Clinical utility of Reticulocyte Hemoglobin and Hypochromic erythrocytes reported by Mindray BC6800 Plus hematology analyzer in the study of erythropoiesis

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RET Channel —— Cell Processing Method

Before Spheroidization

- Laser
- RBC
- RET
- PLT

After Spheroidization

- Laser
- RBC
- RET
- PLT

DR Diluent

Florescent staining

FR Dye

Spheroidization of cells

fluorescence intensity

+++

++

+
HFR: High Fluorescence Reticulocytes

RHE: (reticulocyte hemoglobin content) Provides clinical information for differential diagnosis and monitoring of erythopoiesis

IPF (Immature Platelet Fraction) can guide Platelet transfusion after stem cell transplantation, and may also be used as an index of thrombopoietic activity in bone marrow.
**RBCs 3D 9-square Graph**

**Demands:**
1. Using Mie scatter to calculate the single Corpuscular Hemoglobin (RBCs 3D 9-square Graph) by Siemens is recognized by the industry.
2. Reticulocyte hemoglobin has specific value on clinical treatment of iron deficiency anemia, and differential diagnosis of iron deficiency anemia/thalassemia.

**Present situation:**
- Sysmex XN: FSC calculates HGB
- ADVIA 2120i: Mie scatter calculates RBC volume/HGB

**Advance hematology analyzer:**
Mie scatter calculates Single Corpuscular Hemoglobin Concentration/volume

Ballistic Interpolation Algorithm
Based on SFS to HC(Vol) Grid
<table>
<thead>
<tr>
<th><strong>Meaning</strong></th>
<th><strong>Advanced Hematology analyzer</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Corpuscular Hemoglobin Concentration</td>
<td>MCHC</td>
</tr>
<tr>
<td>Single Corpuscular Hemoglobin Concentration</td>
<td>MCH-O(RUO)</td>
</tr>
<tr>
<td>HGB Distribution Width</td>
<td>HDW</td>
</tr>
<tr>
<td>Reticulocyte Hemoglobin Expression</td>
<td>RHE (MCHr,RH,RCH)</td>
</tr>
<tr>
<td>Mean Reticulocyte Volume</td>
<td>MVCr (RCV,RMCV)</td>
</tr>
<tr>
<td>Mean Platelet Component Conc,</td>
<td>MPC</td>
</tr>
<tr>
<td>Microcyte count</td>
<td>Micro#</td>
</tr>
<tr>
<td>Microcyte percentage</td>
<td>Micro%</td>
</tr>
<tr>
<td>Macrocyte count</td>
<td>Macro#</td>
</tr>
<tr>
<td>Macrocyte percentage</td>
<td>Macro%</td>
</tr>
<tr>
<td>The percentage of hyperchromic red blood cells</td>
<td>HYPER%</td>
</tr>
<tr>
<td>The percentage of hypochromic red blood cells</td>
<td>HYPO%</td>
</tr>
<tr>
<td>Reticulocyte Production Index</td>
<td>RPI</td>
</tr>
</tbody>
</table>

**RBCs 3D 9-square Graph**

![Graphs showing RBC scatter, volume, RBC volume, RBC HC, and RET CH plots.](attachment:image)
Innovative erythrocyte parameters

These innovative parameters most typically include automated reticulocyte and nucleated RBC counts, **hemoglobinization of reticulocytes and RBC** reticulocyte maturation, automatic analysis and **calculation of microcytic and hypochromic RBC**

The various combination of these different parameters not only may be useful to complement clinical history, physical examination and results of more conventional laboratory investigations for investigating the underlying cause(s) of anemia.

G Lippi & M Plebani. Recent developments and innovations in red blood cells diagnostics
*Journal of Laboratory and Precision Medicine* 2018 DOI: 10.21037/jlpm.2018.07.09  AOP
<table>
<thead>
<tr>
<th>RBC extended parameters and Reticulocyte Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hypochromic RBC</strong></td>
</tr>
<tr>
<td><strong>Reticulocyte Hb</strong></td>
</tr>
<tr>
<td><strong>Hypochromic RBC</strong></td>
</tr>
<tr>
<td><strong>Mean Reticulocyte Hb</strong></td>
</tr>
<tr>
<td><strong>Hypochromic RBC</strong></td>
</tr>
<tr>
<td><strong>Equivalent Reticulocyte Hb</strong></td>
</tr>
<tr>
<td><strong>Low Hb Density</strong></td>
</tr>
<tr>
<td><strong>Red Cell Size Factor</strong></td>
</tr>
<tr>
<td><strong>Reticulocyte Hb Content</strong></td>
</tr>
<tr>
<td><strong>Reticulocyte Hb Expression</strong></td>
</tr>
<tr>
<td><strong>Hypochromic RBC</strong></td>
</tr>
</tbody>
</table>


Bessman JD et al. Improved classification of anemias by MCV and RDW
%MIC, %MAC (microcytic & macrocytic RBC) proportion of RBC with volume < 60 fL and > 120 fL

%HPO, %HPR (hypochromic & hyperchromic RBC) proportion of RBC with CHC < 280 g/L and > 410 g/L

more sensitive marker because small changes in the number of RBC with inadequate Hb can be accurately measured
RBC Extended parameter graphics
RBC cytogram Mie Map

RBC classified by volume & Hb concentration
Reticulocytes are immature erythrocytes precursors and their conversion to erythrocytes takes 3 to 4 days, first in the bone marrow and in the last 1-2 days in the circulation.

Circulating reticulocytes do not synthesize hemoglobin, unlike reticulocytes in the bone marrow.

The measurement of their Hb content reflects the amount of iron immediately available for erythropoiesis and provides direct information on iron-deficient erythropoiesis over the previous period of 3-4 days.
Flow Cytometry

LASER BEAM

High angle Side scatter Hb

Low angle Forward scatter VOLUME

Absorbance Fluorescence RNA content
Reticulocyte derived parameters

Reticulocyte count is a quantitation of bone marrow activity, and the base to classify anemia as regenerative.

Reticulocyte Volume and Hb content, describe the quality of erythropoiesis:
- Adequate supply of nutrients for Hb synthesis
- Early detection of negative iron balance requirements/supply
Reticulocyte Hemoglobin Expression-RHE

RET-Reticulocyte:
the most newly released precursor cells from the peripheral blood of the bone marrow, and short life cycle, and it can respond to the hematopoiesis of bone marrow in time.

**RHE (Reticulocyte Hemoglobin Expression):**
Early index of functional iron deficiency / iron deficiency erythrohemopoiesis / iron deficiency anemia;
RHE gradually decreases with the increase of iron deficiency, and is better than HGB/MCV for detection of iron deficiency, especially pure iron deficiency in underaged females.

**RHE decreases:**
α-thalassemia/β-thalassemia / chronic anemia

**RHE increases:**
megalocytic anemia: such as folic acid and vitamin B12 deficiency
influence factors: recent blood transfusion / iron therapy etc.
Macrophages that normally recycle iron, as a consequence of inflammation, sequester it
Consequently, decreased serum iron is available for erythropoiesis

**Functional Iron deficiency**: Lack of availability of pool iron storage in relation to the demands,
to maintain a erythropoiesis level adapted
The Clinician’s Need for Reliable Laboratory Tests

Recombinant human erythropoietin (rHuEpo) for the treatment of patients with anemia related CKD has been available since 1989

Monitoring erythropoietin treated patients’ iron status is important to detect iron deficiency and avoid the adverse effects of iron medication

To evaluate iron available for erythropoiesis:

- transferrin saturation
- percentage of hypochromic red cells (Siemens) 6 %
- reticulocyte hemoglobin content (Siemens) 30 pg

Guideline for the laboratory diagnosis of functional iron deficiency
Br J Hematol 2013; 161:639-648

- MCH, MCV 1B
- % Hypochromic RBC 1B
- Reticulocyte Hb 1B
- Protoporphyirin Zn 1B
- Bone Marrow 1B
- Ferritin 1 A 1 B
- sTfR 1A
- Sat transferria 1 A 1 B
- Erythropoyetin 1 A
- Hepcidin UE

Recommendation

- The %HRC is the best-established variable for the identification of functional iron deficiency (FID) and thus has the greatest level of evidence (Tessitore et al, 2001). CHr is the next most established option. Both tests have limitations in terms of sample stability or equipment availability. Other parameters may be as good but there is no evidence that they are any better, and generally there is less evidence for newer red cell and reticulocyte parameters.

- A CHr value <29 pg predicts IRE in patients with iron deficiency anaemia, FID and those receiving ESA therapy. A Ret-He value <25 pg predicts FID in those receiving ESA therapy. Among reticulocyte variables, a Ret-He value <30.6 pg appears to be the best predictive value for response to intravenous iron in CKD patients on haemodialysis.
The evolution of erythropoiesis in response to therapy

Aids clinicians in decision making
Automated, cheap, fast
Reliable measurement

Turning technology into better caring
Clinical utility of Reticulocyte Hemoglobin and Hypochromic erythrocytes reported by Mindray BC6800 Plus Hematology Analyzer in the study of erythropoiesis

330 samples collected in K2EDTA anti-coagulant were run sequentially on both Sysmex XN-20 and Mindray BC 6800 Plus Analyzers.

The scope of the pathology included a variety of diseases representative of the daily workload: 80 healthy subjects, 84 iron deficiency anemia IDA, 87 anemia of chronic disease ACD, 79 thalassemia carriers.

C reactive protein, S- Iron, Transferrin saturation, s-Ferritin, soluble transferrin receptor (sTfR)

Kolmogorov-Smirnoff was used to verify normality.

Correlation between CHr and Ret He was assessed with Spearman’s coefficient; a polynomial equation for non-linear correlations was applied (HYPO and Hypo He).

ROC was used to assess the diagnostic performance of CHr, and HYPO for detecting iron deficient erythropoiesis. Gold standard for low iron availability was sTfR >52 nmol/L.
Clinical utility of Reticulocyte Hemoglobin and Hypochromic erythrocytes reported by Mindray BC6800 Plus Analyzer in the study of erythropoiesis

Sweked distribution was proven for CHr, Ret-He, HYPO and Hypo He

Median and 25-75th quartiles in healthy subjects
CHr 33.3 pg, 32.0-34.5 pg; HYPO 0.1 % 0.1-0.3%
Ret He 30.0 pg 29.3-32.3 pg Hypo He 0.2 % 0.1-0.6%

Whole range RetHe 20.6-42.5 pg, CHr 25.0-46.1 pg
HypoHe 0.1-14 % %Hypo 0.1-30 %

Linear correlation Ret-He and CHr
y=1.054x-1.86. (95%CI -5.2-1.7 slope; .95-1.1 intercept)

Correlation between HypoHe and HYPO can be described by a 2nd degree polynomial equation
y=0.0082x² +0.765x+0.446
We aimed to study the diagnostic performance of these parameters of hemoglobinization of red cells in the detection of functional iron deficiency

<table>
<thead>
<tr>
<th></th>
<th>AUC</th>
<th>95%CI</th>
<th>CutOff</th>
<th>Sensitivity%</th>
<th>Specificity%</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHr</td>
<td>0.934</td>
<td>0.892–0.963</td>
<td>29.0 pg</td>
<td>82.9</td>
<td>98.6</td>
</tr>
<tr>
<td>Hypo</td>
<td>0.918</td>
<td>0.872–0.951</td>
<td>6.0 %</td>
<td>76.4</td>
<td>97.3</td>
</tr>
</tbody>
</table>
Technological advances in automated full blood count analysers allows the hemoglobin content of individual red cells to be measured by flow cytometry, so it is possible to calculate the number of individual red cells with low hemoglobin content.

CHr is a reliable marker to provide an estimation of the iron available for erythropoiesis improving the evaluation of iron requirements identifying iron deficient erythropoiesis.

RetHe and CHr are directly comparable.
Reticulocyte Hemoglobin (CHr) reported by Mindray BC6800 Plus in the study of anemia

416 samples were run on Mindray BC 6800 Plus analyzer. The scope of the pathology included a variety of diseases representative of the daily workload:
- 80 healthy subjects
- 202 microcytic anemia: thalassemia carriers and IDA
- 91 normocytic anemia: hematology malignancies and ACD
- 37 macrocytic anemia: lack of vitamin B12 or folate and MDS

C reactive protein,
Serum iron, t
Transferrin saturation,
Ferritin
Soluble transferrin receptor (sTfR)
Folate/vitamin B12

Kolmogorov-Smirnoff was used to verify normal distribution of data. Differences among groups were assessed using analysis of variance, considering \( P < 0.05 \) to be significant. For post hoc comparisons of outcomes between each pair of groups, Scheffé correction was applied.

Correlation coefficient between erythrocyte indices and CHr was calculated using the method of Pearson.

Receiver operating characteristic analysis was used to assess the diagnostic performance of CHr for detecting iron deficient erythropoiesis. Gold standard for iron deficiency was sTfR >52 nmol/L.
Healthy subjects CHr 33.2 pg, 28.9-37.5 pg
Microcytic anemia mean 23.7 pg, SD 2.75 pg
Normocytic anemia Mean 32.3 pg, SD 3.84 pg
Macrocytic anemia Mean 37.9 pg, SD 4.41 pg

In the microcytic group, the values in patients with IDA (CHr mean 23.8 pg, SD 1.7pg) and thalassemia carries (CHr mean 23.1 pg, SD 3.0 pg) were not significantly different P=0.0756.
➢ Patients with restricted erythropoiesis, due to lack of iron or globin, had similar low values

Values over the reference range in the macrocytic group is not related to iron status, reflects the megaloblastosis
Reticulocyte Hemoglobin (CHr) reported by Mindray BC6800 Plus in the study of anemia

<table>
<thead>
<tr>
<th>AUC</th>
<th>95%CI</th>
<th>CutOff</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHr</td>
<td>0.934</td>
<td>0.906 - 0.936</td>
<td>29.0 pg</td>
<td>82.9 %</td>
</tr>
</tbody>
</table>

Correlation MCH/CHr $R^2=0.9232$  
$P<0.0001$ (95%CI 0.906-0.936)
Reticulocyte Hemoglobin (CHr) reported by Mindray BC6800 Plus in the study of anemia

✓ Disturbances in erythropoiesis and iron metabolism may occur in many patients, the challenge is to identify these patients as early as possible

✓ Technological advances in automated full blood count analysers allows the hemoglobin content of individual red cells to be measured by flow cytometry, so it is possible to calculate the number of individual red cells with low hemoglobin content

✓ CHr provides a sensitive method for quantifying the hemoglobinization of reticulocytes

✓ It is a reliable marker to identify iron deficient erythropoiesis, CHr may allow the complete scope of disorders of iron metabolism to be identified quickly and managed
Disorders Characterized by Microcytosis

Iron deficiency
Anemia of inflammation

Acquired or genetic?

Iron

Protoporphyrin

Heme

Iron deficiency
Anemia of inflammation

Globin

Sideroblastic anemia

Hemoglobin

Thalassemia

Ineffective erythropoiesis

Erythroblast

Excess α-globin chain synthesis with reduced β-globin synthesis

Hemichromes

Unpaired α-globin chains form hemichromes

Inclusion bodies

Apoptosis

Mutations and deletions, chromosomes 11 (β-thal) and 16 (α-thal)

β-thalassemia

SPECTRUM OF IRON DEFICIENCY
Screening of Hb disorders must rely on inexpensive methods

“Suspicious” samples can be selected to confirm diagnosis: allow an efficient use of the resources

Improvement the Laboratory workflow and efficiency: lean system of high throughput

CBC  Appropriate screening, detection of carriers, and counsel of couples
Clinicians reach a prompt accurate diagnosis: reduces unnecessary diagnostic testing and avoid inappropriate treatment

*Turning technology into better caring*
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value 1</th>
<th>Value 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>5.34</td>
<td>5.03</td>
</tr>
<tr>
<td>Hb</td>
<td>124 g/L</td>
<td>118 g/L</td>
</tr>
<tr>
<td>MCV</td>
<td>74.3 fL</td>
<td>75.1 fL</td>
</tr>
<tr>
<td>MCH</td>
<td>23.2 pg</td>
<td>22.7 pg</td>
</tr>
<tr>
<td>MCHC</td>
<td>312 g/L</td>
<td>301 g/L</td>
</tr>
<tr>
<td>RDW</td>
<td>16.4 %</td>
<td>17.8 %</td>
</tr>
</tbody>
</table>
## Erythrocyte Indices
differential diagnosis of microcytic anemia

<table>
<thead>
<tr>
<th>Indices</th>
<th>Year</th>
<th>IDA</th>
<th>β Thalassemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mentzer = MCV / RBC</td>
<td>1973</td>
<td>&gt; 13</td>
<td>&lt; 13</td>
</tr>
<tr>
<td>Srivastava = MCH / RBC</td>
<td>1973</td>
<td>&gt; 3.8</td>
<td>&lt; 3.8</td>
</tr>
<tr>
<td>England &amp; Fraser = MCV - RBC - 5*Hb - 3.4</td>
<td>1976</td>
<td>&gt; 0</td>
<td>&lt; 0</td>
</tr>
<tr>
<td>Ricerca = RDW / RBC</td>
<td>1987</td>
<td>&gt; 4.4</td>
<td>&lt; 4.4</td>
</tr>
<tr>
<td>Green &amp; King = MCV^2 * RDW / 100* Hb</td>
<td>1989</td>
<td>&gt; 65</td>
<td>&lt; 65</td>
</tr>
<tr>
<td>MH ratio (Technicon) = %micro / %hypo</td>
<td>1992</td>
<td>&lt; 1</td>
<td>&gt; 1</td>
</tr>
<tr>
<td>Sirdah = MCV - RBC - 3*Hb</td>
<td>2008</td>
<td>&gt; 27</td>
<td>&lt; 27</td>
</tr>
<tr>
<td>MH ratio (Siemens)</td>
<td>2008</td>
<td>&lt; 3.4</td>
<td>&gt; 3.4</td>
</tr>
<tr>
<td>Ehsani = MCV - (10* RBC)</td>
<td>2009</td>
<td>&gt; 15</td>
<td>&lt; 15</td>
</tr>
<tr>
<td>ThalIndex=(0.615<em>MCV)+(0.518</em>MCH)+(0.446*RDW)</td>
<td>2012</td>
<td>&gt; 59</td>
<td>&lt; 59</td>
</tr>
<tr>
<td>MH ratio (Abbott)</td>
<td>2015</td>
<td>&lt; 6.4</td>
<td>&gt; 6.4</td>
</tr>
</tbody>
</table>
Discriminant indices for distinguishing thalassemia and iron deficiency in patients with microcytic anemia: a meta-analysis
Clinical Chemistry & Laboratory Medicine 2015; 53(12):1883-94

Table 3: Diagnostic performance of the 12 discriminant indices, arranged in order of diagnostic odds ratio (DOR) with 95% confidence intervals (95% CI).

<table>
<thead>
<tr>
<th>Discriminant Index</th>
<th>DOR (95% CI)</th>
<th>PLR (95% CI)</th>
<th>NLR (95% CI)</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>M/H ratio</td>
<td>100.8 (39.6–256.3)</td>
<td>6.8 (4.8–9.8)</td>
<td>0.07 (0.03–0.2)</td>
<td>0.92 (0.87–0.98)</td>
<td>0.86 (0.81–0.91)</td>
<td>0.956</td>
</tr>
<tr>
<td>RBC</td>
<td>47.0 (29.5–74.9)</td>
<td>8.1 (5.8–11.4)</td>
<td>0.17 (0.13–0.22)</td>
<td>0.85 (0.80–0.88)</td>
<td>0.90 (0.86–0.93)</td>
<td>0.923</td>
</tr>
<tr>
<td>Sirdah</td>
<td>46.7 (23.4–92.9)</td>
<td>8.6 (4.8–15.5)</td>
<td>0.18 (0.12–0.27)</td>
<td>0.83 (0.75–0.89)</td>
<td>0.90 (0.83–0.95)</td>
<td>0.903</td>
</tr>
<tr>
<td>Ehsani</td>
<td>44.7 (26.8–74.7)</td>
<td>5.1 (3.7–7.0)</td>
<td>0.11 (0.10–0.18)</td>
<td>0.91 (0.85–0.94)</td>
<td>0.82 (0.76–0.87)</td>
<td>0.925</td>
</tr>
<tr>
<td>England and Fraser (E&amp;F)</td>
<td>34.7 (25.0–48.2)</td>
<td>9.5 (7.2–12.6)</td>
<td>0.27 (0.23–0.32)</td>
<td>0.75 (0.70–0.79)</td>
<td>0.92 (0.90–0.94)</td>
<td>0.887</td>
</tr>
<tr>
<td>Green and King (G&amp;K)</td>
<td>29.8 (18.5–47.8)</td>
<td>7.2 (5.2–10.0)</td>
<td>0.24 (0.2–0.3)</td>
<td>0.79 (0.73–0.83)</td>
<td>0.89 (0.85–0.92)</td>
<td>0.898</td>
</tr>
<tr>
<td>Jayabose (RDWI)</td>
<td>28.6 (17.8–45.9)</td>
<td>5.6 (4.4–7.1)</td>
<td>0.20 (0.14–0.27)</td>
<td>0.83 (0.78–0.88)</td>
<td>0.85 (0.81–0.88)</td>
<td>0.902</td>
</tr>
<tr>
<td>Mentzer</td>
<td>27.6 (20.7–36.6)</td>
<td>5.6 (4.6–6.8)</td>
<td>0.20 (0.17–0.24)</td>
<td>0.82 (0.79–0.86)</td>
<td>0.85 (0.82–0.88)</td>
<td>0.896</td>
</tr>
<tr>
<td>Shine and Lal (S&amp;L)</td>
<td>15.7 (8.8–28.0)</td>
<td>1.6 (1.3–2.0)</td>
<td>0.10 (0.07–0.16)</td>
<td>0.96 (0.93–0.97)</td>
<td>0.41 (0.27–0.56)</td>
<td>0.885</td>
</tr>
<tr>
<td>Ricerca</td>
<td>15.6 (7.9–30.9)</td>
<td>2.0 (1.4–2.7)</td>
<td>0.12 (0.07–0.22)</td>
<td>0.93 (0.88–0.97)</td>
<td>0.52 (0.36–0.67)</td>
<td>0.850</td>
</tr>
<tr>
<td>Srivastava</td>
<td>15.0 (10.9–20.6)</td>
<td>4.1 (3.3–5.1)</td>
<td>0.28 (0.23–0.34)</td>
<td>0.78 (0.72–0.82)</td>
<td>0.81 (0.77–0.85)</td>
<td>0.850</td>
</tr>
<tr>
<td>Bessman (RDW)</td>
<td>6.8 (4.0–11.7)</td>
<td>5.1 (4.2–6.2)</td>
<td>0.21 (0.17–0.27)</td>
<td>0.62 (0.61–0.63)</td>
<td>0.66 (0.65–0.68)</td>
<td>0.778</td>
</tr>
</tbody>
</table>

AUC, area under the ROC curve; NLR, negative likelihood ratio; PLR, positive likelihood ratio. The higher DOR values, the better discriminatory test performance is present. Positive and negative likelihood ratios >10 and <0.1 indicate that the test generates strong evidence to rule in or rule out a thalassemia diagnosis, respectively.
### Differential Diagnosis of Microcytic Anemia

<table>
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#### M/H ratio

<table>
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<th>Sensitivity</th>
<th>Specificity</th>
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</thead>
<tbody>
<tr>
<td>0.918</td>
<td>0.871 -0.966</td>
<td>90.3</td>
<td>81.1</td>
</tr>
</tbody>
</table>
Extended RBC parameters
✓ Expand information at a cellular level
✓ Correlate with the pathophysiology of disease

Improve the clinical relevant information
Quality of erythropoiesis Aids clinicians in

➢ Assessing true iron status
➢ Detect Functional Iron Deficiency = patients who can benefit from therapy
➢ Differential diagnosis anemia genetic or acquired