Laboratory assessment of iron status and reticulocyte parameters in differential diagnosis of iron deficiency anemia and heterozygous β-thalassemia

Recebido em 14/11/01
Aceito para publicação em 05/03/02

Avaliação laboratorial do estado do ferro e parâmetros reticulocitários no diagnóstico diferencial da anemia ferropriva e β-talassemia heterozigótica

Gisélia A.F.M. de Lima[1]
Helena Z.W. Grotto[2]

key words

Soluble transferrin receptor

Iron metabolism

Microcytosis

Reticulocyte fractions

abstract

Introduction: The soluble form of transferrin receptor (sTfR) has been pointed as a useful parameter to assess the iron status and erythropoiesis activity. Immature reticulocytes present high concentration of membrane transferrin receptor. We tested the correlation between sTfR and reticulocyte parameters in iron deficiency anemia (IDA) and heterozygous β-thalassemia (hetero β-thal) patients. Laboratory parameters related to iron status and reticulocytes were studied in order to establish their clinical value to distinguish both anemias. Material and Methods: Reticulocyte measurements were obtained using a semi-automated analyzer and serum concentration of sTfR was determined by an immunoenzymatic technique. Forty-nine IDA and 43 hetero β-thal patients were studied. Results: Reticulocyte count and sTfR values were significantly higher in IDA than in hetero β-thal group, but the best parameter to distinguish both anemias was sTfR index, obtained by the ratio sTfR/ferritin level. Transport compartment was better evaluated by transferrin dosage than by transferrin iron binding capacity (TIBC) determination. The association of serum iron with transferrin measurements (transferrin index) improved the accuracy of the transferrin test. Discussion: The correlation between highly immature reticulocytes and sTfR level was observed only in IDA group, suggesting that cellular iron deprivation is the main responsible factor for up regulation of the sTfR synthesis in immature red blood cells. High sTfR values in hetero β-thal patients reflect a degree of ineffective erythropoiesis in this hemoglobinopathy. Conclusion: We concluded that sTfR, ferritin and transferrin measurements are useful and precise parameters to discriminate IDA from hetero β-thal patients.

unitermos

Receptor solúvel da transferrina

Metabolismo do ferro

Microcitose

Imaturidade de reticulócitos

resumo

Introdução: A forma solúvel do receptor da transferrina (sTfR) tem sido indicada como um parâmetro útil na avaliação do estado do ferro e da atividade eritropoiética. Reticulócitos imaturos apresentam alta concentração dos receptores de transferrina na sua membrana. Estudamos a correlação entre sTfR e parâmetros reticulocitários em pacientes com anemia ferropriva (AF) e com β-talassemia heterozigótica (β-tal hetero). Os parâmetros laboratoriais relacionados ao estado do ferro e reticulócitos foram estudados a fim de se estabelecer a utilidade clínica dos mesmos na distinção entre os dois tipos de anemia. Material e métodos: As medidas reticulocitárias foram obtidas usando-se um analisador hematológico semi-automático, e as concentrações de sTfR foram determinadas por técnica imunoenzimática. Foram estudados 49 pacientes com AF e 43 com β-tal hetero. Resultados: As contagens de reticulócitos e os valores de sTfR foram significativamente mais elevados na AF do que na β-tal hetero, mas o melhor parâmetro para diferenciar as duas anemias foi o índice de sTfR, obtido pela razão sTfR/ferritina. O compartimento de transporte foi mais bem avaliado pela dosagem de transferrina do que pela capacidade de ligação do ferro à transferrina (TIBC). A associação do ferro sérico à medida de transferrina (índice de transferrina) melhorou a acurácia do teste de transferrina. Discussão: A correlação entre reticulócitos imaturos e nível de sTfR foi observada apenas no grupo com AF, sugerindo que a falta de ferro intracelular seja o principal fator responsável pelo estímulo à síntese de sTfR nas células sangüíneas imaturas. Os valores elevados de sTfR nos pacientes com β-tal hetero refletem um certo grau de eritropoiese ineficaz nessa hemoglobinopatia. Conclusão: Concluímos que as medidas de sTfR, ferritina e transferrina são parâmetros úteis e precisos para diferenciar AF de β-tal hetero.

1. Post-graduate student.
2. Ph.D. Department of Clinical Pathology, School of Medical Sciences, State University of Campinas (Unicamp), Campinas, São Paulo, Brazil.
This work was supported by "Fundo de Apoio ao Ensino e à Pesquisa", from Unicamp (Faep 1123/97).

Introduction

New and reliable laboratory measurements of iron status have been used for detecting and assessing different stages of iron deficiency. Furthermore, there is a consensus that iron status should be interpreted using a group of parameters and not only one single test. It is also pertinent to consider costs and time spent to achieve a correct diagnosis.

Some authors have suggested the use of reticulocyte parameters to distinguish iron deficiency anemia (IDA) from heterozygous β-thalassemia (hetero β-thal) (26), both presenting hypochromia and microcytosis of erythrocytes and both with a high incidence in Brazil (21). The clinical value of the reticulocyte quantitation was renewed by the automation of the reticulocyte count together with the possibility of determining the immaturity of reticulocyte population as erythropoiesis activity analysis (8). Yoldi *et al.* (26) evaluated samples from hetero β-thal and IDA using flux cytometry, and established that a value < 2% for highly fluorescent reticulocytes (HFR) would be used to discriminate between these pathologies. Membrane reticulocyte presents transferrin receptor and the receptor concentration is higher in more immature cells. The soluble form of transferrin receptor (sTfR) was first described by Kohgo *et al.* (15) and corresponds to a truncated form lacking the cytoplasmic and transmembrane domains of the intact receptor (6). The sTfR determination has been pointed as a useful parameter to assess the iron status and erythropoiesis activity (1).

The objective of this study was to test laboratory parameters related to reticulocytes, iron metabolism, including sTfR determination, in order to determine their use to distinguish hetero β-thal from IDA.

Material and methods

Subjects

A group of adult patients (n = 92) with hypochromic and microcytic anemia was studied. IDA (n = 49) was defined as serum ferritin (SF) level below 30ng/ml for men and 12ng/ml for women (minimum normal ferritin levels in our laboratory). Patients with SF levels higher than the above mentioned and Hb A2 level higher than 3.4 % were considered as hetero β-thal (n = 43). Fifty-seven nonanemic subjects were used as controls.

Methods

Hematological measurements including reticulocyte parameters (absolute reticulocyte count and high fluorescent reticulocytes – HFR) were obtained using a Cell Dyn 3500 analyzer (Abbott – USA). Reticulocyte was identified by a non-fluorescent method using New methylene blue as dye. Fractions of reticulocyte maturity were calculated depending on the absorption intensity and they were classified as: mature, midmature and highly immature reticulocytes fractions.

Hemoglobin A2 was quantified spectrophotometrically after cellulose acetate electrophoresis (16).

Determination of iron status: determination of serum iron (SI) and the transferrin iron binding capacity (TIBC) were done with a Mira Plus Cobas analyzer (Roche – Switzerland) using Unimate 5 Iron and Unimate 7 UIBC kits (Roche Diagnostic Systems – Switzerland). SF was determined by a chemiluminescence system (kit Immulite – Diagnostic Products Co. – USA)

Transferrin concentration (TFR) was quantified by nephelometric method (Beckman – Ireland). The serum concentration of sTfR was measured by an immunoenzymatic technique (Quantikine – R&D Systems – USA).

Statistical analysis: In order to compare the variables between groups, the Kruskal-Wallis test was employed. The Spearman correlation coefficient test was used for assessing the association between variables, with level of significance set at < 0.05. The capacity of the tests to discriminate between the groups was studied by means of ROC curves that defined the optimal decision limit of each parameter and the accuracy of each method. The protocol of this study was approved by the Committee of Ethics of the School of Medical Sciences, University of Campinas, São Paulo, Brazil.

Results

The distribution of laboratory values among the patients and control groups are shown in **Table 1**. Two indicators, TI and sTfRI, were included, obtained by the ratio SI/TRF and by the ratio sTfR/SF, respectively (2, 20). Comparing the variables between groups, a significant difference was observed between normal and hetero β-thal, normal and IDA, and hetero β-thal and IDA in reticulocyte indices and sTfR concentrations (p = 0.0001). TFR, TI and sTfRI were significantly different between IDA

Table 1 Hematological and iron parameters in different groups of patients and control. Values are means ± SD and range

Parameters	Groups		
	Control (*n* = 57)	Hetero β-thal (*n* = 43)	IDA (*n* = 49)
Rtc* (x 10^6/l)	67.9 ± 17.6 (28.8-134.0)	160.19 ± 137.8 (64-808)	93.7 ± 52.43 (31.0-305.0)
HFR (%)	6.2 ± 2.9 (2.9-18.9)	7.33 ± 3.74 (6.6-23.6)	6.96 ± 3.55 (1.9-21.2)
SI (µg/dl)	96.7 ± 29.3 (42.0-149.0)	86.8 ± 29.7 (24.0-163.0)	26.6 ± 17.45 (2.0-76.0)
TIBC (µg/dl)	352.4 ± 58.5 (245.0-467.0)	402.9 ± 99.9 (264.0-689.0)	409.1 ± 105.8 (210.0-630.0)
TS (%)	28.0 ± 11.0 (12.0-60.0)	24.0 ± 12.0 (10.0-60.0)	8.0 ± 9.0 (1.0-6.0)
SF (ng/ml)	103.8 ± 72.4 (17.7-334.0)	142.24 ± 156.2 (24.0-682.0)	7.4 ± 4.75 (1.5-23.5)
TRF* (mg/dl)	249.8 ± 28.7 (202.0-317.0)	231.1 ± 55.7 (125.0-366.0)	337.5 ± 61.3 (111.0-427.0)
TI*	0.41 ± 0.11 (0.20-0.65)	0.39 ± 0.15 (0.18-0.76)	0.08 ± 0.05 (0.01-0.26)
sTfR* (nmol/l)	16.88 ± 3.1 (12.5-27)	27.1 ± 15.82 (15.0-91.0)	50.26 ± 21.4 (23.5-108.5)
sTfRI*	27.7 ± 23.60 (5.2-99.4)	46.51 ± 48.92 (3.0-279)	1287.5 ± 1329.2 (119.0-5812.0)

Rtc: reticulocyte count; HFR: high immature reticulocyte fraction; SI: serum iron; TIBC: transferrin iron binding capacity; TS: transferrin saturation; SF: serum ferritin; TRF: serum transferrin; TI: transferrin index = SI/TRF; sTfR: soluble transferrin receptor; sTfRI: sTfR index = sTfR/SF. *$p < 0.05$.

and hetero β-thal and also between IDA and control groups ($p = 0.0001$).

The correlation between HFR and sTfR was modest ($r = 0.349$), but significant ($p = 0.025$) only in IDA group. Transferrin saturation (TS) and TI indices measure iron transport compartment and the correlation coefficients were positive in all groups (**Table 2**). However, TI was more accurate than TS to distinguish IDA from hetero β-thal, with 100% of specificity for TI (**Table 3** and **Figure 1**). Two of 41 IDA patients showed TI > 0.17: one of them showed TRF concentration below normal limit and the other one presented a normal SI concentration. The latter one was iron depleted (low ferritin level), although SI concentration was normal. Comparing TIBC and TRF accuracies, we could observe that TRF had a better performance than TIBC to recognize IDA patients (Table 3). Four IDA patients showed TRF values lower than 278.0mg/dl.

Table 2 Correlation between variables in patients with hetero β-thal, IDA and controls

Variables	Groups		
	Control	Hetero β-thal	IDA
HFR x sTfR	$r = 0.049$	$r = 0.007$	$r = 0.349$
	$p = 0.795$	$p = 0.963$	**$p = 0.025$**
sTfRI x SI	$r = -0.081$	$r = 0.087$	$r = -0.140$
	$p = 0.670$	$p = 0.611$	$p = 0.381$
TS x TI	$r = 0.904$	$r = 0.628$	$r = 0.675$
	$p = 0.0001$	**$p = 0.0001$**	**$p = 0.0001$**
TIBC x TRF	$r = 0.688$	$r = 0.169$	$r = 0.517$
	$p = 0.0001$	$p = 0.322$	**$p = 0.0005$**
sTfR x SF	$r = 0.178$	$r = 0.0117$	$r = -0.384$
	$p = 0.345$	$p = 0.945$	**$p = 0.013$**
sTfR x Rtc	$r = 0.262$	$r = 0.2883$	$r = 0.170$
	$p = 0.1615$	$p = 0.0881$	$p = 0.286$
sTfR x Hb	$r = 0.121$	$r = -0.4249$	$r = -0.1117$
	$p = 0.523$	**$p = 0.0098$**	$p = 0.4867$

Hb: hemoglobin (g/dl).

Table 3 **Accuracy, sensitivity, specificity and cutoff values to distinguish IDA from hetero β-thal (ROC curve results)**

Parameter	Accuracy (%)	Sensitivity (%)	Specificity (%)	Decision limit
Rtc	76.2	68.0	74.4	≤ 93 x 10^6/l
HFR	54.1	82.0	30.2	≤ 8.3%
TIBC	53.6	60.0	62.8	≥ 401µg/dl
TS	94.2	84.0	95.3	≤ 10%
TI	99.2	95.1	100	≤ 0.17
TRF	90.1	87.8	88.9	≥ 278mg/dl
sTfR	87.6	100	69.4	≥ 23nmol/l
sTfRI	99.7	97.6	97.2	≥ 140

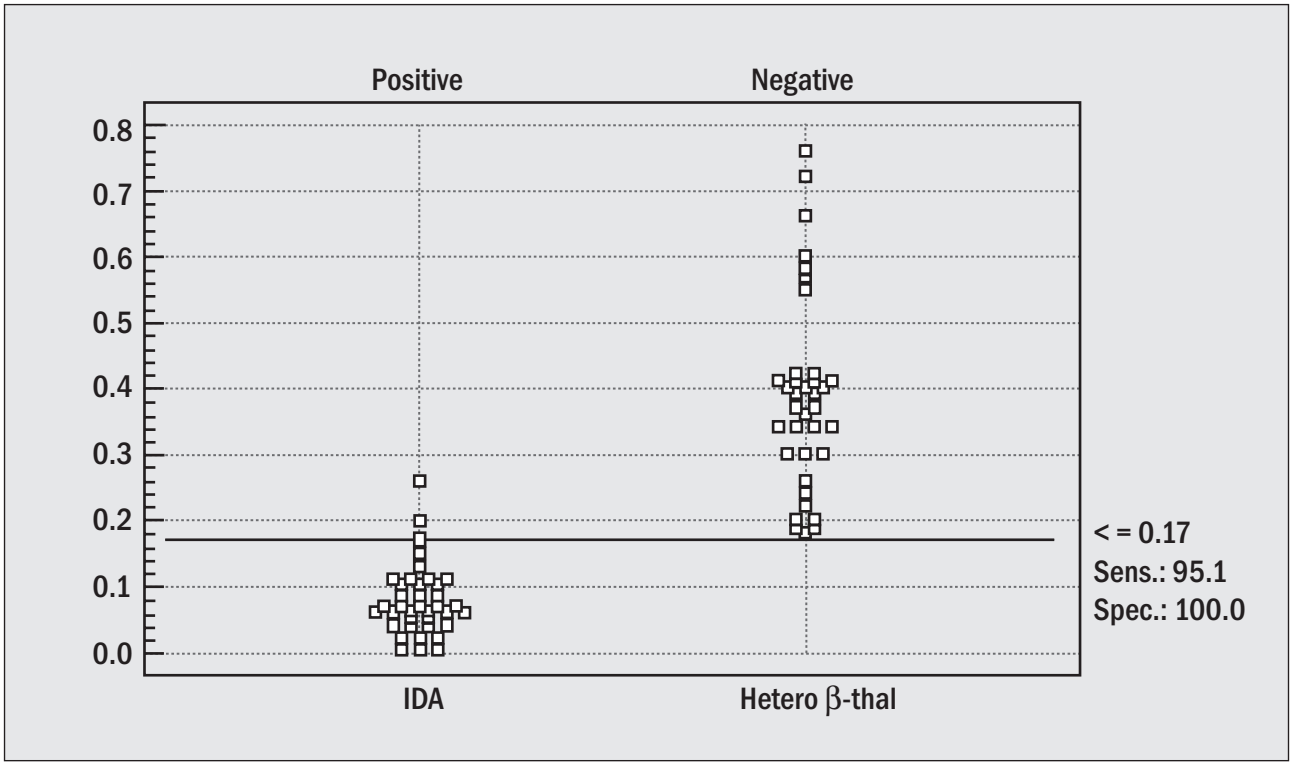

Figure 1 – *Transferrin index (SI/TRF) in IDA and hetero β-thal. The discrimination limit is set at 0.17*

Three of them could be identified as IDA by TI value. The other one presented TRF concentration below normal limit and sTfRI compatible with IDA. We did not investigate if the patient had some associated clinical condition that could explain a decreasing synthesis or loss of TRF. Ten of the 43 hetero β-thal patients presented TIBC values higher than the superior normal limit. One of them showed results compatible with iron deficiency, although ferritin level was normal (SF = 27 mg/l). Transferrin saturation, sTfR and sTfRI values (10%, 72nmol/l and 270, respectively) indicated an iron depletion, but TRF concentration, contrary to expected, showed normal values (212mg/dl). The second patient was firstly classified as hetero β-thal, according to hematological values, HbA_2 and ferritin determinations. However, the film examination revealed nucleated red blood cells, punctuate basophilic, poikilocytes and cell fragments. In fact, clinical features suggest that this individual is β-thalassemic intermedia patient. Medical records reported a splenectomy when the patient was 7 years old and repetitive transfusion proceedings from 7 to 22 years old, due to anemia. Nowadays the patient presents an associated chronic liver disease. The high TIBC value (476mg/dl), low transferrin

saturation and serum iron determinations suggest an associated iron deficiency anemia, a diagnosis which has been reinforced by an elevated sTfR value (41nmol/l). The other eight patients did not present additional laboratorial results that could explain high TIBC values. sTfR measurement showed an accuracy of 0.876 (**Figure 2**) and this result was improved by the ratio sTfRI (accuracy = 0.997) (**Figure 3**). Only one IDA and one hetero β-thal showed values out of limits, providing 97.6% sensitivity and 97.2% specificity for sTfRI parameter.

Discussion

Transferrin receptors are membrane glycoproteins responsible for binding transferrin during the endocytosis of iron. The density of surface transferrin receptor is proportional to the iron requirement of the cell. Then, the transferrin is abundant in cells of organs with the highest iron requirements, such as the erythron marrow and the placenta. After internalization by endocytosis, the endosome containing the transferrin-receptor complex becomes acidified and iron loses its affinity for transferrin. Receptor and transferrin return to the cell surface, where the transferrin is released while the iron is transported to the cytosol (7). The initial study about circulating transferrin receptor was reported by Kohgo *et al.* (15). They demonstrated that the concentration of this protein was significantly elevated in enhanced erythropoiesis and iron deficiency. The clinical use of the sTfR has been reported in several situations, especially to distinguishing iron deficiency from the hypoproliferative anemia that is associated with chronic diseases (10). The serum receptor increases with the severity of the iron deficiency anemia, but not in chronic diseases. In addition, sTfR provides an assessing of erythropoiesis status, because an increase in the sTfR concentration is proportional to the expansion of the erythroid marrow (13). In hemolytic anemias with efficient erythropoiesis there is a close parallel rise in sTfR and absolute reticulocyte count (7). On the other hand, in disorders in which there is an ineffective erythropoiesis, such as myelodysplasia, an increase in sTfR is observed, whereas discret reticulocyte count occurs (3). Patients with hetero β-thal have increased erythroid marrow activity, although they present various degrees of an ineffective erythropoiesis (4).

The association of the sTfR and the serum ferritin by the sTfR/ferritin index has been indicated as a helpful parameter to identify patients with iron deficiency and to distinguish those with iron deficiency from hetero β-thal (5). Gimferrer *et al.* (11) studied 35 hetero β-thal and 10

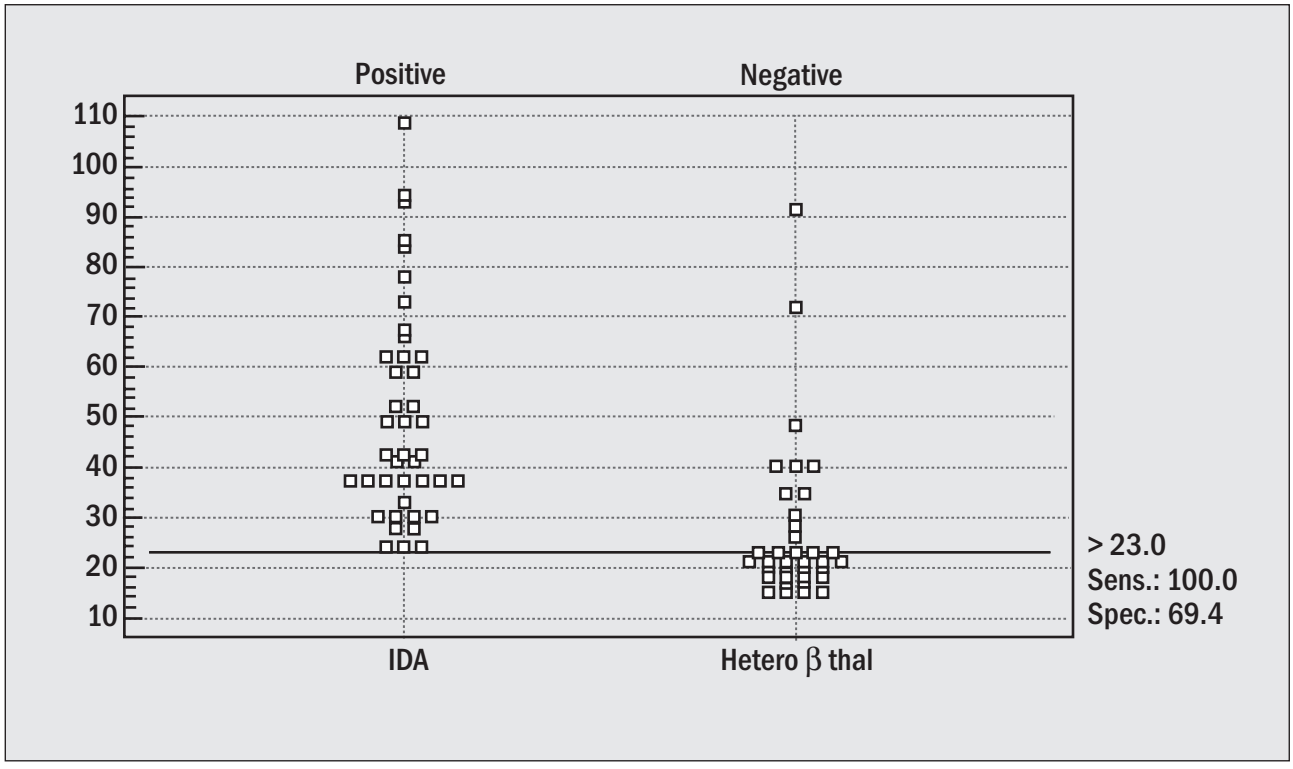

Figure 2 – *sTfR in IDA and hetero β-thal. The discrimination limit is set at 23.0nmol/l*

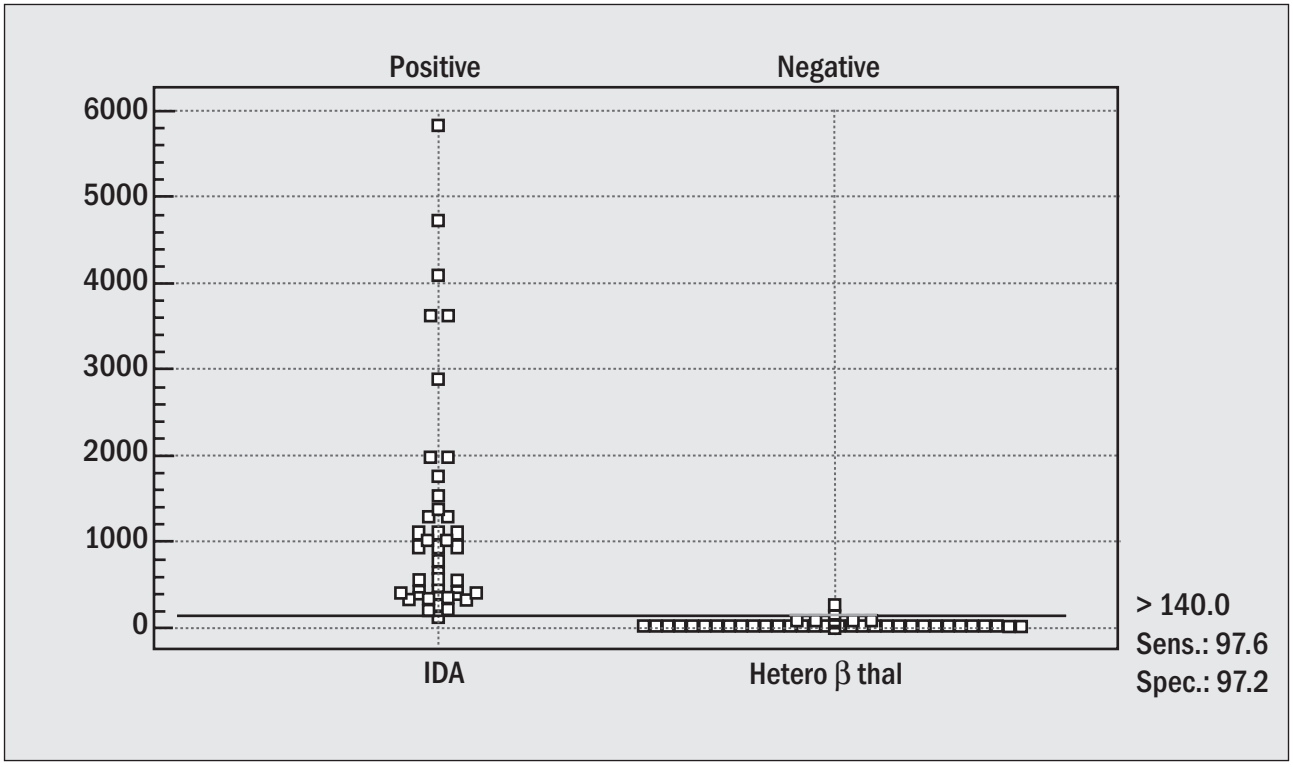

Figure 3 – *sTFRI index in IDA and hetero β-thal. The discrimination limit is set at 140.0*

hetero β-thal associated to IDA and observed that sTfR was significantly higher in hetero β-thal than in normal controls, but significantly lower than in IDA. sTfR was not useful in diagnosing associated IDA in hetero β-thal patients.

In our study we tested parameters related to sTfR, transferrin and reticulocyte counts in patients with IDA and hetero β-thal. Reticulocytes are good indicators of erythropoiesis activity. Automated methods using flow cytometry and RNA dyes resurrected the confidence in reticulocyte counts and introduced new approaches concerning bone marrow response by the quantification of reticulocyte maturity (19). Thus, immature reticulocyte fraction has been reported as an early predictor of the regenerative activity of bone marrow after bone marrow transplantation (8, 12).

Yoldi *et al.* (26) described high fluorescent reticulocytes as a good discriminant between hetero β-thal and IDA, since the value in IDA is higher than in hetero β-thal. Such difference has not been found in our study. An explanation for that fact could be that the used methodology has not included fluorescence to identify reticulocyte maturity. However, we carried out a similar study using flow cytometry and thiazole orange as dye and the results did not change (HFR mean = 5.11 ± 4.47 to hetero β-thal and HFR mean= 4.39 ± 4.55 to IDA, $p > 0.05$ – data not published). It has been reported that reticulocytes in iron deficient conditions may contain increased amounts of transferrin receptor (18). Then, a positive correlation between sTfR and HFR was expected. We observed a modest but significant correlation between these parameters in IDA, but not in hetero β-thal. Iron deficiency causes an increase in the rate of transferrin receptor production, due to both increased synthesis and increased stability of transferrin receptor mRNA (17). Iron deficient rats showed receptor density 74% over the normal rats in function of an increasing in erythroid cells population and an increase in transferrin receptor on the individual cell (14). We tested the diagnostic use of sTfR for IDA and we could determine a value where the test presents sensitivity of 100% and specificity of 69.4% in distinguishing IDA from hetero β-thal. The specificity increased to 97.2% when sTfR value was combined with ferritin value, confirming the results reported by Cermak (5). sTfR and sTfRI detected IDA patients that could not be identified by other laboratory parameters as TIBC, TS or TFR rates, demonstrating the value of the sTfR measurement for identifying functional iron deficiency. We observed a negative correlation between sTfR and ferritin levels. Skikne *et al.* (20) determined the sTfR changes during the

evolution of iron deficiency in a phlebotomy program. They demonstrated that there was a progressive elevation in sTfR when serum ferritin fell below the normal range. Before this stage there was no significant change in sTfR levels. All of our patients had serum ferritin below the normal range and due to this fact we could observe an inverse correlation between sTfR and ferritin. In the phlebotomy study the mean sTfR/ferritin ratio was 100 at baseline stage and a ratio of 500 identified the point at which iron stores were fully exhausted. Our minimum sTfR/ferritin ratio value was 110.0. Up to 140.0 hetero β-thal patients could be identified.

Plasma iron transport is made by transferrin, a monomeric glycoprotein that binds two atoms of Fe^{3+} with high affinity (23). The TIBC is a measurement of transferrin concentration and was described by Ramsay forty years ago. Despite TIBC being indispensable for determining iron status, technical problems found by Bill Ramsay have not been solved until now (9, 24). An alternative approach would be measuring transferrin directly by immunological assay. In our study, the nephelometry was used to measure the concentration of transferrin, which showed to be a more accurate method than TIBC. It also has the advantages of requiring a small sample and being nonsusceptible to iron contamination (22). We could notice the apparent limitation of TIBC estimation when some of hetero β-thal patients showed high TIBC values, although transferrin determination was normal. Probably a spuriously high value was due to a non-transferrin iron measurement (25).

We conclude that the interpretation of iron status is based on a group of laboratory findings and, according to our results, the association of sTfR and ferritin measurements is more precise to distinguish hetero β-thal from IDA. However, in a screening survey where it is pertinent to consider costs, transferrin evaluation may be adopted, since it showed a good performance to discriminate both microcytic hypochromic anemias.

Acknowledgments

We thank the Statistics Committee, FCM, Unicamp, especially Dr. Helymar C. Machado and Dr. Cleide A.M. Silva, for the statistical analyses.

References

1. Beguin, Y. The soluble transferrin receptor: biological aspects and clinical usefulness as quantitative measure of erythropoiesis. *Haematologica*, *77*: 1-10, 1992.
2. Beilby, J. *et al*. Transferrin index: an alternative method for calculating the saturation of transferrin. *Clin. Chem*., *38*: 2078-81, 1992
3. Bowen, D.T. *et al*. Estimation of effective and total erythropoiesis in myelodysplasia using serum transferrin receptor and erythropoietic concentrations, with automated reticulocyte parameters. *Leukemia, 8*: 151-5, 1994.
4. Centis, F. *et al*. Soluble transferrin receptor following bone marrow transplantation from donors heterozygous for beta thalassemia. *Haematologica*, *79*: 448-51, 1994.
5. Cermak, J. & Brabec, V. Transferrin receptor-ferritin index: a useful parameter in differential diagnosis of iron deficiency and hyperplastic erythropoiesis. *Eur. J. Haematol*., *61*: 210-12, 1998.
6. Cook, J.D.; Skikne, B.S. & Baynes, R.D. Serum transferrin receptor. *Ann. Rev. Med*., *44*: 63-74, 1993.
7. Cook, J.D. The measurement of serum transferrin receptor. *Am. J. Med. Sci., 318*(4): 269-76, 1999.
8. Corberand, J.X. Reticulocyte analysis using flow citometry. *Hematol. Cell. Ther., 38*: 487-94, 1996.
9. Fairbanks, V.F. Laboratory testing for iron status. *Hosp. Pract. off Ed*., *26*(3): 17-24, 1991.
10. Ferguson, B.J. *et al*. Serum transferrin receptor distinguishes the anemia of chronic disease from iron deficiency anemia. *J. Lab. Clin. Med*., *119*: 385-90, 1992.
11. Gimferrer, E.; Ubeda, J. & Remacha, A.F. Serum transferrin receptor levels are "physiologically" high in heterozygous β-thalassemia. *Haematologica*, *82*: 728-34, 1997.
12. Grotto, H.Z.W. *et al*. Immature reticulocyte fraction as a criterion for marrow engraftment. Evaluation of a semi-automated reticulocyte counting method. *Clin. Lab. Haem., 21*: 285-87, 1999.
13. Huebers, H.A. *et al*. Intact transferrin receptors in human plasma and their relation to erythropoiesis. *Blood, 75*: 102-7, 1990.
14. Intragumtornchai, T. *et al. In vivo* transferrin-iron receptor relationships in erythron of rats. *Am. J. Physiol., 255*: R236-31, 1988.
15. Kohgo, Y. *et al.* Circulating transferrin receptor in human serum. *Brit. J. Haematol., 64*: 277-281, 1986.
16. Marengo-Rowe, A.J. Rapid electrophoresis and quantitation of haemoglobin on cellulose acetate. *J. Clin. Pathol., 18*: 790-92, 1965.
17. Mullner, E.W. & Kuhn, L.C. A stem-loop in 3' untranslated region mediates iron-dependent regulation of transferrin receptor mRNA stability in cytoplasma. *Cell, 53*: 815-25, 1988.

18. Rouault, T. *et al*. Hemin, chelatable iron and the regulation of transferrin receptor biosynthesis. *J. Biol. Chem., 260*: 14862-66, 1985.

19. Rowan, R.M.; Cavill, I. & Corberand, J.X. The reticulocyte count: progress toward the resurrection of a useful clinical test. *Clin. Lab. Haem., 18*: 3-8, 1996.

20. Skikne, B.S.; Flowers, C.H. & Cook, J.D. Serum transferrin receptor: a quantitative measure of tissue iron deficiency. *Blood, 75*: 1870-76, 1990.

21. Sonati, M.F. *et al*. Hereditary hemoglobinopaties in a population from southest Brazil. *Hemoglobin, 20*(2): 175-79, 1996.

22. Woo, J. & Henry, J.B. Metabolic intermediates and inorganic ions. *In*: Henry, J.B. (ed.). *Clinical diagnosis methods*. Philadelphia: WB. Saunders Company, 1996. p. 190-91.

23. Workwood, M. Influence of disease on iron status. *Proc. Nutr. Soc., 56*: 409-19, 1997.

24. Workwood, M. The laboratory assessment of iron status: an update. *Clin. Chim. Acta, 259*: 3-23, 1997.

25. Workwood, M. Iron deficiency anaemia. *In:* Dacie, J.V. & Lewis, S.M. (ed.). *Practical Haematology*. London: Churchill Livingstone, 1995: 437-44.

26. Yoldi, F. *et al*. Automatización del recuento de reticulócitos. *Sangre*, *33*: 219-22, 1991.

Correspondence to

Helena Z. W. Grotto
Departamento de Patologia Clínica
Faculdade de Ciências Médicas – Unicamp
CEP 13083-970 – Campinas-SP–Brasil
Caixa Postal 6111
Fax: (55) (19) 3289-3273
Phone: (55) (19) 788-7064
e-mail: grotto@fcm.unicamp.br