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Factors determining the percentage of hypochromic red blood cells in hemodialysis patients

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Factors determining the percentage of hypochromic red blood cells determines iron status in hemodialysis patients.

Background. Determination of the percentage of hypochromic red blood cells (RBC; %HYPO) has been advocated as a sensitive index of functional iron deficiency during erythropoietin (EPO) therapy in hemodialyzed patients.

Methods. The significance of %HYPO in chronic renal failure was evaluated in 64 chronically hemodialyzed patients. The linear correlation was determined between %HYPO and 13 variables, including age, sex, weight, C-reactive protein (CRP), ferritin, transferrin (Tf), Tf saturation, soluble Tf receptor (sTfR), serum iron (SI), urea, parathormone, dialysis dose (Kt/V), dose of EPO administered (EPO), and absolute reticulocyte count. Multiple regression analyses were then performed to select the parameters that jointly provide the best prediction of %HYPO.

Results. Univariate analysis showed significant correlations between %HYPO and iron parameters (sTfR, Tf saturation, SI, and ferritin, in decreasing order), EPO, reticulocyte count, and CRP. Multivariate analysis yielded an equation showing that the variation of %HYPO is essentially associated with the combined changes in sTfR, CRP, and EPO dosage.

Conclusions. %HYPO is a meaningful and inexpensive parameter that reflects the integrated effects of iron stores, inflammation, and erythropoietic stimulation on iron availability in hemodialyzed patients. Among iron exchange parameters, sTfR is the best predictor of %HYPO, followed by Tf saturation, SI, and ferritin.

Recombinant human erythropoietin (EPO) is widely used to correct the anemia of chronic renal failure and hemodialysis patients. The accelerated erythropoiesis associated with this treatment increases iron requirements and may thus result in functional iron deficiency, the main cause of EPO resistance. Intravenous iron supplementation is often necessary to improve the response to EPO treatment [1–6].

In order to monitor iron requirements during EPO therapy, sensitive and specific parameters of functional iron deficiency are required. Ferritin and transferrin (Tf) saturation have generally been used to assess iron availability. The ferritin concentration adequately reflects iron stores when iron metabolism is in equilibrium. However, the ferritin level varies independently from iron stores in such conditions as vitamin or folate deficiency, inflammatory and infectious diseases, and malignancies [7]. Moreover, the recommended target ferritin level in hemodialyzed patients is still under discussion and has been variously estimated as 100 to 300 ng/ml [8, 9]. Finally, functional iron deficiency may exist in the presence of adequate, although nonmobilizable, iron stores. Tf saturation is a more sensitive index of iron availability. However, this parameter is strongly disturbed when great variations in serum iron (SI) concentration occur. This is the case under EPO therapy because of the increased iron uptake by the bone marrow and the intravenous iron supplementation [10]. For these reasons, ferritin and Tf saturation are not optimal methods for monitoring iron requirements in hemodialyzed patients treated with EPO.

In the last few years, a determination of the percentage hypochromic red blood cells (RBCs; %HYPO) has been advocated as a sensitive and early marker of functional iron deficiency (abstracts; Macdougall et al, *J Am Soc Nephrol* 3:427, 1992; Golan et al, *Nephrol Dial Transplant* 9:1030, 1994) [11–16]. Several studies have shown an increase in %HYPO in the course of EPO therapy [11, 13]. This increase was consistently reduced by iron supplementation [11, 13, 14]. However, the dependence of %HYPO on biological parameters has not yet been investigated in a complex clinical setting in which hemoglobin (Hb), iron metabolism, and inflammation parameters varied concurrently in the function of disease evolution and therapy. The aim of this study was to determine

Key words: erythropoietin, dialysis, iron deficiency, chronic renal failure, soluble transferrin receptor, red blood cells, anemia.

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Table 1. Mean and SD (or percentages) of patient characteristics

Variable	Patient data
N patients (*males/females*)	64 (37/27)
Age *years*	55 ± 15
Time on dialysis *months*	75.4 ± 75.6
N treated with rHuEPO	56
EPO dose *U/kg/week*	187 ± 142
Hb *g/dl*	11.3 ± 1.1
N (%) with Hb < 10 g/dl	8 (12%)
N with iron supplementation	53
Ferritin *ng/ml*	267 ± 179
N (%) with ferritin < 200 ng/ml	28 (44%)
CRP *mg/liter*	12.5 ± 21.1
N (%) with CRP > 6 mg/liter	27 (42%)
%HYPO	5.3 ± 7.5
N (%) with %HYPO > 3.7%	24 (37%)

Abbreviations are: *N*, number; rHuEpo, recombinant human erythropoietin; Hb, hemoglobin; CRP, C-reactive protein; %HYPO, percent of hypochromic red blood cells.

quantitatively this dependence in chronically hemodialyzed patients.

METHODS

Control subjects

Reference values for %HYPO were recorded in 537 blood donors who were sampled immediately following blood donation and in 32 additional donors whose hematological parameters were compared before and immediately following blood donation.

Patients

Sixty-four chronically hemodialyzed patients were studied. Their characteristics are described in Table 1. Patients with malignancy and those who had experienced an acute fall in Hb levels in the three months before the start of the study were excluded. Fifty-six patients were treated with EPO three times per week, aiming at target Hb values of 11 g/dl. These patients were studied while in the maintenance phase of the treatment with stable EPO dosage and Hb values. Intravenous iron was administered as ferric hydroxide-sucrose complex (Venofer; Vifor, St. Gallen, Switzerland) 100 to 600 mg/month and was adjusted to reach a target ferritin level of 300 ng/ml.

Blood cell parameters

These were determined using the H*2 cell counter (Bayer, Tarrytown, NY, USA). In the latter, the amount of laser light scattered by sphered RBCs as they travel through the counting orifice is a function of both cell volume and Hb concentration. The H*2 counter measures each RBC at two scatter angles, making it possible to determine the volume and Hb concentration of individual RBCs (Fig. 1). %HYPO was recorded as the percentage of RBCs with a Hb concentration inferior to 28 g/dl. The analytical coefficient of variance (CV) of %HYPO determination was 5.2 to 8.2% for values in the 1.8 to 18.5% range. The absolute reticulocyte count was determined by cytofluorometry using a thiazole orange analogue as described previously [17].

Other parameters

A total of 13 parameters were studied, including age, sex, weight, dialysis dose (Kt/V), C-reactive protein (CRP; mg/liter), urea (g/liter), parathormone (pg/ml), EPO dose administered (U/kg/week), ferritin (ng/ml), transferrin (Tf; g/liter), Tf saturation (expressed as decimal fraction), soluble Tf receptor (sTfR; nmol/liter), and serum iron (SI; μmol/liter). Serum ferritin was measured by enzyme-linked immunoadsorbent assays. CRP was measured using a nephelometric method. sTfRs were measured by enzyme-linked immunosorbent assay (Quantikine™ IVD™; R&D Systems, Minneapolis, MN, USA).

Statistics

The statistical comparison of hematological parameters before and after blood donation was performed using paired Student's *t*-test. The influence of iron status and inflammatory state on %HYPO levels was examined by two-way analysis of variance (ANOVA). Values of ferritin, sTfR, CRP, weight, parathormone, and EPO dosage were not normally distributed in the patient population, as judged by the Kolmogorov-Smirnov test. These variables were therefore entered into the correlation and regression analyses following normalization by decimal logarithmic transformation. For CRP and EPO, a constant equal to one was added before the log transformation in order to avoid the use of the invalid log 0. Logit %HYPO, defined as ln [%HYPO/(100 − %HYPO)] was used in place of %HYPO to both normalize data and avoid prediction of values less than 0 or greater than 100 in the regression studies. The correspondence between %HYPO and logit %HYPO is illustrated in Figures 2 and 3. Where logit %HYPO is predicted by a multiple regression equation of the form logit $\%HYPO = C_0 + C_1X_1 + \ldots C_nX_n$, %HYPO can be obtained as $\exp (C_0 + C_1X_1 + \ldots C_nX_n)/[1 + \exp (C_0 + C_1X_1 + \ldots C_nX_n)]$. All statistical analyses were performed using the SigmaStat and SigmaPlot programs (SPSS Science, Erkrath, Germany).

RESULTS

Control subjects

The 99% reference interval for %HYPO determined in 537 blood donors who were sampled following blood donation was 0.0 to 3.7%. In 32 additional donors whose

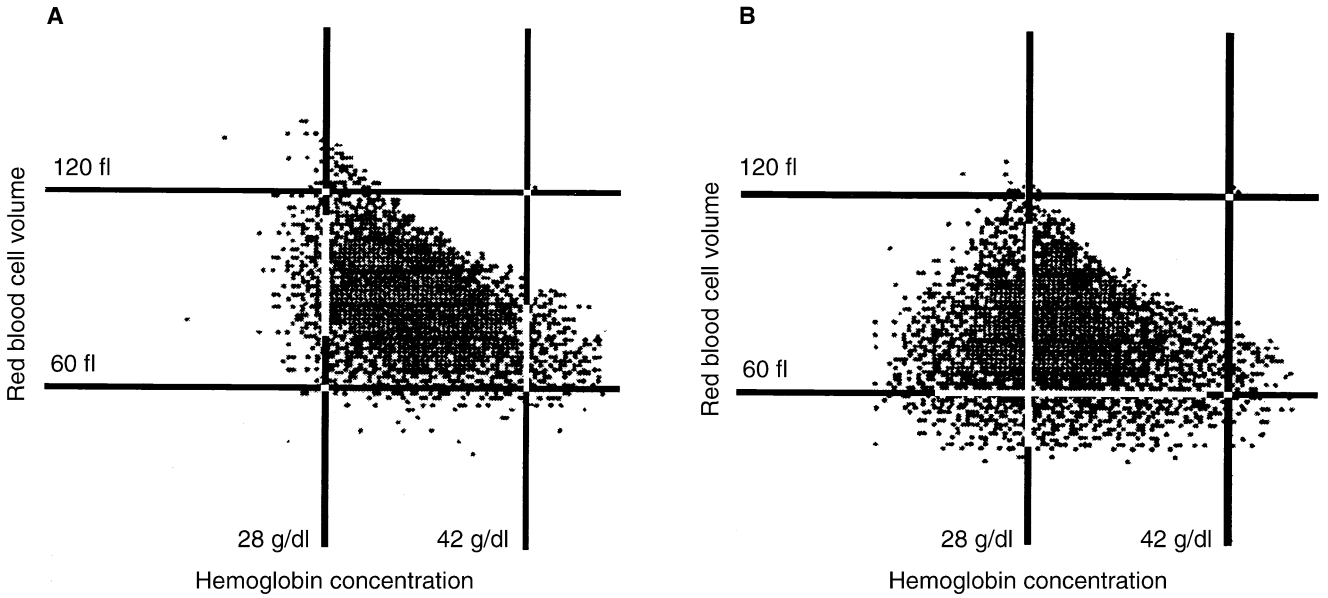

Fig. 1. Cell by cell determination of hemoglobin (Hb) concentration (abscissa) and red blood cell (RBC) volume (ordinate). Horizontal and vertical bars delineate regions with hypochromic (<28 g/dl), hyperchromic (> 42g/dl), microcytic (<60 fl), and macrocytic (>120 fl) RBC. %HYPO was 0.9% (*A*) and 24.7% (*B*).

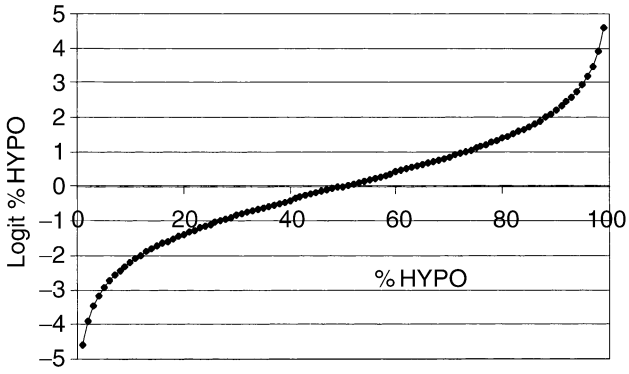

Fig. 2. Relationship between %HYPO and logit %HYPO.

hematological parameters were determined before and immediately following blood donation, including four subjects with an increased %HYPO level, no significant change in the values of hematological tests was detected (Table 2).

Correlations between patient characteristics

In view of the large number of analogous parameters measured, four classes were grouped to evaluate iron exchange (SI, Tf saturation, ferritin, sTfR), inflammation (CRP), erythropoiesis (reticulocyte count), and EPO dose administered. The values of these variables were entered into the correlation analysis displayed in Table 3 and Figure 3. This analysis showed that: (*a*) the iron parameters were significantly correlated. (*b*) sTfR, Tf saturation, and SI, in that order, were highly significant predictors ($P < 0.001$), and ferritin was a significant predictor ($P < 0.05$) of %HYPO. (*c*) %HYPO was also significantly correlated with reticulocyte count ($P < 0.01$), EPO ($P < 0.01$), and CRP ($P < 0.05$).

Of interest was the observation that values of %HYPO greater than 3.7% (the 99.5th percentile of the reference population) could be found at all ferritin levels up to 1000 ng/ml (Fig. 3). In contrast, all subjects with Tf saturation ≥ 0.30 or SI > 12 μmol/liter had %HYPO inferior to 3.7%.

Additional studies were performed to discriminate the influence of iron status and inflammatory state on %HYPO levels. Subjects were classified into four categories, based on Tf saturation (> 0.30 or ≤ 0.30) and the presence (CRP > 6 mg/liter) or absence (CRP ≤ 6 mg/liter) of an inflammatory state. Subjects with no inflammatory state and high Tf saturation had the lowest %HYPO values (0.8 ± 0.6, $N = 9$), whereas those with inflammation and low Tf saturation had the highest value (7.2 ± 8.4, $N = 20$). Patients with no inflammation and low Tf saturation ($N = 23$) or those with inflammation and high Tf saturation ($N = 4$) had intermediary %HYPO values (2.5 ± 1.4 and 5.2 ± 6.0, respectively). By two-way analysis of variance, %HYPO was found to be significantly influenced by both classification factors, Tf saturation ($P = 0.003$), and CRP ($P = 0.03$).

Prediction of %HYPO from other variables

The dependency of %HYPO on the 13 variables, including age, sex, weight, CRP, ferritin, Tf, Tf saturation,

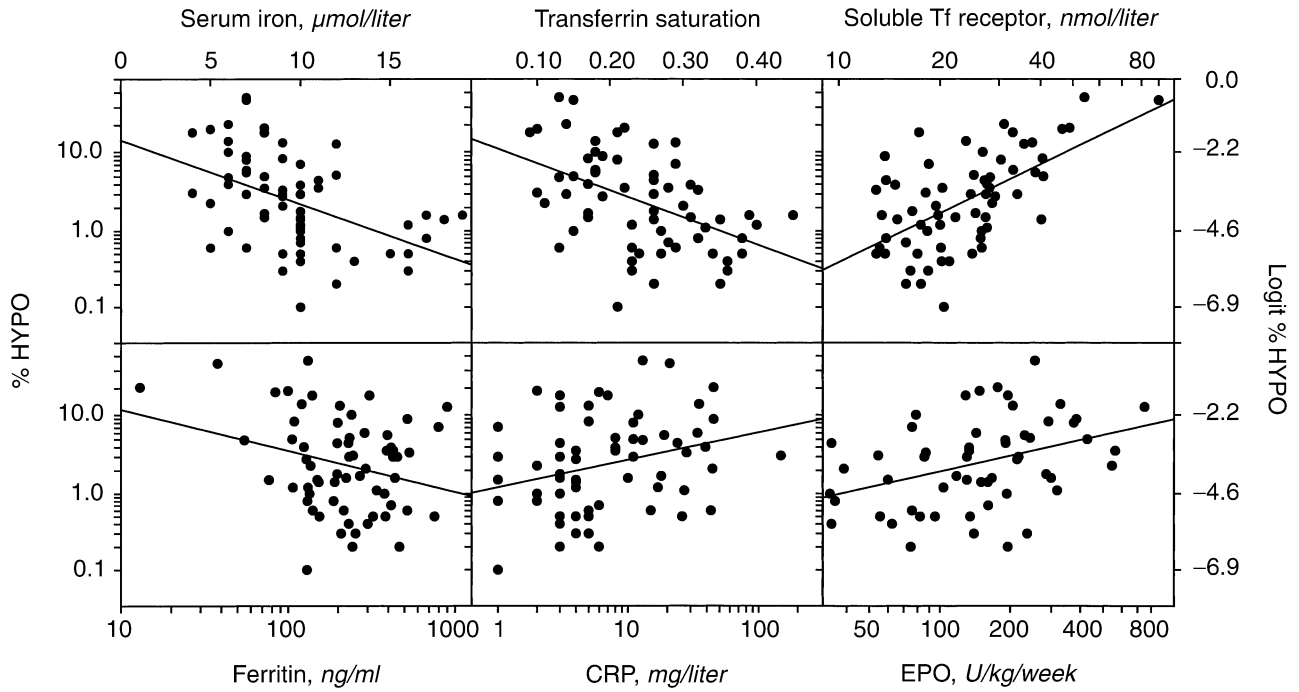

Fig. 3. Correlation studies between %HYPO and iron parameters [serum iron (SI), transferrin (Tf) saturation, soluble transferrin receptor (sTfR), ferritin], C-reactive protein (CRP), and erythropoietin (EPO) administered. To normalize variables as explained in the **Methods** section, sTfR, ferritin, CRP, and EPO have been displayed on a log scale on the abscissa. The ordinates have been expressed as both %HYPO on a logit scale (left) and logit %HYPO on a linear scale (right) to show the correspondence between these two variables. *P* values are expressed as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. R values are: SI, $r = -0.46***$; TF saturation, $r = -0.48***$; sTfR, $r = 0.60***$; ferritin, $r = -0.28*$; CRP, $r = 0.29*$; EPO, $r = 0.38**$.

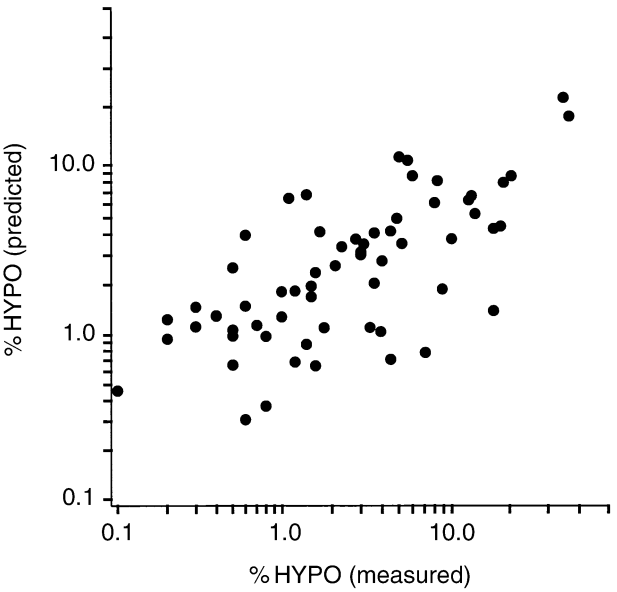

Fig. 4. Relationship between measured %HYPO and predicted %HYPO obtained from the regression equation logit %HYPO = −11.76 + 4.87 log sTfR + 0.40 log (EPO + 1) + 0.75 log (CRP + 1), ($r = 0.69$).

Table 2. Comparison of hematological parameters before and after blood donation in 32 donors

Variable	Before donation	After donation
%HYPO	5.61 ± 6.65	5.40 ± 7.23
Hb *g/dl*	13.8 ± 1.4	13.8 ± 1.3
RBC 10^6*/liter*	4.79 ± 0.46	4.77 ± 0.43
Hematocrit %	41.6 ± 3.7	41.5 ± 3.2
MCV *fl*	87.1 ± 3.2	87.0 ± 3.6
Reticulocyte 10^6*/liter*	130 ± 34	129 ± 34

All comparisons were nonsignificant at the 5% level.

Abbreviations are: %HYPO, percent of hypochromic red blood cells; Hb, hemoglobin; RBC, red blood cells; MCV, mean corpuscular volume.

sTfR, SI, urea, parathormone, Kt/V, dose of EPO administered, and absolute reticulocyte count, was determined by stepwise multiple regression analysis. Only the equations in which all coefficients differed from zero at the 5% level were retained. Because intercorrelations among iron parameters ("multicollinearity") would tend to reduce the efficiency of regression if they were simultaneously entered in the regression equation, %HYPO was related to alternative sets of nine variables in which only one of the iron parameters (Tf saturation, SI, or sTfR) was entered. The most significant equation ($P < 0.001$) (Fig. 4) was:

Table 3. Correlation matrix of the variables studied

	Tf	SI	Tf SAT	Ferritin	sTfR	CRP	RETIC	EPO	%HYPO
Tf		0.10	**−0.34**	**−0.37**	**0.36**	−0.11	**0.37**	0.25	0.11
SI	0.10		**0.89**	0.22	**−0.34**	**−0.40**	−0.11	−0.03	**−0.46**
Tf SAT	**−0.34**	**0.89**		**0.36**	**−0.46**	**−0.34**	−0.22	−0.16	**−0.48**
Ferritin	**−0.37**	0.22	**0.36**		**−0.43**	0.07	−0.11	−0.14	−0.28[a]
sTFR	**0.36**	**−0.34**	**−0.46**	**−0.43**		−0.06	**0.46**	0.31[a]	**0.60**
CRP	−0.11	**−0.40**	**−0.34**	0.07	−0.06		0.03	−0.02	0.29[a]
RETIC	**0.37**	−0.11	−0.22	−0.11	**0.46**	0.03		0.34[a]	**0.41**
EPO	0.25	−0.03	−0.16	−0.14	0.31[a]	−0.02	0.34[a]		**0.38**
%HYPO	0.11	**−0.46**	**−0.48**	−0.28[a]	**0.60**	0.29[a]	**0.41**	**0.38**	

P values are expressed as follows: [a] $P < 0.05$; bold characters $P < 0.01$; bold characters underlined, $P < 0.001$. Iron parameters have been grouped inside the shaded area to highlight their mutual correlations.

Abbreviations are: TfSAT, Tf saturation; RETIC, absolute reticulocyte count. Other abbrevations are in the legends of Tables 1 and 2.

Logit %HYPO = −11.76 + 4.87 log sTfR + 0.40 log (EPO + 1) + 0.75 log (CRP + 1) ($r = 0.69$).

Other, less significant equations confirmed that %HYPO was essentially determined by parameters of iron exchange, including sTfR, Tf saturation, SI, or ferritin, as well as by CRP and the EPO dose administered. Stratifying the patients on the basis of normal or elevated CRP did not alter the significance of iron parameters in predicting %HYPO.

In an attempt to determine the reason that sTfR had a higher correlation with %HYPO than the other iron parameters, the equation log sTfR = −0.34 − (0.79 * Tf saturation) + (0.40 * log reticulocyte count) ($r = 0.59$) was derived. The inference that the level of sTfR was independently determined by iron status and erythropoietic activity may therefore explain why this parameter was the best single predictor of %HYPO.

DISCUSSION

Erythropoietin therapy has considerably improved the quality of life of hemodialyzed patients. The response to this treatment is highly conditioned by iron delivery to erythropoiesis. It has been demonstrated that the assessment of iron stores is not sufficient to monitor iron requirements and that the major factor determining the response to EPO therapy is iron availability [6, 11, 18–21]. Because hyporesponsiveness to EPO bears economical in addition to clinical relevance, a sensitive and early marker of functional iron deficiency is needed.

The data confirm the dependence of %HYPO on iron parameters and EPO dosage, and they establish, to our knowledge for the first time, its relationship with inflammation. The dependence on EPO dosage reflects the increase in iron requirement and functional iron deficiency resulting from erythropoietic stimulation [11, 13, 14]. Inflammation is known to create functional iron deficiency by blocking iron release by the reticuloendothelial system [22]. It is therefore not surprising to find a positive correlation between CRP and %HYPO. Interleukin 1 (IL-1) stimulates intracellular ferritin biosynthesis by a translational mechanism that is independent of iron status and results in intracellular trapping of iron and reduced iron availability for Hb synthesis [23]. In addition, IL-1 and tumor necrosis factor-α (TNF-α) induce secretion by liver cells of serum ferritin, an iron-poor, glycosylated molecule with a different amino acid sequence from intracellular ferritin [24], which plays a negligible role in iron exchange.

Ferritin has been shown to be negatively correlated with %HYPO [13, 14], an observation that has been confirmed in this study (Table 3 and Fig. 3). This observation has prompted the guideline to maintain patients with iron supplementation at ferritin levels as high as 300 ng/ml. However, Figure 3 demonstrates that elevated %HYPO values have been recorded at ferritin levels ranging from 12 to 900 ng/ml, in agreement with published data showing that high iron stores may be associated with low iron availability and functional iron deficiency [1, 11, 18–21, 23]. Although sufficient iron stores are required, ferritin provides no information on the quantity of iron available to erythroblasts. Confusion between size of iron stores and iron availability may explain why the target ferritin level in hemodialyzed patients has been set at such variable levels as 100 to 300 ng/ml [8, 9]. A recent study suggests that the target ferritin level may depend on the amount and frequency of iron supplementation (abstract; Ahmed et al, *J Am Soc Nephrol* 4:423, 1993) [25].

The present study confirmed the partial correlation between %HYPO and Tf saturation or SI (abstract; Golan et al, *Nephrol Dial Transplant*, ibid). All subjects with Tf saturation ≥ 0.30 or SI > 12 μmol/liter had a %HYPO inferior to 3.7%. However, caution must be exercised because Tf saturation and SI are reliable parameters of iron availability only when iron status is in equilibrium. This condition is not always fulfilled during EPO therapy because erythropoietic stimulation, blood losses, and intravenous iron supplementation tend to cause fluctuations in SI levels. Furthermore, Tf saturation shows large circadian variations caused by wide

fluctuations in reticuloendothelial iron release [10]. For these reasons, %HYPO is likely to provide a more stable index of iron availability than Tf saturation.

In our study, sTfR was found to be the best predictor of %HYPO. Its high positive correlation with %HYPO (0.60, $P < 0.001$) was not surprising because sTfR is a reliable parameter for both functional iron deficiency and erythropoietic stimulation [26, 27]. This statement is supported by the regression equation linking sTfR to both Tf saturation and reticulocyte count. Although %HYPO and sTfR have somewhat similar clinical meanings in the setting of hemodialysis patients treated with EPO, determination of %HYPO is to be preferred in the routine monitoring of hemodialyzed patients, as it is much less expensive and more easily available.

The value of 10% hypochromic red cells has been used in several studies as the cut-off for functional iron deficiency [11, 14, 16], and was found to have a sensitivity of 43% to predict the reticulocyte response to iron administration in hemodialyzed patients [28]. However, the 10% cut-off may be inappropriate to monitor incipient functional iron deficiency, as %HYPO was less than 3.7% in 99.5% of the 537 normal blood donors measured in this study. That the upper reference value may be much lower than previously believed is confirmed by the observation that, even in patients with %HYPO less than 5%, EPO dosage could be decreased by 8.5% under iron supplementation [14].

In recent studies, reticulocyte Hb content (CHr) was found to increase within two days following iron supplementation in hemodialyzed patients, demonstrating that CHr is an earlier marker of functional iron deficiency than %HYPO [28]. However, the fact that CHr closely reflects fluctuations of iron levels may be a disadvantage and suggests that %HYPO is a better index of chronic iron requirements. In the future, CHr and %HYPO should be considered complementary, just as determinations of glucose and glycated Hb are necessary for adequate control of diabetic patients [16].

In conclusion, %HYPO is a meaningful and inexpensive parameter that quantitatively reflects the integrated effects of iron stores, inflammation, and erythropoietic stimulation on iron availability in hemodialyzed patients. Our study provides additional support to protocols advocating the determination of %HYPO, together with ferritin and CRP, for iron monitoring during EPO treatment. Resistance to EPO has been defined by the National Kidney Foundation (DOQI) as the failure to achieve target Hb within four to six months following the administration of 450 U/kg/week intravenously or 300 U/kg/week subcutaneously or as the failure to maintain target Hb at that dose [29]. Nevertheless, we believe that patients requiring doses higher than 150 U/kg/week should be tested and, if found functionally iron deficient, treated. Values of %HYPO lower than 3.7% signify that EPO resistance does not result from iron deficiency. Values higher than 3.7% are indicative of an inflammatory state or iron deficiency, be it absolute or functional. If iron supplementation is required, ferritin must be controlled to detect iron overload. These practical guidelines document the central role of %HYPO in the monitoring of iron requirements in hemodialyzed patients. %HYPO is also useful in detecting iron deficiency in thalassemia [30–34] and hemolytic anemias [35], as well as in the general population [36–38].

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APPENDIX

Abbreviations used in this article are: CHr, reticulocyte hemoglobin content; CRP, C-reactive protein; EPO, erythropoietin; Hb, hemoglobin; %HYPO, percentage of hypochromic red blood cells; IL-1, interleukin-1; Kt/V, dialysis dose; MCV, mean corpuscular volume; RBC, red blood cells; SI, serum iron; sTf, soluble transferrin; sTfR, soluble transferrin receptor; Tf, transferrin; TNF-α, tumor necrosis factor-α.

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