Diagnosis of iron deficiency (ID) or functional iron deficiency (FID) is particularly challenging in patients with acute or chronic inflammatory conditions because normal biochemical markers for iron metabolism are affected by the acute-phase response (APR) (1). The hemoglobin content of reticulocytes (CHR) is an early and sensitive indicator of FID (2). Recently, we presented a novel approach to provide insights into the diagnosis of FID in APR by use of the CHR (3). ID and FID were defined as a CHR <28 pg based on the distribution of CHR and biochemical markers of iron status in healthy controls. When a CHR <28 pg was used for identification of ID and FID in anemic patients, the values of ferritin, soluble transferrin receptor (sTfR), and the sTfR-F index (sTfR/log ferritin) (4) performed significantly better in patients without APR [based on a C-reactive protein (CRP) cutoff of 5 mg/L]. In ID combined with inflammation, the cutoff value for the sTfR-F index was 0.8, and in simple ID, it was 1.5. A diagnostic plot was developed that combined CHR and sTfR-F index, which allowed identification of four major categories of ID: (a) iron repletion, normal erythropoiesis; (b) patients with reduced iron supply, but not yet in an iron-deficient erythropoietic state; (c) depletion of storage and functional iron with decreased hemoglobinization of erythrocytes, classic ID; and (d) FID in an iron-replete state, with decreased hemoglobinization of erythrocytes. The plot provided a useful approach to the diagnosis of iron-deficient states.

To date, the measurement of reticulocyte hemoglobin has been restricted to the analyzers of a single manufacturer. Now a second manufacturer has produced what would appear to be a comparable index, the so-called RET-Y (5, 6) generated by the Sysmex XE-2100 analyzer. The RET-Y is the mean value of the forward-scattered-light histogram within the reticulocyte population. A corresponding value, the RBC-Y, is the mean value of the forward-scattered-light histogram within the mature erythrocyte population. Preliminary studies (see below) have demonstrated a good correlation between RBC-Y and mean cell hemoglobin (MCH), better, in fact, than with mean cell volume (MCV). A mathematical transformation applied to RBC-Y can therefore produce a hemoglobin equivalent for erythrocytes (RBC-H₂) expressed in picograms. Applying the same transformation to the RET-Y gives a reticulocyte hemoglobin equivalent expressed in picograms. An appropriate name for this index would be reticulocyte hemoglobin equivalent (RET-H₂).

The objectives of this study were twofold: (a) to establish the diagnostic equivalence of the RET-Y (RET-H₂) with the CHR; and (b) to describe its clinical assessment in the diagnostic plot combining the RET-Y with the sTfR-F index as a tool for the diagnosis and therapeutic monitoring of iron-restricted erythropoiesis.

During a 6-month period, we studied 474 adult anemic patients (221 men and 253 women) with hemoglobin <140 g/L for men and <120 g/L for women. Specimens were collected for complete blood cell count, CHR, Ret-Y, sTfR, ferritin, and CRP within 24 h of admission. The patient diagnoses included 162 cancer-related anemias, 142 anemias of end-stage renal failure, 49 anemias of inflammatory disorders, 34 anemias of pregnancy, and 87 anemias of heterogeneous origin.

Blood counts were performed with the Advia 120 (Bayer Diagnostics) and Sysmex XE-2100 (Sysmex Corporation) automated hematology analyzers. In the reticulocyte channel of the Sysmex XE-2100, the sample, stained by a polymethine dye specific for RNA/DNA, is analyzed by flow cytometry by use of a semiconductor laser. A two-dimensional distribution of forward-scattered light and fluorescence is presented as a scattergram indicating mature erythrocytes and reticulocytes. Ret-Y is the mean value of the forward-scattered-light histogram of the
reticulocyte population, currently expressed in arbitrary units (AU). CRP was measured with the Vitros clinical chemistry analyzer (Ortho Diagnostics), ferritin was measured with the Elecsys 2010 analyzer (Roche Diagnostics), and the sTfR was measured with the BN ProSpect immunoanalyzer (Dade Behring). The reproducibility for erythrocytes, hemoglobin, hematocrit, MCV, reticulocyte count, Ret-Y, and CHr showed within-run imprecision (CV) of 0.9–1.6% for the Sysmex XE-2100 and 0.7–1.2% for the Advia 120 and between-run imprecision of 1.3–4.5% for the Sysmex XE-2100 and 1.3–1.6% for the Advia 120.

To evaluate which cellular features had a major impact on the forward-scattered-light signal, we compared RBC-Y with MCV and MCH. Although the results showed good correlation between RBC-Y and MCV ($r^2 = 0.85$), an even better correlation existed between RBC-Y and MCH ($r^2 = 0.97$). The regression curve for both comparisons, however, showed a nonlinear shape expressed by the exponential functions $y = 25.933e^{0.001x}$ for MCV and $y = 5.5569e^{0.001x}$ for MCH. The regression formula (function $y = 5.5569e^{0.001x}$) might also be used to transform the arbitrary channel numbers of the RBC-Y and RET-Y into their respective hemoglobin equivalents, expressed as picograms and denoted as RBC-He and RET-He, respectively. These results showed that the forward scatter signal provided a better measurement of the cell content (and thus hemoglobin) than the cell volume.

Method comparison between CHr and Ret-Y demonstrated a curvilinear relationship as shown in Fig. 1. The 95% central range of the CHr (28–35 pg) published recently by our group (3) corresponded to a range for Ret-Y of 1630–1860 AU. When we transformed RET-Y data to RET-He (in picograms) and compared those values with CHr, we found a good correlation between the two measurements ($r^2 = 0.92$). The 95% central range of the RET-Y (1630–1860 AU) corresponded to a calculated range for RET-He of 28.2–35.7 pg. This indicated that both methods are measuring the same cellular components and that the conversion function for this patient data set is correct.

Comparison between CHr and Ret-Y showed that a Ret-Y value <1630 AU corresponded to a CHr <28 pg. We then evaluated the ability of the sTfR-F index to indicate ID, using the Ret-Y value instead of CHr as the cutoff for iron-deficient erythropoiesis. The sTfR-F index performed significantly better in the absence of APR; the cutoff was 1.5 in patients without and 0.6 in patients with a CRP concentration >5 mg/L.

The diagnostic plot combines the CHr or the Ret-Y with the sTfR-F index to assess the relationship between iron supply for erythropoiesis (sTfR-F index) and FID (CHr and Ret-Y). The data point distribution of the 474 patients with and without APR showed a distribution pattern as shown in our previous report (3). When we replaced the Ret-Y by CHr on the $y$ axis of the diagnostic plot and reanalyzed the two patient groups with and without APR, the results were identical except for 25 of the 474 patients (5.3%).

The final study involved assessment of the selectivity of the Ret-Y compared with that of CHr in disease-specific anemias. Mismatches ranged from 2.9% in pregnancy to 6.2% in cancer-related anemia (Table 1).

In conclusion, the reticulocyte channel of the Sysmex XE-2100 series offers a new reticulocyte index, the Ret-Y, which shows good correlation with the CHr. When we transformed the RET-Y into RET-He (in picograms), based on the equation of the regression curve obtained from the comparison of RBC-Y and MCH, the values for RET-He and CHr showed excellent correlation and a linear fit of the regression line. When we replaced CHr by Ret-Y in the diagnostic plot and reanalyzed the 474 patients with different disease-specific anemias, placement of individual patients in the correct quadrant was virtually identical. This shows that the Ret-Y is dependent only on iron metabolism and is not influenced by disease-specific factors and that the indices CHr, Ret-Y, and consequently, RET-He, are measuring the same phenomenon.

### Table 1. Mismatches of specific anemias between CHr and RET-Y in 474 patients.

<table>
<thead>
<tr>
<th>pregnancy</th>
<th>ERF*</th>
<th>CRA</th>
<th>Infection</th>
<th>Hetero</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>1/34</td>
<td>7/142</td>
<td>10/162</td>
<td>2/49</td>
</tr>
<tr>
<td>%</td>
<td>2.9</td>
<td>4.9</td>
<td>6.2</td>
<td>4.1</td>
</tr>
</tbody>
</table>

* ERF, end-stage renal failure; CRA, cancer-related anemia; Hetero, heterogeneous diseases.
index, Ret-Y (RET-H2), appears to be clinically equivalent to the CHr and offers an attractive potential tool for the diagnosis and monitoring of iron-restricted erythropoiesis.

References

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Denaturing HPLC-Based Assay for Molecular Screening of Nondeletional Mutations Causing α-Thalassemias, Valentina Guida,1,2 Alessia Colosimo,1,2 Mirella Fiorito,1,2 Enrica Foglietta,2 Ida Bianco,4 Giovanni Ivaldi,5 Marco Fichera,5 and Bruno Dallapiccola1,2 (1 Istituto di Ricovero e Cura a Carattere Scientifico [IRCCS]-Casa Sollievo Sopferenza [CSS], San Giovanni Rotondo and CSS-Mendel Institute, Rome, Italy; 2 Department of Experimental Medicine and Pathology, University of Rome “La Sapienza”, Rome, Italy; 3 Department of Biomedical Sciences, University “G. D’Annunzio”, Chieti, Italy; 4 Centro Microcitemia Associazione Nazionale Microcitemia Italiana-ONLUS, Rome, Italy; 5 Human Genetic Laboratory, Galliera Hospital, Genoa, Italy; 6 Laboratorio di Patologia Genetica IRCCS Oasi Maria Santissima, Troina, Italy; * address correspondence to this author at: CSS-Mendel Institute, Viale Regina Margherita 261, 00198 Rome, Italy; fax 39-06-44160548, e-mail v.guida@css-mendel.it)

α-Thalassemias (OMIM 141850 and 141800; GenBank accession no. NT037687) are recessively inherited hemoglobin disorders caused by loss of function of either one of the two duplicated α-globin genes (α1 and α2), both located on chromosome 16p13.3 (1, 2). More than 95% of α-thalassemia phenotypes result from meiotic unequal recombinational events between the highly homologous α1- and α2-globin loci, which lead mainly to large genomic deletions (3–100 kb), which remove one to four α-globin genes, and rarely to α-gene triplcation or quadruplication. Although less frequent, at least 48 different nondeletional mutations (including point mutations and deletions/insertions of a few nucleotides), mostly located in the α2-globin gene, have also been reported as causative mutations of α-thalassemia (3, 4). At present, molecular identification of this type of nucleotide mutation is carried out by specific PCR amplification of the α2 or α1 gene, followed by methods that have only a limited rate of detection (60–80%) and are technically demanding, such as single-strand conformation polymorphism (SSCP) analysis and denaturing gradient gel electrophoresis (5, 6), or are costly, cumbersome, and time-consuming, such as direct sequencing and reverse dot-blot analysis (7, 8).

We have evaluated the performance of a relatively simple and semiautomated technique, denaturing HPLC (DHPLC), that separates heteroduplex and homoduplex molecules on a stationary phase under partially denaturing conditions (9, 10). We tested a Transgenomic Wave DHPLC-based protocol for the molecular identification of α-globin gene nondeletional mutations in 50 wild-type individuals and 50 heterozygous carriers of Italian origin whose genes had previously been molecularly defined by restriction endonuclease digestion of PCR fragments and/or reverse dot-blot analysis.

Blood samples were collected from heterozygous individuals and healthy controls at the Centro Studi Microcitemia (Rome), the Galliera Hospital Genova, and the Oasi ONLUS Troina after informed consent was given. Genomic DNA was isolated from leukocytes in peripheral blood by salting out procedures (11). Purified DNA was solubilized in Tris-EDTA buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8) and stored at −20 °C. Four overlapping primers (DHPLC1 through -4), were designed to amplify the entire region of both α-globin genes, including the 5′ and 3′ noncoding regions, at the same temperature. Because the third exon and the 3′-untranslated region of both α-globin genes are considered mutational hot-spot regions, we chose two pairs of specific primers located in regions of sequence dissimilarity, thus allowing the selective amplification and analysis of α1 or α2 genes. Furthermore, because the α-globin gene cluster is located within a GC-rich DNA region, which makes clean amplification quite difficult, we explored the use of different Taq DNA polymerases, also changing the composition of the Mg2+-containing buffer. After testing several commercially available DNA polymerases, including AmpliTaqTM (PE Applied Biosystems), Taq GoldTM (PE Applied Biosystems), and Optimase DNA polymerase (Transgenomic; data not shown), we used Platinum® Taq DNA Polymerase High-Fidelity (InvitrogenTM). After empirical optimization, we performed PCR amplifications in a GeneAmp PCR system 9700 (PE Applied Biosystems); the PCR mixture (total volume, 25 μL) contained 250 μM each of the deoxynucleotide triphosphates, 100 ng of template DNA, 0.5 μM each of the primers, and 1.25 U of Platinum Taq DNA Polymerase High Fidelity (Invitrogen) in 1× reaction buffer [60 mM Tris-SO4 (pH 8.9), 18 mM (NH4)2SO4, and 2.0 mM MgSO4]. To preserve the DNAsep column, we replaced the invitrogen buffer with the 1× Transgenomic Optimase buffer (2 mM MgSO4), obtaining the same results. The PCR conditions consisted of an initial denaturation step (94 °C for 3 min), followed by 30 cycles at 94 °C for 30 s, 62 °C for 30 s, and 68 °C for 45 s, with a final extension step of 7 min at 68 °C.

Each amplicon was heated at 95 °C for 5 min and cooled