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Original Article

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Mature erythrocyte parameters as new markers of functional iron deficiency in haemodialysis: sensitivity and specificity

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Abstract

Background. The percentage of hypochromic red blood cells (RBCs) (%HYPO) has been demonstrated as the best predictor of response to iron loading in haemodialysis patients treated with recombinant human erythropoietin (rHuEPO). However, we have previously shown that this parameter is positively influenced by erythropoietic activity since reticulocytes are considered hypochromic by cell counters. New cell counters are able to determine cell volume and haemoglobin (Hb) concentration separately on reticulocytes and mature erythrocytes. The aim of this study was to assess the sensitivity and specificity of mature erythrocyte parameters in detecting functional iron deficiency (FID).

Methods. A total of 32 stable chronic haemodialysis patients in the maintenance phase of rHuEPO therapy were included. Classical parameters of iron monitoring and mature erythrocyte parameters were measured after a 4-week iron-free period. Patients were classified as responders (R) or non-responders (NR) to an iron load of 100 mg iron sucrose at each dialysis session for 4 weeks, according to whether their Hb increased by >1 g/dl at the end of iron loading.

Results. Twelve patients were identified as responders. Receiver operating characteristic (ROC) curve analysis demonstrated %HYPO and its corresponding parameter on mature erythrocyte, %HYPOm, as the best predictors of FID. The other parameters were ordered as follows: tranferrin saturation (TSAT), ferritin (FRT), mature RBC Hb content (CHm), mean corpuscular Hb concentration (MCHC), percentage of mature erythrocytes with a low CHm (%lowCHm), mean content in Hb (MCH) and reticulocyte Hb content CHr. Comparing the parameters at different cut-offs, the best sensitivity, specificity and efficiency were demonstrated for %HYPOm > 6%.

Conclusion. The best efficiency to predict FID was found for %HYPOm > 6%. The predictive value of %HYPO was quite similar. The clinical impact of %HYPOm in iron monitoring should also be tested in the induction phase of rHuEPO treatment because of its independence from erythropoietic activity.

Keywords: anaemia; haemodialysis; iron monitoring; mature erythrocyte parameters; percentage of hypochromic red blood cells

Introduction

The treatment of renal anaemia with recombinant human erythropoietin (rHuEPO) has consistently improved the quality of life and outcome of haemodialysis patients [1–4]. The efficacy of this therapy depends in part on the identification and correction of resistance factors such as vitamin deficiency, inflammation or hyperparathyroidism. The major cause of resistance to rHuEPO is iron deficiency. Absolute iron deficiency is easy to detect. However, functional iron deficiency, defined as a positive response to further iron supplementation in the absence of absolute iron deficiency, remains a daily challenge for nephrologists. It is not only of pharmaco-economical but also of clinical importance since inappropriate intravenous iron administration can lead to acute and long-term complications. First, anaphylactic reactions have been described following intravenous iron administration, mainly associated to iron dextran [5,6]. Second, the demonstration of *in vitro* and *in vivo* oxidation of lipids and proteins could lead to oxidative damage [7–9]. However, the clinical significance of oxidative stress remains uncertain. Finally, an increased risk of infection has been suggested to be associated to iron overload and iron administration [10,11]. Nevertheless, Hoen *et al.* [12], in a prospective study, demonstrated that

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the risk of bacteriemia was neither associated with ferritin level, nor with iron administration.

Routine parameters usually measured for iron monitoring, such as ferritin (FRT), transferrin saturation (TSAT) or serum iron (SI) have shown low sensitivity and specificity in the prediction of response to intravenous iron supplementation in the haemodialysis population under rHuEPO therapy. The percentage of hypochromic red blood cells (%HYPO) has been demonstrated as the most sensitive and specific parameter for functional iron deficiency [13]. However, %HYPO is positively correlated to the erythropoietic activity, as reflected by the reticulocyte count and soluble transferrin receptors (sTfRs) [14]. Indeed, an increased %HYPO following erythropoietic stimulation with rHuEPO may partly be due to increased erythrocyte volume (MCV) with a stable haemoglobin (Hb) content (CH), in a younger erythroid population, resulting in a decrease in mean Hb concentration (CHCM) [15]. Usually, rHuEPO therapy produces both increased erythropoietic activity and functional iron deficiency, two mechanisms resulting in elevated %HYPO.

An ideal marker of functional iron deficiency should thus be independent of the erythropoietic activity, the influence of which could be minimized through the measurement of mature erythrocyte parameters, without the confounding effect of reticulocytes. The Advia 120 cell counter (Bayer Diagnostics, Tarrytown, NY, USA) is capable, in addition to providing routine parameters measured on the whole red cell population, to separate mature erythrocytes from reticulocytes and to measure cell volume and haemoglobin concentration of each individual cell in these two distinct populations.

The aim of this study was to assess the sensitivity and specificity of mature erythrocyte parameters and to compare these new parameters with our current clinical practice.

Methods

Patients

A total of 32 patients with end-stage renal disease (ESRD) were included in the study. Of these 13 were females and 19 were males. Mean age was 65 ± 18 years. The origin of ESRD was diabetes in seven, hypertension in seven, glomerulopathy in seven, tubulo-interstitial disease in six and other origins in five. All patients were chronically haemodialysed three times a week for 3.5 or 4 h. They were all treated with rHuEPO for at least 3 months and were in the maintenance phase of their rHuEPO treatment with stable rHuEPO doses for at least 4 weeks. Prior to the study, iron supplementation was administered in order to meet the recommendations of the European Best Practice Guidelines (EBPG; FRT > 100 ng/ml and %HYPO < 10%). Exclusion criteria were adult dominant polycystic kidney disease, malignancy, haemoglobinopathy, evidence for chronic or acute bleeding, blood transfusion in the previous 3 months, hyperparathyroidism [parathyroid hormone (PTH) > 500 ng/ml], vitamin B12 or folate deficiency, chronic or acute inflammation [C-reactive protein (CRP) > 30 mg/l].

Study design

Patients were screened on the basis of inclusion and exclusion criteria. The screening period continued for 4 weeks after the first screening visit, during which time neither rHuEPO treatment nor iron supplementation were modified. Patients in whom Hb levels varied by more than 1 g/dl during this 4-week period were excluded from the study.

The study began at the end of the screening period with a 4-week iron washout period. At the end of the iron-free period, haematologic and iron monitoring parameters were measured: Hb, haematocrit (Hct), reticulocytes (retic), %HYPO, reticulocyte Hb content (CHr), mature erythrocyte Hb content (CHm), percentage of hypochromic mature erythrocytes (%HYPOm), percentage of mature erythrocytes with a low Hb content (%lowCHm), mean content in Hb (MCH), mean corpuscular Hb concentration (MCHC), FRT, TSAT and sTfRs. Patients in whom Hb levels increased by more than 1 g/dl during the iron-free period were excluded from the study.

During the second part of the study, an iron load was administered to all patients. They received a total dose of 1200 mg iron sucrose, administered intravenously at the dose of 100 mg at the end of each dialysis session during 4 weeks. Haematological parameters were followed weekly during the whole study. The dose of rHuEPO remained unchanged during the whole study period.

Patients were classified into responders (R; presumably functionally iron deficient) and non-responders (NR; presumably iron replete) according to whether their Hb increased by more or less than 1 g/dl during the 4-week iron-loading period.

The protocol was approved by the Ethics Committee of the University Hospital of Liège. An informed consent was obtained from each patient included in the study.

Laboratory analyses

Red cell parameters were measured with the Advia 120 cell counter (Bayer Diagnostics, Tarrytown, NY, USA). Reticulocytes are stained with oxazine 750 and oxazine-negative cells are identified as mature erythrocytes. Routine parameters are measured in the pooled population of reticulocytes and mature erythrocytes (Hb, Hct, MCH, MCHC). In addition, the cell counter derives in the two populations separately, the Hb content (CH), as well as the percentages of microcytic cells (%micro: cell volume < 60 fl), macrocytic cells (%macro: cell volume > 120 fl), hypochromic cells (%HYPO: Hb concentration < 28 pg/ml), hyperchromic cells (%HYPER: Hb concentration > 41 pg/ml), cells with low CH (%low CH: Hb content < 27 pg) and cells with high CH (%high CH: Hb content > 31 pg).

The coefficient of variation (CV) was computed from replicate measures of patient samples (intra-run CV, $n = 8$) for %HYPO, %HYPOm, CHr, CHm and %low CH. All parameters showed excellent reproducibility, with CVs < 5%. (Table 1)

Serum FRT and sTfRs (Quantikine™ IVD™, R&D Systems, Minneapolis, MN, USA) were measured

Table 1. Reproducibility of measure for the Advia parameters. The coefficient of variation (CV) results are shown

Parameters	CV (%)
%HYPO	3.57
%HYPOm	4.05
CHr	0.64
CHm	0.13
%low CH	1.95

by enzyme-linked immunosorbent assays. CRP was measured using a nephelometric method, serum iron with a ferrozin method and transferrin with an immunoturbidimetric method.

Statistical methods

In the text, values are expressed as mean ± standard deviation (M ± SD). For more readability in the graphs, values are shown as mean ± standard error of the mean (M ± SEM). Comparisons between groups were performed using Student's t tests. P-values < 0.05 were considered as statistically significant. ROC curve analyses were performed with MedCalc (Mariakerke, Belgium). The Kappa coefficients for sensitivity (κ_{sens}), specificity (κ_{spec}) and efficiency (κ_{eff}) were calculated to calibrate these results to prevalence and level of the test. Kappa coefficients were calculated as follows: $(\kappa_{sens}) = (SE - Q)/Q'$; $(\kappa_{spec}) = (SP - Q')/Q$ and $(\kappa_{eff}) = (EFF - PQ - P'Q')/(1 - PQ - P'Q')$, where P is the prevalence of the disease in the studied population, $P' = 1 - P$, Q is the level of the test (percentage of false positive) and $Q' = 1 - Q$. SE is the sensitivity, SP the specificity and EFF the efficiency of the test.

Results

Table 2 shows baseline haematological and iron parameters after the iron washout period. Among the 32 patients, 12 (37.5%) were defined as responders (functionally iron deficient). Responders had significantly lower Hb, FRT, TSAT, CHr, CHm and significantly higher %HYPO, %HYPOm and %lowCHm. Reticulocyte count was significantly higher in responders. After iron loading, Hb levels remained constant (12.9 ± 0.8 *vs* 12.9 ± 0.7 g/dl) in NR (iron replete), but increased significantly from 11.7 ± 0.9 to 12.9 ± 1.1 g/dl in R to become similar to those of NR. The rHuEPO doses were not significantly different between the groups. However, R tended to require higher doses than NR (189 ± 129 *vs* 118 ± 75 UI/kg/week; NS).

Figure 1 summarizes the evolution of the studied parameters along the 8 weeks of follow-up. In responders, FRT levels, already lower than in non-responders at baseline, further decreased significantly after the iron-free period (248 ± 162 *vs* 417 ± 249 ng/ml; $P < 0.01$). This was accompanied by a significant decrease of TSAT from 18.8 ± 3.7 to 16.2 ± 3.5%; $P < 0.05$). During this period of time, %HYPO, %HYPOm, CHr, CHm, MCH and MCHC remained constant. Only %lowCHm increased significantly (26.4 ± 16.1 *vs* 23.6 ± 14.1%; $P < 0.03$). In the same group of patients, after iron loading, FRT and TSAT increased significantly (773 ± 381 *vs* 248 ± 162 ng/ml, $P < 0.001$; and 23.9 ± 6.8 *vs* 16.2 ± 3.5%, $P < 0.001$, respectively). CHr increased significantly from 31.2 ± 2.3 to 34.1 ± 2.0; ($P < 0.05$), while %HYPO (8.6 ± 6.2 *vs* 14.1 ± 6.5%; $P < 0.001$) and %HYPOm (8.6 ± 5.9 *vs* 12.9 ± 6.5%; $P < 0.001$) decreased significantly. MCH increased significantly (31.2 ± 2.4 *vs* 30.1 ± 2.3 pg; $P < 0.001$). MCHC and CHm did not change significantly. %lowCHm increased during the iron-free period (26.4 ± 16.1 *vs* 23.6 ± 14.1%; $P < 0.05$) but decreased in the iron-loading period (18.0 ± 11.6 *vs* 26.4 ± 16.1%; $P < 0.05$). Its level at the end of the follow-up was lower than at the start of the study (26.4 ± 14.1%) despite its increase during the iron-free period.

Table 2. Parameters as measured at the end of the iron-free period before iron loading (baseline)

	NR	*R*	*P*
Hb (g/dl)	12.9 ± 0.7	11.7 ± 0.9	<0.001
Hct (%)	39.7 ± 2.1	37.8 ± 3.3	0.06
Retic (%)	1.6 ± 0.4	2.1 ± 0.5	<0.01
Retic abs (10^3/mm^3)	64.6 ± 19.1	85.1 ± 17.2	<0.01
CHr (pg)	33.4 ± 1.3	31.2 ± 2.3	<0.01
CHm (pg)	31.9 ± 1.2	30.0 ± 2.3	<0.01
%LowCHm (%)	11.3 ± 4.5	26.4 ± 16.1	<0.001
%HYPO (%)	4.5 ± 2.5	14.1 ± 6.5	<0.001
%HYPOm (%)	3.9 ± 2.4	12.9 ± 6.5	<0.001
FRT (ng/ml)	446 ± 146	248 ± 162	<0.01
TSAT (%)	25.6 ± 6.6	16.2 ± 3.5	<0.001
rHuEPO dose (UI/kg/wk)	118 ± 75	189 ± 129	NS

In iron-replete patients, during the washout period, TSAT, as well as %HYPO, %HYPOm, CHm, MCH and %lowCHm, remained constant. The only significantly different parameters were FRT (446 ± 146 *vs* 524 ± 148 ng/ml; $P < 0.001$), CHr (33.4 ± 1.3 *vs* 31.9 ± 2.3 pg; $P < 0.05$) and MCHC (32.5 ± 0.8 *vs* 32.1 ± 0.9 pg/ml; $P < 0.05$). After iron-loading, FRT (987 ± 250 *vs* 446 ± 146; $P < 0.001$), TSAT (33.5 ± 8.0 *vs* 25.6 ± 6.6; $P < 0.001$), MCH (32.6 ± 1.9 *vs* 31.9 ± 1.7 pg/ml; $P < 0.01$) and CHr (34.7 ± 1.5 *vs* 33.4 ± 1.3; $P < 0.001$) increased significantly, %HYPO decreased significantly (3.3 ± 1.8 *vs* 4.5 ± 2.5; $P < 0.05$), while MCHC, CHm, %HYPOm and %lowCHm remained constant.

The results of ROC curve analyses are shown in Figure 2. All studied parameters had an area under the curve (AUC) significantly >0.5. Their discriminative abilities to predict functional iron deficiency were, in this order: sTfR (AUC: 0.989; SE: 0.034), %HYPO (AUC: 0.937; SE: 0.051), %HYPOm (AUC:0.935; SE:0.052), TSAT (AUC: 0.896; SE: 0.060), FRT (AUC: 0.834; SE: 0.076), CHm (AUC: 0.808; SE: 0.076), MCHC (AUC: 0.804; SE: 0.077), %lowCHm (AUC: 0.792; SE: 0.088), MCH (AUC: 0.723; SE:0.090)

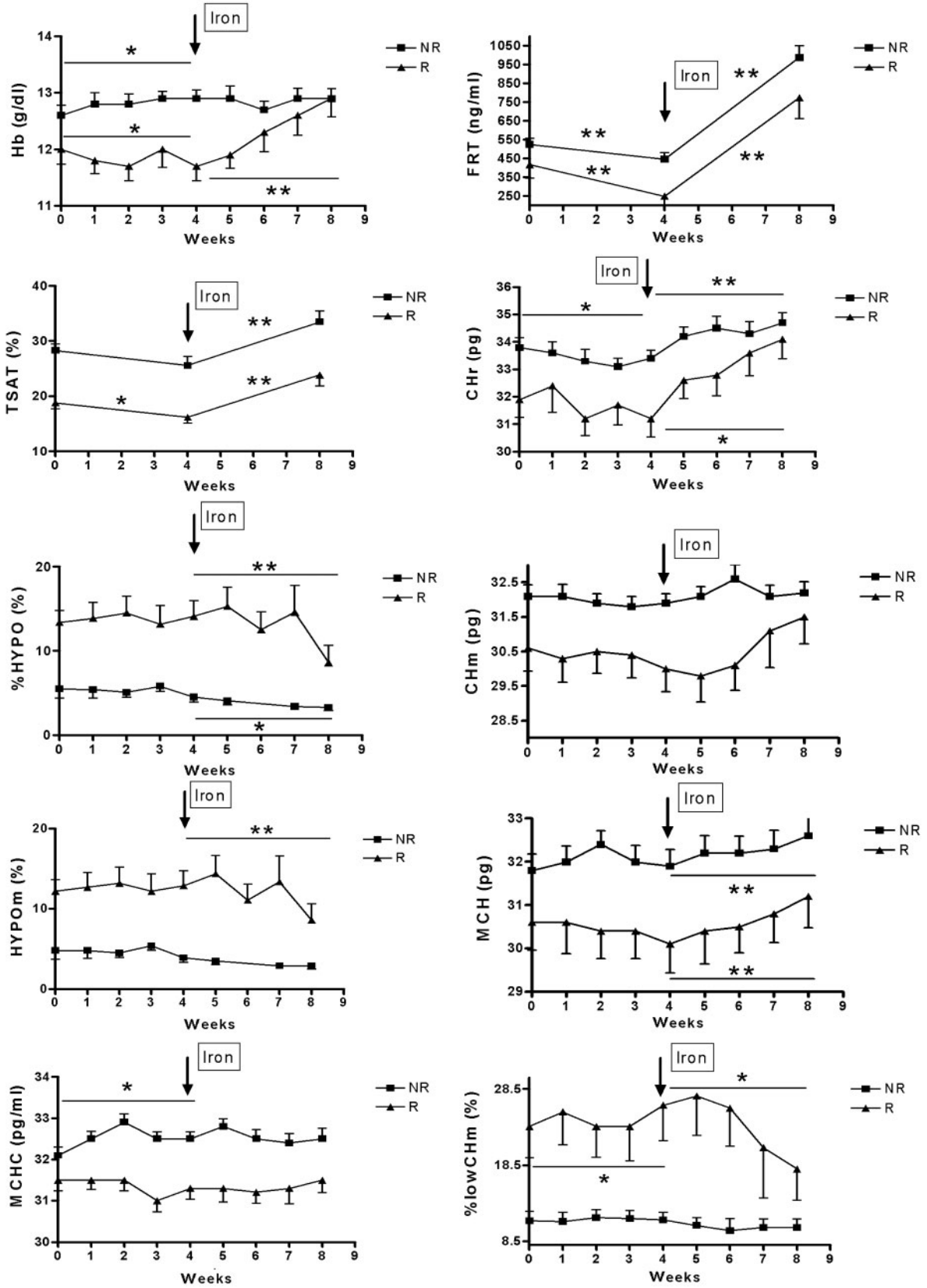

Fig. 1. Evolution of the studied parameters during the study. Comparisons are shown between end of screening period and baseline and between baseline and end of iron-loading period. Responders (R) and non-responders (NR) are depicted separately. $^{*}P < 0.05$; $^{**}P < 0.01$.

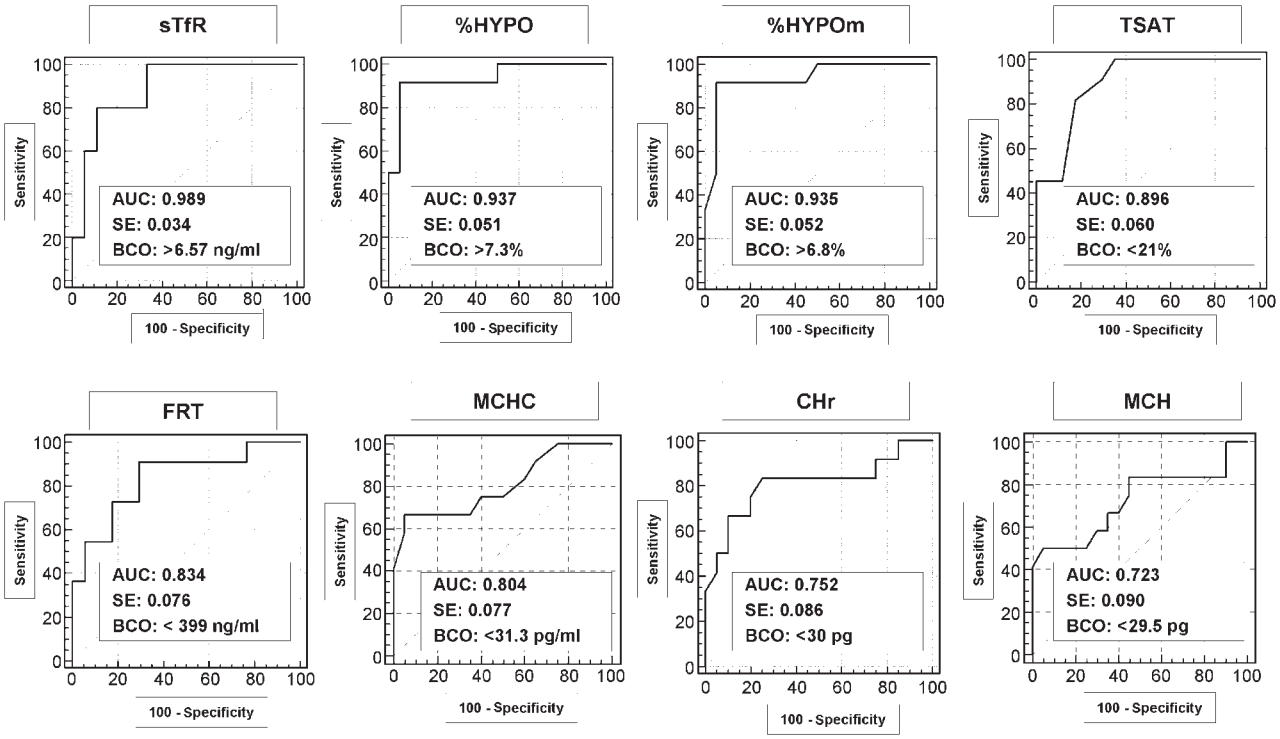

Fig. 2. Results of the ROC curves. AUC, area under the curve; SE, standard error; BCO, best cut-off.

and CHr (AUC: 0.752; SE: 0.086). However, there were no statistically significant differences between any of these parameters.

The best threshold values for prediction of iron response, calculated by ROC curve analysis, were >6.6 mg/l for sTfR, > 7.3% for %HYPO, > 6.8% for %HYPOm, < 21% for TSAT, < 400 ng/ml for FRT, < 32.4 pg for CHm, < 31.3 pg/ml for MCHC, > 19% for %lowCHm, < 29.5 pg for MCH and <30 pg for CHr.

We calculated the specificity, sensitivity and efficiency of these various tests at different cut-offs, as well as combinations thereof as proposed by the European and American guidelines. [16,17] These results are shown in Table 3, where parameters are classified following the best calibrated efficiency (κ_{eff}). %HYPOm > 6% was the best predictor of response to iron loading. At this threshold, %HYPOm was able to correctly classify 87.5% of the cases. The efficiency of criteria proposed by the European Best Practice Guidelines (EBPG) [16] (FRT < 100 ng/ml or %HYPO > 10%) was similar, with correct classification in 85.7% of the cases. Parameters recommended by the DOQI guidelines [17] (FRT < 100 ng/ml or TSAT < 20%) were a little less efficient with a correct classification in 78.6% of the cases.

Discussion

Stimulation of erythropoiesis with rHuEPO leads to a rapid decrease of FRT, TSAT and CHr and a subsequent increase of %HYPO [15,18,19], a pattern highly suggestive of functional iron deficiency. Nutrition is the source of 1–2 mg iron a day in normal subjects. This source of iron is far insufficient to meet the requirements of a stimulated bone marrow. Furthermore, oral iron absorption is impaired in patients suffering from chronic and ESRD [20] and still more so when phosphate binders are used. Intravenous iron supplementation has proven much higher efficacy for this purpose [21] and is now widely prescribed. However, iron administration and iron overload can bear negative consequences. Intravenous iron administration has been shown to induce lipid [10] and protein [11] oxidation. Moreover, iron overload may lead to direct organ damage [21] and an increased risk of infection [7,8], but this association remains controversial. For all these reasons, assessment of iron requirements and monitoring of iron therapy require accurate markers.

Current European [16] and American [17] guidelines may not be optimal for the detection of functional iron deficiency. In a previous prospective trial [23], iron supplements were increased in an unselected cohort of haemodialysis patients. Data suggested that response to rHuEPO continues to improve when one targets the normal range for %HYPO (<2.5%) and that such an approach was cost-effective. FRT levels required to achieve lower %HYPO values were much greater than the current recommended range. Unfortunately, this could produce a significant degree of iron overload.

Tessitore *et al.* [13], in a study similar to ours, demonstrated that the best single predictor of functional iron deficiency was %HYPO and that the

Table 3. Sensitivity, specificity and efficiency of the studied iron parameters at different thresholds. Cut-off values were chosen from ROC curves as well as levels currently recommended in the literature. Parameters are ordered by efficiency

Parameter	Sensitivity (%)	κ_{sens} (%)	Specificity (%)	κ_{spec} (%)	Efficiency (%)	κ_{eff} (%)
%HYPOm > 6%	91.7	85.2	85.0	65.7	87.5	74.2
FRT < 100 ng/ml or %HYPOm > 6%	90.9	84.3	85.0	64.2	87.1	72.9
FRT < 100 ng/ml or %HYPO > 10%	72.7	59.8	94.1	81.6	85.7	69.0
%HYPO > 10%	66.7	53.7	95.0	82.2	84.4	65.0
FRT < 400 ng/ml	90.9	81.8	76.5	53.0	82.1	64.2
%lowCHm > 20%	58.3	46.6	100.0	100.0	84.4	63.7
%HYPO > 6%	91.7	83.4	75.0	50.0	81.3	62.6
TSAT < 20%	90.9	80.4	70.6	45.1	78.6	57.8
FRT < 100 ng/ml or TSAT < 20%	90.9	80.4	70.6	45.1	78.6	57.8
CHm < 30 pg	50.0	38.5	100.0	100.0	81.3	55.7
%HYPOm > 10%	50.0	36.0	95.0	77.1	78.1	49.0
%HYPO > 4%	100.0	100.0	50.0	27.3	68.8	42.9
%HYPOm > 4%	91.7	77.9	55.0	28.0	68.8	41.3
%lowCHm > 15%	66.7	43.9	75.0	38.5	71.8	40.8
MCH < 29.5 pg	42.0	7.2	95.0	86.7	75.0	40.7
CHm < 29 pg	33.3	23.8	100.0	100.0	75.0	38.5
CHr < 30 pg	33.3	23.8	100.0	100.0	75.0	38.5
FRT < 100 ng/ml	27.3	18.6	100.0	100.0	71.4	31.2
CHm < 31 pg	66.7	37.3	65.0	25.3	65.5	30.1
CHr < 29 pg	25.0	17.2	100.0	100.0	71.8	29.2
MCHC < 31.3 pg/ml	75.0	42.9	55.0	20.0	62.5	27.3
%lowCHm > 10%	91.7	70.5	40.0	16.5	59.4	26.8
TSAT < 30%	100.0	100.0	29.4	14.1	57.1	24.6

threshold for that diagnosis was 6%, lower than the 10% currently recommended. Moreover, the sensitivity and specificity of TSAT and FRT in that study were much lower. Even though the value of %HYPO is well established, we have shown in previous studies that erythropoietic activity positively influenced %HYPO [14,24] and that %HYPO could be just increased due to the production of a younger population of erythrocytes with an increased cell volume and a normal Hb content, thereby leading to a decreased Hb concentration in this cell population that includes reticulocytes [15].

In this study, ROC curve analyses confirmed the value of several parameters for functional iron deficiency. In these analyses, sTfR had the largest AUC, directly followed by %HYPO and %HYPOm. However, the sensitivity, specificity and efficiency could not be estimated for sTfR since results were available in only five iron-deficient patients. In addition, despite its possible discriminative value, sTfR assays are not available everywhere and the cost associated to its measurement is much higher compared with that of red cell parameter determination that is part of CBC.

In the order of decreasing AUC, TSAT came after hypochromy with a good sensitivity (90.9%) but quite a low specificity (70.6%). The specificity of FRT levels < 100 ng/ml was perfect. However, its sensitivity was unacceptable from a clinical point of view. Actually, at that threshold, only about 25% of iron-deficient patients could be identified. From the ROC curve analyses, the best threshold for FRT was 400 ng/ml. At such a high cut-off, the sensitivity, specificity and efficiency were similar to those of %HYPO > 6%.

Parameters based on the Hb content of mature erythrocytes, CHm and %lowCHm, could have been of interest since, excluding the effect of reticulocytosis, they further exclude volume variations associated with the younger age of the erythrocyte population resulting from a stimulated erythropoiesis. However, their sensitivity, specificity and efficiency were lower than that for %HYPOm. The efficiency of calculated parameters on the global population (MCH and MCHC) was very low (75 and 62.5%, respectively), much lower than EBPG and DOQI guidelines and usual parameters of the iron monitoring. They thus do not provide any further information or advantage. This is probably due to the 'dilution' of hypochromic cells by the majority of the normochromic erythrocyte population. In this point of view, the %HYPO presents the advantage to detect hypochromic erythrocytes as soon as they leave the bone marrow. %HYPOm and %HYPO are then the more early markers of iron deficiency.

Despite their similar AUC, %HYPO and %HYPOm showed slight differences in terms of efficiency for the intended purpose, but they were not statistically significant. We believe that the stability of the patients in the steady state of the treatment of anaemia is, at least in part, responsible for the non-significance of these differences. The best efficiency was found for %HYPOm at a cut-off value > 6%. At that threshold, 87.5% of the patients were correctly classified as iron deficient or iron replete according to the defined criteria.

Therefore, %HYPOm slightly improves the detection of iron-deficient patients compared with the combination of FRT < 100 ng/ml and %HYPO > 10% [16] or FRT < 100 ng/ml and TSAT < 20% [17]. However, these differences were not statistically significant.

In this study, in spite of stability during the maintenance phase of rHuEPO treatment, about one-third of the patients improved their Hb levels with iron supplementation. This observation underlines the fact that functional iron deficiency remains underdiagnosed. Correct diagnosis and treatment could lead to rHuEPO dose sparing and cost saving in anaemia management.

Mature erythrocyte parameters, in particular %HYPOm, appear to be at least as efficient as %HYPO for iron monitoring. As this study was performed in a perfect steady state of anaemia therapy, erythropoietic activity was quite low as demonstrated by low reticulocyte counts. The induction phase of rHuEPO treatment represents a much stronger erythropoietic stimulation and an increased ratio between young and old erythrocyte that could further increase the efficiency of %HYPOm compared with %HYPO, but this remains to be studied.

Conflict of interest statement. None declared.

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