggesting alpha thalassemia, or besides MCV below 80 fl, hemoglobin electrophoresis with $HbAA_2$ pattern and HbA_2 levels over 3.5%[2], a picture compatible with beta thalassemia minor. Iron deficiency anemia was excluded from this group based on the ferritin levels within the reference range. All the patients with malignant and inflammatory/infectious diseases were excluded from the group, based on clinical data and personal information obtained from the clinical report.

After signature of the informed consent, 5 ml of venous blood was collected in ethylenediaminetetraacetic acid (EDTA). This sample was used for the genetic study. Clinical and laboratorial data were obtained from patients' records, including serum ferritin levels (method: chemiluninescence; DPC Immulite® analyzer), hemoglobin electrophoresis at alkaline pH (method: electrophoresis at pH 8.6 using Sebia's semi-automated Hydrasys® system), HbA_2 measurement (method: elution after cellulose acetate electrophoresis with relative determination of several hemoglobin types), hemoglobin F (HbF) quantitation (method of Betke), reticulocyte count (brilliant cresyl blue), HbH-inclusion body test (brilliant cresyl blue), and CBC (automatic counter Sysmex® XE-2100 of Sysmex®).

In the second group, 197 patients older than 18 years were assessed. They presented hemoglobin of less than 12 g/dl and 13 g/dl for men and women, respectively, MCV below 80 fl and ferritin lower than 6 ng/ml for women and 28 n/ml for men (kit Ferritin, Access®), characterizing iron deficiency anemia. They were recruited at Hospital Governador Israel Pinheiro (Instituto de Previdência dos Servidores do Estado de Minas Gerais [IPSEMG]) in Belo Horizonte (MG). All patients with malignant and inflammatory/infectious diseases were excluded from this group.

A total of 5 ml of venous blood was collected in EDTA, which was used for molecular analysis. The participants in this group did not sign the informed consent, according to an exemption granted by the research ethics committee of Hospital Governador Israel Pinheiro, because just surplus samples of the selected patients were used, with no need for additional blood collection.

Besides the venous blood sample, CBC (automatic counter ABX Pentra DX-DF 120 of Horiba Medical®) and ferritin measurement (method chemiluminescence immunoassay; Access® Immunoassay System of Beckman Coulter®) of these individuals were also performed.

Molecular analysis

The deoxyribonucleic acid (DNA) was extracted by means of leukocytes obtained from whole blood collected in EDTA, using the kit Gentra Puregene Blood (Qiagen), according to the manufacturer's instructions.

The molecular analysis encompassed detection of the main deletional and non-deletional mutations that cause alpha thalassemia, according to the frequency reported in epidemiological articles published on this disorder[16, 17].

The investigation of deletional mutations 3.7, 4.2, MED, 20.5, SEA, FIL and THAI was carried out by Multiplex polymerase chain reaction (PCR). The protocol used for the reaction followed the methodology described by Tan *et al*.[17]. After amplification, the PCR product underwent electrophoresis on a 1.5% agarose gel.

The protocol detecting α^{HphI} mutation followed the methodology by Foglietta *et al*.[16]. For the analysis of non-deletional α^{HphI} mutation, the PCR-amplified product was digested with the *Hph*I restriction enzyme by a PCR-restriction fragment length polymorphism (PCR-RFLP). The digestion product underwent electrophoresis on a 1.5% agarose gel.

Statistical analysis

Normality assessment of hematological parameters was conducted using software Minitab 14, by means of Anderson-Darling test.

The statistical analysis to investigate the difference between hematological parameters of the three patient groups (iron deficiency anemia, thalassemia minor with concomitant iron deficiency anemia and just thalassemia minor) was conducted with software Prism 5 and Kruskal-Wallis test, followed by employment of post-hoc Dunn's test. Values of $p < 0.05$ were considered significant.

RESULTS

Assessment of mutations causing alpha thalassemia

Among the 30 patients of group 1, 24 suffered from beta thalassemia minor. The others were supposed carriers of alpha thalassemia in its minor form. In this group, of supposed carriers of alpha thalassemia trait, 3.7 deletion was found at the homozygous state in five patients (16.7%). The non-deletional HphI mutation was observed in only one (3.3%) patient at the heterozygous state (**Table 1**).

Among the 197 patients of group 2, with confirmed iron deficiency anemia based on reduced ferritin levels, 42 presented concomitant alpha thalassemia, confirmed by molecular exams; 3.7 deletional mutation was detected in 41 (20.8%) – in homozygous state in three (1.5%) and in heterozygous state in 38 (19.3%) patients. The non-deletional HphI mutation was found in only one (0.5%) patient in heterozygous state (Table 1).

Analysis of hematimetric parameters

After search for mutations, a statistical analysis of several CBC parameters was conducted. This analysis was aimed at verifying the possible significant differences that could characterize the groups iron deficiency anemia, thalassemia minor with concomitant iron deficiency anemia, and thalassemia minor, according to hematological parameters, especially RDW. The following CBC parameters were analyzed: number of erythrocytes (red blood cell [RBC]), Hb, MCV, mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), RDW and number of platelets (**Table 2**).

TABLE 1 – Frequency of 3.7 and *Hph*I mutations in individuals diagnosed with thalassemia minor and iron deficiency anemia

Mutation	IDA (n = 197)	Thal (n = 30)	Total (n = 227)
3.7 deletion	41 (20.8%)	5 (16.7%)	46 (20.3%)
Homozygotes	3 (1.5%)	5 (16.7%)	8 (3.5%)
Heterozygotes	38 (19.3%)	0	38 (16.7%)
*Hph*I	1 (0.5%)	1 (3.3%)	2 (0.9%)
Homozygotes	0	0	0
Heterozygotes	1 (0.5%)	1 (3.3%)	2 (0.9%)

IDA: iron deficiency anemia; Thal: thalassemia minor.

TABLE 2 – Hematological parameters of patients with iron deficiency anemia, iron deficiency anemia with concomitant thalassemia minor, and just thalassemia minor

Parameters	IDA (n = 155)	IDA + Thal (n = 42)	Thal (n = 30)
RBC (10^6/µl)	4.45 (4.18; 4.71)	4.6 (4.28; 4.94)	5.4 (5.09; 5.72)[a, b]
Hb (g/dl)	10 (9.3; 10.7)	10.5 (9.5; 11.1)	10.9 (10.5; 11.8)[a, b]
Ht (%)	32.2 (30.3; 33.8)	33 (31; 35)	34.6 (33.2; 36.2)[a, b]
MCV (fl)	73 (67; 76)	72 (69; 76)	63.8 (61.6; 68.5)[a, b]
MCH (pg)	22.7 (20.8; 24.1)	23 (21; 23.9)	20.4 (19.7; 21.6)[a, b]
MCHC (g/dl)	31.2 (30.4; 31.8)	31.5 (30.4; 32.4)	31.8 (31.2; 32.6)[a]
RDW (%)	17.9 (16.6; 19.4)	18.5 (16.2; 19.9)	15.9 (15.3; 16.9)[a, b]

Data were presented as median and interquartile range.
IDA: iron deficiency anemia; Thal: thalassemia minor, RBC: number of erythrocytes; Hb: hemoglobin; Ht: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red blood cell distribution width; a - significant difference compared to group IDA (p < 0.05); b - significant difference compared to group IDA + Thal (p < 0.05).

Among the analyzed hematological parameters, none presented difference between the group with only iron deficiency anemia and the group with concomitant diseases. In the comparison between iron deficiency anemia and thalassemia minor, all the analyzed parameters showed significant difference between the groups. In the comparison between thalassemia minor and the group with concomitant iron deficiency anemia, just the parameter MCHC did not present difference.

DISCUSSION

Assessment of mutations causing alpha thalassemia

In this study, a high frequency of 3.7 deletion (main causing mutation of alpha thalassemia) was observed. Some studies on the frequency of mutations causing alpha thalassemia were conducted in the Brazilian population. By means of molecular analysis, Sonati *et al*.[18] studied 47 individuals of African ethnicity and demonstrated that 10 (21.3%) were heterozygotes and one (2.1%) was homozygote for 3.7 deletion. Adorno *et al*.[19] carried out a study about hemoglobinopathies in newborns from Salvador. They analyzed samples from 514 newborns for mutations 3.7 and 4.2 of alpha thalassemia and observed that, among them, 22.2% had 3.7 deletion, with 19.7% heterozygotes and 2.5% homozygotes. Another study, conducted by Souza *et al*.[20], assessed the presence of 3.7 deletion in a population from Pará. Among the 103 anemic patients the authors found a percentage of 19.4% of heterozygous and 1% of homozygous individuals for the cited mutation, accounting for 20.4% of the patients. The results of these studies are in accordance with the frequency observed in this study: 20.3% of the 227 patients are carriers of 3.7 deletion.

CBC parameters

The highest number of erythrocytes was observed in thalassemia minor, what is related to the disease physiopathology. Globin chain precipitation in erythroid precursor cells and circulating erythrocytes leads to a discrete inefficacious erythropoiesis, causing increased erythrocyte production in an attempt to compensate for anemia, what is observed in the large number of erythrocytes in the blood[3, 21, 22]. Similarly, the highest hemoglobin levels were found in the thalassemia minor group, what may be also explained by the mechanism associated with thalassemia minor. In fact, thalassemia minor cases produce mildly decreased hemoglobin concentration[7].

MCV is clearly higher in iron deficiency anemia, reflecting a lower microcytosis, that is, the disturbance in hemoglobin synthesis is slighter compared with that in thalassemia minor. Analyzing the parameters RBC, Hb and MCV as a group, we may observe that the thalassemia patient presents the highest values for RBC and Hb, and the lowest for MCV.

Another parameter in which a significant difference was observed between the groups with thalassemia minor and iron deficiency anemia was RDW. This parameter is suggested for distinction between the conditions associated with microcytosis. It was first described by Bessman and Feinstein[15], and nowadays is part of automated counter analyses. This index reflects the heterogeneity in size distribution of erythrocytes measuring the coefficient of variation around MCV[5]. Bessman and Feinstein[15] observed that erythrocytes of thalassemia minor patients were more homogeneous than those of iron deficiency anemia patients. Consequently, RDW tends to be higher in iron deficiency anemia than in thalassemia minor[5], although the role of RDW as an auxiliary parameter in the differentiation of types of anemia is controversial. In some studies[23, 24], a significant difference was observed for RDW between patients with iron deficiency anemia and beta thalassemia minor, while in others this parameter presented low diagnostic accuracy[25-32]. In Brazil, Lima *et al*.[33] concluded that a correct discrimination between these disorders could not be done based on just RDW. Besides this study, Matos *et al*.[34] did not verify significant difference in RDW between the three groups of assessed microcytic anemias, indicating that this test has limited usefulness for differentiating iron deficiency anemia, beta thalassemia minor, and chronic disease anemia.

A limitation of Matos et al.[34] and other studies that assessed RDW efficacy is the lack of utilization of molecular biology methods for the diagnosis of alpha thalassemia trait, what could influence RDW performance due to the concomitant diseases. And, according to the observed in this study, the frequency of alpha thalassemia mutations in our environment is high, and must be considered. It is well known that in certain conditions of concomitant diseases (for example, iron deficiency anemia and chronic disease anemia), even the results of gold standard tests may suffer the interference of the intercurrent disease, making diagnosis more difficult. Therefore, the results of our study indicate a limited usefulness of RDW as an auxiliary parameter for differentiation of these types of anemia, because a better discrimination between both nosological entities by RDW would depend on the absence of other disorders favoring microcytosis. In other words, our data suggest the idea that the use of RDW in the discriminatory process between the microcytic anemias in question is valid just when which microcytic anemia does not present other concomitant clinical alterations. In the absence of intercurrent disorders, it may be an important tool for differential diagnosis between iron deficiency anemia and thalassemia minor (alpha and beta), and it must be valued as an auxiliary laboratory parameter for this purpose.

RESUMO

Introdução: *A anemia ferropriva e as talassemias menores são anemias microcíticas e hipocrômicas comumente encontradas em nosso meio. A correta diferenciação entre essas anemias é de grande importância clínica, embora muitas vezes seja dificultada pela concomitância de doenças que podem alterar os resultados dos exames padrão, além dos custos significativos de tais testes.* ***Objetivo:*** *O objetivo deste estudo foi investigar o poder discriminatório do índice de anisocitose eritrocitária (RDW) entre anemia ferropriva e talassemias menores.* ***Método:*** *Foram comparados os parâmetros hematológicos de 227 pacientes portadores de anemia ferropriva e/ou talassemia menor após diagnóstico firmado por biologia molecular ou por dosagem de hemoglobina alfa 2 (HbA2), no caso de traço alfa ou betatalassêmico, respectivamente. Foi também determinada a frequência das talassemias menores em uma amostra populacional proveniente de dois hospitais públicos de Minas Gerais.* ***Resultado e conclusão:*** *Observou-se que o RDW foi capaz de diferenciar as talassemias menores da anemia ferropriva, indicando que este parâmetro do hemograma é uma ferramenta útil, desde que excluídos distúrbios concomitantes. Além disso, foi verificada uma elevada frequência da mutação -$\alpha^{3.7}$ na população estudada (20,3%), o que justifica a sua pesquisa na ausência de outra anemia microcítica.*

Unitermos: *anemia ferropriva; talassemia menor; índice de anisocitose eritrocitária.*

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