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Reticulocyte parameters for monitoring anemia in patients with chronic kidney disease on peritoneal dialysis

Parâmetros reticulocitários para monitoramento de anemia em pacientes com doença renal crônica em diálise peritoneal

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ABSTRACT

Introduction: Anemia is a complication with impact on morbidity and mortality in chronic kidney disease (CKD) patients. Current markers for the diagnosis and monitoring of anemia in CKD are limited by the interrelation between erythropoiesis, iron stores, inflammation, and the resistance to treatment with erythropoiesis stimulant agents (ESA). **Objective:** The aim of this study was to analyze the role of immature reticulocyte fraction (IRF) and hemoglobin concentration in reticulocytes (RET-He) by the hematology analyser Sysmex XN-5000 in the monitoring of CKD anemia in peritoneal dialysis patients. **Methods:** This was a prospective, observational multicenter study which compared IRF and RET-He with parameters recommended by the guidelines. Inflammatory biomarkers were analyzed by the Luminex® Multiplexing Instruments system. Thirty-five patients (59 ± 13 years old; 51% men) were included in the analysis. **Results:** Hemoglobin was 12.2 ± 2 g/dl; 87% had resistance to ESA. Patients with erythropoietin resistance index (ERI) in the upper quartile presented a significantly higher of IRF and a lower percentage of iron deficiency (12%) compared to ferritin (82%) and transferrin saturation index (STI) (51%). Interleukin-6 (IL-6) levels correlated with the percentage of medium fluorescence reticulocyte (MFR) ($r = 0.45, p < 0.03$). Hemoglobin values after 60 and 180 days were consistently higher in the group of patients with a IRF% lower than 10.5. **Conclusion:** IRF and RET-He may add value in the iron deficiency investigation, as well as in the identification of patients with ERI. Due to the restricted number of patients analyzed in this study, future studies should be encouraged in larger populations and with prospective follow-up, to validate our findings.

Key words: anemia; reticulocytes; peritoneal dialysis; kidney failure chronic.

RESUMO

Introdução: *Anemia é uma complicação com impacto na morbidade e na mortalidade de pacientes com doença renal crônica (DRC). Os biomarcadores utilizados no diagnóstico e no monitoramento de anemia na DRC são limitados devido à inter-relação entre eritropoiese, estoque de ferro, inflamação e resistência à terapêutica com agentes estimuladores da eritropoiese (AEE).* ***Objetivo:*** *O objetivo deste estudo foi analisar o papel dos marcadores fração de reticulócitos imaturos (IRF) e concentração de hemoglobina nos reticulócitos (RET-He) do analisador hematológico Sysmex XN 5000 no monitoramento da anemia em pacientes em diálise peritoneal.* ***Métodos:*** *Estudo prospectivo, observacional e multicêntrico que comparou IRF e RET-He com parâmetros laboratoriais recomendados pelos guidelines. Biomarcadores inflamatórios foram analisados pelo sistema Luminex® Multiplexing Instruments. Este estudo incluiu 35 pacientes (59 ± 13 anos; 51% homens).* ***Resultados:*** *Os valores de hemoglobina foram 12,2 ± 2 g/dl; 87% apresentaram resistência a AEE. Pacientes com índice de resistência à eritropoietina (IRE) no quartil superior apresentaram valores significativamente maiores de IRF e menor porcentagem de deficiência de ferro (12%) em comparação com pacientes*

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*com ferritina (82%) e índice de saturação de transferrina (IST) (51%). Os níveis de interleucina 6 (IL-6) correlacionaram-se com a porcentagem de reticulócitos de fluorescência média (MFR) (*r = *0,45,* p < *0,03). Valores de hemoglobina após 60 e 180 dias foram consistentemente mais altos no grupo de pacientes com IRF% menor que 10,5.* **Conclusão:** *IRF e RET-He podem agregar valor na investigação da deficiência de ferro, bem como na identificação do índice de existência à eritropoietina (ERI). Devido ao número restrito de pacientes analisados neste trabalho, estudos futuros devem ser estimulados em populações maiores e com acompanhamento prospectivo, para validação dos nossos achados.*

Unitermos: *anemia; reticulócitos; diálise peritoneal; insuficiência renal crônica.*

RESUMEN

Introducción: *La anemia es una complicación con impacto en la morbimortalidad en pacientes con enfermedad renal crónica (ERC). Los biomarcadores usados para el diagnóstico y seguimiento de la anemia en la ERC están limitados por la interrelación entre eritropoyesis, depósitos de hierro, inflamación y resistencia al tratamiento con agentes estimulantes de la eritropoyesis (AEE).* ***Objetivo:*** *El objetivo de este estudio fue analizar el papel de la fracción de reticulocitos inmaduros (IRF) y el equivalente de hemoglobina en reticulocitos (RET-He) mediante el analizador hematológico Sysmex XN-5000 en el seguimiento de la anemia por ERC en pacientes en diálisis peritoneal.* ***Métodos:*** *Estudio prospectivo, observacional multicéntrico que comparó IRF y RET-He con los parámetros recomendados por las guías. Los biomarcadores inflamatorios fueron analizados por el sistema Luminex® Multiplexing Instruments. Este estudio incluyó a 35 pacientes (59 ± 13 años; 51% hombres).* ***Resultados:*** *La hemoglobina fue de 12,2 ± 2 g/dl; el 87% tenía resistencia a AEE. Los pacientes con índice de resistencia a la eritropoyetina (IRE) en el cuartil superior tenían un IRF significativamente más alto y un porcentaje más bajo de deficiencia de hierro (12%) en comparación con la ferritina (82%) y las ITS (51%). Los niveles de interleucina-6 (IL-6) se correlacionaron con el porcentaje de reticulocitos de fluorescencia media (MFR) (*r = *0,45,* p < *0,03). Los valores de hemoglobina después de 60 y 180 días, fueron consistentemente más altos en el grupo de pacientes con IRF% inferior a 10,5.* ***Conclusión:*** *IRF y RET-He pueden agregar valor en la investigación de ferropenia, así como en la identificación de pacientes con ERI. Debido al número limitado de pacientes analizados en este estudio, se deben impulsar estudios futuros en poblaciones más grandes y con seguimiento prospectivo, para validar nuestros hallazgos.*

Palabras clave: *anemia; reticulocitos; diálisis peritoneal; insuficiencia renal crónica.*

INTRODUCTION

Anemia in chronic kidney disease (CKD) is multi-factorial, but mainly driven by erythropoietin (EPO) production deficiency and depletion of iron reserves[1-3]. Inflammation is also an important determinant in a sub-group of patients, as it interferes directly with iron mobilization and determines EPO resistance[1, 4]. Inflammation in CKD increases the concentration of inflammatory cytokines, interleukin-6 (IL-6) being the principal cytokine responsible for the activation of hepcidin expression[5-8]. Iron stores can be monitored by ferritin and serum levels by iron transferrin saturation (STI), as well as by hemoblobin[9]. Thus, in inflammatory situation, high levels of hepcidin directly and negatively interfere with the availability of iron in erythropoiesis, and may cause anemia, mainly due to iron functional deficiency. Indeed, relative and absolute reticulocyte count is the principal marker the evaluation of the erythropoietic activity of the bone marrow[10]. Although all of these markers are used in clinical practice, they are far from ideal real-time indicators.

Different parameters have been described in the literature, but not much yet evaluated in the CKD population, especially in patients undergoing peritoneal dialysis (PD). Equivalent of hemoglobin concentrations in reticulocytes (RET-He) represents the degree of hemoglobinization that has occurred in the bone marrow which is dependent on iron availability[11, 12]. Thus potentially allows an early evaluation, for clinical diagnosis, monitoring and define intervention, particularly in patients with CKD using erythropoiesis stimulating agents (ESA) and iron supplementation[13]. Additionally, immature reticulocytes fraction (IRF) is an excellent marker of nearly real-time erythropoietic

activity since it represents the proportion of younger reticulocytes in the peripheral blood and rises much earlier than the total number of reticulocytes[3, 11, 14]. These parameters may be useful in the monitoring and management of patients with renal anemia, particularly those on PD.

Due to their conceptual characteristics and because they do not suffer direct analytical interference from inflammation, these reticulocytes markers may identify earlier and better the general iron status and erythropoietic activity of the bone marrow in patients with CKD on PD when compared to conventional markers such as ferritin, transferrin saturation index and reticulocytes count.

OBJECTIVE

Analyze the role of the hematological parameters IRF and RET-He in the monitoring and identification of ESA resistance of CKD anemia in peritoneal dialysis patients.

METHODS

This was a prospective, multi-centric study, including patients with stage 5 CKD on continuous ambulatory peritoneal dialysis (CAPD/APD) for at least 30 days, over 18 years of age, with or without therapeutic use of ESA and iron supplements, and who signed the informed consent (project with approval at Comitê de Ética em Pesquisa envolvendo Seres Humanos (CEP)-Universidade Estadual de Londrina (UEL) under number: 357/2011). The venous blood samples for the laboratory tests and for blood counts were obtained in tubes with ethylenediamine tetraacetic acid (EDTA)-K_2 and in dry tubes with no anticoagulant, using separator gel and clot activator for measuring inflammatory biomarkers.

Complete blood count (CBC) were processed on the same day of collection for up to a maximum of 4 hours in a hematological analyzer (Sysmex, model XE-5000) to obtain hemoglobin parameters, RET-He, relative reticulocyte count (RET%), immature reticulocytes fraction (IRF%), and their fractions [low fluorescence reticulocyte fraction (LRF%), medium fluorescence reticulocyte (MFR%) and high fluorescence reticulocyte fraction (HFR%)]. Inflammatory biomarkers were measured using the Luminex® Multiplexing Instruments (Luminex® xMAP® Technology), in a single sample for all of the following markers: interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and monocyte chemoattractant protein-1 (MCP-1).

The demographic and clinical data of the participants were obtained from the medical records, using data from six months before and after the date of sample collection. To evaluate the association between two quantitative variables, the Pearson or Spearman correlation coefficients were estimated. Student's *t*-test or the non-parametric Mann-Whitney tests were used for independent samples *t* for comparison between two groups defined by the erythropoietin resistance index (ERI) that was calculated using the mean weekly dose of the last six months of EPO (micrograms) by their weight (kg) and hemoglobin (Hb) concentration (dose EPO/weight/Hb).

The normality of the variables was evaluated by the Shapiro Wilks test and *p*-values of 0.05 indicated statistical significance. The data were analyzed using the IBM SPSS Statistics v.20 program.

RESULTS

Population characteristics and clinical and laboratorial data

Thirty-five patients under peritoneal dialysis, at least 30 days previously, accepted to participate and were included in the study. Demographic, clinical and laboratory data are described on **Table 1**.

Analysis of iron deficiency using ferritin, TSI% and RET-He

Ferritin, TSI%, and RET-He parameters were used to evaluate the availability of iron, following the cutoff criteria proposed by KDIGO (2012)[15] of below 500 µg/l for ferritin, less than or equal to 30% for TSI and less than 29 pg for RET-He. The results are shown in **Table 2**.

Analysis da erythropoietin resistance

We calculated erythropoietin resistance index (ERI) for 23 individuals and 86.9% (20/23) presented EPO resistance (values greater than 0.02 µg according to KDIGO, 2012[15]). However, when the ERI values were classified by quartiles (the cut-off of the 3rd quartile being 0.102 µg) 30.5% (7/23) were above this cut-off. For those who were in the fourth quartile, there was a significant difference for RET-He ($p < 0.05$) when compared to other quartiles.

TABLE 1 – General characteristics of the studied population

Variable	Values
Age (years)	59.7 ± 13.4
Male gender	51.4%
Causes of CKD	
Diabetes mellitus	42.9%
Polycystic kidneys	8.6%
Nephrosclerosis	8.6%
Other causes	40%
Dialysis time (months)	32.8 ± 30.5
APD	100%
BMI	27.4 ± 4.9
ESA use	67.6%
ESA dose	6,223 ± 3,525 (UI/week)
	51.7 ± 29.3 (µg/week)
ERI (ESA/Weigth/Hb)	0.071 ± 0.039 (µg)
ERI > 0.02 µg	86.9%
Hemoglobin	12.2 ± 2 (g/dl)
Hemoglobin < 11 g/dl	32.4%
Ferritin	277.5 ± 272.7 (µg)
STI	32.5 ± 10.7 (%)

CKD: chronic kidney disease; APD: continuous ambulatory peritoneal dialysis; BMI: body mass index; ESA: erythropoiesis stimulant agents, ERI: erythropoietin resistance index; Hb: hemoglobin; STI: transferrin saturation index.

TABLE 2 – Deficiency of iron availability and stores, using the parameters ferritin, STI% and RET-He

Iron deficiency	*n*	%
Ferritin < 500 µg	28	82.1
STI ≤ 30%	29	51.7
RET-He < 29 pg	34	11.8

STI: transferrin saturation index; RET-He: reticulocyte hemoglobin equivalent.

Comparative analysis of erythropoietic activity by RET%, RET#, IRF%, RET-He and ERI

Erythropoietic activity of the bone marrow was evaluated by the reticulocyte parameters (RET%, RET#, IRF%, LFR%, MFR% and HFR% and RET-He), the results of which are shown on **Table 3**. For each of the reticulocyte variables, we tested the null and alternative hypotheses that the means would be the same or different in the groups, respectively, defined by the fourth quartile (≤ 0.102 or > 0.102). There was a significant difference for the IRF%, MFR% and LFR% ($p < 0.05$) (**Table 4**, **Figures 1** and **2**).

Analysis of inflammation by biomarkers, reticulocytes parameters and ERI

The values for the inflammatory biomarkers, IL-1β, IL-6, MCP-1 and TNF-α, found in each sample, without exception, were above the maximum reference value reported for each biomarker. IL-6 showed the inverse correlation with reticulocyte parameters (**Table 5**).

TABLE 3 – Values found for reticulocyte counts. RET%, RET#, IRF%, RET-He

Variable	*n*	Mean
RET%	34	1.49 ± 0.55
RET#	34	56,585 ± 16,367
IRF%	34	9.22 ± 5.51
LFR(%)	34	90.8 ± 5.5
MFR(%)	34	8.19 ± 4.66
HFR(%)	34	1.03 ± 0.95
RET-He (pg)	34	34 ± 4.8

RET%: relative reticulocyte count; RET#: absolute reticulocyte count; IRF%: immature reticulocyte fraction; LFR(%): low fluorescence reticulocyte fraction; MFR(%): medium fluorescence reticulocyte fraction; HFR(%): high fluorescence reticulocyte fraction; RET-He: reticulocyte hemoglobin equivalent.

TABLE 4 – Values found for reticulocyte counts. RET%, RET#, IRF%, and RET-He

Variable	ERI (cut at 4th quartile)	*n*	Mean	4th quartile	*p* value*
RET%	≤ 0.102	16	1.53 ± 0.58	1.9	
	> 0.102	7	1.45 ± 0.64	2.04	0.766
RET#	≤ 0.102	16	54,875 ± 18,457	67,800	
	> 0.102	7	58,386 ± 18,350	74,900	0.678
IRF%	≤ 0.102	16	7.5 ± 4.61	11.45	
	> 0.102	7	13.76 ± 8.15	21.1	0.028
LFR(%)	≤ 0.102	16	92.5 ± 4.61	96.4	
	> 0.102	7	86.24 ± 8.15	95	0.028
MFR(%)	≤ 0.102	16	6.66 ± 3.91	10.05	
	> 0.102	7	11.86 ± 6.87	18.3	0.03
HFR(%)	≤ 0.102	16	0.84 ± 0.79	1.65	
	> 0.102	7	1.9 ± 1.35	3	0.076

**Student's t-test for independent samples [RET%, RET#, IRF%, LFR(%) and MFR(%), HFR(%)] or non-parametric Mann-Whitney test;* $p < 0,05$.

RET%: relative reticulocyte count; RET#: absolute reticulocyte count; IRF%: immature reticulocyte fraction; ERI: erythropoietin resistance index; LFR(%): low fluorescence reticulocyte fraction; MFR(%): médium fluorescence reticulocyte fraction; HFR(%): high fluorescence reticulocyte fraction.

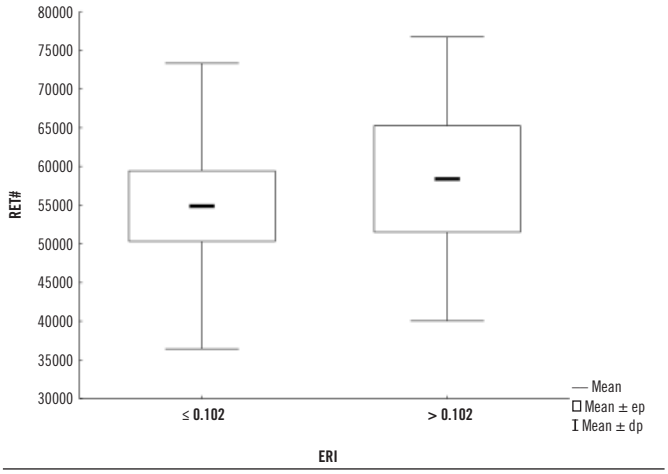

FIGURE 1 – *RET# by ERI, 4th quartile versus other quartiles (0.102)*

RET%: count of reticulocytes; ERI: erythropoietin resistance index.

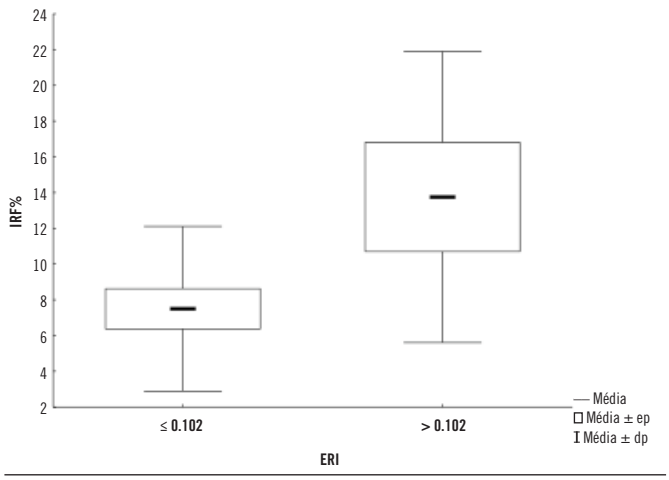

FIGURE 2 – *IRF% by ERI, 4th quartile versus other quartiles (0.102)*

IRF%: number of immature reticulocyte; ERI: erythropoietin resistance index.

TABLE 5 – Correlation analysis between IL-6 and erythropoietic activity

Variables	*n*	Correlation coefficient	*p* value
RET% × IL-6	23	0.14*	0.52
RET# × IL-6	23	0.36*	0.093
IRF% × IL-6	23	0.39*	0.062
LFR(%)× IL-6	23	-0.39*	0.062
MFR(%) × IL-6	23	0.45*	0.03
HFR(%) × IL-6	23	0.21*	0.344

**Spearman correlation coefficient.*

RET%: relative reticulocyte count; RET#: absolute reticulocyte count; IRF%: immature reticulocyte fraction; LFR(%): low fluorescence reticulocyte fraction; MFR(%): médium fluorescence reticulocyte fraction; HFR(%): high fluorescence reticulocyte fraction; IL-6: interleukin-6.

Correlation analysis between hemoglobin and IRF%

Hemoglobin values at the moment of analysis, and after 60 and 180 days were consistently higher in the group of patients with a IRF% lower than 10.5 (**Table 6**).

TABLE 6 – Correlation analysis between Hb values and IRF%, considering values ≤ 10.5 and > 10.5

Variable	IRF%	*n*	Mean	*p* value*
Hb (current)	≤ 10.5	21	12.8 ± 2.1	
	> 10.5	13	11.3 ± 1.4	0.029
Hb pg/dl (60 days)	≤ 10.5	17	12.7 ± 2	
	> 10.5	11	11.6 ± 0.7	0.045
Hb pg/dl (180 days)	≤ 10.5	11	13 ± 2.7	
	> 10.5	8	11.1 ± 1.3	0.02

**Student's t-test for independent samples (Hb pg/dl – 60 and 180 days later) or non-parametric Mann-Whitney test;* $p < 0,05$*; Hb: hemoglobin.*

DISCUSSION

Anemia is an early and frequent complication in patients with CKD, especially for those on dialysis, and is associated with high morbidity and mortality. However, resistance to ESA treatment associated with chronic inflammation in these patients is an important cause of persistent anemia. In view of limited clinical and laboratory tools currently used for the diagnosis, monitoring and treatment of anemia in CKD, in the present study we analyzed the potential of hematological parameters of reticulocytes, IRF and RET-He, that could improve this process[8, 13, 15].

In the present study, 32.4% (11/34) of the individuals presented Hb < 11 g/dl, corroborating the findings of Gonçalves & Pecoits-Filho (2013)[16] (38% in a similar population), and Alves *et al*. (2015)[13] presenting 34% in patients with CKD on hemodialysis (HD). However, it was lower than that related by Kanbay *et al*. (2010)[17], who reported about 90% of patients with a glomerular filtration rate of less than 25-30 ml/min with anemia. In view of these findings, the studied population seemed to represent a typical, clinically stable population on peritoneal dialysis, and therefore, adequate for a study of anemia with new diagnostic perspectives.

Iron deficiency is prevalent in 50% of patients with CKD. Ferritin and TSI% are the main available markers of iron stores and systemic iron, however, their values can be overestimated in situations of chronic inflammatory disease such as anemia in CKD and in PD. For this reason, the goal of this study was use reticulocytes parameters IRF and RET-He as markers of iron and erythropoietic activity that are not influenced by inflammation and may better reflect the real status of these two factors. RET-He has been demonstrated to be a marker with good sensitivity and precocity for the assessment of iron deficiency, and the adequacy of iron stores and availability[18]. Although there are several studies that suggest reference or cutoff values for RET-He, few refer specifically to patients with CKD and PD. The cut-off value proposed in the present study was suggested by the NKF-KDOQI (29 pg), in agreement with the British Guidelines for Laboratory Diagnosis of Functional Iron Deficiency and with the work of Piva *et al*. (2014)[19], who demonstrated a higher positive predictive value, when compared with ferritin values below 50 µg/l and STI% < 13%. We observed that only 11.8% (4/34) were below recommended values, which contrasts with the high prevalence of iron deficiency assessed by TSI% or ferritin.

Future studies should establish a reference or cutoff value for monitoring iron availability in based in RET-He. This will

potentially add information in clinical practice with no additional cost, since RET-HE are included in the report generated by modern analyzers[11, 13].

When evaluating the ESA resistance in the complex context of inflammation and iron mobilization, we observed that 86.9% (20/23) of the individuals presented an ERI greater than 0.02 µg, but there was no significant difference when compared to ERI values with those of RET%, RET#, IRF% (LFR%, MFR%, HFR%) and RET-He. However, when ERI was evaluated in quartiles, it seems to be more adequate approach. In this case, there was a significant difference ($p < 0.05$) when comparing the ERI values of the 4th quartile and the values for IRF%, MFR% and LFR%, but not for RET% and RET#. This finding is striking, since RET# is the recommended exam for monitoring the management of patients with CKD on dialysis using ESA. As suggested by the International Society of Laboratory Hematology, IRF% and RET# may be useful used together because IRF% means response or acceleration and RET# effectiveness of erythropoiesis activity. Butarello *et al*. (2016) [20] hypothesized that this relationship is variable, independent, and concordant. Elevated IRF% compared to normal or decreased RET# is the standard finding that identifies ineffective erythropoiesis. In the present study we standardized reference values for the parameters RET%, RET#, IRF%, LFR%, MFR% and HFR%, in Sysmex analyzer, model XE 5000, 0.43%-1.36%; 23,000-70,100/µl; 1.6%-10.5%; 89.9%-98.4%; 1.6%-9.5%; 0.0%-1.7%, respectively[21].

In addition, in the present study, there was a significant difference between IRF% (values ≤ 10.5% and > 10.5%) and values of current Hb, 60 days and 180 days ($p < 0.05$). We can see that the mean value of Hb was lower for those individuals in whom the IRF% was greater than 10.5%. This fact may suggest two possible and different clinical situations for each group specifically: in the group where IRF% is lower than 10.5% and higher values for hemoglobin, it may indicate a stable and effective erythropoiesis, corroborated by the ERI value, less than 0.102; in the group in which IRF% is higher than 10.5% and with lower hemoglobin values may indicate accelerated but ineffective erythropoietic activity or erythropoietic stress, since in this group ERI values are higher than 0.102, that is, are in the 4th quartile of resistance to EPO.

Finelly, our study describes IL-6 as the inflammatory marker that demonstrates a correlation with the variables MFR% ($p = 0.03$) and tendency of association with IRF% and LFR%, both with p values = 0.062, the latter being an inverse correlation. Ganz *et al*. (2003)[22] positions IL-6 as the main route of activation of hepcidin, directly interfering in the balance of mobility and availability of iron for erythropoiesis. IL-1β and TNF-α suppress the expression of the EPO gene – Canziani *et al*. (2006)[23].

CONCLUSION

We conclude that the new reticulocytic hematological parameters IRF and RET-He, add value for the identification of iron mobilization and deficiency, as well as in the identification of patients with erythropoietin resistance induced by inflammation. Due to the small number of patients evaluated in this study, future studies should validate our findings in larger populations and with prospective follow-up.

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