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Reticulocyte hemoglobin equivalent (Ret He) and assessment of iron-deficient states

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Summary Direct measurement of the reticulocyte hemoglobin content provides useful information for the diagnosis and treatment of iron-deficient states. We have examined direct measurements of reticulocyte and red cell hemoglobin content on the Sysmex XE 2100 (Ret He and RBC He respectively) and the Bayer ADVIA 2120 (CHr and CH respectively) analyzers. Good agreement was found between Ret He and CHr ($Y = 1.04X - 1.06$; $r^2 = 0.88$) and between the RBC He and CH parameters ($Y = 0.93X + 1$; $r^2 = 0.84$ $n = 200$) in pediatric patients and in normal adults (Ret He and CHr; $Y = 1.06X - 0.43$; $r^2 = 0.83$; $n = 126$; RBC He and CH; $Y = 0.94X + 1$; $r^2 = 0.87$; $n = 126$). In 1500 blood samples from patients on chronic dialysis, Ret He was compared with traditional parameters for iron deficiency (serum iron <40 μg/dl, Tsat <20%, ferritin <100 ng/ml, hemoglobin <11 g/dl) for identifying iron-deficient states. Receiver operator characteristic (ROC) curve analysis revealed values of the area under the curve for Ret He of 0.913 ($P < 0.0001$). With a Ret He cutoff level of 27.2 pg, iron deficiency could be diagnosed with a sensitivity of 93.3%, and a specificity of 83.2%. Ret He is a reliable marker of cellular hemoglobin content and can be used to identify the presence of iron-deficient states.

Keywords CHr, reticulocyte hemoglobin content, r-HuEpo (recombinant human erythropoietin), Iron replacement therapy, Ret He (reticulocyte hemoglobin equivalent)

Introduction

By directly measuring the mean hemoglobin content (pg) of red blood cell (RBC) precursors (Reticulocytes), early stages of iron deficiency may be identified, at a time that other traditional biochemical parameters are non-informative (Brugnara, 2000, 2003). The measurement of reticulocyte hemoglobin content is a direct assessment of the incorporation of iron into erythrocyte hemoglobin and thus a direct estimate of the recent functional availability of iron into the erythron (Brugnara *et al.*, 1994c, 1997; Buttarello *et al.*, 2004). The mean intracellular hemoglobin content of erythrocytes (MCH) is an all inclusive measure of both the availability of iron over the preceding 90–120 days, and of the proper introduction of iron into intracellular hemoglobin (Brugnara *et al.*, 1994b,c). The absence of significant interferences with the exception of concomitant alpha- or beta-thalassemia or macrocytosis, provides another potential advantage of these hematological markers over indirect biochemical testing, which is affected by a variety of factors, including inflammation. The CHr (mean cellular hemoglobin content of reticulocytes) parameter provided by the ADVIA 120 and 2120 (Bayer Diagnostics, Tarrytown, NY, USA) was cleared by the FDA in August 1997, for clinical application within the USA (K#971998). Since May 2005, an equivalent parameter, the Ret He (reticulocyte hemoglobin equivalent) has been available for use on the Sysmex XE 2100 (Sysmex Corporation, Kobe, Japan), broadening the availability of a tool for assessing reticulocyte hemoglobin content (K#050589).

Cellular content of Hb can be precisely assessed by flow cytometry in mature erythrocytes as well: the RBC He (Sysmex) and CH (Bayer) parameters express (in pg) the mean erythrocyte hemoglobin content. Examining both the precursors, and mature red cells provides an oppor-

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tunity to detect and monitor acute and chronic changes in cellular hemoglobin status.

In this report, we compare reticulocyte and red cell measurements of Hb content provided by the ADVIA 2120 and Sysmex XE 2100 analyzers in adult and pediatric patients and assess the diagnostic value of Ret He in a large population of adult patients on chronic dialysis.

Materials and methods

Studies in pediatric and adult samples

Samples collected in EDTA anti-coagulant were randomly extracted from the routine workload and run sequentially on both the XE 2100, and ADVIA 2120. Samples were run within 6 h of collection. The scope of the pathology included a variety of diseases considered retrospectively as being representative of the daily workload. Renal disease contributed 19% of the samples, Sickle Cell Anemia 4.5%, and Thalassemias 7%. Reference Range data were gathered from 126 normal adult subjects, all first year medical students participating in a blood drawing/analysis exercise. Studies were carried out under IRB approved protocols S-04-07-080 and NS05-05-005 (comparative analysis of hematology/coagulation analyzers).

Precision analysis for Ret He and RBC He was carried out according to NCCLS EP5 as follows: after a device familiarization period, Quality Control material (E-Check) was run in duplicate, twice daily, over a 20-day period. This allowed calculation of within run precision, SD of the run means, SD of the daily means and total imprecision.

Stability analysis for Ret He and RBC He was carried out as follows: 10 samples were selected, five normal and five abnormal. Aliquots of the selected samples were stored at room temperature, and 4 °C respectively. All samples were tested at baseline, 6, 12, 24 and 48 h in order to assess changes over time.

Studies in patients on maintenance dialysis

Blood samples (1500), utilizing residual material from the monthly blood draw from patients undergoing routine hemodialysis in an outpatient dialysis settings were analyzed at Satellite Laboratories on a Sysmex XE 2100 analyzer. This observational study was performed on left-over material, after all the clinically indicated analyses had been performed. Samples were collected anonymously; no identifying information was recorded during this study. No exclusion criteria were applied during this study. The patients were a representative sample of US ESRD patients with a mean age of 63.5 ± 15.4 years and a male/female ratio of 56/44%.

Patients were managed according to the recommendations of the NKF-K/DOQI (National Kidney Foundation, Kidney Disease Outcomes Quality Initiative) anemia guidelines (NKF-K/DOQI, 2001). All patients were treated with a variety of erythropoietin doses given three times a week, at the time of hemodialysis treatment. In addition, the majority of patients were treated with a maintenance dose of intravenous iron (100–200 mg of iron gluconate) weekly or every other week in order to maintain iron stores at the recommended levels. Forty-eight percent of the patients sampled had significantly elevated C-reactive protein values (>5 mg/l), consistent with the presence of underlying inflammation, a common finding in this patient population (Yeun *et al.*, 2000). Absolute iron deficiency in patients with kidney disease has been defined as serum ferritin levels <100 ng/ml and serum transferrin saturation (Tsat) <20% (NKF-K/DOQI, 2001).

Statistical analysis

Statistical analysis was conducted with the use of Analyse-it® statistical software add-in for Microsoft Excel (for Windows). Receiver operating characteristic (ROC) analysis was utilized to illustrate the diagnostic performance of ret He with ROC curves. Traditional diagnostic criteria for Iron deficiency anemia diagnosis, included as criteria for presence of disease in ROC curve generation: Serum Iron < 40 micrograms/dl, Tsat <20%, Ferritin <100 ng/ml, and Hemoglobin <11 g/dl). The traditional diagnostic criteria for 'Functional iron-deficiency' diagnosis used for ROC curve generation were Tsat <20%, Ferritin 100–800 ng/ml, and Hemoglobin <11 g/dl. The significance of areas under the ROC curves for ret He was assessed in comparison with no discrimination. A ret He cutoff was established based on the optimal combination of sensitivity (≥80%) and specificity (≥50%), decided *a priori* as minimum requirements of a screening test for iron deficiency. Values below this cutoff were considered to be abnormal.

Results

Pediatric samples and normal adult controls studies

Figure 1 presents correlation data for reticulocyte and red cell hemoglobin content respectively. Data were obtained from 200 clinical samples and show good correlation between the Ret He and ADVIA CHr ($Y = 1.04X - 1.06$; $r^2 = 0.88$) and between the RBC He and ADVIA CH parameters ($Y = 0.93X + 1$; $r^2 = 0.84$). Figure 2 shows

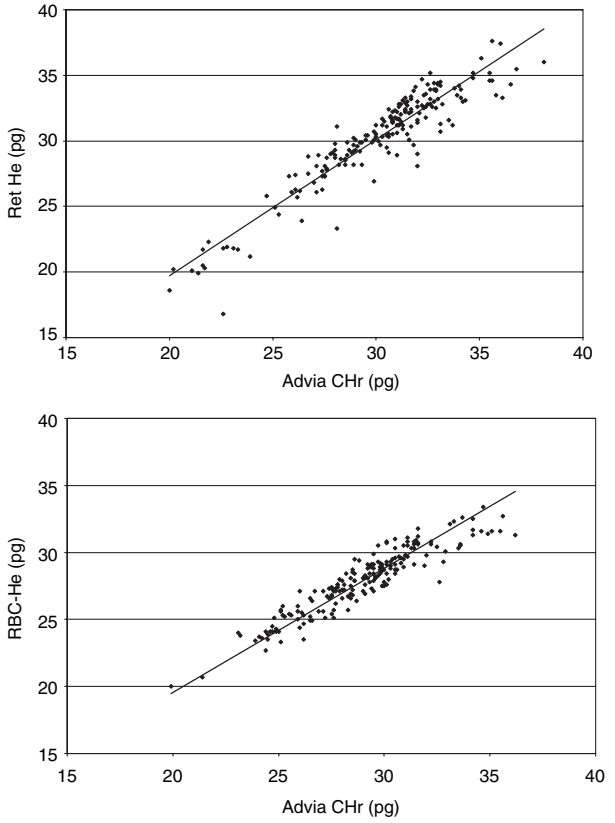

Figure 1. Method comparison data, XE 2100 and ADVIA 2120: top, Ret He *vs.* CHr; bottom RBC He *vs.* CH. Data were obtained from 200 clinical samples and show good correlation between the Ret He and ADVIA CHr ($Y = 1.04X - 1.06$; $r^2 = 0.88$) and between the RBC He and ADVIA CH ($Y = 0.93X + 1$; $r^2 = 0.84$).

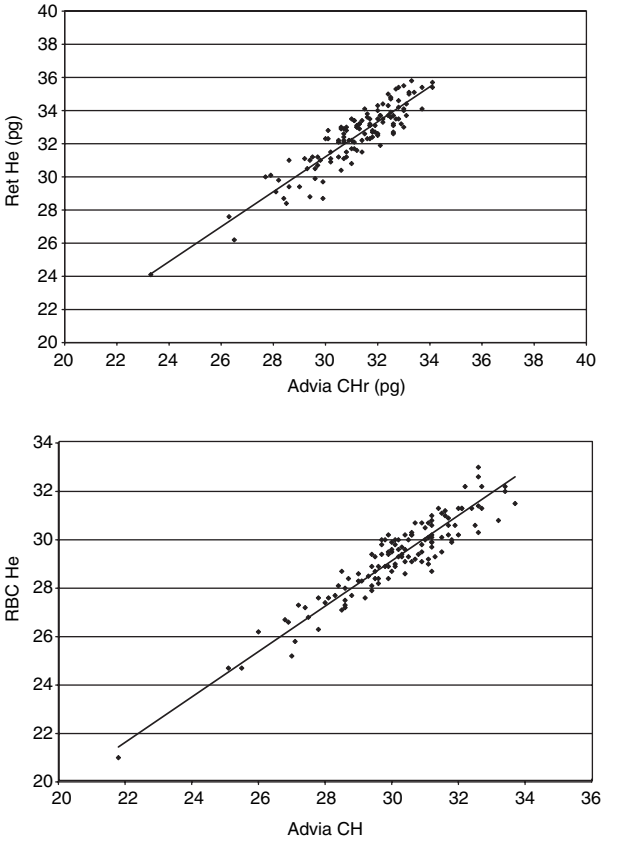

Figure 2. Method comparison data, XE 2100 and ADVIA 2120: top, Ret He *vs.* CHr; bottom RBC He *vs.* CH. Data were obtained from 126 normal adults, and show good correlation between the Ret He and ADVIA CHr ($Y = 1.06 - 0.43$; $r^2 = 0.83$; $n = 126$) and between the RBC He and ADVIA CH ($Y = 0.94X + 1$; $r^2 = 0.87$; $n = 126$).

similar correlation data obtained in 126 normal adult controls. Figure 3 presents a plot of the difference between ADVIA and Sysmex parameters, for both reticulocyte and erythrocyte hemoglobin content. There is a tendency for Ret He to yield values higher than CHr above 30 pg/cell, whereas RBC He tends to provide values lower than CH above 25 pg/cell.

Reference range data

Table 1 presents reference interval data gathered from 126 normal adult subjects.

Precision data

Precision data were collected over 20 days and revealed a total Ret He Imprecision with a SD of 0.85 pg (CV 3.92%), and a total RBC He Imprecision with a SD of 0.29 pg (CV 1.33%; Table 2).

Stability

Five normal and five abnormal samples were selected. Aliquots of the selected samples were stored at room temperature and 4 °C respectively. All samples were tested at baseline, 6, 12, 24 and 48 h in order to assess changes over time. Data show that both Ret He and RBC He are very stable, with <1SD change over 48 h in all samples tested (room temperature and refrigerated; Table 3).

Iron-deficiency diagnosis study

The goal was to determine the diagnostic performance of the Ret He parameter against the existing diagnostic tests for iron-deficiency diagnosis (serum iron < 40 μg/dl, Tsat <20%, ferritin <100 ng/ml and hemoglobin <11 g/dl; Figure 4). ROC curve analysis revealed that by using a Ret He cutoff level of 27.2 pg, iron deficiency could be diagnosed with a sensitivity of 93.3%, and a specificity of 83.2%. The area under the curve was 0.913 ($P < 0.0001$).

A similar exercise was undertaken to determine the diagnostic performance of the Ret He parameter when

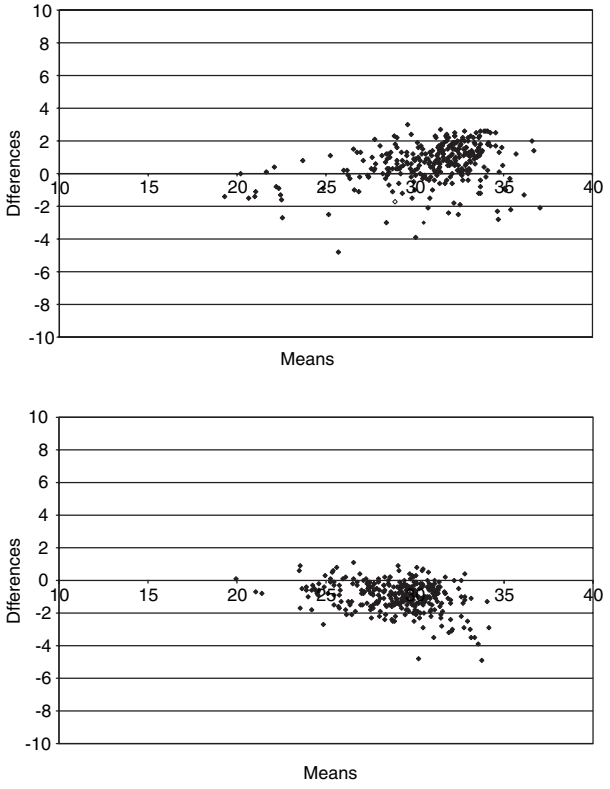

Figure 3. In the top panel, the difference between Ret He and CHr is plotted *vs.* the mean value for the two parameters. In the bottom panel, the difference between RBC He and CH is plotted *vs.* the mean value for the two parameters. Data were combined for the 200 clinical samples and the 126 normal controls.

Table 1. Reference range intervals for reticulocyte and erythrocyte hemoglobin content parameters in 126 normal adults

	CHr (pg)	Ret He (pg)	CH (pg)	RBC He (pg)
Mean	31.18	32.47	30.16	29.29
SD	1.67	1.93	1.82	1.74
Min	23.3	24.1	21.8	21
Max	34.1	35.8	33.7	33

Table 2. Precision analysis for Ret He and RBC He parameters

	Ret He SD (pg)	Ret He CV (%)	RBC He SD (pg)	RBC He CV %
Within run precision	0.32	1.49	0.27	1.23
SD of the run means	0.29	1.32	0.16	0.74
SD of the daily means	0.79	3.66	0.19	0.86
Total imprecision	0.85	3.92	0.29	1.33

compared with the traditional parameters for diagnosis of functional iron deficiency (FID). Functional iron deficiency was identified as Tsat <20%, ferritin 100–800 ng/ml, and hemoglobin <11 g/dl. Using a ret He cutoff level of

Table 3. Room temperature and refrigerated stability for Ret He and RBC He

	Change at 24 h		Change at 48 h		
Parameter	Room temp.	Refrig	Room temp.	Refrig	2× Baseline SD
Ret He	0.13	0.12	0.35	0.25	5.78
RBC He	0.2	0.11	0.12	0.03	3.74

Units, picograms of hemoglobin.

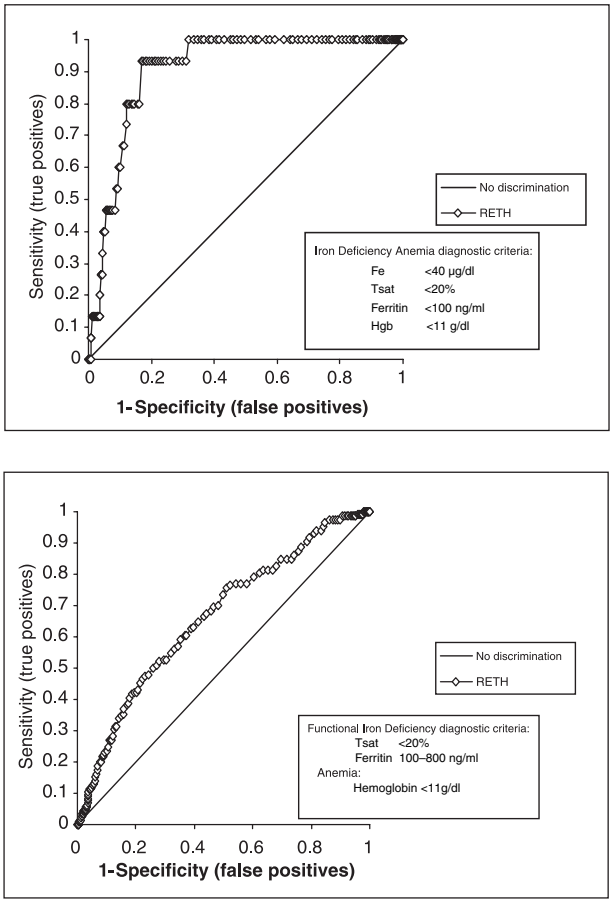

Figure 4. Top: ROC curve analysis for Ret He and the diagnosis of iron-deficiency anemia in hemodialysis patients. The diagnostic performance of Ret He in identifying iron-deficient states was compared with that of traditional parameters for iron deficiency (serum iron <40 µg/dl, Tsat <20%, ferritin <100 ng/ml, hemoglobin <11 g/dl). By using a Ret He cutoff level of 27.2 pg, iron deficiency could be diagnosed with a sensitivity of 93.3%, and a specificity of 83.2%. The area under the curve was 0.913. Bottom: ROC curve analysis for Ret He and the diagnosis of functional iron deficiency in hemodialysis patients. Functional iron deficiency was defined tests as Tsat <20%, Ferritin 100–800 ng/ml, and HGB <11 g/dl. The diagnostic performance of ret He is less favorable (area under the curve 0.657).

27.9 pg, FID could be diagnosed with a sensitivity of 40.2% and a specificity of 80.3%. The area under the curve was 0.657 ($P < 0.0001$).

Discussion

Measurements of reticulocyte hemoglobin content have been shown to provide useful information in diagnosing FID during r-HuEPO therapy (Brugnara *et al.*, 1994a), iron-deficient states in infancy (Brugnara *et al.*, 1999; Ullrich *et al.*, 2005) and response to iron therapy (Brugnara *et al.*, 1993, 1997; Thomas & Thomas, 2002). A limitation of this approach has been the fact that these parameters were available only on Bayer instruments. Recently, cellular hemoglobin content parameters have now become available on the Sysmex instruments (Canals *et al.*, 2005; Thomas *et al.*, 2005).

This study shows a very good level of agreement between the cellular Hb measurements performed with the Sysmex XE 2100 and those performed with the Bayer ADVIA instrument. The precision and stability of both Ret He and RBC He are excellent: minimal variations are seen over time in either room temperature of 4 °C storage. Normal range values for the reticulocyte and red cell hemoglobin content parameters are superimposable between the two different methods.

In patients on maintenance dialysis, the diagnostic performance of Ret He, when compared to traditional parameters for iron-deficiency anemia diagnosis, was excellent, with an ROC area under the curve of 0.913 ($P < 0.0001$).

Iron stores estimates do not provide an assessment of the availability of iron for introduction into erythrocyte hemoglobin. This lead to the establishment of a concept called 'functional iron-deficiency', and has particular relevance in patients receiving recombinant human erythropoietin (rHuEPO; Macdougall *et al.*, 1992; Horl, 1995; Macdougall, 1995). In anemic patients receiving therapeutic doses of rHuEpo, the cellular requirements for iron are increased, and can lead to decreased iron incorporation into cellular hemoglobin, which is thought to hamper the effectiveness of rHuEpo therapy. Patients deemed to be 'functionally iron-deficient' during rHuEpo therapy, are given additional iron, in an effort to compensate for the relative deficiency (Macdougall *et al.*, 2000; Valderrabano *et al.*, 2000). In diagnosing 'functional iron-deficiency', the diagnostic performance of Ret He, when compared with traditional parameters was not as impressive, with a much smaller ROC area under the curve of 0.657 ($P < 0.0001$). However, in the absence of assessing the response to IV iron, there are no uniformly accepted criteria to identify functional iron-deficiency (Fishbane *et al.*, 1997). The presence of inflammation and uremia makes this diagnosis particularly challenging for dialysis patients (Sunder-Plassmann, Spitzauer & Horl, 1997). Attempts to use the baseline Ret He results as a tool to identify potential responders to increased IV iron therapy were unsuccessful in the 'functional iron-deficiency' subgroup (data not shown). This finding suggests that more than one sampling may be required in order to adequately monitor responses to therapy.

In conclusion, this study shows a very good level of agreement between cellular hemoglobin measurements performed by two different analyzers in both reticulocytes and erythrocytes. The expanded availability of these parameters should allow further assessment of their role in diagnosing disturbances of iron metabolism and assessing response to iron supplementation therapy.

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